SPSD II

# PLATFORM FOR SCIENTIFIC CONCERTATION: FOOD SAFETY

# TOWARDS A SAFER FOOD SUPPLY IN EUROPE

Edited by Carlos Van Peteghem, Sarah De Saeger and Els Daeseleire

SUPPORTING ACTIONS

PLATFORM FOR SCIENTIFIC CONCERTATION: FOOD SAFETY

OA-22 CLUSTER

# TOWARDS A SAFER FOOD SUPPLY IN EUROPE

**Edited by**

**Carlos Van Peteghem and Sarah De Saeger**
Ghent University
Laboratory of Food Analysis
Harelbekestraat 72
9000 Ghent
Belgium

**Els Daeseleire**
Institute for Agricultural and Fisheries Research
Technology and Food Unit
Brusselsesteenweg 370
9090 Melle
Belgium

**In collaboration with the following sub-coordinators:**

Lieve Herman and Marc Heyndrickx, ILVO, Brusselsesteenweg 370, 9090 Melle

Marc De Loose, ILVO, Burg. Van Gansberghelaan 115 bus 2, 9820 Merelbeke

Edwin De Pauw, ULg-Cart, Allée de la Chimie B6c, 4000 Liège

Philippe Delahaut, CER, Rue Du Carmel 1, 6900 Marloie

Luc Pussemier, CODA-CERVA, Leuvensesteenweg 17, 3080 Tervuren


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Rue de la Science 8
Wetenschapsstraat 8
B-1000 Brussels
Belgium
Tel : +32 (0)2 238 34 11 - Fax : +32 (0)2 230 59 12
http://www.belspo.be

Contact person :
Mrs Christine Mathieu
Secretariat : +32 (0)2 238 37 61

# Preface by The Belgian Science Policy Office

The Belgian Science Policy Office is a Department active in all domains of science: from space to biodiversity, from Antarctica to art history.

For years, the Belgian Science Policy Office has been financing research projects in the field of agriculture and food issues, and this in a context of standardisation as well as of a sustainable development.

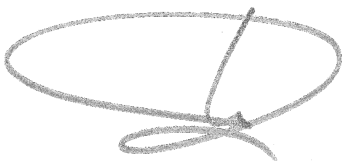
Sustainable food and more specifically the sustainable production and consumption patterns are essential for the implementation of sustainable development. In this way, the Belgian Science Policy Office has created a 'cluster' initiative in the framework of the Scientific Support Plans for a Sustainable Development Policy I and II (SPSD I and II). This cluster initiative enables the researchers and the stakeholders to discuss and interact about the common themes of the programme.

The cluster "Platform for Scientific Concertation: Food Safety" was coordinated by the Ghent University and carried out by six leader teams. It was an ambitious project of two years, and it has contacted, by a multidisciplinary approach, 23 partners for discussing research topics with Belgian and foreign researchers.

This project was carried out by the organisation of five thematic workshops in 2006 and by the redaction of this scientific publication: **'Towards a safer food supply in Europe'**.

This publication is the result of a willingness for scientific collaboration of many of the research teams active in the field of the agro-food theme and whose work was financed during SPSD I and II.
The result of this work is of great scientific quality and is valuable for a whole group of stakeholders like agricultural organisations, consumers, industrial federations, AFSCA/ FAVV (the Belgian Federal Agency for the Safety of the Food Chain), and EFSA (the European Food Safety Agency).

I hope that this publication will offer you a clear insight in the agro-food research and wish you a pleasant reading.



Dr Philippe Mettens
President of the Directory Board of the Belgian Science Policy

# Preface by the editors

Recent research has substantially increased the awareness of the importance of healthy and safe food and also the consumer is more and more concerned about the food he daily consumes. Indeed, food crises generally led to a reduced consumption of certain foodstuffs resulting in severe economic losses. Therefore, research has been further supported at national and international level in order to improve food safety. The Belgian Federal Science Policy finances many projects dealing with food safety, e.g. in the research programmes Scientific Support Plan for a Sustainable Development Policy (SPSD) and Science for a Sustainable Development (SSD).

Three years ago we decided to gather expertise on food safety in Belgium in one cluster project "Platform for Scientific Concertation: Food Safety"**.** This cluster was created in the framework of the SPSD II Support Actions, mobilising 31 research partners during a two-year project. It consisted of 5 sub clusters: 1. microbiological food safety; 2. GMO; 3. environmental contaminants; 4. veterinary drug residues and farm to fork approach; 5. chemical contaminants and sustainable agriculture. Five workshops were organised covering specific food safety topics (cfr. sub clusters). The main objectives of the workshops were to enlarge the scope of the scientific expertise of the project partners, to strengthen cooperation and communication between Belgian food safety experts and to develop multidisciplinary approaches. Furthermore, an international conference was organised in Antwerp on May 16, 2006 as the satellite congress of the 5$^{th}$ International Symposium on Hormone and Veterinary Drug Residue Analysis. With 200 participants, representing different countries, this conference was very successful and contributed to the global valorisation of Belgian research.

This book presents the final deliverable of the cluster project. The six chapters include peer-reviewed scientific papers on advances in microbiological food safety; GMO detection in the EU; analysis and risk assessment of environmental contaminants and veterinary drug residues; pesticides and indicators for sustainable farming systems; chemical contaminants and sustainable agriculture. We firmly believe that the excellence of the papers and the holistic approach will draw the attention of not only the scientific community but also of policy makers, agricultural organisations, food and feed industry, consumer organisations, etc. We sincerely wish to thank the Belgian Federal Science Policy, all the authors and especially the sub cluster coordinators for their continuous support and enthusiasm for the cluster project.

The editors:
Carlos Van Peteghem
Els Daeseleire
Sarah De Saeger

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# Advances in microbiological food safety

# Detection, identification and typing of bacterial pathogens in animal food products

**B. Possé[1], G. Rasschaert[2], M. Heyndrickx[2], L. De Zutter[1] and L. Herman[2]**

*[1] Ghent University, Faculty of Veterinary Medicine, Department of Veterinary Public Health and Food Safety, Laboratory of Hygiene and Technology. Salisburylaan 133, 9820 Merelbeke, Belgium*
*[2] Ministry of the Flemish Community, Institute of Agricultural and Fisheries Research, Unit Technology & Food, Brusselsesteenweg 370, 9090 Melle, Belgium*
*e-mail: bjorn.posse@ugent.be*

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Bacterial pathogens, molecular tools, food products

## SUMMARY

Molecular tools are applied as a complement or as an alternative to conventional microbiological techniques in bacterial detection, identification and typing. The choice of an appropriate target and technique for taxon-specific identification is an important issue as all methods have their advantages and drawbacks. All identification techniques offer great specificity for identification of pathogenic bacteria which were isolated and purified from samples by conventional culture methods. Bacterial typing is a valuable tool: it allows investigation of the population structure of pathogenic bacteria and allows investigating the origin, transmission and persistence of pathogenic types throughout time and space. However, the application and interpretation of bacterial typing tools in epidemiologic studies requires understanding of both the strengths and limitations of the chosen bacterial typing technique as well as the epidemiologic study design to answer the research question. Application of molecular tools for the detection of pathogenic bacteria in food products can cause an array of problems, depending on the stage of the conventional culture method where the PCR method is introduced. Due to the inherent drawbacks of molecular tools (inhibition, detection of non-viable cells, contamination), conventional microbiological culture techniques remain an important and indispensable element in the detection of pathogenic bacteria in food products. However, advances in amplification techniques, in particular real-time PCR along with the development of automated nucleic acid extraction methods, have made these techniques attractive to end-users.

## INTRODUCTION

Foodborne illness poses a significant economic burden for nations worldwide; it damages consumer confidence and impacts international trading of food products. Worldwide, the number of cases of gastroenteritis associated with food is estimated to vary between 68 million and 275 million cases per year [1]. One of the inherent difficulties in the detection of food pathogens is that they are generally present in very low numbers (< 100 cfu $g^{-1}$) among up to a million or more other bacteria. These pathogens may be lost among a background of endogenous microbiota, and substances in the foods themselves may hinder recovery for detection. There is also the difficulty of demonstrating that the strains recovered from a food sample are,

indeed, pathogenic to humans and/or responsible for a foodborne illness. During the past decade, there has been an increased awareness among food producers and legislators regarding food safety. Quality assurance systems, including Hazard Analysis of Critical Control Points (HACCP), have been introduced by food producers to meet legislative requirements and this has led to a significant increase in food microbiology testing.

While conventional microbiological (phenotypic) methods are still required to obtain pure cultures from samples, many of these conventional techniques can be laborious and time-consuming, especially for identification purposes. Genotypic (molecular) methods are useful to detect and identify bacteria either as a complement or alternative to phenotypic methods; besides enhancing the specificity of the identification process, they reduce much of the subjectivity inherent to interpreting morphological and biological data. Basically, DNA is invariant throughout the microbial life cycle and after short term environmental stress factors. Thus, molecular methods targeting genomic DNA are generally applicable.

A major technological breakthrough in molecular biology came in 1983 when the Polymerase Chain Reaction (PCR) method was described by Saiki *et al.* [2], offering an in vitro technique for enzymatic amplification of target nucleic acid sequences using a specific pair of primers and a heat-stable DNA polymerase. The technology of PCR has become one of the most influential discoveries of the molecular biology revolution and one for which Mullis received the Nobel Prize in 1993. Because of the impact of PCR and the thermostable *Taq* DNA polymerase (the enzyme responsible for the PCR revolution), *Taq* DNA polymerase was named as the first “Molecule of the Year” by Science in 1989 [3]. Molecular techniques, including PCR-based assays, were developed to detect virtually every clinically relevant bacterial pathogen.

Although molecular techniques have improved food microbiology to a great extent, there is a significant difference between the theoretical possibilities of PCR application (sensitivity of one target copy) and the practical application of molecular tools. A schematic overview of some molecular techniques and their application are presented in figure 1.

Molecular tools offer great specificity for the **identification** of pathogenic bacteria which were isolated and purified from samples by conventional culture methods. In this way, molecular identification of pathogenic bacteria replaces the final step in conventional microbiological methods namely the biochemical and/or serological confirmation. **Typing** of pathogenic bacteria refers to the differentiation of subtypes, strains or clones within a single species. From epidemiological point of view, bacterial typing is a valuable tool as it allows investigation of the population structure of pathogenic bacteria and allows investigating the origin, transmission and persistence of pathogenic types throughout time and space. Besides many advantages inherent to molecular techniques, care must be exercised when using these tests for **detection** purposes (discussed later).

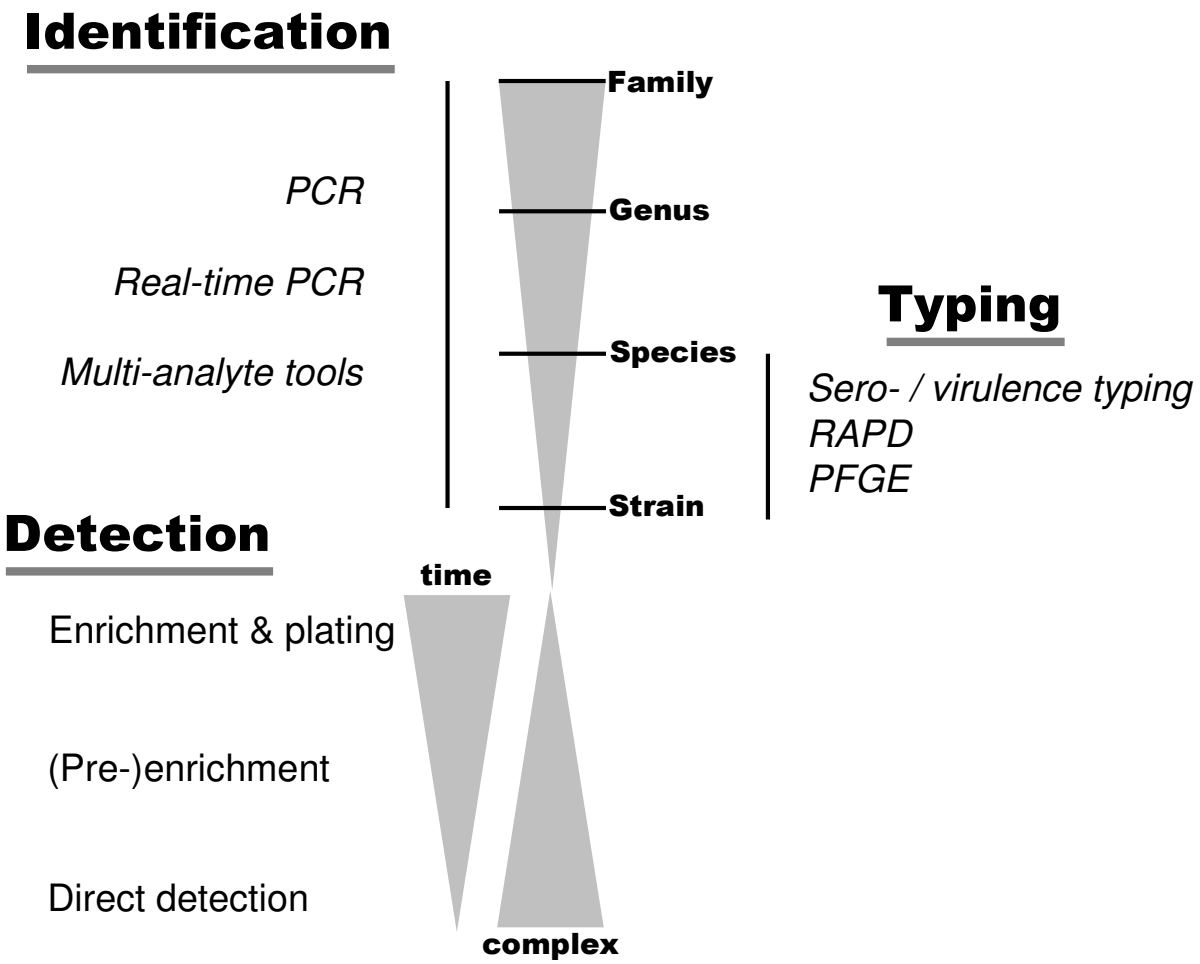


**Figure 1. schematic overview of some molecular techniques and their application**

Shiga toxin-producing *Escherichia coli* (STEC) were used as an example to demonstrate the application of molecular techniques discussed in this chapter. Since its identification as a food borne pathogen in 1982, STEC O157:H7 has been identified as the cause of several outbreaks [4-7]. Besides O157, non-O157 STEC serogroups, most commonly O26, O103, O111 and O145, have been shown to cause diarrhoea in humans. As pathogenic STEC strains are characterized by the presence of virulence genes and only a very low infectious dose is required to cause severe disease, both conventional culture based methods and molecular techniques have to be combined to achieve identification, typing or detection of pathogenic STEC strains.

## MOLECULAR IDENTIFICATION OF BACTERIAL PATHOGENS

Quick and reliable identification of pathogens occurring in the food chain is an indispensable tool in the framework of good manufacturing practices. Molecular identification techniques are applied on pure cultures, e.g. as a final confirmation step in the isolation procedure. According to bacterial nomenclatural rules, bacterial identification is restricted to genus, species and eventually subspecies level. However, beyond these pure taxonomic identification levels, further internationally recognized identification levels, associated to established identification schemes, exist. Most prominent examples for foodborne pathogens are serotypes and phage types.

Furthermore, for foodborne pathogens identification can even be extended to the recognition of certain pathogenic groups within a species or a serotype, such as STEC and EHEC within *E. coli* O157. This is only possible when a delineation of these pathogenic groups is based on a common level of agreement within the scientific community. In this section, molecular identification will be described for the application from genus down to pathogenic group level. Generally speaking, identification thus refers to the application of a recognized name or names to a bacterial isolate.

Identification schemes in conventional methods are based on biochemical and morphological characteristics observed in known and reference strains, with described properties under optimal growth conditions. As these phenotypic characteristics are often variable and can change with test condition, growth, stress or evolution, reliance on phenotypes can compromise accurate identification. Although conventional methods are still an important tool and even a prerequisite in bacterial taxonomy, molecular identification methods offer a lot of advantages (e.g. objectivity, accuracy, reliability and phylogeny inference) compared to conventional biochemical and morphological identification.

As many molecular targets for bacterial identification techniques have been described, the choice of the best target for taxon-specific PCR identification is an important issue. Molecular identification systems are based mostly on rRNA gene sequencing or the use of unique oligonucleotide sequences either as probes in hybridisation (micro-arrays) or as primers for enzymatic amplification of RNA and DNA in conventional PCR (either mono- or multiplex), reverse transcriptase PCR (RT-PCR) and nucleic acid sequence-based amplification (NASBA). Some more advanced methods, including sequencing, real-time PCR and multi-analyte techniques, offer a wide range of current and future technologies for bacterial identification.

**Basic nucleic acid amplification methods**

The **PCR** method involves the selected amplification of a region of DNA delineated by a set of oligonucleotide primers. By successive cycling at different temperatures for several fixed time intervals, a series of annealing, extension, and dissociation steps can be carried out with the net result of exponentially amplifying the sequences flanked by these primers. By amplifying a specific region of DNA over the rest of the genome, the signal to noise ratio increases greatly. The most common visualisation method is agarose gel electrophoresis by which the PCR product is separated by amplicon size and then visualized by ethidium bromide staining [8]. The inherent sensitivity of PCR assays makes detection of a contaminating nucleic acid a potential problem as discussed below (‘detection’ section).

Molecular identification of pathogenic bacteria based on RNA requires a reverse-transcriptase PCR (**RT-PCR**) or another method starting from RNA instead of DNA (e.g. NASBA, see below). This technique uses RNA as a template to produce complementary DNA (cDNA), which is subsequently amplified by PCR. Sensitivity and specificity of this technique is affected by the specimen type used, nucleic acid extraction method and quality of the primers or probes used [9].

Nucleic acid sequence-based amplification (**NASBA**) is an isothermal, transcription-based amplification method which amplifies RNA from either an RNA or DNA target and employs three enzymes: a reverse transcriptase, RNaseH and T7 RNA polymerase [10, 11], which act in concert to amplify sequences from an original single-stranded template. Oligonucleotide primers, complementary to sequences in the target

RNA, are incorporated in the reaction. One primer also contains a recognition sequence for T7 RNA polymerase. The reaction contains both dNTP's (deoxyribonucleotide triphosphate) and NTP's (nucleotide triphosphate). The first primer binds to the RNA, allowing the reverse transcriptase to form a complementary DNA (cDNA) strand. Then the RNase digests away the RNA and the second primer binds to the cDNA, allowing the DNA dependent DNA-polymerase to form a double-stranded cDNA copy of the original sequence. This double-stranded DNA is transcribed by the T7 RNA polymerase to produce thousands of RNA transcripts. The reaction is performed at a single temperature, normally 41°C. At this temperature, the genomic DNA from the target microorganism remains double-stranded and does not become a substrate for amplification. This eliminates the necessity for DNase treatment. The product of a NASBA reaction is mainly single-stranded RNA. This may be detected by gel electrophoresis followed by ethidium bromide staining, but to ensure product specificity, a confirmatory step, generally involving probe hybridization, is usually employed.

**Targets for nucleic acid amplification-based identification**

Over the past decades, many gene targets have been recognized as useful tools for bacterial identification. The target for molecular identification of foodborne pathogenic bacteria depends highly upon the heterogeneity within the taxon and the phylogenetic distance to other taxa. Another important element in selecting the appropriate target for the identification of foodborne bacteria is whether all strains of a certain taxon are considered to be potentially pathogenic, or whether only strains bearing certain virulence genes have the potential to become pathogenic. Finally, the target choice also depends on the research question asked: identifying an unknown isolate or identifying or confirming a specific pathogen obtained through an isolation protocol.

Both the highly conserved regions and the variable regions of the 16S and 23S **ribosomal RNA (rRNA) genes** have been used as target for molecular identification. Highly conserved regions are very useful for analysing the relationship between phylogenetic distant taxa due to the slow pace of variation. More variable regions of these genes are preferred targets for genus and species-specific PCR [12-16]: although they are functionally constant, they serve as molecular clocks of microbial evolutionary change [17]. The intergenic rRNA gene spacer regions are non-coding regions under minimal selective pressure and can therefore vary significantly. These regions have been widely applied for the construction of species-specific probes and primers [18-25].

Besides the identification of specific pathogenic species based on rRNA genes, some species were described where only **virulence factor**-harbouring strains or serogroups are considered pathogenic [26-32], for example, pathogenic Shiga-toxin producing *E. coli* (STEC) strains belonging to serotypes such as O157, O26, O103, O111 and O145 [33-42]. Conventional serotyping is based on three principal antigens: O, K (if applicable), and H antigens. Identification of these *E. coli* surface structures relate to components of somatic, lipopolysaccharide (LPS)-associated O-antigen, capsular polysaccharide (K), and flagellar (H)-antigen. The O-antigen is an important component of the outer membrane of Gram-negative bacteria. It acts as a receptor for bacteriophages and is also important in the host immune response. It consists of a number of repeats of an oligosaccharide, which makes the O-antigen extremely variable: currently 186-O antigens have been documented in *E. coli* typing schemes. Combined with the different H- and K-antigens, a wide array of serotypes can thus be formed.

Genes involved in the synthesis of the O-specific polysaccharide are located in the O antigen gene cluster between the *galF* and *gnd* genes on the *E. coli* chromosome (*rfb*-gene cluster) [43-47]. Amongst others, this region contains *wzx* and *wzy* genes, which show distinct serotype-specific variation. Knowledge of the DNA sequence of the cluster permits identification of unique genes or sequences that can be used to design serogroup-specific PCR assays. These assays can be employed for detection, as well as typing, of *E. coli* as an alternative to serotyping. Primers for detection of O157, O26, O91, O103, O111, O121 and O145 have been described [48].

In order to reliably differentiate pathogenic *Escherichia coli* strains (e.g. enterotoxigenic, enteropathogenic, uropathogenic, Shiga-toxin producing (STEC) or enterohaemorrhagic (EHEC)) from the normal commensal *E. coli* flora, the presence of virulence characteristics needs to be identified [35, 49-55]. During the last decade, the use of PCR based technology to identify virulence factor genes has become widely adopted [50, 56-63].

The main feature of STEC strains is the production of *Stx1* and/or *Stx2*, two major antigenically distinct groups of the Shiga-toxin. The genes for the *Stx* group are either located on lambdoid bacteriophages or on the chromosome. In addition, STEC may express more than one *Stx* if carrying more than one *Stx*-encoding bacteriophage [64]. The *eae* gene that codes intimin is a 94-to 97-kDa outer membrane protein produced by all attaching and effacing (A/E) enteric pathogens. It is the only bacterial adherence factor identified thus far as important for intestinal colonization in animal models. Another putative virulence factor is enterohaemolysin, coded by the EHEC *hly* operon. STEC serotypes may also possess additional virulence factors such as secreted proteins for signal transduction encoded by *espA*, *espB* and *espD* and the translocated intimin receptor encoded by *tir*.

In some cases a specific sequence with an unknown function is used as a target for the molecular identification. This is the case for the Salmonella-specific PCR described by Aabo et al. [65], in which the primers were deduced from a cryptic, 2.3 kb DNA fragment.

**Remark**

In choosing the appropriate target for bacterial identification, one must consider that many virulence factors are located on plasmids, which are often unstable and easily lost during laboratory manipulation, leading to possible false-negative results when detecting plasmid-encoded virulence genes by PCR. Moreover, not all primers described in the literature have been adequately evaluated for their specificity and sensitivity. Many of them should be evaluated against a suitable panel of relevant strains before being used in routine laboratories.

**Identification based on rRNA gene sequencing**

Identification of bacteria by gene sequencing is a suitable method when no or limited information about the potential identity is available. The gene target that is most commonly used for bacterial identification is the 16S rRNA gene (or 16S rDNA): partial (500–base pair) 16S rRNA gene sequencing has emerged as an accurate and fast method to identify pathogenic bacteria [66-72]. A limitation of the 16S rDNA sequence is its inability to discriminate among all bacterial taxa. For example, *Bacillus cereus* and *Bacillus anthracis* have nearly identical 16S rDNA sequences: both species can not be

separated reliably at the molecular level by their genomic DNA, because their differences primarily lie in the acquisition of virulence plasmids [73, 74]. Also the identification of closely related species of bacteria, for example, *Shigella* spp. and *E. coli*, is difficult to achieve through 16S rDNA analysis solely [75]. Sequencing of alternative gene targets (housekeeping genes, invasion genes) can aid discrimination between closely related species (e.g. *rpoB, tuf, gyrA*) [76-80].

Accurate assignment of gene sequences to a particular genus or species requires analysis with a high-quality, comprehensive reference library. For example, GenBank is a large, public database with 1200,000 named 16S rRNA gene sequences. Nucleotide sequences are generally reported in terms of "percent identity." This term refers to the number of identical nucleotide bases shared by the query and reference sequences divided by the number of nucleotide bases sequenced. For bacteria identified by the 16S rRNA gene, most taxonomists accept a percent identity score of at least 97% and preferably 99% to classify a microorganism to species or species group level. Sequence similarity can be assessed further by constructing a relatedness diagram (phylogenetic tree) that estimates the evolutionary distances among sequences [17].

**Real-Time PCR**

Real-time PCR has become an increasingly popular technique for the identification of pathogenic bacteria [81-84] and specific virulence factors [85-92]. The advancement provided by real-time PCR is due to its unique ability to monitor the complete DNA amplification process: real-time PCR refers to a collection of technologies and chemistries that monitor the accumulation of PCR product in the reaction while it is taking place compared to endpoint detection of the PCR product in conventional PCR. In the mid 1990s, researchers showed that the 5′ nuclease activity of the Taq DNA polymerase could be exploited as a method to indirectly assess the level of DNA amplification with the use of specific fluorescent probes [93], eliminating the need for electrophoresis and off-line detection. In general, analysis of amplification during real-time PCR has been achieved by detecting the fluorescence that is either directly or indirectly associated with the accumulation of the newly amplified DNA. Most real-time PCR formats offer the option of melting curve analysis, which allows the amplification product to be discriminated from non-specific product or primer–dimers. Furthermore, real-time PCR offers many advantages over conventional PCR such as rapidity, broader dynamic range, elimination of post-amplification handling steps, and higher throughput conductive to automation. Real-time PCR assays may use the intercalating fluorescent dye (SYBR Green), dual labelled probes (TaqMan) or hybridization probes (LightCycler) as means of detecting amplification.

SYBR Green, the first commercially available dye for real-time PCR, is still used commonly in research applications. SYBR Green is a sequence-aspecific cyanine dye that binds specifically to the minor groove of double-stranded DNA and, as a result, intercalates between the 2 strands of double-stranded DNA. Once bound, the dye emits a fluorescent signal more than 1000 times greater than that emitted by the unbound dye [94]. Sequence confirmation of the amplified product is performed by post-PCR melting curve analysis. Advantages of SYBR Green include the relative ease with which it can be applied to existing PCR assays (SYBR Green is added to an already optimized PCR reaction, however, as real-time PCR usually generates 100-200 bp fragments, primers of the existing conventional PCR should comply to this requirement), relatively lower cost, and ease of assay design. SYBR Green assays do not require the added complexity and cost of designing and manufacturing probes labelled with fluorescent dyes. One drawback, however, is the non-specific nature of

SYBR Green, which allows any non-specific amplification during a PCR reaction (primer dimers, mispriming, … ) to artificially increase the fluorescent signal and incorrectly increase sample values [95].

LightCycler chemistry is based on the phenomenon of fluorescence resonance energy transfer (FRET), in which the energy from an excited fluorophore is transferred to an acceptor moiety, causing the quenching of the fluorophore emission.

Another real-time PCR detection system is the "Taqman" system [96], which makes use of the 5'-3' nuclease activity of *Taq* DNA polymerase to digest a probe. Probes used during Taqman-based real-time PCR are labelled both with a fluorescent reporter dye and a non-fluorescent quencher dye. Fluorescence from the reporter dye is efficiently quenched by the quencher dye on the same probe molecule. As Taq polymerase extends from the primer, it displaces and cleaves the probe, separating the reporter dye from the quencher dye. As a result of this probe hydrolysis and subsequent dye separation, the fluorescence intensity increases.

**Multi-analyte and automated techniques**

Vast knowledge on the specific DNA sequences of pathogenic bacteria has led to the development of many different variants of multi-analyte identification techniques. These techniques are specifically designed to identify multiple nucleic acid targets in bacteria using classical PCR (multiplex PCR), electrical signal-based biosensors or gene-array techniques. Recent technologies have allowed miniaturisation of these systems into micro- and nanometer scaled laboratories.

**Multiplex PCR** involves the simultaneous amplification of more than one target gene per reaction by mixing multiple primer pairs with different specificities. The resulting PCR amplicons of different molecular weight can be separated by agarose gel electrophoresis and visualized by ethidium bromide gel staining. In order to guarantee the specificity of the system (an unique target sequence per primer pair), it is fundamental to design primers which are longer and have a higher melting temperature compared to the primers used in conventional monoplex PCR [9]. Primer-dimer formation, due to primer extension either on itself or on the other primers, should be avoided since self-primer annealing reduces the availability of primers for the correct amplification reaction(s). Multiplex PCR is now widely applied in bacterial identification assays because of the relative ease of use and the increased speed compared to monoplex PCR. Several species-specific multiplex PCR's were also described before [97-104]. Recently, Monday et al [105] described a multiplex PCR to identify enterohaemorrhagic *E. coli* (EHEC). The assay simultaneously detects genes for Shiga toxin (*stx*) and intimin (*eae*), including allelic variants of both genes, 16S internal amplification control, as well as unique sequences in the *wzx* genes that are specific for serotypes O157, O26, O111, O103, O121 and O145. Osek et al. [106, 107] also described multiplex PCR's for identification of Shiga-toxin producing *E. coli* (STEC). These assays used primer pairs that identified the sequences of Shiga toxins 1 and 2 (*stx1* and *stx2*, including the *stx2c*, *stx2d*, *stx2e* and *stx2f* variants), intimin (*eae*), and enterohaemorrhagic *E. coli* enterohaemolysin (*EHEC-hly*). Typing of STEC strains based on variants of virulence genes and serotyping of STEC based on multiplex PCR will be discussed later.

**DNA microarrays** consist of a range of specific probes for the identification of relevant foodborne pathogens to genus, species or subspecies level. Probes are immobilized on a solid surface such as specially treated glass. The first practical demonstrations of

microarrays were treated glass slides with probes deposited in spots onto the surface. Hybridizations are performed by application of labelled nucleic acid target in a liquid state to the microarray surface. Following appropriate hybridization and washing steps, target nucleic acid bound to probes on the array surface are visualized using a microarray scanner. Some progress has been made with the identification of food pathogens from genomic DNA using microarrays under laboratory conditions [108-112].

Diagnostic **biosensors** are a group of analytical devices and technologies that use a biologically derived material (enzyme, antibody, nucleic acid, microorganism or cell) immobilized on a detection platform to measure the presence of one or more analytes [110-112]. Advances in molecular biology have expanded the range of biological recognition elements, while developments in fibre optics and microelectronics have expanded the capabilities of signal transducers. Application areas include the identification/detection of bacterial pathogens through the hybridization of species-specific sequences of DNA. Although still at an early stage, this research appears to offer promising routes for further investigation.

During the last few years, the advancement of silicon technology based on micromachining and biological micro-electromechanical systems have led to the development of micro PCR which have become a central part of a lab-on-a-chip [113, 114]. **Lab-on-chip** applications rely either on a stationary system with cycling temperature [115-117] or a flow system with three zones at different temperatures [118].

## MOLECULAR TYPING OF BACTERIAL PATHOGENS

Bacterial typing refers to discrimination beyond the species and eventually subspecies level. Hence, it includes the internationally recognized typing levels such as serotyping and phage typing, associated with an accepted identification scheme and naming, as well as arbitrary types delineated on laboratory level or types for which international recognition is not yet available or under consideration. To the latter belong for example clones (based on internationally standardized typing techniques such as PFGE and MLST, see further) and virulent subtypes. This section on molecular typing will mainly deal with the latter categories of undefined or provisionally defined but still unnamed types, while named types (especially serotypes and pathogenic groups) have been dealt with in the section on molecular identification.

Typing of bacterial pathogens is necessary in epidemiological studies where origin, transmission and persistence of pathogenic strains are investigated. Foodborne outbreaks often require different types of the pathogen involved to be distinguished and linked to suspected foodstuffs. Pathogenic strains which occur coincidental but independent from an epidemic caused by another strain also have to be identified using bacterial typing techniques to identify the real source of infection. This information is crucial as it directly affects the preventive and hygienic measures to be implemented. Molecular typing techniques are also applied for monitoring contamination cycles from the production site, throughout the processing plants and packaging units to the end-user, the consumer.

During the last decades, the ability to discriminate among bacterial strains, even from the same species, has increased, enhancing outbreak investigations and surveillance, studies of the natural history of infection, and our understanding of the transmission,

pathogenicity and population structure of bacterial pathogens. Traditional typing systems are based on phenotypic typing techniques such as antibiogram typing, biotyping, serotyping based on antibodies, phage typing and multilocus enzyme electrophoresis (MLEE). Many of these classical methods are still frequently used to gather timely information which can be used for epidemiological investigations of foodborne pathogens. Drawbacks of these techniques are high costs, long handling time and the occurrence of non-typable strains. Nevertheless, classical typing techniques produce internationally accepted data as standardised protocols are available [119].

Molecular methods have emerged based on indirect and direct measures of genetic sequence. Common typing techniques used in epidemiologic studies rely on sequencing one or more genetic regions, for example multi-locus sequence typing (MLST), or use enzymes to cut the genome into pieces, for example, pulsed-field gel electrophoresis (PFGE). The number and size of the pieces correspond to the number and location of restriction sites cut by the enzymes, and thus are an indirect measure of sequence similarity. Other common techniques use the polymerase chain reaction targeted to either specific or random sequences, for example in **RAPD-PCR**; the resulting reactions yield fragments of different sizes, which can be used to discriminate between bacterial types. Generally speaking, sequence-based methods are most repeatable and reproducible.

Most typing techniques are DNA-fingerprinting techniques. The fingerprints can also be used for numerical cluster analysis using specialized software, which can be useful to delineate clonal relatedness. Molecular typing techniques are often characterized by their simplicity of performance, reproducibility or their high resolution enabling the differentiation between individual strains of a species. However, not all of these benefits can be combined into one single molecular typing technique [119]. A major drawback of molecular typing techniques is the scarce availability of internationally standardised protocols, which limits the international comparison of fingerprints. However, some advances towards international standardisation have been made, especially regarding the development of PulseNet Pulsed Field Gel Electrophoresis (**PFGE**) protocols [46, 120-123]. Sequence based typing methods like MLST do not suffer from lack of methodical standardisation as sequence data are absolute, meaning classification based on sequence data may be consistent with observed phylogenetic relationships. Understanding the strengths and weaknesses of the chosen bacterial typing technique enhances interpretation and generalization of study results. Generally speaking, sequence-based methods are most repeatable and reproducible. Gel-based fingerprinting methods are less so, because of the inherent variability of the technique [124].

Besides DNA-fingerprinting techniques, molecular typing techniques based on the detection of several variants of **virulence genes**, or of **serotypes** have also been described. Typing based on virulence gene variants will be discussed below using STEC as an example.

**Random Amplification of Polymorphic DNA (RAPD)**

Among the numerous techniques developed to detect DNA polymorphisms by PCR, random amplification of polymorphic DNA (RAPD) [125] is one of the quickest and easiest to perform. This technique involves the use of arbitrary GC-rich decamers as single primers. The issue of the reproducibility and usefulness of the RAPD fingerprinting technique has been widely discussed by many authors [126-130].

Moreover, Jin et al. [131] found RAPD analyses to be faster, easier to perform and more economical compared to multilocus enzyme electrophoresis (MLEE), ribotyping and restriction fragment length polymorphism (RFLP). Although RAPD is known not to be suited for defining the evolution of genetic relationships between organisms, it has significant reliability under strictly defined conditions such as typing a collection of isolates which do not necessitate interlaboratory comparisons. As this typing technique does not require knowledge on the DNA sequence of the target taxon, a number of random decamer primers can be evaluated by trial and error to identify suitable primer sequences. Reproducibility can however be a problem when performing RAPD analysis due to the low annealing temperature that is required for short-length primers. As a consequence, band intensities and positions are known to vary between assays, which complicate interpretation of the fingerprints. The use of a combination of oligonucleotide primers in a single RAPD reaction and eventually a standardised PCR-format can aid in reducing most of these reproducibility issues and increases fingerprint detail [132-135].

During a study by the Institute of Agricultural and Fisheries Research and Ghent University, an RAPD protocol using two decamer primers was optimized for genetic typing of a collection of commensal *E. coli* and pathogenic STEC strains belonging to serotypes O26, O103, O111, O145 and O157. The RAPD analysis protocol yielded fingerprints composed of amplified fragment sizes ranging from 0.4 to 6.0 kb, which resulted in clusters corresponding well to serotype specificity. Cluster analysis generated 15 groups when using an arbitrary cut-off at 79.4 % similarity. Jack-knife analysis was performed using Bionumerics: strains serotyped as O26, O103, O111, O145 and O157 were placed in a serotype-specific RAPD cluster for 81.8 %, 97.7 %, 76.3 %, 92.7 %, 77.5 % of the strains respectively [136].

**Pulsed Field Gel Electrophoresis (PFGE)**

Pulsed-field gel electrophoresis is one of the most important discriminatory methods in genetic characterization of bacterial strains and a commonly used approach to assess the relatedness of bacterial isolates. PFGE is recommended worldwide as a gold standard molecular typing method, that provides a chromosomal overview scanning >90% of the chromosome. The use of rare-cutter restriction enzyme(s) enables detection of chromosomal differences between isolates, and the macro-restriction patterns of large DNA fragments reflect the distances between restriction sites around the chromosome. Although minor genetic changes may go undetected, PFGE has enabled discriminative determination in the molecular comparison of STEC strains, and in the investigation of EHEC O157 and non-O157 infections such as O26, O103, O111, and O118, PFGE has been widely used [43-47, 128, 137]. PFGE showed higher level of discriminating power than RAPD: this reflects the fact that the molecular basis of RAPD and PFGE are different. Isolates with indistinguishable macrorestriction patterns are generally considered to be associated with each other.

Standardization of the PFGE protocols and electronic submission of gel images have created a genetic database where macrorestriction patterns for unknown isolates could be compared for epidemiological purposes, outbreak investigation and definition of clonal relationships. The one-day Pulsenet Pulsed Field Gel Electrophoresis (PFGE) protocol aims at detecting food borne disease case clusters by Pulsed Field Gel Electrophoresis (PFGE) to facilitate early identification of common source outbreaks [46, 120-123].

### Virulence typing of STEC

Within many serogroups identified as STEC, however, both Shiga toxin-positive and Shiga toxin-negative strains can be found; therefore, knowledge of the serogroup is not always sufficient and further virulence typing is needed to characterise a strain as a STEC. PCR assays for the identification of STEC strains by detection of several virulence genes have been described above. Once certain virulence genes have been found, a virulence profile can be composed by determination of the different variants of the respective genes: Shigatoxin encoding genes (*stx1, stx1c, stx1d, stx2, stx2c, stx2d, stx2e* and *stx2f*), intimin encoding genes (*eae, eae α, eae β, eae γ, eae θ, eae ε* and *eae ζ*), Shiga toxin auto agglutinating adhesin gene (*saa)*, enterohaemolysin (*EHEC-hlyA)*, katalase antiperoxidase gene (*katP)*, and extra cellular serine protease (*espP)* [49, 50, 136, 138-145]. Among the *Stx1* and especially *Stx2*, several variants have been identified, the main variants being *Stx1c*, *Stx1d*, *Stx2c*, *Stx2dac* (i.e. activatable by elastase), *Stx2e*, *Stx2f*, or *Stx2g* [64, 141, 144, 146, 147]. *Stx1* and *Stx2* toxins are approximately 59% homologous at the amino acid sequence level, while the variants of *Stx2* share 84-99% similarity with the *Stx2* [64].

### Conclusion

The application and interpretation of bacterial typing tools in epidemiologic studies requires understanding of both the strengths and limitations of the chosen bacterial typing technique as well as the epidemiologic study design to answer the research question. Beyond standard reliability, validity and cost considerations, key characteristics of a typing technique are the ability to discriminate between strains and to form a biologic basis for grouping strains with apparently different types which can not be achieved using classical techniques. The level of discrimination required and need to be able to group strains depends on the research question.

## MOLECULAR DETECTION OF BACTERIAL PATHOGENS

The molecular methods described for bacterial identification can also be applied for the detection of pathogenic bacteria in food products. Detection of the PCR products is performed by gel electrophoresis, by non-radioactive hybridisation or by fluorescence in real-time PCR applications. Depending on the stage of the conventional culture method where the PCR method is introduced, more problems or limitations can be expected as shown in figure 2.

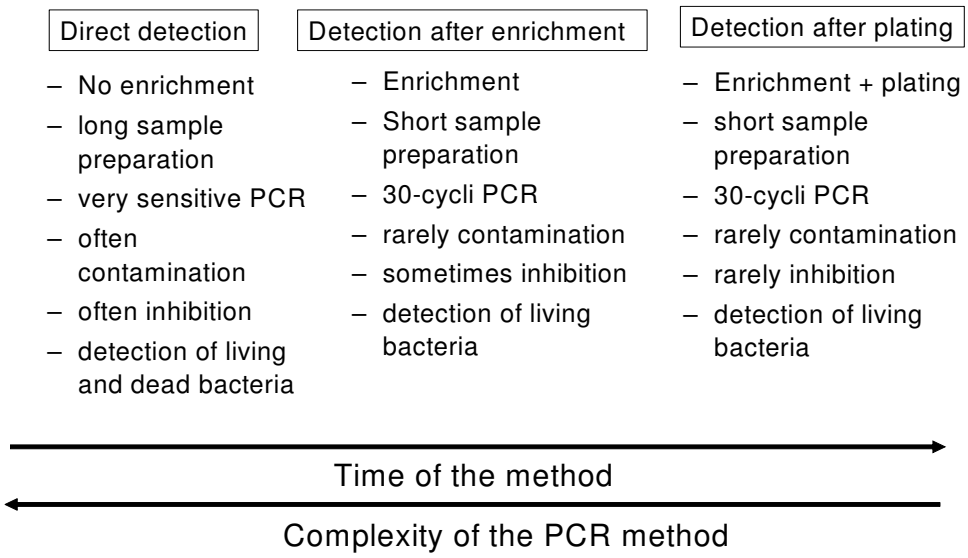


**Figure 2. limitations of PCR in food analysis**

Molecular techniques can be applied to identify strains that were isolated and purified by conventional culture methods: these identification methods (described above) are used as a replacement for the final biochemical or serological confirmation step in conventional methods [58, 148-156]. Application of molecular techniques on these pure cultures (either fluid or on general agar media) will rarely results in problems concerning PCR sensitivity or inhibition. A short sample preparation involving concentration and lysis of the bacteria is required for PCR.

If molecular techniques are applied on non purified isolates grown on selective agar media, possible PCR inhibition due to components present in the selective medium has to be taken into account when designing an appropriate sample preparation routine. However, this type of problems can usually be neutralised by application of one of more washing steps prior to DNA extraction.

More problems can occur when molecular techniques are applied for detection of pathogenic bacteria directly in the sample or from (pre-) enrichment media. These problems concern the requirement of an adequate sample preparation strategy, the occurrence of false-positive results due to contamination of the PCR, false-negative results due to PCR inhibition or bacterial count below PCR detection threshold, detection of dead bacteria and lack of quantitative results. Different strategies to overcome these problems are discussed in the paragraphs below.

**Thresholds for molecular detection**

The intrinsic sensitivity of the PCR procedure allows detection of very low numbers of bacteria. In addition, DNA is always present in the bacterial cell, even if it is sublethally injured. Theoretically this means bacterial growth (and thus enrichment) is no longer necessary. Direct application of PCR for detection of pathogens in foods, however, is restricted because of the physical enclosure of the target cell in the food and because of inhibitory food components. Direct detection of pathogenic bacteria in food products without (pre-) enrichment procedures is possible for certain food matrices as e.g. milk [157-161], and implies the use of very sensitive PCR assays (nested PCR or 40 cycle

PCR) to meet the criteria set for the control of pathogenic bacteria in food products: the strictest criterion being the absence of pathogenic bacteria in 25 g or 25 ml of the food product. Direct detection of pathogenic bacteria also requires the development of complex sample preparation strategies to quantitatively recover the bacteria and efficiently remove any components that may inhibit the enzymatic reaction [62, 152, 162-168].

The need for and the length of the (pre-) enrichment procedure depends on the pathogen involved and the food product: a 30-cycle PCR assay requires in most of the cases a minimum of $10^3$ CFU/ml for detection of the pathogen [157]. In many cases, pathogenic bacteria will be present in food samples in a sublethally injured or stressed state [169]. Selective or elective components used in enrichment media (e.g. bile salts, antibiotics, $MgCl_2$ …) suppress or interfere with the process of repair in sublethally injured cells extending the recovery phase. Resuscitation of these bacteria will result in prolonged enrichment times needed to achieve the $10^3$ CFU/ml level. Enrichment procedures therefore have to be optimized for each food borne pathogen and food item combination as a compromise: selective components are required to reduce the competitive exclusion due to naturally occurring micro organisms present in the sample, but can also cause delayed growth [157]. Also, the level of competition of naturally occurring microorganisms and the pathogenic bacteria depends upon the selectivity of the enrichment media used.

**Sample preparation and inhibition of PCR**

A suitable sample preparation method concentrates most often the bacteria, extracts their DNA and efficiently removes any component that may inhibit the enzymatic reaction. As an alternative, methods can also be described extracting DNA from food products or enrichment media without bacterial concentration. Bacterial cells are lysed using enzymes (lysozyme and/or proteinase K) or by boiling or heating in water or denaturating solutions. Because food samples can vary significantly in consistency, composition and level of endogenous background bacteria, each sample type requires a different approach.

The use of positive control reactions is indispensable when molecular detection methods are applied in routine laboratories because of the possible problem of PCR inhibition. The positive controls can either be amplified in a separate reaction tube, or can be included in the reaction tube for co-amplification with the target DNA. However, caution is required as the latter can result in destabilisation of the system due to competition between the amplification of the positive control DNA and the target DNA.

The easiest way to overcome PCR inhibition problems is to simply dilute the sample. As this procedure also decreases sensitivity, this dilution is often combined with a short enrichment procedure using the enrichment broth as the diluting agent. In many cases, it is necessary to implement a bacterial concentration or immobilization step to reduce the risk for PCR inhibition due to the presence of inhibitory food particles or enrichment components. Several general approaches have been described for selective removal of cells from a fluid food system. Centrifugation, filtration or combinations of both, have been used to remove food particles or for physical concentration of pathogenic bacteria [157]. The affinity of bacteria to solid phases can be applied as a concentration technique: coated magnetic particles can dramatically increase the concentration of pathogenic bacteria. Immunomagnetic separation (IMS) is one such technique that employs antibodies linked to magnetic beads that are added to the sample suspension and allowed to interact with specific epitopes on the bacterial cell surface. The bead

suspension is then exposed to a magnetic field that essentially pulls the beads and attached bacteria out of suspension for plating or molecular-based detection/enumeration [170-174]. PCR inhibition can also be overcome by including several washing steps in the protocol to remove proteins and other macromolecules. Drawback of washing sample suspensions is the high affinity of some bacteria for food particles or the entrapment of bacteria inside particles.

DNA that has been released from bacterial cells can be separated from PCR-inhibitory substances by using glass beads, affinity columns, or commercially available extraction kits. In some samples, inhibition is relieved by addition of bovine albumin serum (BSA) or certain enzymes such as trypsin inhibitor. BSA was reported to bind to the phenolic groups of some PCR-inhibiting substances and haem, thereby preventing their binding to DNA polymerase [175]. Some detergents such as Tween-20 reverse the inhibiting effect of ionic detergents used in lysing solutions [176-178].

**Exclusive detection of viable cells**

A major drawback of conventional PCR is that both viable and dead cells may be detected: as PCR is based on intact nucleic acids rather than intact viable cells, a false positive signal may occur from dead cells [159, 179]. Because the bacteriological safety of food products is determined by the presence of viable pathogenic bacteria at the time of analysis, this poses a problem. Besides application of enrichment procedures to increase the viable/dead ratio prior to DNA purification, also molecular techniques are investigated to asses the viability of bacterial pathogens.

Recently, RNA molecules have been used as an indicator of bacterial cell viability [180]. NASBA and RT-PCR are convenient techniques for the RNA-based amplification. Not all three RNA groups (ribosomal, transfer and messenger RNA) are useful in discriminating viable from dead bacterial cells. Several studies indicated rRNA and tRNA are not to be used as indicators of viability [181-184]. Messenger RNA (mRNA) is a short-lived molecule: it is digested rapidly due to the presence of nucleases. The presence of mRNA can be regarded as a valid and convincing criterion for assessing cell viability [119, 157, 185-187]. In dead cells, mRNA synthesis is likely to be slow and nuclease activity will continue to degrade any mRNA present. The factors controlling mRNA longevity in dead cells are not understood, but presumably mRNA would disappear most rapidly from cells killed by treatments that do not inactivate the degradative RNase enzymes. Conversely, mRNA may remain intact for longer periods in cells killed by treatments that also inactivate RNases or render the RNA resistant to attack [185]. For mRNA based detection of viable cells, the choice of an appropriate target is of great importance for the reliability of the test. On one hand the target has to be expressed constitutively in the pathogenic bacteria and on the other hand it has to be degraded by RNases with an intermediate speed, stable enough to allow extraction from the bacterial cells and not too stable that it can be used as an indicator for viability. Therefore housekeeping genes as the elongation factor (*tufB*) or the sigma factor of the RNA polymerase (*rpoD*) have been studied [188].

**Anti-contamination precautions**

PCR reactions are extremely susceptible to contamination due to the high sensitivity. Care must be undertaken to avoid false-positive reactions, which can result from sample-to-sample contamination or from carry-over of DNA from a previous PCR reaction.

The use of separated rooms for sample preparation, pre-PCR, PCR reaction and post-PCR treatments is highly advisable [189]. All reagents used in the PCR must be prepared, divided in to aliquots and stored in an area free of PCR-amplified products; all oligonucleotides used for amplification should be synthesised and purified in a PCR-product-free area [190].

Centrifugation is a major source of sample-to-sample contamination, but can be avoided by using appropriate aerosol-tight centrifuge tubes. For highly sensitive PCR assays, a layer of sterile mineral oil on top of the PCR reaction decreases contamination problems because it avoids evaporation during thermal cycling. Work areas should be treated with sodium hypochlorite as it destroys both nucleic acids and bacteria.

Pre- or post-amplification sterilisation applications to reduce the incidence of PCR contamination have been published previously. Several approaches have been reported to eliminate contamination by amplicons from previous reactions in the PCR mix of a subsequent amplification. In the widely used pre-PCR sterilisation method using uracil DNA glycosylase (UNG), all PCR's in a laboratory are performed with dUTP instead of dTTP. In contrast to the genomic template DNA, containing no uracil residues, PCR products can be selectively destroyed by the enzyme UNG in the closed vessel prior to PCR [191-193]. Shortwave UV irradiation procedures have also been described as a pre-amplification sterilization process [194] and to sterilize laboratory surfaces, racks, pipets and other laboratory equipment. In the post-PCR method, amplification is done in the presence of the photochemical isopsoralen compound 10 (IP-10). IP-10 is added to the reaction mixture prior to amplification. Following PCR but before the reaction tube is opened, the vessel is exposed to UV light, which activates the IP-10 to form adductors between the pyrimidines on the amplicons. If these amplicons contaminate future PCR assays, these adductors stop *Taq* polymerase from processing along the amplicons and thus prevent subsequent reamplification of any of these contaminating amplicons [195-199]. This allows only the template DNA, added to the PCR mixture, to be amplified.

Finally, a good lab hygiene protocol, eventually combined with an anti-carry-over system (using separate areas for different steps in the PCR protocol) can overcome most of the contamination problems when a single 30 cycles PCR is applied. More serious problems can occur when more sensitive PCR protocols are applied in routine laboratories. Contamination control strategies have been reviewed on several occasions [157].

**Evaluation and validation of PCR based methods**

As DNA based methods are used more frequently to detect pathogenic bacteria in food products, there is a great need for evaluation of numerous protocols which are being published. The difficulty of this evaluation lies within the inherent uncertainty of food analysis which is even enhanced with new variables introduced by rapid detection methods. If conventional culture methods are generally regarded as reference methods, molecular methods can fairly easy be validated by comparing results obtained by molecular techniques to the results obtained by conventional methods. More problems are encountered if conventional methods lack sensitivity (e.g. *Yersinia enterocolitica* [200]) or no generally accepted standard method is available. This is the case for Shigatoxin producing *E. coli* (STEC). Besides the availability of an ISO method for conventional isolation of O157 sorbitol negative strains, no standard methods are available for sorbitol positive O157 or non-O157 serotypes like O26, O103, O111 and

O145 which are regarded as emerging pathogens. As DNA-based methods can reach higher sensitivity compared to conventional methods, demonstrating the validity of the results is essential because very sensitive PCR assays can increase occurrence of false positive results due to contamination or the detection of small quantities of dead bacteria [195, 201-204]. Therefore, for most applications positive PCR results have to be confirmed by repeat analysis of the original enrichment medium using cultural techniques.

During validation experiments, the conventional culture method is compared to a molecular detection method using both naturally and artificially contaminated (spiked) samples. Spiking samples with pure, well grown bacterial cultures, can lead to overestimation of the sensitivity of molecular techniques, as bacteria in naturally contaminated samples are likely to be in some kind of stress condition. Validation of molecular techniques on naturally contaminated samples offers a possible solution to this problem. For some food products or pathogens, however, this is impossible due to the low prevalence of contamination resulting in insufficient samples to be evaluated. This problem can be overcome by evaluating the methods using food samples spiked with artificially stressed bacterial cells. For several foodborne pathogens these stressed cells are available as certified testing material.

**Fluorescence in situ hybridisation**

Besides sequencing or PCR based amplification of 16S rRNA regions for identification purposes, these genetic regions are also used for detection of bacterial pathogens by fluorescence in situ hybridization (FISH) [138, 148, 150, 154, 205-210]. The suitability of fluorescently labelled rRNA-targeted oligonucleotide probes for culture-independent detection of pathogenic bacteria was reported for the first time during the early 1990's [211]. The major advantage of in situ hybridization is the association of the nucleic acid hybridization with phenotypical information such as shape and size of bacterial cells and grouping of these cells. Several hundred rRNA-targeted oligonucleotide probes suitable for FISH have been described, together with a large online database providing an encompassing overview of over 700 published probes and their characteristics [211].

In FISH, either the small or the large-subunit rRNA is typically selected as the phylogenetic marker [212, 213] and probed in situ with fluorophore-labelled DNA oligonucleotides. FISH experiments are successful only if the fluorescent signal received from target cells is sufficient for discrimination of these cells from the background and non-target cells. Because a significant proportion of newly designed DNA probes fail to give satisfactory signal intensity [214], sensitivity is considered one of the major challenges in FISH [215-217]. Microbial cells are first treated with appropriate chemical fixatives and then hybridized under stringent conditions on a glass slide or in solution with oligonucleotide probes. Generally, these probes are 15–25 nucleotides in length and are labelled covalently at the 5′end with a fluorescent dye. After stringent washing, specifically stained cells are detected via epifluorescence microscopy or flow cytometry.

**Multi-analyte**

Many molecular applications have been described for the simultaneous detection of different pathogenic bacterial species from food samples. However, the main drawback for the application of multi-analyte technologies for the simultaneous detection of different pathogenic species is the fact that these technologies have to be applied after

an enrichment procedure due to dead/viable issues, inhibition and sensitivity as described above. The need for an enrichment procedure hampers the detection of multiple pathogens within the same sample as every pathogenic species requires a specific enrichment procedure. Due to this requirement, the use of multi-analyte technologies is actually of very limited practical value for pathogen detection in food products.

**Real-Time PCR based quantification**

As real-time PCR allows cycle-to-cycle detection of amplified PCR products, monitoring of the exponential phase is possible, whereas conventional PCR only allows stationary end phase monitoring. In the initial cycles of PCR, there is little change in fluorescence signal: this defines the baseline for the amplification plot. An increase in fluorescence above the baseline indicates the detection of accumulated PCR product. A fixed fluorescence threshold can be set above the baseline. The parameter CT (threshold cycle) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. Quantification of the amount of target in unknown samples is accomplished by measuring CT and using the standard curve to determine starting copy number [95, 218, 219].

Several papers have been published dealing with the quantification of pathogens in food samples by quantitative real-time PCR [96, 220-222]. However, quantitative real-time PCR suffers from some major drawbacks leading to an over- or underestimation of the amount of pathogens in the food products, depending on the case. Wolffs et al. [220] showed that direct quantitative PCR resulted in an overestimation of up to 10 times of the amount of cells in the samples compared to viable counts, due to detection of DNA from dead cells. On the other hand, underestimation of the real quantity of bacterial pathogens in the food products can occurs due to a problem with the quantitative recovery of bacterial pathogens and the partial lysis of the bacterial cells.

**Commercial systems**

Examples of some commercially available molecular detection assays based on PCR for bacterial foodborne pathogens are listed in table 1. Commercially available conventional assays include the BAX® (DuPont Qualicon, DE, USA), and Dr. Food™. (Dr Chip Biotech Inc., Miao-Li, Taiwan) kits. BAX® kits are available for the detection of *Salmonella*, *Campylobacter jejuni*, *C. coli* and *C. lari, Listeria*, *L. monocytogenes*, *E. coli* O157:H7, *Enterobacter sakazakii* and *Staphylococcus aureus*. BAX® tests involve culture-based enrichment of the food sample, cell lysis to release the DNA, followed by PCR amplification with detection of the PCR products. Dr. Food™ kits provide rapid PCR-based methods for the detection of *Salmonella* spp., *Campylobacter* spp., *E. coli*, *L. monocytogenes*, *S. aureus*, *Yersinia enterocolitica*, *Bacillus cereus*, *Clostridium* spp., *Shigella* spp. and *Vibrio* spp. in culture-enriched foods. Identification of the pathogen is achieved following PCR amplification of extracted DNA and post-PCR hybridization to an oligonucleotide probe in a colorimetric reaction [223].

There are an increasing number of commercially available real-time PCR kits for the detection of foodborne pathogens. Roche Diagnostics 'food-proof' real-time PCR assays are available for *Campylobacter*, *Salmonella*, *Listeria*, *L. monocytogenes* and *E. coli* O157. Other companies with kits on the market include QIAGEN (RealArt™ kits for *L. monocytogenes*, *Salmonella* and *Campylobacter*), Applied Biosystems (TaqMan® detection kits for *C. jejuni*, *L. monocytogenes*, *E. coli* O157, and *Salmonella*

enterica), Artus (*L. monocytogenes*, *Salmonella* and *Campylobacter* PCR kits) and Congen (SureFood® Pathogen Kits for *Salmonella*, *Campylobacter* and *Listeria*) [223].

**Table 1. Overview of commercially available PCR kits for bacterial foodborne pathogens**

| Kit name | Format | Bacteria | Manufacturer |
|---|---|---|---|
| BAX® system | Conventional | *Salmonella*, *E. coli* O157:H7, *Listeria* and *L. monocytogenes* and *Campylobacter jejuni, C. coli, C. lari, Enterobacter sakasakii* and *Staphylococcus aureus* | Qualicon |
| Dr Food™ kit | Conventional | *E. coli*, *L. monocytogenes*, *Campylobacter* spp. and *Salmonella* spp. | Dr ChipBiotech Inc |
| PCR Diagnosis-Bacteria Identification Kit | Conventional | *E. coli* | BioChain |
| LightCycler® foodproof | Real-time | *Listeria, Salmonella, E. coli* O157, *Listeria monocytogenes, Campylobacter* | Roche |
| RealArt™*L. monocytogenes* PCR kits* | Real-time | *L. monocytogenes, Campylobacter, Salmonella* | Qiagen |
| Artus *L. monocytogenes* PCR kit | Real-time | *L. monocytogenes, Campylobacter, Salmonella* | Artus |
| TaqMan® *L. monocytogenes* detection kit | Real-time | *L. monocytogenes, Campylobacter jejuni, E. coli* O157, *Salmonella enterica* | Applied Biosystems |
| SureFood® pathogen *Salmonella* | Real-time | *Salmonella* spp., *Campylobacter jejuni, C. lari, C. coli, L. monocytogenes* | Congen |

## Conclusion

While conventional microbiological culture techniques remain an important and indispensable element in the detection of pathogenic bacteria in food products, the application of molecular techniques is making major progress. These molecular methods are mostly used after a decreased enrichment procedure and holds as major advantage a reduced time for final confirmation of the pathogen identity. Advances in amplification techniques, in particular real-time PCR along with the development of automated nucleic acid extraction methods, have made these techniques attractive to end-users. Although costs remain high compared to conventional culture confirmation methods, the reduced handling time has become the main asset for applying these techniques in routine labs for screening food products. However, every conventional or molecular detection technique must demonstrate reproducible sensitivity, marked specificity, low cost per assay, high speed throughput, standardized protocols, straightforward data interpretation and ease of use. No single approach is capable of demonstrating all or even most of these features, forcing the end-user to make a balanced decision based on specific application-based requirements and laboratory funding capacity in order to select the most appropriate and practical method.

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# Predictive modelling tools for the quantitative assessment of two case-studies within microbiological food safety: low numbers of contaminating cells under stressful conditions & lactic-acid mediated microbial interactions

**A.H. Geeraerd[1], F. Devlieghere[2], J. Debevere[2] and J.F. Van Impe[3]**

*[1] Katholieke Universiteit Leuven, Department of Biosystems (BIOSYST), Division of Mechatronics, Biostatistics and Sensors (MeBioS), W. de Croylaan 42, B-3001 Leuven, Belgium.*
*[2] Ghent University, Department of Food Safety and Food Quality, LFMFP - Laboratory of Food Microbiology and Food Preservation, Coupure Links 653, B-9000 Ghent – Belgium*
*[3] Katholieke Universiteit Leuven, Department of Chemical Engineering, BioTeC - Chemical and Biochemical Process Technology and Control Section, W. de Croylaan 46, B-3001 Leuven, Belgium.*
*e-mail: annemie.geeraerd@biw.kuleuven.be*

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Predictive microbiology; microbial interactions; individual cell level; stochastic modelling; mechanistic modelling

## SUMMARY

This chapter reports on the scientific results obtained within the project CP-31 "Microbial Food Safety Assessment: Development and Integration of Generic Predictive Modeling Tools", which has run from December 15, 2001 to June 30, 2006 and was supported by the Second Multi annual Scientific Support Plan for a Sustainable Development Policy (SPSD II), initiated by the Belgian Science Policy. Its general aim was the development and integration of a new generation of predictive models able to predict the behaviour of micro-organisms in foods as generic tools in microbial food safety assessment. Two important shortcomings of existing predictive microbiology models were identified and used as vehicles for model development and validation purposes throughout the project lifetime: (i) *exploring the lag phase at the boundaries of microbial growth*, and (ii) *quantifying the interaction between micro-organisms*. Dedicated predictive modelling methodologies were developed.

## INTRODUCTION

Safe and healthy food is a main issue for modern consumers. Due to some recent food related crises, e.g., in Belgium BSE, dioxin or the *Bacillus cereus* outbreak in Kinrooi, people are more aware of the quality and the safety of the food they are consuming. Different approaches can be followed to evaluate the microbial safety of a food product, including storage experiments, challenge tests and predictive microbiology. The first two approaches are criticised for being very laborious, time-consuming and non cumulative, as the results can only be used for the very specific case tested. The (relatively young) discipline of *predictive microbiology* deals with the design and analysis of quantitative relations (mathematical models) aiming at the prediction of the evolution (growth, inactivation, survival, …) of pathogenic or spoilage micro-organisms

(the so-called target-organisms) during subsequent stages of production, distribution and storage of food products. Predictive microbiology has the advantage to be fast and flexible, able to handle changes in the product properties or the environmental conditions, although also some drawbacks should be mentioned, mainly involving the simplifications and initial assumptions made during modelling.
The research project on which this chapter reports focuses on the development and integration of a collection of generic predictive modelling tools for predictive microbiology, hereby aiming at standardizing and consolidating the promising use of mathematical modelling techniques in the framework of risk analysis of foods. The overall aim is to design and exploit new generation predictive models able to predict the behaviour of micro-organisms in foods, taking into account their complex microbial ecology, as generic tools for microbial food safety assessment. As a vehicle to demonstrate their intrinsic generic nature and applicability, two case studies (that are challenging from both the scientific and technological/economical point of view, as will be motivated below) have been used for development and validation purposes: (*i*) exploring the lag phase at the boundaries of microbial growth, and (*ii*) quantifying interactions between micro-organisms.
Concerning the *exploration of the lag phase at the boundaries of microbial growth*, two approaches regarding modelling the microbial lag phase were explored. In a first part, the lag phase induced by a sudden temperature change was studied through the use of an extensive set of computer-controlled bioreactor experiments making use of *Escherichia coli* K12. For reasons of space limitations, the interested reader is referred to [1-2] for more information on this first part. In the second part, which will be denoted as Case-study 1 for the remainder of this chapter, the behaviour of single cells of *Listeria monocytogenes* was studied in cups of a microtiter plate as influenced by environmental stress factors. The *quantification of lactic-acid mediated interactions between micro-organisms* delineates Case-study 2 of this chapter.

## Case study 1: effect of environmental and precultural conditions on the lag phase of *Listeria monocytogenes* at individual cell level

### *Introduction & motivation*

Within predictive microbiology, the study of the lag phase is an important topic. The lag phase can be defined as a delayed response of the microbial population to a (sudden) change in the environment. During the lag phase, microbial cells adapt to the new surroundings in order to take advantage of a new environment and initiate exponential growth. Commonly, the transient growth phase following the inoculation of a laboratory medium (or, the contamination of a food product) is characterized as the (initial) lag phase; however, (sudden) environmental variations during growth can also result in delayed growth or (additional) lag. Factors inducing lag and/or affecting the lag duration are (i) nutrition changes, (ii) physical environment changes (e.g., temperature, pH, $a_w$), (iii) the presence of an inhibitor, (iv) spore germination, and (v) the physiological state of the microbial population [3].
Accurate mathematical models are indispensable in view of prediction, simulation, optimization and/or model based control of microbial growth processes. At present, most available modelling techniques solely focus on the initial lag phase and take only into account the influence of the actual environment on the lag phase. Any effect of the pre-history of the microbial cells has been neglected by considering constant (usually, optimal) pre-culturing conditions as well as using cells from the same growth phase (usually, the stationary phase). However, it is frequently observed that (i) the pre-culturing conditions (temperature, pH, medium composition, etc.), (ii) the inoculum size,

(iii) the growth stage of the cells, and (iv) the magnitude of change between the past and new environmental conditions, have a crucial influence on the microbial lag phase. More details can be found in the review article [4].
Within this first case-study the focus was placed on the inoculum size effect, studying the lag phase from an individual cell point of view. Robinson and co-workers [5] observed that the variability of detection times of *L. monocytogenes* increased when a lower inoculum level was applied, while also other authors (for example [6-7]) reported effects of a small inoculum size. Moreover, foods are mostly initially contaminated with low numbers of food pathogens, e.g., the initial contamination level of *L. monocytogenes* on cooked meat products and poultry products is mostly between 1/10g and 10/g [8].

*Protocol development for isolation of individual cells [9-10]*

In a first step a protocol was developed to isolate single cells in the cup of a microtiter plate based on standardized dilution principles. The bacteria were subcultured twice to eliminate variance in the pre-cultural conditions. Afterwards the cell count was standardized to $10^8$ cfu/mL using OD measurements at 600 nm. Starting from the standardized inoculum a classical dilution series was made ending up with 1000 cfu/mL. This cell count was controlled by plate counting of 200 µL inoculum on Tryptic Soy Agar (TSA), incubated at 30°C for 24h. Further dilution was performed by adding 200 µL of inoculum to 200 µL broth in each cup of the first column of a microtiter plate. These cups were used to make further ½-dilution series ending up with single cells isolated in the cup of a microtiter plate (Figure 1). This procedure was repeated for 9 plates, resulting in 72 ½-dilution series. The content of each cup was plated on TSA to control the dilution pattern and to locate the single cells.
From the results (not shown), it was clear that individual dilution series do not follow the expected pattern from the theoretical mean values. Sometimes it can even be seen that empty cups are followed by cups containing one or even two cells. In contrast, the mean values do follow the normally expected dilution pattern.
Single cells are mainly located in the last 5 columns, so these columns are taken into account. In the last 5 columns, 75 cups containing cells were counted giving a yield of 75, which is better than observing only the last well of each row showing growth (this would give a yield of 72). From these 75 cups, 60 contained one single cell, while 15 cups did contain two or more cells, resulting in a chance of 80% having a single cell.

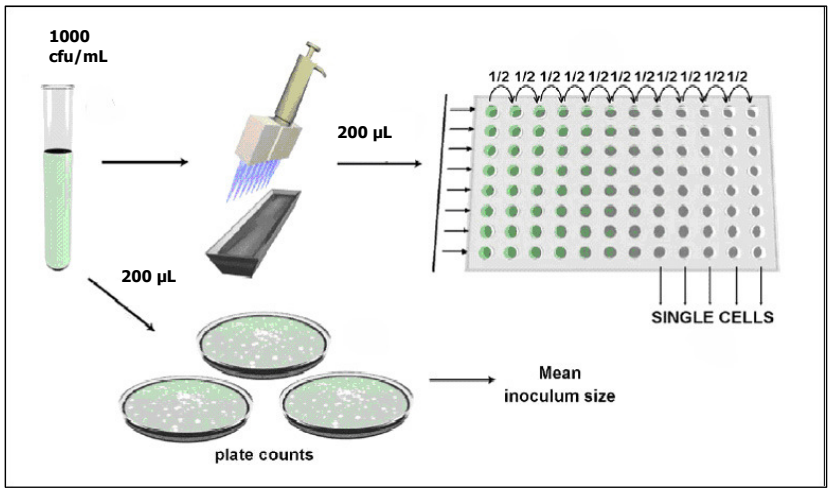


**Figure 1. Overview of the dilution protocol**

*Protocol development for measuring the lag phase of individual cells [11]*
For measuring the lag phase of individual cells, a protocol was developed using optical density measurements in microtiter plates. Microtiter plates were filled as previously described, and incubated at the fixed conditions. The cell density was measured at regular intervals using OD measurements at 600 nm (Versamax microplate reader, Molecular devices, Sunnyvale, CA, USA). The upper part of the growth curve was generated by calculating the cell counts out of the OD values using a calibration curve. To establish this calibration curve, a dataset was generated containing 96 points starting from a cell density of about 5 x $10^9$ cfu/mL and diluted in a ½ way to a cell density of about 5 x $10^6$ cfu/mL. Differences in OD between the blanks and the samples occurred starting from a cell density of 1 x $10^7$ cfu/mL. A logarithmic transformation was done for both the OD values and the cell counts to equalize the differences between the data points. These transformed data were used to fit a linear regression curve. A good correlation was observed ($R^2$ = 0.972).

Using these data, the upper part of the *L. monocytogenes* growth curve could be constructed, consisting of a linear part (the exponential growth zone) moving over to the stationary phase. The method assumes that once the cell lag phase has passed, a cell immediately grows at its maximum growth speed ($\mu_{max}$) until reaching the stationary phase. By extrapolating the linear part of the curve, the individual lag phase ($\lambda_{ind}$) is cut off at the inoculation level (1 cfu/200 µl = 5 cfu/mL) (Figure 2). The generation time (GT) can be calculated from the slope. At least 100 replications were made for each set of conditions.

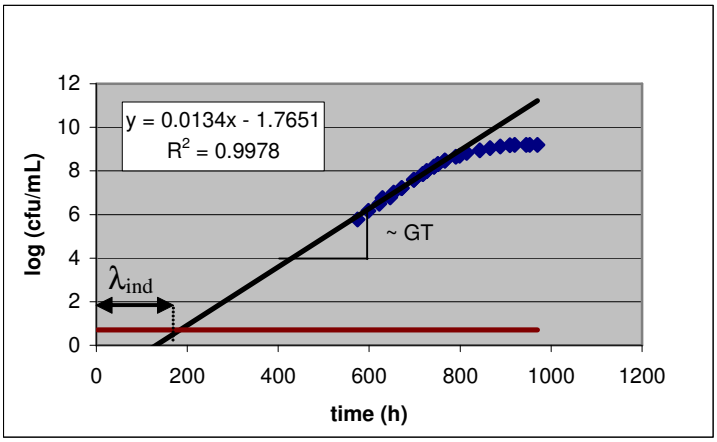


**Figure 2. Linear extrapolation method to calculate individual cell lag phases ($\lambda_{ind}$) and generation times (GT), illustrated on a growth curve of *L. monocytogenes* LMG 13305 at 4°C and at pH 5.55**

However, during the project it was noticed that environmental factors do have an influence on the relation between the optical density and the cell count. Therefore, the effect of environmental stress factors on the relationship between the optical density measured at 600 nm and the plate count results was investigated. Different temperature levels (between 2°C and 30°C), pH levels (7.4 – 4.8), and water activity ($a_w$) levels (0.995 – 0.946) were investigated at nineteen different combinations. *L. monocytogenes* cells were grown in Brain Heart Infusion (BHI) Broth, adjusted to the appropriate growth conditions. When the turbidity in the tube was maximal, a ½ dilution series was made in a microtiter plate, resulting in twelve consecutive dilution steps with

eight replicates of each dilution. The optical density of the cups was measured and consecutively the cell count was determined by classical plate counting on TSA. A logarithmic transformation was performed for both the OD values and the cell count data to standardize the variability and equalize the differences between the data points. These transformed data were used to fit a linear regression curve.
Different stress factors were shifting the calibration curve parallel to the optimal curve. Especially pH was having a main effect (Figure 3), whiles the pure effect of temperature and $a_w$ was less pronounced, although these environmental factors played a more important role when different environmental factors were combined. The parallelism between the different calibration curves was statistically proved by an F-test. As the curves were assumed to be all parallel, a forced regression procedure was performed on all nineteen datasets: all regression lines were "forced" to have the same slope, while the intercept of the regression line was variable as a function of the environmental conditions.

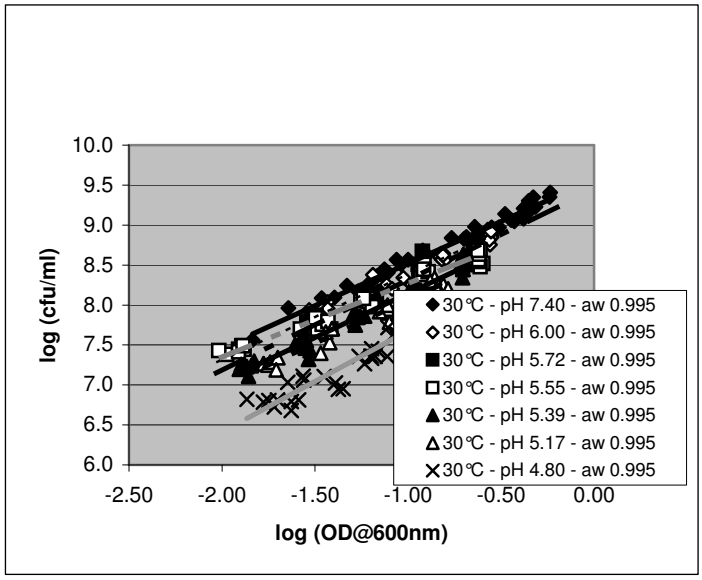


**Figure 3: Effect of low pH-values on the calibration curve between OD measured at 600 nm and viable counts for *L. monocytogenes*, keeping temperature and $a_w$ at their optimal values.**

The forced calibration curve results were used to model the calibration curve shift as a function of the environmental parameters temperature, pH and $a_w$.
Microscopic viability tests showed a viability decrease with increasing stress levels, causing a shift of the calibration curve.
In a last step, a model was developed describing the effect of environmental factors on the calibration curve, making use of a constrained polynomial approach which was also developed within this research project.

*Data collection evaluating the effect of environmental parameters on the individual lag phase of L. monocytogenes [12-13]*
In a third step, the individual cell lag time of *L. monocytogenes* was investigated as a function of temperature, pH and $a_w$. To isolate the single cells in the cup of a microtiter plate, the protocol that was previously developed was used.

In a first step the growth curves of *L. monocytogenes* were determined starting from individual cells, as described previously. A factorial experiment design was made incorporating temperature, pH and $a_w$.

For all performed experiments, the GT and $\lambda_{ind}$ were calculated using the linear extrapolation method. High adjusted correlation factors were obtained for the linear regression (0.99 – 0.98). The results were examined at three levels: firstly the mean values of the GT and $\lambda_{ind}$ were calculated for each set of environmental conditions and these results were compared to the predictions from the Pathogen Modeling Program (US Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, Wyndmoor, Pennsylvania, USA, http://ars.usda.gov/Services/docs.htm?docid=11550); secondly, histograms were made describing the data per set of environmental conditions; and thirdly, a distribution was fitted to the data using @RISK 4.5.2 Professional Edition (Palisade Corporation, Newfield, NY, USA).

All three factors had a significant influence on the distribution of $\lambda_{ind}$ of a contaminating population of *L. monocytogenes*. The influences of temperature and pH are illustrated in Figure 4.

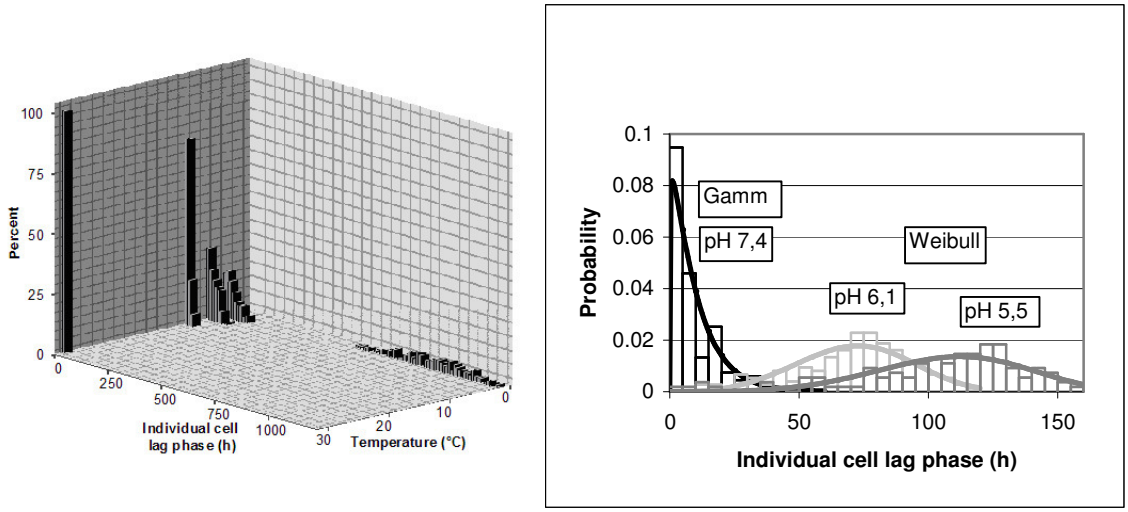


**Figure 4. The effect of temperature stress on the distribution of the $\lambda_{ind}$ of *L. monocytogenes* (left). Distributions fitted on the histograms of $\lambda_{ind}$ of *L. monocytogenes* cultivated at 7°C in BHI. pH was adjusted to 7.4 (no acid added), 6.1 and 5.5 using HCl (right)**

Two types of distributions were necessary to cover the whole range of observed datasets: when dealing with low and intermediate stress levels, the gamma distribution fitted best to the data, while for higher stress levels a Weibull distribution is proposed. When dealing with rather low stress levels – which was often the case if only one type of stress was applied - the gamma distribution should be applied; when only temperature stress was applied, using the non-acidified growth medium, the gamma distribution was applicable for temperatures down to 7°C. When, on the other hand, the effect of pH was tested at 30°C, the gamma function was valid for pH values down to

5.0, although at such high stress levels the distribution fit was rather poor. When more severe stress conditions were applied (2°C or combined pH-temperature stress) the Weibull distribution delivered more acceptable fits for all combinations. This distribution was able to handle the right density shift in the distribution, and was proposed in @RISK as one of the best distributions for all combinations.

### *Modelling individual cell lag time distributions for L. monocytogenes [14]*

An integrated modelling approach was proposed and applied to an existing dataset of individual cell lag time measurements of *L. monocytogenes*. In a first step, a logistic modelling approach was applied to predict the fraction of zero-lag cells (which start growing immediately) as a function of temperature, pH and $a_w$. For the non-zero-lag cells, the mean and variance of the lag time distribution were modelled with a hyperbolic-type model structure. This mean and variance allow identifying the parameters of a 2-parameter Weibull distribution, representing the non-zero-lag cell lag time distribution. The integration of the developed models allows predicting a global distribution of individual cell lag times for any combination of environmental conditions in the interpolation domain of the original temperature, pH and $a_w$. These distributions are further applied to refine the risk assessment concerning *L. monocytogenes* by incorporating intercellular variability.

### *Effect of pre-cultural conditions on the individual cell lag phase of L. monocytogenes [15]*

In this part of the project, the impact of the precultural temperature and pH on $\lambda_{ind}$ of *L. monocytogenes*, incubated at 7°C, is assessed.
In a first step, the pure temperature effect (37, 15, 10, 7, 4 and 2°C) was investigated on a subsequent growth at 7°C and pH 7.4. In a second step, low precultural temperatures (10, 7 and 4°C) were combined with a controlled pH at 7.4 and 5.7 with a subsequent growth at 7°C and different pH values (7.4, 6.0 and 5.5). Again, growth was monitored by OD measurements at 600 nm.

For all temperature-pH combinations, $\lambda_{ind}$ and GT were determined using a three phase linear growth model. Around 100 replications were made for each set of conditions. The results were shown as histograms, and distributions were fitted to those data. In most cases the exponential distribution gave the best fitting results.
It was observed that at low precultural temperatures, a high proportion of *L. monocytogenes* cells were able to grow with almost no lag phase. The lower the precultural temperature, the shorter the mean lag phase and the higher the proportion of cells showing no lag phase. Regarding to the pH effect, the pH transition from the precultural to a growth media was proportional to the mean values of the lag phases. There was no remarkable effect observed on the GT.

### *Practical implications of the individual cell approach on the level of challenge tests [16]*

In this part of the project the focus of this research shifted from experiments in broths to tests in real food data: the variability in growth between individual *L. monocytogenes* cells was investigated on liver pâté and ham. These results were compared to simulations based on previous data obtained in the project. Single cells were isolated by a dilution protocol which was a slight modification from the protocol previously described and inoculated on 15 g samples of liver pâté and ham, pasteurized in the packaging. 250 samples were inoculated of each product, of which 50 samples were analyzed on each analysis day. The results are illustrated for pâté in Figure 5. Results were compared to Monte Carlo simulations performed in @RISK 4.5, based on

distributions that describe the variability of GT and $\lambda_{ind}$ of *L. monocytogenes*. Based on the same simulation techniques, the variability effect was investigated for different inoculum levels (10, 100, 1000 and 10000 cells). It was demonstrated that the expected variability of the outgrowth of *L. monocytogenes* in a challenge test is very high for low inoculum levels.

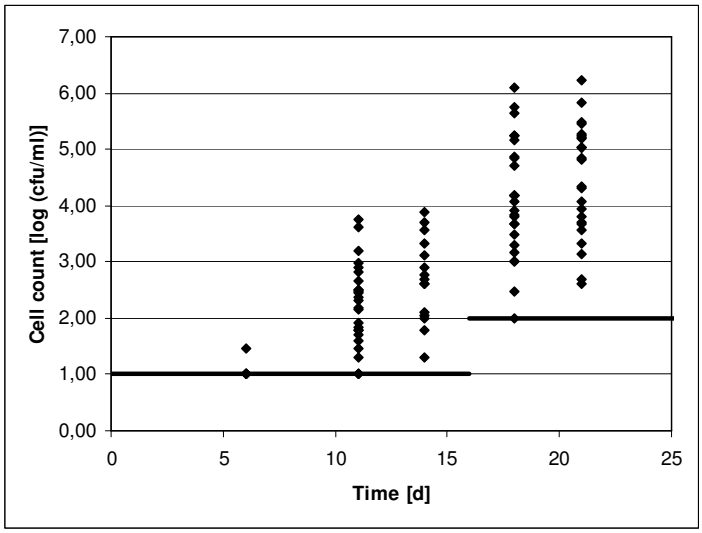


**Figure 5. Cell density of *L. monocytogenes*, grown in liver pâté at 7°C, as a function of time. Single cells were used to inoculate samples of 15 g of liver pâté. 50 samples were analyzed per day, except for day 14 (35 samples) and day 21 (48 samples)**

The variability in growth characteristics observed between different single *L. monocytogenes* cells on foods appeared to be very large. The simulations based on the previously collected OD data in broths, could be confirmed by foods inoculated with single *L. monocytogenes* cells. The large variability between different individual *L. monocytogenes* cells has serious consequences for the experimental design of a challenge test. 1000 cells have to be inoculated to a food sample in order to reduce the variability to acceptable levels and quantify the behaviour of the pathogen consistently.

### *Practical implications of the individual cell approach on the risk assessment level [17]*

In this part, the effect of the variability of $\lambda_{ind}$ was encapsulated in a risk assessment study for *L. monocytogenes* in liver pâté. A basic framework was designed to estimate the contamination level of the pâté at the time of consumption, taking into account the incidence levels and the initial contamination levels at retail. Growth was calculated on liver pâté units of 150 g, comparing an individual based approach to a classical population based approach. The two different protocols were compared using @RISK 4.5 simulations.

Two approaches were developed. Firstly, for all factors (except the individual cell lag phase variability) relevant values were determined and the factor was fixed at that level (e.g., four levels were tested for the inoculum level: 1/100 g, 1/10 g, 1/g and 10/g and one value for the time of consumption: eight days). In this case, important differences were observed between the individual based approach and the classical approach, especially at low inoculum levels, where high variability was emerging when using the individual based approach. Secondly, when all factors were considered variable,

including the inoculum level or the time of consumption, no significant differences were observed between the individual based approach and the classical approach. As such, it can be concluded that the variability of $\lambda_{ind}$ was overruled by the global variability of the exposure assessment framework. Even if the simulated conditions became harsher, by lowering the inoculum level and lowering $a_w$, no differences were created between the individual based approach and the classical approach.
This means that the variability of $\lambda_{ind}$ of *L. monocytogenes* has important consequences when studying specific growth cases, especially when the applied inoculum levels are low, but when performing more general exposure assessment studies, the variability of $\lambda_{ind}$ is too limited to have a major impact on the total exposure assessment.

## Case study 2: Quantifying inhibition and inactivation phenomena due to microbial interaction

### *Introduction & motivation*

With the increasing consumer's demand for fresh-like food products with high sensorial and nutritional quality, there is a growing interest in the food industry for alternative food processing and preservation techniques. By replacing the traditional thermal technologies such as sterilisation and pasteurisation, these (non-thermal) techniques result in minimally processed foods with a high safety *and* quality level.
In this respect, *organic acids* (e.g., lactic acid, acetic acid, citric acid) are popular preservatives. They are effective against a broad spectrum of micro-organisms, even at low concentrations. According to their origin, two groups of organic acids can be found in food products: (*i*) organic acids added to the product as an antimicrobial additive, and (*ii*) organic acids produced *in situ* in the food product. The latter group originates from the fermentative metabolism of starter cultures used in the production of fermented food products, or is part of the control mechanisms used by protective cultures to prevent outgrowth of pathogenic or spoilage organisms in minimally processed food products. The antimicrobial activity of the organic acid comprises (*i*) the lowering of the extracellular *pH* to unfavourable values, and (*ii*) a metabolic inhibition through the undissociated form of the acid. These effects can result in microbial growth retardation, early induction of the stationary phase (i.e., *inhibition*) or decrease of the microbial cell concentration (i.e., *inactivation*).

### *Antagonistic inhibition phenomena through a single metabolic product: experimental set-up and results [18-20]*

To allow for an unambiguous qualitative and quantitative analysis, a well-defined experimental system was designed as simple as possible. This system involves a two species population, in which *1* antagonist, a lactic acid bacterium, interferes through *1* antimicrobial metabolite, lactic acid, with *1* target, a foodborne pathogen. Two examples of this *1:1:1* system are considered:

- *Lactococcus lactis* and *Listeria innocua* (Co-culture #1), and
- *Lactobacillus sakei* and *Yersinia enterocolitica* (Co-culture #2).

The selection of the antagonistic and pathogenic species, the metabolite and the further experimental implementation is guided by the following considerations.

*Antagonist.* The casting of a lactic acid bacterium as antagonist is self-evident, on the basis of its safety and antimicrobial potential. To preserve the single mechanism aspect, the lactic acid bacterium must be homofermentative. In addition, it may not produce bacteriocins or other metabolites that may be toxic towards the pathogen.

*Pathogen*. For the same reason as the antagonist, the pathogen must operate a homolactic metabolism. The non pathogenic *L. innocua* is chosen as a model for the foodborne pathogen *L. monocytogenes*.

*Metabolite*. Since the antagonist is a homofermentative lactic acid bacterium, the single antimicrobial metabolite is automatically lactic acid. Lactic acid is produced by all lactic acid bacteria. Next, it is the only compound that appears as a single metabolite: production of other metabolites is always accompanied by lactic acid formation.

*Medium*. For reasons of convenience and reproducibility, the use of a commercially available undefined rich growth medium is evident. In view of the desired homofermentative metabolism, the use of glucose as a carbon source is desirable. Further, to preclude competition for available nutrients, a possible second interaction mechanism, these nutrients must be present in excess at all times during mono- and co-culture incubation. Considerable effort has been devoted to the establishment of a medium meeting these requirements.

*Environmental conditions*. In order to maintain the homofermentative metabolism of *L. innocua*, experiments are performed in an anaerobic atmosphere. Other environmental factors are not critical with respect to the *1:1:1* system and are indicated further in the text.

*Experimental plan*. Growth curves of the L. lactis/L. innocua case study were collected in 1L erlenmeyer flasks, filled with 550 mL of medium, which were closed with screw caps containing a septum. The medium used was a modified BHI broth, containing 37 g/L BHI, 18 g/L glucose, 4 g/L yeast extract, 1mL/L Tween 80, 0.2 g/L $MgSO_4 \cdot 7H_2O$ and 0.04 g/L $MnSO_4 \cdot H_2O$. Before inoculation, the medium was flushed with $N_2$ to obtain anaerobic conditions and pH correction to a value of 6.2 was performed with HCl 4N. At regular time intervals, samples were taken with a sterile syringe and needle through the septum. In these samples, growth was quantified through determination of cfu/mL by selective plate counting. After filtration of the sample to remove the cells, the total lactic acid concentration (gas chromatography), the pH (pH sensor) and glucose concentration (Granutest, Merck) are monitored.

All experiments are performed in duplicate. An assessment of the influence of (*i*) the inoculum concentrations of antagonist and pathogen, and (*ii*) the temperature on the interaction effects is aimed at. The selected levels of the influencing factors can be motivated as follows.

*Inoculum concentration*. For the monoculture experiments, a fixed inoculum level of $10^3$ cfu/mL is selected. It is widely accepted that for single species growth, the maximum specific growth rate and the maximum cell concentration, which are important growth parameters in this research (see further), are not (or only negligibly) influenced by the inoculum size (if not too low or too close to the maximum cell concentration), see, e.g., [21]. An investigation of different initial cell concentrations is thus not necessary. However, many research reports mention the influence of the antagonist's inoculum on the interaction effect experienced by the pathogen, see, e.g., [22]. Therefore, we have opted to test a number of antagonist/pathogen inoculum ratios ($10^3/0$, $0/10^3$, $10^3/10^3$, $10^4/10^3$, $10^5/10^3$, $10^6/10^3$, $10^7/10^3$). The selected ratio levels differ from each other with respect to the cell concentration of the antagonist.

*Temperature*. Each set of mono- and co-culture experiments is performed at different temperatures. The tested temperature levels include values typical for fermentation processes on the one hand (37, 35 and 22°C), and for cool storage -with a possible temperature abuse- on the other hand (12, 7 and 4°C). Co-Culture #1 was investigated at 12 and 35°C, while Co-Culture #2 was tested at 4, 7, 12, 22 and 37°C.

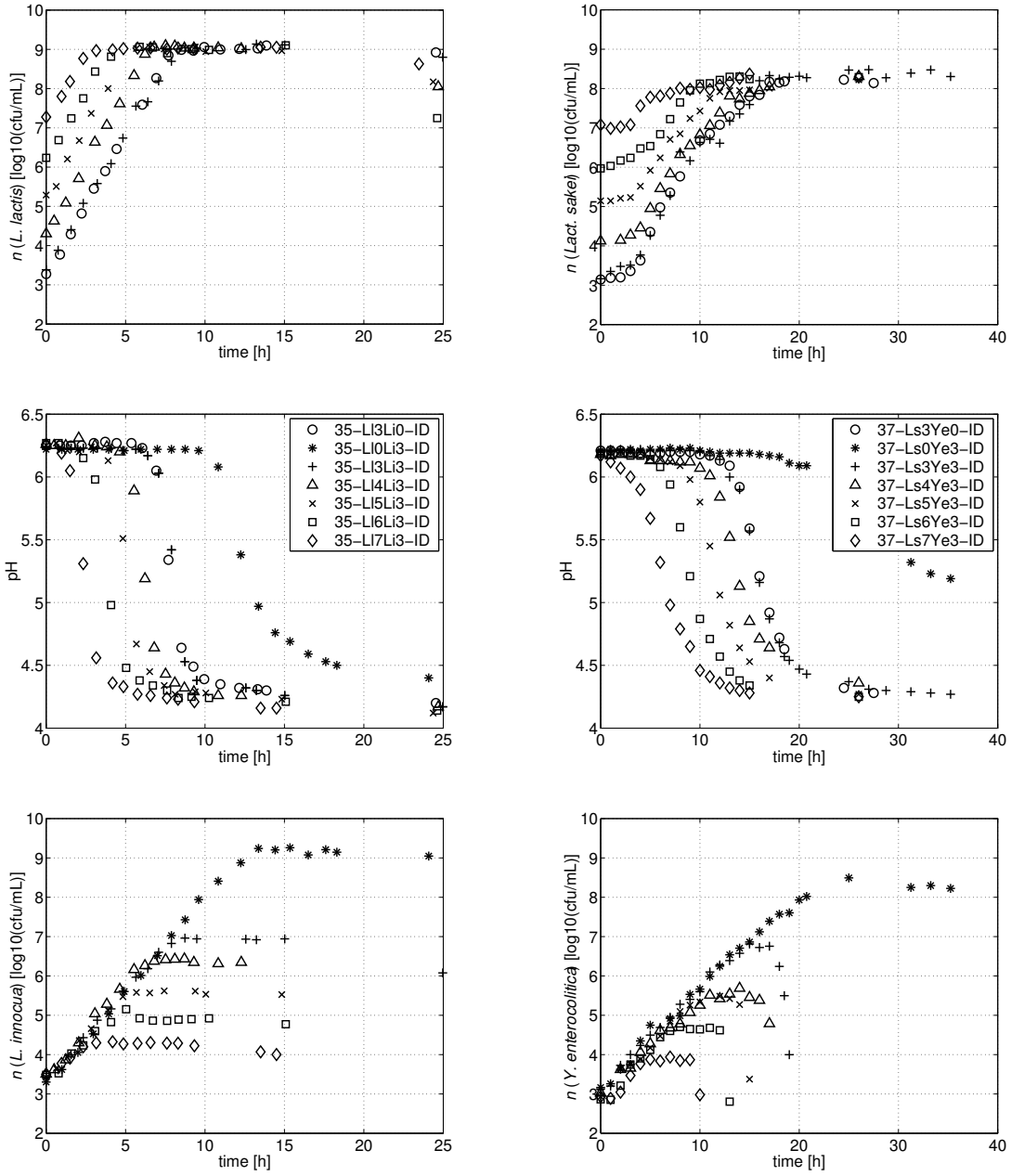


**Figure 6. Cell concentration and pH versus time for *L. lactis/L. innocua* at 35ºC (left) and *Lact. sakei/Y. enterocolitica* at 37ºC (right). Each experiment is referred to with a code, e.g., 37-L4Y3-ID indicates the experiment at 37ºC with an inoculum of $10^4$ cfu/mL of *Lact. sakei* and $10^3$ cfu/mL of *Y. enterocolitica***

Figure 6 represents the cell concentration and the pH as a function of time for Co-culture #1 at 35℃ (left panel) and for Co-culture #2 at 37℃ (right panel). For the monoculture experiment, a clear exponential growth phase and a stationary phase can be observed, whereas a lag phase is barely present (except for *Lact. sakei*). Significant acid production (not shown) (and corresponding pH reduction) is only apparent from the late exponential phase on. In the co-culture experiments, lactic acid is formed in a larger amount as compared to the monoculture experiments because of the additional production by the lactic acid bacterium. For both case studies, two distinct antagonistic

effects emerge, namely, an early initiation of the stationary phase and a decline phase, where the cell concentration is reduced to beneath the detection level.
For Co-culture #1, the growth of *L. lactis* (Figure 6, left panel) always proceeds at the same rate, evolving to the same stationary level, irrespective of the co-incubation with *L. innocua*. On the other hand, in the different experiments, the inhibition of *L. innocua* occurs at a gradually earlier time instant for increasing antagonist inocula, but always synchronous with the abrupt increase in lactic acid (not shown in the figure) and decrease in pH. In addition, a complete inactivation (i.e., to below the detection limit of $10^{2.8}$ cfu/mL) is obtained at the end of some experiments. For the *Lact. sakei/Y. enterocolitica* cocultures in Co-culture #2 (Figure 6, right panel), the same features as for the *L. lactis/L. innocua* curves can be recognised: the growth characteristics (i.e., lag phase duration, maximum specific growth rate and maximum cell concentration) of *Lact. sakei* remain unchanged, while the *Y. enterocolitica* growth curves are distorted by inhibition and inactivation effects. As for the previous case study, the stationary phase of *Y. enterocolitica* starts earlier when the initial cell concentration of *Lact. sakei* increases. However, in contrast to *L. innocua* in Co-culture #1, *Y. enterocolitica* cannot maintain the stationary cell level for a long period. In all experiments, a pronounced inactivation takes place quite rapidly after inhibition. Furthermore, from the curves, it is clear that the inactivaton rate is significantly larger than the preceding growth rate.
It appears that two threshold concentrations of lactic acid exist, of which the first one is bacteriostatic, and the second one -only attained in the co-culture- is bactericidal. It should be noticed here that the data of glucose concentration (not shown) reveal that there is never substrate limitation. By consequence, all intra- and interspecific interaction effects, in casu induction of the stationary phase and/or the decline phase, can only be ascribed to the increasing lactic acid concentration, which is in agreement with the particular intoxication mechanism, postulated above.

### *Antagonistic inhibition phenomena through a single metabolic product: model construction [18-20]*

A *first approach* consists of exploiting predictive modelling knowledge for pure cultures in order to quantify interaction phenomena in mixed cultures, see, e.g., [23]. A classical single species model, namely, the model of Baranyi and Roberts [24] is used to fit the experimental data of the pathogenic organism both in pure and mixed culture. Discrepancies in the estimated values for the growth parameters (lag phase, maximum specific growth rate and maximum cell concentration) are quantified by means of statistical techniques and can be regarded as a measure of the degree of interaction.
Application of this method to the experimental data reveals, as expected, a pronounced reduction of the parameter denoting the maximum cell concentration in co-culture as compared to its value in monoculture (results not shown).
Positive aspects of this approach are its simplicity and descriptive quality. However, interaction effects are only *reflected* in the numerical values of the parameters and not (mechanistically) *explained*.

Therefore, a *second approach* is proposed, in which interaction effects are embodied in the model's structure.

As a first step, a reaction scheme for the *1:1:1* type interaction is outlined in Figure 7, including available mechanistic knowledge. In this scheme, the full line arrows indicate the different subprocesses, i.e., the growth and lactic acid production by the antagonist and the pathogen, and the equilibrium dissociation reaction of lactic acid in the applied medium. The dashed line arrows express the negative influence of the undissociated form of lactic acid [*LaH*] [M] and the protons [$H^+$] [M] on the growth and production processes. From literature, it is known that the toxic activity of lactic acid is mediated through these components in particular, e.g., [25].

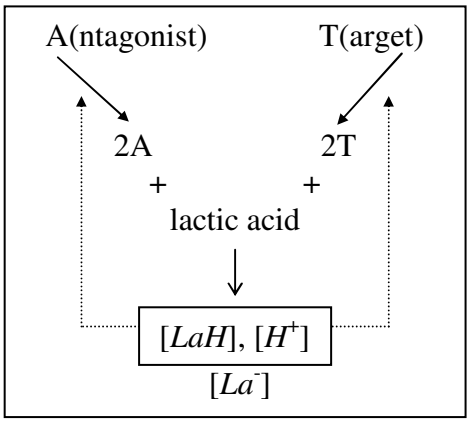


**Figure 7. Reaction scheme of the experimental system**

As a global *modelling framework* in which the reaction scheme can be enclosed, we propose the following set of differential (*balance models*) and algebraic equations (with *i*=*A*,*T*):

$$\frac{dN_i}{dt} = \mu_i \cdot N_i = \mu_{\max,i} \cdot \mu_{Q,i}(Q) \cdot \mu_{LaH,H^+,i}\left([LaH],[H^+]\right) \cdot N_i \quad (2.1)$$

$$\frac{dLaH_{tot}}{dt} = \sum \pi_i \cdot N_i = \sum \pi_{\max,i} \cdot \pi_{LaH,H^+,i}\left([LaH],[H^+]\right) \cdot N_i \quad (2.2)$$

$$[H^+] = f(LaH_{tot}, \text{buffer}) \quad (2.3)$$

$$[LaH] = g(LaH_{tot}, [H^+]) \quad (2.4)$$

with *t* [h] the time, $N_i$ [cfu/mL] the cell concentration, $\mu_{max,i}$ [1/h] the maximum specific growth rate, $\pi_{max,i}$ [mmol/(cfu · h)] the maximum specific production rate and $LaH_{tot}$ [M] the total lactic acid concentration (i.e., [*LaH*] + [$La^-$]). In this set, the *differential equations* quantify the growth of and the lactic acid production by the organisms, comprising the *biochemical* subprocesses of the experimental system. Since the specific growth and production rates are dependent on [*LaH*] and [$H^+$], interaction effects will be described as a consequence of an increasing concentration of [*LaH*] and [$H^+$] in the environment. In contrast to the differential equations, the *algebraic equations* account for the purely *chemical subprocess* of the experimental system, i.e., the dissociation of lactic acid in the aqueous medium.

In a first modelling phase, the chemical subprocess of lactic acid dissociation is taken into account [equations (2.3) and (2.4)]. To start, two mechanistic models out of literature [26-27] are analysed and compared. Although these models -which are based on classical chemical equilibria, mass and charge balances- are not directly applicable to the experimental data, they have played an inspiring role in the establishment of an alternative method. This novel method, which builds upon the results reported in [18], consists of two reversible algebraic equations, relating [*LaH*] to $LaH_{tot}$, and pH to [*LaH*] respectively:

$$[LaH] = \alpha_1 \cdot LaH_{tot} - \frac{\alpha_1 \cdot \beta_1}{2 \cdot (\beta_1 - \gamma_1)} \cdot \left[ (LaH_{tot} + \beta_1) - \sqrt{(LaH_{tot} + \beta_1)^2 - 4 \cdot (\beta_1 - \gamma_1) \cdot LaH_{tot}} \right] \quad (2.5)$$

$$pH = \frac{1}{2 \cdot \alpha_2 \cdot \gamma_2} \cdot \left[ (\beta_2 - 2 \cdot \gamma_2) \cdot [LaH] - \sqrt{\beta_2^2 \cdot [LaH]^2 + 4 \cdot \alpha_2 \cdot \beta_2^2 \cdot \gamma_2 \cdot [LaH]} \right] + pH_0 \quad (2.6)$$

Starting from the increasing $LaH_{tot}$-values, the equations provide an accurate description of the acidifying profiles measured in the media of the two case studies.

In a second phase, the main achievements are the development of model components for the biochemical subprocesses of (*i*) growth and (*ii*) lactic acid production of antagonist and target.
The specific *growth rate* in equation (2.1) incorporates an inhibition function dependent of [*LaH*] and [$H^+$] (or equivalently, their negative logarithms, *pLaH* and *pH*), describing the stationary phase in the mono- and co-culture growth curves. A suitable inhibition function is selected out of a set of candidate equations found in literature. Further, the function is adapted in such a way that it includes consecutively a decreasing phase and a zero phase when the lactic acid concentration increases:

$$\mu_{pLaH,pH,i}(pLaH, pH) \tag{2.7}$$
$$= \begin{cases} \left(1 - \frac{10^{-pLaH}}{10^{-pLaH_{\min,i}}}\right)^{\alpha} \cdot \left(1 - \frac{10^{-pH}}{10^{-pH_{\min,i}}}\right)^{\beta} & \text{if } pLaH \geq pLaH_{\min,i} \text{ and } pH \geq pH_{\min,i} \\ 0 & \text{if } pLaH < pLaH_{\min,i} \text{ or } pH < pH_{\min,i} \end{cases}$$

with $pLaH_{\min,i}$ and $pH_{\min,i}$: the values of *pLaH* and *pH*, respectively, at which growth ceases; parameters $\alpha$ and $\beta$ are free (but constrained to $\alpha > 1$, $\beta > 1$) or fixed (both at a value of $1 + 10^{-6}$).
Next, the submodel for the growth model is applied to the experimental data of the two case studies. The resulting parameter estimates and confidence intervals (not shown) indicate that not all parameters can be estimated in a reliable way. It is postulated that this is caused by the correlation between the independent variables of the specific growth rate function *pLaH* (or [*LaH*]) and *pH* (or [$H^+$]), which is inherently present in (natural) fermentation processes. The problem can be relaxed by using a reduced version of the novel model, containing four free parameters, namely the initial cell concentration $N_0$, the lag phase duration $\lambda$, the maximum specific growth rate $\mu_{max,i}$ and a growth limiting concentration of undissociated lactic acid $pLaH_{\min,i}$ (which corresponds to the negative logarithm of $[LaH]_{max,i}$, i.e., $-\log([LaH]_{max,i})$). Parameters $\alpha$ and $\beta$ are both fixed at a value of $(1 + 10^{-6})$ during the estimation procedure. For parameter $pH_{\min,i}$, two methods are suggested. In the first, $pH_{\min,i}$ is put equal to the minimum pH for growth at the ambient temperature in a rich medium acidified with a strong acid, as available in literature. For the second method, the parameter $pH_{\min,i}$ is related to $pLaH_{\min,i}$ by means of equation (2.6). In this case however, the model is only appropriate if acidification results from lactic acid production only (and not, for example, from addition of a strong acid).
The specific *production rate* in equation (2.2) comprises growth and non-growth associated (maintenance) production of lactic acid, and the negative influence of [*LaH*] and *pH* on these metabolic processes. It is demonstrated that the experimental system under study does not obey the classical linear law, which is based on a constant maintenance related production rate. Therefore, a novel expression for the maintenance is proposed, in which the experimentally observed decreasing production rate at higher lactic acid concentrations is accounted for.

$$\pi_{pLaH,pH,i}(pLaH,pH) = Y_i \cdot \mu_i(pLaH,pH) + m_{\max i} \cdot m_i(pLaH,pH)$$

$$m_i(pLaH,pH) = \begin{cases} \left(1-\frac{10^{-pLaH}}{10^{-pLaH_{\min,M,i}}}\right)^{\gamma} \cdot \left(1-\frac{10^{-pH}}{10^{-pH_{\min,M,i}}}\right)^{\delta} & \text{if } pLaH \geq pLaH_{\min,M,i} \text{ and } pH \geq pH_{\min,M,i} \\ 0 & \text{if } pLaH < pLaH_{\min,M,i} \text{ or } pH < pH_{\min,M,i} \end{cases}$$

The descriptive power of this model is illustrated by means of the experimental data of the case studies. Key parameters in the submodel for lactic acid production are the yield of lactic acid over cfu $Y_i$ [mmol/cfu], the maximum maintenance $m_{\max,i}$ [mmol/(cfu·h)], and a metabolism limiting concentration of undissociated lactic acid $pLaH_{\min,M,i}$ [corresponding to $-\log([LaH]_{\max,M,i})$]. Similar to α and β in the model for growth and lactic acid induced inhibition, parameters γ and δ are both kept constant at a value of $(1 + 10^{-6})$ during the parameter optimization procedure. And again, the parameter $pH_{\min,M,i}$, corresponding to $-\log([H^+]_{\max,M,i})$, is related to $pLaH_{\min,M,i}$ by means of equation (2.6).

In summary, combination of the submodels for growth, lactic acid production and dissociation enables to quantify the lactic acid induced inhibition effect on growth *and* metabolism. By means of the unified model, a precise description of the experimental data of the cell concentration, the lactic acid concentration and the pH is obtained for both case studies (see, for example, Figure 8). Next, it is demonstrated that the complete model, in combination with the estimated parameter values yields an accurate prediction of the experimental data of the validation set.

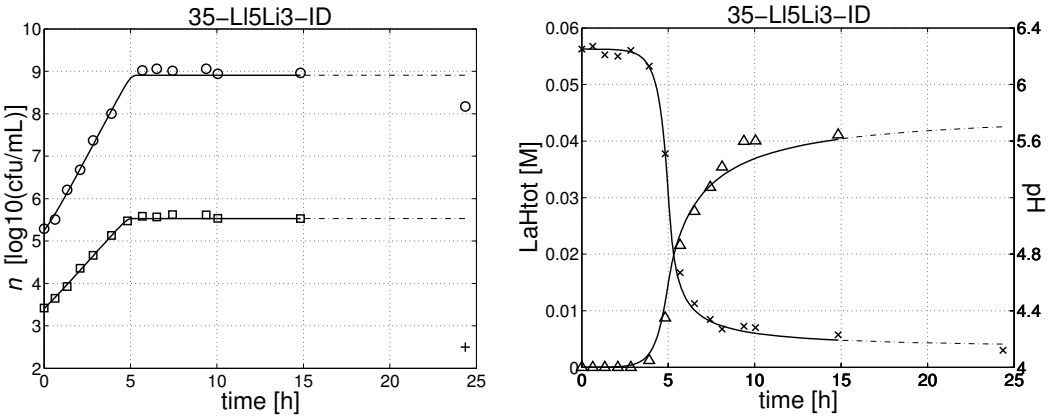


**Figure 8. Application of the unified model to Co-culture #1, experiment with inoculum ratio *L. lactis*/*L. innocua* = $10^5/10^3$ cfu/mL**

Next to this global modelling framework for the two case studies at hand, an extended literature review was made concerning different strategies for modelling *chemical inhibition and inactivation* of micro-organisms [28].

### *Antagonistic inactivation phenomena through a single metabolic product via correlated pH and lactic acid profiles [29]*

The experimental data for Co-culture #2 at 12°C were explored. Similarly to the experimental data of the evolution of *Y. enterocolitica* for the co-cultures performed at 37°C presented in Figure 6, the evolution of *Y. enterocolitica* for the co-cultures performed at 12°C also show growth, early induction of the stationary phase (i.e.,

inhibition) and finally inactivation of the target organism. Data of the experiments 12-Ls0Ye3-ID and 12-Ls7Ye3-ID were not suitable as inactivation of *Y. enterocolitica* in monoculture did not occur within the observed time range. On the contrary, it proceeded too fast in the co-culture experiment 12-Ls7Ye3-ID to obtain some data points in the inactivation phase.

The differential equation for growth and inhibition of the pathogen [equations (2.1) and (2.7)] was extended to describe the subsequent, experimentally observed, inactivation phase of *Y. enterocolitica* in Co-culture #2 as function of the influencing factors pH and undissociated lactic acid. An important structural model requirement is the reduction to growth and inhibition of the pathogen when no inactivation takes place.

Modular extension of the existing model [equations (2.1) and (2.7)] to inactivation can be done in two ways: (*i)* the population N can be divided into a viable and death fraction, or (*ii*) the reaction kinetics can be extended with additional terms (i.e., additive) and/or factors (i.e., multiplicative) in such a manner that it describes the three subsequent growth phases (i.e., growth, inhibition and inactivation). As measurements of the total cell concentration (i.e., viable and death cells) were lacking in the current study, method (*ii*) was preferred. The reaction kinetics in the newly developed model comprises two parts: one for growth and inhibition of the target organism, and one for the subsequent inactivation process.
The selection of a suitable model structure for inactivation is driven by data of the variation of the *specific evolution rate* for *Y. Enterocolitica* $\mu_{Ye}$ with pH and the concentration undissociated lactic acid [LaH]. The data for $\mu_{Ye}$ were constructed by linear regression of every three subsequent data points of the cell concentration. In contrast to the specific growth rate in equation (2.1), $\mu_{Ye}$ comprises both growth *and* inactivation. The evolution of $\mu_{Ye}$ with pH and undissociated lactic acid is presented in Figure 15. As a low pH corresponds to a high [LaH], the evolution of $\mu_{Ye}$ with pH and [LaH] show opposite behaviour. This graphical profile is useful for the identification of critical points in the evolution of $\mu_{Ye}$.
Initially, the pH of the medium equals $pH_0$ ($[LaH]_0 = 0$). As pH decreases to $pH_{inhib}$ (or [LaH] increases to $[LaH]_{inhib}$), the specific evolution rate $\mu_{Ye}$ decreases from a positive value towards zero. Then $\mu_{Ye}$ remains zero over a certain range. From $pH_{inact}$ (or $[LaH]_{inact}$) on, $\mu_{Ye}$ decreases to negative values. These parameters are preferably incorporated into the function describing $\mu_{Ye}$ as they are easily interpretable.

For simplicity, a suitable model structure is initially developed for only one of the two toxic components. Because of the diffuse evolution of $\mu_{Ye}$ with [LaH] at high concentrations (Figure 9, right), the factor pH is preferred. The factor [LaH] does not appear in the equations, but is implicitly taken into account as undissociated lactic acid is directly related to pH by the chemical equilibrium and mass and charge balances for a specific medium having a fixed buffer capacity [equations (2.3) and (2.4), or in full form, equations (2.5) and (2.6)]. Afterwards, the second toxic component [LaH] is taken into account.

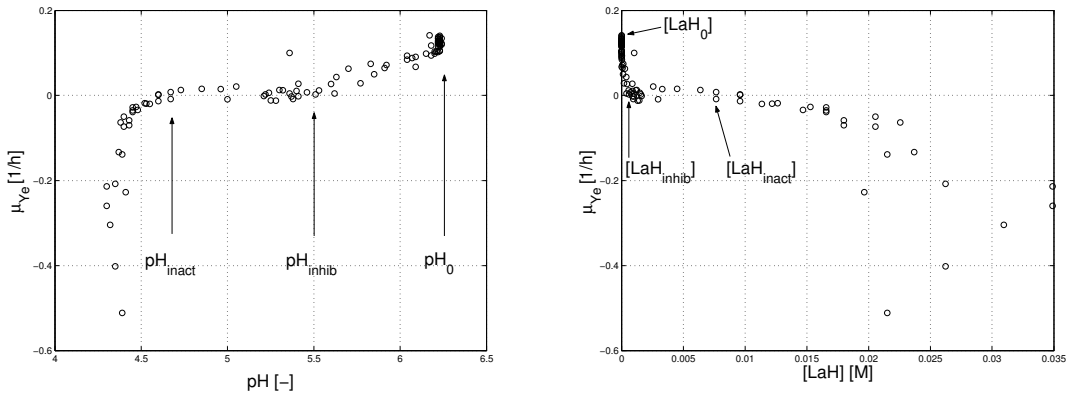


**Figure 9. Specific evolution rate $\mu_{Ye}$ of *Y. enterocolitica* as function of pH (left) and [LaH] (right) with $pH_0$ and $[LaH]_0$: the starting pH and concentration undissociated lactic acid, $pH_{inhib}$ and $[LaH]_{inhib}$: their values at which growth ceases, $pH_{inact}$ and $[LaH]_{inact}$: their values at which *inactivation starts***

Based on the thermal model structure [30] the reaction kinetics $\mu_{Ye}$ finally consists of two parts: $\mu_{growth}(pH, pLaH)$ for description of the growth and inhibition (i.e., positive values of $\mu_{Ye}$), and $\mu_{inact}(pH, pLaH)$ for description of the inactivation (i.e., $\mu_{Ye}$ at negative values). Both parts were formulated as being negatively influenced by the undissociated lactic acid concentration [*LaH*] (or its negative logarithm, *pLaH* = -log([*LaH*])) and *pH*.

$$\mu_{Ye}(pH, pLaH) = \mu_{growth}(pH, pLaH) \cdot F_{trans}(pH) + \mu_{inact}(pH, pLaH) \cdot [1 - F_{trans}(pH)]$$

An expression for $\mu_{growth}(pH, pLaH)$ describing the exponential growth phase and early induction of the stationary phase is equal to $\mu_{max,\,i} \cdot \mu_{pLaH,\,pH,\,i}(pLaH, pH)$. An expression for the latter is taken from equation (2.7).
The transition function $F_{trans}(pH)$ with values between 0 and 1 ensures the smooth transition from growth to inactivation. The function, given in the equation below, has been applied similarly in, for example, Van Impe and co-workers [30], and has also similarities with the well-known modified Gompertz equation for microbial growth [31].

$$F_{trans}(pH) = \exp\{-\exp[\alpha \cdot (pH_{trans} - pH)]\} \qquad \text{with } \alpha > 0$$

with $pH_{trans}$ [-]: the pH-value at which transition takes place, α [-]: the parameter describing the curvature of the transition. Depending on the value of α being finite or infinite, $F_{trans}(pH)$ responds to a continuously differentiable equation or a step function respectively. As a consequence, according to the shape of $F_{trans}(pH)$, the functions $\mu_{growth}(pH, pLaH)$ and $\mu_{inact}(pH, pLaH)$ have to fulfil different conditions to ensure a smooth transition. In contrast to $\mu_{growth}$ and $\mu_{inact}$ who both are dependent on *pH* and *pLaH*, $F_{trans}$ remains dependent on *pH* only since it guarantees the transition between $\mu_{growth}(pH, pLaH)$ and $\mu_{inact}(pH, pLaH)$.

Parameter optimization studies based on experimental data of the cell concentration of *Y. enterocolitica* were performed, in a first phase for *pH* only, but in a second phase the second influencing factor undissociated lactic acid was included. The lactic acid associated parameters in the model structures for the inactivation phase were related to the pH-parameters to circumvent the strong correlation between them. This finally

led to the selection of one possible structure for a good and reliable prediction of the inactivation phase ($L_1$ [-], $L_2$ [-] and $c_B$ [-]: parameters).

$$\mu_{inact}(pH, pLaH) = -\exp\left(-\frac{pH}{L_1} - \frac{pLaH}{L_2} + c_B\right)$$

Parameters $L_1$ [-] and $L_2$ [-] can be interpreted as a pH- and pLaH-value, respectively. Consequently, as already mentioned, the parameter $L_2$ was related to $L_1$ by means of equation (2.6).

Until this point, model equations were applied to each of the co-cultures separately. The resulting parameter values (not shown) for each co-culture were comparable, but not exactly equal. However, when considering undissociated lactic acid and pH as the influencing factors in the current system, there should exist *one unique parameter set valid for all the co-cultures*. Additionally, the present results do not enable the prediction of the inhibition and inactivation of the target organism in co-cultures with intermediary inoculum concentrations for the antagonist (i.e., intermediary concentrations in the range of $10^3$ – $10^6$ cfu/mL). Because of these reasons, the final set of equations was applied simultaneously to the experimental data of all co-cultures showing an inactivation phase. A global model valid for all the co-cultures was obtained. Comparison of the model simulation based on the optimal parameter values and the experimental data is presented in Figure 10.

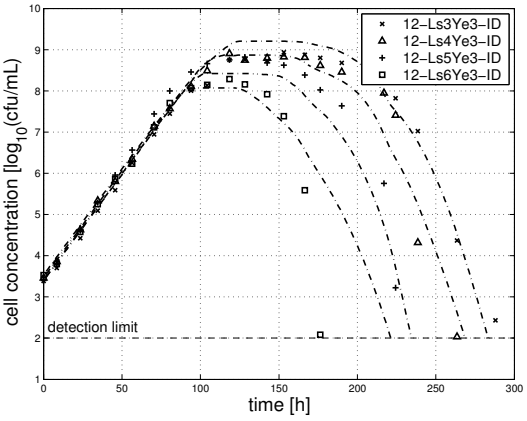


**Figure 10. Simulation of the evolution (growth, inhibition and inactivation) of *Y. enterocolitica* in co-culture with *Lact. sakei* by means of the global model with one unique parameter set valid for all the co-cultures**

The global model and its optimal parameter values gave satisfying results when used to predict the experimental data of the validation set (not shown).
Observe that it could be anticipated that other lactic acid mediated co-culture experiments could also be described using the developed model structure. The resulting overall model can also be seen as a basis for other models describing microbial interactions, as it can be assumed to be transferable to, for example, other organic acids, bacteriocins, etc. Additionally, when no microbial interaction occurs (e.g., no antagonist), the equation for growth of the antagonist and the term for lactic acid production by the antagonist can be omitted. As such, the model reduces in a natural way to growth and lactic acid production and (intraspecies) inhibition in monoculture of the target organism.

*Antagonistic inactivation phenomena through a single metabolic product via decorrelated pH and lactic acid profiles [32]*

The one-to-one interrelationship between pH and undissociated lactic acid are not to be seen as an artefact of the experiments performed, but are inevitably related to each lactic acid production process. To circumvent these difficulties, more knowledge of the *individual* (*separate*) effects of pH and undissociated lactic acid is desirable. Indeed, by studying the individual effects of pH and undissociated lactic acid the model structure could be refined. This information can be obtained by studying the effects of pH and undissociated lactic acid not only at conditions determined by the one-to-one relationship but also at conditions outside this relationship, see e.g., [33].

<u>*Experimental plan.*</u> The inactivation of *L. innocua* of Co-culture #1 was investigated at controlled (static) conditions of pH and [LaH]. Experiments were performed in 1 L erlenmeyer flasks filled with 550 mL of a rich, modified BHI medium. This medium was flushed with $N_2$ to obtain anaerobic conditions. Before inoculation, a combination of initial pH (i.e., $pH_0$) and initial concentration of undissociated lactic acid (i.e., $[LaH_0]$)

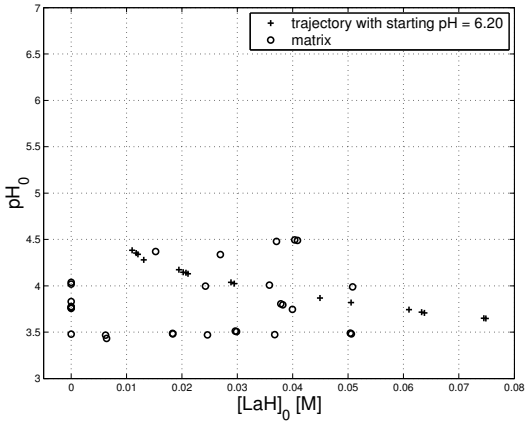


**Figure 11. Overview of the ($pH_0$,$[LaH]_0$)-combinations tested**

was set by addition of the appropriate volumes of strong acid (HCl) or base (KOH), and lactic acid. No extra buffers were added. 30 combinations were tested, which are graphically presented in Figure 11.

√ 11 ($pH_0$, $[LaH_0]$)-combinations situated *on* the traditional (*pH*, [*LaH*])-trajectory: to simulate the effect of co-cultures the evolution of *L. innocua* at artificially created initial total lactic acid concentrations (i.e., both dissociated and undissociated forms) ranging from 0.04 to 0.12 M was followed. Before the addition of lactic acid, the pH was set to 6.20 for these experiments. As such, the ($pH_0$, $[LaH_0]$)-conditions obtained are determined by the same relationship as for the co-cultures in Co-culture #1.

√ 19 ($pH_0$, $[LaH_0]$)-combinations situated outside this trajectory: to be able to separate the effects of *pH* and [*LaH*] combinations forming a rectangular shape in the (*pH*, [*LaH*])-plane were tested. Values of $pH_0$ are ranging from 3.43 to 4.50, while $[LaH]_0$ ranges from 0 to 0.05 M. Differences in inactivation curves with equal $pH_0$ can be ascribed to the variation in $[LaH]_0$, and vice versa.

*L. innocua* was inoculated at a concentration of $10^8$ cfu/mL and all experiments were performed at 12°C.

<u>*Experimental results.*</u> In the evolution of the cell concentration as function of time, two phases could be distinguished: (*i*) a period with a constant cell concentration, i.e., a *shoulder period*, followed by (*ii*) a period in which the cell concentration decreased to values below the detection limit, i.e., a *descent phase*. The latter phase consisted of one or two loglinear part(s) with respective slope(s), or took a concave or convex shape. During the experiments, the glucose concentration remained constant, indicating that nutrient depletion cannot be the cause of the inactivation. As anaerobic conditions prevent *L. innocua* from producing acetic acid next to lactic acid (if any production would occur at all, given the inactivation of the microbe) [34], the prevailing lactic acid and pH conditions were the only explaining factors for the observed inactivation process.

For the experimental conditions situated on the trajectory, it can be concluded that when increasing the initial total lactic acid concentration $LaH_{tot,0}$ (i.e., increasing $[LaH]_0$ and decreasing $pH_0$), the length of the shoulder period was reduced, while the inactivation rate increased. This is illustrated in Figure 12.

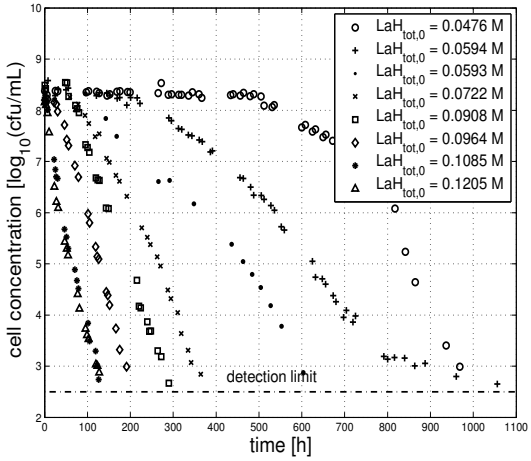


**Figure 12. Inactivation at ($pH_0$,$[LaH]_0$)-combinations on the trajectory**

For the other ($pH_0$, $[LaH]_0$)-conditions, it appeared that $[LaH]_0$ and $pH_0$ have an influence on both the length of the shoulder period and the inactivation rate when considering results at a constant $pH_0$ and $[LaH]_0$ respectively (Figure 13).

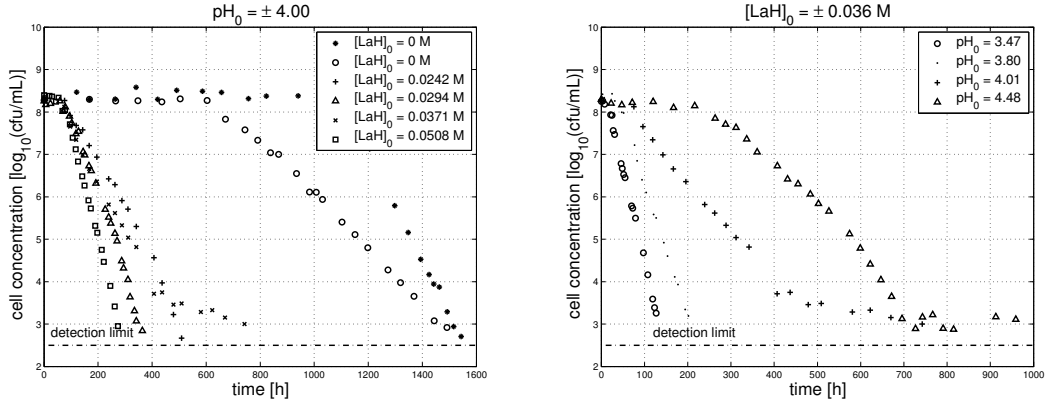


**Figure 13. Inactivation of *L. innocua* at ($pH_0$, $[LaH]_0$)-combinations in the rectangular shape for a constant $pH_0$ = 4.00 (left) and $[LaH]_0$ = 0.036 M (right)**

After comparing the collected inactivation curves, a certain variation became visible: (*i*) inactivation curves for identical conditions (for experiments performed in duplicate) did not show an identical evolution (results not shown), and (*ii*) in the vicinity of the growth/no growth interface the inactivation process seemed to be a rather contradictory process (e.g., for $pH_0$ = 4.00 and $[LaH]_0$ = 0 M in Figure 13). Attempts to locate the cause of this variation did not led to any conclusive results. However, it seems acceptable that an increased variance in the bacterial response due to less favourable conditions might serve as the main reason for the observations made.

*Model development.* In the model development steps, no further distinction is made between the two series of experimental data as the final model has to be valid for *all* ($pH_0$, $[LaH_0]$)-combinations. In addition, because of the observed variability in the inactivation process for some ($pH_0$, $[LaH_0]$)-conditions, all inactivation curves (singular or plural) were taken into account.
First, four types of primary inactivation models were calibrated on the experimental data by means of the Microsoft® Excel Tool GInaFiT [35], a tool which was developed within the frame of this research project. For most of the experimental data, either the loglinear model with shoulder or a Weibull-type model gave the best result and a preference for one or the other model could not be derived on the goodness-of-fit criterium only. Finally, the Weibull-model was preferred as primary inactivation model as it was able to describe the various inactivation curve shapes.
Next, a secondary model was developed to describe the evolution of the parameters of the primary Weibull-type model as function of $pH_0$ and $[LaH_0]$. Suitable model structures and parameter values are identified. Based on combination of the calibrated primary and secondary models, one can predict which conditions of $pH_0$ and $[LaH]_0$ lead to a predetermined inactivation within a predetermined time range.

## CONCLUSIONS

In this chapter, two case-studies related with the microbial safety of food products are introduced: a first case-study related with realistic, low numbers of contaminating *L. monocytogenes* cells under stressful conditions and a second case-study related with lactic-acid mediated microbial interactions. For both case-studies, an experimental setup was carefully designed, a large set of informative data was gathered and dedicated predictive microbiology models were developed. The models constitute valuable tools for the quantitative assessment of microbial food safety problems related with these case-studies. Furthermore, the generic character of the developed modelling tools enables transferability to different (but similar) research questions within food microbiology.

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# Molecular epidemiology of *Salmonella* and *Campylobacter* contamination during poultry production

**G. Rasschaert[1], L. Herman[1], W. Messens[1], L. De Zutter[2] and M. Heyndrickx[1]**

*[1] Ministry of the Flemish Community, Institute for Agricultural and Fisheries Research (ILVO), Unit Technology & Food, Brusselsesteenweg 370, B-9090 Melle, Belgium.*
*[2] Ghent University, Faculty of Veterinary Medicine, Department of Veterinary Public Health and Food Safety, Salisburylaan 133, B-9820 Merelbeke, Belgium.*
*e-mail: geertrui.rasschaert@ilvo.vlaanderen.be*

**Keywords**
*Salmonella*; *Campylobacter*; poultry; transmission; slaughter

## SUMMARY

*Salmonella* and *Campylobacter* are two important causes of gastroenteritis in the industrialized world. Handling or consumption of contaminated poultry meat is considered an important source for the transmission of both pathogens to humans. Chickens can become colonized via several routes and during processing of the chickens the bacteria may be spread out on the carcasses. This paper reviews the sources, the mechanism and epidemiology of poultry flock colonization. In addition, it describes the critical steps in the slaughter process during which gastrointestinal leakage or cross-contamination may occur.

## INTRODUCTION

*Salmonella* and *Campylobacter* are the two major bacterial causes of gastrointestinal diseases in the industrialized world. Both infections are characterized by symptoms such as diarrhea, abdominal pain, fever, headache, nausea, vomiting. Non-typhoidal salmonellosis and campylobacteriosis are usually self-limiting with recovery after a few days to a week. In rare cases, *Salmonella* infections will be followed by bacteremia or by reactive arthritis (ReA). The latter is an immune-mediated inflammation of the joints with symptoms such as pain, stiffness, redness or swelling in the joints of the limbs. *Campylobacter* infections can also lead to ReA but can in addition also cause the more severe Guillain-Barré syndrome (GBS). GBS is an autoimmune disease with the immune system mistakenly attacking myelin or axons of the peripheral nervous system associated with weakness of the limbs, the respiratory muscles and areflexia. Mortality is low and usually confined to elderly patients or patients suffering already from another underlying disease [1-3].
In Belgium, 35 *Salmonella* cases and 54 *Campylobacter* cases per 100 000 habitants were reported in 2006 (Figure 1). The number of *Salmonella* cases increased until 1999 and remarkably decreased since 2000 (with the exception of 2003). This trend has also been observed in other developed countries and is attributable to the occurrence of the serotype Enteritidis. Since the mid 1980s, public health laboratories in Europe and the US reported a dramatic increase in the number of human *Salmonella* Enteritidis cases [4]. The decrease observed recently is probably related to the control programs such as vaccination of laying and breeder hens or the eradication of positive breeder hens [5]. The peak in 2003, also observed in The Netherlands, can be

explained by the avian flu in 2003 during which probably more *Salmonella* contaminated eggs were imported from abroad [6].
For *Campylobacter*, a steady increase in the number of reported cases has been observed in most countries. The increase in the 1980s can be explained by various factors such as increased physician awareness, increased culturing by laboratories, improved detecting methods, whereas the increase observed since the 1990s more probably reflects a true increase in infections [7]. In Belgium, *C. jejuni* subsp. *jejuni* (hereafter called *C. jejuni*) and *C. coli* account for 80% and 12% of all human *Campylobacter* infections, respectively [8].

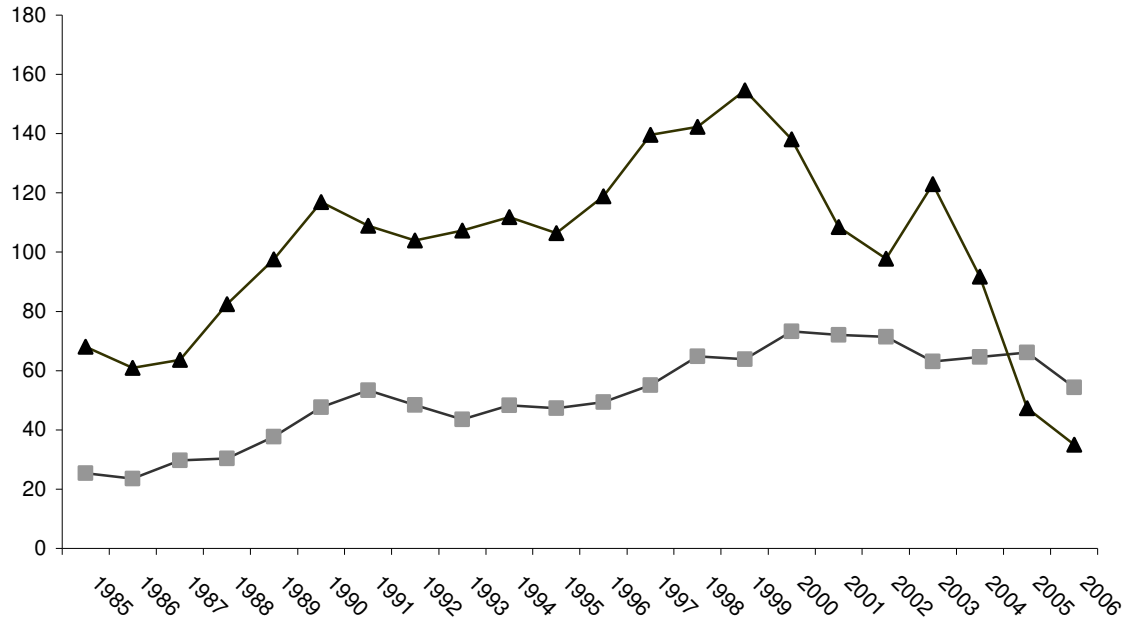


**Figure 1. Reported *Salmonella* (▲) and *Campylobacter* (■) cases in Belgium from 1985 to 2006 per 100 000 habitants [9]**

Due to differences in health care, good laboratory support, monitoring and reporting procedures, it is difficult to compare *Salmonella* and *Campylobacter* incidence rates between different countries. In the EFSA report concerning zoonoses in the EU in 2005, the mean number of reported *Salmonella* cases was 38 per 100 000 persons, ranging from 4 to 322 amongst the different member states. For *Campylobacter*, the mean EU incidence was 52 per 100 000 habitants, but also varied widely between countries ranging from 0 to 303 [10]. Furthermore, the number of cases is probably underreported for both pathogens. For example, the World Health Organization estimates that approximately 1% of the European population is infected with *Campylobacter* each year [11].
Both pathogens affect all age groups, but children younger than 5 years are the most affected age group, probably due to oversampling [7, 9, 12]. The *Campylobacter* incidence is 1.2 to 1.5 times higher in males than in females, which is not noticed for *Salmonella*, and is explained partially by a sex-specific behaviour [7]. A last trend for both zoonoses is a seasonal distribution with a well-defined summer peak. For *Campylobacter*, this peak may vary from country to country and from year to year but remains unexplained [13]. Several hypotheses were suggested: a variation in human behaviour during the summer months such as barbecue, the increased occurrence of

*Campylobacter* in known reservoirs such as poultry flocks and the increased prevalence of flies, which may act as potential source of infection for both humans and reservoirs [7, 14, 15].

## SOURCES OF HUMAN INFECTION

According to Mead *et al.* [16], more than 95% of all *Salmonella* infections are foodborne. In The Netherlands, eggs, poultry meat, pork and beef are believed to be responsible for 39%, 21%, 25% and about 10% of human salmonellosis cases, respectively [17]. Eggs are undoubtedly the most important source of salmonellosis, especially in outbreaks where the serotype Enteritidis is involved [18-21]. Poultry meat also contributes to the transmission of *Salmonella* to humans. For example, there was recently a *Salmonella* outbreak in Spain with more than 2000 cases due to the consumption of pre-cooked chicken of a particular brand [22].

The majority of *Campylobacter* infections are not related to outbreaks but occur as sporadic infections. *Campylobacter* does not multiply on food, which explains the rarity of large outbreaks related to food. Since it is difficult to determine the source of an individual case, several case-control studies have been performed to identify the most likely sources of *Campylobacter* infections [23-28]. Though different techniques applied and the array of hypotheses tested, these studies all indicate the same sources: handling or consumption of poultry meat, barbecue, drinking contaminated water, drinking bird-pecked milk, contact with pets and other animals and overseas travel (travelers' diarrhea). Handling and consumption of poultry meat was the major risk factor for a variable percentage of cases ranging from 10% in Denmark to more than 70% at a US university [7, 24]. Studies other than case-control studies have also shown the association between *Campylobacter* infection and poultry meat. The dioxin crisis in June 1999 resulting in the withdrawal of poultry meat from the Belgian market, caused a decline of 40% in the number of Belgian *Campylobacter* infections [29].

Studies have been performed to determine the presence of *Salmonella* or *Campylobacter* on retail chicken. It is difficult to compare the reported prevalence between different studies, since several sampling and isolation methods were applied. In a Belgian four-year study, *Salmonella* prevalence on poultry carcasses was between 17% and 27% in the period 1993-1996 [30]. Since the last few years, a decline in the *Salmonella* prevalence on poultry meat has been noticed in several studies. Nowadays, the *Salmonella* prevalence on raw retail chicken ranges from 4% to 30% in the UK, the US and Canada [31-38]. However, the prevalence of *Salmonella* on poultry carcasses in Southern Europe is higher than in the rest of Europe. In Spain and Portugal, the reported prevalence was approximately 60% [39-40].

In general, the prevalence of *Campylobacter* contaminated retail poultry meat is much higher than for *Salmonella*. According to Ghafir *et al.* [41], the prevalence of *Campylobacter* contaminated poultry meat in Belgium between 2000-2003 ranged from 19% to 47%, depending on the type of poultry meat and sampling scheme. In the rest of Europe, the prevalence ranged from 38% to 83% [31, 33, 34-36, 38, 42-45]. In the United States and Canada, *Campylobacter* was isolated from 62% to 82% of poultry carcasses [32,37]. Species identification revealed that *C. jejuni* was the most prevalent species isolated from raw poultry meat, with levels ranging from 77% to 98% [32, 43, 44, 46].

Cross-contamination in the kitchen is an important risk factor for acquiring *Salmonella* and *Campylobacter* infections. Cogan *et al.* [47] quantified cross-contamination in a study where participants were asked to cut *Salmonella* or *Campylobacter* contaminated chicken carcasses in pieces. After handling *Salmonella* contaminated chicken, 45% of the hands and 35% of the cutting boards were contaminated with *Salmonella* with 5%

of the cutting boards at levels of more than 1000 CFU. The levels of *Campylobacter* cross-contamination were much higher; 85% of the hands and 80% of the cutting boards were contaminated with 20% of the hands and 45% of the cutting boards at levels of more than 1000 CFU. After cleaning and rinsing, no *Campylobacter* could be detected, whereas significant numbers of surfaces were still contaminated with low levels of *Salmonella*. The packaging can also be considered as a risk factor, since Harrison *et al.* [31] demonstrated that 3% of the outer packaging from raw poultry products was *Campylobacter* contaminated.

## SOURCES OF POULTRY COLONIZATION

In theory, chickens can become colonized with *Salmonella* and *Campylobacter* in two ways: vertical transmission, from the hen through the egg to the chick, and horizontal transmission, from the environment to the chick.

### Vertical transmission

There are two possible routes of *Salmonella* contamination of intact hatching eggs. Via the transovarian route, the yolk, the yolk membrane or the albumen surrounding it becomes contaminated before the eggs are covered by the shell as a result of *Salmonella* infection of the reproductive organs of the laying hen. In the trans-shell route, *Salmonella* penetrates through the egg shell after oviposition via for example fecal contamination on the shell. It is difficult to distinguish between contamination during formation of the egg and contamination after oviposition [48-49]. In some cases where other *Salmonella* strains infect the broiler flock, the vertically transmitted *Salmonella* strain may only appear near the end of the rearing period [50]. However, vertical transmission is nowadays of less importance mainly due to the vaccination of breeder flocks. As demonstrated by Heyndrickx *et al.* [51], horizontal transmission is nowadays the main determinative factor for colonization of broiler flocks.

The theory of vertical transmission for *Campylobacter* has been a controversial issue. *Campylobacter* can be present in the reproductive organs and semen which could theoretically lead to vertical transmission of *Campylobacter* from the hen to the chick [52]. Based on a correlation between hatchery and broiler flock colonization, Pearson *et al.* [53] concluded that vertical transmission was a source of *Campylobacter* broiler flock colonization. Cox *et al.* [54] came to the same conclusions by genotyping the strains isolated from breeder flocks and their progeny. In contrast, other studies in which the strains from the broiler flocks and the parent flocks were compared suggest that there is little likeliness of vertical transmission [55-56]. Moreover, if colonization takes place by vertical transmission, it would be expected that campylobacters would be detected in an affected flock early after hatching as mostly (but not always) observed with the vertical transmission of *Salmonella*. However, there is a delay of two to three weeks before the birds become colonized with *Campylobacter*. It is possible that small numbers of *Campylobacter* may be present in the hatching chick, but that the growth is constrained by maternal antibodies [57-58]. Nowadays, the general tendency is to control the horizontal route that appears to be the major risk for broiler flock colonization and then, to determine the role of vertical transmission if there are still problems [59].

### Horizontal transmission

The houses used for rearing broilers can largely be considered as closed environments. However, *Salmonella* and *Campylobacter* are present in the environment in and around broiler houses. Many studies have been undertaken to

identify the risk factors for *Salmonella* or *Campylobacter* colonization. In the following paragraphs, each potential source is reviewed.

*Feed and drinking water.* Several studies have linked contaminated feed to the occurrence of *Salmonella* in poultry [60]. Analyses of commercially manufactured feeds confirmed that both feed ingredients and dust can be sources of *Salmonella* contamination in feed mills [61-62]. Heyndrickx *et al.* [51] demonstrated that 3.5% of fresh feed samples tested positive for *Salmonella*. In contrast, it is widely accepted that feed is not a potential source of *Campylobacter* transmission to poultry. The dry conditions of feed are considered lethal to *Campylobacter* [63-64].
Although the drinking water in poultry houses of colonized poultry flocks is often contaminated with *Salmonella* or *Campylobacter*, the water contamination usually follows flock colonization rather than preceding it [51, 58, 63]. According to most studies, the water source is a low risk factor for flock colonization with *Salmonella* or *Campylobacter* [59, 64-69]. This is in contrast with a study of Pearson *et al.* [70], in which was reported that a *Campylobacter* serotype persisted for at least 18 months on a poultry farm. The source was shown to be the farm's water system; campylobacters were found from the bottom of the 30-m borehole to the biofilm of the pipework within the poultry house.
According to a recent study, waterborne protozoa have strong potential to act as protective reservoirs for *C. jejuni* in the drinking water systems of poultry houses. Experimental cocultivation of *C. jejuni* with such protozoa appears to reduce the susceptibility of the bacteria to chlorine as well as to certain disinfectants [71].
Many studies have investigated the possibility of acidification of feed or water to reduce *Campylobacter* and *Salmonella* colonization of broilers. These studies, reviewed by Doyle and Erickson [72] and Van Immerseel *et al.* [73], yield conflicting results. Furthermore, if the infection pressure is high or when the chickens are highly stressed, colonization is not always affected by this treatment. It appears that the way of administration, the type of acid and the concentration used are very important. Indeed, in a longitudinal study on a commercial broiler farm with a persistent *Salmonella* Paratyphi B var. Java infection, it was found that a first type of commercial short chain organic acid preparation in the drinking water was able to eliminate the shedding of *Salmonella* by the broilers for the first seven succeeding flocks with acid treatment, but following flocks again became positive. After a switch to a second commercial preparation in the drinking water consisting of a mixture of mid chain organic acids and a short chain organic acid, more than 10 succeeding flocks remained negative for *Salmonella*. It was demonstrated that a *Salmonella* Paratyphi B var. Java strain isolated after the first seven flock treatments with short chain organic acids had become more resistant to the first commercial short chain organic acid product than a strain isolated before this treatment (M. Heyndrickx, unpublished results).

*Broiler house cleaning and disinfection.* The carry-over from a *Salmonella* or *Campylobacter* colonized flock to a new flock in the same house seems an obvious source. *Salmonella* is frequently isolated in poultry houses after the cleaning and disinfection process [51, 74-76]. A fundamental error is over-dilution or inconsistent application of disinfectants [75]. Despite this, there are no published reports of *Campylobacter* isolation from emptied, cleaned and disinfected poultry houses. Consequently, infection is not predictable from the *Campylobacter* status of the previous flock in the house. Negative flocks can follow positive flocks, positive flocks can occur in newly constructed houses, and sequential positive flocks can be colonized by different genotypes [59, 65, 77-78]. These studies suggest that routine house cleaning and disinfection are largely adequate for *Campylobacter* decontamination.

Even more, in the case that the litter is not removed from the poultry houses and that the poultry houses are not cleaned and disinfected between flocks as in the United States, this does not cause an increase in *Campylobacter* colonized flocks [59]. Payne *et al.* [79] showed that chicks reared on litter removed from a *Campylobacter*-positive house did not become colonized over a 7-week period. However, flock positivity is linked to a too short down period between rotating flocks. It is advisable to maintain a down period of at least two weeks [80-81].

*Human traffic and activities.* The staff is the main human traffic in and out of the broiler house for the purpose of routine animal husbandry. *Salmonella* and *Campylobacter* can be carried into the house via boots, clothes and equipment [51, 63, 82]. Therefore, introducing a hygiene barrier with an anteroom and walk-over benches, using boot dips or better house dedicated footwear, washing hands with antiseptic soap, changing clothes, cleaning the equipment that is brought into the house, and minimizing visits are all measures to improve hygiene and to reduce the possibility of flock colonization [51, 59, 64-65, 68-69, 80, 83-84]. The extent of *Campylobacter* contamination in the environment of the broiler house will obviously contribute to the risk of introducing *Campylobacter* into the broiler house. Studies of Hiett *et al.* [85] and Bull *et al.* [86] have demonstrated that *Campylobacter* isolates from puddles, recovered before flock colonization, were of the same genotype as isolates subsequently isolated from the broiler flock. Therefore, clean and intact concrete aprons around the broiler house can reduce the risk of flock colonization [59].
Thinning of the flock, which is reducing bird density within the broiler house, is a common procedure in many European countries, including Belgium. This practice enables higher productivity and provides the market of birds of different weights. Thinning or partial depopulation occurs normally at the age of 35 days, depending on the size and weight of the birds. During thinning, the doors of the poultry house are opened and the catching crew and the catching equipment enter the poultry house without any hygiene measures. Ramabu *et al.* [87] found that trucks, forklifts, pallets, crates and drivers' and catchers' boots were all contaminated with *Campylobacter*. Whether thinning is a risk factor in the introduction of *Campylobacter* into the broiler house, is not clear as several studies have reported conflicting results [65, 88-89].

*Rodents, insects and wild birds.* Though conventionally reared poultry flocks are kept in closed poultry houses, some animals such as rodents and insects may have free access to the house. The significance of rodents, mice in particular, as vectors and reservoirs of *Campylobacter* and *Salmonella* has been shown by several studies [75-76, 85, 90-92]. Henzler and Opitz [90] have shown that less than 15 *Salmonella* bacteria are enough to infect a mouse and that mice droppings can contain up to 2.3 x $10^5$ CFU/dropping. Since most farms apply rodent control programs, some studies consider rodents not longer a significant risk factor for introducing *Salmonella* and *Campylobacter* in poultry houses [83].
Insects are more difficult to control. Several hundreds of flies per day pass trough the ventilation system into the broiler house [93]. Flies and beetles in and around poultry houses have been reported to carry *Campylobacter* and *Salmonella* [93-101]. Under experimental conditions, flies can become infected by *Campylobacter* colonized chickens and are able to transmit the bacteria to *Campylobacter*-free flocks [102]. Chickens consuming one *Campylobacter* or *Salmonella* contaminated beetle became infected by *Campylobacter* or *Salmonella*, respectively [95, 101]. However under non-experimental conditions, it is still not clear if insects cause *Campylobacter* colonized birds or *vice versa*. Templeton *et al.* [103] demonstrated that *Campylobacter* does not survive for extended periods (less than 72 h) in or on darkling beetles, which are

consequently not a source of carry-over of *Campylobacter* in flocks subsequently raised in the same poultry house.
Wild birds have, under good management practices, no access to the broiler house. However, contaminated droppings can be brought into the house by footwear, clothing or material. Though many wild birds are colonized with *Campylobacter* or *Salmonella* [104-105], the genotypes of the isolates from wild birds and from broilers are seldom the same [105-106]. This suggests that the importance of wild birds as a reservoir of infection is limited.

*Pet animals and livestock.* A last risk that should be considered is the presence of pet animals and livestock such as pigs, cattle and sheep around the broiler house. These animals are unlikely to enter the house, but they may excrete *Campylobacter* or *Salmonella*. This can result in the contamination of boots, clothes or equipment taken into the house. Liebana *et al.* [107] found that the cattle located in the proximity of the feed mill of a broiler house were colonized with the same *Salmonella* strain as the broilers. The same strain was also isolated from the feed mill. Identical *Campylobacter* strains were found in cattle next to the broiler house and subsequently in the broiler house [59, 77]. However, transmission of *Campylobacter* between pigs and poultry on mixed-species farms occurs infrequently according to Boes *et al.* [108]. Moreover, replacing the livestock by new broiler houses to avoid loss of income is not an option, since it has been demonstrated that an increasing number of poultry houses are associated with a higher risk of colonization with *Campylobacter* and/or *Salmonella* [72, 100, 109].

## MECHANISM AND EPIDEMIOLOGY OF POULTRY FLOCK COLONIZATION

The pathogenicity of *Salmonella* depends on the serotype, the strain, and the susceptibility and age of the birds [110]. *Salmonella* Pullorum and *Salmonella* Gallinarum are responsible for the Pullorum disease and fowl typhoid disease, respectively. Pullorum disease causes weakness, white diarrhea and a high mortality rate (50% to 100%) among embryos and young chicks. Fowl typhoid is a disease of mature fowl that results in either acute enteritis with greenish diarrhea or a chronic disease of the genital tract that reduces egg production. Certain strains of other serotypes are also able to cause disease. After natural infection with *Salmonella* Enteritidis of broilers, indurated yolk sac remnants, pericarditis, necrotic foci and petechiae in the liver have been observed [111]. As the birds age, they become more resistant to *Salmonella*, though *Salmonella* can colonize the intestines or cause a systemic infection in the absence of disease. Experimental infection of adult birds results in fecal shedding which is much lower than after infection of young chickens. It has been supposed that the birds are more resistant to *Salmonella* due to the presence of a more complex intestinal flora when the birds become older [110]. The intestines, especially the ceca are the primary sites of colonization for *Salmonella* [112]. *Salmonella* can also be isolated from a variety of organs including the spleen, liver, gall bladder, heart, ovaries and oviducts [113].
*Salmonella*-positive birds can become (apparently) free of infection. In a Belgian study in which 18 flocks were followed from hatching to slaughter, ten flocks received a *Salmonella*-positive status. Nine of these flocks were already positive after two weeks of rearing, the remaining flock became positive after four weeks. The number of positive flocks dropped to six after six weeks of rearing [51]. In a study of Bolder *et al.* [114], 21 broilers were inoculated with $10^8$ CFU *Salmonella* and sampled for six weeks. During this period, the number of *Salmonella* shedding birds decreased from 21 in the beginning of the experiment to 14 birds at six weeks. When *Salmonella* is undetectable

in the feces of the birds, it is possible that they are still carriers [115]. They can become shedders again when the immune response of the chicken is lowered due to stress or concurrent diseases. Rigby and Pettit [116], for example, have shown that birds can change from *Salmonella* carriers to shedders during transport.
Several studies have determined the prevalence of *Salmonella* colonized flocks in Belgium. In the study of Heyndrickx *et al.* [51] 50% of the examined broiler flocks were shedding *Salmonella* at an age of two weeks and 33% of these flocks at an age of six weeks. In the study of Rasschaert *et al.* [117], 13% of the broiler flocks were colonized with *Salmonella* at slaughter. In Table 1, an overview of the prevalence of *Salmonella* colonized flocks in Belgium and other industrialized countries is given. As birds can be carriers without shedding the organisms and as flock prevalence decreases with age, the type of sample and the age of the birds are included in this Table. The within flock-prevalence for *Salmonella* is variable, ranging from 5 to 43% in Japanese broiler flocks [118].
Flocks can become colonized with *Campylobacter* from the age of two weeks [66, 126]. Once introduced, campylobacters spread very quickly throughout the broiler house, probably via the drinking water system and by coprophagic behavior [59, 65, 127]. Within a few days, all birds within the flock become colonized and shed campylobacters until slaughter age which is between five and six weeks [59, 66]. Colonized chickens usually show no observable clinical symptoms of infection. *Campylobacter* colonizes the mucus overlying the epithelial cells primarily in the ceca and the small intestine but may also be recovered from elsewhere in the gut and from the spleen and liver [59]. Experimentally, the dose of campylobacters required to colonize chickens can be as low as 40 CFU, though it is dependent on the bacterial strain [128]. Campylobacters can rapidly reach extremely high numbers in the cecal contents. Numbers in the region of $10^5$-$10^9$ CFU/g intestinal contents have commonly been observed [129, 130, 131].

**Table 1. Prevalence of *Salmonella* colonized broiler flocks in various industrialized countries**

| Country | Prevalence (%) | Type of sample | Age of birds | Reference |
|---|---|---|---|---|
| Belgium | 50 | overshoes | 2 weeks | [51] |
| | 33 | overshoes | 6 weeks | [51] |
| | 13 | cecal content | at slaughter | [117] |
| The Netherlands | 27 | cecal content | at slaughter | [119] |
| | 25 | fecal samples | 3 - 4 weeks | [120] |
| | 12 | fecal samples | 6 weeks | [120] |
| France | 70 | environmental samples | 4 - 6 weeks | [67] |
| Spain | 30 | fecal samples | at slaughter | [121] |
| Denmark | 17 | cecal content | 3 weeks | [109] |
| | 6 | overshoes | 3 weeks | [81] |
| | < 5 | fecal samples | 3 - 4 weeks | [122] |
| Sweden | 0 | cecal content<br>fecal samples | 4 - 5 weeks | [123] |
| Canada | 77 | litter and water samples | 1 - 8 weeks | [124] |
| USA | 5 | fecal samples | 6 - 7 weeks | [125] |
| Japan | 64 | cecal content | at slaughter | [118] |

The proportion of broiler flocks colonized with *Campylobacter* varies among countries, ranging from 3% in Finland [132] to more than 90% in the UK [78] (Table 2). However, this variation may reflect, at least in part, different sampling and isolation methods applied. In Belgium, the prevalence of *Campylobacter* colonized flocks ranges from 39% at the farm to 67% at slaughter [63]. In this study, the same flocks were sampled and the discrepancy between the prevalence at the farm and at the slaughterhouse is probably due to an acquired infection during transport. In the study of Rasschaert *et al.* [117], in which flocks were sampled at the slaughterhouse, a prevalence of 73% was detected. There appears to be a lower prevalence of *Campylobacter* colonized flocks in the Nordic countries compared to the other European countries and the United States. The reason for this is still unknown, though climatic conditions, the distance between farms, and less intensive rearing practices may influence flock prevalence. Moreover, the poultry industry in the Nordic countries is more strictly regulated than elsewhere in Europe [59]. In contrast to *Salmonella*, a seasonal variation in the prevalence of *Campylobacter* colonized flocks has been mentioned [81, 83, 119]. This seasonal variation is expressed by a higher rate of colonization in summer than in winter. The reason for this seasonal variation is unknown but may reflect levels of environmental contamination.

**Table 2. Prevalence of *Campylobacter* colonized broiler flocks at slaughter age in various industrialized countries**

| Country | Prevalence (%) | Reference |
|---|---|---|
| Belgium | 39[a]-67[b] | [63] |
| | 73 | [117] |
| The Netherlands | 82 | [119] |
| | 45 | [120] |
| United Kingdom | 76 | [64] |
| | > 90 | [78] |
| Denmark | 45 | [80] |
| | 46 | [133] |
| | 43 | [81] |
| | 50 | [134] |
| Finland | 3 | [132] |
| Norway | 18 | [83] |
| Sweden | 27 | [65] |
| | <10 | [59] |
| | 17 | [135] |
| Canada | 60 | [136] |
| United States | 88 | [137] |

[a] : prevalence determined by collecting cecal droppings in the poultry house at the age of 6 weeks just before slaughter
[b] : prevalence determined by collecting ceca (from the same flocks as in a) in the slaughterhouse

Only few studies have investigated the possible correlation between colonization with *Salmonella* and *Campylobacter*. A positive correlation between the concurrent colonization within Dutch flocks was found by Jacobs-Reitsma *et al.* [119] and Jacobs-Reitsma [138], meaning that *Salmonella* free flocks were more often *Campylobacter* free, and *Salmonella* positive flocks were more often also *Campylobacter* positive. In contrast, in the studies of Wedderkopp *et al.* [81] and Rasschaert *et al.* [117] no significant correlation between *Campylobacter* and *Salmonella* colonization was found.

## TRANSPORT OF POULTRY FLOCKS

At the age of approximately six weeks, broilers are loaded in containers and transported to the slaughterhouse. Different studies have shown that the cleaning and disinfection process is often inadequate in eliminating *Salmonella* and *Campylobacter* from transport containers.
In the study of Rigby *et al.* [139], 99% of the cleaned and disinfected containers examined were still contaminated with *Salmonella*. More recently, *Salmonella* was isolated from 13% to 87% of cleaned and disinfected containers at eight Danish poultry slaughterhouses [140]. According to Rigby *et al.* [141] and Corry *et al.* [142], more crates were contaminated with *Salmonella* after the cleaning and disinfection process than before. Even more, during this process, the crates may become contaminated

with other *Salmonella* serotypes than originally present in the crates [142]. Rigby *et al.* [141] and Rigby and Pettit [116] have shown that the transport of broilers in *Salmonella* contaminated containers could lead to the contamination of the exterior of the birds and even to the colonization of the birds.
In the study of Slader *et al.* [143] and Hansson *et al.* [144], 60% of the cleaned and disinfected transport containers sampled were contaminated with *Campylobacter*. However, in these studies the containers were examined immediately after cleaning and disinfection. As *Campylobacter* is sensitive to dryness, the number of campylobacters present in the containers can decrease or certain genotypes can die off in the time span between washing the containers and loading the birds into them. Therefore, it is more meaningful to determine the number of campylobacters in the containers just before using them. In a Belgian study of Rasschaert *et al.* [145], 71% of the sampled containers were contaminated with *Campylobacter*, though they were examined just before loading the birds and at least 12 hours after the cleaning and disinfection process. From individual containers, up to 90 CFU/cm$^2$ and up to four different genotypes were isolated. Analogous as for *Salmonella*, chickens transported in *Campylobacter* contaminated transport containers can become externally contaminated with the same strains as recovered from the containers (145-146). Few studies have investigated if birds can become colonized during transport in *Campylobacter* contaminated containers. However, these studies yielded conflicting results [144-145, 147].
Mulder [148] identified catching, loading and transport as stress factors. Transport-induced stress may occur as a result of factors such as crowding, motion, temperature fluctuations and feed and water deprivation. Stressed animals have increased peristaltic movements and excrete pathogenic microorganisms more frequently [149]. Consequently, a significant increase in *Campylobacter* contamination of the exterior of the birds after transport may be observed [150]. Therefore, if the contamination level on the exterior of the birds is extremely high before slaughter, the bacteria on the bird exteriors could contribute to the levels found on fully processed carcasses [150].
Birds are subjected to feed withdrawal before slaughter. Although the intestines are the primary site of *Campylobacter* and *Salmonella* colonization of poultry, feed withdrawal may influence the crop colonization. Several studies from the same research group [151-153] demonstrated that feed withdrawal in market-age broilers resulted in an increased incidence of *Salmonella*-positive crops and less pronounced in *Salmonella*-positive ceca. The number of *Salmonella*-positive crops may even exceed the number of positive ceca. Byrd *et al.* [154] showed that following feed withdrawal significant more crops were *Campylobacter* contaminated than ceca. In contrast, Rasschaert *et al.* [117] demonstrated that -at flock level- the duodena were most often found positive for *Salmonella* and *Campylobacter*, followed by the ceca and finally by the crops.

## SLAUGHTER PROCESS

### *Description of a Belgian poultry slaughterhouse*

Modern Belgian slaughterhouses have processing capacities of 6000 to 10000 birds per hour. The live birds are manually hanged by their legs on shackles on a moving line. They are stunned by electrical shock and killed by bleeding. An alternative is to stun the broilers by $CO_2$ before hanging the birds on the slaughter line. During scalding, the feathers are loosened by submerging the carcasses in a water bath at a temperature of ± 51°C. The feathers are subsequently removed on a plucking machine by means of a series of rotating discs, each with several rubber fingers. The head of the bird and the feet are removed before the carcasses are hung over on a second moving line, the evisceration line. Together with different procedures to remove crop,

neck and internal organs, the carcasses are eviscerated mechanically by spoons or clamps. At frequent intervals along the line, water is used to wash both the carcasses and the equipment. The most important washing point is immediately prior to chilling when the carcasses are washed inside and outside. Finally, the carcasses are chilled by air-chilling.
A control measure to reduce contamination of carcasses with *Salmonella* is logistic slaughter which is applied in Belgium since 1999. This means that on a daily basis *Salmonella*-free flocks are slaughtered before the *Salmonella*-positive ones. The *Salmonella* status is determined by analysis of fecal samples collected in the poultry house maximum three weeks before slaughter. At present, no such control measure is implemented for *Campylobacter* in Belgium.

### *Contamination of carcasses during slaughter*

Most recent studies focus on *Campylobacter* which is at present of more importance than *Salmonella* given the higher flock prevalence. Despite the use of different methods for sampling and quantification, the same observations were made in most studies. Campylobacters are present on the carcasses throughout the whole slaughter process, but the levels may decrease during scalding, chilling and freezing and may increase during defeathering and evisceration.
As explained above, it is likely that campylobacters are present in large numbers on the skin when a broiler enters the processing plant, especially when the flock is colonized with *Campylobacter*. This is demonstrated by several studies, in which campylobacters have been recovered from broiler carcasses prior to entering the scalding tank [130, 155-156]. Though the numbers present on the exterior of the birds are reduced by scalding, survival of *Campylobacter* has been reported [155, 157].
Spilling of the intestinal content of colonized flocks is the most important factor contributing to carcass contamination during slaughter and is difficult to prevent [129,157-158]. Berrang *et al.* [159] have shown that even small amounts (5mg) of intestinal content can cause a significant increase in the numbers of *Campylobacter* on broiler carcasses. Herman *et al.* [63] demonstrated that a significant correlation exists between the *Campylobacter* colonization of the broilers at the farm and the contamination of the carcasses after processing. In this study, no Belgian slaughterhouse was able to avoid contamination of carcasses when flocks colonized with *Campylobacter* were processed. During the different stages of the slaughter process, the crop or intestines may be damaged or the content may leak and cause an additional contamination on the carcasses. Feather removal by the mechanical picker may remove bacteria that are associated with the feathers and the skin of the bird [160]. On the other hand, the rubber fingers applied in the defeathering process exert pressure on the carcasses, forcing potential contaminated fecal material out and spreading it on the carcasses and the slaughter equipment [157]. Berrang *et al.* [161] showed that carcasses plugged with tampons and sutured were significantly less contaminated with campylobacters just after defeathering than control carcasses which were unplugged and unsutured. During evisceration, the intestines may rupture and leak fecal material. Hargis *et al.* [151] demonstrated that in the processing plant examined the crops of the birds were 86 times more likely to rupture than the ceca. Several studies have shown that *Campylobacter* contamination levels increase during the evisceration and decrease during air and water chilling and freezing [155,157,162]. Despite the fact that water chilling may lead to cross-contamination, this method washes off bacteria from the surface of the carcasses. The drying effect of air chilling causes physical stress for *Campylobacter*. In the study of Rosenquist *et al.* [162], the reductions obtained by water and air chilling were very similar, therefore none of the methods could be preferred to the other. Several studies have reported a reducing effect of freezing [157]. Therefore, this technique has been implemented as

intervention in broiler processing in Norway, Iceland and Denmark. In practice, this means that *Campylobacter*-positive flocks are used for the production of frozen chicken meat [162].
In addition to carcass contamination due to leakage from the crop or intestinal content from the *Salmonella* or *Campylobacter* colonized flock itself, cross-contamination to carcasses from the same flock or other flocks is also an important source of carcass contamination. That way, the number of *Salmonella* or *Campylobacter* contaminated carcasses increases during processing. Carramiñana *et al.* [121] isolated *Salmonella* from fecal material from 30% of the incoming birds and from 60% of the air-chilled carcasses. Jones *et al.* [163] demonstrated the same for *Campylobacter*. *Campylobacter* was recovered from 20% of birds entering the plant and from 52% of the carcasses following immersion chilling. The three potential routes of cross-contamination are direct contact between carcasses, indirect contamination via slaughter equipment and processing water, and airborne spread via aerosols. In a Belgian study of Heyndrickx *et al.* [50-51] no correlation was found for *Salmonella* -in contrast to the results for *Campylobacter* as mentioned above- between the colonization of the flocks at the farm and the contamination of the end product. It was shown by molecular typing that *Salmonella* strains contaminating broiler carcasses do not predominate in the preharvest environment [50]. The presence of fecal material in the transport containers and especially the identity of the slaughterhouse were determining factors for carcass quality. This was also demonstrated by another Belgian study of Rasschaert *et al.* [164] in which two out of the three examined slaughterhouses delivered *Salmonella* contaminated carcasses, while the flocks were not colonized and slaughtered first that day. In these two slaughterhouses, the slaughter line was contaminated with *Salmonella* before the onset of slaughter despite the daily cleaning and disinfection process. The same *Salmonella* strains were isolated from the slaughter line and the processed carcasses clearly indicating cross-contamination. In other studies, *Salmonella* was also recovered from the slaughter line before processing [140,142]. Certain *Salmonella* strains were demonstrated to persist on the slaughter line for five days [140]. In contrast, an inadequately cleaned and disinfected slaughter line does not seem to play an important role in the contamination of carcasses with *Campylobacter*. Only one study demonstrated that *Campylobacter* contaminated equipment due to poor sanitation at the end of the day (only washed with potable water) may be the source of contamination on broiler carcasses the next day [165]. In other studies, no campylobacters were isolated from the slaughter equipment after cleaning and disinfection [166-167]. In several studies cross-contamination from one flock to a following flock slaughtered the same day was observed by means of molecular methods [146,167-170]. Only very rarely, strains were isolated from processed carcasses which originated from flocks slaughtered maximal one day before [170] indicating the limited role of cross-contamination from the slaughter line. The excessive use of water during the slaughter process produces a lot of aerosols which may be a vector for airborne transmission. Posch *et al.* [171] isolated campylobacters from the aerosols in a poultry slaughterhouse with an average of $3.6 \times 10^3$ CFU/$m^3$ air in the scalding area and $1.3 \times 10^4$ CFU/$m^3$ air in the evisceration area. According to Allen *et al.* [172-173] the microbial cross-contamination of broiler chicken carcasses during defeathering occurs mainly via the airborne route.
A comparison of the level of *Campylobacter* contamination of carcasses between different studies is difficult due to the wide variety of methods used, such as examination of an area of the skin (the skin itself or swabs), a certain weight of skin or meat, or whole carcass rinses. Overall, the slaughter process may reduce the level of contamination with 2 or 3 log. Mead *et al.* [2] found that during processing of broiler flocks colonized with a mean of 6.8 log CFU *Campylobacter* per g of cecal content, there was a reduction in the numbers of campylobacters on the neck skins samples

from 3.7 log CFU/g after exsanguination to 1.8 log CFU/g after slaughter. Izat *et al.* [155] found a reduction from log 3.5/1000 $cm^2$ skin on broilers entering the plant to log 1.8/1000 $cm^2$ skin on carcasses just before package.

## CONCLUSIONS

*Campylobacter* and *Salmonella* account for over 90% of all reported cases of bacterial food poisoning. As poultry meat is an important source for the transmission to humans, it is advisable to control this vehicle. Attempts to sensibilize the public on the proper handling and preparation of poultry meat have limited influence. Another approach to decrease the risk for human infection is to avoid that contaminated poultry meat reach the consumer. This can be achieved by the reduction of the number of colonized broiler flocks by vaccination or eradication of positive breeder flocks, improved hygiene measures at the farm and treatment of water and feed. Although these control measures have proven their use in the reduction of *Salmonella* colonized flocks, they seem of limited use in the control of *Campylobacter.* As it is difficult to prevent contamination of the carcasses during processing, a possibility lies in the decontamination of the end product. However, chemical decontamination and irradiation are still forbidden in the EU and other decontamination methods cause changes in the appearance of the product or are still in development. Therefore, further research on decontamination methods of the end product is still necessary.

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# GMO detection in the EU: past, present and future

# General introduction

**I. Taverniers[1], A. Leunda[2] and M. Van den Bulcke[2]**

*[1]Institute for Agricultural and Fisheries Research (ILVO), Unit Technology and Food, Burg. Van Gansberghelaan 115 bus 2, B-9820 Merelbeke, Belgium*
*[2]Scientific Institute of Public Health, Division of Biosafety and Biotechnology, 14 rue J. Wytsmanstraat, B-1050 Brussels*
*e-mail: isabel.taverniers@ilvo.vlaanderen.be*

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## GENERAL INTRODUCTION

The recombinant DNA technology emerged in the late seventies as a result of several discoveries of different types of DNA-enzymes and the existence of autonomous replicating DNA molecules in bacteria. The opportunity to develop novel combinations of DNA elements and transferring these into bacteria for functional testing, created a revolution in the understanding of the molecular mechanisms of many elementary biochemical processes at the cellular level. In the late-seventies, several groups invoked the role of DNA as principle basis in the generation of novel tissue structures, crown galls also called tumors at the time, by a soil bacterium known as *Agrobacterium tumefaciens* [1-4].

Merely a decade later, the first plant transformations mediated by *Agrobacterium tumefaciens* were produced through modulating the natural transfer DNA in the laboratory, resulting in genetically modified (GM) plants in 1985 [5]. In 1994, the first GM food, derived from the Flavr Savr tomato (Calgene, USA) modified for increased shell-life [6], was launched on the market in the USA. In Europe, the introduction of GM food and feed derived from herbicide tolerant Roundup Ready soy GTS-40-3-2 (Monsanto, US) and from an insect resistant maize Bt176 (Ciba-Geigy, Switzerland) took place in 1996 [7]. In October 1997, due to strong political pressure in several EU Member States (including France and Austria), a *'de facto'* moratorium was imposed upon the authorization of any new GM-crop in the EU. Within this period, several adaptations of the initial legislations were made, mainly through the establishment of measurable restrictions and monitoring programmes. To date, the EU is gradually adapting to this novel legal situation. In the coming years, the EU will probably be urged to adapt a more pragmatic attitude towards GM food/feed, considering the globalisation of the GMO production for food/feed usage [8].

Indeed, genetically modified (GM) crops have become a reality in agriculture and the food/feed market [8-9]. This worldwide increase of commercially available GM crops, especially as commodity products, has created a novel global market situation. In many countries (including the European Union), threshold levels for labelling products with GMO presence were established as to guarantee consumer information on the application of biotechnology in the generation of seed/grain (derived) raw materials for food/feed purposes [10-11]. The detection, the identification and the quantification of the GMO presence in certified seed lots and along the food/feed production chain is essential to properly fulfill downstream labeling and traceability requirements.

The European Union has decided to label GM food/feedstuffs containing GM plants or GMO-derived ingredients and additives when present in an amount above 0.9 % [10-11]. Analytical tools to detect and quantify GMOs are thus required in order to be able to comply with the European legislation requirements. There is a wide variety of techniques available to detect GM material but the most universal ones are DNA-based techniques because the only common feature between different GMOs is a modification of their genomic material obtained through recombinant DNA technologies. Among the DNA-based methods, the most common technique used by enforcement laboratories is the polymerase chain reaction (PCR). This technique allows the detection of specific recombinant DNA sequences. Recently, the European Commission recommended that "the results of quantitative analysis of GMOs should be expressed as the number of target DNA sequences per target taxon specific sequences calculated in terms of haploid genomes" [12]. In addition to detection methods, also reference materials are essential, especially in the view of a need of precise quantification of the GM-material present in a product.

Foreseeing such rapid evolutions at the global level and understanding the need and importance for science-based methodology in GMO detection in support of monitoring and control activities, the Belgian Federal Science Policy (then SSTS–DWTC) included a major GMO research program within its Scientific Support Plan for Sustainable Development (SPSD) during the period 1998-2004. Two integrated GMO projects were funded within the SPSD program: the SPSD I project entitled 'Detection and authentication of food/feedstuffs based on genetically modified organisms' (1998-2001) and the SPSD II entitled 'Tracing and authentication of GMOs and derived products in the agro-food sectors' (2002-2004). The Division of Biosafety and Biotechnology of the Institute of Public Health (IPH) in Brussels, under the responsibility of Dr W. Moens, acted as a co-ordinator for both projects. The other partners in both projects were the Centre for Agricultural Research (CLO) (now Institute for Agricultural and Fisheries Research (ILVO) in Merelbeke and the Centre de Recherches Agronomiques (CRA) in Gembloux (now Centre wallon de recherches agronomiques (CRA-W)). In the second project the Veterinary and Agrochemical Research Centre (CODA-CERVA) in Tervuren and the University of Namur were two additional partners.

In view of harmonization of methodologies, it was considered at that time that reference materials play a central role in the identification/quantification of GMO in products. Based on the research performed at CLO [13], it was considered most straightforward to extend the concept of plasmid-based reference materials towards a standard norm as GMO detection. This research has contributed towards revision of the European regulatory framework for introducing and commercializing new GMO events. In the first article by Van den Bulcke et al., an overview of the main legal framework setup in the EU on GMO food/feed is presented. In addition, the concept of normalisation as a basis for harmonized enforcement in support of a common internal market in the EU, is described.

The development of a "plasmid marker library" within the two SSTC projects has been the catalyser towards the current acceptance of plasmid DNA as a reference material. However, considering the diversification of plasmid markers construction, an important aspect was to maintain a certain degree of harmonization and standardization between the different constructed plasmids. For this, the ISP introduced two essential criteria: the final cloning of the targeted sequence has to be done in a uniform background (e.g. a pUC-vector) and the plasmid has to be documented by reporting essential scientific information of the construction. This has resulted in a common procedure of producing,

documenting and "making available to third parties" the plasmid reference markers (discussed also in the last article).

It was also recognized that the quality of the analyte isolated from a sample will determine the outcome of the analysis. During these SSTC projects, several parameters influencing the PCR kinetics have been addressed by the CRA-W (see article 2). This was in fact a first though still restricted approach to tackle what became the broader concept of “modular approach” that was put to the fore by the team of Arne Holst-Jensen of the Norwegian Veterinary Institute [14].

Finally, the correct identification of the different GMOs present in a sample is a key towards a regulated enforcement. CLO-ILVO has therefore developed novel technologies allowing to isolate unique event-specific sequences covalently linked to the GM-event [15]. This concept, anchor PCR fingerprinting or transgene display technology is by now generally accepted as the central "dogma" of detection of GM-material in a product. Anchor PCR amplification of an event-specific sequence, starting from a known DNA region in the T-DNA inserted in the event, allows to develop event-specific primers and PCR assays to be used further in routine GMO detection (see also article 3).

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# Regulatory/legal framework of GMO detection

**M. Van den Bulcke and M. Sneyers**

*Scientific Institute of Public Health, Division of Biosafety and Biotechnology, 14 rue J. Wytsmanstraat, B-1050 Brussels*
*e-mail: e-mail: gmolab@sbb.ihe.be*

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## SUMMARY

In the EU, the production and commercialization of GMOs and GMO-containing or -derived food and feed products are regulated by the 'Food and Feed' Regulation (EC) No 1829/2003 and the 'Labeling and Traceability' Regulation (EC) No 1830/2003. Labelling is mandatory above a 0.9% threshold GMO percentage, relative per ingredient (and translated as plant taxa for analytical purposes). Analytical methods for detection and accurate quantification of GMO contents in derived food and feed products, require robust detection methods and adequate reference materials as identifier and quantifier controls.

## INTRODUCTION

Detection, authentication and quantification of GM plants and of their derived products used in the food/feed-processing area are a major challenge. In the European Union, according to the recent GM Food/Feed Regulation (EC) No 1829/2003 [1] and 1830/2003 [2], it has been decided to label GM food/feedstuffs containing GMO or GMO-derived ingredients and additives. During the last decade, the political will prevailed in a vast majority of the EU member states to provide full transparency on the nature, origin and manufacturing of food/feed products present on the European market. Also, driven by environmental concerns, the EU committed itself to apply the so-called "Precautionary Principle" as from the Treaty of Maastricht. Finally, the EU-consumers explicitly requested for "freedom of choice" with respect to food/feed products. All together, the EU has decided that in case of GM food/feed products a "Technology label" is to be introduced allowing to trace down the presence of GM-material in a product or the use of GM products during the manufacturing of a product.

The present analytical threshold for labeling has been set to 0.9 % GM-material /ingredient [2]. Such threshold level definition for labellng implied *de facto* the need to develop detection and quantification tools able to comply with the legal requirements.

Thus, several critical elements needed to be established at the EC level: i) the "Community Reference Laboratory for GMO detection" was inaugurated in 2003, ii) a scientific platform supporting the CRL in its mission was also established at the same time (the so-called "European Network of GMO Laboratories" (ENGL)) and iii) a concrete "unit of GMO measurement" has been proposed at the EC level [3].

All the above elements, somehow modulated the EU policy with respect to the introduction of GM materials in food/feed products on the EU market. The installation of

such legal framework on GMO commercialisation implied however the need for effective but harmonized enforcement measures. For this, the enforcement on GMO detection was organized within the Regulation (EC) No 882/2004 [4], applying the ISO-17025 norm as basis of logistic harmonization throughout the EU.

Here, an overview is presented of the different key regulatory elements within the EU legal framework for commercialisation of GM food/feed products on the EC market. The legal basis as presented in the Regulations (EC) No 1829/2003 [1] and 1830/2003 [2] (and to some extend in the Directive 2001/18/EC, [5]) will be discussed. Also, the CRL-GMO and the role of the ENGL in the process of GMO enforcement will be documented.

## Overview of the legal framework for GMO Food/Feed commercialisation in EU

GMO as or part of food/feed products required the establishment of a European legal framework to address the particular issues within this industrial sector. Initially, GMO use as food/feed products was assessed within the framework of the Directive 90/220/EEC [6]. In 1997, the Regulation (EC) No 258/97 on Novel Foods [7] came into force and GM Foods were retained within that legal framework. However rapidly, it became clear that food/feed products comprising or containing GMO, represent a particular type of "Novel Foods", requiring a particular risk assessment, especially due to the introduction of a genetic modification in the host organism.

For this reason, the EC established the Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed [1]. In this regulation all aspects on risk assessment (and a number of elements on risk management) are stipulated. In addition this Regulation provided a role for the "European Food Safety Authority" (EFSA). The EFSA was created as stipulated in Regulation (EC) No 1642/2003 [8], as a response to various food crises at the EU level. The EFSA acts as an independent Food Safety Authority in the EU and is entitled to provide expert opinions on the safety of foods in general, and for GM food and feed. The EFSA constitutes 20 different expert panels on different fields in food safety; the secretariat to the GMO Expert Panel is currently based in Parma (Italy).

Next to Regulation (EC) No 1829/2003 [1], the Regulation (EC) No 1830/2003 stipulating the labeling and traceability measures to be applied in the EU when commercialising GMO or GM-derived materials for food/feed purposes [2] became effective. Regulation (EC) No 1830/2003 is an EU response towards the consumer's request for "freedom of choice " in food/feed products. Within the scope of this regulation, threshold levels for obligatory labeling requirements for presence of GM-material(s) in a product have been established. To date, the treshold level for GM-labeling is at 0.9% GM/ingredient present in the food/feed product (see further below).

Next to the above regulations, the EC also established a framework for the normalisation of the enforcement measures and procedures. Such framework was to take into account international trade agreements (GATT, WTO,...), the subsidiarity principle on implementation of adequate, appropriate measures at a Member State level and the "state of the art" in the scientific field. Moreover, such global framework needs to remain sufficiently flexible and fast-responsive, guarantee robustness and nevertheless provide reasonably cheap tools. Therefore, multiple technical platforms at the EC and at the international level have been addressing the different aspects of the

GM-commercialisation, including the Technical Committees at the "Centre Européen de Normalisation" (CEN), the GM Taskforces at the CODEX Alimentarius level and at the OECD level, the ISO-normalisation platform and the EU governmental stakeholders at the competent authorities of the Member states and at the European Commission: the JRC-IHCP, the JRC-IRMM, the EFSA, the ENGL,...).

## Regulation (EC) No 1829/2003

In the period 1997-2004, the stipulations described within the Novel Food Regulation (EC) No 258/97 were applicable to notifications for commercialisation of GMO as food/feed products. This regulation covered "Novel foods and novel food ingredients", among which GM materials. The GM approval system within the Regulation (EC) No 258/97 [7] was modelled to the *ad hoc* procedures applied within the Directives 90/220/EEC [6] and 2001/18/EC [5] for risk assessment of GMO. The Regulation foresaw a "Fast-track" approval for GM foods which are deemed to be “substantially equivalent” to non-GM food (see Art. 5 of Regulation (EC) No 258/97). The concept of "substantial equivalence" was a source of confusion and controversy and has by now *in se* been abandoned and modified towards more appropriate concepts.

The Regulation (EC) No 1829/2003/EEC [2] replaced the Novel Food regulation and applied to larger extend the "One door, one key" principle, meaning that a single risk assessment is performed and a single authorisation is being granted for a GMO and all its uses (cultivation, importation, processing into food/feed or industrial products). In this evaluation the EFSA plays a pivotal role; the experts of the scientific "GMO Panel" perform the scientific risk assessment for each application covering both the environmental risk and the human and animal health safety assessment of the GM product(s) and their uses as or in food/feed products. Authorisations are now granted for a maximum of 10 years and the so-called "simplified" procedures (Art. 5 of the Regulation (EC) No 258/97) have been omitted. With respect to environmental risk assessment, a bridging procedure towards the Directive 2001/18/EC [5] has been incorporated.

The procedure described within art. 4 of the Novel Food regulation has in essence been retained, except that the EFSA is included as a central evaluation body within the assessment process. In short, the notifier introduces a novel application for a GM Food/Feed at the competent authority of a Member State, who immediately transmits the dossier to the EFSA. Within 6 months, EFSA will formulate an opinion to the European Commission (DG SANCO), who will, upon consultation with the competent authorities of the Member States, prepare a Commission proposal for approval by the competent authorities of the Member States. Upon approval by qualified majority vote (QMV) at the Standing Committee level, the GM product becomes authorized from the moment the EC decision is published in the European Journal.

The above described procedure has to date merely figured as theory as at various steps during the process a number of technical and/or political hurdles emerged. To date, no single GM product has been approved by QMV, meaning that the authorization process for a GM food/feed product in the EU remains a time-consuming process.

The list of authorized GM food/feeds within the EU can be found a.o. on the website of the Belgian Biosafety Server (www.biosafety.be)

## The labelling and traceability legal framework in the EU

The Regulation (EC) No 1830/2003 [2] concerning "the traceability and labeling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organism" aims at establishing the conditions of management and/enforcement set by the Regulation (EC) No 1829/2003 [1]. The main objective of this regulation is to facilitate the control and the verification of the labeling claims for GMO food/feed products. Furthermore, this regulation puts forward the necessity of targeted monitoring of potential effects on health and the environment, where appropriate, and stipulates the obligatory withdrawal of products where an unforeseen risk to human health or the environment is established.

The regulation applies the "From fark to fork" principle to all products consisting of or containing GMOs and products produced from GMOs (thus covering products authorized under the Directive 2001/18/EC [5] and/or the Regulation (EC) No 1829/2003 [1].

In order to identify the different GMO products, a "Unique identifier" system has been established through which a unique code is assigned to each particular GMO (to be mentioned in the case of products containing or consisting of GMOs). In this way, along the food chain, any product becomes traceable to the operator by whom and to whom the product has been made available.

The operators handling the GM product must have a system in place allowing to trace the information concerning the GM products residing or passing their facilities for up to 5 years after the transaction.

The regulation stipulates specific labeling requirements in line with the introduced principles within the Novel Foods Regulation (EC) No 258/97 [7] in order to ensure proper information for the final consumer. In essence, any presence in foods and food ingredients of protein or DNA resulting from a genetic modification resumes into a labeling requirement except in cases when the GM material is present at levels below a certain threshold. The Regulation (EC) No 49/2000 [9] established a 0.9 % level of adventitious GMO presence (ingredient-based) as threshold value for obligatory labeling of food/feed products containing or comprising food/feed products.

The labeling obligation applies to all food and feed products consisting/containing GMOs, or produced from GMOs (i.e. oil, gluten …), but does not apply to products (such as meat, milk or eggs) obtained from animals fed with genetically modified feed or treated with genetically modified medicinal products.

Labeling is moreover obligatory regardless of whether DNA or proteins derived from genetic modification are contained in the final product or not. In other words, the labeling informs the consumer that the product is or contains material that has been obtained from material generated through genetic modification.

## The EC standardization/certification policy and GMO

In 1985, the Council adopted a new approach towards technical harmonisation and standardisation which allowed Member States to reach an appropriate level of legislative harmonisation through the adoption of so-called "essential safety requirements" for products, if to be acceptable for free movement within the EU. These

requirements are laid down in the decisions of the European Commission or the Council and stipulate which technical specifications a manufacturer has to comply with in order to be allowed to put a product on the market.

The key to the implementation of this approach to technical harmonisation is the common standardisation policy, through the adoption of standards, that determine the specifications of industrial production. These standards are adopted by official European bodies who must assure that the standards comply with the specification as laid down in the harmonisation directives and are in agreement with all parties concerned: producers, users, consumers, administrations, etc. The normalisation body implicated in the GMO regulations is the European Committee of Standardisation (CEN).

With respect to GMO analysis, the CEN has developed a number of norms, in co-operation with the International Standardisation Organisation (ISO). The international standards ISO/DIS 21569, ISO/DIS 21570, ISO/DIS 21571, and ISO/DIS 24276 [10-13] represent the key norms applicable to the methods of analysis for the detection of genetically modified organisms and derived products, the extraction procedures and general considerations/terminologies in the case of molecular analysis in GMO detection.

DNA as the target analyte for detection of GMO is by now globally accepted. In the Recommendation 2004/787/EC, the EU has proposed the "Haploid Genome Equivalent" (HGE) as the 'unit of measurement' for GMO presence in a sample. Also, a specific method of detection for each GM-event is (becoming) a standard requirement for obtaining market authorization (see e.g. legislation in the EU, Japan). Within the EU, the Community Reference Laboratory for GMO analysis (CRL) at the Institute of Health and Consumer Protection (IHCP) at the JRC-Ispra (Italy) is supervising the validation of the GMO detection methods (see further below).

Reference material represents another key requirement for harmonisation and mutual recognition of analysis by the Member States of the EU (and at a global level). The reference materials should by definition represent an unambiguous equivalent of the analyte as present in a certain sample (see [13]). Applied to DNA as a standard and taking into account the requirements set out by the international standards ISO/DIS 21569 [10] and ISO/DIS 21570 [11], 'equivalence' in GMO reference plasmids should pertain to the DNA sequence as present in the analytical sample. The Institute of Reference Materials and Methods at the JRC-IRMM (located in Geel, Be) (http://www.irmm.jrc.be) has been producing a number of reference materials (certified for different weight % GM-material presence) for the major GM-events commercialized to date.

The establishment of a harmonized environment for GMO detection within the EU invokes enforcement measures. As the control of food/feed present on the EU market is subject to subsidiarity, the EU Member States are in charge for the management of such controls. A certain level of uniformity within the control measures as to safeguard a harmonized internal market is essential. Recently, the notification of the accidental release of some non-authorized GMO in the US, has urged the EC Commission to develop a concerted procedure of action in case of such emergencies (see Decisions 2005/317/EC [14] and 2006/578/EC [15]). Considering the diverging timeframes of authorisation of GM-events within the different parts of the world, the accidental occurrence of non-EU authorized GM-events on the EU market is to be managed. For

this, at Codex level, a global policy is being discussed on the documentation of low-level presence of non-globally authorized GMO.

## The Community Reference Laboratory for GMO detection at EC-Joint Research Centre (Ispra, It)

To support the vigilance on complying with the GMO labelling legal rules as stipulated in the Regulation (EC) No 1828/2003 on GM Food/Feed, the establishment of the 'Community Reference Laboratory (CRL) for Genetically Modified Food and Feed' has been laid down. The CRL has been attributed to the 'Biotechnology and GMOs Unit' at the 'Institute of Health and Consumer Protection' (IHCP, JRC-Ispra, Italy) (http://gmo-crl.jrc.it). Core tasks of the CRL with respect to experimental GMO detection methodology are: 1°) the international validation of event-specific detection and quantification methods for GM food and feed products destined for the market approval, and 2°) the reception, preparation, storage, maintenance and distribution to national enforcement laboratories of the appropriate positive and negative control samples. The CRL is supported in its mission by the ENGL (http://engl.jrc.it/), a consortium of laboratories officially assigned by the Competent Authority of all 27 EU Member States. The ENGL provides expertise and logistic support to the CRL with respect to e.g. the assessment of detection methods in CRL-organized ring trials and position papers on critical issues related to GMO.

The CRL has focused on the validation of the methods provided by the notifiers within the Regulation on GM Food/Feed. The validation of these methods is performed through ring trials. A ring trial consists of the parallel testing of a method in different laboratories using uniformized reference materials, protocols and statistical interpretation. The CRL has conducted so far the validation of quantitative detection methods for more than 25 GM products in crops such as maize, oilseed rape, cotton, rice, potato, sugar beet and soy (status August 2007). The results of these analyses and the protocols of the detection methods are published on the internet (http://gmo-crl.jrc.it).

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# Analytical sample preparation steps for GMO analysis

**G. Berben, F. Debode and E. Janssen**

*The Walloon Agricultural Research Center (CRA-W), Department Quality of Agricultural Products, Chaussée de Namur 24, B-5030 Gembloux, Belgium*
*e-mail: berben@cra.wallonie.be*

**Keywords**
Sample reduction; grinding; test portion; extraction and purification, DNA quality

## SUMMARY

This article is reviewing the state of the art in the successive sample preparation steps that are required for DNA-based detection of genetically modified organisms or GMO derived products. The steps considered are those of sample preparation up to the DNA sample that is yielded through the procedure : sample reduction, grinding, uptake of the test portion, DNA extraction and purification. Finally a section is devoted to assessing the quality of the DNA extract that is obtained by this way with different methods and matrices and the problem of the definition of criteria or parameters that could be used for validation of extraction methods.

## INTRODUCTION

Techniques to detect genetically modified organisms (GMO) or GMO-derived products may be of different type. They are either based on genomic or phenotypic characteristics like for instance a bioassay that can detect the tolerance of seedlings towards an herbicide in order to test a seed lot, detection of a new protein linked to the new trait (e.g. [1]) or even detection based on NIR infrared spectroscopy (e.g. [2], [3]). Nevertheless as a GMO has basically undergone a modification in its genome, the most universal technique of detection should target the genomic level through a DNA-based approach. This does not mean that for some well-defined purposes the other methods are no longer useful, they certainly still are valuable for screening purposes.

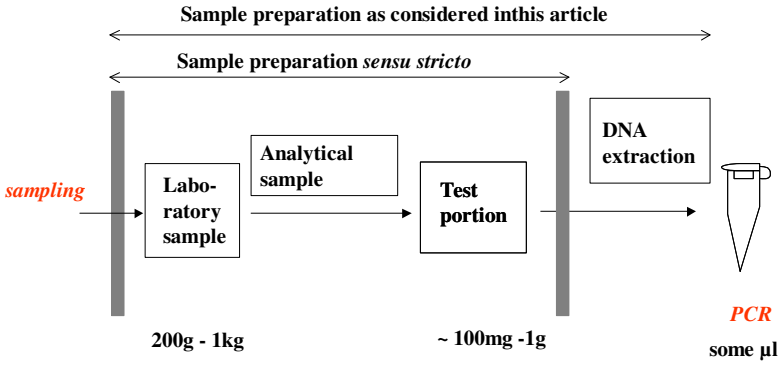


**Figure 1. Steps handled in this review include the strict sample preparation steps (from laboratory sample to the test portion) and the DNA extraction procedure**

Nevertheless, if the event has to be identified the most straightforward methods are DNA-based. Application of such methods require that DNA has to be isolated from the organism or the product to be analysed and therefore sample preparation steps are to be considered. This article will focus on this topic based on review of the available literature and on some results obtained within projects financed by the Belgian Federal Science Policy services (Belspo) during the period 1998-2004 [4], [5].
Strictly taken the sample preparation steps in GMO detection are all the analytical steps that go from the reception of the laboratory sample to the test portion submitted to DNA extraction (see Figure 1) but in this review we will also include the DNA extraction steps in the sample preparation steps.
These steps include sample reduction processes : if the laboratory sample is too large, it should be reduced to an analytical sample that is representative of the laboratory sample. It may include comminution steps [6] which aim at reducing the particle size of the solid constituents of the sample, this happens generally by grinding or milling. Out of the ground analytical sample a test portion is taken (normally in duplicate according to ISO standards [7]) and this test portion will then be submitted to a DNA extraction procedure. The so yielded DNA might finally itself be submitted to purification steps. In what follows, each of these steps will be highlighted.

## SAMPLE REDUCTION

Subsampling at laboratory level aims at reducing the size of the sample received because, for instance, the capacity of the grinder is limited to a certain volume. Even if it is advised to indicate some guidelines to customers about sample size, generally this happens through minimal requirements like the ones that were clearly given in the AFNOR experimental standards [8]. This does not preclude the laboratory to give a maximum size above which the sample can no longer be accepted. Although still being within an acceptable size range, a sample can be close to the maximum size given and may therefore need to be reduced as some flexibility should be given in defining the maximum size. To be correctly done subsampling should be performed in a statistically sound way to be representative of the laboratory sample.
A review on this topic has been done by Berben & Janssen [9] as a chapter within deliverable D4.3 of the European FP6-project Co-Extra and this text should become available to public soon. Here we just will summarize the main guidelines given within this latter review:
- grab sampling methods and quartering methods which are among the most common ones in GMO analyses are those which can lead to the most severe sampling errors,
- mechanical subsampling devices like a rifle splitter or a spinner rifle are better from a sampling point of view but generally have the big disadvantage that they are difficult to clean so that if a qualitative detection is crucial (essentially for unauthorised events) then these devices are not appropriate,
- the sampling tools (shovels, spoons and spatula) should comply to some recommendations (square shape) to avoid over- or underestimations of some particles which could lead to biased results,
- the paper cone rifle splitter which is used in soil analysis [10] might probably be a very efficient tool in GMO analysis by combining the requirements of a probabilistic correct sample reduction and the fact that contamination problems can be mastered.

## GRINDING

One of the most important steps in sample preparation for GMO detection is grinding, even if on some matrices it will not be possible neither required to do it (e.g. on sojadrink). However the most common samples in GMO analysis can be ground. Sometimes the matrix cannot be ground directly. Especially fresh plant tissues like leaves should first be freeze-dried before grinding. Some matrices like bread need first to be dried in an oven [4] before grinding. Kernels (seeds or grain) and compound feed may generally be ground directly.

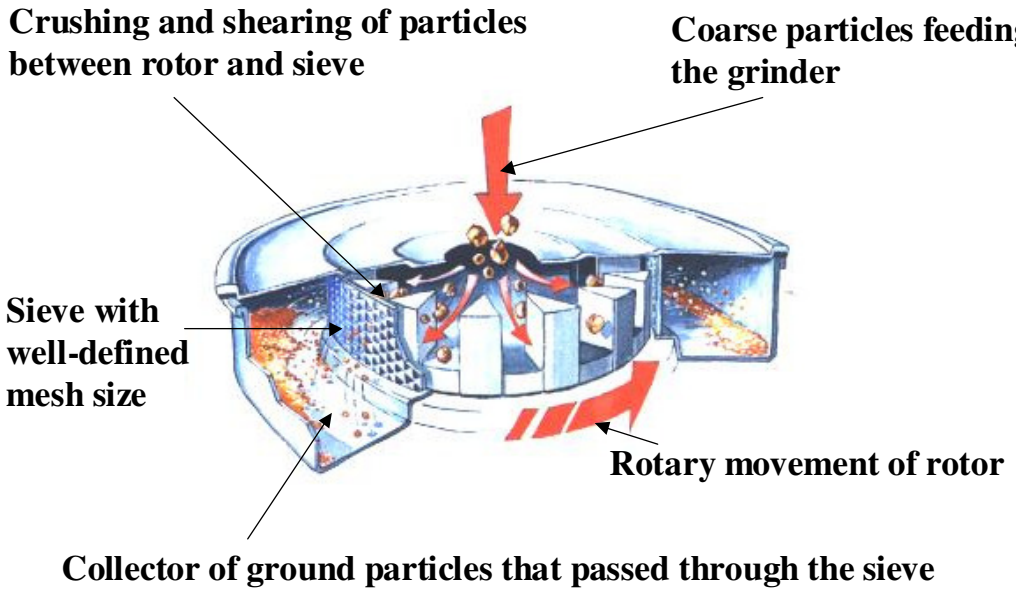


**Figure 2. Grinding chamber of the ZM200 Retsch grinder (drawing adapted from RETSCH)**

There is a huge diversity of grinding systems, some being more appropriate for one or another type of matrix. When performing analyses on a wide variety of matrices, all of them cannot have been analysed in detail through validation dossiers. In such a situation, a centrifugal mill like the Retsch ZM200 seems very fit for purpose. Indeed in this type of mill the ground material is collected after passage through a sieve with a known mesh size (see Figure 2); this will guarantee that most of the resulting particles don't exceed the mesh size of the used sieve (e.g. 0.5 mm). Moreover the checking of the performance level of the grinder can then be reduced to a granulometry check performed on a well-mastered matrix.

It has been documented in the literature (e.g. [11]) that DNA yield is directly linked to particle size (with as a rule of thumb : the smaller, the better) but also the sampling error is linked to the mean size of particles of the flour [9, 12]. With a Retsch ZM200 grinder, different mesh sizes of sieves can used. It is advised by Retsch to proceed successively through mesh sizes decreasing by a factor of four (e.g. beginning with a mesh size of 2 mm and continuing with a mesh size of 0.5mm). For some matrixes (e.g. rapeseed kernels), the 0.5 mm mesh size is too small, it will result in heating up the flour that sticks to the sieve with, as result, a possible destruction of DNA targets.

## UPTAKE OF THE TEST PORTION

The test portion size may vary from 100 mg to 5 g according to the used extraction protocol [13]. The material taken from the ground analytical sample is generally taken through simple grab sampling which seems accurate enough for this purpose. The ISO standards [14] advice a test portion of at least 200 mg or a minimum of 10 000 particles. However, there are still gaps in the rationale that should underpin this approach [9].

## DNA EXTRACTION PROCEDURES

There is a very wide variety of DNA extraction procedures available for preparation of DNA for GMO detection. Detailed protocols are given in the ISO standards [15]. The possible methods go from solvent-based ones like the phenol-chloroform method and the CTAB-method to a huge range of commercial kits. The CTAB method generally yields sufficient amounts of DNA which are pure enough for analysis but it is generally time-consuming. However, for some matrices like cotton, it does not work well. On the other hand commercial kits are generally more user-friendly and faster. Nevertheless, some of these latter ones, especially when used on highly degraded DNA, may give rise to bias problem when performing quantification [16].
According to [17] the CTAB-method, the DNA-binding silica column method (various commercially available kits) or a combination of the two are the most commonly used for plant material or plant derived material.

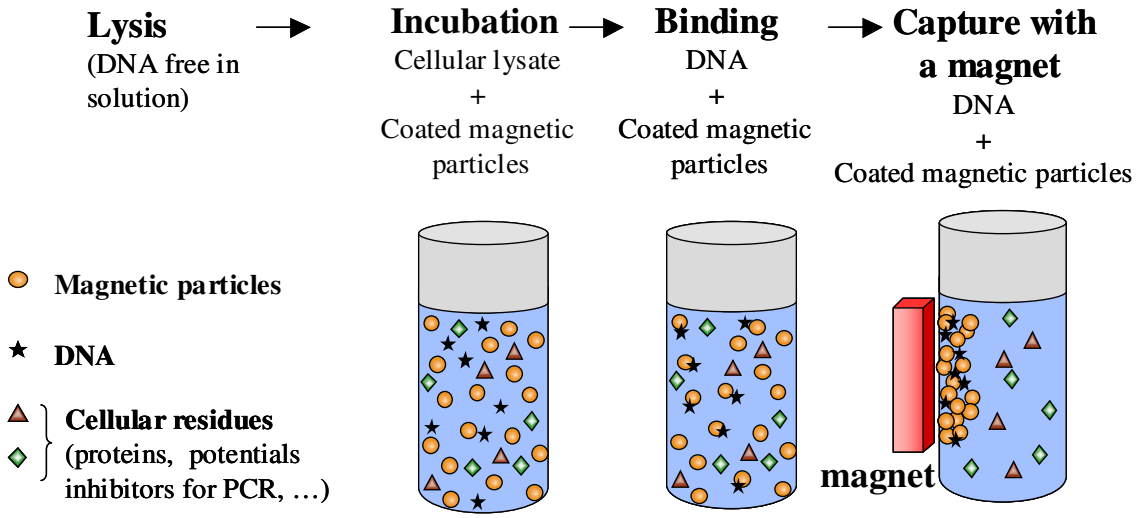


**Figure 3. Principle of the use of coated magnetic beads for DNA extraction (drawing adapted from Thermo Life Sciences)**

All these DNA extraction methods begin by a lysis step within an appropriate buffer. Presence of detergents (like SDS: sodium dodecyl sulphate or CTAB : cetyltrimethylammonium bromide) help in disruption of cell membranes and cell walls. The buffer also most generally contains EDTA (ethylendiaminetetraacetic acid) acting as a chelator in order to inactivate nucleases by mobilizing ions like $Mg^{2+}$ or $Zn^{2+}$ that are essential for these enzymes. The lysis step aims at bringing the DNA freely available in solution, the other consecutive steps may have two objectives, either trying

to isolate specifically DNA or nucleic acids in general (e.g. through ethanol precipitation in presence of salts like $Na^+$, $K^+$ or $NH_4^+$) or to eliminate other compounds (e.g. with chloroform or phenol-chloroform extraction the purpose is to get rid of proteins that appear at the interphase between the solvent and the aqueous supernatant containing the DNA). Some kits rely on the affinity of DNA to some compounds like silica (at least at well-defined pH, ionic strength and temperature conditions) and such compounds are used to coat magnetic beads. The advantage of such an approach is that centrifugation steps may then be replaced by just attracting the magnetic beads with a magnet to collect the beads and the DNA linked to it (Figure 3) which is then afterwards released from these beads. This reduces cross-contamination problems as centrifugation is a step that might be at the origin of cross-contamination through aerosol formation.
Purification of the DNA may also be achieved during these extraction procedures through enzymatic steps: for instance proteinase K is used to digest proteins, RNAse A to eliminate RNA and a-amylase to suppress starch compounds.

Comparison of several DNA extraction methods with respect to yield, advantages and disadvantages next to applicability to different matrixes may be found in [5, 13, 18, 19, 20, 21].

## PURIFICATION OF THE DNA EXTRACT

Generally, the extraction procedure already integrates purification steps as described above. However, in some cases, extra purification may be required. This is generally achieved by a combination of methods like a CTAB method followed by a purification of the extract on a silica-based column. Examples of such combinations and the scope of their application can be found in [13].

## ASSESSMENT OF THE QUALITY OF THE EXTRACTED DNA

Once the DNA is extracted from a defined matrix with a certain method, one can raise the question if it is of a suitable quality for the purpose of GMO detection, especially for quantification. This is a difficult question but it is a crucial one to guarantee as well qualitative as quantitative sound results.
A parameter that is frequently considered but that can be of rather limited interest is the DNA yield. Of course, the better the yield, the more targets can be isolated and the more trust could be gained in quantitative results. However, correct determination of total DNA quantities is not easy at all [20]. Spectrophotometric measurements of the DNA concentration by using the absorbance at 260 nm might largely overestimate the DNA quantity (up to a factor of 10 times [4]). On the other hand, fluorometric measurements with the help of picogreen may also be completely biased due to interfering compounds that can bind the fluorescent dye or in the other way round that impair its binding to DNA (for instance DNA obtained with the CTAB method will be underestimated through fluorometric measurements because CTAB still bound to DNA interferes on the binding of picogreen to the DNA and thus on the intensity of the fluorescent signal emitted).

One of the aims in the BELSPO projects cited was to characterize the DNA extract quality [5]. It appears that presently the best way to do it, is through PCR analysis itself [5, 20, 21] to define presence of PCR inhibitors, amplification efficiency, extend of the dynamic range of quantification of the extract. During one of the BELSPO research projects [5] it was for instance shown that from a kinetic viewpoint there were two types of PCR inhibitors which act probably in a different way (e.g. inhibitory compounds that

can interfere with the Taq DNA polymerase will decrease their influence through dilution of the DNA extract while compounds that impair PCR through binding to DNA target itself will still inhibit the PCR even if the DNA is diluted). A recent article [22] clearly points out that different extraction methods applied on a same matrix lead to results that are apparently small in difference but which are nevertheless significant and thus method-linked. In the future, it will be possible to relate these PCR-mediated results to some intrinsic properties of the extract that might be measured independently of a PCR result. Focussing on matrices with important interfering effects is probably the best way to get aware of the underlying mechanisms. Such an understanding of the involved phenomena will be important to define appropriate validation parameters for DNA extraction methods, so that validity of a method on a particular matrix may be tested. This is one of the most urgent needs in present GMO detection, as once that this problem is solved, then the next steps at the beginning of the process of sample preparation and the sampling schemes may also be analysed in the concept of the modular approach of validation [23].

## CONCLUSIONS

This article gives a small overview of the successive steps to which a laboratory sample is submitted to finally lead to a DNA extract that will be analysed through PCR for GMO detection. Research in this topic is still required to be sure that the obtained extract is suitable for PCR but also that the measurement uncertainty linked to each of the successive steps remains within acceptable limits.

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# Detection, characterisation and quantification of GMOs

**I. Taverniers, N. Papazova and M. De Loose**

*Institute for Agricultural and Fisheries Research (ILVO), Unit Technology and Food, Product Quality and Innovation, Burg. Van Gansberghelaan 115 bus 2, B-9820 Merelbeke, Belgium*
*e-mail: isabel.taverniers@ilvo.vlaanderen.be*

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GMO characterization, qualitative PCR, quantitative real-time PCR, screening, event-specific identification, fingerprinting, method validation, quality assurance

## INTRODUCTION

As mentioned also by Berben et al. (this subchapter), different methodologies and techniques exist for GMO detection. Basically, we can distinguish biological, chemical, and genetic or bioanalytical methods. Bioanalytical methods are either protein- or DNA-based. Protein-based techniques, more in particular immunoassays such as ELISA, are fast and cheap means for the detection of proteins in raw products such as beans and kernels, and are the methods of choice in the USA. In Europe to the contrary, PCR is accepted as the reference technique for regulatory compliance. Innumerable types and categories of PCR methods exist, classified either as qualitative or quantitative, according to the level of target specificity and as simplex or multiplex, depending on the number of targets simultaneously amplified in a single assay. Besides immunoassays and PCR techniques, high throughput techniques such as macro- and microarrays, nanoscale technologies and microfluidics are gaining interest and attention world-wide. The latter may become of high value, given the expected growth of GMO events and matrices containing GMOs.
In general, a GMO method can be evaluated based on a number of criteria:

- Applicability in terms of number of GM events;
- Applicability in terms of range of products/matrices;
- Value, utility and completeness of the obtained result. Here, according to the level of information that can be extracted from the test result, three 'categories' of methods are distinguished:
    1. 'Screening methods' detect the presence of a range of different GMOs but do not identify the type of GMO [1]. They cannot be used to identify neither to quantify GMOs [2, 3].
    2. 'Identification methods' are 'event-specific methods' that can make a distinction between different GMO events based on a unique, unambiguous signature for each event [4].
    3. In order to be in compliance with labelling requirements, 'quantitative methods' are needed. The EU legislation stipulates that all food and feed products containing or derived from GMOs should be labelled per individual ingredient, above 0.9 % for authorized events, and above 0.5 % for non-authorized events with a favourable risk evaluation [5].

  The above described categories, valid for each type of method, can be seen as objectives as such. In addition, they can be regarded as sequential steps.
- Sensitivity and risk for false results and reliability/robustness;
- Installation and working costs (equipment and reagents needed, availability, etc.);

- Time needed for one analysis and general user friendliness [4].

In a GMO analysis scheme, typically the first aim is to determine whether a product contains GMO(s) or not. This is achieved in a screening test. If the result is positive, the second objective is identification of the GMO(s), in order to know whether it (they) is (are) authorized within the EU or not. Finally, for compliance with EU threshold regulations, the precise GMO concentration(s) in the product should be determined with an appropriate quantitative method [6].

## GENERAL PRINCIPLE OF PCR

The essence of DNA detection methods is the complementarity of two strands of a DNA double helix and the very specific hybridization between them [7]. DNA always needs to be isolated from the sample first, which means that sampling and sample preparation precede the proper DNA detection. These steps in the GMO analytical procedure are described by Berben et al. (this subchapter).
Amplifying a specific target DNA sequence can make it detectable above the background of other DNA sequences. This is the principle of the polymerase chain reaction (PCR) and declares the extremely high sensitivity of this technique. By thermocycling the target DNA, mixed with a thermostable Taq enzyme, deoxynucleotides (the building blocks of the DNA), two specific primers and other reagents, as low as one single copy can be amplified [1]. The two primers are designed to hybridize on opposite strands of the specific sequence of interest. One primer is called the forward primer and is sense or 5 $\rightarrow$ 3' directed, whereas the second primer is called the reverse primer, antisense or 3' $\rightarrow$ 5' directed.

For detection of the amplified DNA fragments, a list of techniques exist. The most common is separation by agarose gel electrophoresis, followed by ethidiumbromide staining or blotting to a membrane and colour-detection. Other separation techniques include high performance liquid chromatography (HPLC), capillary electrophoresis (CE), surface plasmon resonance (SPR) and biosensor technologies and mass spectrometry (MS). A second, nested PCR or a sequencing reaction can be performed for confirmation of the formed amplicons. The PCR can also be combined with an ELISA in a PCR-ELISA assay. The latter involves selective immobilisation of PCR products by biotin-labelling one of the primers and capturing on streptavidin-beads, followed by a specific antibody binding and colouring reaction [1, 7].

## PCR FINGERPRINTING METHODS FOR GMOs

The anchor-PCR or T-DNA display technique, developed at ILVO [8], is a type of genome walking PCR method of high added value for GMO detection and characterization.
Anchor PCR is an adaptation and modification of the well-known amplified fragment length polymorphism (AFLP) technique [9]. The basic anchor PCR exists of the following steps (Fig. 1): (1) DNA extraction; (2) restriction digestion; (3) ligation of restriction site-specific adaptors to the cleaved ends; (4) PCR with a radioactively-(33P-) labelled transgene primer and an unlabelled adaptor primer and (5) visualization and detection of the labelled anchor PCR fragment by polyacryl amide gel electrophoresis followed by autoradiography [8]. Fig. 1 shows that, in contrast to conventional PCR where a known sequence is amplified between two target-specific primers, anchor PCR amplifies an unknown sequence, adjacent to a known region in the genome.

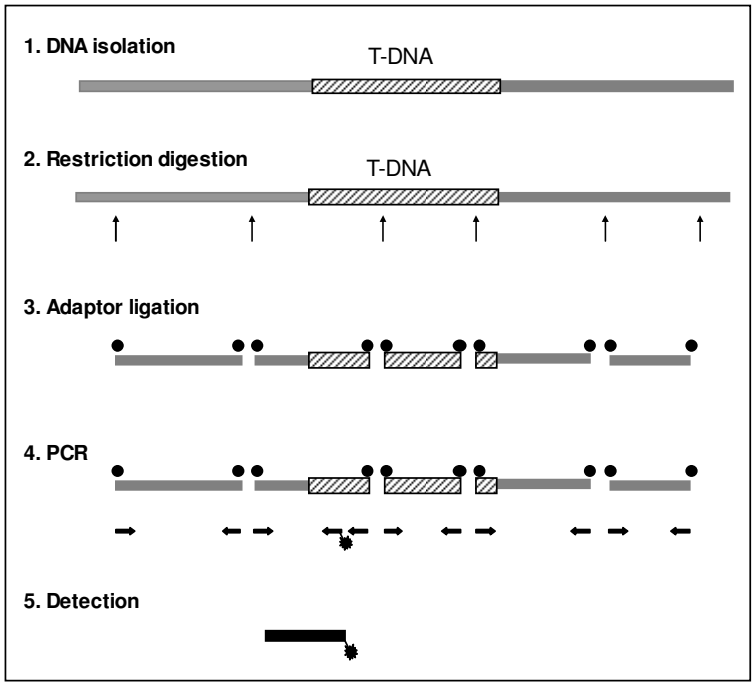


**Figure 1. Schematic presentation of anchor PCR or T-DNA display [8]**

The number and length of the fragment(s) obtained by anchor PCR depend on the distance between the used transgene primer and the first restriction site. This means that for each event and for each combination of transgene primer/adaptor primer, a different fingerprint pattern is obtained. In practice, a wealth of information is gained for an unknown sample, by combining different transgene or anchor primers with different restriction enzymes, the most frequently used being tetra- and hexacutters. The selection of appropriate transgene primers requires knowledge of sequence data of at least one border. Depending on the sequence distance from the anchor primer to the first cleavage site of the used enzyme, different patterns may be observed on the resulting fingerprint.

## REAL-TIME PCR FOR GMO QUANTIFICATION

The qualitative or quantitative character of a PCR has to do with the relationship between the amount of PCR product formed and the initial concentration of DNA target which is being amplified. In order to know whether there is such a relationship, we have to look to the stage of the PCR, which refers to the PCR efficiency. In the beginning of the PCR, products are being formed after each amplification cycle with a constant rate or amplification efficiency. In the later cycles however, reagents become limiting and products are formed with a changing rate and in a non-logarithmic way. PCR efficiency is not constant anymore at the end of the PCR. Quantitative data will only be obtained if the effect of the efficiency is nihil, i.e. if one measures in the early phase of the PCR, where the efficiency is still constant. Such PCR methods are 'kinetic methods', also called real-time PCR. In real-time PCR, a 'fluorescence threshold value' is fixed. In this early stage, the PCR is exponential and not subjected to the effects of changing amplification efficiency or inhibitors. The point at which the fluorescence signal crosses the predetermined threshold value is called the 'threshold cycle number' ($C_T$). This value is directly related to the initial target concentration.

A range of real-time PCR methods exists, thanks to the fast evolution in the development of chemistries and instrumentation. The most popular chemistry is the

TaqMan or 5' exonuclease assay, using a fluorogenic probe labelled with a reporter and a quencher. A fluorescent signal is generated only if the probe has hybridized to its complementary target sequence. Thanks to the 5' exonuclease activity of Taq polymerase, the reporter is cleaved from the probe so that it is not quenched anymore. Other real-time PCR chemistries are fluorescence resonancy energy transfer (FRET) probes, molecular beacons, Scorpion probes and SYBR Green I. The latter differs from the other chemistries, in that it is a non-specific, ds-DNA binder.

Whereas the first PCR methods for GMOs were simplex, amplifying a single target in a tube, later on also duplex and multiplex PCR methods have been developed. Duplex PCR is relevant in particular for relative quantification purposes, where both a GM-specific target and a species-specific target sequence may be amplified simultaneously in the same tube. Duplex PCRs are described for quantification of different events based on the p-35S element, a gene- or constuct-specific or an event-specific DNA sequence [10-15]. Multiplex PCR allows to amplify and detect several target DNA sequences at the same time, in one and the same tube.

## CONTROL AND REFERENCE MATERIALS FOR GMOs

Real-time PCR has proven to be very suitable for GMO quantification. Measured fluorescence signals are converted into quantitative estimates by special software [16, 17]. On the basis of the quantitative estimate lies the setting up of a reliable standard curve and thus the use of suitable control materials or calibrants. Different $C_T$ values will be obtained for a number of calibrators with different, precisely known concentrations of target-DNA. By plotting those $C_T$ values as a function of the logarithm of the initial concentrations, a standard curve or calibration line is obtained. The concentration of an unknown sample can then be calculated by extrapolating this curve [15, 18].
Real-time PCR is a very fast, sensitive, specific and accurate technique, with a lower risk for PCR carry-over contamination and with possibilities for high-throughput analysis. The latter is possible thanks to the 96-well plate format of most instruments and to the ability of simultaneously multiplex amplifications. Amplification is combined with product detection and with quantification in a wide dynamic range (usually 7-8 logarithmic decades), all in a 'closed tube' system [19-21]. A pitfall of real-time PCR is that it demands extensive and accurate optimization in order to be reliable. Main factors affecting the quantitative power of real-time PCR are the amplification reaction as such (used primers, reaction conditions), the calibrators and the detection chemistry (used probes). Another critical point is that quantification of low amounts of targets (below 10 copies) becomes very difficult and unreliable, because of the stochastic behaviour of the target molecules in the reaction [19, 22]. All these aspects must be taken into account especially when considering quantitative analysis of GMOs.

As control materials for GMO analysis, both matrix reference materials (RMs) and pure analyte RMs exist. The first commercially available certified reference materials (CRMs) for GMO analysis were matrix RMs, consisting of a mass fraction of GM powder, prepared from GM seeds or beans, present in a mass of non-GM powder, prepared from non-GM seeds or beans [23-26]. In the nineties, these CRMs have proven to be of great benefit in Europe. However, DNA and/or protein isolated from matrix RMs may be subject to degradation and thus instability. Moreover, given the expansion of GMO events and products, numerous types of matrix CRMs would be required to be produced, this process being a very cost intensive and cumbersome activity. Finally, as discussed by Van den Bulcke et al. (elsewhere in this subchapter), legal thresholds for mandatory labelling of GMO products are being highly

recommended to be expressed as genome percentages. This means that legal limits now correspond to analytical limits, i.e. numbers of haploid genome copies or equivalents [27]. Pure DNA calibrants are perfectly in line with legal recommendations and moreover, with the modular validation approach (see also Berben et al., above in this subchapter).

Different types of genomic and plasmid DNA standards have been used as calibrators for quantitative real-time PCR in other applications, as reviewed by several authors [1, 22, 28, 29]. Genomic DNA needs to be extracted from a matrix first and thus is liable to matrix effects and processing influences such as degradation. Plasmid DNA vectors, containing the sequence of interest, are preferred because of their simple and cheap production process, their stability as well as universality and wide applicability.
Since 2000, the cloning of GMO-specific PCR fragments in a vector, and the production of plasmid DNA markers for GMOs has been carried out in the frame of the OSTC-SPSD I project (see General introduction). It has been demonstrated that dilution series of both a GMO-specific and a plant-specific plasmid molecule, expressed in absolute copy numbers, can be used as calibrators in real-time PCR, allowing the accurate and sensitive quantification of GMO events [12, 30-32]. Further evolutions and outcomes on the production and use of pure plasmid DNA calibrants for GMO quantification are described in the introductory as well as conclusion parts of this subchapter on GMOs (common research carried out within SPSD I and II projects).

## VALIDATION, QUALITY ASSURANCE AND ACCREDITATION

Credibility of analytical data has never caught the public's eye more than today. Rather than on the used techniques and methodologies themselves, attention is nowadays paid to the quality and reliability of the final results. This is influenced by a higher demand for regulatory compliance, a higher consciousness of the customer – the client wants to know the level of confidence of the reported result – and under impulse of new, more exigent European and international standards such as the ISO/IEC 17025 norm for laboratory accreditation [33]. The underlying key principle is comparability of results between laboratories and on a wider, international basis. In order for results to be comparable, they must be reported with a statement of measurement uncertainty (MU) and they must be traceable to common primary references. Methods must be validated to show that they actually measure what they are intended to measure; that they are fit for their specific purpose.
Because validation and quality assurance (QA) apply for a specific analytical method, it is important to approach each method on a case–by-case basis. An analytical method is a complex, multi-step process, starting with sampling and ending with the generation of a result. As a GMO analysis involves a PCR-based analytical procedure, this will have consequences for MU estimation, method validation, and QA in general. Some pecularities for GMO analysis:

- *Modular nature of a GMO analysis*. Each step in the procedure can be considered as an independent 'analytical system', characterized by an input, a well-defined protocol, a particular output and a specific purpose [3]. However, the validity of the final result depends on the whole procedure and is determined chiefly by the weakest links in the process. Also, the MU on the final result represents the overall MU of the procedure. As a consequence of modularity, validation of the whole procedure is only possible by validating each module separately.
- *DNA analytes are determined through an in-vitro amplification process.* This refers to the special nature of the analyte (DNA) and its determination. An

amount or concentration of DNA cannot be measured directly but gives rise to an observed 'signal'. The success of DNA analyte determination by means of PCR depends on the amplification efficiency, which on its turn relies on the characteristics of the template DNA. Factors of influence on the yield and quality of this DNA, which need to be considered, are the history of the sample (raw material versus processed product), the matrix composition, the presence of PCR inhibitors in the DNA solution, and the degree of degradation and accessibility for PCR of the targeted sequence(s). An additional particularity for GMO analysis is that not one but two target sequences must be amplified in the PCR reaction. A quantitative estimate is always relative to the total amount of species present, i.e. on the ingredient level. This additionally requires that PCR amplification efficiencies for both targets are equal.

- *Real-time PCR data are not normally distributed but need to be transformed*. Quantitative results of GMO analyses, e.g. from proficiency testing (PT) schemes, are skewed around the assigned value or the robust mean, and not normally distributed until log-transformed. For this reason, it is not straightforward to assess the quality of GMO results based on precision characteristics. It was illustrated that for the quality assessment of data generated in a PT round, other means were needed. Powell and Owen [34] summarized that log-transformation of the data, as well as prescribing a value for RSD according to enforcement purposes, are appropriate for processing and interpretation of data from interlaboratory validation studies and PT schemes on GMO methods of analysis.
- *GMO concentrations and analyte levels may be exceptionally low.* The outcome of quantitative PCR methods does not follow a normal distribution around the zero value, however shows stochastic variations due to molecular fluctuations in the DNA template. Those statistical variations are of high relevance for very low DNA target concentrations [35]. This has a significant influence on GMO quantification near legal threshold limits. Small changes in calibration curve settings, influenced by e.g. the number of replicates analyzed and the accuracy of the used calibrator samples, may become increasingly important if GM concentrations below the legal threshold of 0.9 % are to be detected [36]. Affected by processing steps, target DNA sequences may be degraded to a significant level, such that DNA traces may be present below detection limits of about 10 absolute template copies or 0.1 % relative GMO concentrations.

Despite those intricacies for PCR-based GMO analysis, today a common understanding exists regarding the validation of PCR methods for GMOs and the implementation of general QA principles in laboratories detecting GMOs on a routine scale. At the European level, the CRL (EC-JRC) is in charge of the international validation of event-specific quantitative methods for all newly authorized GMOs and coordination of the production and distribution of GMO control materials. For its tasks, the CRL is sustained by the ENGL laboratories, in addition to national NRL bodies and laboratories. In this framework, NRL laboratories need to be accredited according to the international ISO:IEC 17025 standard [33]. In Belgium, the three NRL laboratories are accredited for PCR-based GMO analysis in a wide range of matrices and for a wide range of events, by BELAC. They are working at the highest quality level possible and perform quality control measures needed to maintain their accreditation status [37-38].

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# Challenges for future research in GMO detection

**G. Berben[1], F. Debode[1], M. De Loose[2], E. Janssen[1], N. Papazova[2], M. Sneyers[3], I. Taverniers[2], A. Leunda[3], A. De Schrijver[3] and M. Van den Bulcke[3]**

*[1]The Walloon Agricultural Research Center (CRA-W), Department Quality of Agricultural Products, Chaussée de Namur 24, B-5030 Gembloux, Belgium*
*[2]Institute for Agricultural and Fisheries Research (ILVO), Unit Technology and Food, Burg. Van Gansberghelaan 115 bus 2, B-9820 Merelbeke, Belgium*
*[3]Institute of Public Health (IPH), rue Juliette Wytsmanstraat 14, B-1050 Brussels, Belgium*
*e-mail: berben@cra.wallonie.be*

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## SUMMARY

This article reviews several research challenges for GMO detection as some pending questions still exist and need more research efforts to be solved. The question of how to apply validation in a modular scheme is handled. It is followed by the problem of how to manage the detection of an always increasing number of new events and the fact that in such a context screening will probably be more and more important. The issue of detection of unauthorized GMOs with special attention to unknown GMOs is considered. Some technical limitations in result expression with respect to botanical impurities or stacked events are also addressed. Finally the establishment of plasmid reference calibrants as alternative to the current plant-derived certified reference materials, their distribution and utilisation are discussed.

## INTRODUCTION

GMO detection analyses in food, feed or seeds are now routinely performed for some years in a wide number of laboratories throughout the world, thus not only in the European Union. It covers screening, identification of events and when necessary quantification. Polymerase chain reaction (PCR) is the most widely used detection method (screening, identification and quantification) because it can focus on the recombinant DNA target and especially on the junction between the incoming construct and the plant DNA as this is a unique identifier of an event. Next to that, protein-based methods are generally feasible but are unable to identify an event as they are only trait-specific but they may be quantitative or semi-quantitative. Both these methods are covered by the ISO-standards [1-6] that were developed in order to enhance the harmonization of methods and to ensure that the delivered results meet some quality criteria.
Existence of valid detection methods and strategies are necessary for the enforcement of the European legislation that was progressively set up in this field. Briefly outlined, it requires labelling of food and feed products containing authorized GMOs or GM derived material except if, on an ingredient-basis, the level of 0.9% is not exceeded and provided that this presence was the result of an accidental contamination (dilution

is thus forbidden). Due to the existence of this tolerance level, the detection methods of authorized events have to be quantitative. For seeds there is no such tolerance level. Furthermore, in food and feed, there is a zero tolerance for presence of unauthorized GMOs or for material derived from them. Another important point to highlight is the fact that as PCR is the major detection method, the European Union recommends that the unit for expression of results should be adapted to the analyte detected by this method. That is why ideally quantitative results should be expressed in copy numbers of the event-specific target per haploid plant genome equivalent of the considered crop.
One could therefore believe that presently GMO detection as such is no longer a research topic. A conclusion like that is far from true, even if, in most cases, routine analyses run smoothly. Of course, as there are new events, new methods (essentially PCR methods) have to be developed but next to that there are still a lot of pending questions which need research efforts to be solved. The aim of this chapter is to present several of the remaining challenges for research in GMO detection.

## MODULARITY OF THE ANALYTICAL PROCEDURE AND OF VALIDATION

An important goal of analytical laboratories performing GMO detection is to provide reliable results. Therefore methods to be used have to be fit for purpose. This means that appropriate methods have to be used for each step of the procedure, starting already from the sampling step. Use of suitable validated methods is thus highly recommended. However, validation studies generally don't integrate all steps of the analytical procedure but mainly focus on the final steps of the analysis. This results in an underestimation of the global measurement uncertainty. It is indeed generally accepted that the sampling errors are much larger than the analytical errors [e.g. 7].
It is in this context that the Norwegian Veterinary Institute proposed the concepts of modularity and of upstream validation in a modular approach [8]. The modular concept is based on the fact that the whole analytical procedure is made of successive steps each of which can be considered as a module (see Figure 1). Ideally each step should be assessed independently taking into account parameters of the input material and parameters of the output material to set performance criteria for this module as this could highly facilitate the way of performing validations.

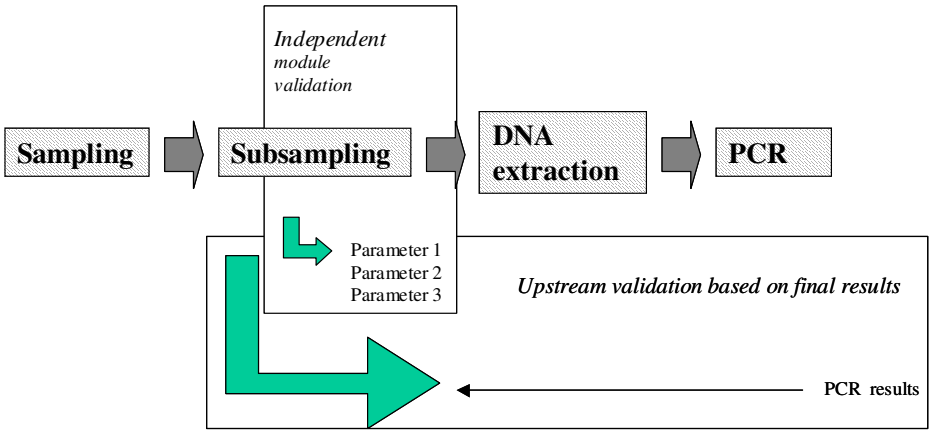


**Figure 1. Schematic presentation of successive steps, each being a module, in the analysis of a food or feed product. It is a simplified example as there is but one subsampling step. The diagram also shows that the aim of the modular approach would be to consider the validation of each module independently by defining specific parameters for each step (which might be dependent on the type of matrix considered), with appropriate performance criteria. While in the upstream validation approach the module is assessed with the final results of the most downstream module**

Sometimes or mostly (this is already a research question) however assessment of a well-defined step can only be done through the final analytical results. That is why in that case the validation can only be done in an upstream manner. For instance one will first need a validated PCR method before assessing the previous step of extraction that will release the targets submitted to PCR.
Such an upstream assessment stream between the PCR step and the extraction module was in fact already considered during the SPSDII program within the project "Tracing and authentication of GMOs and derived products in the agro-food sectors' (2002 - 2004)" [9]. It was already a kind of modular approach but with only two modules.
The problem of trying to validate an extraction method independently is nevertheless not yet solved, it is still a pending question. Reaching that goal would be of great help because it is impossible to repeat validation studies on a wide variety of matrixes. It would be much more convenient to be able to say, this extraction method is valid for that type of matrix because the resulting extracted material meets the performance criteria for the following parameters.
One will then reasonably understand that if the integration of the two last modules, i.e. PCR and extraction, is not easy at all, it will be more difficult if one adds even more upstream modules (e.g. assessment of sampling plans). That is why putting more research efforts in developing the modular validation approach is really crucial to gain more reliable analytical results. Presently indeed the full measurement uncertainty of a GMO analysis is certainly not known and there is much chance that the sampling protocols used are far from being fit for purpose as already concluded in the KeLDA study [10]. At laboratory level also the final effects on uncertainty of various subsampling processes and of test portion size are still not well known [11].

## FACING AN INCREASING NUMBER OF AUTHORIZED EVENTS TO BE DETECTED

At the time of writing this chapter the number of authorized GM events to be detected in food or feed was as follows : 1 soybean line, 8 maize lines, 8 rapeseed lines and 3 cotton lines (this list does not take into account stacked events[1] nor withdrawn events for which presence in a product is still acceptable). This list will increase in the near future as other GMO lines are awaiting approval. Therefore, even if next to the addition of such new lines there might be some counterbalancing effect due to withdrawal of some older lines [e.g. 12, 13], the present trend is still an increase of the total number of events to be detected at laboratory level. This raises the crucial question of how to organize cost-effective analyses with an increasing number of events. To face this challenge two possibilities will be considered: i) with the help of PCR-based methods and ii) without the help of PCR-based methods.

### A. *Facing the problem of detection of an increased number of events with PCR*

If PCR is to be used, then the screening step will become a more and more important first line analysis. Generally, a screening method is defined as 'a method that will rapidly and reliably eliminate (screen) a large number of negative (or positive) test samples and restrict the number of test samples requiring the application of a rigorous method' [5]. Screening methods are thus used for routine purposes, i.e. the screening of a large range of samples for GMO presence [14]. In the case of GM material, screening should be better defined as the search for the presence of a wide range of

[1] STACKED EVENTS AS HANDLED FURTHER ON IN THIS CHAPTER ARE LINES OBTAINED BY GATHERING SEVERAL SINGLE EVENTS THROUGH E.G. CROSSING BY CLASSICAL BREEDING TECHNIQUES (E.G. MON810 X NK603 IS A STACKED EVENT MADE OUT OF THE SINGLE EVENTS MON810 AND NK603)

events in a sample through the detection of a set shared elements in the considered events.
Ideally, a screening should be performed in a rapid, simple, cheap and rigorous way, and should deliver a set of results representing the 'whole image' of the sample with respect to the studied issue (here GMO presence).
Important factors in the set up of generic screening methods are: (1) the **specificity** and sensitivity of the detection method(s), (2) the 'fitness-for-purpose' of the detection method, (3) the requested **output** i.e. **qualitative** and/or **quantitative** detection of the analytes, and (4) the **detection format** of the screening assay.
In most cases, GMO screening elements are seen as genetic target sequences that are present in a large number of GMOs, such as e.g. promotors, terminators and genes coding for resistance to antibiotics. In a broader sense, however, screening can also be based on gene- or trait-specific, construct-specific, and transgene event-specific target elements. Here, only the use of DNA-based PCR methods in GMO detection will be discussed.
A 'GMO screening method' could thus be defined as "a tool for detecting, typing and/or characterizing a large set of GMOs present in a sample". Given the ongoing and further expected worldwide expansion of GMO acreages and markets, the development of PCR assays that target event-specific sequences is essential for identification of the GMO. Event-specific sequences should allow unequivocal identification of a GM event, based on the junction region between the transgene insert and the flanking plant genomic sequences or another sequence that is specific for the particular event such as a unique rearrangement within the construct. Such a sequence target is unique for each GMO event and therefore acts as a 'unique molecular identifier' of the GMO event**.**
The identity of any detected target sequence can be verified by an appropriate method such as melting curve analysis (after SYBR Green I real-time PCR), by applying sequence-specific probes (e.g. "Taqman" technology), by means of restriction analysis, hybridisation, and ultimately by DNA sequence analysis. The specificity of the primers/probes shall always be experimentally evaluated by testing the possibilities to discriminate between the target and other, closely related sequences.
The use and development of molecular screening tools (commonly used to date are the CaMV 35S promoter and/or the *Agrobacterium* NOS terminator) is only possible in well-designed screening and detection strategies. Technical performances (sensitivity, specificity, …) of this kind of tools are important but their potential has to be exploited in scientifically sound decision trees taking into consideration effectiveness, quickness and cost. In this context, it will be essential to develop new screening tools (to complete the existing ones) in the context of standardized screening strategies.
Thus, so-called "open-source screening methods" will have to be developed to obtain a whole image of the GMO composition and content of a sample (i.e. what can be considered as an 'open source'). Such an approach however raises a lot of issues. Which **criteria** should be established for **analytical-grade DNA** as input in PCR assays? Which parameters determine the purity (e.g. presence of inhibitors) and integrity (e.g. DNA degradation) of analytical-grade DNA and how can these be evaluated in a simple and straightforward way? What should a screening method test or look for? Which criteria are valid for **screening elements** to be included in a generic screening model? Which decision criteria to consider in the choice of screening elements? What should be the **result(s)** of a generic screening methodology and how will these results allow creating a whole image of the sample composition? Which **decisions** are to be made based on generic screening results and how especially should combinations of results of elements be handled for event-based interpretations? How can a minimum set of methodological criteria for GMO screening be **validated**?

All these issues need to be discussed in depth and formalized into concrete experimental assessments. The final outcome should be preferentially incorporated into a (matrix-based) screening format, allowing an assisted decision-making in GMO detection.

B. *Facing the problem of detection of an increased number of events without PCR*

For the time being, the only theoretical alternative to try to solve the detection of a high number of events without PCR would be the use of DNA arrays provided that direct hybridisation would be possible. Nowadays, biochips can only work if the hybridisation is done with DNA having gone through an amplification reaction - not necessarily PCR but to be detected each possible target of the array should be multiplied - and thus not directly on a DNA extract. In this latter case the number of targets which might hybridise with the capture probes of the array and then be detected is generally too low to give rise to signals above the background noise. Maybe with more research this drawback could be lifted and DNA arrays would then become much more powerful tools.

## THE ISSUE OF DETECTION OF UNAUTHORIZED GMOs

To date, most GMO detection analyses are focused on authorized GMOs and in particular on compliance with product labelling requirements (especially in Europe and Japan). However, the detection of unauthorized GMOs, certainly if they have not been submitted to a thorough satisfying safety assessment, is by far much more important at least from public health perspective.

Events of recent years, like the BSE crisis, have led to set up alert systems in case of emergencies like the “rapid alert” procedure put in place whenever something harmful for the food chain is to be found within the EU territory. Within this scope, the unintended release of some unauthorized GMOs on the US market (e.g. the Bt10 maize and LL601 rice crises[2], see chapter on legislation) activated the rapid alert process. In those cases, the European Community Reference Laboratory (CRL) for GMOs (housed at JRC-IHCP, Ispra, Italy) plays a key role in providing as quickly as possible a validated method and control materials for enforcement purposes. Moreover, generally the matrixes in which the presence of the event can be suspected are also more or less defined to limit the analyses (for instance in the Bt10 crisis the matrix with the highest probability of occurrence was maize glutenfeed while presence in food products was said to have a very low chance).

The main problems that can exist with unauthorized GMOs are the following: lack of an available validated detection method (if not even lack on data about sequences of the construct or the borders) or lack of control material. These limitations may seriously hamper reliable detection according to quality assurance standards. This is not necessarily the case for all unauthorized events because some being authorized elsewhere in the world may therefore be very well described, be detectable with published validated methods and available also in the form of control material or even better as certified control material. The worst case scenario is of course the appearance of a totally unknown GMO because then all information is lacking, including design of a PCR test as well as control material. Therefore, development of specific techniques or strategies for detection of unknown GMOs is one of the biggest challenges for future research.

[2] THE BT10 MAIZE CRISIS OCCURRED IN SPRING 2005, WHILE THE LL601 RICE CRISIS OCCURRED AT THE END OF SUMMER 2006.

While some ideas for tackling this problem can be put forward, coming up with a solid methodology that can be applied in a systematic way, will need much more research. Especially the advent of 'whole genome' sequencing programs allows to better define what kind of small DNA sequence patterns are possible to be found in an organism (although there is always some individual variability to be taken into account). Such an approach is being envisaged at the Norwegian Veterinary Institute [15] where an array-based strategy was designed that might flag any artificial modifications because of its absence in the known natural genome (*Arabidopsis thaliana* being taken as model). Another possibility is the use of the anchor-PCR method developed at ILVO [16, 17] in which one of the primers is based on a frequently used element of GM constructs, while the other primer is based on a linker element attached in a kind of random way to the sticky ends of DNA fragments obtained with a 4-base cutter endonuclease (see also in chapter on characterization and quantification). Moreover, the applicability of this technique for detection of low DNA target amounts was also shown [18].
The greatest pitfall in the detection of unknown GMOs, whatever the technique or strategy used, will always be related to the probability of detection of such an event. While this probability should of course need to be as high as possible, one will never be able to guarantee a 100% success rate. Moreover, in some way, any conclusion on the finding of an unknown GMO in a product will have to be supported by sound DNA sequencing data in order to provide concise proof of a non-natural origin that is presumed to arise from recombinant DNA technology. Availability of huge databases of known natural DNA sequences should furthermore be needed to provide support to the whole analytical process.

## BOTANICAL IMPURITIES

In animal feed products, the problem of cross-contamination is a challenge. A non-GM animal feed could be cross-contaminated with a GM feed (compound). According to GM food and feed labelling Regulation 1829/2003/EC, the mandatory labelling of food/feed products above the fixed threshold of 0.9%, is to be considered at the level of each single ingredient. Applied to mixed feeds, this means all components present in the feed and declared as "feed material" or feed ingredient in accordance with Regulation (EC) N° 178/2002 [19]. Consider e.g. a practical example of a maize feed in which 0.8% (0,8kg/100kg) of 100% GM soybean is present as an impurity. As long as the soybean is not considered as an ingredient (i.e. when presence is below a mass fraction of 5%), the GM soybean quantity is to be calculated in relation to the total feed, i.e. maize, and thus labelling in this case is not mandatory. Once the soybean is considered as an ingredient however, labelling of the maize feed as "contains GM soybean" is necessary. The challenge here is thus how to determine if the impurity exceeds or not the 5% in mass fraction and to decide if the GMO content should be calculated in terms of the total feed rather than per ingredient (in this case % GM soybean / total (maize) feed versus % GM soybean / total soybean content).

## STACKED GMO EVENTS

Until recently, the dossiers handed in for market authorisation mainly covered single GM events. Nowadays, there is a clear trend, at least in maize, to combine two or more transgenic traits present in single events through traditional breeding. Next to conventional crossing, stacked events can be obtained also by cotransformation – transformation with two or more transformation vectors – or by re-transformation of a single transgene plant with additional transgenes. The obtained plants are referred to as GM stacked events. According to current regulatory practice within the EU, GM stacked events are considered as new GMOs and therefore should be traceable throughout the food chain. If the sole purpose of testing is to determine if GM material

is present, then it may not be necessary to test for stacking. However, if for instance the gene stack is legally considered distinct from the parental GMOs and it is necessary to discriminate between authorised and non-authorised GM material, then identification of stacked material may immediately become necessary. Specific detection and quantification methods are available for most commercialised single-event GMOs ("unique events", according to Holst-Jensen *et al*. [20], and these methods may be used to identify and quantify commercialised gene stacks. Up-to-date, once the material to be analysed is no longer available as kernels, it becomes impossible to technically differentiate between materials derived from GM stacked events (e.g. MON810 x MON863) and material derived from a mixture of the GM parental lines (e.g. the separate GM events MON810 and MON863).
Food/feed product labelling in the EU with respect to GMOs is defined as the "% GM material present per ingredient". The official detection methods internationally validated and published by the CRL-JRC in case of GM stacked events have been based merely on the detection of both the parental lines in the same sample. Such an approach might result in difficulties with interpretation of quantitative results of grain or seed samples. Indeed, while on weight basis, a hybrid seed will represent a single unit, on HGE[3]-basis (i.e. copy number basis) the unit will be higher (according to the number of stacked events present in a single kernel). Compared to a weight or particle (e.g. seed/kernel) based approach, a haploid genome based approach may result in GMO quantities exceeding 100%, since the presence of e.g. two modified sequences in a single haploid genome would yield an estimated GMO concentration of 200%. The hemizygous offspring GM maize seed will yield an estimated GMO concentration of 40-60% depending on the paternal or maternal origin of the transgenic trait [21-23]. Conversely, if seed number is the prevailing unit, the same seed is deemed 100%. Thus, the measured GMO content will be lower or higher depending on the applied approach. To date, the technical solution for this issue is not foreseeable and at the EC-level propositions for a pragmatic but secure solution to the problem are being evaluated.
Related to quantification also, an important issue is whether or not a quantitative analysis of a multiple event (obtained e.g. by co-transformation) should be treated differently from a quantitative analysis of a stacked event (obtained by crossing single events), if the modified sequences in both events are the same [24].

## PLASMID REFERENCE MATERIALS

With the diversification and extension of the plasmid markers construction, an important aspect is to maintain the harmonization or standardization of the different constructed plasmids. For this, three points have to be respected: the final cloning of the targeted sequence has to be done in a uniform background (e.g. a pUC-vector) and the plasmid has to be documented by reporting essential scientific information of the construction. Finally plasmids responding to the standard procedure and presenting complete information must be safely deposited at an official collection.

[3] HGE : HAPLOID GENOME EQUIVALENT, SEE ELSEWHERE IN THIS SUBCHAPTER ON GMOS

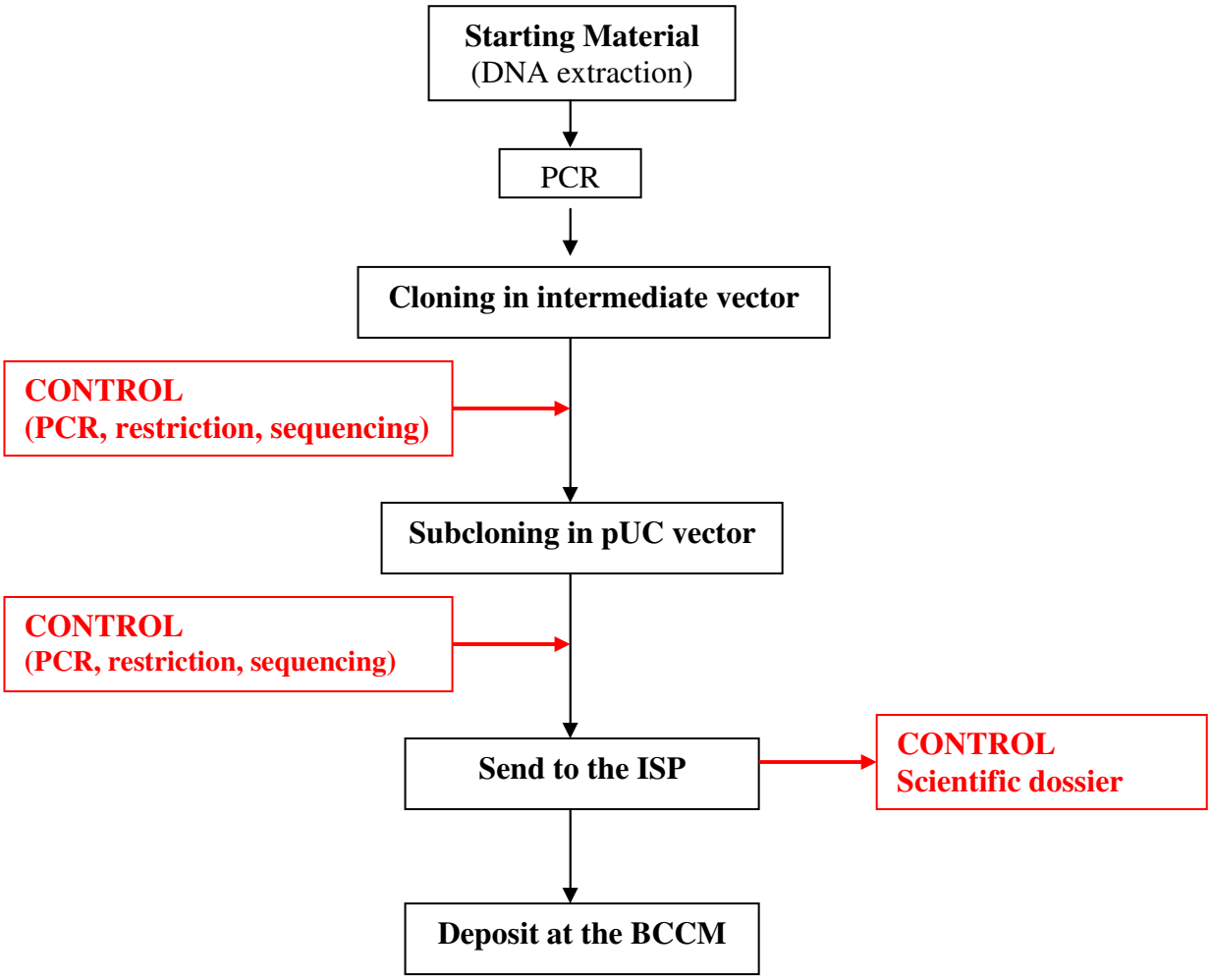


**Figure 2. Procedure for construction, documentation and deposit of a GM-marker plasmid at the BCCM collection**

During the OSTC project, the IPH has been responsible for the deposition of the developed plasmid markers at the BCCM (BCCM-LMBP[4]). Based on this experience, it will be a challenge to establish in the near future a platform allowing the rapid distribution of such GM-marker plasmids to a larger group of stakeholders.
In short, the current procedure to deposit a GM-plasmid at the BCCM collection is described in Figure 2. Once a plasmid constructed by a laboratory involved in the project is ready to be deposited at the BCCM collection, the IPH checks the authenticity of the material and the standard information included in the scientific dossier accompanying the plasmid. Then, the plasmid and the documentation are transmitted for deposit at the BCCM collection.
It is the aim to make such a plasmid collection widely available for research and enforcement purposes. Also, this platform could be envisaged to support the delivery of

[4] BCCM: BELGIAN COORDINATED COLLECTION OF MICROORGANISMS, THIS COLLECTION INCLUDES MICROORGANISMS AND PLASMIDS. IT IS MANAGED BY DIFFERENT INSTITUTIONS. THE PLASMID COLLECTION (BCCM-LMBP) IS THE ONE CONSIDERED HERE AND IS MANAGED BY THE UNIVERSITY OF GHENT (BELGIUM).

other interesting GM markers. Major players and stakeholders within the EU regarding the organisation, supervision, and management of such a platform could be the ENGL, the EC-JRC and the competent authorities of the Member States.

## CONCLUSIONS

The topics outlined above are part of a non-exhaustive list of the existing and remaining problems in the field of GMO control and monitoring. We hope to make clear to deciders or research funding organizations that, in what may appear as a very narrow topic, GMO detection, there are still a lot of interesting future research challenges which once solved will benefit to everybody.

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# Environmental contaminants: latest developments in analytical strategies and risk assessment

# Toxicological aspects of emerging environmental contaminants

**G.E.R. Schoeters**

*VITO, Flemish Institute for Technological Research, Unit of Environmental Toxicology, Boeretang 200, 2400 MOL, Belgium and University of Antwerp, Dept of Biomedical Sciences, Antwerp, Belgium*
*e-mail: greet.schoeters@vito.be*

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## SUMMARY

Concerns about persistent organic pollutants in our environment have resulted in regulations such as the Stockholm POPs convention which has been shown effective in reducing the production, release in the environment and presence of 12 harmful compounds in wild life and in the human body. Nevertheless EU guidelines are running behind the facts since meanwhile other persistent chemicals such as brominated flame retardants (BFR) and perfluoralkylcompounds (PFAS) are being found in biota, in the food chain and in the human population. Humans are also exposed to non persistent chemicals such as bisphenol A and phthalates which are used in plastics and are present at low levels in foods and drinks. Despite the worldwide distribution no adequate data on behavior in the environment and on the toxicity of most of these chemicals are available. The EU- REACH program which came into force in June 2007 should fill these gaps of knowledge and provide a scientific basis for further regulations of industrial chemicals.

## INTRODUCTION

Environmental contaminants are potentially harmful substances that have been released into the environment due to accidental spilling or to human activities such as mining, industrial emissions, agricultural practices, waste disposal, heating, traffic etc. . Contaminants can be divided into four categories: chemicals (inorganic and organic); biologicals (bacteria, viruses, moulds/fungi, dust mites and parasites); radiation; and physical (light, noise). We will deal here only with chemicals and human health risks. Chemicals are the basis of most of our products. Since 1930, global production of chemicals has risen from 1 million tonnes to over 400 million tonnes annually. In the EU, 100 000 chemicals were already registered in 1981. The current number of existing substances marketed in volumes above 1 tonne is estimated at 30000 [1]. For 99 % of chemicals (by volume), information on properties, uses and risks is sketchy. High production volume chemicals (above 1 000 tonnes per year) have been examined more closely. Still, there are no data for about 21 % of those, and another 65 % come with insufficient data [2].
The physico-chemical properties are important determinants for the behavior of chemicals in the environment [reviewed in 3]. Substances with a volatility of <1000 Pascals readily vaporize into air. Chemicals with low water solubility such as PCBs and dioxins adsorb to particles in soil, sediments and air. Another characteristic is the persistence of compounds to resist physical, chemical or biological degradation. Compounds that are halogenated, highly branched, have multiple rings, or are acyclic

are more likely to be persistent. Persistency is measured by the half life of the chemicals in environmental compartments. Chemicals that have a half life in air of more than 2 days can be transported over several thousand kilometers (UNECE LRTAP [4]) and may reach distances far away from their sources. Chemicals with half-lives in soil, water or sediments between 2 and 6 months are considered as persistent and may be taken up by organisms and transported in the food chain. The octanol/water partition constant (Kow) is another determinant of the environmental behavior of chemicals. It is a surrogate parameter for lipid/water partitioning of persistent organic pollutants (POPs). In general chemicals with a log Kow above 5 have a preference for lipophilic media and accumulate in biota[5].

## Environmental chemicals entering the food chain

Humans can be exposed to environmental chemicals by inhalation of gaseous air and fine dust particles, by dermal contact and by ingestion of contaminated food, water and dust.

There are different pathways for environmental chemicals to enter the food chain. Of special concern are those contaminants that are persistent in the environment such as heavy metals and organohalogen compounds. They may recycle between air, soil, waste, sediments and water. Persistent organic pollutants (POPs) such as dioxins, polychlorinated biphenyls (PCBs) and brominated flame retardants bind to fine particles in air and are deposited on fields and plants. They are taken up by animals and enter the food chain. These compounds also bind to soil particles and sediments and bioaccumulate in aquatic animals. The bioconcentration factor is the ability of a chemical to bioaccumulate in living tissues to levels higher than those in the surrounding environment and is expressed as the ratio of the concentration in the target tissue to the environmental concentration. Chemicals with high Kow concentrate in lipid fraction of biota, and are biomagnified in the food chain. Biomagnification can be regarded as a special case of bioaccumulation in which the chemical concentration in the organism exceeds that in the organism's diet, due to dietary absorption [6]. Organisms at higher trophic levels in the food chain contain higher concentrations of lipophilic contaminants: levels of dioxins measured in 2001 in cows milk in Belgium are 1 pg TEQ/ g milk fat, while in the same period human breast milk samples showed a mean value of 29.4 pg TEQ/g fat [7]. Humans are top predators and as such human breast milk accumulates high levels of persistent organic pollutants. These pollutants are transferred to the newborn which is highly exposed in a sensitive stage of life.

PCBs and dioxins are well studied compounds. Regulatory measures have been taken over the last decades (eg. Stockholm POPs convention) and this has resulted in decreasing concentrations of these chemicals in the environment, in feed and food and in the human body. Concentrations of other persistent substances such as brominated flame retardants are increasing in the environment. Brominated flame retardants (BFRs) are added to polymers to improve fire resistance of electronic equipment, plastics and textiles. There are five major classes of BFRs: brominated bisphenols, diphenyl ethers, cyclododecanes, phenols and phthalic acid derivatives. The first three classes represent the highest production volumes, with the major BFRs tetrabromobisphenol A (TBBPA), hexabromocyclododecane (HBCD), and three commercial mixtures of polybrominated diphenyl ethers (PBDEs), namely deca-BDE, octa-BDE and penta-BDE [8] (Fig 1). The technical mixture of decabromodiphenyl ether (DecaBDE), in which the bromodiphenyl ether 209 (BDE-209) is the dominating congener, now dominates the market among the PBDE products [9]. In 2001 the global market demand was estimated as 203740 metric tonnes. These compounds have entered the food chain and levels in fish have increased exponentially over the last years. Total PBDE concentrations in human blood, milk, and tissues have increased by

a factor of approximately 100 during the last 30 years; this is a doubling time of approximately 5 years [10].
Another class of persistent organic compounds which recently became of concern are fluorinated surfactants. These compounds have been used extensively in numerous commercial applications including surfactants, lubricants, paper and textile coatings, polishes, food packaging and fire-retarding foams since the 1950s and are now detected in environmental and human samples worldwide [11,12]. They can be divided in three major groups, which include the perfluoroalkylsulfonic acids and derivates, the perfluorinated carboxylic acids and the fluorotelomers. The perfluorinated compounds (PFAS) are fully fluorinated in the hydrophobic tail, whereas the fluorotelomers contain non-fluorinated sites, typically methylene groups, near the head group (Fig 1). It is primarily the area tied to the head group that is subject to degradation in the environment. The perfluoroalkylsulfonic acid derivates include primarily the perfluorooctanesulfonic acid (PFOS) salts and sulfonamide derivates of PFOS, such as perfluorooctanesulfonamide (FOSA) and the alkylated perfluorooctane-sulfonamidoethanol (FOSE). The PFOS derivates are prepared by electrochemical fluorination of octansulfonyl fluoride. The major perfluorinated carboxylic acid is perfluoroctanoic acid (PFOA), which is used in the aid of manufacturing PFTE and polyvinylidinefluoride [13]. Among other uses, PFOA is also employed in the manufacture of grease-resistant packaging for candy, pizza, microwave popcorn and hundreds of other foods. It is a component of Teflon which is used in cooking ware. Bioaccumulation of perfluoralkylated compounds is a function of carbon length. PFAS are hydrophobic but also oleophobic and will therefore not accumulate in fatty tissues as is usually the case with other persistent halogenated compounds. Their environmental behaviour is not well understood but they have entered the food chain and are of growing concern for their potential environmental and human impact [11].
Non persistent chemicals may pose a risk for contaminating the food chain as well. Examples are chemicals used in plastics and food packaging material. Examples of emerging chemicals are bisphenol-A and phthalates (Fig 1). These compounds are not persistent in the environment but continuous exposure results in the increasing presence of these chemicals in humans. Bisphenol-A (BPA) is extensively employed in the production of epoxy resin and polycarbonate plastics for use in food and drink packaging industries. Resins containing bisphenol-A are commonly used to coat metal products such as food cans, bottle caps and water supply pipes. This chemical and its derivatives may leach from such polycarbonate and epoxy resin products leading to exposure of humans predominantly [14].
Phthalates are the dialkyl- or alkylesters of 1,2-benzenedicarboxylic acid. Phthalate diesters are the most commonly used plasticisers in the world, primarily to make polyvinylchloride (PVC) soft and flexible. In soft PVC they can account for up to 40%. Phthalates have been widely used for more than 50 years and are contained in a large variety of industrial and consumer applications. Typical products containing phthalates are automotive applications, building and construction materials, cables and wires, floorings, food contact material, toys, paints, adhesives, medical devices, pharmaceuticals and cosmetics. Most of the phthalates are non-volatile clear liquids with little or no odour. Diethylhexylphtalate (DEHP) is the most abundant compound. More than 2Mt of DEHP are produced annually worldwide. Phthalates are not covalently bound in the products into which they have been incorporated and therefore relatively freely leach from them during manufacturing, use and after their disposal [15]. In general the primary route of human exposure to phthalates (and DEHP) is through ingestion, particularly ingestion of phthalate-containing foods. Their presence in foods is not only because of their movement up the food-chain, but also due to their migration from plastic containers, wrapping and other packaging into lipid-rich foodstuffs. Children may be particularly exposed to phthalates through breast milk and infant

formula, but also through swallowing of saliva containing phthalates that migrate out of mouthing and teething toys and drinking bottle teats [16].

## Toxicokinetics

Compounds may directly interact with skin, with respiratory airways and with the gastro intestinal tract. This can cause irritation and sensitization. Most compounds are only toxic if they cross the external barriers and enter into the blood circulation. The physicochemical characteristics determine whether chemicals are well absorbed. Lipophilic compounds are in general well absorbed and are distributed preferentially to body fats. They are transported over the placenta and over the blood brain barrier reaching the developing fetus and the brain tissue respectively. Bioaccumulation depends on the metabolization rate. Rapidly metabolized compounds have short biological half lives and are rapidly excreted. Contaminant exposure itself can result in the induction of metabolizing enzymes such as Phase I cytochrome P450 monooxygenase (CYP) enzymes and Phase II conjugation enzymes (e.g., glucuronosyltransferases, sulfotransferases, and glutathione-S-transferases). In mammals, CYP1, CYP2, and CYP3 are of importance for the primary metabolism of anthropogenic compounds [17]. The most common inducers of CYP1A are planar aromatics [e.g., polynuclear aromatic hydrocarbons (PAHs) and coplanar polychlorinated biphenyls (PCBs)], while CYP2B and CYP3A are induced by globular molecules (e.g., ortho-chlorine substituted PCBs). The most common reaction catalyzed by cytochrome P450 is a monooxygenase reaction, i.e. insertion of one atom of oxygen into an organic substrate (RH) while the other oxygen atom is reduced to water. Phase I metabolites (e.g., hydroxylated (OH)) are subsequently metabolized via Phase II conjugation and excreted, although competition with protective mechanisms such as protein binding may result in tissue retention (e.g., OH–PCBs).

When the toxicokinetics of a compound are known, the best estimate of exposure is obtained from biomonitoring studies. Biomarkers of exposure are measured in human tissues or fluids and provide the most relevant measure of dose. Concentrations of lipophylic chemicals can be measured directly in fat extracted from breast milk or blood samples. Exposure to compounds with short half lives can be evaluated by measuring concentrations of metabolites or parent compounds in urine. Other matrices such as exhaled air or hair can be used as well depending on the properties of the compounds. These biomarkers of exposure reflect the actual dose and take into account the accumulated intake over time, absorption, metabolism and individual variability in these physiological parameters.

BFRs have been shown to be susceptible to several metabolic processes including oxidative debromination, reductive debromination, oxidative CYP enzyme-mediated biotransformation, and/or Phase II conjugation (glucuronidation and sulfation). The apparent half-lives of deca- to heptabrominated diphenyl ethers in serum increase with decreasing number of bromine substituents. In comparison with the lower brominated mixtures, oral studies in rats found that decaBDE is minimally absorbed (0.3–2%), has a relatively short half-life (<24 hours), and is rapidly eliminated via fecal excretion (>99% in 72 hours). TBBP-A is likely to be fully absorbed from the gut and can undergo rapid metabolism (conjugation) via glucuronidation and/or sulphation [18].

Perfluoralkylcompounds are also readily bioavailable via both the gastro-enteral tract and the lungs. Metabolism does not seem to play a relevant role for their elimination. They are accumulated in the liver and in the blood and bind to specific proteins. In humans, PFOS has a very long elimination half-life. There are reports ranging from 2.3 to 21.3 years with a mean value at 8.7 years. PFAS can be transferred to the fetus via the placenta and later to the offspring via lactation [19]

Systemic bioavailability of BPA via oral route is lower than following subcutaneous (sc) or intraperitoneal (ip) routes of administration. BPA undergoes rapid extensive first

pass metabolism in the liver, the main metabolite being the monoglucuronide conjugate of BPA [20]. BPA can be transferred across the placenta to the rat fetus, but concentrations are much lower than in the mother. BPA glucuronide is transferred to pups in rat maternal milk, but again only in very small amounts [21].
The primary route of human exposure to phthalates (and DEHP) is through ingestion, particularly ingestion of phthalate-containing foods [16]. Phthalates undergo rapid metabolism upon entering the human body and do not appear to bioaccumulate. Phthalates are lipid soluble. High molecular weight phthalates such as DEHP are rapidly converted to polar metabolites, which may then be rapidly cleared from the body. They are not stored in body fat. Since phthalates are rapidly and completely metabolized by hydrolytic cleavage of at least one ester group, the most appropriate biomarker for human exposure to phthalates is the urinary concentration of their primary monoesters [22].

## Toxicity

Toxicity is next to bioaccumulation and persistence a major evaluation criterion for characterization of chemicals. Toxicity is multidimensional and multifaceted. Chemicals can interact with different molecular targets in human cells and tissues and may adversely affect diverse physiological functions. According to a technical guidance document a well defined set of animal tests approved by OECD should be used to identify whether toxic effects are acute or chronic and whether the effects are transient or reversible. Any assessment of toxicity requires an assessment of dose. Substances of moderate toxicity may cause concern because they are present in significant doses. Of most concern is the potency of environmental chemicals for sensitization, carcinogenicity, reproduction toxicity and neurotoxicity. An inventory of the available toxicological information from animal studies for high production volume chemicals (HPVC) showed that information on sensitization is only available for 48.3 %, on carcinogenicity only for 43.9%, on reproduction toxicity for 26%, on developmental toxicity and teratogenicity only for 32% of the HPVC [2].
Human epidemiological and biomonitoring studies raised concerns regarding substances with endocrine disrupting potency. Declining sperm counts have been observed over the last 50 years in some, but not all Western countries. The prevalence of congenital malformations in children such as hypospadias (a congenital abnormality of the urethra in the penis) and cryptorchidism (undescended testes) is increasing in humans[23]. Increased incidences of hormone-related cancers of both women (breast & ovary) and males (testes & prostate) have been observed in the West and in countries adopting Western lifestyles[14]. Some studies report that adolescents in polluted areas have changed rates of reaching puberty[24]. Studies in Denmark, Germany and USA have suggested that children born in polluted areas have some impairment of memory and intelligence[25]. Different life style factors are known to be important in the aetiology of these adverse effects. A causal link with chemical pollution has often been suggested.
Some chemicals have endocrine-disrupting properties, which means they mimic or inhibit hormones. This has been demonstrated in experimental animals and wild life such as frogs, birds, fish and molluscs. They have produced infertility and gender changes. Whether current environmental levels may be linked to similar effects in the human population is still under debate.
For the twelve POPs which are regulated under the Stockholm POPs convention it is generally agreed that there are significant potential risks to man and the environment at present levels in exposed populations. Production is banned and levels in humans and in the environment are declining. Less information is available for some of the emerging chemicals listed in table 1. Human epidemiological data are lacking except

for some occupational studies. The listed compounds show structural similarities to endogenous hormones and mimic or inhibit their binding to receptors and transporter molecules. Alternatively they may interfere with hormone metabolism or with the expression of the hormone receptors. *In vitro* screening indicates that BFRs have endocrine disrupting properties such as antagonizing binding to the androgen and progesterone receptors [26]. The structural resemblance between TBBPA and PBDEs to thyroxine (T4), which is the precursor of the active thyroid hormone 3,3',5-triiodothyronine (T3) may explain the reported interaction of these BFRs with the thyroid axis and puts forward concerns for toxic effects on neurodevelopment [26]. *In vivo* decreases in plasma T4 levels have been observed and may be linked to the developmental neurotoxic and behavioral effects described for rodents exposed to PBDEs and HBCD [27]. The estrogenic receptor seems to be activated by lower brominated PBDEs and inactivated by higher PBDEs. TBBPA has been shown estrogenic in the mouse uterotrophic assay [28]. Developmental exposure of rats with penta PBDE showed reduced sex hormones in male offspring and interference with sexual development and sexually dimorphic behavior [29]. The toxicity of deca-BDE is generally much less pronounced than for octa- and pentaBDE commercial products following acute and repeated-dose exposures. This dissimilar toxicity is likely related to the preferential accumulation of lower brominated congeners in the body, due to their greater partitioning and retention in lipid-rich tissues and lower rates of metabolism and elimination relative to decaBDE. On the other hand, Deca-PBDE has induced liver tumors in rodents and is considered as a potential carcinogen [30].

PFOS and PFOA exhibit hepatoxicity, developmental and reproductive toxicity in experimental animals even at relatively low dose levels. Changes in serum triglycerides, serum cholesterol and serum triiodothyronine are the most sensitive effects after subchronic exposure of Cynomolgus monkeys and occur at serum PFOS levels of 65 µg/mL. After PFOA exposure, increased liver weight is the most sensitive effect that has been observed in several species [19].

BPA is oestrogenic both *in vitro* and *in vivo* as shown by increases in uterus weight after exposure. BPA has been shown to have effects on spermatogenesis in male adult rats and delays puberty onset in offspring from female mice exposed during gestation [31,32].

Phthalates are well known anti-androgens and teratogens. They have been shown to produce adverse effects on reproduction and development in animals. The reproductive abnormalities in offspring range from diminished birth weight and reduced survival rate to malformations of the external genitalia, undescended testicles (cryptorchidism), retention of nipples/areolae or reduced anogenital distance in male rodents, impaired spermatogenesis and a general reduction of male fertility [33]. Most of these effects are probably caused by a modulation of testicular testosterone levels. Phthalates are suspected of acting as endocrine disrupters also in humans, affecting male reproductive tract development [34].

## Risk

Any risk assessment on chemicals is composed of two distinct elements, [1] an evaluation of the properties which are intrinsic to the chemical, called *hazard assessment*, and [2] an estimation of the *exposure* which depends on the use of the chemical. Evaluation of risks associated with food intake are carried out by committees such as the Joint Evaluation Committee on Food Additives and the World Health Organisation (JECFA/WHO) and European Food Safety Agency (EFSA) and are based on expert judgement of available information on toxicity and on exposure. The latter is often obtained by monitoring or mathematical modelling. Depending on the outcome,

the risk evaluation may lead to risk management including banning of production, restricted use and limit values for feed and food.
The above mentioned compounds are all produced in large quantities and are present in our environment, while risk assessment studies are ongoing and data remain scarce. Based upon current toxicity data and upon the precautionary principle, the EU (and the United States) has banned since 2004 the production and use of penta and octa-BDE. However, exposure to these congeners will continue during the coming decades, from products manufactured before 2004, and from imported products. Based upon its deca-BDE risk assessment, the EU has considered it to be unnecessary to ban the use of deca-BDE [35]. DecaBDE is still in use. JECFA was unable to allocate a provisional maximum tolerable daily intake (PMTDI) or provisional tolerable weekly intake (PTWI), this being the normal approach for non-genotoxic substances. Based on the limited available toxicity data, the Committee concluded that there appeared to be a large margin of exposure (MOE) (apparently around 25000) between the dose of the more toxic PBDEs considered unlikely to produce adverse effects in rodents (100 µg/kg bw/day) and the mean intake of roughly 4 ng/kg bw/day for consumers. Thus, despite the inadequacy of the data on toxicity and intake, the Committee was reassured that intakes of deca-BDE are not likely to be a significant health concern [36].
The EU has classified three phthalates, di(2-ethylhexyl)phthalate (DEHP), Di-n-butylphthalate (DnBP), and benzylbutyl phthalate (BBP), as toxic to reproduction. Thus these phthalates may cause harm to the unborn child and should be regarded as if they cause developmental toxicity to humans. Additionally they may impair the fertility in humans. These phthalates are no longer allowed in toys and childcare articles of plasticized material (Directive 2005/84/EC). For di-isononylphthalate (DiNP) di-isodecyl phthalate and di-n-octylphthalate (DNOP) a restricted usage in toys is allowed only in such toys that can not be placed in the mouth of children (Directive 2005/84/EC). The European Food Safety Authority (EFSA) has regularly been assessing phthalates. In 2005 the agency re-evaluated five phthalate chemical compounds used in plastic packaging, resulting in the raising, lowering or maintenance of acceptable daily intake limits [16].
The EU risk assessment report of bisphenol A has been published in 2003 and concluded that for consumers and for humans exposed by the environment, there is need for further information and/or testing in relation to developmental toxicology [21]. The European Scientific Committee on Food (SCF) has set a maximum limit for human daily intake of bisphenol A (BPA), which provides guidance on the use of the chemical to regulators and processors as this can be used as the basis for scientific risk assessments on whether it can be used, reduced or banned. No regulations are yet in place [37].
Perfluoralkyl compounds are not yet regulated. Several risk assessments are being carried out, but data are scarce especially data on intake by food are missing. Meanwhile the major producing companies have on a voluntary basis partially phased out the production of PFOA and PFOS. No regulation is yet in place.
To speed up the process of risk assessment and to prevent that chemicals are further introduced in the environment without prior adequate testing, the EU has recently introduced (June 1, 2007) the REACH programme on Registration, Evaluation, Authorisation and restriction processes for CHemical substances (Regulation (EC) No 1907/2006, Directive 2006/121/EC). The REACH Regulation gives greater responsibility to industry to manage the risks from chemicals and to provide safety information on the substances. Manufacturers and importers will be required to gather information on the properties of their substances. The European Chemicals Agency will act as the central point in the REACH system and will register the information in a central database and run a public database in which consumers and professionals can

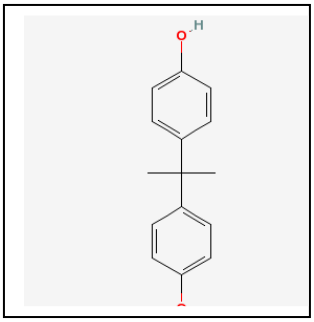
find hazard information. The Regulation also calls for the progressive substitution of the most dangerous chemicals when suitable alternatives have been identified.

**Table 1. Screening Criteria for Identifying Chemicals that are persistent bioaccumulating and toxic (PB&T)**

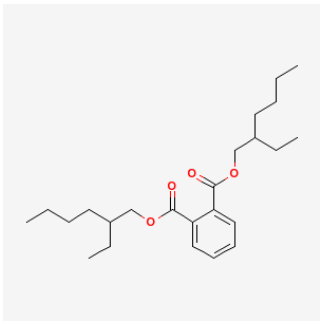
| Compounds | VPa (Pa) | Water solubility | Persistence t 1/2 (d) | Log Kow | Levels in biota | Levels in human milk |
|---|---|---|---|---|---|---|
| 2,3,7,8 TCDD | 2,0 $x10^{-7}$ (25°C) | 1,93.$10^{-5}$ mg/L | 25 to 100 years in subsurface soil | 5.8 | 0.7 ng WHO TEQ/ g fat median 152 milk samples [38] | 8.9 ng WHO TEQ/ g fat median in human milk samples of 3rd WHO study [39] |
| PCB153 | 7,55. $10^{-4}$ (25°C) | 5,25. $10^{-3}$ mg/L | | 7.14 | 0.67–2.29 (P10-P90) ng/ g fat ( milk and milk products) [40] | 67.8 ng/ g fat human milk 3rd WHO study [39] |
| TBBPA | <100 | 4.2 mg/L | 48-84 (soil/water) | 4.5-5.3 | 0.3-136 ng/ g lipid weight, mean values in aquatic biota [41] | 0.01-11 ng/g lipid from 3 studies [42] |
| HBCD | < 133 | 8 µg/L | 60 (soil) | 7.74 | 35-2945 ng/g lipid weight, mean values in aquatic biota [41] | 0.35 and 1.1 ng/g lipid [43] |
| Deca-BDE | < $10^{-4}$ | insoluble | | 9.97 | | 0.3-9.2 ng/g serumlipid [44] |
| Octa –BDE | 1.2-2.2 x $10^{-7}$ | 20-30 µg/L | | 8.4-8.9 | | |
| Penta-BDE | 4.69 $x10^{-5}$ | 2.4 µg/L | 182 (soil) | 7.88 | 22-41 µg/kg wet weight in fish [45] | 2-4 ng/g lipid in human milk and adipose tissue [45] |
| PFOA | 100 | 3.4 g/L | | Not measurable | 2.2 µg/ kg ww fish and fishery products [46] | Up to 6 µg/L serum [47] |
| PFOS | 3.31 × $10^{-4}$ | 519 mg/L | | Not measurable | 83.7 µg/kg ww fish and fishery products [46] | Up to 65 µg/L serum [47] |
| Bisphenol A | 5.3 $x10^{-6}$ | 300 mg/L | 15 (water) | 3.4 | 7 ng/g canned food [48] | 3.1 ng/mL maternal plasma 2.3 ng/mL cordplasma, placental tissue 12.7 ng/g [49] 1.4 µg/g urinary creatinine [50] |
| Phtalates | 3.4x 10-2 | 3µg/L (DEHP-22°C) | | 7.50 (DEHP) | 0.1 - 25 mg/kg food 31 samples [51] | 5oxo MEHP 22-60 µg/ L urine |
| | | | | | | 5 OH MEHP 29-75 µg/L urine [52] |

**Table 2. Toxicological characteristics**

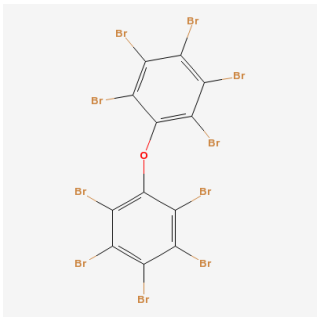
| Compounds | T1/2 Experimental animals | T1/2 Human workers | Endocrine activity as tested in vitro | Sensitive endpoints observed in animal studies |
|---|---|---|---|---|
| 2,3,7,8 TCDD | 20-60 days (rodents) [53] | 7. 5 years | Binding to Aryl hydrocarbon receptor (Anti) estrogenic [54] | Developmental neurotoxicity, immunotoxicity, reproductive toxicity, endometriosis, binding to CYP1A2 [53] |
| PCB153 | 113 days (rats) [40] | 10 years [40] | estrogenicity | Liver toxicity, thyroid toxicity, neurobehaviour, immunotoxicity [40] |
| TBBPA | < 3 days [42] | 2.2 days [42] | Weakly estrogenic Inhibits TTR binding of thyroxin Strong Inhibitor of E2 sulfation [26] | Lipid metabolic disorder and hepatic toxicity after prenatal and postnatal exposure of mice. Serum concentrations of total-cholesterol and liver weights of treated dams and offspring were higher than those of the control mice [55]. |
| HBCD | 2 hrs[56] | | Anti estrogenic Anti androgenic anti progestagenic Antagonistic for dioxin receptor activity [26] | |
| deca-BDE | | 11-18 days 11-19 [57] | | Non genotoxic carcinogenicity, neurotoxicity, [35] |
| Octa -BDE | | 37 – 91 days [57] | DR antagonistic ER antagonistic activity [26] | Increased liver weight, thyroid toxicity , reproduction toxicity, developmental toxicity, neurobehaviour [58] |
| Penta-BDE | 25-47 days [45] | Months to years [45] | DR antagonistic PR and AR antagonistic activity [26] | Increased liver weight, thyroid toxicity , neurobehavioural effects [45] |
| Bisphenol A | minutes | | Estrogenic Androgenic [26] | male fertility early onset of puberty I females mammary and prostate cancer [14, 31, 32] |
| PFOA | < 9 days (rats) | 3.8 yrs | | Liver toxicity Immunotoxicity Non genotoxic carcinogenicity Reproductive toxicity[19] |
| PFOS | > 90 days (rats) | 5.4 yrs | | Effects on liver lipids, serum cholesterol and thyroid levels Reproductive toxicity[19] |
| DEHP | | A few hrs [59] | Anti androgenic | Testicular toxicity, changes in male sexual differentiation after perinatal exposure [33, 34, 60] |



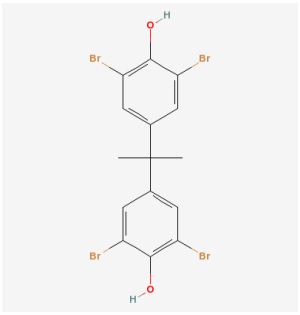
Bisphenol A



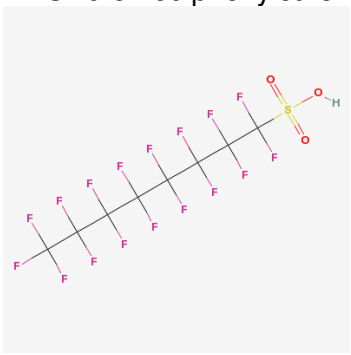
Bis(2-ethylhexyl)phthalate



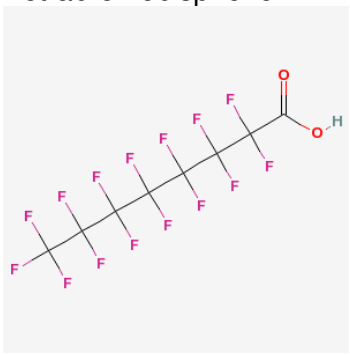
DECAbromodiphenylether



Tetrabromobisphenol A



Perfluorooctane sulfonate



Perfluoroctanoic acid

**Figure 1. Molecular structures of selected new emerging chemicals which are present in the environment and in the human body**

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# Human dietary exposure assessment to environmental contaminants

**I. Sioen[1,2], S. De Henauw[1], J.L. Willems[1] and J. Van Camp[2]**

*[1] Ghent University, Department of Public Health, UZ 2 Blok A, De Pintelaan 185, B-9000 Ghent, Belgium.*
*[2] Ghent University, Department of Food Safety and Food Quality, Coupure links 653, B-9000 Ghent, Belgium.*
*e-mail: stefaan.dehenauw@ugent.be*

**Keywords**
Exposure assessment; Environmental contaminants; Dietary intake; Modelling; Risk assessment

## SUMMARY

Food contains a wide range of substances which are either desired, such as nutrients and other components such as bioactive compounds, or undesired, such as environmental contaminants. When considering the undesired substances risk assessments can be executed to ensure that consumers risk no harm caused by the presence of those substances. In this article, we focus on exposure to environmental contaminants via food. So, no other exposure routes than food were considered. Exposure assessment is the third of four steps in the risk assessment process and includes the qualitative and/or quantitative evaluation of the intake of the contaminant under consideration. When only food is considered, the term dietary exposure assessment or intake assessment is used. Three important aspects are related to dietary exposure assessment: (1) the collection of food consumption data, (2) the collection of contamination data, and (3) the methodology needed to combine both data. In this article, we tried to give an introduction to the existing methods that can be used to estimate dietary intake of environmental chemicals and the different aspects related to those methods. Additionally, examples related to the Belgian populations are added to illustrate the methods introduced.

## INTRODUCTION

Food contains a wide range of substances which are either desired (nutrients, additives and other components such as bioactive compounds) or undesired, such as natural toxins, pesticide residues, mycotoxins, or contaminants. For all these substances, excessively high but in some cases also insufficiently low amounts can create a risk [1]. In the EU Regulation (EC) No 178/2002, risk is defined as 'a function of the probability of an adverse health effect and the severity of that effect, consequential to a hazard' [2]. This article focuses on the presence of environmental contaminants as undesired substances in foods. To ensure that no harm results to consumers, a risk assessment can be executed.

Risk assessment is defined as a four-step process: hazard identification, hazard characterisation (dose-response relationship), exposure assessment, and risk characterisation. The hazard identification involves the determination of the different compounds to include in the analysis. Hazard characterisation involves the quantitative description of the level at which a compound has potential to cause adverse effects

(based on dose-response relationships). Exposure assessment is defined by the WHO as the qualitative and/or quantitative evaluation of the likely intake of biological, chemical or physical agents via food as well as exposure from other sources if relevant [3]. When only food is considered as source, the term 'exposure assessment' is often replaced by 'intake assessment' or 'dietary exposure assessment'. The latter step, risk characterisation, integrates the information collected in the preceding three steps [2]. It interprets the qualitative and quantitative information on the toxicological properties of a chemical in combination with the assessed amount to which individuals (parts of the population, or the population at large) are exposed [1]. This text focuses on the third step of the risk assessment process: the dietary exposure assessment and this related to exposure to environmental contaminants.

In the case of environmental contaminants, exposure assessment is based on three major aspects: (1) how to determine the consumption patterns of the individual foods containing the relevant contaminants; (2) how to determine quantitatively the presence of a contaminant in individual foods and diets, including its fate during the processes within the food production chain; (3) how to integrate both the contaminant concentrations and the consumption patterns to estimate dietary exposure [1, 4]. Several methods can be used to assess the intake of a contaminant, and the choice will depend on the information available and how accurate and detailed the estimate needs to be [5]. No single method can meet all the criteria that refer to cost, accuracy, time frame, etc [1, 6]. In this article, we tried to give an introduction to the existing methods that can be used to estimate dietary intake of environmental chemicals and the different aspects related to those methods. Additionally, examples related to the Belgian population are added to illustrate the methods introduced.

## Collection of consumption data

In this paragraph epidemiological techniques to measure dietary habits and food consumption patterns of individuals or groups of people are described. In principle, to assess food consumption four different types of data can be used: food supply data, data from household consumption surveys, data from individual dietary surveys of individuals, and the collection of duplicate diets [1]. The first three are described in more details below. The fourth, duplicate diets, are described in the paragraph about the collection of contaminant concentration data because this method also includes the analysis of the contaminant content.

### *Food supply data*

Food supply data provide gross annual estimates of the national availability of food commodities. They are calculated in food balance sheets (FBSs), which are accounts, on a national level, of annual production of foods, changes in stocks, imports and exports, and agricultural and industrial use. The result is an estimate of the average value per head of the population, irrespective of, for instance, age and gender. Food supply data refer to food availability, which gives only a crude impression of potential average consumption. FBSs are especially used for assessing trends over time and for comparing the food availability of different countries [1]. The FAO has published FBSs since 1949, available on www.fao.org. When using this information for a dietary intake assessment, only a crude estimate on population level is obtained giving a very general view but no information on individual level. Such information is mostly used to study trends over time or to compare the exposure for different countries. No examples were found in published literature where FBSs were used for exposure assessment of the Belgian population to environmental contaminants.

*Household budget surveys*

Household budget surveys (HBSs) give information on the purchase of food in terms of expenditure and is used for economic policy. The amount of foods and drinks brought into the household are recorded. In most cases, only the expenditures of meals taken at home are noted. In general, household surveys do not provide information on how food is handled within the household, or on actual consumption by its members. A period of two weeks is usual for this kind of surveys [1]. This information is mostly used to compare the use of food items between different socio-economical groups and geographical regions within a country and to monitor changes in dietary patterns over time.

Post-harmonization of data already collected at national level, in the context of HBSs was accomplished through the Data Food Networking (DAFNE) initiative. The DAFNE project exploits food and socio-demographic data collected in the HBSs, aiming at the development of a cost-effective food databank that allows monitoring of food availability both within and between European populations [7]. However, no examples of using these data for exposure assessments to environmental contaminants could be found.

*Individual dietary surveys*

Dietary surveys of individuals can be divided in two categories: **record** and **recall** methods. Record data collect information on current intake over one or more days and are the most accurate and detailed when measuring the dietary intake of individuals. Recall methods reflect past consumption, varying from intake over the previous day (24-hour recall) to usual food intake (food frequency questionnaire or dietary history) [1, 8]. Moreover, record methods are also distinguished in **long-term** and **short-term** methods. Long-term methods are used to describe the usual food intake over an extended period of time (e.g. a season, a year). In contrast, short-term methods are used for the collection of consumption data of a limited period of time (one to seven days).

Four different methods are distinguished:

(1) **Food records** (also called dietary records or food diaries) are kept for a specified time period, usually 1 to 7 days. If total daily intake is required, the food records should include all foods and beverages consumed at meals and in between, in quantified amount [1, 8]. Respondents need to record all the food consumed at home and outside on the moment of consumption. This method leads to quantitative and qualitative data on individual level. The strengths of the food record include (1) a high accuracy of portion size, (2) a high level of detailed information, and (3) that the method asks no effort from the memory which minimizes the mistakes. In contrast, a quite high effort is asked from the respondent in terms of collaboration and time. Some other weaknesses included that (1) it can disturb the normal eating pattern and (2) the analysis of the collected data is quite expensive and time-consuming. It is known that the correctness of this method is decreasing when the number of days increases [8]. As an example, food record data from Flemish adolescents were used for the intake assessment of dioxin-like substances by Vrijens et al. [9]. More details about this intake assessment is given further on in this article.

(2) In the **24-hour recall** method the subject is asked by a trained interviewer to recall and describe the kinds and amounts of all foods and beverages ingested during the immediate past, mostly a 24- or 48-hour period. Food quantities are usually assessed by using household measures, food models, or photographs [1, 8]. This information can be used to describe the mean intake of a group of people. The strengths of this method

include that (1) it asks a low workload for the respondents, (2) the respondents do not need to be able to read and write, and the approach can therefore be used in clinical settings, (3) it asks for information in the recent past, which is not so hard for the memory, (4) it leads to detailed information, and (5) there is only a small chance of disturbing the eating pattern. Nevertheless, a first weakness of this method is the chance of inaccurate reporting caused by a memory gap, the tendency to give the most desirable answer, and the method of asking. Second, this method only records information during a short period of time and as such does not reflect the usual diet.
The most recent and national representative consumption data for the Belgian population were collected in 2004 by a 24-hour recall on two different non-consecutive days as well as with a general food frequency questionnaire (see next paragraph). The report of this National Belgian Food Consumption Survey is available on http://www.iph.fgov.be/epidemio/epinl/index5.htm [10]. No publications about the exposure to environmental contaminants based on this food consumption database are published yet.

(3) A **food frequency questionnaire** (FFQ) consists of a structured list of individual foods or food groups. The aim of the FFQ is to assess the frequency with which these items are consumed during a specified period (e.g. daily, weekly, monthly, yearly). FFQs may be qualitative, semi-quantitative or completely quantitative. Qualitative FFQs only obtain the usual number of times each food is eaten during a specified period. Semi-quantitative methods allow estimation of a standard portion or ask respondents to indicate how often they consume a specified common amount. A quantified FFQ allows the respondent to indicate any amount of food typically consumed. The FFQ is often used to rank individuals by food or nutrient intakes and also by food group intakes so that high and low intakes may be studied [1, 8]. The strengths of the FFQ are that (1) it focuses on usual intake, (2) the results are useful to study diet-disease relationships, (3) an FFQ is relatively easy to compete, code and scan, and (4) it is a relatively inexpensive method. In contrast, an FFQ fails to measure details of dietary intake. Moreover, quantification of the intake is not always very accurate since it is difficult to report food items consumed in mixtures and respondents have difficulties with recalling frequencies of intake. It is known that FFQs with a long food list tend to overestimate food intake [8].

(4) With the aid of a **dietary history method**, a trained interviewer assesses an individual's total usual food intake and meal pattern. The respondent is asked to provide information about his/her pattern of eating over an extended period of time (often a typical week) and also to recall the actual foods eaten during the preceding 24 hours. In addition, the interviewer completes a checklist of foods usually consumed. Finally as a cross-check, the respondent is often asked to complete a 3-day estimated record [1, 8]. This method has potential to assess usual mean patterns together with details of food intake and preparation. Moreover, it reflect quite accurate long-term food intake. However, disadvantages of this method include that (1) this method leads to high respondent burden, (2) highly trained interviewers are needed, and (3) the results are difficult and expensive to code.

It should be noted that there is no single ideal method to assess food consumption. The choice depends on the objectives of the study, the foods of primary interest, the need for group versus individual data, the characteristics of the population, the time frame of interest, the level of specificity needed for describing foods, and available resources. Currently, most methods to assess food consumption are not developed explicitly from the perspective of risk assessment, and the available data are used for other purposes than the original ones as well [1].

## Collection of concentration data of environmental contaminants

Heavy metals, PCBs and dioxins are well-known environmental contaminants. Most of these contaminants are due to environmental pollution; however, for some heavy metals also natural sources play a role. Contamination of products of animal origin with environmental contaminants is largely due to the pollution of the animal feed or the pollution of the living environment (e.g. the aquatic environment in the case of fish and other seafood) [1]. Another source of contamination can be misuse of components, e.g. the Belgian dioxin crisis in 1999 caused by the use in animal feed of recycled fats which had been contaminated with discarded synthetic materials containing PCBs and dioxins. Many scientific papers are available describing the effects and consequences of the Belgian dioxin crisis [9, 11, 12]. Some of them are described in more detail below.

A key component in dietary exposure assessment to environmental contaminants is the determination of the amounts of the considered contaminants in foods at various stages of processing and factors affecting the levels and characteristics of these substances. Therefore, the collection of quantitative data being accurate (i.e. agreeing with the actual concentration), and representative (i.e. reflecting the concentration of the whole group) is crucial. First, **food sampling procedures** can critically determine how close the measured value is to the real value. Relevant aspects in this regard are the representation of sampling, the completeness of sampling in relation to the distribution of the chemical within the sample, and the variation in concentrations between samples. Second, the accuracy of the **analytical methods** is important. Data collected at different time points may be affected by improvements of the available methods but also by changes in actual composition. Another aspect is lab-to-lab variation depending on the expertise of the analysts involved and differences in equipment and reagents [1]. No explicit details are given to these aspects, since this will be the topic of other articles of the chapter. However, one specific methodology of collecting concentration data are the total diet studies, which is handled in the following paragraph.

### *Total diet studies: exposure of the population*

The FAO/WHO recommends the use of total diet studies for estimating dietary exposure of the population. In total diet studies, representative samples of widely consumed foods are collected and analysed for the substances of interest. The accuracy of population intakes estimated using total diet study results depends on the extent to which the foods analysed represent important dietary sources of the chemical [1]. Three different approaches in total diet studies are distinguished. First, the **market basket** approach is based on the dietary intake of a defined population group. All food items, which are part of the average diet, are purchased, prepared according to standard household procedures, and aggregated into a number of food groups. Each food group is subsequently analysed for a number of additives, contaminants and nutrients. Second, in the **individual food items** approach, a list of foods representing the products most commonly consumed is composed based on national food consumption surveys for several age-sex groups. All selected food items are prepared according to methods most commonly consumed and analysed. Third, in the **duplicate diet approach**, the individual daily diet as consumed is analysed. The duplicate diet method is the only method where individual consumption data and contamination data are directly related [1]. Although not statistically based, total diet studies yield data useful in assessing food chemical intake. Total diet study results are used mainly for identifying trends in concentrations of pesticides residues, contaminants and nutrients

in the food supply and population intakes. However, total diet studies use only a certain number of foods to represent thousands of foods, and therefore they are not appropriate to assess intakes on individual level.

An example of a Belgian market basket approach to assess the intake of a certain environmental contaminant is the market basket study executed by Voorspoels et al. [13] to estimate dietary polybrominated diphenyl ether (PBDE) intake. Shortly, a food market-basket, representative for the general Belgian population, containing various meat, fish and dairy food products, was assembled and analysed for its PBDE content. Additionally, fast food samples were also investigated. Based on the measured PBDE levels, an average daily dietary intake estimate of PBDEs was calculated. PBDE intake calculations were based on the average daily food consumption in Belgium and were estimated between 23 and 48 ng/day of total PBDEs (lower and upper bound), being in accordance with what was previously reported for diets from geographical distinct areas, such as Canada, Finland, Spain, Sweden and the UK. The intake calculations were based on a theoretical estimate of the average daily food consumption (KB 03.03, 1992). The fast food intake estimate was based upon one restaurant visit each two weeks. Intake calculations did not include any fruit or vegetables, which (should) contribute to a great extent to the total daily diet. Levels in these foods however, are expected to be very low, seeing the low fat content of these products and the lipophilicity of the pollutants under investigation. Consequently, the intake assessment results are not comprehensive, but rather indicative [13].

Robberecht et al. [14] used a duplicate diet approach to assess the daily dietary total arsenic intake by adults in Belgium. The mean intake value was below the 30 µg/day-detection limit of the method, except for fish-based diets. Arsenic analysis of various foodstuffs revealed that rice products, but especially fish and fish products are important sources of arsenic.

## Methodologies for dietary exposure assessment

When one wants to use on the one hand consumption data and on the other hand contamination data to calculate the exposure to environmental contaminants, a method for combining both data is needed. In its broadest sense, the model to represent dietary exposure can be considered as: *Consumption x Concentration/Residue = Dietary exposure*. There are, however, three different approaches for combining the consumption data with contaminant concentrations: deterministic modelling, simple distributions, and probabilistic analysis.

*Deterministic modelling*
Deterministic modelling involves using a single estimate of each variable within the model [15]. In the context of intake assessment, the term ‘point estimate’ refers to a method whereby a fixed value for food consumption (such as the average or high level consumption value) is multiplied by a fixed value for the concentration and the intakes of all sources are then summed. Deterministic modelling is commonly used as a first step in exposure assessment because it is relatively simple and inexpensive to carry out. However, this approach does not provide insight into the range of possible exposures that may occur in a population [1, 16] and it also obscures the ability to determine which scenarios present a risk that is likely to occur [17]. In fact, in the total diet studies previously described, deterministic modelling is often applied in order to assess the intake of the considered population.

An example of deterministic intake assessment to environmental contaminants is the study of Focant et al. [18]. They analysed 7 polychlorinated dibenzo-p-dioxins (PCDDs), 10 polychlorinated dibenzofurans (PCDFs) and 4 non-ortho (coplanar) polychlorinated biphenyls (cPCBs) in 197 foodstuffs samples of animal origin from Belgium during years 2000 and 2001. Based on levels measured in the samples, an estimation of the dietary intake was made, being 65.3 pg WHO-TEQ/day for PCDD/Fs only (1.00 pg WHO-TEQ/kg bw/day, for a 65 kg person) and 132.9 pg WHO-TEQ/day if cPCBs were included (2.04 pg WHO-TEQ/kg bw/day, for a 65 kg person) [18].

*Simple distributions*
'Simple distributions' is a term used to describe a method that employs distributions of food intake but uses a fixed value for the concentration variables. The results are more informative than those of the point estimates because they take account of the variability that exists in food consumption patterns [1, 16]. This approach is widely used for the intake assessment of macro- and micronutrients, where one value is used to represent the content of each nutrient in each food. No Belgian example was found where this methodology was used to assess the exposure to environmental contaminants.

*Probabilistic analysis*
In contrast to the deterministic approach, probabilistic analysis involves incorporation of the variability and/or uncertainty for the different parameters. As such, it takes into account all the possible values that each variable could take and weights each possible model outcome by the probability of its occurrence [15]. Food consumption and nutrient/contaminant concentration data can be entered in probabilistic models by two different approaches: non-parametric or parametric. The choice of the approach depends largely on the data resources available. In the **non-parametric** approach all the individual data are used as such, without assuming any underlying probability model. In the **parametric** approach probability distributions are fitted to the available data. This process involves making assumptions about the underlying mathematics of the distribution. In a next step, random values are drawn from these distributions and used as input for the mathematical model which describes the intake assessment process [16]. The probabilistic approach requires appropriate modelling software. The probabilistic analysis for intake assessment permits the exposure assessor to consider the whole distribution of exposure, from minimum to maximum, with all modes and percentiles [1].

An example of a probabilistic intake assessment to environmental contaminants is the study of Vrijens et al. [9]. The objective of the study was to perform a dioxin intake (and dioxin body burden) estimate based on a probabilistic intake assessment of PCDDs, PCDFs and dioxin-like PCBs because of the so-called 1999 `Belgian dioxin incident'. Monte Carlo simulation techniques were used to combine detailed 7-day food intake data on the individual level from a sample of 14-18-year-old adolescents with `background' and `incident-related' food contamination data. A non-parametric approach was used both for the consumption data as for the contamination data. The results showed that in background conditions, 3% of the adolescents had an intake < 1 pg TEQ/kg bw/d, while 85% had < 4 pg TEQ/kg bw/d. During the dioxin incident, the estimated median dioxin intake showed a moderate increase. On the basis of their results, Vrijens et al. concluded that the 1999 Belgian dioxin incident most likely did not increase dioxin body burden in the Belgian population and did not affect public health in a measurable way, although exceptions remain possible on the individual level [9].

## CONCLUSIONS

Several methods can be used to assess the food consumption pattern of a certain study population, to measure the level of contamination in the food item under consideration and to combine both datasets collected in order to assess the dietary exposure. Many factors will influence the choice of the method. No single method can meet all the criteria that refer to cost, accuracy, time frame, etc [1, 6]. Moreover, once a certain method is chosen, many factors will influence the representativeness of the collected data.

*Acknowledgement*
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# Consumer risk perception with regards to food products

**W. Verbeke**

*Ghent University, Department of Agricultural Economics, Coupure links 653, B-9000 Gent, Belgium.*
*e-mail: wim.verbeke@ugent.be*

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Consumer; Perception; Quality; Risk; Safety

## SUMMARY

This contribution introduces basic principles of consumer behaviour and consumer decision-making with relevance to food safety risks. Food safety is one of the product attributes that consumers can use during their evaluation of alternative products. Safety is usually considered a credence attribute, specifically one that consumers are hardly willing to compromise on. A perception filter, shaped by individual, environmental and situational factors, is responsible for the often seen gap between scientific reality and consumer perception. Whereas scientific reality pertains to manageable, measurable and repeatable practices, it is consumer perception that determines beliefs, attitudes, preference and the ultimately choice or behaviour. First, the Coca-Cola case with a relatively minor amount of initial chemical contamination, and the resulting incidence of mass sociogenic illness, illustrates the strong potential impact of attitudes and emotions with respect to risk perceptions. It also illustrates how valuable a strong brand can be in restoring consumer confidence. Second, the meat case demonstrates the strong potential impact of negative press relative to positive news. Although traceability and labelling were expected to solve part of the (real and perceived) quality and safety problems confronting the meat chain, it remains debatable whether consumers are after all interested in this type of additional information. This contribution herewith aims at providing insights and raising stakeholder's interest in a wide range of contemporary consumer behaviour issues relevant to food risk and food safety debates in the agri-food chain.

## INTRODUCTION

In a typical food consumer decision-making process, food safety is a non-negotiable product attribute. Consumers expect all food to be intrinsically safe, and a well-informed and rational consumer would never knowingly purchase or consume unsafe food. In order to shed some light on consumer risk perception with respect to agri-food products, this contribution provides some basic principles of consumer behaviour, and a selection of topical case studies. First, this paper envisages introducing basic principles of consumer behaviour and consumer decision-making that are applicable in food consumer research. To this end, consumer motivation for food choice and a classical model of consumer decision-making with related information processing concepts and influencing factors are presented. Particular attention is paid to the potential role of risk perception in shaping consumer attitudes and behaviour. Second, this contribution presents selected cases about consumer perception of risks in the agri-food chain.
Food safety scares have substantially increased consumer concerns towards food consumption and potential human health risks. Some of the recent issues of consumer

concern and consecutive research focus include BSE, dioxins, genetic modification, or specific outbreaks of food poisoning from microbiological or chemical contamination. Without exception, former real or perceived food safety problems extended into food scares after extensive mass media coverage. A wide diversity of studies consistently reported declining consumer confidence, deteriorating perception and decreasing consumption rates after exposure to adverse food-health communication. Ultimately, consumers vote for products with their available budget and, in accordance with perceived product value, consumers pay prices that makes up the profit of all previous agri-food chain participants. Hence, understanding consumer behaviour is critical to making the right managerial and marketing decisions, including strategic choices with respect to risk management, risk assessment and risk communication.

## CONSUMERS' FOOD CHOICE BEHAVIOUR

### *Motives, decision-making and influencing factors*

Motives or consumer motivations perform a central role in consumer behavioural processes. Motives are defined as enduring predispositions that direct behaviour towards attaining specific goals or objectives [1]. Motives function both to arouse behaviour and direct it to certain ends [2]. Given the primary role of motives in arousing needs and generating specific behaviour, motives or consumer motivation are discussed first. The best known classification of motives was presented by Abraham Maslow [3], who introduced a hierarchy of motives ranging from physiological, over safety, social, esteem to self-actualisation motives. The idea is that consumers will satisfy the basic motivational level first, before trying to satisfy higher levels in the hierarchy of motives.

Von Alvensleben [4] provided an overview of the major motives for food demand. In the specific case of food choice behaviour, satisfying hunger and thirst emerge as the basic physiological motives. In today's affluent society with plentiful of food, the physiological motive mainly pertains to optimum satisfaction of nutritional needs, hence avoiding over nutrition and related problems with overweight or obesity. Physiological motives may help explaining consumer choice of low fat diets or functional foods, for instance. Safety constitutes the second level motive, which in the case of food is quite straightforward. Consumers may decide to accept organic foods and reject GM foods for safety motives. The physiological and safety motives in food choice are strongly linked to health. The third level motive, social motive, includes belongingness, love, friendship and affection. Specific food choices for special occasions, or food choice in compliance with important referent persons (social norms, religious motives) fit with this social motive. Also environmental or political motives for food choice are related to the social motive.

Once physiological, safety and social motives are satisfied, consumers will aim at esteem, prestige and status. Food choices in accordance with this type of motivation are for instance the purchase luxury foods or goods, or food choice for specific hedonistic motives (e.g. full fat products). Clearly, conflicts between esteem motives and lower level motives, like safety and health, may emerge. The ultimate level is self-actualisation or self-fulfilment. To some extent, consumer choice for convenience foods fits with this motive. Time-savings realised in shopping, food preparation and consumption provide more room for activities, like learning, spiritualism or sports, that allow consumers actualising or fulfilling the self.

Consumer behaviour is defined as "those acts of individuals directly involved in obtaining, using and disposing of economic goods and services, including the decision processes that precede and determine these acts" [2]. From a micro-economics point of view, much emphasis has traditionally been placed on consumer decision-making

and choice behaviour. Most of the presented schemes are so-called stage models, which assume that consumers move through a problem solving process, ranging from the recognition of needs, over information search and the evaluation of alternatives, to reach the final stage of choice or purchase. Studying consumer behaviour based on stage models is also referred to as the decision-making perspective in consumer behaviour research. From this decision-making perspective, purchase is considered as one point in a particular course of actions undertaken by a consumer. In order to understand that ultimate point, examination of preceding events, such as problem or need recognition, the search and processing of information and the evaluation of product alternatives, is needed. Typically, *needs* are defined as discrepancies between the actual versus the desired state of being or feeling. Consumers feel thirsty and need a drink. It should be noted that a *want* is more specific than a need, e.g. a consumer needs a drink, and wants a particular branded soft drink. *Demand* can be considered as a want that is backed up by spending power and willingness to pay.

After realising a need, consumers can start searching for information about potential solutions to satisfy the need that was recognised. Both internal and external sources can be consulted. Internal sources typically pertain to previous experience and memory, whereas external sources include commercial or non-commercial stimuli in the consumers' environment.

The following step is the evaluation of alternative solutions on criteria that are relevant for the individual consumers in the specific situation. Such criteria are referred to as attributes, about which consumers hold specific beliefs. Beliefs about attributes, combined with attribute importance weights, result in product preference, which logically is translated into purchasing intentions. In most cases, purchasing intentions yield actual purchasing or behaviour, unless the consumer is confronted with events like out-of-stocks or in-store promotions.

Attributes are product characteristics that are either intrinsic, like taste, texture or colour or extrinsic, like packaging, brand or label, to the product. Another attribute classification distinguishes between search, experience and credence attributes. Search attributes are available for product evaluation before purchase. Typical examples are price, appearance, brand and packaging. Experience attributes can only be evaluated upon or after purchase and/or product use. Examples are taste and texture. Credence attributes are attributes that consumers cannot evaluate or verify themselves. Instead they have to put trust in people or institutions, like government controls or industry claims. Attributes relating to production (e.g. organic), processing (e.g. free of additives, free of GM ingredients) and product contents (e.g. nutrient or contaminant content) are typically of the credence type. Safety as a product attribute is mainly of the credence type. However, when safety is guaranteed through trustworthy branding of labelling, it may reach the status of a search attribute. Safety can also be of the experience type, e.g. when safety pertains to some type of microbiological risk like *Salmonella* or *E. coli*, which eventually result in immediate illness. The stage of evaluation of alternatives is also where perception comes into play, since consumers' beliefs about product attributes are strongly determined by their perception.

The classical four-stage model of the decision-making process forms the point of departure in many consumer studies. The model can be extended and integrated, first, with a "hierarchy of effects"-model as initiated by Lavidge and Steiner [5] and revisited by Barry and Howard [6]. Second, concepts related to information processing as presented by McGuire [7] and more recently discussed by Scholten [8] can be supplemented. Finally, a classification of factors or variables that potentially influence consumer the decision-making process is adopted [9] (Figure 1).

Since, in the current food situation, specific attention is to be paid to potential influences on consumer decision-making that result from communication and marketing, an information-processing concept is included in the framework. The

concept identifies communication effects in terms of ordered stages: exposure and attention to communication, comprehension, persuasion, which refers to attitude change, and finally, retention of a new attitude. This type of model was advanced as a framework for the study of persuasion in the field of social psychology, with a specific focus on the impact from persuasive communication.

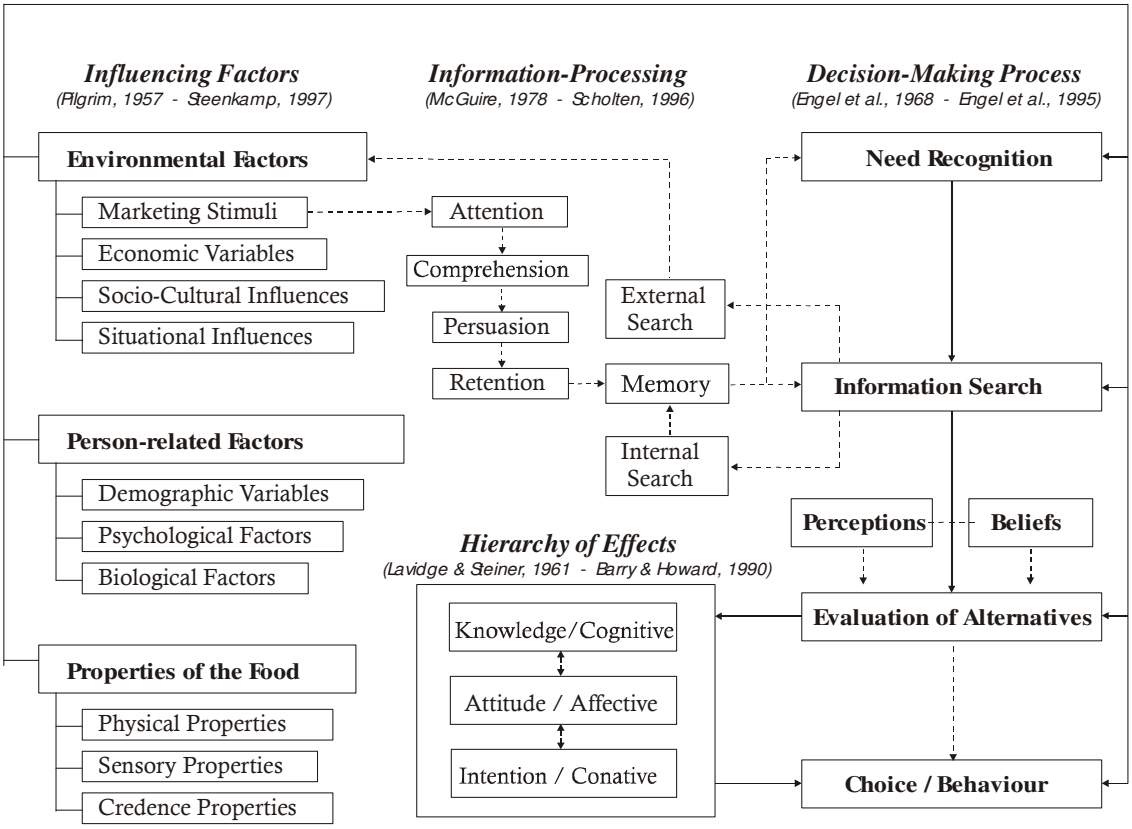


**Figure 1. Framework for consumer decision-making towards food; based on: [10]**

At any point in time or throughout the decision-making process, judgements and choices are affected by a variety of stimuli from the environment, as well as by internal processes and characteristics from the consumers themselves. Numerous classifications of stimuli have been set forth in literature. Also, it has generally been recognised that boundaries between groups of stimuli are fuzzy and that factors can be mutually exchangeable between groups. Marketing stimuli, the economic and socio-cultural environment, as well as situational influences constitute the consumers' environments. Person-related factors or individual difference variables relate to demographic, psychological and biological characteristics of the individual consumer. Classifications of food properties, like intrinsic versus extrinsic, or search, experience and credence attributes, have yet been discussed before. Combinations of these factors explain why some consumers go through all steps of the decision-making process for particular products or in particular cases, whereas or they don't in others.

*Safety risks and their perception by consumers*

In recent years, it seems that consumers are overall uncertain about the safety and quality of their food, despite the fact that our food has never been safer before. Safety is one of the factors that determine food purchase intentions. Under normal conditions,

the majority of consumers are not anxious about product safety, although a certain fear is always present in a latent state. However, the perceived safety can drop dramatically when new information is provided even without medical or scientific evidence as demonstrated by the recent events concerning BSE, GM food, Coca-Cola or acrylamid. Even when the fear appears disproportionate to food scientists, it is not the objective safety that is important to food quality perception, but it is the perceived safety that is critical.

Research shows that the public tends to judge the relative risks from food safety issues differently than experts expect them to do. Even more, there is often little relationship between the perceived hazard of a food safety concern and its actual hazard. Consumers often place much importance on factors that are of little or no relevance, whilst ignoring factors that in reality pose a substantial threat to safety [11]. Food- and lifestyle-related heart and coronary diseases, as well as lung cancer from smoking, are relatively large risks, which however are largely underestimated by consumers. Simultaneously, newly emerging food processing technologies or food-borne illnesses caused by microbiological or chemical contamination are examples of overestimations of relatively small actual risks.

Although major crises date back several years, the Eurobarometer survey from Autumn 2005 indicates that European consumers persist in expressing concerns about residues in meat, together with pesticide residues in fruits and vegetables, as well as new zoonoses such as avian influenza [12] (Figure 2).

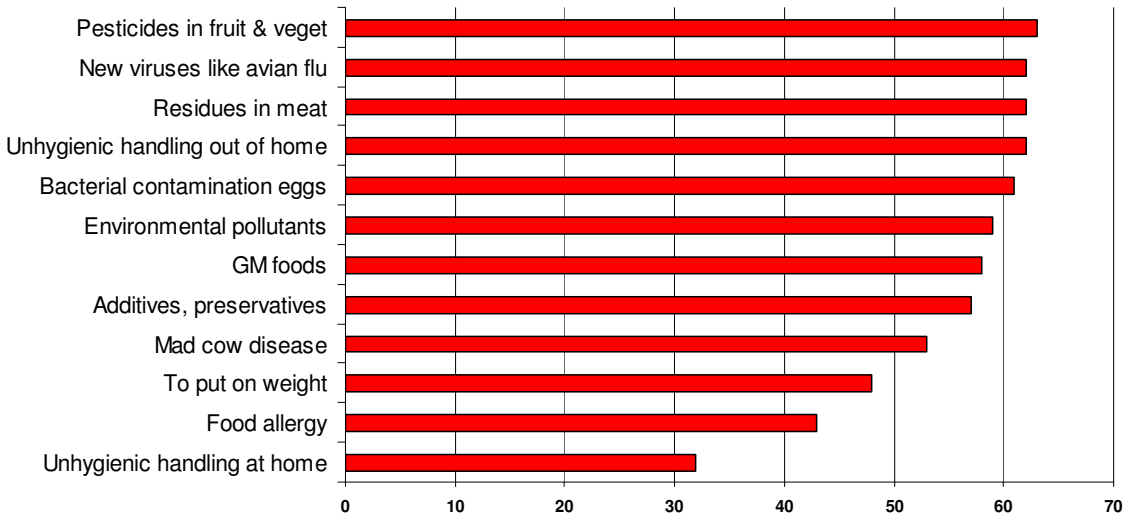


**Figure 2. Average "worry index" for possible risks associated with food. Source: pan-European consumer survey, Eurobarometer 2006 [12]**

Furthermore, the Eurobarometer findings indicate that chemicals, pesticides and toxic substances in food rank second after food poisoning as things that come to consumers' minds when thinking about possible risks associated with food in general (Figure 3). Note that "bad diets", which are in principle under control of the consumer her/himself rank very low in terms of personal worry.

A so-called perception filter is responsible for the bias between reality, scientific evidence or facts on the one hand, and consumer perception of these facts on the other hand (Figure 4). Facts result from scientific objectivity, and pertain for instance to product properties like quality, safety, nutritional value or price. These attributes or characteristics are manageable, measurable and repeatable throughout the agri-food

chain. Consumer perceptions relate to human subjectivity, and as indicated before, they often deviate from the expert view on facts and reality. The perception filter between reality and perception is to be considered as some of kind of mirror that reflects, deflects or distorts factual information. Communication, situational and individual factors are the main factors that mediate between scientific objectivity and human subjectivity. Ultimately, perceptions determine the development of attitudes and preferences, based on which buying and consumption choices are made.

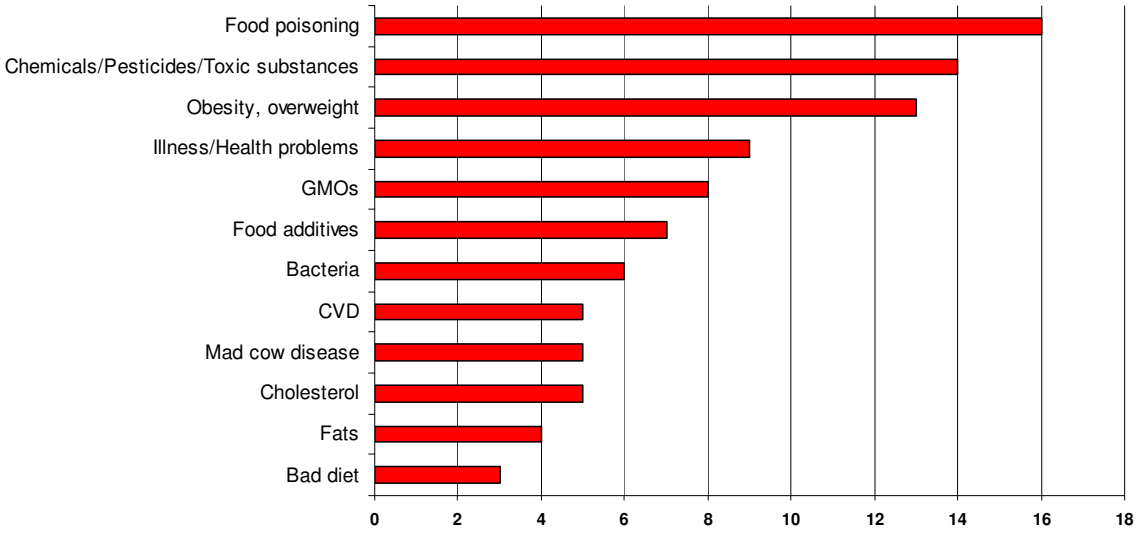


**Figure 3. What comes to consumers' mind when thinking about possible problems or risks with respect to food; % of consumers associating a hazard with food? Source: pan-European consumer survey, Eurobarometer 2006 [12]**

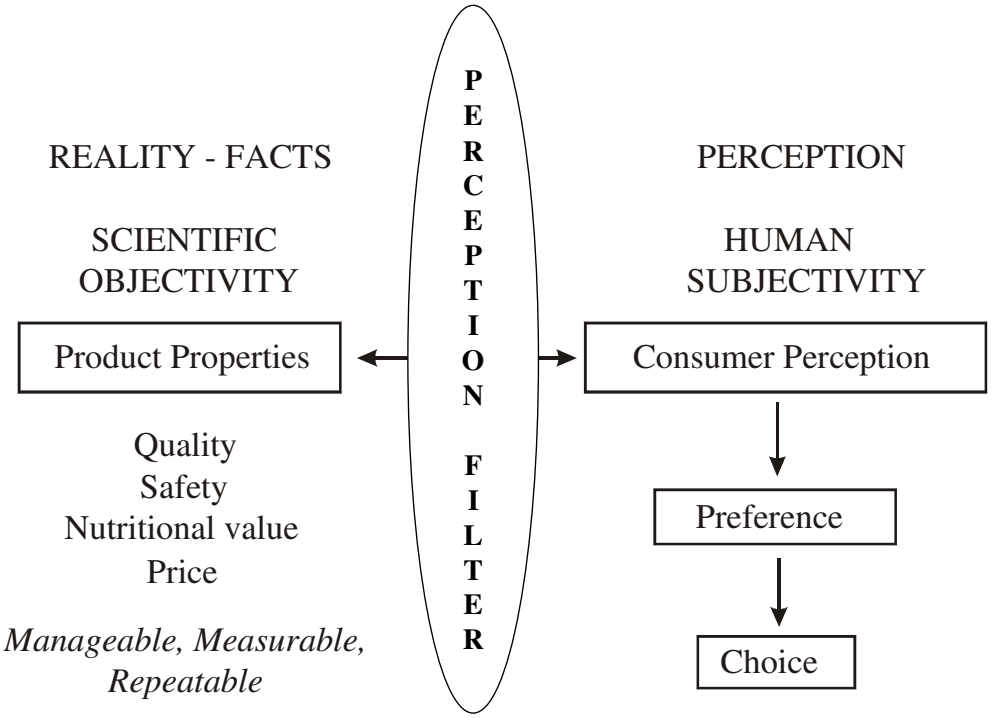


**Figure 4. Perception filter: the gap between reality and consumer perception**

*Understanding consumer's perception of safety risks*

Given the premise that food crises have emerged frequently during the last decade, a logical question relates to the pathways of food risk perception and food scare development. For instance, why did smoking never evolve into a crisis whereas BSE, dioxin, Coca-Cola in Belgium or acrylamid in Sweden did? As explained in the previous section, perceptions or beliefs about risks, which often differ from technical risk estimates, drive individual responses to risks. A good starting point for understanding consumer risk perception is provided by the psychometric paradigm developed by Paul Slovic and his co-workers [13], which has demonstrated that psychological factors determine a person's response to different hazards, including those in the area of food safety. Psychological factors of relevance include, for example, whether the risk is perceived to be *involuntary* (i.e. in terms of personal exposure), *catastrophic* (i.e. affecting large numbers of people at the same time), or *unnatural* (i.e. technological in nature). These psychological factors increase or reduce the threat value of different hazards.

Another key to understanding food scares pertains to the theory of social amplification of risk [14], providing insight in the problem why some relatively minor risks elicit often-strong public reactions. A related key to a better understanding of the pathway of crisis development pertains to a classification of three types of factors that are crucial for any problem to evolve into a crisis. Besides several psychological fright factors and panic elements, as indicated in the previous paragraph, the classification also includes media triggers. Table 1 provides a qualitative assessment of the presence or absence of these facilitating factors or catalysts for the example of risks that were imposed by BSE, dioxin, Coca-Cola and high fat diets.

**Table 1. From food safety problem or risk to crisis or scare; qualitative assessment of catalysts' presence (indicated with a number of "+"). Based on: [15, 16]**

| Catalysts | BSE | Dioxin | Coca-Cola | High-fat diets |
|---|---|---|---|---|
| Fright factors | | | | |
| Involuntary risk | + | | | |
| Inevitable risk | ++ | + | | |
| Contradictory messages | + | ++ | + | |
| Difficult to understand | +++ | + | +++ | |
| Panic elements | | | | |
| Universal risk, catastrophic | + | | | |
| New risk | +++ | + | + | |
| Believable risk | + | + | ++ | + |
| Uncertainty | ++ | ++ | ++ | |
| Unnatural, technological | + | +++ | +++ | |
| Media triggers | | | | |
| Accused / Suspects | + | ++ | | |
| Personalities | ++ | ++ | | |
| Crime | | + | | |
| Visual impact | +++ | | | + |

Fright factors pertain mainly to the individual's perception of the seriousness of a risk. Fright relates to a risk that is run involuntary, for instance, for human beings eating in the strict sense is not a voluntary decision. Contrary to generic or unbranded products like most meat, fruit or vegetables, brand choice is a voluntary decision, hence risky branded products can more easily be avoided. Furthermore, fright increases when the problem is perceived as inevitable, e.g. it can not be avoided or eliminated through personal precautions like careful cooking. This is the case with BSE or dioxin, though not with most microbiological contaminants such as *Salmonella* and *Campylobacter*. Finally, fright increases when the problem is subject to contradictory messages from different stakeholders, e.g. opposing views held by science and policy, and also resulting from a difficult understanding of the real problem or risk, even by scientists.
Panic elements pertain to the nature of the risk itself, including whether the risk is universal, new, believable and unsure. Universal does not necessarily refer to a global or world-wide exposure, though to a large potential exposure (probability), as is again more the case with generic or unbranded food products. In consumer's perception, any beef could be suspect to BSE, whereas only chicken from Belgium or the one Coca-Cola brand were suspicious during the crises in 1999 (see further). Furthermore, newness, believability and uncertainty heightened the panic value of the crises at hand. Finally, media triggers are crucial in the development of a crisis. Some elements like the presence of accused – or even better so – suspects, a link with personalities (e.g. ministers) or crime, and a strong visual impact attract mass media. BSE was exemplary in terms of visual impact, with the mad cow and UK government on stage, whereas the Belgian government and the accused animal feed component suppliers acted as ideal media triggers for the dioxin crisis in 1999. Clearly, few or none of the aforementioned fright factors, panic elements or media triggers are fulfilled in well-known risk behaviours such as smoking, alcohol or the intake of high-fat diets.

## SELECTED CASES IN FOOD RISK PERCEPTION

### *The 1999 Coca-Cola crisis*

The Belgian Coca-Cola crisis emerged on June 8, 1999, two weeks after the outbreak of the major (meat) dioxin crisis. School children complained of general malaise, nausea, headache and abdominal pain after having drunk bottled Coca-Cola. The following days, the company announced a product recall of all suspected products. Despite the product recall, the first outbreak was soon followed by more school outbreaks, alleged both to bottled and canned products. Inquiries indicated hydrogen sulfide ($H_2S$) and carbonyl sulfide (COS) in bottled Coca-Cola as the cause of odor and taste abnormalities, and identified traces of p-chloro-m-cresol on pallets and cans. However, due to the lack of adequate epidemiological or toxicological evidence, only a small number of health complaints could be directly attributed to those product-related causes. Additionally, numerous elements fitted within the configuration of epidemic hysteria outbreaks as summarised by Sirois [17] (i.e. symptoms, school setting, age group, occurrence during the last month of the school year, and type and amount of rumour). As a consequence, the hypothesis of mass sociogenic illness (MSI) has been set forth [18, 19]. A mass sociogenic illness is defined as "the occurrence in a group of people of a constellation of physical symptoms suggesting an organic illness but resulting from a psychological cause, with each member of the group experiencing one or more of the symptoms that can not be explained biologically". Frequently used synonymous terms are: epidemic hysteria, mass hysteria, and mass psychogenic illness. Only in March 2000, the Belgian Health Council formally confirmed the diagnosis of mass sociogenic illness during the Coca-Cola crisis, with the exception of the first cases.

Verbeke and Van Kenhove [20] executed behavioural research related to this food safety crisis. The basic aim was to investigate the role of the personality trait 'emotional stability' and attitude toward the brand on behaviour, i.e. the restoration of consumer trust as exemplified in the decision to cease consumption during and restart after the crisis. The findings of this research were threefold.
First, a direct and positive effect of attitude toward the brand on the behavioural response (restoration of Coca-Cola consumption after the crisis) was found (Figure 5). Both the drop at the moment of the crisis, and the restoration immediately after the crisis, of Coca-Cola's share within the soft drink expenditures of consumers with a strongly positive attitude towards this brand were significantly different from those of consumers with a less strong attitude. This finding is in line with most previous research in behavioural and consumer sciences, indicating the existence of a direct link between attitude and behaviour. Second, no direct effect of the personality trait 'emotional stability' on behaviour was revealed. However, an indirect effect from personality to behaviour was discovered. Emotional stability correlated positively with attitude, which in turn associated with a faster restoration of Coca-Cola drinking. Thus, in this specific case of a premium branded product, the impact of personality was mediated by attitude toward the brand. Third, differences in information perception and importance were found between consumers with low versus high emotional stability scores. Lower emotional stability associated with a higher need for information (or communication), and with higher importance attached to information during this (perceived) food safety crisis. Therefore, these consumers with lower emotional stability require specific attention in future communication. It is in this respect important to realise that the specific target audience of consumers with low emotional stability can not readily be identified through behavioural or socio-demographic variables as our analyses have shown.

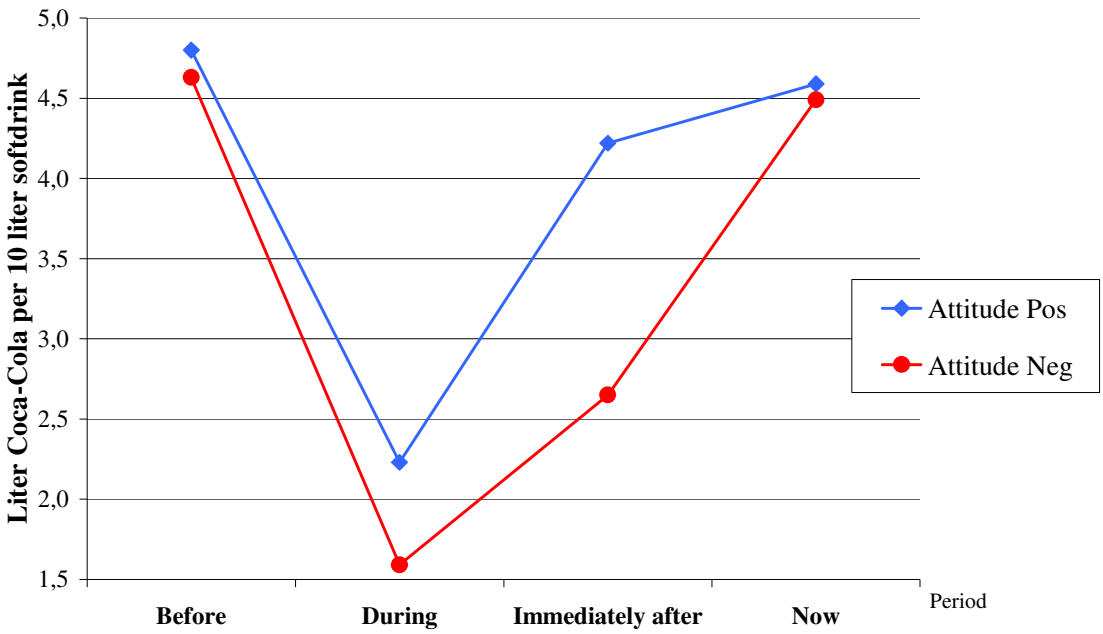


**Figure 5. Impact of consumer attitude on restoration of Coca-Cola consumption Source: [20]**

This branded food product crisis revealed that with respect to future risk and health communication, fast provision of adequate, reliable and sufficient information to consumers is crucial. Despite experience with numerous previous crises, this conclusion appeared not so obvious in practice. In the specific case of Coca-Cola, it took a whole week before the public was addressed by the company about eventual health risks, eventual causes of the problems and the actions undertaken (June 15, 1999). Government and health practitioners (through the Scientific Society of General Practitioners) received detailed information only on June 17, 1999. This meant that reliable and scientifically based information could be spread to the public only 10 days after the first incident. Uncertainty about the assessment and management of the risk and speculative mass media reports stirred up the crisis in the meantime. Furthermore, fragmentary and incomplete information from the company to government prevented targeted public health interventions, instead leading to the massive product recall enforcement guided by precautionary principles.

The Belgian Health Council drew three lessons from the Coca-Cola case. First, immediate government action should install a cell responsible for crisis management, with a single contact person who receives and spreads information to policy makers, health practitioners and the press. Second, epidemiological investigations should start immediately. Third, an ad hoc task force under auspices of the Health Council or the newly established Federal Agency for Safety in the Food Chain should be installed. It is noteworthy that mainly responsibilities, risk assessment and risk management tasks are dealt with, while the communication task itself is largely ignored. Based on the experience with the crises in Belgium, the following key elements of crisis communication were identified: background information, details about the incident, actions undertaken, sympathy, and consumer re-assurance. Clearly, it should not take days to communicate the first four elements, and doing so raises the chance of avoiding a large-scale scare. Consumer re-assurance, however, can hardly be provided immediately since it will only be successful when based on scientific evidence resulting from careful investigation. This is where the previous Health Council recommendations come into play, at least when communication is not ignored in the meantime.

Last but not least, this case of a premium branded food product facing a safety crisis demonstrated that full recovery of market share and consumer acceptance is feasible. Brand and image strength, together with favourable consumer attitudes, and appropriate (despite being delayed) marketing communications accounted for restoration of consumer trust. It is a case many generic fresh food products facing a safety crisis can only dream of.

### *The meat safety crises*

Meat production and consumption have been under heavy criticism during all the last decade. Many organisations including consumers, industry, producers and governments, as well as scientists from a plethora of disciplines, have recently been involved in debates that were initiated by numerous occurrences and stirred up by conflicting motivations and influencing factors. Meanwhile, meat has been referred to as the food item in which consumer confidence decreased most during the last decade. Distinct changes at the consumer level increasingly determine the present and future outlook of the meat chain. Meat has traditionally constituted a substantial part of the Western-European diet. Increasing economic and social welfare since the 1950's resulted in increasing amounts of animal protein intake. Top meat consumption levels were noticed during the first half of the nineties in most of the European countries, but ever since the BSE crisis, fresh meat consumption levels generally decreased. The Bovine Spongiform Encephalopathy (BSE) epidemic peaked in the UK in 1993 and

emerged into one of the major food scares in Europe from 1996 on. Public concern was driven less by the risks of BSE per se, but rather the failure of the UK government to acknowledge the *uncertainty* about BSE as a potentially causative agent of the human form of the disease, Creutzfeldt-Jakob Disease (nvCJD), prior to 1996. Public risk perception was also affected by the failure to provide information relevant to the actual concerns of consumers about food hazards, as was also seen again later with the 1999 dioxin crisis in Belgium.

The impact of mass media communication during the BSE crisis was investigated through two empirical studies in Belgium, one based on cross-sectional survey data, and the other based on time series data. Consumers who attended mass media coverage of fresh meat issues, reported significantly higher meat consumption decreases with reference to the past as well as stronger intentions for decrease in the future. It was also found that consumers who pay a high level of attention to media reports expressed higher health consciousness, more *mis*perception of health risks and higher levels of concern about potential health hazards that were frequently reported in mass media. Most importantly, the negative press effect was strongest among the younger consumers, who cut their meat consumption after attending mass media coverage in a similar way as 60+ aged consumers [21].

The negative impact from television publicity was confirmed through econometric time series analysis. Probabilities to cut fresh meat consumption were boosted as consumers reported to have paid high attention to television coverage of meat issues. Similarly, parameters of television coverage indices were largely significant and negative in an Almost Ideal Demand System for fresh meat, contrary to the estimates of the advertising expenditure variables, which were insignificant. In the case of beef in Belgium during the second half of the nineties, a negative press over advertising impact ratio of five to one was found [22]. It means that five units of positive news are needed to offset the impact of one similar negative message (Figure 6).

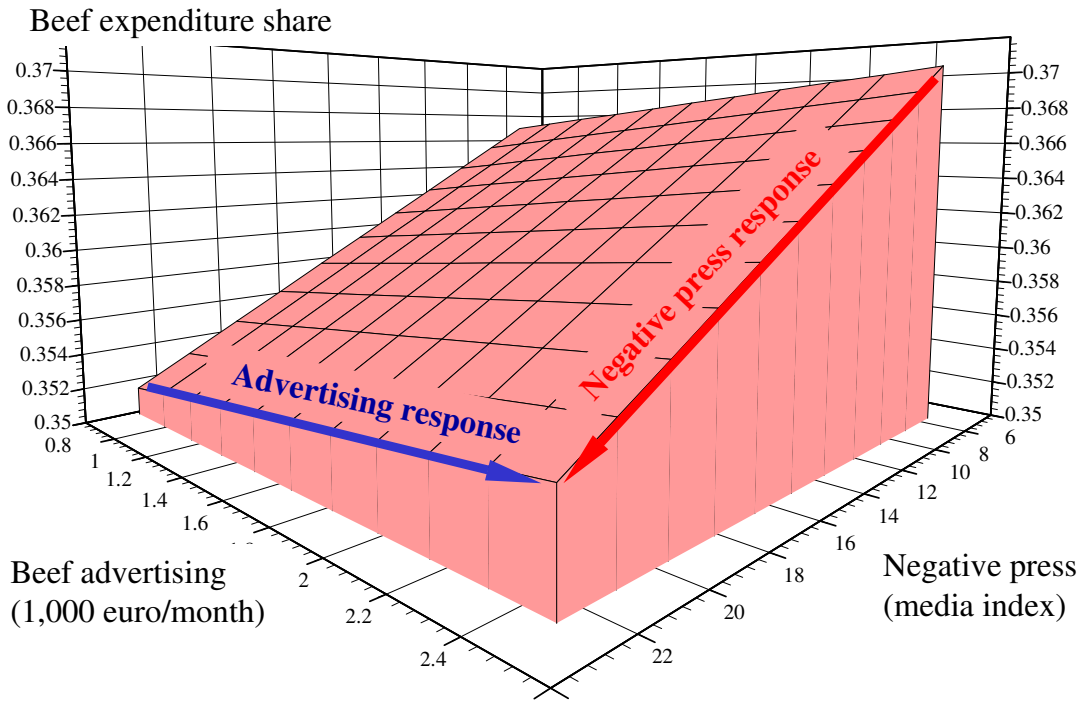


**Figure 6. Negative press versus generic advertising impact on beef expenditure share during the BSE crisis in Belgium. Source: [22]**

Traceability and beef labelling have been issues in Europe since the BSE crisis starting in 1996 and are currently also prominent in the regulatory debate in the US. The beef safety crises and subsequent decline in beef consumption, particularly in Europe, have forced governments and the meat industry to react and to work toward restoring consumer confidence. Traceability systems and subsequent quality and origin labelling of beef were considered as major instruments for addressing the problem [23]. The systems are fully operational and traceability information has been placed on meat labels. Nevertheless, consumer interest in this kind of information can not be taken granted. Communication efforts aiming at informing consumers about the existence and meaning of beef traceability failed to evoke active information search by consumers, and consumers are by far least interested in traceability cues on beef labels [24, 25].

Apparently, and despite considerable meat safety crises, consumer interest is low for cues directly related to traceability and product identification while much higher for others like readily interpretable indications of quality such as certified quality marks or seals of guarantee, as well as for mandatory standard information like expiration date. Hence, although traceability has to be in place for legal purpose and in order to help guaranteeing product quality or origin, consumers are not interested in the traceability information per se. The obvious conclusion is that the role of traceability information is mainly in the defensive, aiming at guaranteeing a safe product to the next level in the agri-food chain. Its potential in the offensive, i.e. its potential usefulness from a marketing perspective, is highly questionable. As a result, while possibly useful for legal purposes and quality management in the agri-food chain, traceability does not have to be predominant on the food label.

## CONCLUSIONS

This contribution provided basic principles of consumer behaviour and consumer decision-making with relevance to food safety risks. The paper also introduced a number of selected case studies about consumer perception of safety in the agri-food chain. Clearly, food purchasing and consumption decisions can be driven by safety-related motivations. In this case, concerns or uncertainty about food quality and safety may trigger problem or need recognition, information search and information processing.

Food safety is one of the product attributes that consumers can use during their evaluation of alternative products. Safety is usually considered a credence attribute, especially when safety related to the absence of for instance chemical residues or GM ingredients that have no immediate health impact. Notable exceptions are when safety is guaranteed and trusted through control certificates, labels or brands (in that case, safety can become a search attribute, e.g. food allergy labelling), or when safety leads to immediate health problems (in that case, safety becomes an experience attribute, e.g. presence of microbiological contamination). A perception filter, shaped by individual, environmental and situational factors, is responsible for the gap between scientific reality and consumer perception. Whereas scientific reality pertains to manageable, measurable and repeatable practises, it is consumer perception that determines beliefs, attitudes, preference and the ultimately choice or behaviour. Some food safety or lifestyle risks evolve from a problem into a crisis, while others don't. Catalysts for the evolution from problem to crisis can be classified as psychological fright factors and panic elements, and media triggers. When several of these catalysts are present to a high degree, the problem or risk stands a reasonable chance to emerge into a crisis or scare.

The Coca-Cola case with a small amount of initial chemical contamination, and the resulting incidence of mass sociogenic illness illustrates the strong potential impact of emotions and psychological with respect to food quality and safety perceptions. Relevant lessons were drawn with respect to future risk communication. The case also exemplifies the recovery potential of a strong brand that can build on its past image and mainly favourable consumer attitude. The fresh meat demonstrates the strong potential impact of negative press relative to positive news during the meat safety crises at the end of the nineties. Although traceability and labelling were expected to solve part of the quality and safety (real and perceived) problems confronting the meat chain, it remains debatable whether consumers are after all interested in this type of additional information. Some consumer segments may be, whereas the majority by far prefers more direct indications of food quality and safety like expiry date, and even brand names or price information. As indicated before, this contribution aimed at providing insights and raising stakeholder's interest in a wide range of contemporary consumer behaviour issues relevant to food risk and food safety debates in the agri-food chain. It hopefully contributes to an understanding for why consumer perceptions often deviate from expert opinions or from the way involved stakeholders would prefer consumers to perceive and behave in relation to food in general, and food safety and quality in particular.

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# Methodologies for measurement of dioxins in food

**J.-F. Focant[1], G. Eppe[1], M.-L. Scippo[2] and E. De Pauw[1]**

*[1]University of Liège, Faculty of Sciences, Department of Chemistry, Laboratory of Mass Spectrometry, CART (Centre of analysis of residues in trace), Allée de la Chimie, 6, Bat B6c, Sart Tilman, B-4000 Liège, Belgium.*
*[2]University of Liège, Faculty of Veterinary Medicine, Department of Food Sciences, Laboratory of Food Analysis, CART (Centre of analysis of residues in traces), Boulevard de Colonster, 20, Bat B43-bis, Sart Tilman, B-4000 Liège, Belgium.*
*e-mail: J.F.Focant@ulg.ac.be*

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Dioxins; Sample Preparation; Gas Chromatography; Mass Spectrometry, CALUX

## SUMMARY

This report focuses on the last development in terms of analytical strategies for dioxin measurement in biological samples. The extraction step is described through the various available methodologies available to perform efficient and reliable analyte extraction from complex matrices. Emphasis is given on the clean-up part that permits to isolate analytes of interest from co-extracted matrix interferences. The automation and coupling of the extraction and clean-up step is briefly discussed. The major part of the manuscript is dedicated to the various mass spectrometric techniques that are usable in the field of dioxin measurement. Next to the high resolution mass spectrometry (HRMS), quadrupole ion storage MS (QISTMS) and time-of-flight MS (TOFMS) are described as alternative tools. Those methods are compared in terms of potentialities for the analysis of food samples. A biological method (the DR-CALUX) is also briefly discussed to complete the analytical picture.

## INTRODUCTION

Humans all over the world are exposed to chemicals during their life time. Among the thousands of existing anthropogenic compounds, some are persistent and remain in the environment for years once generated. Polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs) are the persistent organic chemicals that are the most often measured in various types of matrices during food safety programs, environmental monitoring, and epidemiological studies. All together, they represent more than 400 individual molecules (congeners), which have to be separated from each other to ensure distinctive quantification of the target ones. Information on their toxicities and levels at which they can be measured can be found elsewhere [1,2].
PCDDs, PCDFs and dioxin-like PCBs are found at levels as low as pico- or femtogram per gram of matrix depending on the investigated food sample. In addition, matrix-related interferences are present in concentrations at orders of magnitude higher than the analytes of interest. For those reasons, accurate measurement of dioxins and related compounds at ultra-trace level in food requires high standard analytical strategies. A complex multi-step approach is required to 1) extract the analytes from the matrix core, 2) separate undesirable interferences and, 3) finally isolate, separate

and quantify analytes of interest under strict quality assurance/quality control (QA/QC) criteria. In terms of cost per sample and sample throughput, it is not only the final measurement of the analyte concentration, but – maybe even more importantly - the complex sample preparation procedure, which makes this measurement possible. Details on sample preparation for dioxin analyses are available in review articles [3,4].

## DISCUSSION

### Extraction

The extraction device presented by Professor von Soxhlet in 1879, which re-circulates the extraction solvent while accumulating extracted analytes in a heated flask, is still used in the dioxin field and is often considered as the reference extraction method, at least for solid samples such as soils, sediments and fly ashes [5].

For liquid samples, liquid-liquid extraction (LLE) has been used for a long time, mainly for biological fluids [6]. Although easy to set up, several critical drawbacks such as phase emulsions, required quantities of solvent and intensive handling make it unappealing to use.

Extractions of biological tissues using supercritical fluid extraction (SFE) have also been reported using supercritical $CO_2$ [7]. During an extraction cycle, static (fluid immobilized with the sample in the closed vessel) and dynamic (percolation of the fluid through the cell) modes can be used together to ensure both penetration of the matrix by the fluid and to avoid saturation of the fluid. Co-extracted lipids may be eliminated in part by directly adding adsorbents inside the cell [8,9]. SFE may be coupled with various analytical methods like gas chromatography (GC) but very fine tuning is then required [10].

Since first reports on the use of domestic microwave ovens to carry out extraction of organic compounds, microwave aided extraction (MAE) has been applied to organochlorine pesticides (OCPs) and PCBs present in solid matrices [11]. Selected extraction solvents usually have high dielectric constant to absorb the microwave energy efficiently.

In pressurized liquid extraction (PLE), also named PFE (pressurized fluid extraction), organic solvents are used in the liquid phase at temperatures above their boiling point. The stainless steel extraction cell can generally be heated up to 200°C and pressurized up to 3000 psi. A minimum extraction time of 10 min is usually required to ensure efficient transfer of analytes out of the sample matrix [12]. For extraction from high-fat content biological samples, quantitative recovery rates can be obtained using classical organic solvents but hexane is usually preferred to solvent mixtures because less matrix-related interferences are co-extracted under mild temperature conditions [13]. Drying of the sample can be carried out using lyophilization prior to extraction. This reduces the risk of extracting traces of water, which may lead to over estimation of the lipid content of the sample. The pre-lyophilization freezing process can be accelerated by cryo-homogenization of the samples using liquid nitrogen [14]. The dry material can then be easily homogenized before extraction.

Among potential alternative techniques, solid phase extraction (SPE) constitutes the alternative of choice for extraction of dioxins from liquid samples. Bonded-silica and styrene-divinyl benzene synthetic polymer [15] are well suited for isolating groups of compounds from sample matrix components. The use of vacuum manifold and automated SPE workstation easily regulates flow rates through the cartridges. In the case of dioxins and related compounds, the use of $C_{18}$ cartridges has been reported for water, serum and milk [16]. Good recovery was achieved when fat globules were efficiently disrupted in order to release the pollutants from the lipoproteins. It has successfully been applied in the dioxin field. Solid phase dispersion on diatomaceous

earth can also be used in SPE cartridge format. This is a valuable SPE method for the extraction of milk samples without the inconvenience of losing part of the lipids during the extraction step. Lipids are therefore quantitatively extracted from the milk matrix and isolated for gravimetric determination prior to further clean-up.

## Clean-up

Highly efficient clean-up procedures are required to purify samples issued from the extraction step prior to the final analysis and quantification. Classical solid-liquid adsorption chromatographic separations based on sorbents such as silica, alumina and Florisil, have long been regarded as important in the field [17]. For biological samples, sulfuric acid silica or gel permeation chromatography (GPC) columns are used to remove the bulk of the lipids and other oxidizable components. Basic alumina columns are then used to separate dioxins from pesticides and PCBs [18]. Activated carbon sorbent can join the column set as a complementary fractionation tool to alumina [19]. Due to its affinity for certain planar aromatic compounds, especially those with adjacent aromatic rings and electronegative substituents, carbon-based sorbent can fractionate the planar dioxins, furans and PCBs from other classes of aromatic compounds, improving sample clean-up.

The multi-sorbent clean-up procedure can be automated. The fractionation procedure can be tuned to allow the collection of different fractions [20]. To accommodate foodstuffs analysis, generally characterized by low levels of persistent organic pollutants (POPs) and high lipid content, high capacity disposable silica columns have been developed to accommodate 4-5 g of lipids on-column [14].

Coupling the extraction and clean-up steps can further be done to reduce sample and extracts handling [21]. Recent reports proposed on-line PLE extraction and clean-up for PCB analysis in solid food and feedingstuffs by directly adding sorbents such as acidic silica and activated carbon inside the extraction cell [22]. Another approach for the sample preparation of solid samples is the use of a modified version of the automated clean-up system described earlier. Either an SPE or a PLE system is directly connected to the clean-up instrument and the resulting integrated system is fully automated [21]. This system was studied for the on-line extraction and clean-up of different foodstuffs types.

## Measurement

Because of the semi-volatility of the analytes of interest, gas chromatography (GC) is the preferred approach for the final separation stage prior measurement of the individual species. The chromatographic separation relies on capillary GC columns made of appropriate lengths of specialty phases (polar and apolar) and allows to differentiate between the different congeners inside the fractionated sub-groups of compounds. Although micro electron-capture detectors (μECD) offer the required sensitivity for the measurement of selected PCBs, accurate peak identification can sometimes be difficult and mass spectrometric (MS) detectors are usually preferred.

### *GC-HRMS*

High resolution (HR) MS based on sector instruments has long been, and still is, the reference measurement method for PCDD/Fs [22]. It offers the required sensitivity and specificity in addition to valuable mass spectral information. The high sensitivity (down to the low femtogram level) is achieved using electron impact (EI) ionization, which produces abundant molecular ions, but also by operating the MS in selected ion monitoring (SIM) mode. In SIM, a restricted number of relevant masses corresponding to the analytes of interest are selected, this increases the time spent on particular masses (dwell time) and consequently improve the sensitivity. The high selectivity

results from the elevated mass resolution (c.a. >10,000, 10% valley definition) of sector instruments. This allows mass discrimination at the 0.03 to 0.05 mass unit (dalton) level in the tetra- to octa-substituted congeners mass range. The use of isotope dilution (ID) based on commercially available $^{13}C_{12}$-labeled internal standard offers accurate peak identification by means of retention time comparisons between native ($^{12}C$) and labeled ($^{13}C$) compounds, as well as accurate peak quantification by comparison of peak areas/heights [23]. This technique consists of spiking samples with an ideal internal standard, which is the isotopically labeled standard (e.g. $^{13}C_{12}$ 2,3,7,8 TCDD), showing almost identical characteristics to the compound of interest (e.g. $^{12}C_{12}$ 2,3,7,8 TCDD) The small mass difference (e.g. 12 m/z) enables the discrimination between the compound of interest and its internal standard (Table 1). A calibration performed for all the PCDD/Fs and DL-PCBs with known amounts of native and internal standard congeners allows to calculate the Relative Response Factor (RRF). The RRF takes into account the discrepancy that can be observed during MS ionization between natives and internal labeled standards. Thus, the RRF value directly affects the congener quantification. The measurement of the two most intense ions in the molecular cluster of native and labeled compounds allows to measure the theoretical isotope ratio and serves as a confirmatory procedure for peak identification.

Because the use of HRMS instruments requires high investment cost and highly skilled personal, their operation implies large investments, which results in high analysis prices. Moreover, as one observed during the 1999 Belgian dioxin crisis, rapid high throughput and cost-effective analytical methods are requested for emergency response. In addition, the completion of large scale monitoring programs requires affordable analytical methods to fit the limited budgets. This can barely be attained using HRMS instruments and alternative measurement methods are desirable. From the “MS islands” presented by Brunée in 1987 [24], quadrupole ion storage mass spectrometry (QISTMS) as well as time-of-flight mass spectrometry (TOFMS) appear to be the most promising ones when coupled to suitable GC methods such as large volume programmable temperature vaporizer injection (PTV-LV) GC, fast GC (FGC) or comprehensive two-dimensional gas chromatography (GCxGC). The potential decrease in selectivity due to the low mass resolution, relatively to HRMS sector instruments, can be counterbalanced by operating the instrument in tandem mode or by improving the chromatographic separation.

### *GC-QISTMS/MS*

QIST mass spectrometers have the capability to store selected ions [25]. The lack of selectivity due to the unit mass resolution is compensated by operating the instrument in the tandem mode (MS/MS or $MS^2$). This is referred as tandem-in-time mass spectrometry because the process takes place in 3 successive steps: 1) selected precursor (parent) ions are isolated in the ion trap after ionization, 2) their dissociation by collision-induced dissociation (CID) occurs, and 3) the product ions (daughters) are sequentially ejected from the trap according to their mass and further detected by an electron multiplier.

The use of ion trap MS/MS for PCDD and PCDF analysis is based on the specific loss of a $COCl^{\bullet}$ fragment through a unique fragmentation reaction that produces the daughter ions [26]. For each analyte, it is necessary to monitor the production of at least two different daughter ions to check the isotope ratio. Precursor species containing at least one $^{37}Cl$ atom ($[M+2]^{+\bullet}$) must be isolated to ensure the production of both $[M\text{-}CO^{35}Cl^{\bullet}]$ and $[M\text{-}CO^{37}Cl^{\bullet}]$ ions for both native and labeled compounds (Figure 1).

**Table 1. Target masses for PCDD/Fs and non-*ortho* PCBs in SIM mode for HRMS**

| | Window | monitored ions | | Ion dwell time | interscan time | Theoritical | 15% for |
|---|---|---|---|---|---|---|---|
| | (min) | Quantitation ion | Confirmation ion | (ms) | (ms) | isotopic ratios | isotopic ratios |
| **TCB** | 20-26 | 291.9194 [M+2] | 289.9224 [M] | 110 | 10 | 0.77 | 0.65-0.88 |
| **TCB $^{13}C_{12}$** | | 303.9597 [M+2] | 301.9626 [M] | 40 | | 0.77 | 0.65-0.88 |
| **lock mass** | | 316.9824 [l] | 316.9824 [l] | 50 | | | |
| **TCDF** | 26-30 | 305.8987 [M+2] | 303.9016 [M] | 100 | 10 | 0.77 | 0.65-0.88 |
| **TCDF $^{13}C_{12}$** | | 317.9389 [M+2] | 315.9419 [M] | 15 | | 0.77 | 0.65-0.88 |
| **TCDD** | | 321.8936 [M+2] | 319.8965 [M] | 100 | | 0.77 | 0.65-0.88 |
| **TCDD $^{13}C_{12}$** | | 333.9339 [M+2] | 331.9368 [M] | 15 | | 0.77 | 0.65-0.88 |
| **TCDD $^{13}C_6^x$** | | 331.9078 [M+6] | | 85 | | | |
| **PeCB** | | 325.8804 [M+2] | 327.8775 [M+4] | 100 | | 0.64 | 0.56-0.75 |
| **PeCB $^{13}C_{12}$** | | 337.9207 [M+2] | 339.9177 [M+4] | 15 | | 0.64 | 0.56-0.75 |
| **lock mass** | | 330.9792 [l] | 330.9792 [l] | 50 | | | |
| **PeCDF** | 30-35 | 339.8597 [M+2] | 337.8627 [M] | 120 | 10 | 0.61 | 0.53-0.71 |
| **PeCDF $^{13}C_{12}$** | | 351.9000 [M+2] | 349.9029 [M] | 15 | | 0.61 | 0.53-0.71 |
| **PeCDD** | | 355.8546 [M+2] | 353.8576 [M] | 150 | | 0.61 | 0.53-0.71 |
| **PeCDD $^{13}C_{12}$** | | 367.8949 [M+2] | 365.8978 [M] | 15 | | 0.61 | 0.53-0.71 |
| **HxCB** | | 359.8415 [M+2] | 361.8385 [M+4] | 100 | | 0.81 | 0.69-0.94 |
| **HxCB $^{13}C_{12}$** | | 371.8817 [M+2] | 373.8788 [M+4] | 15 | | 0.81 | 0.69-0.94 |
| **lock mass** | | 380.9760 [l] | 380.9760 [l] | 50 | | | |
| **HxCDF** | 35-42 | 373.8207 [M+2] | 375.8178 [M+4] | 150 | 10 | 0.81 | 0.69-0.94 |
| **HxCDF $^{13}C_{12}$** | | 385.8610 [M+2] | 387.8580 [M+4] | 15 | | 0.81 | 0.69-0.94 |
| **HxCDD** | | 389.8156 [M+2] | 391.8127 [M+4] | 150 | | 0.81 | 0.69-0.94 |
| **HxCDD $^{13}C_{12}$** | | 401.8559 [M+2] | 403.8530 [M+4] | 15 | | 0.81 | 0.69-0.94 |
| **lock mass** | | 380.9760 [l] | 380.9760 [l] | 50 | | | |
| **HpCDF** | 42-47 | 407.7818 [M+2] | 409.7788 [M+4] | 150 | 10 | 1.04 | 0.88-1.20 |
| **HpCDF $^{13}C_{12}$** | | 419.8220 [M+2] | 421.8190 [M+4] | 15 | | 1.04 | 0.88-1.20 |
| **HpCDD** | | 423.7767 [M+2] | 425.7737 [M+4] | 150 | | 1.04 | 0.88-1.20 |
| **HpCDD $^{13}C_{12}$** | | 435.8169 [M+2] | 437.8140 [M+4] | 15 | | 1.04 | 0.88-1.20 |
| **lock mass** | | 430.9728 [l] | 430.9728 [l] | 50 | | | |
| **OCDD** | 47-52 | 459.7348 [M+4] | 457.7377 [M+2] | 150 | 10 | 0.89 | 0.75-1.01 |
| **OCDD $^{13}C_{12}$** | | 471.7750 [M+4] | 469.7780 [M+2] | 15 | | 0.89 | 0.75-1.01 |
| **OCDF** | | 443.7398 [M+4] | 441.7428 [M+2] | 150 | | 0.89 | 0.75-1.01 |
| **OCDF $^{13}C_{12}$** | | 455.7801 [M+4] | 453.7830 [M+2] | 15 | | 0.89 | 0.75-1.01 |
| **lock mass** | | 466.9728 [l] | 466.9728 [l] | 50 | | | |

*: syringe standard added prior to GC-HRMS analysis and used for recovery

The use of multiple reaction monitoring (MRM) allows to monitor the production of selected daughter ions for chromatographically coeluting native and labeled compounds and perform ID [27]. The instrument alternatively scans in the native and label MS region and ion current can be reconstructed from those channels. After the ionization (EI), the isolation of molecular ions from the produced ions has to be optimized for each congener. Depending on the elution order (the chlorination level), segments are defined and specific isolation parameters are applied for each of them. The isolation of both native ($^{12}C$) and $^{13}C$-labeled precursors is optimized to satisfy to ID requirements.

Although at least $[M+2]^{+\bullet}$ species have to be considered as precursor (Figure 1), the choice of the parent ions is not only related to the relative isotope abundances (the more parents you isolate, the more daughters you potentially produce), but also by the isotopic ratio of the produced daughter ions. In fact, because the isotopic ratio check is carried out on the daughter ions, it is desirable to get similar abundances for both daughters to ensure accurate measurement of both isotope species at low

concentrations. Therefore, as illustrated in Table 2, $[M+2]^{+\bullet}$ or $[M+4]^{+\bullet}$ parent ions are often selected. The loss in abundance of the parent ion gets limited when moving up in the chlorination level and is counterbalanced by better daughter ratios. The reconstructed ion current (RIC) permitted to reach instrumental limit of detections (iLODs) of 200 fg.

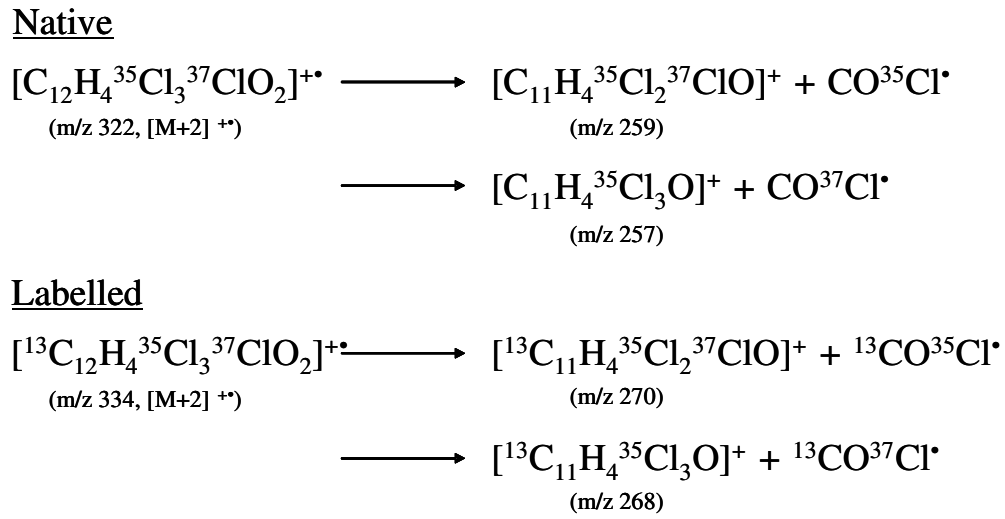


**Figure 1. Production of daughter ions from both native and $^{13}$C-labeled TCDD species using QISTMS/MS**

The CID process, responsible for the fragmentation of precursor ions in product ions, mainly depends on the excitation mode, the CID time, the excitation voltage and the stability parameter $q_z$, which is issued of the Mathieu second-order differential equation that accounts for the ion motion in the trap [28]. Most of the MS/MS experiments described in the literature use resonant excitation mode. That provides the adequate internal energy to allow fragmentation reactions involving rearrangements via the breakage of multiple chemical bonds (loss of $COCl^{\bullet}$). The excitation voltage and the stability parameter $q_z$ are closely linked and an optimum of the couple (CID voltage, $q_z$) has to be found for each congener [29]. Table 2 summarises the optimised MS/MS parameters for the PCDD/Fs segments.

The MS/MS approach has been used to measure PCDD and PCDF levels in various types of matrices. The limited sensitivity can be improved by the use of a large volume programmable temperature vaporizer injection (PTV-LV) GC-QISTMS/MS method for measurement of dioxins in food and feed [30]. It appeared that a 10 µl injection volume of toluene extracts was the maximum to avoid facing excessive presence of toluene in the trap for hours and subsequent sensitivity drop. Such a limitation point out a drawback of QISTMS/MS compared to triple-stage quadrupoles (TSQ) MS/MS where species are separetd in space rather than in time. If similar sensitivity is attained using QISTMS/MS and TSQMS/MS [31], the later suffers less from matrix effects due to the intrinsic difference in the CID process.

Figure 2 illustrates the very good compound-specific correlation between HRMS and MS/MS data, even if higher standard deviations for the MS/MS method were obtained. In TEQ, the results indicate that no bias between the methods was observed in the range of 0.2 to 25 ngWHO-TEQ/kg using different matrices. Other reports also demonstrated the efficiency of QISTMS/MS for the measurements of PCDD/Fs in foodstuffs at low picogram level [32].

QISTMS in tandem mode can also be used to measure PCB levels in biological samples. Selectivity is ensured by monitoring the loss of a $Cl_2$ fragment through a unique fragmentation reaction that produces the daughter ions [20].
A QISTMS-based method has also been developed for the measurement of PBDEs in biota samples [33]. EI was also used, instead of the more commonly used negative chemical ionization (NCI), to ensure the monitoring of $^{13}C$-labeled species for ID. Mass spectra are dominated by $M^{+\bullet}$ and $[M\text{-}Br_2]^{+\bullet}$ species for low and high degrees of bromination, respectively. The dissociation of the parent in daughter ions by CID was also congener-dependent, with loss of $Br_2$ (such as PCBs lose $Cl_2$) or loss of $COBr^{\bullet}$ (such as dioxins lose $COCl^{\bullet}$).

**Table 2. Principal parameters for the MS/MS measurement of PCDD/Fs using QISTMS/MS**

| | | Isolation[a] | Dissociation[b] | QA/QC | | |
|---|---|---|---|---|---|---|
| Segment # | Congeners | Molecular ions (m/z) | Excitation amplitude (Volts) | Isotope ratios | Daughter ions (m/z) | Validity (+/- 20%) |
| 1 | TCDD $^{12}C$ | 322 (M+2) | 1.3 (5)[c] | 0.33 | 257/259 | 0.26<0.33<0.4 |
| | TCDD $^{13}C$ | 334 (M+2) | 1.3 (5) | 0.33 | 268/270 | 0.26<0.33<0.4 |
| | TCDF $^{12}C$ | 306 (M+2) | 1.6 (5.5) | 0.33 | 241/243 | 0.26<0.33<0.4 |
| | TCDF $^{13}C$ | 318 (M+2) | 1.6 (5.5) | 0.33 | 252/254 | 0.26<0.33<0.4 |
| 2 | PeCDD $^{12}C$ | 358 (M+4) | 1.3 (6) | 0.66 | 293/295 | 0.53<0.66<0.8 |
| | PeCDD $^{13}C$ | 370 (M+4) | 1.3 (6) | 0.66 | 304/306 | 0.53<0.66<0.8 |
| | PeCDF $^{12}C$ | 342 (M+4) | 1.6 (6) | 0.66 | 277/279 | 0.53<0.66<0.8 |
| | PeCDF $^{13}C$ | 354 (M+4) | 1.6 (6) | 0.66 | 288/290 | 0.53<0.66<0.8 |
| 3 | HxCDD $^{12}C$ | 392 (M+4) | 1.3 (6) | 0.5 | 327/329 | 0.4<0.5<0.6 |
| | HxCDD $^{13}C$ | 404 (M+4) | 1.3 (6) | 0.5 | 338/340 | 0.4<0.5<0.6 |
| | HxCDF $^{12}C$ | 376 (M+4) | 2 (6) | 0.5 | 311/313 | 0.4<0.5<0.6 |
| | HxCDF $^{13}C$ | 388 (M+4) | 2 (6) | 0.5 | 322/324 | 0.4<0.5<0.6 |
| 4 | HpCDD $^{12}C$ | 426 (M+4) | 1.5 (6) | 0.4 | 361/363 | 0.32<0.4<0.48 |
| | HpCDD $^{13}C$ | 438 (M+4) | 1.5 (6) | 0.4 | 372/374 | 0.32<0.4<0.48 |
| | HpCDF $^{12}C$ | 410 (M+4) | 2 (6) | 0.4 | 345/347 | 0.32<0.4<0.48 |
| | HpCDF $^{13}C$ | 422 (M+4) | 2 (6) | 0.4 | 356/358 | 0.32<0.4<0.48 |
| 5 | OCDD $^{12}C$ | 462 (M+6) | 1.5 (6) | 0.6 | 397/399 | 0.48<0.6<0.72 |
| | OCDD $^{13}C$ | 474 (M+6) | 1.5 (6) | 0.6 | 408/410 | 0.48<0.6<0.72 |
| | OCDF $^{12}C$ | 446 (M+6) | 2 (6) | 0.6 | 381/383 | 0.48<0.6<0.72 |
| | OCDF $^{13}C$ | 458 (M+6) | 2 (6) | 0.6 | 392/394 | 0.48<0.6<0.72 |

[a]The $q_Z$ values were 0.3 and 0.45 when the damping gas flow was 0.3 ml/min and 1.7 ml/min, respectively.

[b]The excitation time was 10 ms.

[c]Values in brakets are voltages at damping gas flow of 1.7 ml/min and using an external source QISTMS.

*FGC-TOFMS*

Reports on general principles and developments of time-of-flight mass spectrometry (TOFMS) are available in the literature [34]. In TOFMS instruments, ions are accelerated to high velocity by an electric field in a flight tube. Since all ions have the same kinetic energy, the time ions take to traverse the flight tube is proportional to their masses. Light mass ions traveling faster than high mass ions. The time to acquire a complete mass spectrum is limited by the flight time of the highest mass under

analysis. A full mass spectrum can therefore be collected in less than 100 µs. A unit *m/z* resolution TOFMS instrument is capable to acquire 500 complete mass spectra/s for the mass range from 10 to 1000 *m/z*. Conversely to sector and quadrupole instruments, which offer limited scanning rates (c.a. < 20 scans/s) due to either the time required for electromagnets to change field strength or the limited ring electrode voltage ramp to be applied to maintain QISTMS unit m/z resolution, TOFMS analyzers are a non-mass-scanning device because all ions are virtually collected at the same time.

Fast GC (FGC) type separations are appealing in terms of sample turnover but also because sharper and taller peaks are produced with potential subsequent improvement of the method sensitivity. The use of TOFMS as the detection device permits the accurate characterization of those narrow peaks without the drastic loss in peak resolution usually observed when using low scan rate instruments and SIM or MS/MS mode.

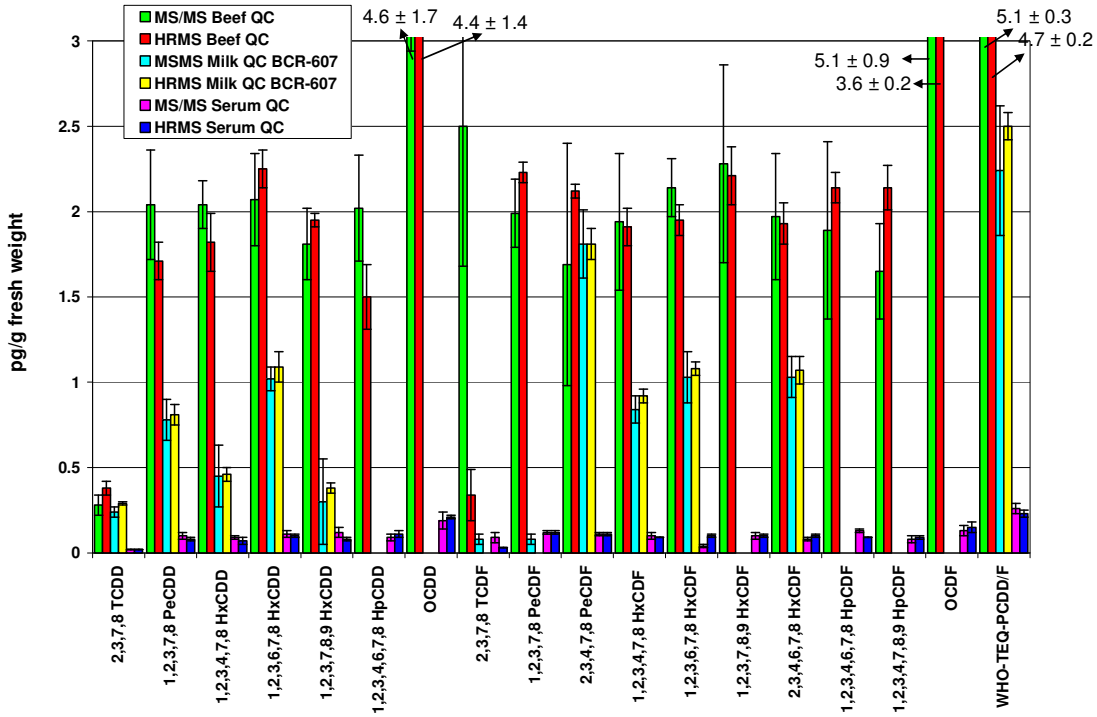


**Figure 2. Comparison of PTV-LV-GC-MS/MS and GC-HRMS for the measurement of PCDD/Fs at the low pictogram level in biological matrices**

TOFMS matches the speed of fast gas chromatographic separations and allows reliable reconstruction of resulting chromatograms. Oppositely to the use of SIM mode with sector or quadrupole instruments, which consists in pre-selection of masses that will be collected during the analysis, a reconstructed ion chromatogram (RIC) can be extracted based on any ion included in the collected mass range once data collection is completed. By comparison to scanning MS, it is as if full scan data had been collected and that only few masses (native and labeled for example) were used to reconstruct the current, no SIM descriptors are required to improve sensitivity. Additionally, because all ion fragments represent the same time point on the chromatographic peak profile, there is no concentration bias and the ion ratio remains the same, ensuring spectral continuity. This important feature allows MS deconvolution of overlapping

peaks if the fragmentation pattern is different. This backs up potentially poor chromatographic resolution situations [35].

Deconvoluted ion current (DIC) can thus be used to solve chromatographic co-elution problems that might arise while time-compressing the chromatograms. A method for high-throughput analysis of human serum for the 38 most prevalent PCBs in 8 min has been developed, based on the use FGC-IDTOFMS (Figure 3) [36]. The separation of the congeners was carried out either chromatographically or using MS deconvolution. The instrument and the method (5 ml of serum) limit of detections (LODs) were 0.5 pg/µL and 20 pg/µL, respectively (S/N greater than 3), which is not as good as the one achieved using HRMS but allows the detection and quantification of the prevalent PCBs present in real human serum samples. Isotope ratio verification ($^{35}Cl$, $^{37}Cl$) was carried out during the data processing using the two most intense masses for all native and $^{13}C_{12}$-labeled PCBs and several characteristic masses were summed for quantification. The dynamic range covered 3 orders of magnitude (0.5 pg/µl up to 1000 pg/µl). In terms of analyte concentration, the comparison with the HRMS reference method was good. Identical sample preparation steps were performed for the methods comparison. However, it appeared that the TOFMS instrument required less maintenance than the sector instrument in terms of ion source cleaning although fullscan data obtained with the TOFMS instrument pointed out the poor quality of the extracts. The FGC-TOFMS method allows the analysis of 100 samples per day per instrument. Furthermore, because other POPs were present in the PCB cleanup fraction, we extended the measurement procedure to selected organochlorine pesticides (OCPs) [36]. The many new co-elutions between PCBs and OCPs were easily solved by MS deconvolution because the characteristic ion clusters were different.

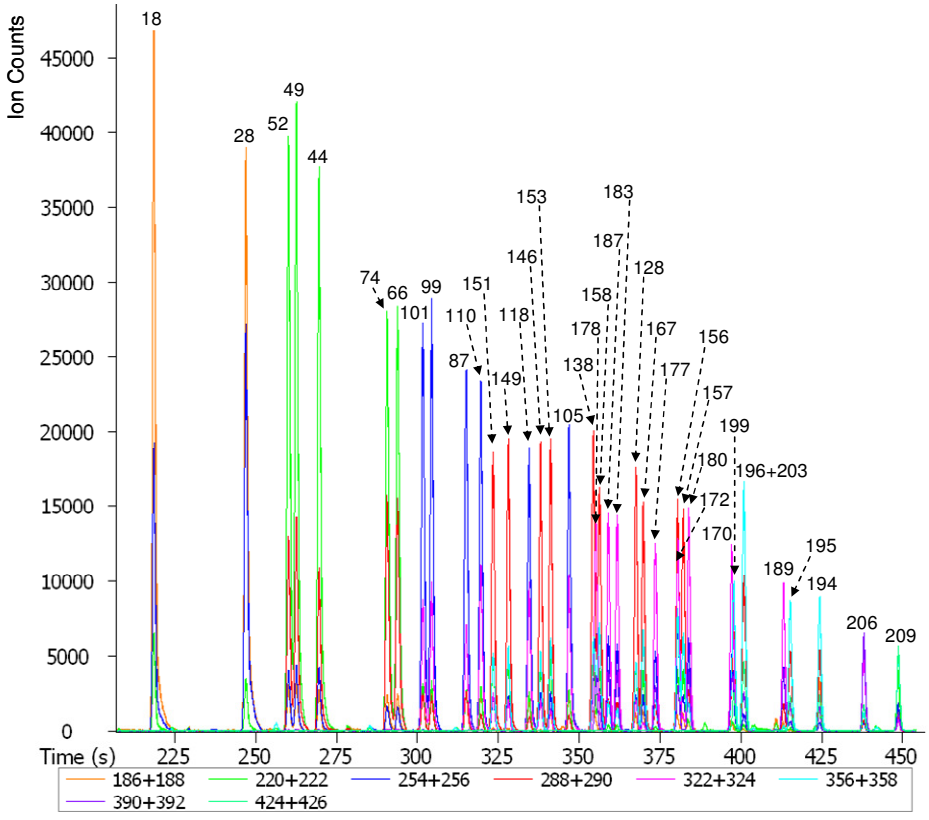


**Figure 3. FGC-IDTOFMS RIC chromatogram of 38 prominent PCBs found in human samples using a DB-XLB column**

One could also mention the use of FGC-IDTOFMS as a screening tool capable to sort-out large biological sample batches prior further investigation. In fact, it appears that most of the total toxic equivalency (TEQ) of those samples is due to very few congeners. The use of 2,3,7,8-TCDD, 2,3,7,8-TCDF, 1,2,3,7,8-PeCDD and 2,3,4,7,8-PeCDF as 'screening congeners' can allow the use of simplified $^{13}C$-labeled standard mixtures, the time compression of the GC run and slight simplification of the samples preparation step [37]. Figure 4 shows the type of separation that can be achieved in few minutes using classical GC injector and oven.

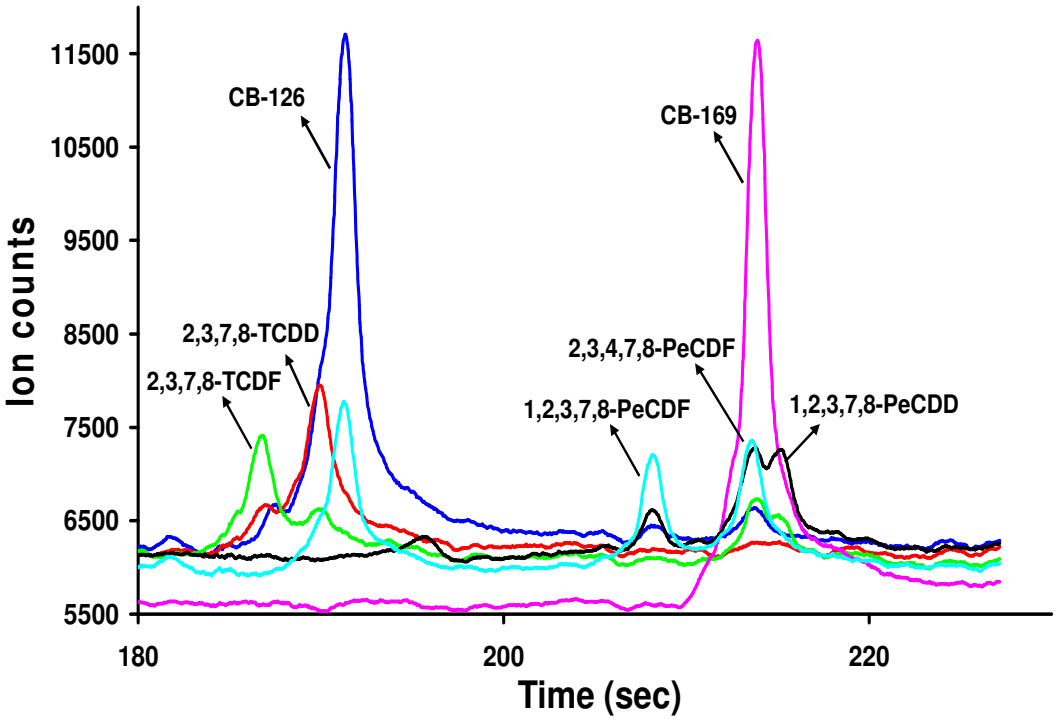


**Figure 4. Monitoring of selected 'screening congeners' by FGC-IDTOFMS using a DB-5MS (15m x 0.10mm I.D. x 0.10µm df) (J&W) column [49]. The cycle time was less than 10 min**

*GCxGC-TOFMS*

As we saw, TOFMS is well suited for the analysis of toxicants like PCBs. However, this type of instrument has a rather limited sensitivity, which does not allow low picogram detection with sufficient reliability. Comprehensive two-dimensional gas chromatography (GCxGC) is a relatively new technique that rests on the use of two different GC column phases to improve the chromatographic separation efficiency. Its comprehensive aspect is due to the fact that all eluents from the first dimension column ($^1D$) are re-injected into the second dimension column ($^2D$) with conservation of the resolution already achieved in $^1D$. Extensive review of the principles of the technique is available in the literature [38]. GCxGC offers several advantages over classical GC. Among them, on the side of the significant peak capacity enhancement, an increase in peak intensity is obtained after zone compression due to the modulation of the eluents of $^1D$. Because narrow peaks are produced after modulation, mass conservation ensures higher peak intensities [39]. This is of prime interest when LODs of a detector need to be improved, as it is the case for TOFMS.

The coupling of GCxGC with TOFMS has been presented as a comprehensive three-dimensional system in which gas sample components go through three dissimilar separation mechanisms based, for example, on analyte volatility, polarity, and mass. Early work on GCxGC-TOFMS were limited by the data handling and processing but more recent reports presented GCxGC-TOFMS as a promising tool for the analysis of complex mixture of analytes at the picogram level. Classically, $^{2}$D peak widths are 100-200 ms. The coupling between GCxGC and TOFMS is thus symbiotic because the GCxGC component allows signal enhancement and improvement of the TOFMS LODs, although TOFMS is the fast mass analyzer of choice for the description of narrow $^{2}$D peaks. Very recently, a robust GCxGC-TOFMS instrument has been launched on the market. This contributed to move the technique from its childhood stage to a more mature status, making it a tool to be evaluated in various areas of separation science.

The use of GCxGC-TOFMS for the isotope dilution measurement of dioxins and related compounds in environmental matrices such as soils and ashes showed to be correlated to GC-IDHRMS data [40].

Recently, a GCxGC-IDTOFMS experimental setup was also tested for the measurement of 7 PCDDs, 10 PCDFs, 4 NO-PCBs, 8 MO-PCBs, and 6 indicator PCBs (Aroclor 1260) in foodstuff samples [41]. A 40m RTX-500 (0.18 mm ID x 0.10 µm df) was used as the first dimension ($^{1}$D) and a 1.5m BPX-50 (0.10 mm ID x 0.10 µm df) as the second dimension ($^{2}$D). The unique GCxGC chromatographic separation was completed in 45min (Table 3 and Figure 5). Isotope ratios of the selected quantification ions were checked against theoretical values prior to peak assignment and quantification. The dynamic working range spanned three orders of magnitude. The lowest detectable amount of 2,3,7,8-TCDD was 0.2 pg. Fish, pork, and milk samples were considered. On a congener basis, the GCxGC-IDTOFMS method was compared to the reference GC-IDHRMS method and to the alternative GC-IDQISTMS/MS. PCB levels ranged from low picogram (pg) to low nanogram (ng) per gram of sample and data compared very well between the different methods. For all matrices, PCDD/Fs were at a low pg level (0.05 pg–3 pg) on a fresh weight basis. Although congener profiles were accurately described, RSDs of GCxGC-IDTOFMS and GC-QISTMS/MS were much higher than for GC-IDHRMS, especially for low level pork and milk.

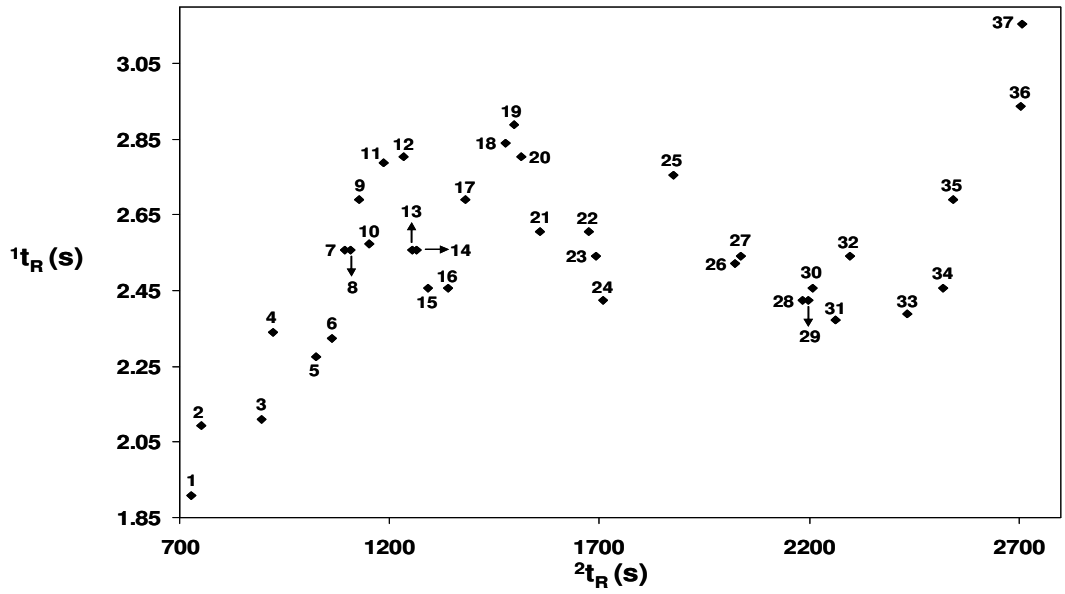


**Figure 5. GCxGC-IDTOFMS apex plot based on the retention data of the 37 compounds**

As for GC-IDHRMS and GC-IDQISTMS/MS, ID based on the use of $^{13}C$-labeled compounds was used for quantification. The ratios of $^{12}C$-native areas over $^{13}C$-label areas were calculated and corrected by RRF values issued from the calibration curve calculations. The major difference using GCxGC-IDTOFMS was that areas of 2 to 4 $^{2}D$ peaks had to be summed up prior to quantification. This exercise significantly increases the processing and reviewing time, but is important for accurate quantification [42].

**Table 3. Principal chromatographic and mass spectrometric parameters for the GCxGC-IDTOFMS separation of the selected PCBs and PCDD/Fs**

| Peak Number | Congener[a] | $^{1}t_R$ (s) | $^{2}t_R$ (s) | Quantification Masses | | | | Theoretical Isotope Ratios | Acceptable Range (20%) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | $^{12}C_{12}$-natives | | $^{13}C_{12}$-labels | | | |
| 1 | TriCB-28 | 727 | 1.91 | 258 | 256 | 270 | 268 | 0.98 | 0.78-1.18 |
| 2 | TeCB-52 | 751 | 2.09 | 290 | 292 | 302 | 304 | 0.77 | 0.62-0.92 |
| 3 | TeCB-80[b] | 895 | 2.11 | - | - | 302 | 304 | 0.77 | 0.62-0.92 |
| 4 | PeCB-101 | 923 | 2.34 | 328 | 326 | 340 | 338 | 0.65 | 0.52-0.78 |
| 5 | TeCB-81 | 1025 | 2.27 | 290 | 292 | 302 | 304 | 0.77 | 0.62-0.92 |
| 6 | TeCB-77 | 1061 | 2.32 | 290 | 292 | 302 | 304 | 0.77 | 0.62-0.92 |
| 7 | PeCB-123 | 1094 | 2.56 | 328 | 326 | 340 | 338 | 0.65 | 0.52-0.78 |
| 8 | PeCB-118 | 1106 | 2.56 | 328 | 326 | 340 | 338 | 0.65 | 0.52-0.78 |
| 9 | PeCB-114 | 1126 | 2.69 | 328 | 326 | 340 | 338 | 0.65 | 0.52-0.78 |
| 10 | HxCB-153 | 1150 | 2.57 | 362 | 360 | 374 | 372 | 0.82 | 0.66-0.98 |
| 11 | PeCB-105 | 1186 | 2.79 | 328 | 326 | 340 | 338 | 0.65 | 0.52-0.78 |
| 12 | HxCB-138 | 1233 | 2.81 | 362 | 360 | 374 | 372 | 0.82 | 0.66-0.98 |
| 13 | 1,2,3,4-TeCDD[b,c] | 1252 | 2.56 | - | - | 328 | - | - | - |
| 14 | 2,3,7,8-TeCDF | 1264 | 2.56 | 304 | 306 | 316 | 318 | 0.77 | 0.62-0.92 |
| 15 | 2,3,7,8-TeCDD | 1292 | 2.46 | 320 | 322 | 332 | 334 | 0.76 | 0.61-0.91 |
| 16 | PeCB-126 | 1340 | 2.46 | 328 | 326 | 340 | 338 | 0.65 | 0.52-0.78 |
| 17 | HxCB-167 | 1381 | 2.69 | 362 | 360 | 374 | 372 | 0.82 | 0.66-0.98 |
| 18 | HxCB-156 | 1476 | 2.84 | 362 | 360 | 374 | 372 | 0.82 | 0.66-0.98 |
| 19 | HxCB-157 | 1496 | 2.89 | 362 | 360 | 374 | 372 | 0.82 | 0.66-0.98 |
| 20 | HpCB-180 | 1512 | 2.81 | 396 | 394 | 408 | 406 | 0.98 | 0.78-1.18 |
| 21 | 1,2,3,7,8-PeCDF | 1559 | 2.61 | 342 | 340 | 354 | 352 | 0.65 | 0.52-0.78 |
| 22 | 2,3,4,7,8-PeCDF | 1675 | 2.61 | 342 | 340 | 354 | 352 | 0.65 | 0.52-0.78 |
| 23 | 1,2,3,7,8-PeCDD | 1691 | 2.54 | 358 | 356 | 370 | 368 | 0.66 | 0.53-0.79 |
| 24 | HxCB-169 | 1711 | 2.42 | 362 | 360 | 374 | 372 | 0.82 | 0.66-0.98 |
| 25 | HpCB-189 | 1875 | 2.76 | 396 | 394 | 408 | 406 | 0.98 | 0.78-1.18 |
| 26 | 1,2,3,4,7,8-HxCDF | 2025 | 2.52 | 376 | 374 | 388 | 386 | 0.82 | 0.66-0.98 |
| 27 | 1,2,3,6,7,8-HxCDF | 2037 | 2.54 | 376 | 374 | 388 | 386 | 0.82 | 0.66-0.98 |
| 28 | 1,2,3,4,7,8-HxCDD | 2185 | 2.42 | 392 | 390 | 404 | 402 | 0.82 | 0.66-0.98 |
| 29 | 1,2,3,6,7,8-HxCDD | 2197 | 2.42 | 392 | 390 | 404 | 402 | 0.82 | 0.66-0.98 |
| 30 | 2,3,4,6,7,8-HxCDF | 2209 | 2.46 | 376 | 374 | 388 | 386 | 0.82 | 0.66-0.98 |
| 31 | 1,2,3,7,8,9-HxCDD | 2264 | 2.37 | 392 | 390 | 404 | 402 | 0.82 | 0.66-0.98 |
| 32 | 1,2,3,7,8,9-HxCDF | 2296 | 2.54 | 376 | 374 | 388 | 386 | 0.82 | 0.66-0.98 |
| 33 | 1,2,3,4,6,7,8-HpCDF | 2436 | 2.39 | 410 | 408 | 422 | 420 | 0.98 | 0.78-1.18 |
| 34 | 1,2,3,4,6,7,8-HpCDD | 2519 | 2.46 | 426 | 424 | 438 | 436 | 0.98 | 0.78-1.18 |
| 35 | 1,2,3,4,7,8,9-HpCDF[b] | 2543 | 2.69 | - | - | 422 | 420 | 0.98 | 0.78-1.18 |
| 36 | OCDD | 2703 | 2.94 | 458 | 460 | 470 | 472 | 0.88 | 0.70-1.05 |
| 37 | OCDF | 2707 | 3.15 | 442 | 444 | 454 | 456 | 0.88 | 0.70-1.05 |

[a]Numbering of PCBs according to IUPAC. [b]Congeners used for recovery calculation. [c]This congener is $^{13}C_6$-1,2,3,4-TeCDD only.

To reduce the influence of sample extraction and clean-up on the comparison exercise, the same sample sizes were extracted and identical sample preparation steps were performed for the three MS techniques.

Recovery rates, based on the addition of recovery (surrogate) standards prior to GC-MS injection, were therefore similar for all methods. They complied with the requirements of the European Commission Directive 2002/69/EC [43], in which a range of 60% to 120% has been defined for confirmatory methods and a range of 30% to 140% for screening methods. Although GC-IDQISTMS/MS and GCxGC-IDTOFMS are strictly defined as screening methods in the Directive, recovery rates ranged in the interval defined for confirmatory methods. Blank (BC) analyses were performed by carrying out the entire analytical procedure to which unknown samples were exposed. Because BC levels are mainly dependent on sample preparation procedure, levels were similar and no significant influences of MS measurement on BC levels were recorded during the study. All data reported here were BC-corrected.
For PCDD/Fs (Figure 6), levels in the unfortified matrices were much lower than for PCBs and can be considered as the background levels currently encountered in the EU. For fish, because of the relatively high levels and the relatively large sample sizes (15 g), both GCxGC-IDTOFMS and GC-IDQISTMS/MS compared well with GC-IDHRMS. However, although the RSDs for GC-IDHRMS were 7–14%, GCxGC-IDTOFMS and GC-IDQISTMS/MS RSDs ranged from 10 to 60% and from 5 to 30%, respectively. In practice, such concentrations were very close to the lower end of the working range defined by the calibration standards and on the edge of the LOQs. Increasing sample sizes is not feasible in practice because the larger the sample size, the larger the quantities of solvents and sorbents, the higher the BC levels, and the higher the LOQs.
Conversely, in some cases, large standard deviation might be attributed to the fact that the system was measuring outside the working range. This is the case for 2,3,7,8-TeCDF for which the 3.1 pg/g fw values represent 8.4 pg injected (15 g sample size, 75% recovery rates, 1.2µl injected out of 5µl), although the highest point of calibration was 7.5 pg. Increasing the sample size would accentuate the problem. The calibration standard concentrations were selected to cover as much as possible of the working range but out-of-calibration situations can always arise, depending on the congener distribution in the sample. From this study, it appeared that GCxGC-IDTOFMS was more affected by this type of out-of-calibration situation. In the case of pork (30 g sample size) and milk (130 g sample size), which are characterized by low background levels, the RSDs were higher (up to 90%). Such variations were not acceptable. Despite the poor precision, the congener distribution was still well defined for all matrices and can be used to describe specific matrix patterns for contamination source tracking or fingerprinting of sets of samples.
Finally, because of the resulting zone compression after modulation, another field of application for GCxGC is its use as a signal enhancer, rather than to increase the peak capacity of the chromatographic separation. A current area of efforts is the coupling between GCxGC and sensitive sector HRMS instruments. In the case of PCDD/Fs, where a good separation of the 17 2,3,7,8-substituted congeners can be achieved in less than 40 min with classical GC, the use of the GCxGC modulator with a short piece of open tube as $^{2}$D can improve instrument LODs. Early promising results were in the low attogram range for 2,3,7,8-TCDD [44]. Improvements of some aspects like sector MS scanning rate and data handling still need to be carried out to offer the robustness required for routine use of this extremely sensitive tool for ultra-trace analysis.

### *Cell-based assays*

The most used cell-based assay to detect dioxin and dioxin-like compounds is named CALUX (Chemically Activated LUciferase gene eXpression). The CALUX assay is based on the use of eukaryotic cells, genetically modified to contain the firefly luciferase gene under the control of a promoter containing at least one DRE (Dioxin Responsive Element). When these cells are exposed to dioxins, dioxins enter into the

cells by easily crossing the phospholipidic membrane of the cells and bind to the cytoplasmic Ah receptor. The complex dioxin-AhR is then translocated into the nucleus of the cell and bind to DREs, inducing the expression of the luciferase gene, and subsequently of the synthesis of the firefly luciferase protein. After substrate (ATP and luciferin) addition, one can measure the emission of light, which is correlated to the concentration of dioxin. The first CALUX assay was described by Aarts and coworkers in 1993 [45]. Nowadays, at least two commercial systems exist, using either rat (DR-CALUX®, BDS [46]) or mouse cells (CALUX®, XDS [47]). The application of the CALUX bioassay for the monitoring of dioxins in feed and food has recently been reviewed [48,49].

The same fish, pork, and milk samples as previously reported were run on the DR-CALUX (two independent sets of replicates) and data were compared to the MS-based results. As shown in Figure 7, a large discrepancy appeared between the GC-IDHRMS data and the raw DR-CALUX data.

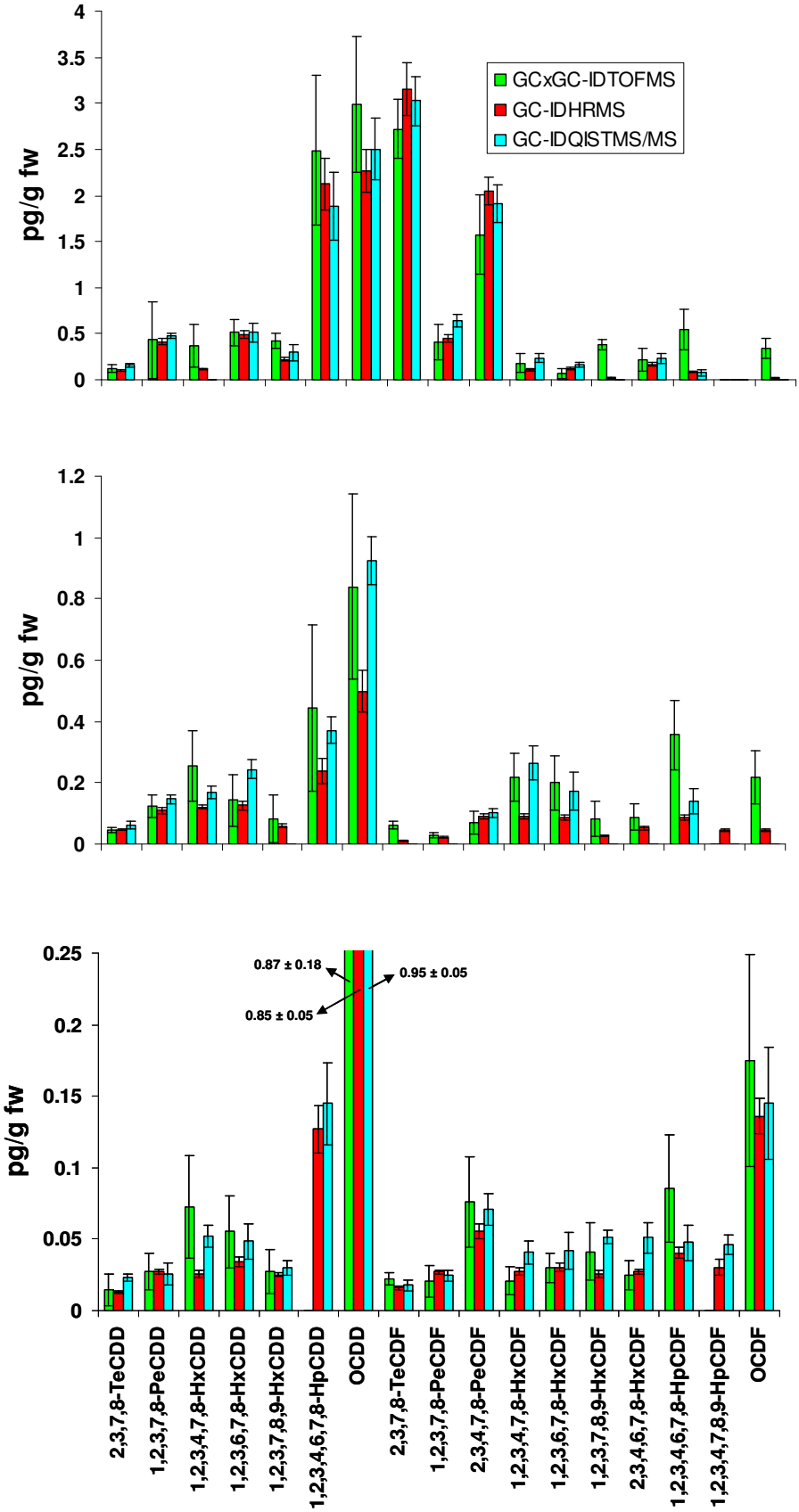


**Figure 6. Comparison of GC-IDHRMS with GCxGC-IDTOFMS and GC-IDQISTMS/MS for the measurement of PCDD/Fs in fish (A), in pork (B), and in milk (C) samples (n=6)**

The biological method clearly and systematically underestimated the total TEQ (PCDD/Fs and dioxin-like PCBs) concentrations in each case. For the biological measurement, because the use of ID based on $^{13}C$-labelled internal standards is not possible, it is difficult to account for the loss of analytes during the sample preparation procedure and an underestimation of sample burden is likely to happen. Two approaches were investigated to correct the raw DR-CALUX data.

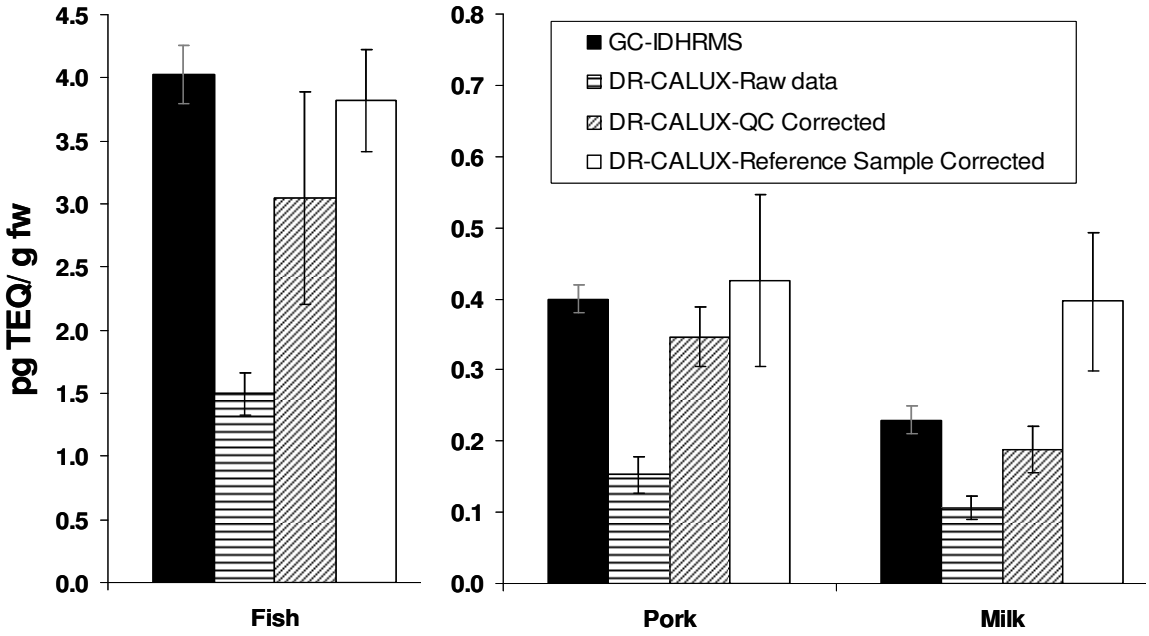


**Figure 7. Raw and corrected responses of the DR-CALUX assay versus GC-IDHRMS for the investigated samples**

First, the biological data were corrected by a factor taking into account the ratio between results obtained for a well characterized fortified (PCDD/Fs and dioxin-like PCBs) beef fat quality control (QC) sample included in both GC-IDHRMS and DR-CALUX series. This spiked beef fat QC contained 3.1 pg WHO-TEQ per g (34% of PeCB-126, 17% of PeCDD, 17% of TeCDD, 9% of 2,3,4,7,8 PeCDF), as measured by GC-IDHRMS, and an average of 1.8 pg CALUX TEQ/g. This significant variation accounted for the rather large difference we reported earlier [50] between the relative potencies (REP) of WHO-TEF and the DR-CALUX for PeCB-126 and PeCDD, two major components of the QC sample. The QC corrected DR-CALUX thus gets closer to the reference GC-IDHRMS data but the trend of underestimation was still present for all samples. Additionally, because the QC samples consisted of fat, they were not submitted to the entire sample preparation procedure (no extraction step required). Therefore, correcting raw DR-CALUX data using a factor based on this fat QC did not account for potential analyte losses during the extraction step.

The second approach was based on the use of matrix-specific reference samples for raw DR-CALUX data correction. Each reference sample followed unknowns through the entire matrix-specific sample preparation procedure. This constitutes a better approach because similar congener distributions, and thus similar assay responses, can be expected in identical matrices. Also, because the reference value is calculated by GC-IDHRMS, having a matrix specific reference sample helps to reduce the effect of the differences between TEFs and REPs. The ratio of the total TEQ (sum of PCDD/Fs and dioxin-like PCBs) concentration measured by GC-IDHRMS over the DR-CALUX response was used as a correction factor applied to the raw DR-CALUX data. Results for the congener-specific GC-IDHRMS measurement of those DR-CALUX

matrix-specific reference samples were the following: for the fish reference sample, 42% of PeCB-126, 27% of 2,3,4,7,8-PeCDF and 10% of 2,3,7,8-TeCDF; for the pork reference sample, 24% of 2,3,4,7,8-PeCDF, 15% of PeCB-126, 11% of 2,3,7,8-TeCDD, 10% of both HxCB-156 and 157; for the milk reference sample, 40% of PeCB-118, 28% of PeCB-126, and 9% of 2,3,4,7,8-PeCDF. Both pork and fish reference samples had a congener profile corresponding to a classical background contamination (similar to the pattern of the analyzed sample) and good correlations with the GC-IDHRMS data were observed (Figure 7). The situation was not as good for milk because the pattern observed in the reference sample (PeCB-118 was unusually high) was different from a classical background congener distribution for milk and this influenced the raw data correction, as though a non-matrix specific reference sample had been used. The direct consequence led to an unexpected low recovery for the milk sample, inducing an over-estimation of the corrected CALUX data.

Figure 8 summarizes the comparison of all methods in terms of TEQs. Quite surprisingly, although we previously pointed out much higher variations in the GCxGC-IDTOFMS and GC-IDQISTMS/MS responses for PCDD/Fs on a congener basis, as well as the difficulty for those methods to detect the low pg levels of analytes, the TEQ results compared favorably with GC-IDHRMS (lower part in the bar graph in Figure 7). In fact, a rather good description of the TEQ contributors (2,3,7,8-TCDD [TEF=1], 1,2,3,7,8-PeCDD [TEF=1], 2,3,4,7,8-PeCDF [TEF=0.5], see [51] for complete list of the TEFs) was achieved using the alternative methods. The PCB contribution to the TEQ was similar for the three MS-based methods. The lower GCxGC-IDTOFMS value for pork was due to the lower reported concentration for PeCB-126, the most important PCB contributor (TEF=0.1) to the TEQ. The MS-based method TEQs and the DR-CALUX reference sample corrected TEQ compared well (see earlier for milk discrepancy), although DR-CALUX RSDs were significantly higher (10–28%), which is acceptable for a screening method [43].

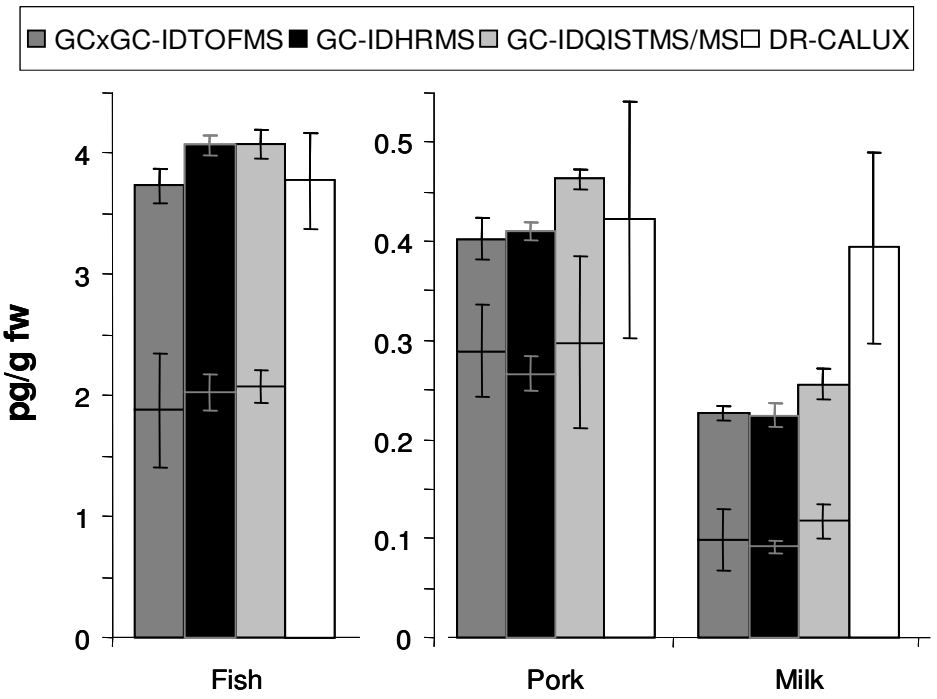


**Figure 8. TEQ comparison of GC-IDHRMS with GCxGC-IDTOFMS, GC-IDQISTMS/MS, and DR-CALUX (reference sample corrected) for the measurement of PCDD/Fs (bottom part) and dioxin-like PCB (non-*ortho* and mono-*ortho*-PCBs) (upper part) for the investigated samples**

## CONCLUSIONS

The implementation and feasibility of efficient measurement campaigns depend on several factors among which the versatility of the analytical methods to be used is of prime importance. The cost, the rapidity, and the robustness of a method have to be optimized for it to be a commercially viable tool. However, efforts in that direction are confined in a working area where the quality of the results can not be compromised. Alternative MS tools exist in addition to the reference sector HRMS instruments for the measurement of dioxins and related compounds. PTV-LV-GC-IDMS/MS based on QISTMS, FGC-IDTOFMS and GCxGC-IDTOFMS are among the most investigated ones. Although none of them offers the sensitivity usually attained by GC-IDHRMS, they consist in viable approaches in terms of versatility, sample turnover, and cost. PTV-LV-GC-IDMS/MS and GCxGC-IDTOFMS have similar iLODs (0.2 pg) but the later is the most suited to fulfill both selectivity and speed requirements simultaneously (Table 4). Additionally, TOFMS instruments, especially when coupled to GCxGC, seem to be able to handle more matrix interferences than QISTMS instruments, potentially reducing the cleanup requirements of the method. The various techniques have advantages and limitations (Table 4) and their applicability depends on the specific field of application and the set of analytes to be reported.
Under specific criteria described elsewhere [41], a cost estimate can be drawn for the different methods investigated. In Table 5, a relative cost comparison is shown and indicates that the costs involved in alternative techniques are not much lower than for GC-IDHRMS. A closer look indicates that the cost distribution is however different. Only the DR-CALUX permits the cutting of prices by half, making it appealing for screening, but it does not offer congener specific data and pattern description, which is crucial for source identification in case of contamination.

**Table 4. Comparison between the main characteristics of the MS-based analytical methods**

| | GC-IDHRMS | PTV-LV-GC-IDMS/MS | FGC-IDTOFMS | GCxGC-IDTOFMS |
|---|---|---|---|---|
| Investment cost (€) | 350,000 | 140,000 | 170,000 | 240,000 |
| Operating cost | +++ | ++ | + | + |
| Sample turnover | + | + | +++ | + |
| # of analytes per unit of time | + | + | +++ | +++ |
| iLODs | +++ | ++ | + | ++ |
| PCDD/F measurement | +++ | ++ | - | ++ |
| PCB measurement | +++ | +++ | +++ | +++ |
| Unknown measurement | - | - | ++ | +++ |

**Table 5. Estimated percent distribution of the cost of the various stages of the measurement methods in the case of feed samples**

| | GC-IDHRMS | GCxGC-IDTOFMS | GC-IDQISTMS/MS | DR-CALUX[a] |
|---|---|---|---|---|
| **Scientist employment** | **23** | **35** | **35** | **36** |
| **Extraction** | **11** | **8** | **11** | **7** |
| **Clean-up** | **28** | **27** | **33** | **29** |
| **Measurement** | **38** | **30** | **21** | **8** |
| **Licensing and royalties** | **-** | **-** | **-** | **20** |
| **Cost per sample (Relative)** | +++++ | +++++ | ++++ | +++ |

[a]Cost based on duplicate measurements.

The coming years will show us how those alternative techniques will evolve and which place they can reach in the field of dioxin and related compounds measurement.

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# Veterinary drug residues

# Veterinary Drug Residues: Regulatory Aspects

**G. Maghuin-Rogister**

*University of Liège (Belgium); Department of Food Sciences; Center of Analysis of Residues in Traces*
*e-mail: g.maghuin@ulg.ac.be*

**Keywords**
Veterinary drugs, European legislation

## EUROPEAN LEGISLATION

### *Council Regulation EEC N°2377/90*

laying down a Community procedure for the establishment of maximum residue limits *(MRLs)* of veterinary medicinal products in foodstuffs of animal origin.
This regulation involves 5 annexes:
Annex I: substances for which MRLs have been fixed by EMEA (European Agency for the Evaluation of Medicinal Products)
Annex II: substances not subject to maximum residue limits (generally recognized as safe)
Annex III: substances for which provisional MRLs have been fixed
Annex IV: substances for which no maximum levels can be fixed (ex. chloramphenicol, nitrofurans,…).
Annex V: information and data needed for the establishment of the MRL (identity of the substance, toxicological research, metabolism, residues)

The position adopted in this regulation is thus that substances with insufficiency of data are not authorised to be used for food-producing animals in the EU.

### *Directive 96/23/EC*

on measures to monitor certain substances and residues thereof in live animals and animal products.
Some parts of this directive merit a special attention:

- In CHAPTER II. *Monitoring plans for the detection of residues or substances*
  *Article 3 :* … "detecting the presence of residues and substances listed in ANNEX I in live animals, their excrements et body fluids, and in tissue, animal products, animal feed and drinking water"

ANNEX I is subdivided in two groups: A (banned substances), B (tolerated substances)

GROUP A

Substances having anabolic effect and unauthorized substances

1. Stilbenes and derivatives *(DES, …)*
2. Antithyroid agents *(tapazol, thiouracil,…)*
3. Steroids *(estrogens, androgens, gestagens)*
4. Resorcylic acide lactones *including zeranol*
5. ß-agonists *(clenbuterol, salbutamol,…)*
6. Compounds included in Annex IV to Council Regulation N°2377/90 (MRL)

GROUP B

Veterinary drugs* and contaminants (* including unlicensed substances which could be used for veterinary purposes)

1. Antibacterial substances, including sulphonamides, quinolones
2. Other veterinary drugs
    a) Anthelmintics
    b) Anticoccidials, including nitroimidazoles
    c) Carbamates and pyrethroids
    d) Sedatives
    e) Non-steroidal anti-inflammatory drugs (NSAIDs)
    f) Other pharmacologically active substances
3. Other substances and environmental contaminants
    a) Organochlorine compounds including PCBs
    b) Organophosphorous compounds
    c) Chemical elements *(Pb, Cd, Hg,…)*
    d) Mycotoxins *(aflatoxins, ochratoxin,…)*
    e) Dyes
    f) Others

- In CHAPTER II. *Monitoring plans for the detection of residues or substances* *Article 5, §2:"* a) the plan shall provide for detection of groups of residues according to type of animal, in accordance with ANNEX II";

ANNEX II gives a list of residues or substance groups to be detected by type of animal, their feedingstuffs, including drinking water, and primary animal products (table 1)

***Table 1a.*** **Residue or substance group to be detected by type of animal, their feedingstuffs, including drinking water, and primary animal products.**

| **Type of animals, feedingstuffs or animal products Substance groups*** | **Bovine, ovine, porcine, equine, animals** | **Poultry** | **Aquaculture animals** | **Milk** | **Eggs** | **Rabbit meat and the meat of wild** game and farmed game** | **Honey** |
|---|---|---|---|---|---|---|---|
| **A1** | X | X | X | | | X | |
| **2** | X | X | | | | X | |
| **3** | X | X | X | | | X | |
| **4** | X | X | | | | X | |
| **5** | X | X | | | | X | |
| **6** | X | X | X | X | X | X | |

*Refer to ANNEX I for numbering

**Only chemical elements are relevant where wild game is concerned

***Table 1b.*** **Residue or substance group to be detected by type of animal, their feedingstuffs, including drinking water, and primary animal products**

| Type of animals, feedingstuffs or animal products Substance groups* | Bovine, ovine, porcine, equine, animals | Poultry | Aquaculture animals | Milk | Eggs | Rabbit meat and the meat of wild** game and farmed game | Honey |
|---|---|---|---|---|---|---|---|
| **B1** | X | X | X | X | X | X | X |
| **2a** | X | X | | | | X | |
| **b** | X | X | | | | X | |
| **c** | X | X | | | | X | X |
| **d** | X | | | | | | |
| **e** | X | X | | | | X | |
| **f** | | | | | | | |
| **3a** | X | X | X | X | X | X | X |
| **b** | X | | | X | | | X |
| **c** | X | X | X | X | | X | X |
| **d** | X | X | X | X | | | |
| **e** | | | X | | | | |

*Refer to ANNEX I for numbering
**Only chemical elements are relevant where wild game is concerned

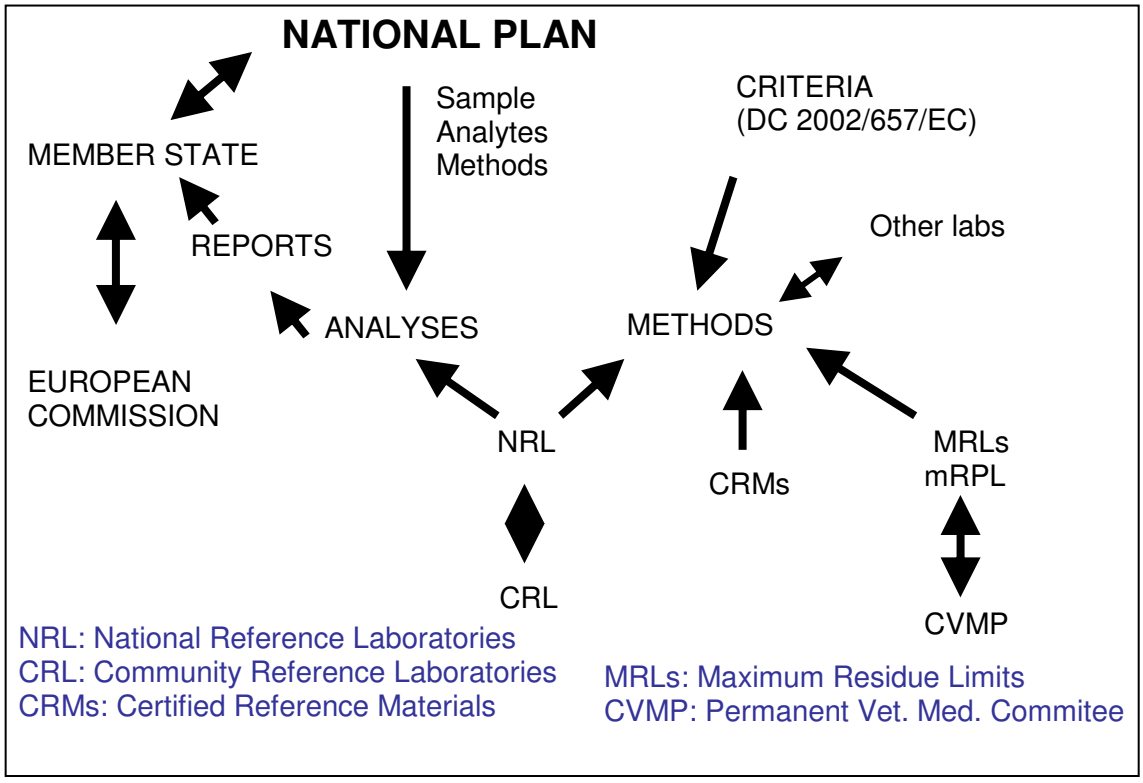


**Figure 1. Summary of the organization concerning monitoring plans for the detection of residues or substances.(Heitzman, 1994)**

- In CHAPTER III. "*Self-monitoring and co-responsability on the part of operators",* Article 10 states rules about registers kept by the stockfarmer and the veterinarian to ensure traceability:
  - *Stockfarmer*

    Must enter in the register:
    - the date and nature of the treatment administered
    - satisfy himself that withdrawal periods have been observed
    - keep the prescriptions to prove it for 5 years

  - *Veterinarian*

    Must enter in the register:
    - the date and nature of any treatment prescribed or administered
    - the identification of treated animals
    - the corresponding withdrawal periods

Beside ANNEX I and II already presented before, directive 96/23/EC involves other annexes:

- *ANNEX III deals with the* sampling strategy

  1. *Aim :* surveying and revealing the reasons for residue hazards in food of animal origin in farms, slaughterhouses, dairies, fish processing plants and egg collecting and packing stations.
  Sampling must be unforeseen, unexpected and effected at no fixed time, and on no particular day of the week. The Member States must take all the precautions necessary to ensure that the element of surprise is constantly maintained.

  2. *For Group A:*surveillance should be aimed at detecting the illegal administration of prohibited substances and the abusive administration of approved substances

  3. *For Group B substances:*surveillance should be aimed particularly at controlling:
  - the compliance with MRLs for residues of veterinary medicinal products fixed in Annexes I and III to Regulation (EEC) No 2377/90, and the
  - maximum levels of pesticides fixed in Annex III to Directive 86/363/EEC, and
  - monitoring the concentration of environmental contaminants.
  - No random sampling but targeted samples!

- *ANNEX IV deals with* sampling levels and frequency

  *Chapter 1* Bovine, porcine, ovine, caprine, equine animals.
  1. Bovine: minimum number of animals at least equal to 0.4% of bovine animals slaughtered the previous year.
  - *Group A (forbidden susbtances):* 0.25%
    - ½ of the samples taken from live animals on the holding.
    - ½ of the samples taken on the slaughterhouse.
  - *Group B (veterinary drugs and contaminants)*: 0.15%
    - 30% : Group B1 substances (antibiotics)
    - 30% : Group B2 substances (other veterinary drugs)
    - 10% : Group B3 substances (contaminants)

- The balance must be allocated according to the situation of the Member State.

- *ANNEX V lists the* Community Reference Laboratories and their tasks:
  - RIVM Bilthoven (NL): *anabolics and antithyroids, veterinary drugs, mycotoxins*
  - CNEVA-LMV Fougères (F): *antibiotics and dyes*
  - BGVV Berlin (D): *beta-agonists, vet drugs*
  - ISS Roma (I): *vet drugs, contaminants*

## Directive 70/524/EEC and amendments

of 23 November 1970 concerning additives used as growth promoters in feeding-stuffs.
These growth promoters are antimicrobial (ionophores and non ionophores) agents.
In 1970, 12 substances were authorized. In 1990, Avoparcin was submitted to a moratorium in EU. It was followed in 1999 by a ban of antibacterial growth promoters used as feed additives except for four substances:

- monensin,
- salinomycin,
- flavophospholipol
- avilamycin.

And finally in 2006, a total ban was applied.

## Commission Decision 2002/657/EC

*(Criteria for Analytical Methods of Determination of Residues)*
of 12 August 2002 « implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results"

The novelties of this decision compared to the previous one are:

- A clear description of the protocol of validation of analytical methods concerning:
  - Screening methods
  - Confirmatory methods
- Quantitative methods for substances with a MRL
- Minimal Required Performance Limit (mRPL) for forbidden substances
- Identification points in MS-based methods
- Definitions, based on statistical considerations, of the Limit of Decision (CC□) and the Capacity of Detection (CC□)

<u>Minimal Required Performance Level (mRPL)</u>

- minimum content of an analyte in a sample, which at least has to be detected and confirmed.
- It is intended <u>to harmonize the analytical performance</u> of methods for substances for which no permitted limit has been established.

<u>Examples</u>:

- Chloramphenicol: mRPL = 0.3 µg/kg*
- Nitrofurans (marker metabolites): mRPL = 1 µg/kg*
- Malachite green and leucomalachite: mRPL = 2 µg/kg**

**Commission Decision 2003/181/EC*
*** Commission Decision 2004/25/EC*

## Belgian Legislation

**AR 08-09-1997.** Arrêté royal relatif aux mesures en matière de commercialisation des animaux d'exploitation en ce qui concerne certaines substances ou résidus de substances pharmacologiquement actives

**AM 10-09-1997.** Arrêté ministériel portant exécution de l'arrêté royal relatif aux mesures en matière de commercialisation des animaux d'exploitation en ce qui concerne certaines substances ou résidus de substances pharmacologiquement actives

**AR 11-10-1997.** Arrêté royal modifiant l'arrêté royal du 9 mars 1953 concernant le commerce des viandes de boucherie et réglementant l'expertise des animaux abattus à l'intérieur du pays

### *Statute H*

It applies to forbidden substances

- hormones and thyrostatics,
- beta-agonists,
- substances listed in Regulation N°2377/90 Annex IV (substances for which no MRL can be fixed)

Penalties :

- Identification of animal farm for a period of 52 weeks
- In case of relapse : 104 weeks
- If animals must be slaughtered:
  - Residue analyses paid by the owner
  - If results are not compliant: complete destruction of carcass and offal without compensation
  - If results are compliant: animals slaughtered in local slaughterhouse (not licensed for exportation)

### *Statute R*

It applies to substances for which residues have been discovered in animal products at concentrations higher than MRL

Penalties:

- Identification of animal farm for a period of 8 weeks
- In case of relapse: 26 weeks
- If animals must be slaughtered:
  - Residue analyses paid by the owner
  - If residue concentration > MRL: waiting period until residue concentration < MRL
  - If results are compliant: animals may be slaughtered in a slaughterhouse licensed for exportation.

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MB 07/10/1997

AR 08-09-1997
Arrêté royal relatif aux mesures en matière de commercialisation des animaux d'exploitation en ce qui concerne certaines substances ou résidus de substances pharmacologiquement actives
MB 07/10/1997

AR 11-10-1997
Arrêté royal modifiant l'arrêté royal du 9 mars 1953 concernant le commerce des viandes de boucherie et réglementant l'expertise des animaux abattus à l'intérieur du pays
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# Development of immunoassays for detecting the coccidiostats halofuginone, nicarbazin and nitroimidazoles in egg and chicken muscle

**A-C. Huet[1], C. Charlier[1], E. Daeseleire[2] and P. Delahaut[1]**

*[1] CER Groupe, Laboratoire d'Hormonologie, Rue du Carmel 1, B-6900 Marloie, Belgium.*
*[2] ILVO, Unit Technology and Food, Brusselsesteenweg 370, B-9090 Melle; Belgium.*
*e-mail: p.delahaut@cergroupe.be*

**Keywords**
Coccidiostats, nitroimidazoles, ELISA, egg, chicken muscle

## SUMMARY

Nicarbazin and halofuginone have been widely used as coccidiostats for the prevention and treatment of coccidiosis in poultry. Nitroimidazoles, the use of which is forbidden in the European Union, were used to treat and prevent as well coccidiosis as histomoniasis. In the past it was shown that accidental cross-contamination of feed can be a major cause of the presence of coccidiostats in eggs and meat. This paper describes the development and validation of a direct competitive assay for the detection of halofuginone and nicarbazin and another one for the detection of nitroimidazole drugs. The methods can be used as screening assays.
Extraction was performed with acetonitrile followed by a washing step with hexane. The assay's detection capabilities (CCβ) for halofuginone were < 0.5 μg kg $^{-1}$ in egg and < 1 μg kg $^{-1}$ in muscle. For dinitrocarbanilide the CCβ was estimated at < 3 μg kg $^{-1}$ in egg and < 10 μg kg $^{-1}$ in chicken muscle. Detection capabilities (CCβ) were also determined for the nitroimidazoles ELISA: dimetridazole, <1 μg kg $^{-1}$ (egg) and <2 μg kg $^{-1}$ (muscle); metronidazole, <10 μg kg $^{-1}$, ronidazole and hydroxydimetridazole, <20 μg kg $^{-1}$; ipronidazole, <40 μg kg $^{-1}$.

## INTRODUCTION

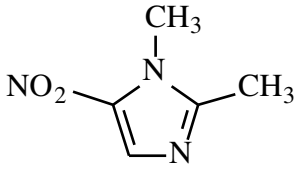
Coccidiosis is a highly contagious animal infection caused by single-celled organisms of the genus *Eimeria* (class: Sporozoa). In poultry, seven *Eimeria* species are known to cause serious clinical disease [1]. Although in most cases repeated infection results in the development of immunity, even asymptomatic infection (sub clinical coccidiosis) leads to economic losses due to reduced egg production or malabsorption (poor weight gain or feed conversion) [2].
The anti-coccidial drugs currently available are used only prophylactic, but continuous prophylactic use of coccidiostats can lead to progressive loss of efficacy due to emerging drug resistance in the parasite [3]. In Belgium and other countries, some compounds are licensed for the use as feed additives at prescribed concentrations and during certain time intervals for broilers and young chickens, but most of them are not licensed for use in egg-laying chickens [4]. Due to carry-over from previously medicated feeds, accidental cross-contamination occurred and this is probably the major cause for the presence of coccidiostat residues in eggs and tissues [5-7].

In the present study we have focused on two chemical coccidiostats, halofuginone (HFG) and nicarbazin (NIC). The last one is a mixture of 4,6-dimethyl-2-hydroxypyrimidine (DHP) and 4,4'-dinitrocarbanilide (DNC) in 1:1 molar ratio. The DHP moiety is excreted rapidly following drug withdrawal, but DNC is less rapidly eliminated. Therefore the FAO/WHO Joint Expert Committee on Food Additives (JECFA) has established DNC as the marker residue for nicarbazin [8].

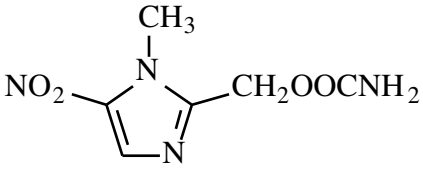
In the European Union, halofuginone hydrobromide has been authorised since 1993 (Council Directive 91/248/EC). According to Directive 70/524/EEC and Commission Regulation (EC) no. 2430/1999, it is still allowed for laying hens until 2009, but no residues may be found in eggs. The use of nicarbazin is not authorised for laying hens. As far as poultry muscle is concerned, the European Agency for the Evaluation of Medicinal Products (EMEA) has not yet established a maximum residue limit (MRL) for halofuginone or nicarbazin. As a consequence, in the EU the zero tolerance principle has to be applied when evaluating the presence of residues in poultry matrices. However different European countries apply different rules. In Belgium for example, the scientific committee of the Belgian Food Agency has proposed an action limit of 10 µg/kg for a group of coccidiostats.

The nitroimidazole antiprotozoal drugs are not regarded as classical coccidiostats but are primarily used to prevent and treat the diseases histomoniasis and trichomoniasis in game birds, turkeys and pigeons. However, they have also been used to treat coccidiosis in poultry and game birds. Two additional effects have been shown: growth promotion and improvement of feed efficiency [9].

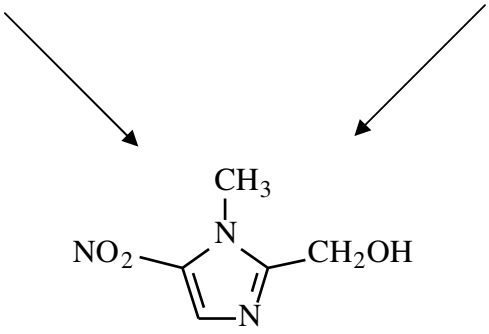
The most frequently used nitroimidazoles, namely dimetridazole (DMZ), ipronidazole (IPZ), metronidazole (MNZ), and ronidazole (RNZ), are suspected of being genotoxic, carcinogenic, and mutagenic, as are their hydroxy metabolites having retained the original nitroimidazole ring (figure 1) [10-11].




Dimetridazole




Ronidazole




Hydroxydimetridazole

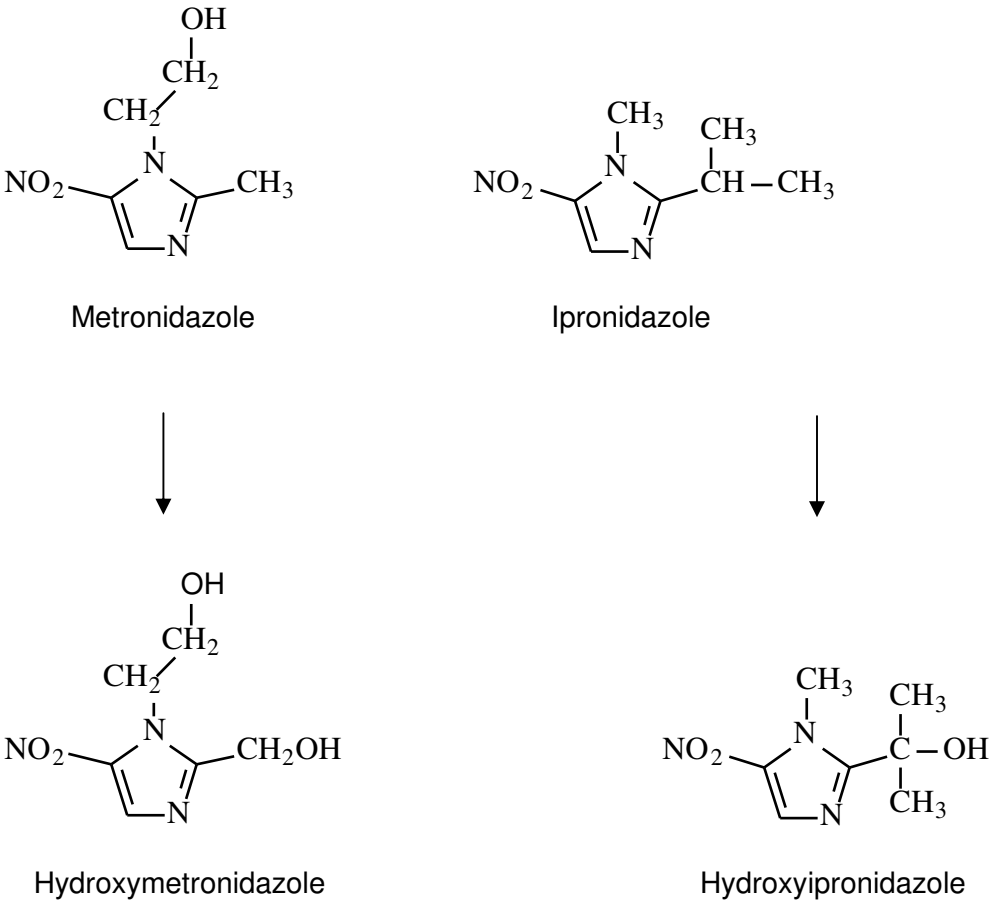



**Figure 1. Chemical structures of the nitroimidazole drugs and their main metabolites**

For this reason, a number of them have already been banned in Europe by Council Regulation 3426/93/EEC for RNZ [12], 1798/95/EEC for DMZ [13] and 613/98/EEC for MNZ [14]. IPZ is not authorised in the EU [15]. 5-Nitroimidazoles are known to be rapidly metabolised, the main metabolites resulting from oxidation of the side-chain at the C2 position of the imidazole ring.

A number of immunoassays have been described for the determination of halofuginone and nicarbazin [16-21]. Only a few papers describe ELISAs for the determination of nitroimidazoles [10], [22-23].

This paper reports on one hand, the development of a competitive enzyme-linked immunosorbent assay (cELISA) for MNZ, DMZ, RNZ, DMZOH, and IPZ in eggs and chicken tissues and on the other hand, a cELISA for detecting HFG and DNC in the same matrices. This is the first example of dual coccidiostat residue detection in a single immunoassay procedure.

## EXPERIMENTAL

### *Standards, reagents and chemicals*

DNC was provided by Sigma (St. Louis, MO, USA) and HFG was from Intervet (Mechelen, Belgium). DMZ, RNZ, MNZ were obtained from Sigma (St. Louis, MO, USA). DMZOH, MNZOH, IPZ, and IPZOH were provided by the EU Reference Laboratory, BgVV (Berlin, Germany). PD-10 Gel filtration columns were purchased from Amersham Biosciences (Uppsala, Sweden). Acetonitrile for HPLC was from Across Organics (Geel, Belgium). Hexane and methanol for HPLC were bought at VWR international (Leuven, Belgium). TMB and $H_2O_2$ were from KPL (Maryland, USA).

### *Preparation of immunogens*

As a nicarbazin-mimicking immunogen, N-succinyl-L-alanyl-L-alanyl-L-alanine 4-nitroanilide (sharing a common substructure with DNC) was used and conjugated with the carrier protein human serum albumin (HSA). The carbodiimide method, used to form the conjugate, has been described in detail [20]

A halofuginone derivative having a succinyl linker was synthesized from halofuginone hydrobromide as described by Rowe *et al.* [24]. Briefly, *N*-(trimethylsily)-imidazole was used to protect the HFG hydroxyl group during reaction of the piperidyl nitrogen with succinic anhydride. The succinyl derivative was conjugated to HSA via an N-hydroxysuccinimide enhanced, carbodiimide-mediated coupling reaction.

The nitroimidazole chosen as a hapten was MNZ; a jeffamine spacer was used to extend the distance between the drug and the carrier protein, human serum albumin (HSA). Disuccinimidyl carbonate (DSC) was used to prepare the immunogen. Further practical details are available in reference [23].

Both immunogens were purified by dialysis against 0.15 M saline (3 x 1L).

### *Preparation of enzyme-labelled drugs*

Two DNC-mimicking compounds, nitrosuccinanilic acid and N-succinyl-L-alanyl-L-alanyl-L-alanine 4-nitroanilide, were conjugated with the enzyme label horseradish peroxidase (HRP) using the same method as described above (carbodiimide reaction).

Halofuginone-hemisuccinate-HRP was synthesised in the same manner as the halofuginone immunogen, but replacing the carrier protein with the enzyme HRP.

Two nitroimidazoles (DMZOH and MNZ) were conjugated to the enzyme label horseradish peroxidase (HRP). For the first conjugate, the isocyanate end of p-maleimidophenyl isocyanate was allowed to react with the hydroxyl group of DMZOH. The maleimide end of the hapten was then allowed to react with amines and sulfhydryls on the protein. For the second conjugate, MNZ was conjugated to HRP by the same method as for the immunogen, but the jeffamine spacer was excluded from the preparation of this label. Further practical details are available in reference [23]. The conjugates were purified by gel filtration on PD-10 columns.

### *Immunisation of rabbits*

Immunogen emulsions were injected subcutaneously into four sites on the animal. Rabbits were immunised every 28 days with 200 µg immunogen, and blood samples were taken from the marginal vein of the ear 10 days after each immunisation (from the third immunisation onward). The antiserum used in the study was harvested 10 days after the seventh immunisation.

### *Preparation of microtitre plates*

For the nitroimidazoles assay, 96-well Maxisorp microtitre plates (Nunc, Roskilde, Denmark) were coated by adding 200 µl diluted antibodies raised against

metronidazole to each well. The antiserum was diluted in coating buffer, 0.1 M sodium hydrogen carbonate pH 9.6 (VWR international - Leuven, Belgium), at a dilution rate of 1:15000. Other microtitre plates were coated with two specific antibodies (one recognising HFG and the other, DNC) in the same well, for the coccidiostat assay. Unpurified antisera raised against HFG and DNC were both diluted 1:48000 in coating buffer.
The diluted antibodies were allowed to adsorb onto the plate for 72 hours at 4°C. This was followed by blocking with 25 g $L^{-1}$ casein hydrolysate solution pH 7 (Calbiochem - San Diego, USA) for two hours at room temperature.
The plates were washed three times with washing buffer (0.15 M NaCl from VWR international - Leuven, Belgium; 0.05% Tween 20 from Merck - Darmstadt, Germany), then, the wells were filled with 1% saccharose (Merck - Darmstadt, Germany). After 10 minutes, the microplates were completely emptied and stored in the dark at 4°C.

### *Competitive ELISA*

After three washings, each well, coated as described above, was filled with 100 µl working standard or sample (diluted or not) or assay buffer ($B_0$). The assay buffer composition was: 0.15 M NaCl, 0.056 M $Na_2HPO_4.2H_2O$, 0.009 M $NaH_2PO4.2H_2O$, 0.2% gelatine, 0.05% Tween 20, 0.01% 8-anilino-1-naphtalenesulfonic acid ammonium salt, and 0.0028 M ascorbic acid.
Per well, 150 µl peroxidase conjugate diluted in assay buffer was added (the conjugate being chosen according to the residue to be detected). The respective dilution rates for enzyme-labelled DNC, HFG and nitroimidazoles were 1:6000, 1:60000 and 1:65000 in assay buffer. The plate was incubated overnight at 4°C. After five washes with washing buffer, antibody-bound conjugate was measured with chromogen TMB and the enzyme substrate $H_2O_2$ (1/1). The average optical density (OD) of $B_0$ wells, containing all components except the competitor, was taken to represent 100 % activity.

### *Sample extraction*

Validation of the method was carried out as follows: whole eggs were homogenised and muscle was minced. Then, 2 g homogenised egg or 2 g minced muscle was weighed into a 50-ml Falcon tube. At this stage, twenty known negative samples (per matrix) were spiked by adding an appropriate amount of standards corresponding to the detection capability (CCß) and allowed to stand for 10 minutes in the dark. Twenty known negative samples were extracted simultaneously with the twenty fortified samples (HFG, or DNC or DMZ). Validation for the other nitroimidazoles was carried out as follows: for each matrix, 2 known negative samples were extracted simultaneously with 20 known negative samples spiked with MNZ, RNZ, DMZOH or IPZ. All known negative samples were declared free of the analytes of interest on the basis of a liquid chromatography-tandem mass spectrometry method developed by our laboratory team [25].
Acetonitrile (8 ml) was added to each sample and the mixtures were immediately vortexed for 1 minute and placed in an ultrasonic bath for five minutes. All tubes were centrifuged for 10 minutes at 4100 g and 4°C. Supernatants were transferred to 10-ml tubes and evaporated to dryness under nitrogen at 40°C. Hexane (1 ml) was added to each sample and mixed, then 1 ml methanol/water (3/1) was added. Samples were vortexed for 10 seconds and allowed to stand in a water bath at 40°C for 5 minutes. After centrifugation at 1000 g for 5 minutes at 4°C, the hexane layer and any traces of emulsion at the interface were removed with a Pasteur pipette. The remaining solution was evaporated to dryness under nitrogen at 40°C, and the dried material was dissolved in assay buffer containing 5% methanol.

It was shown that blank samples extracted at room temperature showed inhibition of antibody binding. So therefore it is important that the samples are well cooled before use in the nitroimidazoles assay; Samples were firstly cooled at 4°C for at least one hour. They were also kept on ice during preparation of the test as it was proven that cooling decreased the matrix effect, making it possible to detect lower concentrations of nitroimidazoles.

## RESULTS AND DISCUSSION

### *Antibody selection*

Enzyme-labelled nitroimidazoles were prepared by conjugation of MNZ or DMZOH with HRP. Blood samples taken from immunised animals were assessed for their antibody content. Each serum was tested with both peroxidase conjugates in the presence of two standards (MNZ and DMZOH). Antibodies perform best as regard to sensitivity when used in the heterologous format (different compounds used to synthesise the immunogen and the peroxidase conjugate). The DMZOH-HRP conjugate gave better results, in agreement with the observations of Fodey *et al.* [23]. Heterologous assays are indeed well known to help improve immunoassay sensitivity and overcome unwanted cross-reactivity [18]. Five rabbits were immunised with the immunogen MNZ-DSC-jeffamine-HSA. The rabbit PC 107 was chosen because it showed both a high antibody titre and an acceptable displacement with MNZ and DMZOH

Two conjugates were tested with anti-nicarbazin antisera: nitrosuccinanilic acid-HRP (Nic-C-HRP) and N-succinyl-L-alanyl-L-alanyl-L-alanine 4-nitroanilide-HRP (Nic-A-HRP). Four out of the five rabbits treated produced antibodies. Plates coated with the various antibody preparations were screened for displacement of the conjugate by free DNC. Nic-A-HRP was not displaced, contrary to Nic-C-HRP. This confirms the finding of Connolly *et al.* [20] that such antibodies work best when used in a heterologous assay format. Polyclonal antibody M 187 was chosen for further experiments, having shown the highest sensitivity for DNC detection.

Five rabbits were immunized with halofuginone-hemisuccinate-HAS. All rabbits, except for one, produced a specific response. Displacement was observed with all four of the corresponding antisera. Polyclonal antibody M 98 was selected, having exhibited the greatest affinity by cELISA.

### *Assay development and antibody characteristics*

To optimise the assay with regard to sensitivity and precision, several key parameters were studied: choice of antibody and conjugate, amount of coating antibody and conjugate, incubation time and temperature. The highest relative affinities for halofuginone, nicarbazin and nitroimidazoles were observed with the assay format described within the experimental section. The assay sensitivity was calculated as 50 % inhibition of control ($IC_{50}$), the concentration of residue necessary to cause 50 % inhibition of antibody binding. The degree of antibody specificity (i.e., the extent of cross-reactivity) was estimated by means of the formula:

$$100 \times \frac{IC_{50} \text{ of compound used to raise antibody}}{IC_{50} \text{ of competing compound}}$$

With the standard curves prepared in buffer, the values of $IC_{50}$ were calculated as well as the cross-reactivity profile of PC 107 antibody within the nitroimidazole family (table 1). Cross-reactions were observed with DMZ, RNZ, DMZOH, MNZ, and IPZ. No significant cross-reactivity was found with any of the following other coccidiostats

commonly used in broiler production: halofuginone, dinitrocarbanilide, diclazuril, and robenidine.

**Table 1. $IC_{50}$ values and cross-reactivity profile of anti-metronidazole antibodies with a range of nitroimidazoles**

| | $IC_{50}$ (ng $ml^{-1}$) | % Cross-reactivity |
|---|---|---|
| **Metronidazole** | 17.5 | 100 |
| **Hydroxymetronidazole** | 137.5 | 12.7 |
| **Dimetridazole** | 1.3 | 1346 |
| **Ronidazole** | 15.3 | 114 |
| **Hydroxydimetridazole** | 24.4 | 71.7 |
| **Ipronidazole** | 17.6 | 99.4 |
| **Hydroxyipronidazole** | 297 | 5.9 |

The antibody chosen showed the highest affinity for DMZ and an equal affinity for all other compounds except for IPZOH and MNZOH, for which the affinity was quite low. It seems that the binding affinity is influenced by different parameters of the molecule. On the one hand, the highest and lowest affinities observed suggest an effect of steric hindrance on antibody binding. The chemical structure of dimetridazole is the simplest, making antibody binding easier. The electron density (around either n° 1 nitrogen or n° 2 carbon), being greater in the other nitroimidazole formulas, makes antibody access for binding more difficult and might explain the differential antibody binding results. On the other hand, the antibody binding is influenced by the presence or absence of a single hydroxyl group and by its position on the imidazole ring. This confirms the finding of Stanker *et al.* [22]. For each metabolite (DMZOH, MNZOH, and IPZOH), a hydroxyl group is added at the same position on the imidazole ring (figure 1). Clearly, addition of this group reduces affinity, suggesting that this position is important for antibody binding.

Wesseling *et al.* [10] have also raised polyclonal antibodies against MNZ and RNZ, but these were not suitable for application in ELISA because of their low sensitivity. Stanker *et al.* [22] generated and isolated 11 monoclonal antibodies making use of hybridoma production. Only one antibody was chosen for the development of a competitive ELISA for detection of DMZ, DMZOH, IPZ, IPZOH, and MNZ. Their $IC_{50}$ values suggest that the antibody binds to nitroimidazoles with a relatively low affinity. The antibodies isolated showed 166-fold-reduced binding to the hydroxyl metabolite of IPZ. The antibody used in our study also showed low affinity to IPZOH. Stanker *et al.* developed an extraction method for turkey muscle, but this procedure was too long and required solvents in large amounts. In the framework of the European project "Poultry-Check", the team of Christopher Elliott reported the production of antibodies and the development of an immunoassay for nitroimidazoles [23]. The results shown for IPZOH were no better than the results obtained in this study, but some of the antibodies used showed acceptable binding to MNZOH.

The $IC_{50}$ were also estimated for the coccidiosats assay, namely 0.08 ng $ml^{-1}$ for HFG and 2.5 ng $ml^{-1}$ for DNC. A typical standard curve for each analyte is shown in figure 2.

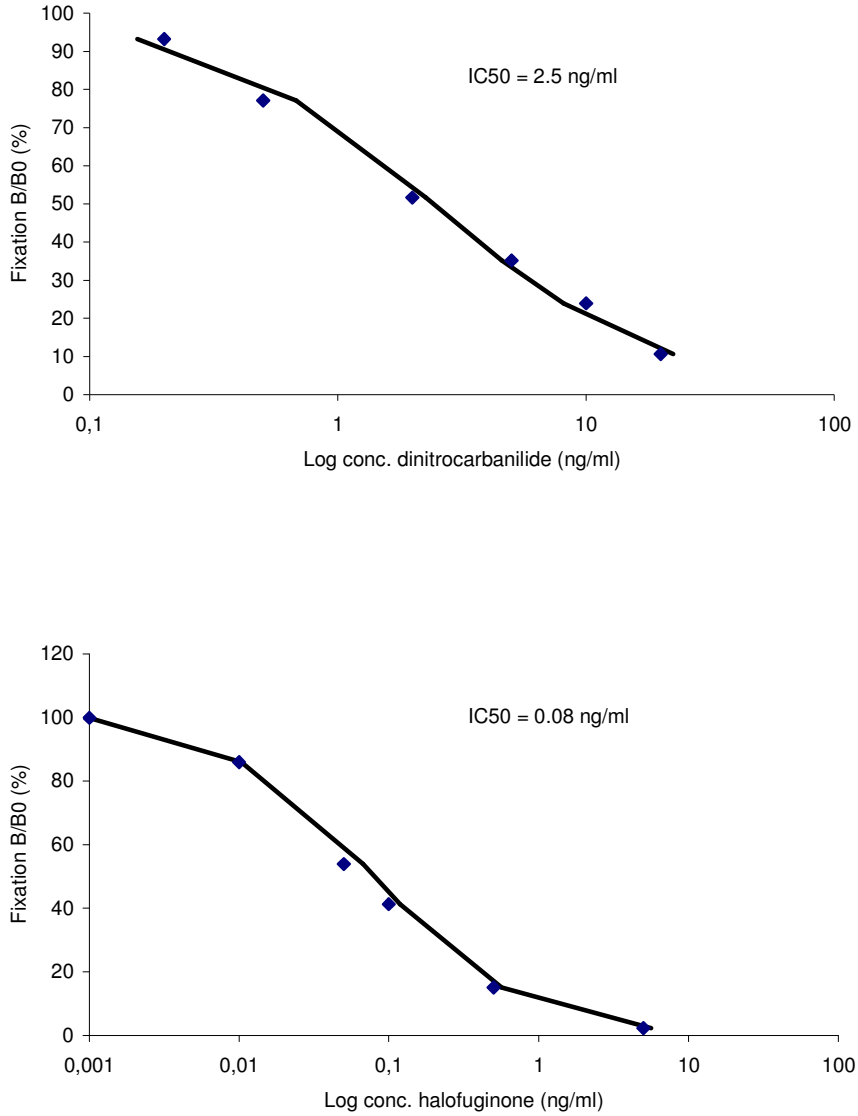



**Figure 2. The ability of anti-DNC and anti-HFG polyclonal antibodies to bind free DNC and free HFG respectively was evaluated using a standard curve analysis**

Only a few papers described the development of ELISA's for the determination of halofuginone and dinitrocarbanilide. In one study, six monoclonal antibodies against halofuginone were produced with $IC_{50}$ values ranging from 1.9 to 120 ng $ml^{-1}$ in ELISA experiments using halofuginone as competitor [16]. Stanker *et al.* [17] developed an immunoassay capable of detecting halofuginone between 6 and 50 µg $kg^{-1}$ in chicken serum when using one of these antibodies. Monoclonal antibodies have also been isolated for the detection of DNC, but the development of this competitive ELISA gave inconsistent results [19]. On the basis of this study, the same researchers developed a cELISA for nicarbazin but the results showed lower sensitivity than in the present study [18]. In the framework of the European project "Poultry-Check", the production of

antibodies and the development of an immunoassay for nicarbazin were also reported. The $IC_{50}$ results of the antibodies ranged from 2.3-7.6 ng $ml^{-1}$ [20].
Since antibodies to DNC and HFG were present in the same well, it was essential to check for cross-reactivity. Cross-reactions were checked for halofuginone, DNC, diclazuril, robenidine and a range of nitroimidazoles (DMZ, RNZ, MNZ, IPZ, DMZOH). The M 98 antibody proved to be HFG-specific and the antibody M 187 DNC-specific. They showed little to no cross-reactivity with any other coccidiostat at the maximum concentration tested; the competitive ELISA was performed with increasing amounts of competitor, ranging up to 1500 ng $ml^{-1}$ for M 98 and 2000 ng $ml^{-1}$ for M 187.

### *Detection in chicken muscle and egg*

The validation study was carried out according to the criteria set by Commission Decision 2002/657/EC [26].

### *Detection capability: coccidiostats assay*

The detection capability (CCβ) is defined as the smallest concentration of analyte that can be identified and/or quantified in a sample with an error probability of β. The β error should be less than or equal to 5%. In the case of substances without an established MRL, the CCβ is the lowest concentration at which a method can detect truly contaminated samples with a statistical certainty of 1-β. Theoretically, if 19 out of 20 fortified samples were declared non-compliant then CCβ = level of fortification. If all of the fortified samples should have been declared non-compliant, then CCβ < level of fortification. In practice, the levels of fortification in each matrix were chosen as to ensure that all of the fortified samples were declared non-compliant. This decision should avoid the problem of false negatives. However, it was kept in mind that the level of fortification should be as low as possible. The detection capabilities were obtained for each matrix by assaying 20 known negative samples and 20 known negative samples being fortified with HFG at 1 μg $kg^{-1}$ (muscle), 0.5 μg $kg^{-1}$ (egg) and with DNC at 10 μg $kg^{-1}$ (muscle) and 3 μg $kg^{-1}$ (egg). The mean values of the absorbance (450 nm) obtained for each sample are divided by the absorbance value of the zero standard ($B_0$) and multiplied by 100. For each group of 20 samples, the mean ratio $B/B_0$ (%) ± two standard deviations was calculated (table 2).

**Table 2. Detection capabilities of HFG, DNC in egg and muscle matrices. The average (n = 20) binding ratio $B/B_0$ (%) was calculated ± two standard deviations for negative samples (blank) and spiked samples (test)**

| Matrix | Residue | Blank Binding $B/B_0$ (%) | Test Binding $B/B_0$ (%) | Detection capability CCβ (μg $kg^{-1}$) | Sample dilution |
|---|---|---|---|---|---|
| Egg | HFG<br>DNC | 62-114<br>81-101 | 45-58<br>53-74 | < 0.50<br>< 3 | 10 x<br>2 x |
| Muscle | HFG<br>DNC | 85-111<br>76-101 | 48-68<br>55-75 | < 1<br>< 10 | 10 x<br>20 x |

An egg sample was considered non-compliant if the binding percentage was below 62% for the HFG assay or 81% for the DNC assay. A muscle sample was considered non-compliant if the binding percentage was below 85% (HFG) or 76% (DNC). For the HFG-spiked matrices, the estimated detection capabilities were < 0.5 μg $kg^{-1}$ (egg) and < 1 μg $kg^{-1}$ (muscle). For the DNC-spiked matrices the values were < 3 μg $kg^{-1}$

(egg) and < 10 μg kg $^{-1}$ (muscle). Indeed, all of the fortified samples were declared non-compliant from which it can be concluded that CCβ < level of fortification.
A matrix effect resulting in inhibition of colour development occurred in some assays. This effect was essentially eliminated by dilution of the reconstituted sample with assay buffer. The matrix effect was greatest when muscle extracts were used in the immunoassay for DNC. In this case, to obtain blank-sample optical density (OD) values near the OD value for $B_0$ (maximal activity), a dilution of 20-fold had to be made on the reconstituted sample.
As reported in the introduction, no MRL (maximum residue limit) has been set for poultry or egg but in practice, in Belgium, an action limit of 10 μg kg$^{-1}$ has been proposed by the scientific committee of the Belgian Food Agency for monensin, salinomycin, diclazuril, lasalocid, maduramycin, narasin, nicarbazin, robenidine and the group of sulphonamides [27]. The screening assay was able to identify HFG and DNC at levels inferior to 10 μg kg$^{-1}$.

*Detection capability: nitroimidazoles assay*

Twenty known negative samples spiked with dimetridazole at 1 μg kg $^{-1}$ for the egg matrix and 2 μg kg $^{-1}$ for the muscle matrix were simultaneously extracted with 20 known negative samples. Validation of the other nitroimidazoles was carried out as follows: for each matrix, 20 known negative samples were fortified with metronidazole at 10 μg kg $^{-1}$, with ronidazole at 20 μg kg $^{-1}$, with hydroxydimetridazole at 20 μg kg $^{-1}$, with ipronidazole at 40 μg kg $^{-1}$ simultaneously with 2 known negative samples.
Table 3 summarizes the results obtained after processing each biological matrix. The data obtained from samples fortified or not were calculated as $B/B_0$ (in percent) as explained above.
For each group of 20 negative samples, the mean ratio $B/B_0$ (in percent) ± two standard deviations was calculated: 80% to 103% for the egg matrix and 81% to 97% for the muscle matrix. Egg samples were declared non-compliant if the binding percentage was inferior to 80%. Muscle samples were declared non-compliant if the binding percentage was below 81%. We observed that the mean percentage $B/B_0$ of negative samples (n = 2) was in the range 80% to 103% range for egg matrix and in the range 81% to 97% for muscle matrix. For spiked samples, the average (n = 20) binding ratio $B/B_0$ (%) ± two standard deviations corresponds to criteria that we determined in order to check the progress of the extraction during routine use of the method.
Because of the matrix effect in muscle tissue, reconstituted extracts had to be diluted twice in assay buffer before they were added to the coated wells. Otherwise, the optical density (OD) values for blank samples were too different from those obtained at maximal binding ($B_0$).

**Table 3. Validation of the screening method: detection capabilities of nitroimidazoles in egg and muscle matrixes. The average (n = 20) binding ratio $B/B_0$ (%) was calculated ± two standard deviations for all spiked samples (test) and negative samples (blank) validated simultaneously with dimetridazole. The average (n = 2) binding ratio $B/B_0$ (%) was calculated for remaining negative samples. Recovery data are also presented**

| Matrix | Compound | Blank Binding $B/B_0$ (%) | Test Binding $B/B_0$ (%) | Detection capability CCβ (μg kg $^{-1}$) | Recovery (%) |
|---|---|---|---|---|---|
| Egg | Dimetridazole | 80 - 103 | 59-76 (1 μg kg $^{-1}$) | < 1 | 28 |
| | Metronidazole | 81 | 42-65 (10 μg kg $^{-1}$) | < 10 | 95 |
| | Ronidazole | 102 | 33-61 (20 μg kg $^{-1}$) | < 20 | 92 |
| | Hydroxy dimetridazole | 87 | 43-61 (20 μg kg $^{-1}$) | < 20 | 58 |
| | Ipronidazole | 93 | 40-69 (40 μg kg $^{-1}$) | < 40 | 18 |
| Muscle | Dimetridazole | 81 - 97 | 57-69 (2 μg kg $^{-1}$) | < 2 | 66 |
| | Metronidazole | 82 | 58-78 (10 μg kg $^{-1}$) | < 10 | 98 |
| | Ronidazole | 90 | 64-78 (20 μg kg $^{-1}$) | < 20 | 82 |
| | Hydroxy dimetridazole | 84 | 52-75 (20 μg kg $^{-1}$) | < 20 | 94 |
| | Ipronidazole | 97 | 58-80 (40 μg kg $^{-1}$) | < 40 | 50 |

*Recovery*

The effectiveness of the extraction procedure was assessed by determining the recovery. On the one hand, three known negative samples (per analyte and per matrix) were fortified before the extraction with the analytes of interest at CCβ level. On the other hand, three known negative samples (per analyte and per matrix) that were called total-count, were also analysed. All these samples were extracted as previously described, except that after the methanol/water layer was evaporated to dryness, the total-count vials were resuspended in assay buffer containing the level of fortification for each residue. Then, all samples were applied to the ELISA plate and the average (n=3) analyte levels were calculated in order to establish the percent recovery. For example: (DMZ levels in samples spiked before extraction divided by DMZ levels in samples spiked after extraction) x 100. The mean percentage recovery of the five nitroimidazoles, from egg and chicken muscle, is given in table 3. The lowest recovery found was for ipronidazole (18% for egg and 50% for muscle) which might explain the highest CCβ recorded for this residue.

The mean percentage recovery of HFG from egg and chicken muscle was 84 % and 71 % respectively. The mean percentage recovery of DNC from egg and chicken muscle was 67 % and 85 %.

*Comparison of the developed immunoassay for coccidiostats with the LC-MS-MS method*

HFG and DNC residues were determined in 14 egg samples, using either incurred or spiked samples. Various levels of DNC and HFG contamination were measured as a result (table 4).

**Table 4. Results of two different assays (ELISA and LC-MS-MS) for the determination of DNC and HFG residues in eggs**

| Sample | Halofuginone | | Dinitrocarbanilide | |
|---|---|---|---|---|
| | ELISA (µg kg $^{-1}$) | LC-MS-MS (µg kg $^{-1}$) | ELISA (µg kg $^{-1}$) | LC-MS-MS (µg kg $^{-1}$) |
| 01 | 100 | 70 | 12 | 10 |
| 02 | 12.50 | 8 | 26 | 15 |
| 03 | 39 | 18 | 60 | 78 |
| 04 | 51 | 21 | 19 | 17 |
| 05 | 0 | 0 | 64 | 83 |
| 06 | 0 | 0 | 24 | 26 |
| 07 | 27 | 23 | 0 | 0 |
| 08 | 140 | 76 | 14 | 13 |
| 09 | 26 | 22 | 87 | 158 |
| 10 | 11 | 9 | 92 | 157 |
| 11 | 123 | 67 | 0 | 0 |
| 12 | 7 | 7 | 70 | 109 |
| 13 | 136 | 63 | 31 | 26 |
| 14 | 3.60 | 7 | 69 | 88 |

The results obtained with the ELISA described here were compared with a method based on electrospray liquid chromatography tandem mass spectrometry (LC-MS-MS) [25]. Differences between results were evaluated by means of the regression coefficient ($r^2$). Good correlations were observed between HFG and DNC screening & confirmatory assays ($r^2$ = 0.95 and 0.95 respectively). No false negatives and no false positives were identified by the immunoassay. The HFG ELISA results, apparently, tend to overestimate the HFG content of samples. This may be due to the fact that to quantify these reconstituted samples, we had to dilute the samples 100-fold in order to reach OD values within the limits of the standard curve or to the fact that the ELISA also measures metabolites that can be present in the incurred eggs.

## CONCLUSIONS

This paper reports the development and validation of an immunoassay for the determination of five nitroimidazoles and another one for the detection of halofuginone and dinitrocarbanilide, both in chicken muscle and egg. It was shown in this study that immunoassay techniques are a suitable alternative approach for the screening of residues in biological matrices. Another advantage of immunoassays is the reduction of the use of expensive and sophisticated equipment [28-29].

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# Case study: coccidiostats

**E. Daeseleire[1], A.-C. Huet[3], G. Huyghebaert[2] and P. Delahaut[3]**

*[1] ILVO, Unit Technology and Food, Brusselsesteenweg 370, B-9090 Melle, Belgium*
*[2] ILVO, Unit Animal, Scheldeweg 68, B-9090 Melle*
*[3] CER Groupe, Laboratoire d'Hormonologie, Rue du Carmel 1, B-6900 Marloie, Belgium*
*e-mail : els.daeseleire@ilvo.vlaanderen.be*

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Coccidiostats, residues, eggs, LC-MS/MS, ELISA

## SUMMARY

Anticoccidials are compounds that are widely used as feed additives to prevent and treat coccidiosis. They are licensed for use in a prescribed concentration and during a certain time interval for broilers and pullets but not for laying hens. It was shown in the past that carry-over at the feeding mill is found to be the main reason for the presence of residues in eggs.
An animal experiment was set up to investigate the effect of carry-over at the feeding mill on the presence of residues of anticoccidials in eggs. For the compounds diclazuril, robenidine, halofuginone and nicarbazin in combination with narasin, two concentration levels were tested: the maximum allowed concentration for broilers (100%) and a concentration corresponding to 5% carry-over during feed preparation. Also dimetridazole was included in the experiment but only at one concentration level. Eggs were sampled during treatment (14 days) and for a period of 30 days after withdrawal of the anticoccidial-containing feed. Residues were determined and deposition and depletion curves were generated. Analyses were performed by ELISA and LC-MS/MS. For all compounds, substantial residues could be found in the 5% groups, which points out the risk of carry-over at the feeding mill. The distribution of the residues between egg yolk and white was determined by analyzing both fractions.

## INTRODUCTION

Anticoccidials are compounds that are widely used to prevent and treat coccidiosis, a contagious amoebic disease affecting livestock and particularly poultry. In warm, humid conditions it causes intestinal lesions, which result in diarrhoea and related health problems in the animal. The disease is carried by unicellular organisms belonging to the genus *Eimeria* in the class Sporozoa. In its acute form coccidiosis causes high mortalities, in its sub-acute form, small numbers of oocysts can adversely affect weight gain, feed conversion and egg production in poultry. Of all domestic animals, industrially bred poultry and rabbits are particularly prone to this disease.
The economic damage caused by coccidiosis in modern poultry production is so serious that practically all poultry farms have resorted to feeding anticoccidial drugs as a feed additive to pullets and broiler breeders for 12 to 16 weeks and to broiler chickens for almost their entire life. Despite the use of anticoccidial drugs, coccidiosis remains one of the biggest causes of losses in poultry production.
A wide range of anticoccidial drugs is available to treat and prevent coccidiosis. Besides the ionophoric anticoccidials, such as narasin, monensin, lasalocid and

salinomycin, there is also a class of chemical anticoccidial drugs. The most common chemical anticoccidials are nicarbazin, halofuginone, diclazuril and robenidine.
According to Regulation 1831/2003/EC, anticoccidials are at the moment licensed as feed additives [1]. They can be used at a prescribed concentration and during a certain time interval for broilers and pullets but not for laying hens. Hence, no residues of anticoccidials should be present in eggs. However, carry-over in the feeding mill is a major problem and can run up to 15% [2]. Therefore, anticoccidials can be present in feed intended for laying hens. With a view to a decision on the phasing-out of the use of anticoccidials as feed additives by 31 December 2012, the European Commission shall submit to the European Parliament and the Council a report on the use of these substances as feed additives and available alternatives before 1 January 2008. In the mean time, there are, except for lasalocid, no Maximum Residue Levels (MRLs) set for eggs, and thus the compounds may not be present in eggs. Hence the zero tolerance principle has to be applied. In practice however, different EU member states apply another approach. In Belgium, an action limit of 10 µg/kg has been proposed by the scientific committee of the Belgian Food Agency for monensin, salinomycin, diclazuril, maduramycin, narasin, nicarbazin, robenidine and the group of sulphonamides [3] . In the United Kingdom on the other hand, an action limit of 100 µg/kg for nicarbazin in eggs has been set and in Sweden, action is take when narasin concentrations exceed 5 µg/kg [4-5].
The occurrence of anticoccidial residues in eggs has been widely reported. In Northern Ireland, 161 eggs were analyzed in 1994 and residues of monensin, salinomycin and narasin were detected [6]. In 1995, a granular formulation of lasalocid was introduced in the United Kingdom. This decreased the carry-over and six months after the introduction of this granular formulation, the incidence of lasalocid residues in eggs had decreased to 21% [7]. Also in Northern Ireland, in 1996 a survey was conducted in which 190 egg samples were analyzed on the presence of nicarbazin. In 39 samples (20.5%), nicarbazin could be detected in concentrations varying from 4 to 342 µg/kg [4]. In Great Britain, the overall incidence of residues of nicarbazin in eggs tested was 10.7% in 1996, 6.8% in 1997 and 4.0% in 1998 [4]. In 1999, 24 egg samples were analyzed on the presence of narasin in Sweden. Twelve samples (50%) contained between 0.2 and 11 µg/kg narasin [5]. Also in our lab, residues of anticoccidials were encountered in eggs. In 2002, 232 samples were analyzed on the presence of dimetridazole, diclazuril, robenidine, halofuginone and nicarbazin. Four samples contained nicarbazin in concentrations varying from 3 µg/kg to 197 µg/kg. Three samples were positive on the presence of halofuginone but concentrations did not exceed 3 µg/kg. In 2003, 245 eggs were analyzed. Nicarbazin and robenidine were encountered in two samples each. In 2004, 190 samples were analyzed. For these samples, also the ionophores narasin, salinomycin, lasalocid and monensin were included in the monitoring. Twelve of the 190 samples contained residues of coccidiostats: robenidine (8 µg/kg), monensin (10 µg/kg), salinomycin (2 and 8 µg/kg) and lasalocid (4 to 90 µg/kg) were found.
In this study an extensive animal experiment was set up to investigate the effect of carry-over at the feeding mill on the presence of residues in eggs. This experiment was carried out in the framework of a project, which had the aim to set up an integrated approach for the detection of residues of anticoccidials in eggs. This integrated approach is based on the use of the pyramid structure: first a screening is carried out so that only positive samples need to be analyzed by the more expensive confirmation methods. To use this approach, it is necessary that the screening method does not produce false negative results. The compounds studied in this project are: diclazuril, dimetridazole, halofuginone, robenidine and nicarbazin. In the first part of the project, immunological screening methods and liquid chromatographic mass spectrometric confirmation methods were developed and validated. An ELISA (enzyme linked

immuosorbent assay) was developed for the detection of halofuginone, nicarbazin and the nitroimidazoles [8-9]. Unfortunately, in spite of many immunization attempts, no ELISA could be developed for diclazuril and robenidine. A liquid chromatographic tandem mass spectrometric (LC-MS/MS) method was developed and validated for the chemical anticoccidials halofuginone, robenidine, diclazuril, nicarbazin and dimetridazole [10] and for the ionophoric anticoccidials narasin, salinomycin, monensin and lasalocid [11].

Nicarbazin is the generic name of the equimolar complex of 4,4'-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP). When chickens are given nicarbazin in the feed, the HDP fraction is absorbed and excreted more rapidly than the DNC fraction and consequently most residue analyses for nicarbazin are based on methods for the DNC molecule [12]. Thus in this experiment, we focused only on the DNC compound.

As mentioned above, dimetridazole or 1,2-dimethyl-5-nitroimidazole belongs to a group of compounds called the nitroimidazoles. The major pathway of elimination of dimetridazole is hydroxylation of the 2-methyl group to 2-hydroxymethyl-1-methyl-5-nitroimidazole [13]. The fact that dimetridazole is metabolized rapidly and that the main metabolite, 2-hydroxydimetridazole, is present in higher concentrations in tissues and eggs emphasizes the need to monitor for both of these compounds when performing residue analysis.

In the animal experiment, 10 groups of 12 laying hens were included. For the compounds diclazuril, robenidine, halofuginone and nicarbazin, two concentrations levels were tested, namely the maximum level that can be present in feed intended for broilers or pullets and a lower concentration level, corresponding to 5% carry-over at the feeding mill. Since 2001, nicarbazin may no longer be administered alone but only in combination with narasin as Maxiban® and hence, narasin was included in the experiment. Dimetridazole is now listed in Annex 4 of Council Directive 2377/90 [14] and is, as a consequence, a banned substance. As a result, carry-over is not likely to occur for dimetridazole. Therefore, only one concentration level was included for this compound. In this way, the use of the ELISA test still could be evaluated. Also a blank control group was included. Residue-containing eggs were sampled during the treatment period and for a period of 30 days after withdrawal of the anticoccidial-containing feed.

## EXPERIMENTAL

### *Reagents and standards*

Diclazuril was from Janssen Animal Health (Beerse, Belgium); dimetridazole, dinitrocarbanilide, narasin (from Streptomyces aurofaciens, approx. 97%), monensin sodium salt (90-95%), lasalocid sodium salt (97%), salinomycin sodium salt 2.5 hydrate (85.8%) and nigericin sodium salt (from Streptomyces hygroscopicus, 98%) were from Sigma (St. Louis, MO, USA). Halofuginone was from Intervet (Belgium) and robenidine was from Alpharma (Technical Center, Willow Island, USA). Dimetridazole-$d_3$ was from RIVM (Bilthoven, The Netherlands). Hydroxydimetridazole was purchased at Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin (BgVV) in Berlin, Germany. Dimethylformamide, dimethylsulfoxide and ethanol were all pro analysi from Merck (Darmstadt, Germany).

Acetonitrile and methanol (MS quality) were from Biosolve (Valkenswaard, the Netherlands) and formic acid (98-100%) was from Merck (Darmstadt, Germany). Water was HPLC grade (generated by an ELGA purification system). Filters for filtration of the extract were from Millipore (Millex GV, 0.22 µm).

*Preparation of standard solutions*

Individual standard stock solutions were prepared by weighing approximately 5 mg in a glass tube and adding an appropriate amount of solvent to reach a concentration of 1 mg/ml. The solvents used for preparing the stock solutions were dimethylformamide for diclazuril, a mixture of acetonitrile-water (50/50, v/v) for dimetridazole, water for halofuginone, dimethylsulfoxide for dinitrocarbanilide, methanol for lasalocid, monensin, salinomycine, nigericin (I.S.) and salinomycin and ethanol for robenidine. In order to obtain a homogenous solution, the mixture was vortex mixed. These stock solutions were stored at 4°C. Working solutions of 10 ng/µl 1 ng/µl and 0.1 ng/µl were prepared daily by diluting the stock solution in a mixture of acetonitrile-water (50/50, v/v) or water. Individual tuning solutions of 1 ng/µl were made in water-acetonitrile (50/50,v/v) containing 0.1% formic acid. Since robenidine is very light sensitive, the solutions were always kept in the dark. To enhance the ruggedness and quantification of the method, 2 internal standard were taken through the whole clean-up procedure, namely deuterated dimetridazole (DMZ-$D_3$) for the chemical coccidiostats and nigericin for the ionophores.

*Sample preparation*

After mixing the egg with an ultra-turrax, 10 g homogenized egg was weighed in a centrifuge tube. Then internal standards were added to all samples in a concentration of 5 µg/kg for DMZ-$D_3$ and 10 µg/kg for nigericine. Also at this stage, samples were spiked if necessary by adding an appropriate amount of standard in water solution. The sample was vortex mixed and allowed to stand for 10 min. Then 10 ml of acetonitrile was added and the sample was vortex mixed for 1 min and placed in an ultrasonic bath for 5 min. The sample then was centrifuged during 10 min at 2000 x g. The supernatant was transferred into a graduated tube and was concentrated to a volume of 4 ml under nitrogen in a water bath at 60°C. After filtration through a 0.22 µm filter, 40 µl of the remaining extract was injected into the LC-MS/MS system.

*Liquid chromatography-tandem mass spectrometry*

A model 2695 Alliance LC system (Waters, Milford, USA) was used. Separation was performed on a Waters Symmetry® $C_{18}$ column (150 x 2.1 mm) with 5 µm particle size protected with a guard column Alltima $C_{18}$ 7.5 x 2.1 mm with 5 µm particle size (Alltech, Deerfield, USA). Column temperature was set at 40°C. HPLC eluent A was water/acetonitrile (95/5, v/v), containing 0.1% formic acid; eluent B was acetonitrile containing 0.1% formic acid. The flow rate was 0.25 mL/min and the injection volume was 10 µL. No split was necessary to introduce the LC effluent in the mass spectrometer. For the ionophores an isocratic elution with A/B (10/90) was used due to the high lipophylic character of the compounds. For the chemical coccidiostats a gradient elution was used. The gradient conditions were as follows: from 0-0.5 min, hold 100% A; ramp over 0.1 min to 55% A and 45% B; ramp over 7.9 min to 35% A and 65% B; ramp over 0.1 min to 100% B; hold for 1 min; ramp over 0.2 min to 100% A. Hold 100% A fro 7.2 min to re-equilibrate the system

The MS equipment consisted of a Waters Micromass Quattro Ultima Pt (Altrincham, Cheshire, UK) equipped with a Z-spray system. The MS system was controlled by version 4.0 of the MassLynx software.

Collision energy was tuned to optimise the fragmentation of the precursor ion into the most abundant product ions. Nitrogen was used as cone gas and desolvation gas at flow rates of 60 L/h and 700 L/h, respectively. The source block and desolvation temperature were set at 120°C and 300°C, respectively. RF 1 was set at 35 V for the chemical coccidiostats and at 50 V for the ionophores. Capillary voltage was set at 3

kV for the chemical coccidiostats and at 1.5 kV for the ionophores. The optimised MS parameters are presented in table 1.

**Table 1. Summary of the mass spectrometric conditions**

| compound | ionisation mode | cone (V) | m/z precursor ion | m/z product ions | collision energy (eV) |
|---|---|---|---|---|---|
| narasin | ES + | 80 | 787.4 | 431.2<br>531.2 | 50<br>43 |
| monensin | ES + | 80 | 693.4 | 461.3<br>479.4 | 48<br>50 |
| lasalocid | ES + | 80 | 613.4 | 377.3<br>359.4 | 35<br>35 |
| salinomycin | ES + | 80 | 773.5 | 431.3<br>265.3 | 48<br>48 |
| nigericin (IS) | ES + | 80 | 747.3 | 703.5 | 52 |
| diclazuril | ES - | 50 | 404.9<br>406.9 | 334.1<br>336.1 | 16<br>16 |
| dimetridazole | ES + | 50 | 142.1 | 96.1<br>81.2 | 12<br>20 |
| 2-OH dimetridazole | ES + | 40 | 158.1 | 140.1<br>55.2 | 9<br>14 |
| dinitrocarbanilide (nicarbazine) | ES - | 35 | 301.1 | 137.1<br>107.1 | 8<br>30 |
| halofuginone | ES + | 50 | 416.0 | 100.2<br>120.1 | 20<br>18 |
| robenidine | ES + | 50 | 334.1 | 155.1<br>138.1 | 18<br>24 |
| DMZ-$D_3$ (IS) | ES + | 50 | 145.0 | 99.2 | 12 |

The LC effluent was connected to the interface via a divert valve to avoid pollution of the mass spectrometer. The valve switched to the mass spectrometer 3 min after the injection of the extract into the LC system. The instrument was operated in MRM mode with dwell times between 0.1 to 0.5 s, an interchannel delay of 0.01 s and an interscan delay of 0.1 s.

*Preparation of the experimental diets*

Experimental diets were prepared at the mill of ILVO-animal unit. For the preparation of the diets containing diclazuril, halofuginone and robenidine, Clinacox® (Janssen Animal Health, Beerse, Belgium), Stenerol® (Intervet, Mechelen, Belgium) and Cycostat® (Alpharma, Antwerpen, Belgium) were used as premix, respectively. Maxiban® (Elanco, Brussel, Belgium) is a mixture of nicarbazin and narasin in a 1/1 ratio. It was used to prepare the nicarbazin and narasin-containing feed. For dimetridazole, we were not able to obtain a premix. Therefore, we used an analytical standard purchased at Sigma (Bornem, Belgium) to prepare the dimetridazole-containing feed. The concentrations corresponding to the maximum allowed concentration for broilers or pullets, further referred to as the 100% concentrations, were 1 mg/kg for diclazuril, 3 mg/kg for halofuginone, 36 mg/kg for robenidine, a combination of 40 mg/kg narasin and 40 mg/kg nicarbazin. For dimetridazole a concentration of 200 mg/kg was chosen. For each compound, except dimetridazole, a second concentration corresponding to 5% carry-over also was prepared. Dimetridazole is listed in Annex 4 of Council Directive 2377/90 and is, as a consequence, a forbidden compound. As a result, carry-over is not likely to occur for dimetridazole. Therefore, only one concentration level was included for this compound.

The appropriate amount of premix was weighed and added to the blank feed. The experimental diets were least-cost formulated according to the requirements of the laying hens during the first half of their production cycle. All feedstuffs were coarsely milled with a hammer mill and carefully mixed in the feed unit. No pelletation was carried out.

### *Animal treatment*

Animal experiments were conducted at the poultry experimental facility of ILVO-animal unit. A flock of medium weight laying hens (ISA-brown) was used for the trial during the first half of their production cycle (31-39 weeks of age).

The hens were randomly divided into 10 groups of 12 animals each. These laying hens were housed in three tier battery pens of four laying hens each, under conventional conditions of ventilation, temperature (18-22 °C) and lighting (16 h light/day). During the study, they were given free access to water and feed. Each group was previously controlled for their laying persistency in order to improve the homogeneity of the entire flock. During the entire experiment, the hens were monitored daily for general health by qualified personnel supervised by a veterinarian. Eggs were collected daily during the complete course of the study. After the animals were placed in their pens, they were allowed to adapt to their environment for 4 weeks. During this adaptation period, all animals were kept on anticoccidial-free feed. The eggs collected during this period were used as blank control material. After the adaptation period, group 1 continued to receive blank feed while the other nine groups received the feed containing an anticoccidial during 14 days (day 1 – 14). From day 15 on, all 10 groups were fed again the anticoccidial-free feed. Collecting of the eggs was stopped at day 44 i.e. 30 days after cessation of administration of the anticoccidial-containing feed.

Of each experimental group, 10 eggs were homogenized daily and stored at –18°C until analysis. On Mondays, also the eggs collected during the weekend were homogenized and frozen. As each group consisted of 12 laying hens, usually 10 eggs per day were available. Moreover, for most groups and at most days, 11 or 12 eggs were available. The remaining eggs were stored refrigerated. At the end of the experiment, in those cases when more than 10 eggs were available for a certain group on a certain day, one egg was used to split the egg yolk from the albumen. They were homogenised and stored separately at –18°C.

During the experiment, the following conventional zootechnical data were recorded : average feed intake (g/day), laying percentage, egg weight (g), daily egg mass (g/hen) and feed efficiency (feed intake/egg mass). To determine these parameters, the experimental period was subdivided into 3 sub-periods: (1) 7 days on the blank reference diet, (2) 14 days on the respective 'anticoccidial' diets, and (3) another 30 days on the blank reference diet.

### *Analysis of the egg samples by ELISA*

The incurred samples were extracted and tested using the screening assays for halofuginone, dinitrocarbanilide and dimetridazole together with its main metabolite. For each extraction, one known negative sample and three known negative samples fortified at CCβ level were included to serve as quality control (halofuginone at 0.5 µg/kg, dinitrocarbanilide at 3 µg/kg and dimetridazole at 1 µg/kg). In addition, a standard curve prepared in egg matrix was extracted simultaneously with the samples. It has to be kept in mind however that the ELISAs were developed to perform a qualitative screening test and not to obtain quantitative results. Therefore, concentrations obtained with the ELISAs should be considered as estimations.

The mean values of the absorbance (450 nm) values obtained for each sample were divided by the absorbance value of the zero standard (Bo) and multiplied by 100. The

extraction was considered as valid if two out of three quality control samples gave a binding percentage ≤ 58% for the halofuginone assay, ≤ 74% for the dinitrocarbanilide assay or ≤ 76% for the dimetridazole assay and if the negative control showed a binding percentage superior to these values.
In some cases, concentrations were so high that the mean value of the absorbance fell out of the range of values obtained with the extracted standard curve. In that case, the dilution factor was increased and the samples were again applied on the ELISA plate until the OD values corresponded to the range of OD values obtained with the standard curve after extraction.

*Analysis of the egg samples by LC-MS/MS*
As well the whole egg samples as the separate yolk and albumen samples were analysed with the LC-MS/MS methods described above. Analyses performed by LC-MS/MS were quantitative. A matrix calibration curve was made using the MRM-data of the transition of the precursor ion into the most abundant product ion. Quantification was conducted by internal calibration using a weighing factor of 1/x. The results were calculated by the TargetLynx software. For each series of samples, a calibration curve was made in a specific concentration range to make sure that the concentrations in the samples of that particular series were covered. Also in each series of samples, 2 unknown samples were included as a control. Additionally, for each series of samples, all criteria set by Commission Decision 2002/657/EC (relative retention time and ion ratios) were checked.
The detection limit, or CCα was 0.5 µg/kg for diclazuril, 1 µg/kg for dimetridazole, halofuginone, robenidine and dinitrocarbanilide, and 2 µg/kg for 2-hydroxydimetridazole. For the ionophores narasin, salinomycin, lasalocid and monensin a CCα of 1 µg/kg was obtained. During validation, recovery rates varied from 88 to 108 % for the chemical anticoccidials and from 90 to 113 % for the ionophores.

## RESULTS AND DISUCUSSION

*Analysis of the feed samples*
The feed samples were analysed with LC-MS/MS and the results are presented in table 2.

**Table 2. results of the analyses of the feed samples**

| experimental group | compound | premix used | theoretical concentration | measured concentration | % of theoretical concentration |
|---|---|---|---|---|---|
| 2 | diclazuril | Clinacox® | 1000 µg/kg | 926µg/kg | 93 |
| 3 | | | 50 µg/kg | 47 µg/kg | 93 |
| 4 | halofuginone | Stenerol® | 3000 µg/kg | 1475 µg/kg | 49 |
| 5 | | | 150 µg/kg | 162 µg/kg | 108 |
| 6 | robenidine | Cycostat® | 36 mg/kg | 39 mg/kg | 108 |
| 7 | | | 1800 µg/kg | 1597 µg/kg | 89 |
| 8 | narasin | Maxiban® | 40 mg/kg | 41 mg/kg | 102 |
| | nicarbazin | | | 41 mg/kg | 102 |
| 9 | narasin | Maxiban® | 2000 µg/kg | 2114 µg/kg | 106 |
| | nicarbazin | | | 2144 µg/kg | 107 |
| 10 | dimetridazole | analytical standard Sigma | 200 mg/kg | 101 mg/kg | 50 |

As can be seen in this table, satisfying results were obtained for diclazuril, robenidine, nicarbazin and narasin. For halofuginone and dimetridazole, results were less satisfying. Remarkably, only for the group with the highest concentration of halofuginone, only about 50% of the intended concentration was achieved while good results were obtained for the 5% group. This indicates that most likely a human mistake during the feed preparation is the cause of the lower concentration achieved. As a consequence, for halofuginone, there was a 50% and a 5% group (instead of a 100% and 5% group). A possible explanation for the result for dimetridazole is that no premix but an analytical standard was used for feed preparation. This analytical standard is less suitable for preparing medicated feed. But since dimetridazole is a forbidden compound, the concentration achieved was less important.

*Analysis of the egg samples*

*Diclazuril*

The depletion curves of both groups receiving diclazuril are presented in figure 1. Diclazuril was detectable in the eggs from birds fed the 1 mg/kg diet from day 2 onwards whereas it was detectable in the eggs from birds fed the 0.05 mg/kg diet from day 3 onwards. Concentrations increased until a plateau concentration of about 100 µg/kg for the 100% group and a plateau concentration of about 5 µg/kg for the 5% group was reached at day 10. This plateau was maintained until day 16 for the 5% group and until day 18 for the 100% group. Thereafter, concentrations started to drop until no more residues were found 22 days and 11 days after the end of the treatment for the 100% and 5% group, respectively. For diclazuril, a clear relationship between feed and egg concentration was observed. Taken into consideration the fact that yolk formation takes about 10 days and that a plateau concentration is reached 10 days after start of the treatment, suggest that residues mainly will be present in the egg yolk. For diclazuril, results of only one study could be found in literature but the limit of detection of the method used was only 50 µg/kg [15].

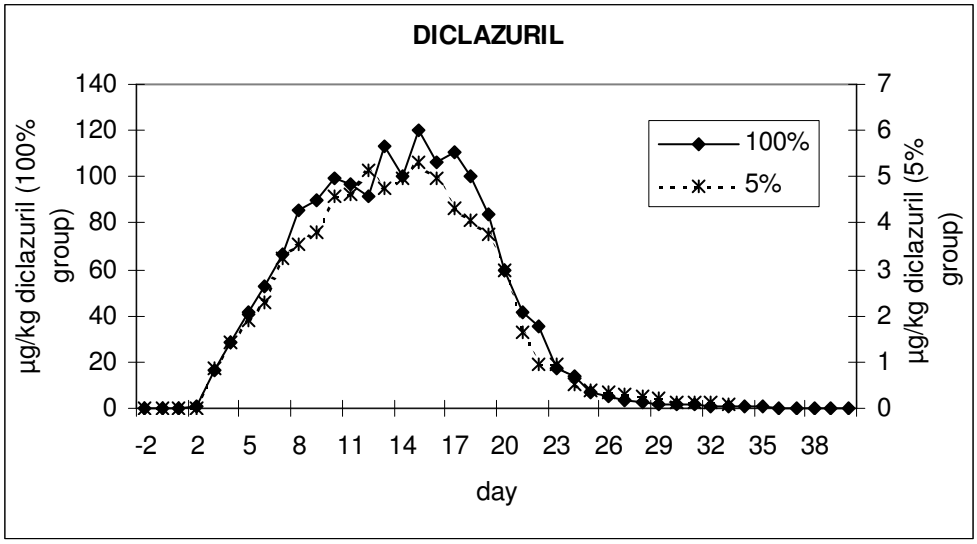


**Figure 1. results of the analyses of the whole eggs of the experimental groups receiving diclazuril**

*Halofuginone*

For halofuginone a similar pattern is observed as for diclazuril. This is shown in figure 2. The first residues appear two and three days after the beginning of the

administration of the halofuginone-containing feed for the 50% and 5% group, respectively. For both groups, a plateau concentration is reached but this happens earlier for the 5% group (day 4) than for the 50% group (day 7). As this plateau is already reached at day 4 and 7, halofuginone residues will probably also be present in the albumen. Plateau concentrations are 450 µg/kg for the highest concentration group and 30 µg/kg for the lowest concentration group. So for halofuginone, the relationship between feed and egg concentration is less obvious. Yakkundi et al. described an animal experiment in which laying hens were fed halofuginone-containing feed in order to establish the relationship between the halofuginone concentration in feed and the residues in eggs. Five groups of six laying hens were fed with halofuginone-containing diets at concentrations ranging between 0.1 and 10% of the therapeutic dose for broilers (3 mg/kg) for 14 days. The group fed the highest dose was then fed with a halofuginone-free diet for a further 14 days. A plateau concentration of about 40 µg/kg was observed after feeding laying hens feed containing 0.3 mg/kg halofuginone [16]. Another animal experiment, in which laying hens were fed halofuginone-containing feed, was described by Mulder et al. Twenty ISA brown laying hens were treated with feed containing 3 mg/kg halofuginone for 14 days. Eggs were collected before, during and after treatment. Residue concentrations were determined in whole egg, as well as in the yolk and albumen. Mulder et al. reported a plateau concentration of 450 µg/kg after administrating feed with 3 mg/kg halofuginone during 14 days [17].

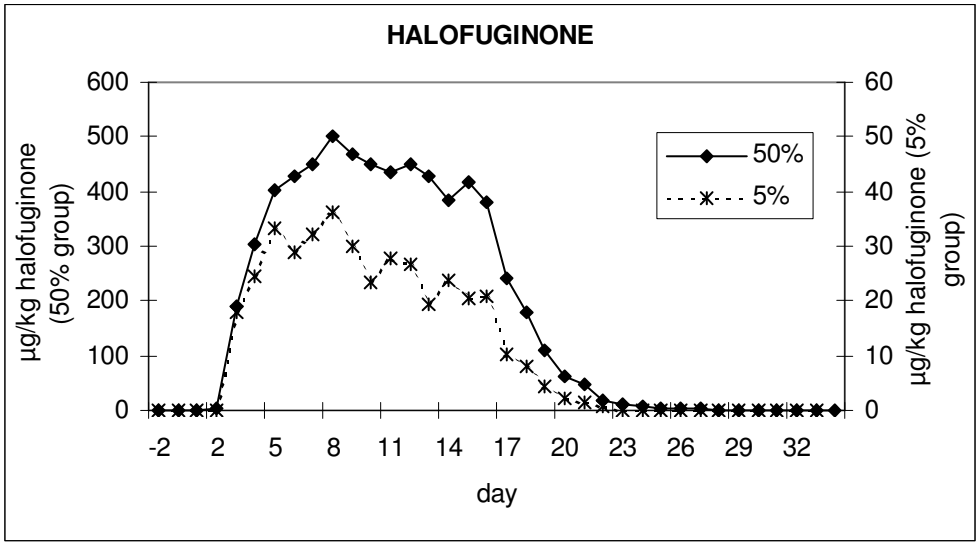


**Figure 2. results of the analyses of the whole eggs of the experimental groups receiving halofuginone**

*Robenidine*

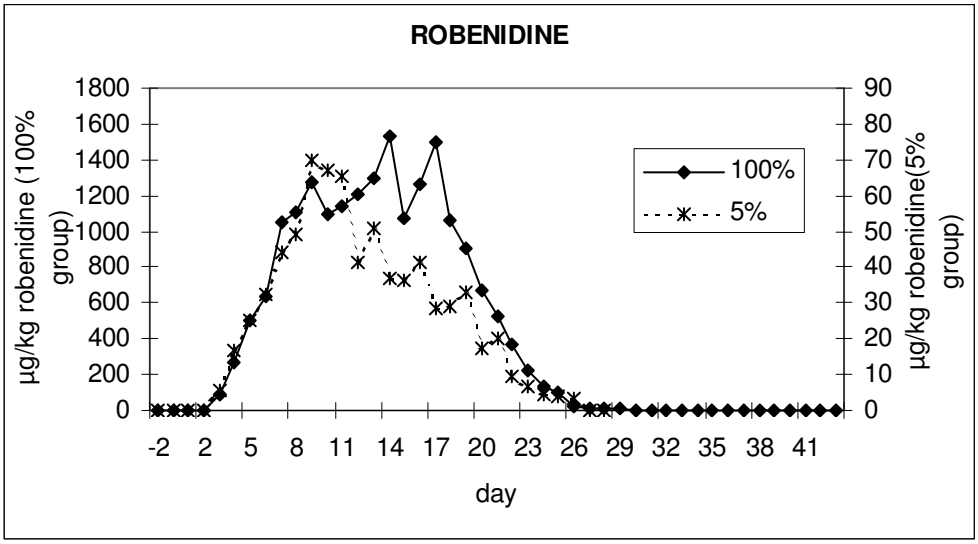


**Figure 3. results of the analyses of the whole eggs of the experimental groups receiving robenidine**

For robenidine (figure 3), only for the 100% concentration group, a plateau is reached i.e. about 1300 µg/kg. For the 5% group, a maximum value of 70 µg/kg is reached on day 9 of the experiment and from that point on, a decrease is observed. For both concentration groups, the first robenidine residues were observed on the third day of the experiment. For the 5% group, no more residues were found 13 days after cessation of the treatment. For the 100% group, it took 26 days to obtain concentrations below the CCα value (1 µg/kg) but until day 29 traces of robenidine could still be found. To our knowledge, no experiments with robenidine were published elsewhere.

*Dinitrocarbanilide (Nicarbazin)*

The administration of nicarbazin to laying hens clearly leads to considerable amounts of dinitrocarbanilide in eggs. This is shown in figure 4. For the highest concentration group from day 11 onwards, a plateau concentration of 6500 µg/kg was observed. This plateau was maintained until day 18 of the experiment. So only 5 days after cessation of the treatment with nicarbazin, dinitrocarbanilide concentrations in the eggs start to drop. Residues can be found more than three weeks (23 days) after nicarbazin-free feed was given. For the 5% group also a plateau is reached: this happens from day 10 until day 18 of the experiment. As was the case with diclazuril, residues are probably present in the egg yolk since it takes ten days to reach the plateau. For the 5% group, it took 15 days to obtain eggs free of residues of dinitrocarbanilide. Blanchflower et al. described a small feeding trial in which a group of 5 laying birds was fed a ration formulated to contain 10 mg/kg nicarbazin for 9 consecutive days. DNC concentrations continued to rise throughout the experiment, reaching a mean level of 309 µg/kg on day 9 [18]. Cannavan et al. designed an experiment to establish the relationship between nicarbazin-containing feed and nicarbazin residues in eggs. Five groups of 6 laying hens received for 16 consecutive days daily 120g feed containing 0.2, 0.4, 1.3, 3.8 and 12.1 mg/kg nicarbazin, respectively [4]. Concentrations of dinitrocarbanilide in the whole eggs increased rapidly until about day 6, and then reached a plateau. The

plateau concentration was 600 g/kg. Twelve days after withdrawal of the nicarbazin-containing feed, DNC was not longer detected in the eggs.

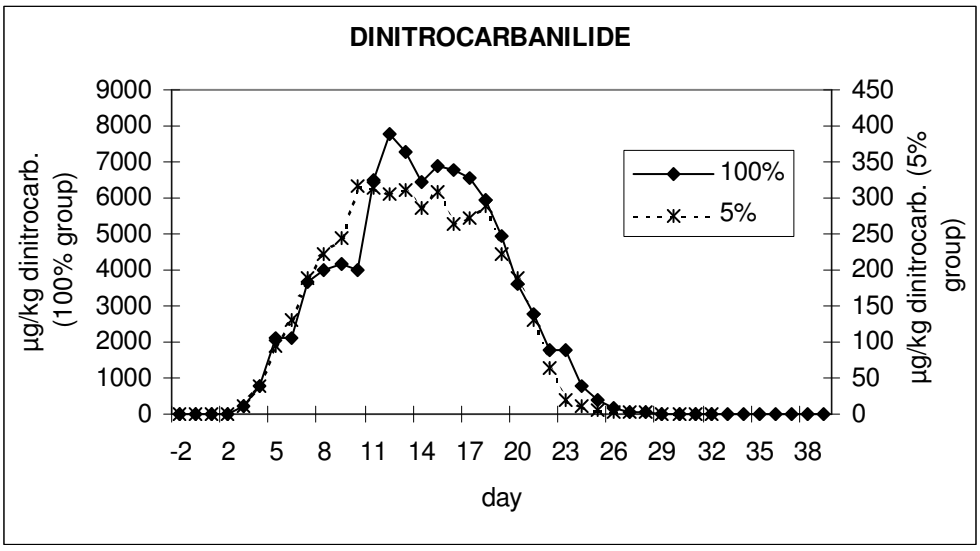


**Figure 4. results of the analyses of the whole eggs of the experimental groups receiving nicarbazin**

*Narasin*

Although narasin is administered as Maxiban and thus together with nicarbazin, a different pattern for narasin and dinitrocarbanilide was observed, as shown in figure 5. The plateau concentration of 90 µg/kg is already reached 8 days after start of the treatment. This plateau is maintained until day 15 of the experiment. From day 16 onwards, i.e. 2 days after the switchover to Maxiban-free feed, narasin concentrations already start to drop. Concentrations below 1 µg/kg are reached 17 days after this switchover. But until after 24 days, still some narasin was detected in the whole egg samples. For the lowest concentration group, a totally different pattern was observed. From day 3 on, it seems like a plateau is reached of about 2 µg/kg but then suddenly a new plateau of 6 µg/kg is reached that is maintained for 7 days. Eight days after the ending the treatment with Maxiban, concentrations fall below 1 µg/kg. For narasin, only one study with laying hens is described. In an experiment carried out by Kolsters three groups of 4 laying hens were fed narasin-containing feed during 7 days at a concentration of 76 mg/kg and another three groups of 4 laying hens at a concentration of 3 mg/kg [19]. In this experiment a detection method was used which had a limit of detection of 10 µg/kg. Therefore, it is difficult to compare the results

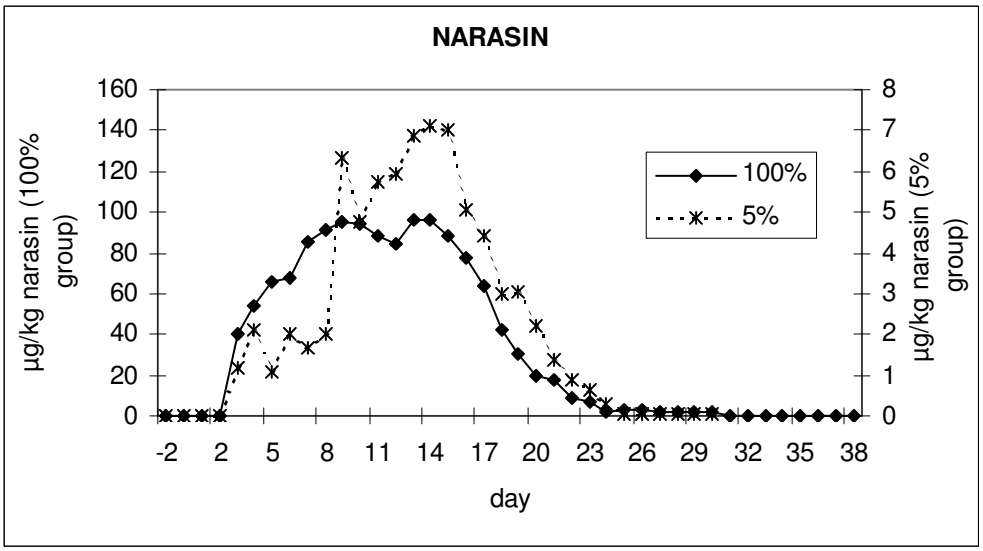


**Figure 5. results of the analyses of the whole eggs of the experimental groups receiving narasin**

*Dimetridazole*

For dimetridazole, only one group was included in the experiment. But as mentioned in the introduction, when performing residue analysis for dimetridazole, also the main metabolite, 2-hydroxydimetridazole has to be monitored. The results are presented in figure 6. For both compounds, the first positive samples are encountered from day 2 onwards and immediately the plateau concentration is reached. This suggests that residues will mainly be present in the egg white since the egg white concentrations can be considered as a measure for the plasma concentration [20]. Clearly higher concentrations of the metabolite are found. Immediately after cessation of the treatment, concentrations drop. During the plateau period, the metabolite/parent compound – ratio equals 2.6 ± 0.2. This clearly endorses that the hydroxymetabolite must be included when performing residue analysis for dimetridazole.

An animal experiment in which laying hens were fed feed containing 10 mg/kg dimetridazole was described by Cannavan et al. [21]. In this trial laying hens received daily 120 g feed containing approximately 10 mg/kg dimetridazole for 7 days. Residues of dimetridazole were found in the eggs taken 1 day after commencement of the dimetridazole diet and in all eggs taken thereafter. The mean concentration in eggs taken after 7 days was 21.6 µg/kg. The samples were not analyzed for 2-hydroxydimetridazole.

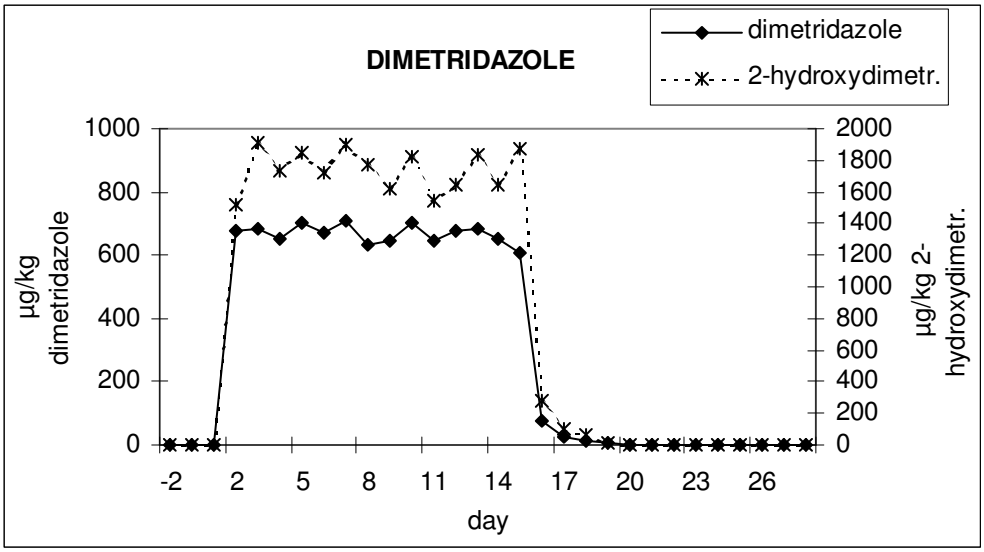


**Figure 6. results of the analyses of the whole eggs of the experimental groups receiving dimetridazole**

*Comparison of the results obtained by ELISA and LC-MS/MS*

The main objective of the development of the ELISAs was to reduce the number of samples that need to be analyzed by the more expensive LC-MS/MS methods. Therefore, it is important that the screening method does not produce false negative results. With the incurred samples of the animal experiment, this could be tested. A comparison of the results between both methods is presented in table 3. The ELISA is perfectly capable of identifying the first positive samples as shown in the first part of the table. But the ELISA for the detection of dinitrocarbanilide clearly overestimates the concentrations. During depletion, the ELISAs detect residues somewhat longer and hence false positive results are generated. Nevertheless, it can be concluded that the ELISAs are perfectly suitable for performing the screening.

**Table 3. comparison of the analyses of the whole eggs samples by ELISA and LC-MS/MS**

| compound | group | ELISA | | LC-MS/MS | |
|---|---|---|---|---|---|
| | | first positive sample | conc. (µg/kg)* | first positive sample | conc. (µg/kg) |
| halofuginone | 5% | day 3 | 15 | day 3 | 17.8 |
| | 50% | day 2 | 3 | day 2 | 3.3 |
| dinitrocarbanilide | 5% | day 3 | 26 | day 3 | 10.7 |
| | 100% | day 2 | 11 | day 2 | 2.7 |
| dimetridazole + 2-hydroxydim. | 100% | day 2 | 926 | day 2 | 676.3 + 1513.1 |

* : estimations

*Results in the perspective of the action limit set by the Belgian Food Agency*

As mentioned earlier, in December 2004 an action limit of 10 µg/kg was set by the Scientific Committee of the Belgian Food Agency for monensin, salinomycin, diclazuril, lasalocid, maduramycin, narasin, nicarbazin, robenidine and all sulphonamides. Especially for the 5% groups, it is interesting to check when concentrations drop below 10 µg/kg. For the 5% diclazuril group, concentrations never exceed the 10 µg/kg value as the plateau concentration equals 5 µg/kg. For the highest diclazuril concentration group, it would take 11 days to obtain residues below 10 µg/kg. Also for narasin, concentrations of the lowest group never exceed 10 µg/kg. For the 5% groups of robenidine and nicarbazin, it would take 8 and 11 days until concentrations fall below 10 µg/kg. For the highest concentration groups, 15, 8 and 19 days are required to obtain concentrations below the action limit for robenidine, narasin and nicarbazin, respectively. Although not included in the advice, for halofuginone it would take 4 and 10 days for the lowest and highest concentration group, respectively.

*Analyses of the yolk and albumen samples*

As mentioned in the animal treatment-section, also some separate yolk and albumen samples were analyzed. The same methods as for the whole egg samples were used. It has to be noted that the yolk and albumen samples were no pooled samples as was the case with the whole egg samples. So variability between animals is not compensated for. Another remark that has to be made is that the egg samples were stored refrigerated for several months before yolk and albumen were separated. This is because the separate analysis of yolk and albumen was originally not planned. Therefore, it cannot be excluded that there was transfer from residues from one egg compartment to the other. However, Kolsters et al. tested this possible transfer for monensin, narasin and salinomycin and no differences in concentration were observed when separating yolk and albumen immediately after laying or after storage [19].

Major differences in distribution between the compounds could be observed. Diclazuril, robenidine, dinitrocarbanilide and narasin are mainly present in the egg yolk. For both groups of diclazuril, about 4 times more residues were found in the yolk than in the albumen. For the highest concentration group of dinitrocarbanilide, concentrations up to 10 mg/kg were found in the yolk, while the maximum concentration in the albumen was 120 µg/kg. For dinitrocarbanilide, similar results were obtained by Cannavan et al.[4] The relatively non-polar component of nicarbazin, was also found to be almost exclusively contained in the more fatty matrix of the yolk. For the highest concentration group of robenidine, 2300 µg/kg was detected in the yolk on day 13 of the experiment while only 7 µg/kg was detected in the white of the same egg. For narasin, about 5 times more residues are found in the yolk. For as well narasin, dinitrocarbanilide, diclazuril and robenidine, residues disappear much faster out of the albumen than the yolk. For halofuginone, initially more residues are found in the albumen. During the plateau period, halofuginone can be found in both compartments. During depletion, residues are longer found in the yolk. All these observations reflect the process of egg formation in the hen. Similar results are obtained for the lowest halofuginone concentration group. These observations are in agreement with those of Yakkundi et al. who reported that the concentrations in the egg yolk were marginally higher than those in the albumen [16]. Mulder et al. on the other hand, reported that residue concentrations were approximately twice that in albumen [17]. This ratio remained more or less constant during the medication and post-medication period. This does not seem to agree with the process of egg formation, which predicts residues to last longer in the yolk. They observed substantial variability with respect to the concentrations determined in the individual egg white and yolk samples (relative standard deviation up to 78%) as well as in the distribution ratio (relative standard deviation up to 74%). They

suggest that this may be due to differences in metabolism between individual hens but mention also that the method performed less well for the egg white and yolk matrices than for whole egg.
For dimetridazole and 2-hydroxydimetridazole, a completely different pattern is observed. Dimetridazole concentration is ten times higher in the albumen than in the yolk. Also for the hydroxymetabolite, higher concentrations are found in the albumen. These observations are in agreement with the curves observed for the whole egg samples.

*Comparison of the concentration ratio in the whole eggs and the feed*
In table 4, for those compounds for which two concentration groups were included in the experiment, a comparison is made between the concentration ratio (highest/lowest concentration) in the feed and in the whole eggs. The concentration reached at the plateau is used for the calculation of the concentration ratio in the eggs. It is clear that for diclazuril and nicarbazin/dinitrocarbanilide, a good agreement is obtained. For halofuginone, relatively higher concentrations are found in the eggs while on the contrary, for narasin, proportionally fewer residues are found in the eggs as the concentration in the feed increases. Of course, care must be taken when evaluating these results since for each compound only two concentration levels were tested. In the experiment described by Yakkundi et al., a linear relationship between the steady-state halofuginone concentration in egg homogenates and the feed halofuginone concentrations was obtained ($R^2$=0.992). In their experiment, 5 concentration levels between 0.003 and 0.3 mg/kg halofuginone in the feed were included. Of course, the concentrations used in our experiment (approximately 0.15 and 1.5 mg/kg) are outside the concentration range of the experiment conducted by Yakkundi et al. A linear relationship between dinitrocarbanilide residues in whole eggs and nicarbazin in the feed was found by Cannavan et al. In that experiment, feed concentrations ranged from 0.2 mg/kg to 12.1 mg/kg (n= 5, $R^2$=0.998).

**Table 4. comparison of the concentration ratio in the whole eggs and the feed**

| compound | "plateau period" | average concentration ratio* (± standard deviation) during plateau in the whole egg | concentration ratio in the feed |
|---|---|---|---|
| diclazuril | day 10 – 16 | 21.2 ± 1.9 | 19.9 |
| halofuginone | day 7 – 14 | 16.7 ± 2.8 | 9.1 |
| robenidine | no plateau for the lowest concentration group | | |
| narasin | day 9 – 15 | 14.9 ± 2.3 | 19.3 |
| nicarbazin / dinitrocarbanilide | day 11 – 18 | 23.1 ± 1.9 | 18.9 |

* : concentration of the highest concentration group / concentration of the lowest concentration group

## CONCLUSIONS

It can be concluded that this experiment has shown that carry-over levels of anticoccidials in feed intended for laying hens leads to the presence of residues in eggs. Even with 5% carry-over, it can take up to 15 days to become residue-free eggs. The analyses of the separate yolk and albumen revealed that differences in distribution between the different compounds are big. The ELISAs developed within the framework of the project (i.e. for halofuginone, dimetridazole and nicarbazin) can be used to perform a screening.

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# Pesticides and indicators for sustainable farming systems

# Certified production systems: a way forward towards sustainability?

**K. Mondelaers[1], F. Garreyn[2], L. Roussel[3], M. Louviaux[3], M. Mormont[3], L. Pussemier[4], W. Steurbaut[2] and G. Van Huylenbroeck[1]**

*[1] Ghent University, Department of Agricultural Economics, Coupure Links 653, B-9000 Gent, Belgium.*
*[2] Ghent University, Department of Crop Protection, Coupure Links 653, B-9000 Gent, Belgium.*
*[3] University of Liege, SEED*
*[4] CODA-CERVA, Tervuren, Belgium*
*e-mail: Guido.Vanhuylenbroeck@Ugent.be*

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Certification schemes; labelling; environmental sustainability; agro-food sector; chain perspective

## SUMMARY

Different public and private policy tools exist to steer food production towards more environmentally sound practices. One of them is certification and labelling. Private actors are increasingly interested because these systems combine a superior ecologic performance with economic opportunities. In this article we explore the contribution of labelling and certification initiatives to sustainability from an integrated chain perspective. The reason is that these systems are multi-stakeholder constructs. Our analysis reveals that, from a strategic perspective, three different types of systems can be distinguished, depending on the strategic objective and the stakeholder composition. We furthermore introduce a tool to evaluate the environmental performance of certification books and an instrument to analyse the willingness of farmers to accept further strengthening of the prescriptions. Finally, a deliberative focus groups approach has enabled us to analyse the consumers' acceptance and bottlenecks of eco-labelled products.

## INTRODUCTION

During the past decades, the request for more sustainable practices in food production has grown. Agricultural practices may have an adverse impact on natural resources. Pollution of soil, water and air, fragmentation of habitats and loss of biodiversity are undesired side effects of inappropriate agricultural practices and land-use. To increase the sustainability of agriculture, several public and private strategies can be pursued. Public strategies may rely on command-and-control policies or on economic instruments. Command-and Control policies are based on the coercive power of the State to change the structure of property rights and to impose regulations to prevent the occurrence of negative externalities or to stimulate the production of positive externalities. Economic instruments rely on market mechanisms. By integrating price mechanisms, such as taxes, retributions, subsidies, trading in property rights, the regulated agents are pushed to internalise the environmental externalities.

However, besides public policies also private actors may have incentives to voluntarily improve their environmental sustainability performance if this creates economic opportunities. Voluntary private labelling and certification strategies are seen as promising tools for the introduction of more sustainable production patterns. The aim of these strategies is to introduce market differentiation by adopting or imposing extra product or production process characteristics going beyond the obligatory legal standards. This differentiation of certified production systems from conventional systems is based on quality, environmental or other standards. This market approach coincides with an increasing interest of consumers in the lifecycle and credence characteristics of food products.

To analyse the performance and long term sustainability of such certification (and labelling) strategies, our research project adopted a chain perspective identifying different types of certification strategies based upon the chains' characteristics. We furthermore focused on the two principally affected stakeholders: the farmers, who have to implement the certification rules, and the consumers, who have to buy the products. From an environmental perspective, we developed a method to assess the ecologic performance of different certification systems in the Belgian fresh fruit and vegetable market. Hence, the issue of sustainability of certification schemes gives rise to following four questions:

1. From a strategic chain perspective: how do certification strategies (and related labels) position themselves in the market?
2. From an environmental perspective: what is their environmental/ecological performance?
3. From a producer (farmer) perspective: how do they perceive the rules?
4. From a consumer perspective: how are labels perceived and how can their acceptance be improved?

For the empirical observations, 8 certification schemes frequently used in the Belgian fruit and vegetable sector and primarily focusing on the reduction of the impact of pesticide use on public health and the environment were selected. These certification schemes are: Biogarantie, Charte Perfect, EurepGAP, Flandria, FlandriaGAP, Fruitnet, Integrated Fruit Production and Terra Nostra. They operate at different levels in the food chain and have a high diffusion in and impact on Flemish and Walloon agriculture.

## CERTIFICATION STRATEGIES

The performance and sustainability of certification systems will be influenced by the objectives, the composition and internal organisation of an initiative. The stakeholder composition of a certification network results in a particular strategy to approach the market. As a result, several types of private certification initiatives can be identified, In order to differentiate these strategies, interviews with stakeholders in the selected networks were conducted to analyse the content of the specifications, the forms of organisation and competition and the power relationships within the networks.

The analysis of interviews and public documents allows us to distinguish three different types of certification:

1. Product certification: This type of certification aims to guarantee a certain premium quality. Products have to comply with certain standards and commercial requirements. Examples of this type of certification are Flandria, Charte Perfect and Terra Nostra

2. Production process certification: This type of certification tries to increase the link between production practices and product quality. Control is at the level of production methods. Examples are organic agriculture and Fruitnet.
3. Rules and procedure certification: Here the emphasis is on compliance with rules and procedures in order to guarantee traceability. It is assumed that there is a link between compliance with rules and the product quality. Examples of this type are EurepGAP and other retail certificates.

Type 1. The Flandria, Charte Perfect and Terra Nostra certifications focus on the final product. This means that a given product must exhibit certain characteristics relating mainly to the appearance of the fruit or vegetable, as well as their preservation and compliance with legal standards. This enables the certifiers to guarantee a higher level of quality for consumers or intermediaries compared to standard products. It is therefore a product-oriented certification that requires compliance with verifiable characteristics. It also means that if the product does not respect these criteria, it is excluded from the premium market. This type of certification therefore implies two parallel certifications: certification of the producer and certification of the product. Criteria mainly relate to the appearance of the product (presence or not of spoilage, colour, compliance with the characteristics of the variety being cultivated), as well as to the size and homogeneity of the product. The pesticides used are traced back to the producer, which enables wholesale markets to decide whether the product is to be exported or not depending on the potential presence or absence of pesticides.

Type 2. This type of certification emphasizes the production method and practices. The main difference between this type of certification and the previous one is the actual content of the certification. Integrated production and organic farming labels attach more importance to environmentally friendly production methods. In terms of organic farming, this means that chemical fertilisers and pesticides are not used and no chemical additives are allowed. Similarly, for integrated production the aim is to reduce the amounts of pesticides used through orchard management and especially by integrated biological control methods and soil management practises. There are as such no claims of premium quality. This is proven by the following sentence from the European legislation on organic production: “No claim may be made on the label or advertising material that suggests to the purchaser that the indication shown in Annex V constitutes a guarantee of superior organoleptic, nutritional or salubrious quality”. A producer is awarded a certification if he has complied with a series of fundamental practices. It are mainly the compliance with these rules that are controlled either directly either by measuring levels of contaminants or residues in the final product. Similarly, “commercial” qualities (taste, appearance) are defined differently, sometimes in quite the opposite way to conventional products. For the Fruitnet certification, e.g., two aspects are measured: sugar content and firmness, and if the fruit is not in conformity, it can be withdrawn from sale. These “commercial” criteria define products using a different scale of values, i.e. one that respects nature.

Type 3. The EurepGAP (and other retail) certification, is awarded without taking into consideration the final product and is based mainly on compliance with national legislation and a number of procedures (traceability, hygiene, identification of plots). There is no link between the certification and the commercial quality of the product or a production philosophy. If the producer complies with the rules, the product will be sold under the EurepGAP certification, no matter how it looks or tastes. Quality characteristics are indicated by the category in which a certified product can be classified (extra, 2 or 3). This type of certification aims to guarantee, above all, traceability and compliance with legislation, rather than to promote a type of product or

quality. The EurepGAP certification is therefore becoming a basic standard or licence to deliver, the aim of which is to enable the movement of products across international markets. It creates a uniform European (and increasingly world) standard based essentially on respect for food safety.

Despite these different types and philosophies behind certification we can observe a certain degree of convergence mainly under pressure of the main distribution channels:

- Type 1 and type 2 certifications converge with mass distribution certification by alignment with the commercial criteria relating to the appearance and size of the products and have introduced product categories in order to allow the retail sector to further differentiate the market. We may therefore speak of convergence or overlapping of quality-based certification with classical commercial standards-based certification.
- A second convergence is the increased visibility of certification through labelling. Although Flandria and other labels were primarily developed as business-to-business labels we see increased efforts to inform the final consumer. The same happens with retail based certificates.
- Because presently farmers often need to have different labels in order to serve different market outlets, also a convergence and alignment of quality labels is noticed. Hereby EurepGAP or other retail labels are more and more perceived as a basic license to deliver and become the standard. Other labels try therefore to incorporate the EurepGAP standards in their certification. Example of this trend is the alignment of Flandria with the EurepGAP standard (FlandriaGAP).

We notice thus both a tendency towards differentiation and a tendency towards standardisation, the latter mainly based on safety criteria and pushed for by mass distribution companies.

However, this does not mean that all certification schemes are the same because still significant differences exist with respect to the extension to producers and the geographical connection with the region. Type 1 certification schemes provide a system of general extension in order to inform farmers about how to reach the required standards. The geographical connection with the region is rather strong and normally only producers within a geographical region may produce under the certificate. In the type II certification the role of extension is important. The rules and prescriptions are the starting point for finding information and to give extension on the required practices. Everybody who complies with the practices and requirements can obtain the certificate (although there are often regional label differences). Finally the certification of type III is not differentiated regionally and the certificate does not provide extension or help. This is provided by private organisations.

Depending on the type of strategy, the equilibrium between the stakeholders involved in the certification network might also differ. The certificates of type I are often developed by farm organisations or they are at least involved in it. Also in the type II certificates there is a high involvement of producers as well as other societal groups. In the type III certificates the involvement is much lower or non-existent. In any case all certification types evolve in time and become more and more stringent (also to keep their position in the market). Therefore we will also consider the farmers' perspective, but first we focus on the environmental performance of labels.

## ENVIRONMENTAL PERFORMANCE

The assessment of the **environmental** sustainability of certification initiatives is based on an in depth analysis of the guidelines within the prescription books of the different certificates. The developed environmental sustainability analysis method is based on an approach already applied in France (Girardin and Sardet, 2002). The procedure is mainly based upon the scoring by experts of each of the relevant certification book rules (as currently encountered in the market place) for their marginal contribution to the different environmental sustainability pillars.

The developed scientific analytical methodology consists of three phases (Figure 1). In a first step the different aspects of environmental sustainability are defined. Environmental as well as human health aspects were emphasised. More specifically, the following aspects were taken into consideration: (1) air quality, (2) climate conservation, (3) biodiversity and landscape, (4) water quality, (5) soil fertility, (6) pest pressure reduction, (7) scarce resource use, (8) waste reduction and management, (9) noise quantity reduction, (10) food safety and (11) worker safety. This list of selected sustainability items is not exhaustive, but based on an extensive literature review of the available scientific literature (Doom, R., 2001; Girardin and Sardet, 2002; Melo and Wolf, 2005; de Snoo and van de Ven, 1999). For each of these aspects a checklist was compiled. All the rules that were thought to have an impact on the sustainability aspect under study were taken up in the list.

In a **second** phase these checklists were submitted to experts in the different disciplines of environmental sustainability. The experts were asked to rank the prescription rules in descending order of importance, starting with the rule that has the lowest positive impact on the sustainability item under study. On the basis of the rankings made by the experts, weights could be attributed to all of the rules mentioned in the checklists by using the revised Simos methodology (SRF) (Simos, 1990a, 1990b; Figueira and Roy, 2002). In a **third** phase the weights of the rules were multiplied with a factor which reflects the mandatory level of the rule. The mandatory level of a particular rule is determined by a code that is attributed to each rule by most certification schemes. Three codes are distinguished. First of all there are 'Major Musts'. These criteria have to be followed at all times. Secondly, criteria can be classified as 'Minor Musts'. This implies that a certain percentage of all those criteria have to be followed by the farmers. This percentage differs according to the certification scheme studied. And finally there are the 'Recommendations', which are only recommended and thus not obligatory. Subsequently a total score for each environmental sustainability item was calculated by adding the individual criterion scores. In a last step the total environmental sustainability scores were determined by multiplying the theme-sustainability scores with weights attributed by experts corresponding to the respective themes of environmental sustainability (also determined by means of the revised Simos procedure). On the basis of the calculated environmental sustainability scores for the specific certification books, one can pass judgements on the contribution of a particular certification book towards environmental sustainability and its pillars. Next, the ecological sustainability of the selected labels and certification schemes is compared and assessed by means of determining the distance to a so called ideal point. This ideal point is represented by an ideal certification book composed of the best rules of the specifications of the selected standards. This ideal point represents the solution where all objectives achieve their optimum value. This implicates a score of 100 for each environmental sustainability aspect. The label or certification scheme contributing the most to environmental sustainability is that standard for which the distance to the ideal point is minimal.

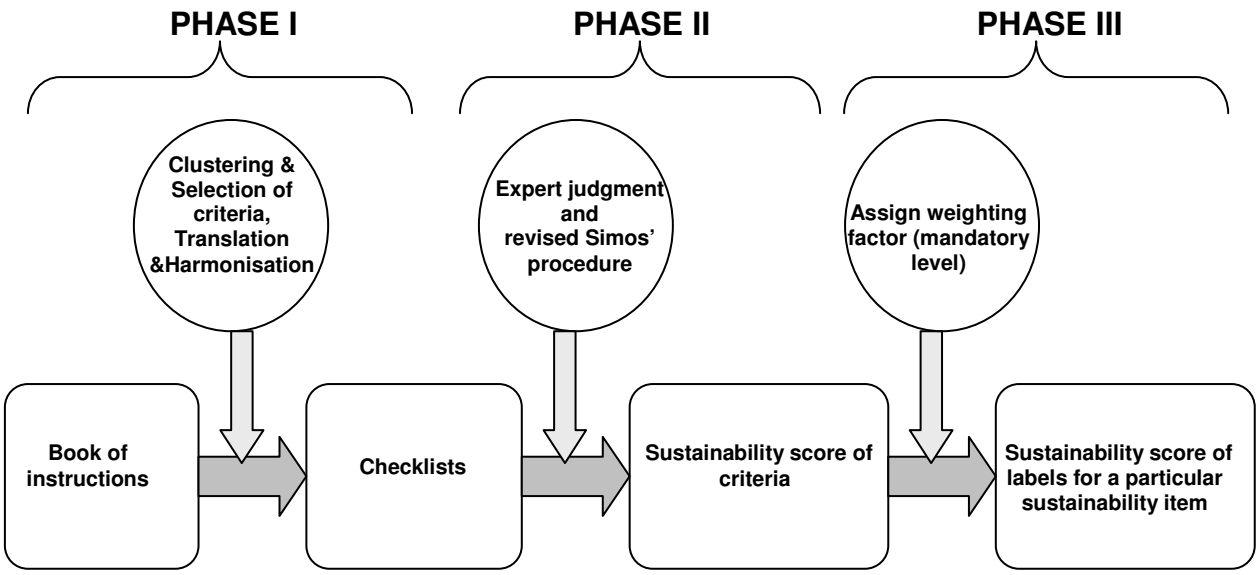


**Figure 1. Overview of the technical-scientific analysis method**

As an example we give in Figure 2 the results for the Fruitnet certification. The full line reflects the scores of the ideal standard, while the dotted line represents the scores for the Fruitnet label. The further the dotted line is from the centre of the graph, the better the scores. The greater the distance between the dotted and the full line, the higher the scope for improvements. Figure 2 also shows the contribution of each item to the overall concept of environmental sustainability. The further the full line is from the centre of the graph, the more important the item is. Water quality is considered the most important, noise quantity reduction the least important with respect to environmental sustainability. The results clearly indicate possible further ways to develop the standard.

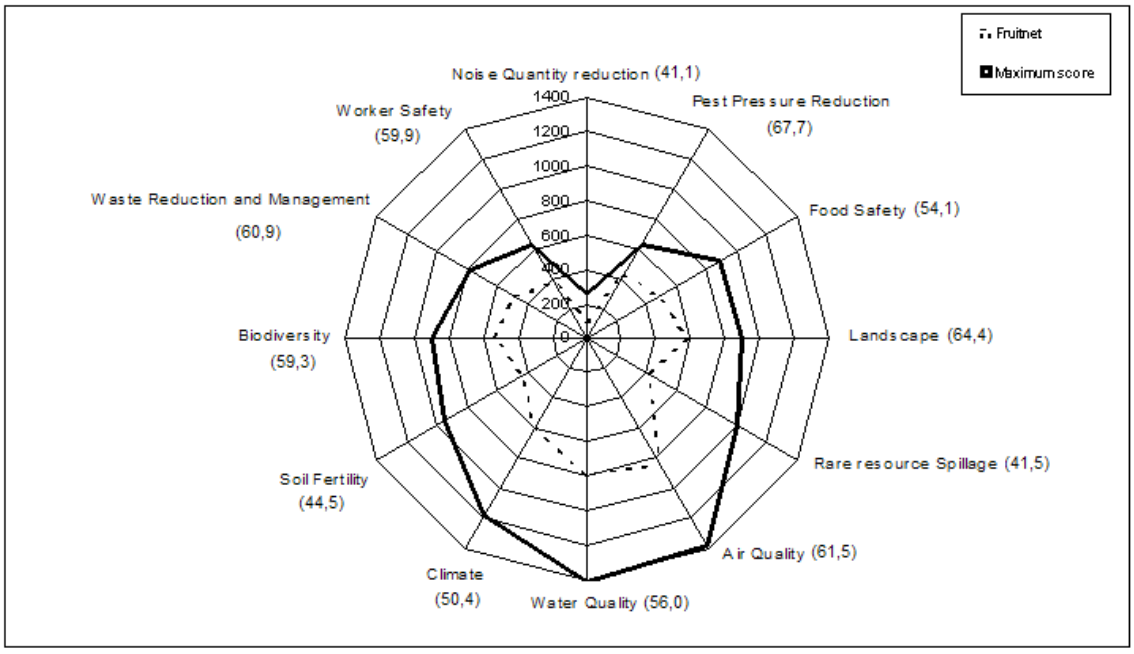


**Figure 2. The environmental performance of the Fruitnet label**

In Table 1 the results of the other analysed certificates are summarized. The scores on the different sustainability items reflect partially the importance given by the different certificates to certain aspects. The Fruitnet standard e.g. imposes stringent specifications regarding pest pressure reduction. This can easily be explained since the main goal of integrated farming is to reduce the quantities of pesticides applied. IPM farmers only intervene when really necessary, relying on analytic and diagnostic procedures before using agrochemicals. Observation systems for pest scouting are used to detect the presence of pests, and more specifically to determine the extent of their population.

**Table 1. Overview of the scores of the analysed certification schemes on the different environmental and human health sustainability aspects**

| Sustainability aspect | Labels | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Bio-garantie | Charte Perfect | Eurep GAP | Flan-dria | Flandria GAP | Geïntegreerd Pitfruit | Fruitnet | Terra Nostra |
| air quality | 55.6 | 62.4 | 45.4 | 49.0 | 56.0 | 54.7 | 61.5 | 32.3 |
| climate conservation | 51.5 | 63.7 | 27.9 | 29.0 | 40.4 | 40.2 | 50.4 | 38.3 |
| soil fertility | 48.7 | 45.9 | 33.5 | 25.7 | 46.1 | 25.7 | 44.5 | 39.1 |
| pest pressure reduction | 63.7 | 53.6 | 55.2 | 38.6 | 60.2 | 62.7 | **67.7** | 46.7 |
| water quality | 52.7 | 56.7 | 44.3 | 35.0 | 54.1 | 36.9 | 56.0 | 32.1 |
| biodiversity | 59.8 | 42.7 | 38.5 | 29.5 | 41.5 | 41.1 | 59.3 | 20.7 |
| landscape | 43.2 | 38.6 | 21.5 | 12.3 | 23.0 | 43.9 | **64.4** | 14.3 |
| waste reduction and management | 61.7 | 65.3 | 25.3 | 6.7 | 27.5 | 20.0 | 60.9 | 0.0 |
| scarce resource spillage | 37.1 | 55.0 | 29.2 | 23.9 | 48.4 | 27.8 | 41.5 | 28.2 |
| noise quantity reduction | 28.0 | 57.5 | 36.9 | 40.1 | 61.1 | 11.6 | 41.1 | 20.0 |
| food safety | 42.7 | 71.0 | 52.6 | 42.5 | 55.5 | 40.0 | 54.1 | 40.6 |
| worker safety | 31.0 | 57.3 | 61.0 | 32.6 | 59.7 | 33.3 | 59.9 | 18.3 |

In general, Fruitnet scores also well on other aspects, indicating a rather balanced and comprehensive certification book. This is e.g. not the case with Eurepgap that scores high on food safety and workers' safety but scores low on e.g. climate conservation and landscape. Also Biogarantie (organic label) scores high on nature protection criteria but has only few rules with respect to noise quantity reduction, food safety and workers' safety.

This shows that our tool is able to indicate which aspects are well developed and underdeveloped in a certification initiative. The tool also allows to evaluate potential improvements of a label as illustrated in Figure 3. In this figure, the original Flandria certificate rules as compared with the FlandriaGAP (this is the EurepGAP aligned version of Flandria) standard and with a proposed improved FlandriaGAP' standard are indicated. The proposed improvements are rules that are discussed in the organisation and therefore realistic for the near future. They include among others adapted rules relating to the application of pesticides, the choice of crop variety, the type of pesticides allowed, the number of treatments and the condition of the propagation material.

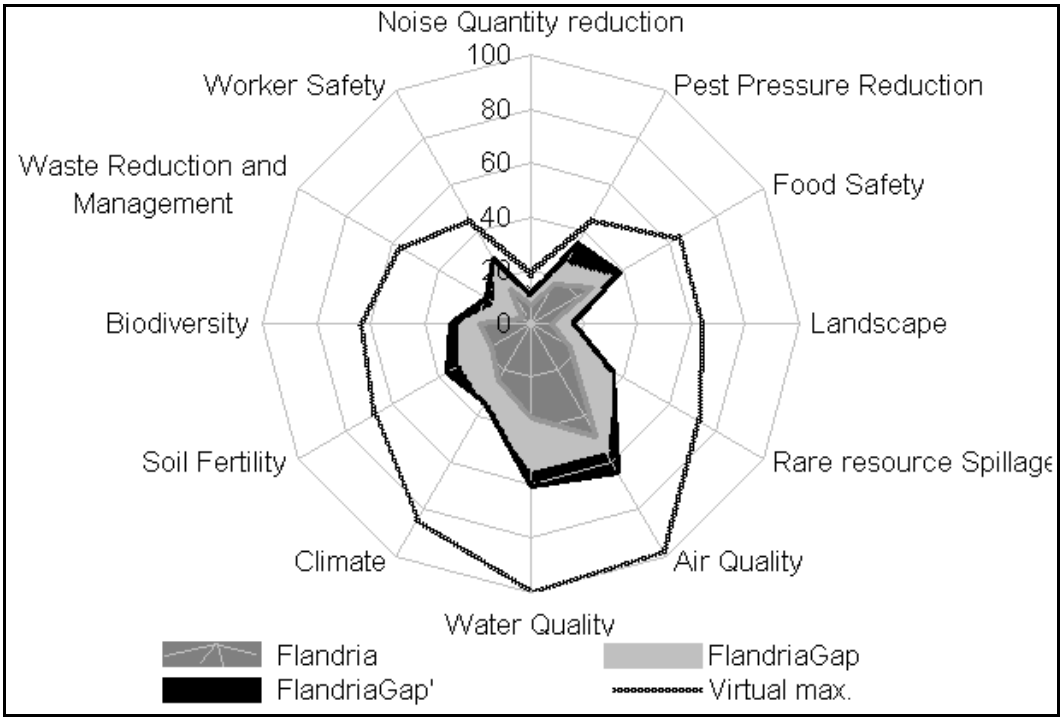


**Figure 3. Environmental performance of the Flandria, FlandriaGAP and the improved FlandriaGAP' label**

Given that certification books are predominantly implemented by farmers and constantly evolving, we assess in the next session the attitude of farmers towards these evolutions. For reasons of comparison with the work on environmental performance, we also concentrated this part of the research on the Flandria label and investigated the opinion of farmers towards the envisaged changes described above.

## Farmer perspective

To remain sustainable, it is clear that environmental certification standards have to maintain a difference with the law, otherwise there is no added value and hence no justification for a price premium. Different drivers for adaptation of the standards are, amongst others, changes in the legislation, fine tuning with other initiatives, changing demands from buyers and the availability of new production techniques. Market actors do not fully agree upon the evolutionary potential of standards, resulting in opposite stakes in the chain.

An important stakeholder hereby are the **farmers** who implement the certification system consisting of different prescription rules. It is the particular combination of rules which in the end results into a differentiated product or production process. Assessing the long term sustainability of certification therefore also requires the measurement of the farmers' willingness to participate in further evolving certification systems.

This requires an ex ante evaluation of the combined effect of changing different certification book rules simultaneously. A technique particularly suited to do so is the 'stated choice preference' method. In this method, a decision maker is confronted with several multi-attribute alternatives and is asked to choose his preferred one. The farmers' preference for the different attributes can then be calculated by means of a maximum likelihood estimation procedure. In order to illustrate the approach an experiment was set-up in which 68 farmers participating in the FlandriaGAP-initiative were surveyed. The questionnaire was mainly built around two choice preference experiments with the first experiment assessing the farmers' attitude towards general changes in the prescription book, and the second focusing on the measures aimed at reducing pesticide use. The different attributes (i.e. changes) selected for choice experiment 1 are summarized in Table 2.

**Table 2. Certification initiative attributes and attribute levels in choice experiment 1**

| **Attributes** | **Attribute levels**** |
|---|---|
| • Origin | *everybody accepted* / only Flemish farmers accepted / only Flemish products accepted |
| • Control | 2 controls per year / *1 control per year* / degressive control system |
| • Certification | group certification / individual certification / *free choice for farmers* |
| • Adherence to measures in certification book* | mm 100% / *mm 80%* / mm 90% |
| • Administration | *½ h per week* / 1 h per week / 1and1/2 h per week |
| • Social component | not integrated / *limited* / extensive |
| • Communication | towards end consumer / towards retail / *depending on preference retail* |
| • Relative change in price | -0,5% / *0%*/+0,5%/+1%/+1,5% |

* mm = minor must
**current level in italic

Today, measures indicated as 'minor musts' in the FlandriaGAP standard are for 80% compulsory, i.e. at least 8 out of 10 measures should be fulfilled. This is the first attribute integrated in the choice experiment to capture (a part of) the evolutionary potential of certification books. It is argued that the measures indicated as 'minor musts' will be the first to evolve, to a 'major must' level, hence uplifting the compulsory level of the minor musts is considered as a good proxy for evolution of the current standard. The results of the experiment indicate that farmers expect for a 1% increase in the minor must level (i.e. increasing the level of compliance from 80 to 81 on 100) corresponds with an increase in the product price (at auction level) of 0,18% (Table 3). With other words it indicates that in order to voluntary accept an increase of the level of the minor musts from 80 % to 90 %, farmers expect a premium of 1,8 %. EurepGAP, FlandriaGAP's challenger, has for example fixed the minor must level at 95%, an increase in the minor must compulsory level of 15%. The average farmer is only willing to accept (WTA) this increase if compensated with a 2,7% increase in the price for his product. The WTA thus reflects the compensation payment a producer expects for a more demanding cahier the charge. The willingness to pay (WTP) on its turn reflects how much a farmer is prepared to pay for a relaxation of the rules in the cahier the charge.

Another cornerstone of certification initiatives is the controlling system. A certification initiative offers an advantage to the individual farmer (whether this is market access,

higher or guaranteed price or something else), to compensate for the extra administrative and productive burden. If not effectively controlled, farmers will evade the certification rules, but will capture the surplus value offered under the initiative. The type and number of controls are hence a major question of debate between the different stakeholders within the initiative. Different types of control exist (group certification versus individual certification). In case of group certification, the farmers' cooperative (in casu the auction) is controlled and certified by an independent control body. This cooperative, on its turn, is responsible for the controls at farm level. When successful, the farmer receives an attestation from the auction's control officer. The independent control body only examines a sample of the farmers (the square root of the total number of participating farmers at cooperative level). This in contrast with individual certification where an independent control body directly controls the production process at individual farm level. The approved farmer then receives an individual certificate. At present, farmers have a free choice, hence the obligation of an individual control means a restriction for the farmer, which is reflected in the rather high WTA of 1,83% for a switch from group to individual certification.

**Table 3. WTP* and WTA* measures for the choice experiment (% change of end product price a farmer is prepared to pay/receive for a change in the rule)**

| Change in the rule | WTP measure | WTA measure |
|---|---|---|
| • Minor Musts more compulsory (currently 80% of the rules need to be fulfilled) | | 0,18% per 1% increase |
| • Group certification → Individual certification | | 1,83% |
| • 1 control → 2 controls | | 0,80% |
| • 1 control → Degressive controls | 0,18% | |
| • Administration (30 min/1h → 1h30min) | | 4,74% |
| • Limited → Extensive social component | | 2,43% |
| • Limited → No social component | 0,33% | |
| • Only Flemish farmers allowed | 0,15% | |
| • Everybody allowed | | 0,84% |

*WTP = Willingness to Pay
*WTA = Willingness to Accept

Regardless of the type of control, the number of controls is also of importance. With an increasing number, evaders have less chance to survive in the system. In the experimental set up, three attribute levels were presented to the farmers: 1 control/year, 2 controls/year and a degressive control system. In the present situation, farmers on average are controlled once a year. The degressive control system rewards farmers with positive control scores by reducing the number of controls in the subsequent years. This system is under consideration in several other European certification initiatives, among which Q&S (Qualität und Sicherheit) and Biogarantie. Farmers clearly favour the degressive control system, they are on average willing to accept this system for a price decrease of 0,18%.
Opposite stakes might also exist with regard to the type of communication. In the choice experiment, farmers were confronted with 3 possibilities: communication towards end consumer (through label), communication towards retail and communication depending on the retailer's preference (label or not). It was hypothesized that farmers derive a higher utility from direct communication towards the consumer, because this creates a pull mechanism (i.e. consumers specifically request the labelled product, which result in better prices or increased market shares). However, this item was found non significant, hence

no WTP or WTA was estimated. The farmers are in general indifferent whether a farmer label is used or not.

Administration is one of the major transaction costs associated with certification. The administrative burden caused by certification books is a major source of complaints by farmers, and it is probably the largest cost factor induced by certification. The choice experiment results confirm the negative utility farmers experience when administration time increases. By far the highest WTA measure is obtained for this item.

The incorporation of a social component attribute may seem strange and not directly linked with certification, but major retailers (mainly from the UK) are increasingly requesting the inclusion of specific social (labour) measures in the rules of prescription. More restrictive measures for this component are clearly not welcomed by farmers. For a change from a certification book with no social component to a certification book with an extensive social component, the surveyed farmers on average expect a financial compensation of 2,43%.

Finally, origin is integrated in the choice set because it is assumed that farmers do see a market advantage in restricting the number of farmers able to participate in their initiative. The different attribute levels presented to the vegetable growers are ‘everybody allowed’; ‘only Flemish farmers allowed’ and ‘only Flemish products allowed’, the latter because some Flemish farmers also possess production units abroad (e.g. in Spain or the United States). We could measure a negative WTA for the system in which there is unrestricted access.

The experiment shows that the method is able to evaluate the attitude of farmers toward proposed changes. It also allows to investigate differences among a group of farmers or to go deeper into detail about specific rules (see for this the final report of the project in Van Huylenbroeck *et al.* (2006)).

## Consumer perspective

When assessing the sustainability of certification schemes and labels, it is of course also important to take into account the consumer’s perspective, in particular for private initiatives where farmers hope to get a price premium as compensation for the extra requirements or extra measures taken. Rather than doing a general survey, the research followed a more qualitative method to assess the way how common consumers perceive labels in general and the issue of pesticides in particular. Although our approach does not allow to generalise the findings, they give an idea about how accepted private initiatives are and in how far the logic behind these certification and label initiatives is understood. The approach taken was the conduction of in total five focus groups. The first two focus groups were restricted to consumers. Next interested consumers were confronted with representatives of two certification stakeholders, the label promoters and the producers. In a last focus group, final conclusions were elaborated (Figure. 4). The aim of the exercise was mainly to see how consumers take deliberations between different interests and aspects into account.

The major result of the participative framework used was that consumers perceived the question of labels and pesticides rather differently from the way the various certification initiatives define the issue and look at the relations with consumers. The focus groups

highlighted a difference between the anxious consumer as often implicitly assumed by regulations on food safety and the moderated opinion of consumers. The experiment showed that consumers when placed in a deliberative situation comprehend the issue from multiple simultaneous dimensions and points of view and are able to understand the position of other stakeholders.

Consumers seem in particular aware of and concerned with the social aspects of certification. A social aspect raised was the possible exclusion of less fortunate consumers or small-scale producers, as well as the possible exclusion of certain types of retail outlets, products and varieties. A parallel to the social and technical irreversibilities brought about by the modernisation process in the industrial fruit and vegetable market was mentioned.

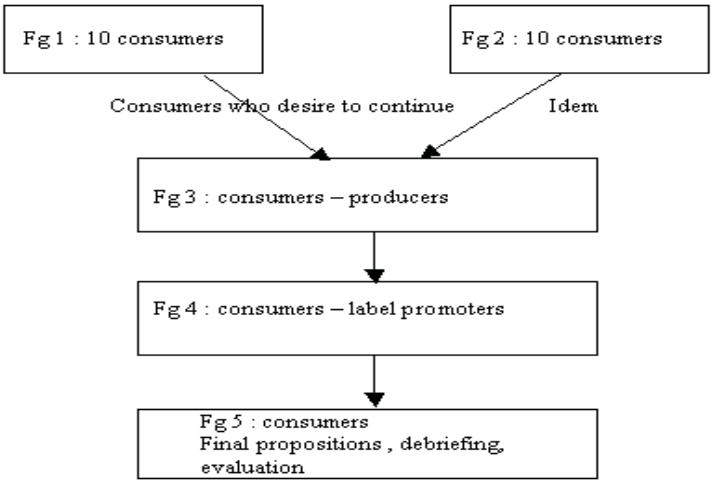


**Figure 4. Focus Group rationale for consumers' perspective on environmental certification**

The consumers also link the social aspect to the economic aspect. The latter is developed on the basis of the cost of labelled products for the consumers, the extra costs for the producers and, finally, the profits and commercial strategies of the intermediaries, in particular the supermarkets. They question in particular the position of retailers and advocate therefore the maintenance of differentiation in standards, preferring diversity to standardisation in both production and consumption practices. In so doing, they put forward a criterion of equity when raising the collective interest justifying public intervention with regard to food safety and quality.

The environmental aspect is questioned in the sense that the usual guarantees based on controls of results are not sufficient for them. They propose to put the environmental issues in other terms - commitments, approach, production project – and to extend them to criteria of origin and production type. The controls, which are the basis of the labels and are supposed to create consumer confidence, are therefore not central to their concerns, which cannot be reduced to mere food safety matters.

Another important aspect raised during the discussions was the mechanisms that are used to pass information. Consumers have a certain reluctance in how far this information is

objective, in particular when coming from stakeholders situated in the delocalised, retail framework. They question whether the present information on labels is effective and propose simplified approaches based on a clear visual message, which is related to a production approach and which could potentially be based on more interpersonal relationships with producers. Consumers are open for realistic compromises between different approaches such as outlet information in supermarkets as well as direct sales situations, even transposing characteristics from one type to the other (e.g. tastings and meetings with producers in supermarkets). But they also broaden the questions of information and education with concerns about training for supermarket departmental heads, supermarket managers, and both current and future farmers.

Although this consumer perspective is far from conclusive, it indicates the complexity of the issue. It reflects how difficult the equilibrium is between strict regulation and freedom of choice, two seemingly contradictory values. This also explains the contradiction between their request for simplification (too many labels) on the one hand and differentiation between labels on the other. If certification initiatives indeed want to move from a business-to-business approach to a business-to-consumer approach, it is certainly an important aspect to take into account.

## CONCLUSIONS

The presented research attempted to look at certification processes from a chain perspective. Methodologies were developed and applied enabling us to evaluate and assess certification of production systems both from the environmental as from the chain, farmer and consumer perspective. Besides the developed methodologies, the research made clear that certification and labels are social constructs in which an equilibrium needs to be found between the interests of different stakeholders but also between the possible ecological improvements and the costs of implementation of these improvements. To be sustainable in the long term, certification processes need to take into account the socio-economic context in which the stakeholders (producers, intermediates, retailers and consumers) involved are operating. Certification may indeed change the mode of operation for these different stakeholders making adaptation difficult with risk of exclusion of those that can not adapt (small producers or shops or less fortunate consumers).

Sustainability is therefore not only a question of more rules or better control of the rules but also of how these rules and prescriptions can be implemented. Certification processes with rules that are not feasible for one or another actor in the chain are not sustainable, bringing us to the question of choice between small incremental steps for a large group or a more radical change but with the risk of concentration and exclusion. In this aspect the role of extension and guidance of producers seems to be crucial.

Another point indicated is that private strategies have only a long term economic perspective if they can maintain a distinction and differentiation with the general regulative framework. This raises the issue of equilibrium between the role of the State and the private sector or in other words how far must the regulator go and from where starts the individual responsibility and choice of stakeholders in the chain. Finally the research revealed also another danger; the recuperation and harmonisation by the retail sector of the different approaches. This may lead to price erosion and more international

procurement and thus reducing the scope for regional initiatives. We therefore make a pledge for more technical-economic research in this area extending the pure instrumental and normative evaluations with socio-economic elements.

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# Development of a pesticide risk indicator for the evaluation of the Belgian reduction plan

**S. Vergucht[1], J. D. Piñeros-Garcet [2], L. Pussemier[2] and W. Steurbaut[1]**

*[1] Ghent University, Department of Crop Protection, Coupure Links 653, 9000 Ghent, Belgium*
*[2] Veterinary and Agrochemical Research Centre (VAR-CODA-CERVA), Department of Quality and Safety, Leuvensesteenweg 17, 3080 Tervuren*
*e-mail: walter.steurbaut@ugent.be*

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## SUMMARY

One of the goals of the present Belgian Reduction Programme for Pesticides and Biocides is to achieve in 2010 (compared to the reference year 2001) a 25% risk reduction for agricultural pesticides. In order to assess the improvements being made, a specific tool complying with the Belgian situation is required. The risk indicator developed in this framework is PRIBEL (Pesticide Risk Indicator for BELgium). By means of PRIBEL the agricultural use of pesticides can be evaluated and the quantitative goals set in the reduction plan can be measured.
PRIBEL is a multi-impact indicator that assesses at the level of Belgium both the human risk from occupational and dietary exposure to pesticides and the risk to the environment from the use of agricultural pesticides. The indicator consists of seven modules: operator, consumer, aquatic organisms (surface water), ground water, earthworms, birds and bees. For each module the potential impact is estimated by indicators based on an exposure toxicity ratio, a legal threshold and coefficients based on expert knowledge. PRIBEL follows an aggregated approach of the risks linked to pesticides because it balances each individual risk of pesticide use by the number of risk events (frequency of use). An aggregation procedure is implemented in PRIBEL to aggregate the risks over different levels (pesticide groups, crop groups, years).
PRIBEL is used to obtain a description of general tendencies in agricultural pesticide impact in Belgium and affords risk managers to gain a better perception of the bottlenecks of pesticide usage in specific crops and hence to tackle particular problems in an efficient way in order to achieve the determined reduction of 25% in 2010.

## INTRODUCTION

### *Need for potential impact indicators*

Since already more than half a century a lot of chemicals are developed as plant protection products (pesticides) for agricultural and non-agricultural use, biocides, plant growth regulators, etc. Those compounds are released in the environment as spray, dust, seed treatment, aerosol or bait and may possibly cause an impact on ecosystems (water,

soil, air, living organisms) and on human health (as well during application as via food contamination).

Because these side-effects only reveal a certain time after their usage and because their impact is only recently earnestly taken into consideration by scientists and the public opinion an urgent need arose for developing tools which can measure the impact of pesticides on human health and environment.

Such indicators which provide a quantitative interpretation of the use and impact of pesticides can be very useful instruments for the evaluation of pesticide applications in function of time in a certain region or country. They are also very advantageous to assess the relative importance of certain pesticides or pesticide groups (insecticides, fungicides, herbicides,…), crops (cereals, potatoes, orchards,…), cropping systems (conventional, organic, integrated pest management,…) or certification and labelling systems (Eurepgap, bio labels,…).

*Development of pesticide usage and impact indicators*

The first approaches to evaluate pesticide impact were based on the applied amount. For example, the first Dutch reduction plan (Meerjarenplan Gewasbescherming) [1] was based on a reduction of annual use (kg/year) and aimed a 50% reduction for pesticides (1990-2000). This approach, albeit very commendable, did not make any difference between the relative impact of (eco)toxic versus less (eco)toxic compounds. Therefore the development of “impact” indicators was needed. In Flanders (Belgium) an indicator for surface water was developed (Seq; spread coefficient [2]) which takes into account the usage (kg/year), the persistence (half-life time) and an ecotoxicological parameter based on the endpoints for water organisms (fish, daphnia and algae). Although the surface water compartment is considered to be a very important aspect for the evaluation of pesticides, such single parameter indicators are rather incomplete and do not allow a global analysis of pesticide impact on human health and environment.

Only recently multiple impact indicators are developed for a more general approach of pesticide usage evaluations. An example is the POCER (Pesticide OCcupational and Environmental Risk) developed at Ghent University [3]. The scientific principle of this indicator set is based on general scientific rules used in risk assessment analysis which uses the quotient between exposure and effect to indicate the risk. That value is then transformed into a dimensionless value between 0 and 1 by a method developed by Beinat & Van den Berg [4]. The risk is acceptable or non acceptable if the risk is respectively $< 1$ or $> 1$. A set of partial indicators has been developed for a series of important (eco)toxicological aspects: operator, bystander, field worker, consumer, aquatic organisms (surface water), groundwater contamination, soil organisms, birds, bees and beneficial arthropods. This concept allows a comparison between the impact of standard pesticide treatments (one application, one hectare, standard dose, worst case approach). Advantages are that the underlying algorithms are not complicated and that the data base only requires a relatively limited number of easy accessible input data. The modelling scenarios described by the indicators can be considered as “realistic worst case”. Changing certain default values in the algorithms allows to implement some more fine-tuning aspects such as drift value, soil type, protective equipment, time of spraying, dosage, formulation type, etc.).

A major challenge related to the use of a set of separate indicators each developed for a specific compartment is the attempt to aggregate these individual values into one or probably two (environmental, human health) values which can then be applied as tools in risk management measures (e.g. reduction plans). Such aggregation was developed in the POCER II approach [5] and the EU-HAIR [6] project where an aggregation system was developed on different levels (field, crop, pesticide group, time scale, etc.) and is a very powerful tool for risk managers in order to develop reduction plans, certification systems, label development etc. In POCER II the risk is no longer transformed into a value between 0 and 1 but is tested against a trigger value. Those trigger values differ per compartment and are official values established in the Uniform Principles of Annex VI of the European Council Directive 91/414/EC [7]. The risk is considered as acceptable when lower than the trigger value.

The value of such pesticide impact indicator largely depends on (1) the algorithms used in the indicator and (2) the underlying input data used in the calculations. A conflict always exists between the applicability of the system (by using simple calculations with a limited number of easy accessible data) on the one side, and using complex calculations and models based on a large set of input data on the other side. A compromise between scientists and policy makers has to be created.

### *Framework of the Belgian Reduction Programme for Pesticides and Biocides*

One of the aims of the present Belgian Reduction Programme for Pesticides and Biocides is to achieve in 2010 (compared to the reference year 2001) an impact reduction of 25% of pesticides used in agriculture [8]. In order to assess the improvements being made, a specific tool complying with the Belgian situation was required. The indicator developed in this framework is PRIBEL (Pesticide Risk Indicator for BELgium). By means of the PRIBEL-indicator the quantitative goals set in the reduction plan can be measured and additional measures and conceptions can be underpinned.

## METHODOLOGY

### *Overall principles and formulas of PRIBEL*

PRIBEL [9] is a multi-impact indicator based on the POCER II indicator [5] which is an extension of the POCER I indicator [3]. The principles are based on the acceptance criteria formulated in Annex VI of the European Council Directive 91/414/EC. PRIBEL takes into account (1) the scientific principles of risk assessment (risk quotient approach), (2) simple algorithms for several impacts, (3) a database with official EU-endpoints (4) a database of usage doses and sales per active substance and (5) an scientific aggregation methodology based on a statistical approach.

PRIBEL assesses at the level of Belgium both the human risk from occupational and dietary exposure to pesticides and the risk to the environment from the use of agricultural pesticides. The indicator consists of seven modules: operator, consumer, aquatic organisms (surface water), ground water, earthworms, birds and bees. As the goal of an indicator is to synthesize as much information as possible into a few numbers and graphical representations, an aggregation procedure involving several steps (spatial aggregation and aggregation of the active substances over the pesticide groups and the crop groups) is employed. In this way, all the information can be concentrated in global

PRIBEL-value per compartment for Belgium, but intermediate results are still available for more refined comparisons, e.g. assessment of the impact of a specific pesticide on a single compartment.

The calculations in PRIBEL were performed using official (eco)toxicological data, data from surveys on usage data and sales. The two keywords of PRIBEL are risk and frequency. The risk indices are calculated using the appropriate algorithm for each risk event: one hazard for one compartment at one moment on one parcel and take into account the (eco)toxicological data of the active substance and the application dose per hectare, whilst the frequency considers the number of application events of one active substance per hectare and the national area per crop. Although toxicological data applied in PRIBEL were established in laboratories, calculations of the risk indices by algorithms integrated several variable parameters simulating the influence of field conditions (e.g. protection of the applicator, growth stage of the crop, etc.). The risk indices reflect a first tier approach, i.e. worst case for exposure is taken into consideration.

### *Approach for risk calculation RI*

For each module, the risk is estimated by the use of risk indices (RI) which are the quotient of the exposure assessment or the Predicted Environmental Concentration (PEC) and the effect assessment or the (eco)toxicological Predicted No Effect Concentration (PNEC). Each risk index is then compared to its corresponding trigger value. The formulas used to calculate the RIs for every compartment and the trigger values are mentioned in the table below (Table 1) [9-10].

**Table 1. Overview of the formulas used to calculate the risk indices in PRIBEL for the 7 compartments**

| Compartment | Risk index | Trigger value |
|---|---|---|
| **operator** | RI = IE / AOEL | 100 |
| **consumer** | RI = MRL * EDI / ADI | 1 |
| **aquatic organisms** | RI = $PEC_{aq.org}$ / MPC | 1 |
| **ground water** | RI = $PEC_{ground\ water}$/0.1 | 1 |
| **earthworms** | RI = $PEC_{earthworms}$ / $LC_{50}$ | 0.1 |
| **birds** | RI = $PEC_{birds}$ / ($LD_{50}$ * BW) | 0.1 |
| **bees** | RI = AR / $LD_{50}$ | 50 |

Where IE: Internal Exposure of the operator (mg/kg BW/workday); AOEL: Accepted Operator Exposure Level (mg/kg BW/workday); MRL: Maximum Residue Limit (mg/kg food); EDI: Estimated Daily Intake (kg food/kg BW/day); ADI: Acceptable Daily Intake (mg/kg BW/day); $PEC_{aq.\ org}$: Predicted Concentration in the water (g/l); MPC: Maximum Permissible Concentration (g/l); $PEC_{ground\ water}$: Predicted Environmental Concentration in groundwater (µg/l); 0.1: drinking water quality standard (µg/L); $PEC_{earthworms}$: fraction of applied a.s. in the upper soil layer (mg/kg soil); $LC_{50}$: Lethal Dose for 50% of the population (mg/kg soil); $PEC_{birds}$: Estimated Daily Pesticide Intake (mg/day); $LD_{50}$: Lethal Dose for 50% of the population (mg/kg BW); BW: Body Weight (kg); AR: Application Rate (g/ha); $LD_{50}$: Lethal Dose for 50% of the population (µg/bee).

### *Approach for frequency calculation F*

The frequency F is obtained by dividing the total used quantity Q (kg a.s.) of a specific pesticide per crop in Belgium by the application rate AR (kg a.s./ha) of that pesticide. The application rate results from surveys on usage data (Van den Bossche et al., 2002 –

2003). In the surveys a certain number of farms representing a fraction of the total crop area were sampled and the results were extrapolated to the total crop area in Belgium. Hence, the number of applications per hectare per growth season of a particular pesticide and the total number of hectares per crop in Belgium are already incorporated in the end results of the survey. The formula below gives the calculation algorithm for the frequency.

$$F = \frac{Q\,[kg / jr]}{AR\,[kg / ha]}$$

With:

- F = frequency of application, signifying the number of risk events (the number of times the AR is sprayed or the number the environment is exposed to the specific risk RI).
- Q = total quantity (kg a.s.)
- AR = application rate (kg a.s./ha)

The total quantity Q is derived from the national sales per active substance and per year and a repartition coefficient. A weighted mean of three years is used in PRIBEL in order to level off peaks in sales due to errors in the sales statistics or to exceptional high sold amounts of certain active substances caused by specific weather conditions. This would obscure overall trends in pesticide use.

$$sales_{2001^*} = \frac{sales_{2000} + sales_{2001} + sales_{2002}}{3}$$

With:

- $sales_{2001^*}$ = the weighted mean of the 2001 sales

The repartition coefficient RP indicates the distribution of the sold amount between crops and is based on usage surveys carried out in different crops and on reports estimating the distinction between agricultural and non-agricultural use of pesticides, for instance by public services.

$$Q\,[kg / jr] = sales\,[kg / jr] * RP[\%]$$

With:

- Q = total quantity (kg a.s.)
- Sales (kg/yr) = weighted mean of the national sales
- RP (%) = repartition coefficient for a particular active substance over the different crop groups

The basic hypothesis for such a calculation is that the amount of pesticide used per hectare is quite constant over years while the sales are varying according to the national area of each crop and the number of doses applied per hectare in a given year.

*Risk aggregation concept*

The data on pesticide usage were grouped according to pesticide groups and crop groups. Five pesticide groups can be distinguished: insecticides, fungicides, herbicides, soil disinfectants and non plant protection products (nppp) such as additives, surfactants and emulsions. The results of the last category are not completely satisfying yet due to a lack of adequate toxicological input data. Nine crop groups have been selected according to the available data and the importance of the culture for the Belgian situation. These are potato, cereal, maize (and corn), sugar beet (and chicory), orchard (apple and pear), vegetables, greenhouse vegetables, industrial crops (flax and colza) and fodder (temporary and permanent grassland). The application type is also taken into account, e.g. spraying, pouring, granules,…

The aggregation concept used in PRIBEL is derived from the approach first developed in the POCER II model [5], and further improved in the EU-HAIR [6] project and the work done by Piñeros-Garcet *et al* [11-12]. Risk estimation is generally the result of various operations: emissions modelling, exposure and toxicity modelling, risk modelling in order to obtain a risk value (such as a ratio exposure/toxicity (most frequently) or a sum or even a more sophisticated relation), and finally sometimes a scoring operation to transform risk values into scores. Each risk value obtained by modelling is associated with a corresponding risk event which is the risk corresponding to a unitary time and area in which an individual is exposed to a single active substance, and for which only one hazard is envisaged (i.e. the highest modelling resolution considered). In a given region or period of time, a number of risk events can be potentially present and can be counted, named the risk frequency. Risk aggregation is the operation devoted to summarise the risk out of a collection of risk values (resulting from various pesticide applications or various organisms, places, dates or regarding various hazards). The number of pesticides, organisms, places, dates and hazards is denominated as the aggregation level. In PRIBEL risk is summarised (i.e. aggregated) for various aggregation levels either by expressing it using a single value, or by the computation of the statistical distribution of risks and frequency for the particular aggregation level.

PRIBEL calculates the risk indices for each active substance corresponding to each crop group (~ 8 000 combinations in Belgium) and then connects the risks to the frequency database. Two aggregation methods are applied: the total PRIBEL value (total risk or impact) and the weighted median risk. The total risk is the result of multiplying the risk index of each application by the frequency of application (F). The sum of these multiplications sum RI*F gives an estimation for one year of the total risk for Belgium, for a pesticide group or a crop group. The weighted median risk is calculated as the weighted $50^{th}$ percentile of the risk indices, having the frequency of application as weights and using the R statistical software and the Hmisc library [13].

The two aggregation types are applied to the following aggregation levels in PRIBEL:

- Level 1: aggregation by pesticide group.
  1 compartment, 1 pesticide group, all crop groups, all applications located in Belgium, year 2001.
- Level 2: aggregation by crop group.
  1 compartment, all pesticide group, 1 crop group, all applications located in Belgium, year 2001.

A third level is considered for future calculations: aggregation by year. This is a total aggregation only providing a total risk value per compartment summarized over all pesticide groups and crop groups, and therefore not appropriate to discuss in the following paragraphs. The third level will be useful for between-years comparisons in the reduction plan (2001- 2010).

*Approach for total risk or impact calculation RI*F*

The impact involves both risk and frequency by multiplying them per pesticide. The risk RI includes an exposure and an effect assessment involving the application rate AR (kg a.s./ha), and indicates the risk for a specific compartment when using a particular pesticide one time on one hectare. As the aim of a national study is to investigate whether a pesticide poses a high risk for Belgium, the number of times the risk is posed to a particular human or environmental compartment in a specific crop in Belgium has to be taken into consideration. This occurs by multiplying the risk with the frequency of use F:

$$total\ risk_{as} = RI_{as} * F_{as}$$

With:

- total $risk_{as}$ = the total risk for Belgium caused by the use of the specific as (impact)
- $RI_{as}$ = the risk of the active substance
- $F_{as}$ = the frequency of the active substance

The total risk for Belgium per pesticide group or per crop group is the result of the sum of the total risk values for all active substances included in the respective pesticide or crop group.

$$total\ risk_{pesticide\ group\ i} = \sum_{i=1}^{n} RI_{as} * F_{as}$$

$$total\ risk_{crop\ group\ j} = \sum_{j=1}^{n} RI_{as} * F_{as}$$

A numeric example for the total risk caused by isoxaben used in apple and pear on operator is elaborated. For isoxaben used in apple for instance (Table 2) it means that 420 times 0.188 kg isoxaben was sprayed in an apple parcel anywhere in Belgium. Hence, 420 times a risk of 0.1058 existed for any Belgian operator. There can be concluded that the impact of applying isoxaben in apple equals a value of 44.43, which is much higher than the total impact of isoxaben used in pear (3.92). Both frequency and risk are lower for isoxaben in pear. The values for apple and pear are counted up per column respectively to attain the total values for orchard. One should notice that the total impact RI*F is calculated by first multiplying the risk and frequency per pesticide, and then adding up all the impact values per pesticide group or per crop group. This implies that the total impact values given in the last column of the following tables 3 and 4 are not the multiplication of

the risk and frequency columns, yet the sum of all the impact values of the different pesticides included in the pesticide or crop group.

**Table 2. Example of calculation method for frequency F, risk RI and impact RI*F for isoxaben in apple and pear**

| Isoxaben | AR (kg/ha) | total quantity Q (kg) | frequency F | risk RI | total risk RI*F |
|---|---|---|---|---|---|
| apple | 0.188 | 78.95 | 419.93 | 0.1058 | 44.43 |
| pear | 0.045 | 6.97 | 154.90 | 0.0253 | 3.92 |
| total orchard | 0.233 | 85.92 | 574.83 | 0.1311 | 48.35 |

It is worthwhile to comment whether risk managers should consider pesticides with a high risk ranking or with a high impact ranking. The example below (Table 3) illustrates the point with two insecticides used in greenhouse vegetables: chlorpyrifos poses a higher risk to the operator when used once on one hectare (RI = 0.5551), whilst deltamethrin causes a much higher impact (122.96) because of a higher frequency. Although chlorpyrifos is a more risky pesticide for the operator than deltamethrin, it is used less in greenhouses in Belgium implying that the total risk or the impact on the Belgian operators will be less. Deltamethrin on the other hand causes a smaller risk to the operator (due to a lower application rate per hectare and safer toxicological values) but is a more popular pesticide in greenhouses entailing a higher total risk when reckoning with all Belgian operators in greenhouses. It is strongly advised to risk mangers to take into account both rankings, dependent on the goals set and on the type of measures intended to impose.

**Table 3. Comparison of risk RI and impact RI*F for operators of chlorpyrifos and deltamethrin applied in greenhouses in Belgium**

| | AR (kg/ha) | RI | F | RI*F |
|---|---|---|---|---|
| chlorpyrifos | 0.652 | 0.5551 | 9.62 | 5.34 |
| deltamethrin | 0.028 | 0.2795 | 439.97 | 122.96 |

## RESULTS AND DISCUSSION

### *Pesticide group aggregation (level 1)*

*General results*

Table 4 summarizes the relative contribution in terms of total risk of the five different pesticide groups to the seven compartments for the reference year of the reduction plan 2001. The total risks (RI*F) are expressed in percentages divided over the five groups. Comparing compartments with each other is not performed in PRIBEL, scientists (risk analysers) cannot equal the importance of one earthworm to one consumer for instance. As this is the task of policy makers (risk managers) they should be entrusted with all possible data to form themselves a picture of the national impact of the use of agricultural pesticides and decide to which compartment(s) priority will be given.

Fungicides pose the highest risk for consumer (e.g. thiram, fenpropimorph, epoxiconazole), operator (e.g. fentin hydroxide, mancozeb, fluazinam and an important soil disinfectant is methyl bromide) and earthworms (e.g. mancozeb, fenpropidin, fentin hydroxide); insecticides persuasively for birds (e.g. aldicarb, carbofuran and carbosulfan), bees (e.g. vamidothion, chlorpyrifos, imidacloprid) and aquatic organisms (e.g. flufenoxuron, lindane, endosulfan), and herbicides for groundwater (e.g. lenacil, atrazine, isoproturon). Between brackets some active substances causing a high total risk for the specific compartment are mentioned.

**Table 4. Total risks RI*F (in percentage of all per compartment) for the aggregation by pesticide group (level 1) for the seven compartments for 2001**

| Pesticide group | consumer | operator | birds | bees | aquatic organisms | worms | ground water |
|---|---|---|---|---|---|---|---|
| **insecticides** | 9 | 38 | **95** | **95** | **61** | 14 | 10 |
| **fungicides** | **60** | **40** | 1 | 3 | 21 | **57** | 33 |
| **herbicides** | 31 | 17 | 0 | 2 | 18 | 29 | **54** |
| **soil disinf.** | 0 | 4 | 3 | 0 | 0 | 0 | 3 |
| **nppp** | 0 | 1 | 0 | 0 | 0 | 0 | 0 |

*Results for operator, aquatic organisms and bees*

One human and two environmental compartments are discussed in detail, namely the pesticide operator, the aquatic organisms and the bees. As pesticides are designed to control or destroy living organisms, they constitute a potential health hazard to agricultural workers who use them or are exposed to them, to aquatic organisms inhabiting Belgian surface waters and to bees looking for nectar in the treated fields.

The acute risk for pesticide operators in Belgium is estimated within the operator module of PRIBEL. Pesticide operators are persons who mix, load and apply pesticides. Since the pesticide handler works with the concentrated product, exposure during mixing and loading can form an important part of the total exposure of the pesticide operator. Operators are not only exposed to pesticides during mixing, loading and spraying but also during seed treatment, application of granules, dipping into pesticide solution or pouring pesticide solution onto plants. The major routes of exposure are inhalation and dermal absorption. The oral exposure in agriculture is of a minor importance when appropriate hygienic measures are taken. In addition, uptake through the eyes is possible when pesticides splash up. This mainly occurs during mixing and loading activities [14-15]. The toxicological endpoint for operators is the Acceptable Operator Exposure Level (AOEL).

Aquatic organisms are exposed to pesticides through different routes. Both direct and indirect losses of pesticides sprayed on the field are taken into consideration in the module, with point source contamination the main form of direct losses whilst indirect losses involve drift, runoff, interflow and drainage. Point source contamination constitutes an important pollution form for surface water because a fraction of the application rate directly lands in the ditch or river. Drift is dependent on the application rate, climatologic conditions (wind, temperature) and the distance between sprayed field and surface water. Runoff is obviously subject to the application rate but also to the amount of pesticide already fade away in the environment by direct losses and drift, and to interception

capacities of the crop. Interflow and drainage are influenced by the soil characteristics and the behaviour of the pesticide in the soil [16]. The sum of all these exposure routes is then divided by the toxicological value for aquatic organisms (Maximum Permissible Concentration of MPC) to achieve the risk index. To compose the toxicological endpoint, acute and chronic values for daphnia, fish and algae are required, including a safety factor.

The acute risk for bees is associated to the risk event of a single pesticide application. One apiary per hectare is considered which often implies an overestimation of the risk but nevertheless fits in the worst case approach of PRIBEL. Only some crop groups are eligible for calculating bee risk bearing in mind that the risk for bees is not considered on non-melliferous crops, that all products are considered as being applied by spraying and that bees can not enter into greenhouses. Having those hypotheses, bee risk is calculated in five crop groups: vegetables (bean and pea), orchard (apple and pear), potato, fodder and industrial crops (flax and colza).

The risk RI, frequency of application F and the total risk RI*F are given in tabular form (Tables 5, 6 and 7 for operator, aquatic organisms and bees respectively). The total risk RI*F for operator caused by the use of fungicides is the highest (39.59%), closely followed by insecticides (38.42%) and then herbicides (17.38%). Soil disinfectants and particularly the non plant protection products contribute for a small part (3.64% and 0.96% respectively). The total risk per pesticide group is the result of the sum of the total risk values for all active substances included in the respective pesticide or crop group.

Concerning the frequency herbicides head the ranking (51.73%), then fungicides (35.82%), insecticides (7.06%), nppp (5.35%) and soil disinfectants (0.04%) successively. Herbicides are used several times per growth season in all crops contrary to the more limited use of fungicides and certainly insecticides. Although rarely used (revealed in the very small frequency) soil disinfectants represent a huge risk for operator (63.78%). Methyl bromide is responsible for the high value, due to the combination of a small toxicological value (AOEL) and a high application rate (441 kg a.s./ha). In addition, methyl bromide is applied in greenhouses, which pose a higher risk for the operator because of the “indoor” situation. Insecticides contribute for 28.05% to the risk, e.g. lindane having a small AOEL and a high dermal absorption resulting in a high risk for the operator. Fentin hydroxide and fenpropimorph are examples of fungicides posing a high risk for the operator; propachlor and isoproturon are on top of the herbicides group, however it has to be mentioned that the risk for the four afore-mentioned compounds is a factor 10 lower than the risk created by the use of some insecticides or soil disinfectants.

**Table 5. Overview of the results (risk, frequency and total risk; expressed in absolute values and in %) obtained per pesticide group for the risk for operator in Belgium, 2001**

| Pesticide group | risk RI | risk RI (%) | frequency F | frequency F (%) | total risk RI*F | total risk RI*F (%) |
|---|---|---|---|---|---|---|
| **insecticides** | 2.01E+04 | 28.05 | 5.71E+05 | 7.06 | 6.88E+07 | 38.42 |
| **fungicides** | 3.87E+03 | 5.40 | 2.90E+06 | 35.82 | 7.09E+07 | **39.59** |
| **herbicides** | 1.96E+03 | 2.74 | 4.19E+06 | **51.73** | 3.11E+07 | 17.38 |
| **soil disinf.** | 4.57E+04 | **63.78** | 3.51E+03 | 0.04 | 6.52E+06 | 3.64 |
| **nppp** | 1.87E+01 | 0.03 | 4.33E+05 | 5.35 | 1.72E+06 | 0.96 |

When considering the results for the aquatic organisms, a different trend is observed for risk and total risk compared to Table 5 (operator results). Both the risk RI and the total risk RI*F are determined to a large extent by insecticides (77.77% and 60.89% respectively). The contrast with the low frequency (7.18%) is conspicuous. This entails some active substances with a high RI within the insecticides group. Many insecticides are toxic to crustaceae, fish and/or algae, perceptible in small MPC-values (Maximum Permissible Concentration). Examples are bifenthrin (used in flax), flufenoxuron (pear) and cypermethrin and lindane (used in a broad spectrum of crops). Also for aquatic organisms frequency is the highest for herbicides (49.57%).

**Table 6. Overview of the results (risk, frequency and total risk; expressed in absolute values and in %) obtained per pesticide group for the risk for aquatic organisms in Belgium, 2001**

| Pesticide group | risk RI | risk RI (%) | frequency F | frequency F (%) | total risk RI*F | total risk RI*F (%) |
|---|---|---|---|---|---|---|
| **insecticides** | 7.30 E+04 | **77.77** | 6.21E+05 | 7.18 | 1.73E+08 | **60.89** |
| **fungicides** | 4.90 E+03 | 8.42 | 3.27E+06 | 37.86 | 6.00E+07 | 21.11 |
| **herbicides** | 7.93 E+03 | 5.22 | 4.29E+06 | **49.57** | 5.04E+07 | 17.73 |
| **soil disinf.** | 1.40 E+02 | 0.15 | 3.27E+04 | 0.38 | 7.70E+05 | 0.27 |
| **nppp** | 8.39 E-02 | 0.000089 | 4.34E+05 | 5.02 | 8.24E+03 | 0.0029 |

Regarding bees (Table 7), the risk is nearly solely determined by insecticides (99.43%); risks linked to fungicides and herbicides are two orders below those for insecticides. Some examples of pesticides that cause such high risk for bees are omethoate (orchard), vamidothion (orchard), cyfluthrin (orchard), bifenthrin (industrial crops), imidacloprid (orchard and potato), chlorpyrifos (orchard and potato), parathion (potato),…The total risk RI*F of insecticides is tempered (94.77%) due to smaller frequency values, but still the principally determined by insecticides. Frequency is again the highest for herbicides. As all soil disinfectants are applied in greenhouses or in leek, risk is not relevant for bees because of non-entering and non-flowering respectively.

**Table 7. Overview of the results (risk, frequency and total risk; expressed in absolute values and in %) obtained per pesticide group for the risk for bees in Belgium, 2001**

| Pesticide group | risk RI | risk RI (%) | frequency F | frequency F (%) | total risk RI*F | total risk RI*F (%) |
|---|---|---|---|---|---|---|
| **insecticides** | 5.98 E+03 | **99.43** | 2.95 E+05 | 7.48 | 8.44 E+06 | **94.77** |
| **fungicides** | 1.77 E+01 | 0.30 | 1.99 E+06 | 42.06 | 2.88 E+05 | 3.23 |
| **herbicides** | 1.65 E+01 | 0.27 | 1.66 E+06 | **50.41** | 1.78E+05 | 2.00 |
| **soil disinf.** | / | / | / | / | / | / |
| **nppp** | 8.20 E-04 | 1.36E-05 | 1.92 E+03 | 0.05 | 1.57 E+00 | 1.76 E-05 |

An interesting way to analyze the situation in Belgium for the risk for operators, aquatic organisms and bees is to observe the bubble chart (Figures 1, 2 and 3). These figures consist of 3 important parameters: on the ordinate (Y-axis) the frequency of use of all the products aggregated in pesticide groups, on the abscissa (X-axis) the median risk ($50^{th}$ percentile) linked to each group, and the size of the bubbles equals the total PRIBEL value

(RI*F). The statistical parameter that was retained here (median risk) represents the RI value for which half of the applications in the pesticide group have a weaker or higher RI. The median risk index characterizes better the studied distributions (i.e. irregular distributions) compared to the other calculated percentiles. The herbicides bubble lies on top of the ordinate corresponding with a high frequency for both operator, aquatic organisms and bees risks (Figures 1, 2 and 3).

Regarding the operator (Figure 1) the size of the insecticide and fungicide bubble is approximately the same and bigger than the others, which complies with a higher total risk for fungicides and insecticides. The soil disinfectants contain only 9 application cases –yet some of them having a high risk, resulting in a high median risk.

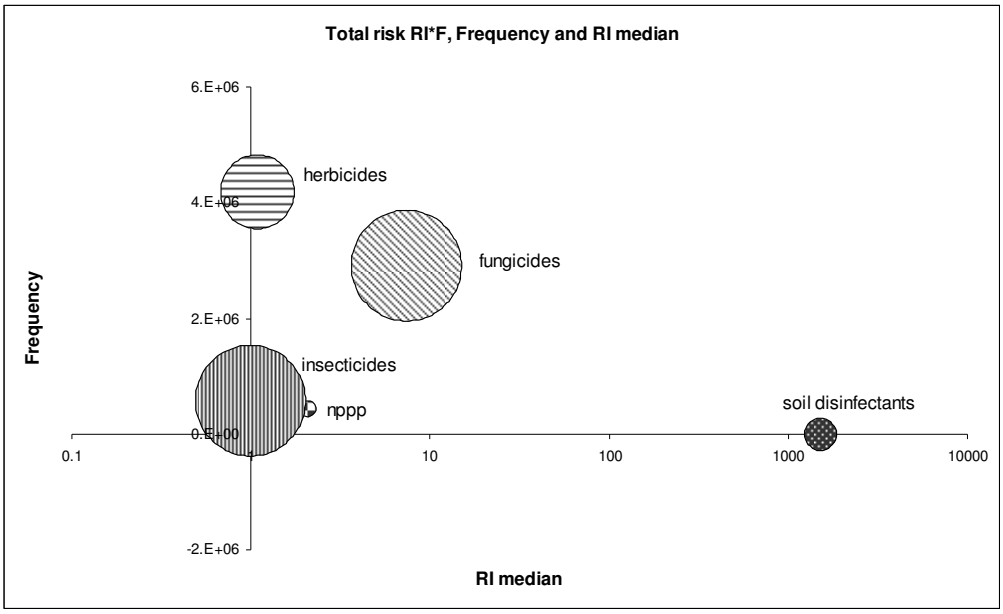


**Figure 1. Median risk on a logarithmic scale (abscissa), frequency (ordinate) and total risk RI*F (bubble size) of each pesticide group for operator in Belgium, 2001**

With respect to the risk evaluation of aquatic organisms in Belgium insecticides obviously represent the highest area RI*F (60.89%) notwithstanding a low frequency (7.18%). Equal to operator risks, soil disinfectants appear to have the highest median risk for aquatic organisms as well. The bubble for nppp cannot be plotted in Figure 2 due to a too small total risk area (8.24E+03).

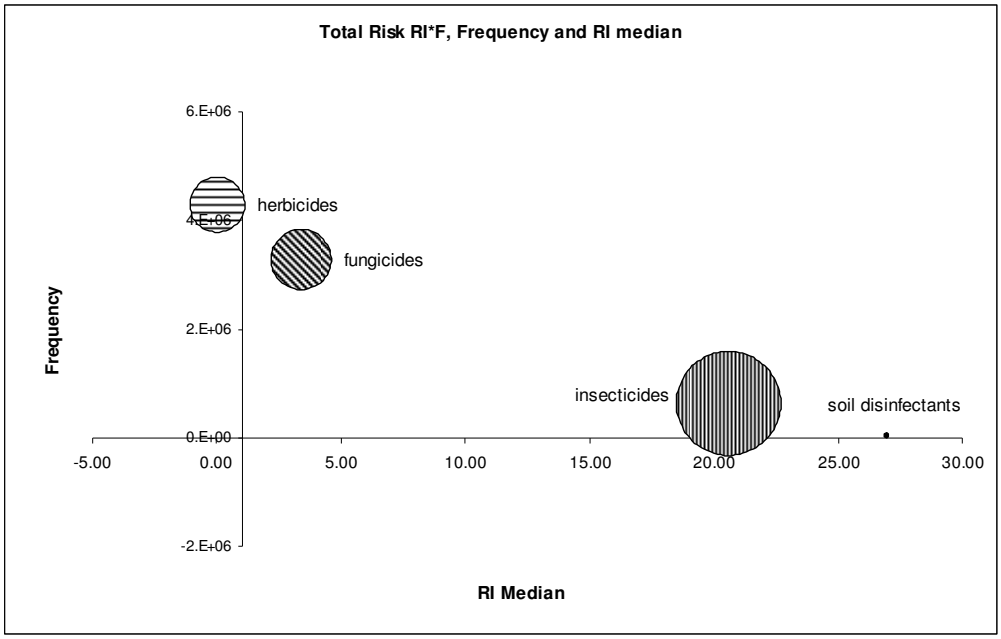


**Figure 2. Median risk (abscissa), frequency (ordinate) and total risk RI*F (bubble size) of each pesticide group for aquatic organisms in Belgium, 2001**

Figure 3 shows the bubble chart for bees and it as could be observed in Table x it is immediately clear that the median and total risk for insecticides is –although less frequent- predominantly the highest for bees. Bubbles for soil disinfectants (risk not relevant) and nppp (too small total risk area) are not represented in Figure 3.

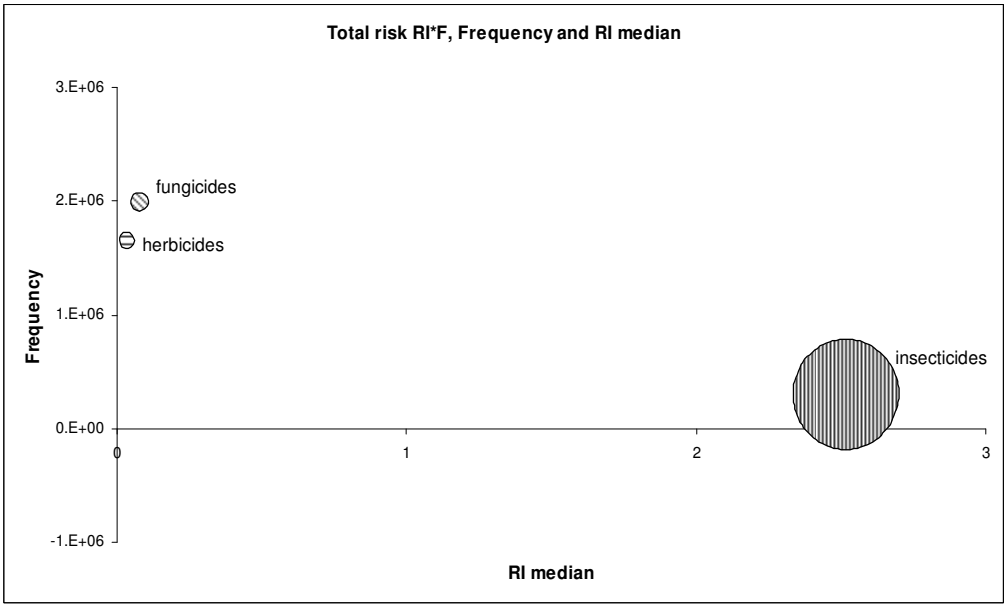


**Figure 3. Median risk (abscissa), frequency (ordinate) and total risk RI*F (bubble size) of each pesticide group for bees in Belgium, 2001**

## *Crop group aggregation (level 2)*

### *General results*

Table 8 reflects the total risk values (RI*F) per crop in percentage of all crops per compartment for the year 2001. Highest total risks for consumer are observed in orchard (e.g. thiram, captan, dodine). Highest impact on bees and aquatic organisms are also caused by the use of pesticides in orchard. For bees vamidothion, imidacloprid and dimethoate are some examples, whilst for aquatic organisms flufenoxuron, dodine and endosulfan are the active substances showing highest total risks. Potato poses the highest risk for operator (due to the great use of mancozeb and maneb in powder form and to fentin hydroxide and fluazinam). Pesticides applied in sugarbeet cause a high risk for birds (the insecticides aldicarb, carbofuran, carbosulfan), earthworms (fenpropidin, aldicarb, carbendazim ) and groundwater (lenacil, ethofumesate, chloridazon ).

**Table 8. Pribel values (sum (RIxF) in percentage of all) for the aggregation by crop groups for the seven compartments for 2001**

| Crop group | consumer | operator | birds | bees | aquatic organisms | worms | ground water |
|---|---|---|---|---|---|---|---|
| **potato** | 11 | **49** | 3 | 26 | 22 | 29 | 12 |
| **maize** | 1 | 13 | 11 | 0 | 5 | 10 | 11 |
| **vegetables** | 1 | 3 | 2 | 3 | 9 | 8 | 11 |
| **orchard** | **44** | 1 | 2 | **59** | **29** | 10 | 17 |
| **sugar beet** | 0 | 16 | **82** | 0 | 13 | **34** | **32** |
| **cereal** | 42 | 11 | 0 | 0 | 18 | 7 | 6 |
| **industrial** | 0 | 0 | 0 | 4 | 3 | 0 | 5 |
| **greenhouse** | 1 | 5 | 0 | 0 | 0 | 0 | 4 |
| **fodder** | 0 | 3 | 0 | 8 | 1 | 1 | 1 |

### *Results for operator, aquatic organisms and bees*

It is particularly interesting to consider an overview of the risk, frequency and total risk results for the different crop groups. With regard to the operator risk (Table 9) applications in greenhouse crops show the highest total risk (64.22%) due to the regular use of soil disinfectants. The reason why greenhouse crops do not manifest a high total PRIBEL value RI*F (3.66%) is their low frequency (0.29%). Soil disinfectants are only applied after harvesting a crop and before sowing another one hence not throughout the whole growth season. In addition, the Belgian area covered with greenhouses is small in comparison to other crops. In cereal, potato, sugar beet and vegetables specific pesticides constituting a reasonably high risk for the operator are used, e.g. lindane (in cereal, potato and sugar beet), parathion (in cereal and vegetables), chlorfenvinphos (in vegetables) and fentin hydroxide (in sugar beet) in the year 2001. Cereal and potato cover more than 50% of the frequency of use, partly attributed to the high number of hectares of those crops in Belgium and partly to repeated treatments of mainly fungicides. The total PRIBEL value RI*F is the highest for potato, followed by sugar beet, maize and cereal.

**Table 9. Overview of the results (risk, frequency and total risk; expressed in absolute values and in %) obtained per crop group for the risk for operator in Belgium, 2001**

| Crop group | risk RI | risk RI (%) | frequency F | frequency F (%) | total risk RI*F | total risk RI*F (%) |
|---|---|---|---|---|---|---|
| **potato** | 5.54E+03 | 7.73 | 1.70E+06 | 21.06 | 7.45E+07 | **41.63** |
| **orchard** | 8.61E+02 | 1.20 | 8.16E+05 | 10.08 | 1.00E+06 | 0.56 |
| **cereal** | 5.67E+03 | 7.92 | 2.51E+06 | **31.05** | 1.97E+07 | 10.99 |
| **sugar beet** | 4.82E+03 | 6.73 | 8.73E+05 | 10.79 | 3.70E+07 | 20.69 |
| **maize** | 3.18E+03 | 4.44 | 7.55E+05 | 9.33 | 2.90E+07 | 16.19 |
| **fodder** | 8.53E+02 | 1.19 | 9.54E+05 | 11.79 | 5.45E+06 | 3.04 |
| **vegetables** | 4.61E+03 | 6.44 | 3.20E+05 | 3.95 | 5.45E+06 | 3.04 |
| **industrial** | 9.56E+01 | 0.13 | 1.35E+05 | 1.67 | 3.64E+05 | 0.20 |
| **greenhouse** | 4.60E+04 | **64.22** | 2.37E+04 | 0.29 | 6.55E+06 | 3.66 |

The frequency pattern for aquatic organisms (Table 10) is analogous to the one for operator (over 50% for pesticides applied in cereal and potato). Concerning the risk RI orchard heads the list (34.14%). Although cereal has the highest frequency, the total risk is preceded by orchard and potato. This implies that a lot of products applied in cereal have rather small risk indices. Indeed, the majority of products used in cereal belongs to the herbicides group (44%) whereas only 18% are insecticides. In orchard and potato the insecticides fraction is much higher (33% and 28% respectively). Active substances on top of the RI ranking of orchard are all insecticides (cypermethrin, flufenoxuron, phosalone, endosulfan, parathion, omethoate, etc.). The top of the RI list for potatoes is a mixture of insecticides, fungicides and herbicides, with cypermethrin, lindane, parathion, diazinon, heptenofos and chloorpyrifos between them.

**Table 10. Overview of the results (risk, frequency and total risk; expressed in absolute values and in %) obtained per crop group for the risk for aquatic organisms in Belgium, 2001**

| Crop group | risk RI | risk RI (%) | frequency F | frequency F (%) | total risk RI*F | total risk RI*F (%) |
|---|---|---|---|---|---|---|
| **Potato** | 1.05E+04 | 12.22 | 1.90E+06 | 21.92 | 6.37E+07 | 22.41 |
| **Orchard** | 2.94E+04 | **34.14** | 7.86E+05 | 9.07 | 8.17E+07 | **28.75** |
| **Cereal** | 1.29E+04 | 15.03 | 2.75E+06 | **31.72** | 5.22E+07 | 18.37 |
| **Sugar beet** | 9.61E+03 | 11.18 | 9.00E+05 | 10.39 | 3.80E+07 | 13.37 |
| **Maize** | 3.78E+03 | 4.40 | 7.53E+05 | 8.70 | 1.31E+07 | 4.61 |
| **Fodder** | 3.05E+03 | 3.55 | 1.02E+06 | 11.77 | 3.77E+06 | 1.33 |
| **Vegetables** | 1.20E+04 | 13.97 | 3.91E+05 | 4.51 | 2.43E+07 | 8.55 |
| **Industrial** | 4.71E+03 | 5.47 | 1.40E+05 | 1.62 | 7.44E+06 | 2.62 |
| **Greenhouse** | 2.65E+01 | 0.03 | 2.61E+04 | 0.30 | 2.73E+03 | 0.001 |

The highest risk for bees (75.48%) is caused by pesticides used in orchard (Table 11). The total risk is also highest for orchard, but not as explicitly as the sum of all the risks, due to a higher frequency of application of pesticides applied in potato and fodder. The national orchard area is small in comparison with the national potato area, but nevertheless the risk

for orchard is much higher because of the use of many risky insecticides for bees. High total risk values are observed for vamidothion, cyfluthrin, imidacloprid, parathion, dimethoate, chlorpyrifos, omethoate and methidathion; all those insecticides having a very small lethal dose ($LD_{50}$) for bees.

**Table 11. Overview of the results (risk, frequency and total risk; expressed in absolute values and in %) obtained per crop group for the risk for bees in Belgium, 2001**

| Crop group | risk RI | risk RI (%) | frequency F | frequency F (%) | total risk RI*F | total risk RI*F (%) |
|---|---|---|---|---|---|---|
| **Potato** | 9.36 E+02 | 15.56 | 1.80 E+06 | **45.60** | 2.27 E+06 | 25.52 |
| **Orchard** | 4.54 E+03 | **75.48** | 7.83 E+05 | 19.83 | 5.25 E+06 | **58.95** |
| **Fodder** | 2.24 E+02 | 3.72 | 1.02 E+06 | 25.79 | 6.96 E+05 | 7.82 |
| **Vegetables** | 4.1 E+01 | 0.68 | 2.07 E+05 | 5.24 | 2.75 E+05 | 3.10 |
| **Industrial** | 2. 73 E+02 | 4.55 | 1.40 E+05 | 3.54 | 4.12 E+05 | 4.62 |

Figure 4 encompasses the frequency of use of the pesticides in the different crop groups (ordinate), the median risk (abscissa) and the total PRIBEL value RI*F (bubble size) for operator. As revealed in Tables 9 and 10 pesticides applied in cereal and potato represent the highest frequency for both the pesticide operator and the aquatic organisms. It is abundantly clear that the highest total risk is attributed to products used in potato (operator) and orchard (aquatic organisms). Pesticides used in potato, maize and greenhouse generate the highest median risk regarding operator risk and sugar beet, potato, orchard and vegetables with respect to the risk posed to aquatic organisms. For spraying events in greenhouses drift is not judged to be a relevant criterion and therefore not taken into account in the risk calculations. This results in a generally lower risk for aquatic organisms caused by pesticides applied in greenhouses when compared to other crops. Hence, the greenhouse bubble is too small to be shown in Figure 4.

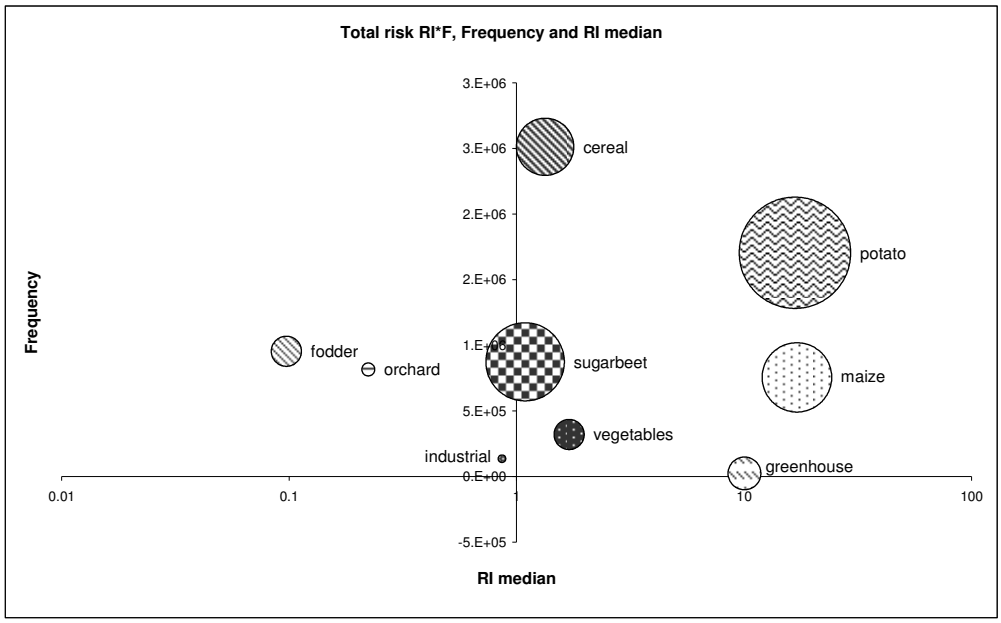


**Figure 4. Median risk on a logarithmic scale (abscissa), frequency (ordinate) and total risk RI*F (bubble size) of each crop group for operator in Belgium, 2001**

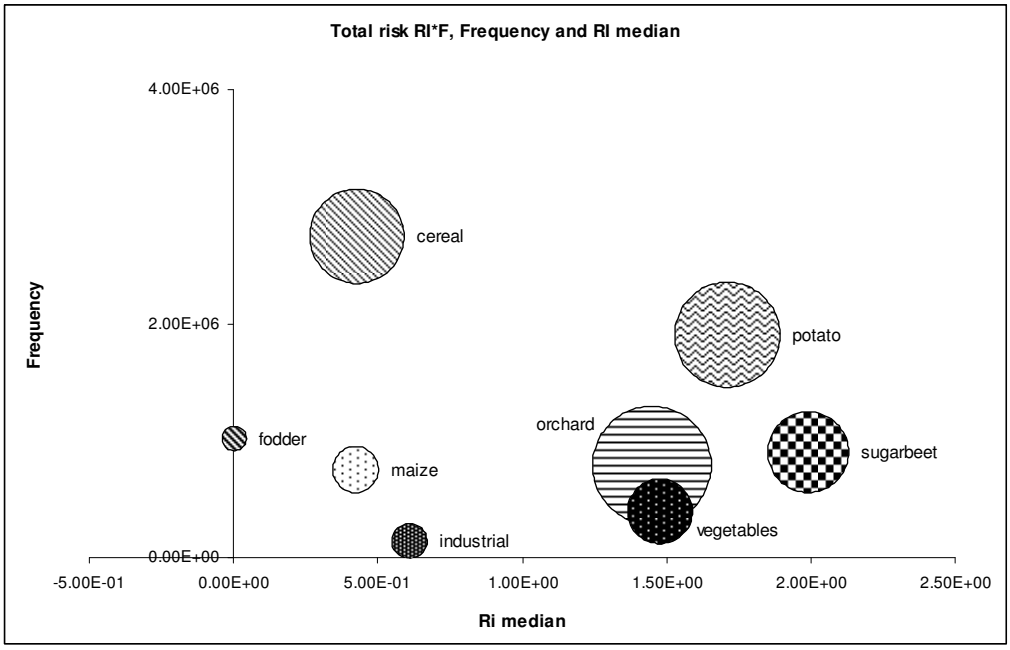


**Figure 5. Median risk (abscissa), frequency (ordinate) and total risk RI*F (bubble size) of each crop group for aquatic organisms in Belgium, 2001**

Analogous to the bubble charts for operator and aquatic organisms, potato shows a high frequency in the bee bubble chart (Figure 6), whereas industrial crops and vegetables represent a small part in the total frequency pattern. The bubble for orchard has the biggest area meaning that the highest total risk is caused by pesticides (principally insecticides) applied in apple and pear. Median risk is the highest for potato but all crops have a median risk in the same range.

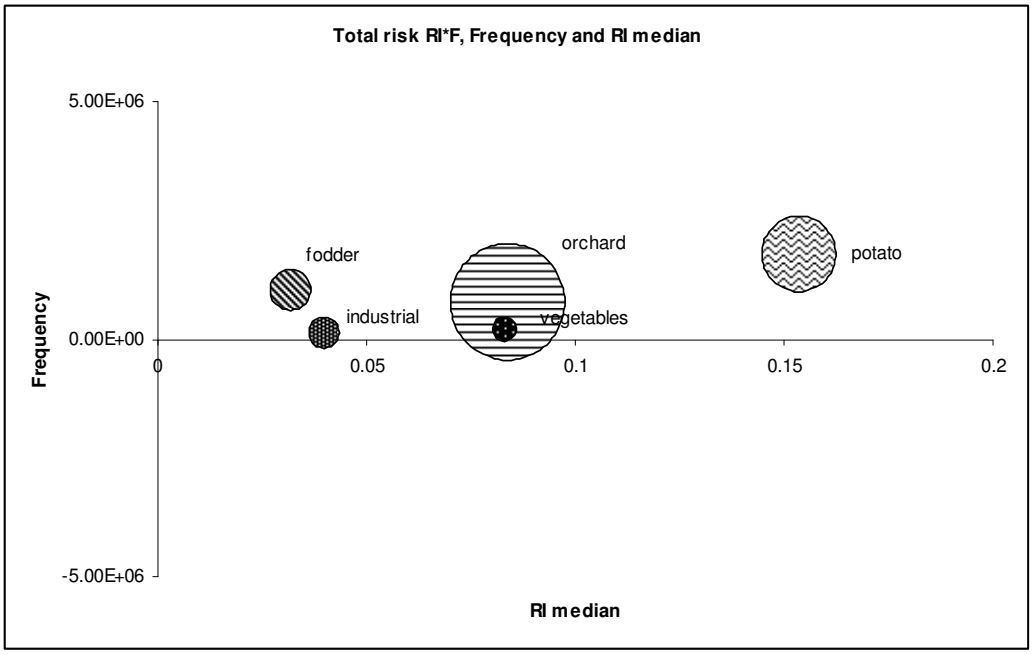


**Figure 6. Median risk (abscissa), frequency (ordinate) and total risk RI*F (bubble size) of each crop group for bees in Belgium, 2001**

## CONCLUSIONS

PRIBEL follows an aggregated approach of the risks linked to pesticides because it balances each individual risk of pesticide use by the number of risk events. Balancing risky situations in relation to frequent situations can not be done if only individual risks (one application at one hectare) are considered without taking the frequency into account.

PRIBEL allows extracting various types of information:

- Comparison of the risk associated with different pesticide or crop groups and regions
- Evolution in time of the risk associated with specific pesticides, pesticide groups or crop groups, regions, etc.
- Identification of the riskiest application cases for specific crops in terms of the risk index and the total risk.

PRIBEL provides calculation software to evaluate the risk for seven human and environmental compartments. Three modules are elaborated in this paper: pesticide

operator, aquatic organisms and bees. Soil disinfectants applied in greenhouses pose a high risk for the operator when considering one application, whilst fungicides and insecticides, primarily applied in potato, sugar beet, maize and cereal manifest high total risks for the operator when the frequency is taken into account. Insecticides applied in orchard, potato, cereal and sugar beet cause –although less applied than herbicides and fungicides- a high risk for aquatic organisms. Insecticides clearly dominate the risk pattern for bees.

The way risk is estimated using PRIBEL contends with some limitations and hypotheses made in order to allow the calculation, i.e. there is only dealt with the active substance of a pesticide product; adjuvants added to formulate the product are not taken into consideration because of the lack on accurate (eco)toxicological data, interactions between two or more active substances are not taken into account, organisms are supposed to inhabit near to a single field with a single pesticide applied and recover after each risk event and chronic risks are ignored for all modules except consumer and aquatic organisms.

PRIBEL is used to obtain a description of general tendencies in agricultural pesticide impact in Belgium and affords risk managers to gain a better perception of the bottlenecks of pesticide usage in specific crops and hence to tackle particular problems in an efficient way in order to achieve the determined reduction of 25% in 2010.

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# Exposure of Belgian consumers to pesticides : a comparison of two indicators

**S. de Voghel and L. Pussemier**

*CODA-CERVA, Leuvensesteenweg 17, B-3080 Tervuren, Belgium.*
e*-mail: Lupus@var.fgov.be*

**Keywords**
Indicator; exposure; consumers; pesticide residues; foodstuffs

## SUMMARY

Through food consumption, Belgian consumers are exposed to pesticide residues on a daily basis. Indeed, application of pesticides on crops is widely used for the production of conventional fruits and vegetables marketed in Belgium. Therefore, it is of utter importance to assess possible harmful effects that pesticide intakes may cause to human health.
The goal targeted by the two indicators presented in this chapter is to provide a tool enabling us to describe with a few figures the situation regarding pesticide residues in food. By comparing the exposure with toxicological endpoints, indicators strive to provide a clear picture of the risks. Each indicator has its own features and the results obtained by calculations are differing in some points, delivering different types of results that must not be strictly compared. It is our interest in this chapter to analyze these differences in terms of accuracy, type and meaning of results as well as handling facilities.
Finally, limits of the methods and possible improvements will be suggested in order to refine the general approach linked to the issue of pesticide residue in the diet.

## INTRODUCTION

The Programme for Reduction of Pesticides and Biocides (PRPB) has been launched in 2001 with the aim of reducing by 50% the risks on environment and human health caused by pesticides uses (25% for agricultural uses). Within this federal programme, several studies were carried out implicating all the stakeholders concerned by pesticide issues. In order to estimate the impact of governmental actions such as specific policies regarding use of pesticides, restricted use or ban of selected pesticides, the PRPB needed a useful tool.

In order to measure variation of risks linked to pesticide uses, development of indicators became essential. The collaboration between the University of Ghent (UGent) and the CODA-CERVA led to the development of different risk indicators related to both environment and human health. The final version named PRIBEL allowed the calculation of risks linked to 9 compartments at national scale and over several years (cfr chapter written by Vergucht et al. in this issue). Among these indicators, PRIBEL*consumers* rely on data such as Maximum Residue Limits (MRL) and international dietary consumption data (GEMS-Food) in order to calculate consumer exposure to pesticide residues in Belgium.

In the framework of the PRPB, another indicator was set out specifically for consumers by the CODA-CERVA. The EXPO*consumers* indicator is based on data from national sampling campaign and food consumption. For this project, the deterministic method was used to estimate consumers' exposure. Results obtained by calculating the ratio Exposure/Toxicological endpoints may be helpful to provide an overview of food safety in Belgium in terms of pesticide residues. Besides, the procedure followed in this project can be repeated as further research could take advantage of the database and codes already created

With the comparison of these two indicators linked to consumers, the way to measure food safety and public health issues will be tackled down in this article. As both adopted approaches give inputs for food safety in Belgium, the discussion will strive to demonstrate the assets of each indicator. Their places in the food chain are described in Figure 1.

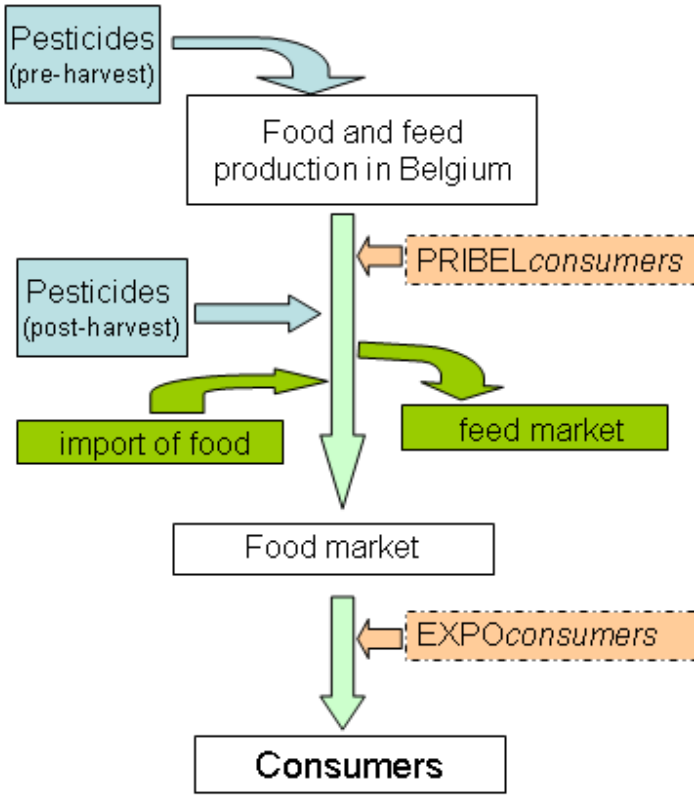


**Figure 1: The two indicators in the food chain**

Choosing a reliable indicator in order to monitor trends can improve risk assessment regarding food safety in Belgium. An assessment of chronic exposure to pesticide residue could be performed using the same methodology year after year. Nevertheless, one should bear in mind that many factors such as the improvement of analysis method,

marketing of new pesticides, and changes in regulation can impede strict comparison of results obtained from two different years.

## The pribel$_{consumers}$ indicator

*Indicator features*

The formula of the PRIBEL*consumers* derives from the previous RI*consumers* (Risk Indicator for consumers) and takes account of the frequency of use in Belgium. Therefore, PRIBEL*consumers* is calculated as such:

$$\text{PRIBEL}consumers = \text{RI}consumers \; X \; Frequency$$

The frequency of use (Frequency) is derived from national Belgian sales data coupled with the amount of pesticide used per crop.

RI*consumers* is given by :

$$\text{RI}consumers = \left( \frac{\text{MRL} \times \text{EDI}}{\text{ADI}} \right)$$

- MRL (Maximum Residue Limit; mg active substance/kg food);
- EDI (Estimated Daily Intake; kg food/kg body weight/day);
- ADI (Acceptable Daily Intake; mg as/kg body weight/day).

Regarding Good Agricultural Practices (GAP), data on food residues obtained in accordance with intensive but adequate pesticides application will be used to set a Maximum Residue Limit (MRL) of residue concentration not to be exceeded. Values of MRLs will differ in accordance with the pesticide considered and with the crop chosen. Some MRLs are set at the European scale whereas some MRLs are set by national authorities.

The Estimated Daily Intake was obtained through the GEMS/FOOD Regional Diets database [1]. Created by the World Health Organization (WHO), it encompasses the average daily consumption of foodstuffs by populations around the world. The cluster selected for the indicator was the cluster “Europe”.

The ADI (Acceptable Daily Intake) is widely used to describe “safe” levels of dietary intake on a daily basis over a lifetime without appreciable risk threatening human health. It is generated from the NOAEL which is the highest dose of a compound that does not provoke any adverse effect on tested animals. The ADI is therefore obtained by applying at least a 100-fold factor [2]. Indeed, two 10-fold factors are applied to the NOAEL. The first one takes into account of interspecies variability (tested animals and human) whereas the second one considers the intraspecies variability [3].

To calculate the results with PRIBEL a lot of input data are required. They are collected in a database owned by the University of Ghent (see chapter written by Vergucht et al. in this issue for more details) and they rely on the following kind of data:

- Amount (kg) of active substances yearly applied in Belgium
- Sales of active substances per year in Belgium (kg)
- Ecological and toxicological values: these data are collected in the database of UGent and were obtained from the European Union (when already available) and several other sources.

*Indicator calculations*

In a previous study, the indicator was calculated for the year 2001 which is considered in the PRPB as reference year for calculations. Results obtained trough the initial configuration of the PRIBEL software led to an underestimation of the risks. Indeed, some application cases (297 out of 1016) lacked a RI*consumers* value. Besides, application cases within crop groups "vegetables" and "greenhouse vegetables" were scarce. This problem was solved by adding in the software, all the MRL default values for commodities for which the pesticides are not authorized.
The PRIBEL software provided all the appropriate values for Frequency, MRL, EDI and ADI.

*Results*

In terms of pesticide groups, fungicides (FUNG) appear to be the riskiest group for consumers (58% of the total risk), followed by herbicides (HERB) (31%) and insecticides (INSE) (10%) (Table 1). Non plant protection products (NPPP) and soil disinfectants (SODE) represent a far more lower risk (less than 1% of the total risk) (Figure 2).

Another way to analyze the situation in Belgium is to observe the bubble chart (Figure 3). The size of each bubble, linked to a pesticide group, gives the importance of the PRIBEL value. Its position on the X-axis is giving the importance of the sum of the frequency of use whereas its position on the Y-Axis is related to the median of the RI*consumers* values for the pesticide groups.

**Table 1. Overview of the results obtained per pesticide group**

| Pesticide group | RIconsumers (mean) | PRIBEL (RI*F) (sum) | % of total risk | # of application cases |
|---|---|---|---|---|
| **FUNG** | 0,059 | 72525 | 58 | 205 |
| **HERB** | 0,012 | 39871 | 31 | 207 |
| **INSE** | 0,027 | 12438 | 10 | 208 |
| **NPPP** | 0,001 | 103 | 0,1 | 2 |
| **SODE** | 0,017 | 9 | 0,01 | 8 |
| **TOTAL** | - | 124945 | 100 | 630 |

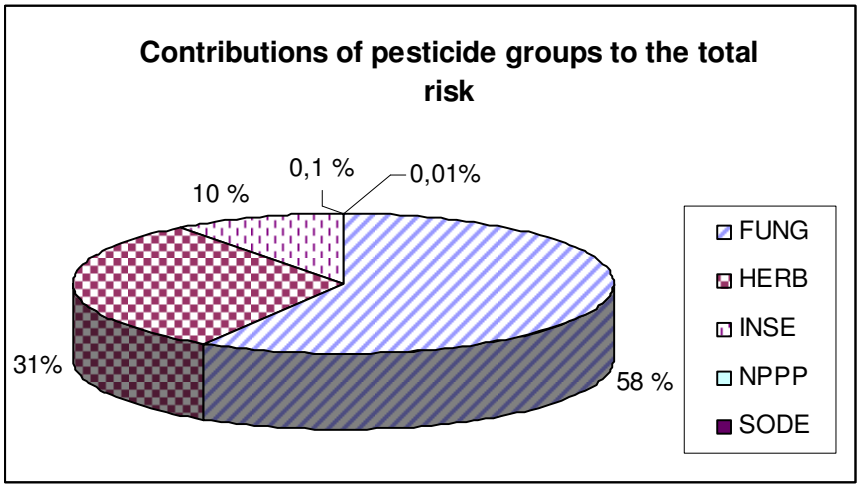


**Figure 2. Contributions of pesticide groups to the total risk in Belgium, 2001**

As it is seen in Figure 3, the fungicide group is accounting for a high proportion to the total PRIBEL for Belgium, both because its frequency and the RIc*onsumers* value are high. For herbicide, its importance is mainly due to the frequency of use. For the insecticide group, the RI*consumers* is the highest value of all pesticide groups, but the frequency is relatively low compared to fungicides and herbicides.

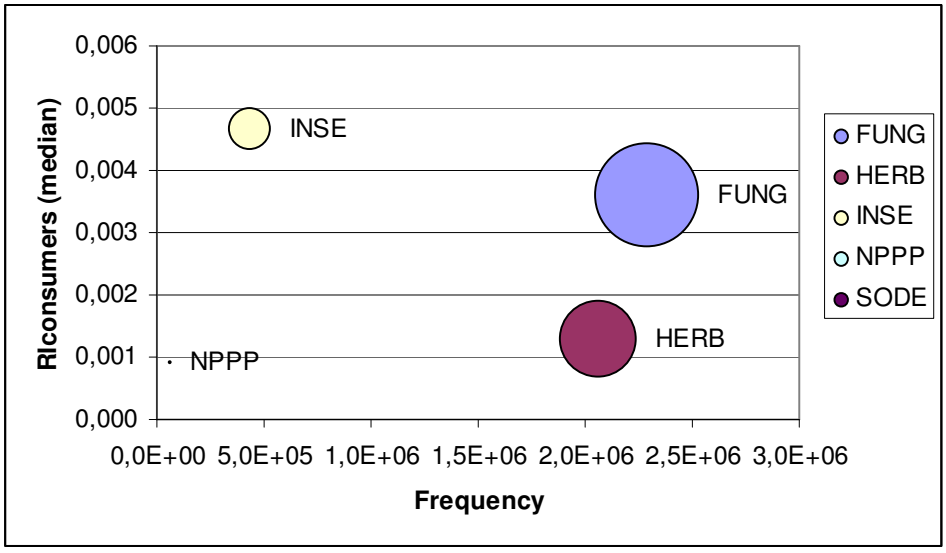


**Figure 3. Median Risk (Y) and Sum of Frequencies (X) of each pesticide group and Contribution of each group to the Total Risk (size of bubble, sum(RIxF)) on Consumers, Belgium, 2001**

The riskiest application cases for each pesticide group can be obtained through PRIBEL software. In terms of crop groups, pesticide applications in the cereal and orchard (fruits) groups show the higher risks of all groups (Table 2). The potato group accounts for 11% of the total risk whereas greenhouse vegetables, vegetables, and maize do not exceed 1 % of the total risk (Figure 4).

**Table 2. Overview of the results obtained per crop group**

| Crop group | RIconsumers (mean) | PRIBEL (RI*F) (sum) | % of total risk | # of application cases |
|---|---|---|---|---|
| **Cereal** | 0,038 | 54256 | 43,4 | 92 |
| **Orchard** | 0,067 | 52936 | 42,4 | 186 |
| **Potato** | 0,022 | 14454 | 11,6 | 80 |
| **Greenhouse veg.** | 0,031 | 1263 | 1,0 | 85 |
| **Vegetables** | 0,004 | 1048 | 0,8 | 108 |
| **Maize** | 0,001 | 989 | 0,8 | 43 |
| **TOTAL** | - | 124945 | 100 | 630 |

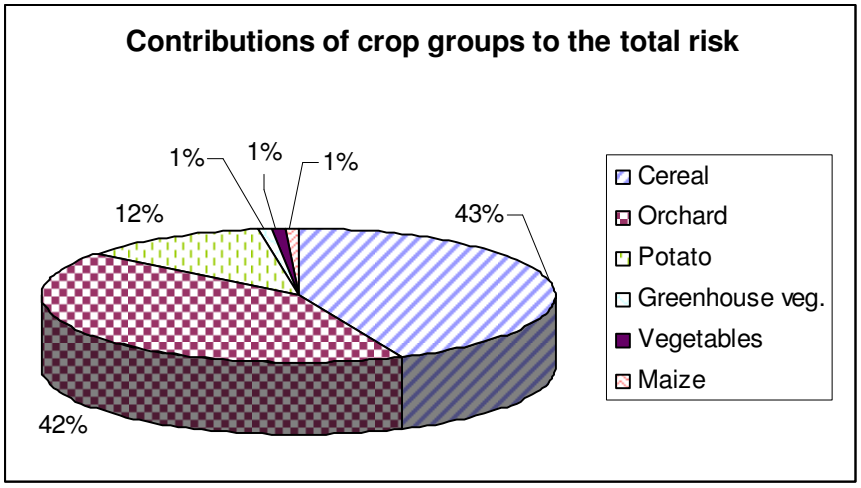


**Figure 4. Contributions of crop groups to the total risk in Belgium, 2001**

Figure 5 gives a clear look on the importance of frequency of use for cereal and potato crop groups. Orchard and cereal crop groups both have a high RI*consumers* median value. Concerning greenhouse vegetables, one can notice that the frequency of use is relatively low but the RI*consumer*s is high. The corresponding bubble is approximately the same size as for maize, whose contribution to total PRIBEL is mainly due to the Frequency of use.

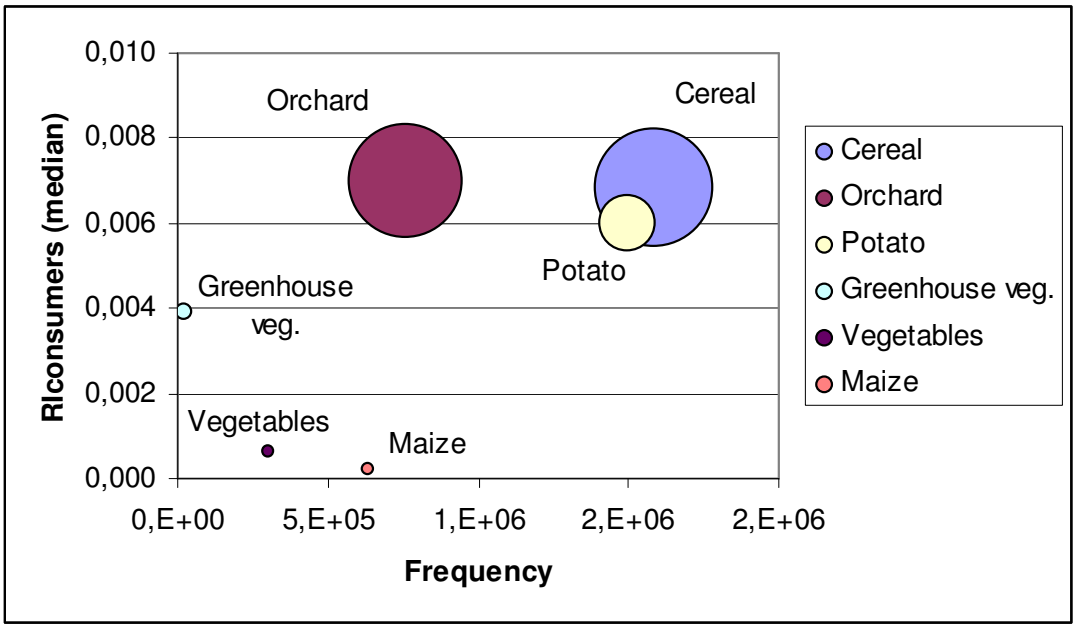


**Figure 5. Median Risk (Y) and Sum of Frequencies (X) of each crop group and Contribution of each group to the Total Risk (size of bubble, sum(RIxF)) on Consumers, Belgium, 2001**

Finally, we ranked with regards to the risk indicator the pesticide applications for the group of fruits and vegetables (i.e. potatoes, orchards, vegetables, and greenhouse vegetables). It appears that, for this group, the applications of sulphur on apples stand for 19% of the total risk (Table 3). Mainly apples in orchard and potatoes account for a high proportion of the total risk of this group. The 3 application cases with a PRIBEL*consumers* value above the P99 of the risk are concerning apples in orchards.

*Discussion*

High PRIBEL values from the riskiest application cases can be explained differently in accordance with their frequency of use or their RI*consumers* values (i.e. the high ratio potential exposure : toxicity). For the application of sulphur on apples, being the riskiest one, it is clearly the high value of RI*consumers* (4,36) that contribute mostly to the high PRIBEL value for consumers during the year 2001. Whereas for chlormequat on winter wheat, the frequency of use seems to contribute largely to the high PRIBEL value (see Figure 5). The PRIBEL risk indicator is calculated on the basis of a worst-case approach. Indeed, consumer exposure is evaluated by the MRL and the EDI, no matter if residue concentrations are lower than the MRL value and if the food consumption is less important than the one used in the model. Risk is therefore calculated taking account of a potential exposure. For reasons cited above, real exposure can be considered lower than the one calculated by PRIBEL. Besides, processing factors (eg. washing, peeling, heating,…) [4] which tend to decrease pesticide residue concentrations in commodities were not taken into account in the calculation.

**Table 3. Riskiest application cases for fruits and vegetables (including potatoes) (*=above the percentile 99th of total risk)**

| Active substance | Crop group | Pesticide Group | RI consumers | PRIBEL (RI*F) |
|---|---|---|---|---|
| sulphur | apple | FUNG | 1-10 | *24062 |
| thiram | apple | FUNG | 0,1 - 1 | *7268 |
| copper hydroxyde | apple | FUNG | 0,1 - 1 | *3468 |
| captan | apple | FUNG | 0,01 – 0,1 | 2375 |
| diquat | potato | HERB | 0,01 – 0,1 | 2355 |
| dodine | apple | FUNG | 0,01 – 0,1 | 2207 |
| mancozeb | potato | FUNG | 0,001 – 0,01 | 1770 |
| thiram | pear | FUNG | 0,01 – 0,1 | 1269 |
| thiram | greenhouse veg. | FUNG | 0,1 - 1 | 1077 |
| linuron | potato | HERB | 0,01 – 0,1 | 1024 |
| fluazinam | potato | FUNG | 0,001 – 0,01 | 923 |
| ziram | apple | FUNG | 0,1 - 1 | 906 |
| dithianon | apple | FUNG | 0,01 – 0,1 | 797 |
| carbendazim | apple | FUNG | 0,01 – 0,1 | 741 |
| deltamethrin | potato | INSE | 0,01 – 0,1 | 734 |

## The Expo$_{consumers}$ indicator

### Indicator features

This indicator was developed by CODA-CERVA in the project named "*Exposure of Belgian adult consumers to pesticide residues through consumption of fresh fruits and vegetables*". Using recent national data both on residue concentrations in foodstuffs and on dietary consumption, the EXPO$_{consumers}$ indicator provides an accurate estimation of consumers' exposure. Risk for consumers is therefore given by comparing this exposure to long-term toxicological endpoint. The lower the ratio exposure/toxicological endpoint will be, the greater food safety will be guaranteed.

Raw data on pesticide residue concentration were provided for the year 2005 by the Federal Agency for the Safety of the Food Chain [5], whereas raw data on food consumption were provided for the year 2004 by the Institute for Public Health (IPH)[6].

### *Indicator calculations*

Concerning pesticides, 200 different pesticide residues were sought on 1322 samples of fresh and frozen fruit and vegetables. During the year 2005, no less than 134940 combinations residue/commodity were analyzed by the FASFC [5].

Related to dietary consumption, the Food Consumption Study[5] performed by the IPH in 2004 gives precise data on foodstuff consumption in Belgium. In total, 3214 Belgian citizens over 15 years old were questioned two times about their last-24-hours-

[5] THE COMPLETE STUDY IS AVAILABLE IN FRENCH (HTTP://WWW.IPH.FGOV.BE/EPIDEMIO/EPIFR/FOODFR/TABLE04.HTM) AND IN DUTCH (HTTP://WWW.IPH.FGOV.BE/EPIDEMIO/EPINL/FOODNL/TABLE04.HTM)

consumption. The selection of interviewed people and the moment of the interview were chosen in order to gather a databank reflecting yearly representative consumption of the Belgian population [6].

Thus, intake (or exposure) is equal to residue concentration in a commodity multiplied by the consumption of this commodity. Following a deterministic approach, the average of residue concentration in a commodity was multiplied by average or percentiles consumption, as presented in Figure 7. For a given combination residue Y/commodity Z, the residue concentration average value was multiplied by the average consumption as well as by different consumption percentiles. Since total exposure for the residue Y can be due to more than one commodity, total exposure for the residue Y was obtained by summing exposures from all combinations residue Y/authorized commodities.

Total Intake *residue Y* = ∑ Intakes from all *commodities*

Intake from the *commodity Z*
**=**
Average [residue Y] in *commodities Z* **X** Consumption of *commodity Z*

*Results*

In Table 4, we can compare these results (ranked by decreasing % of detection) with the data gathered by the FASFC during the sampling campaign in 2005.

A closer look to the Table 4 brings us to the issue of left censored data (i.e. high percentage of samples < Limit of Quantitation (LOQ). Since average values for residue concentrations in samples are calculated on a large amount of samples, the amount of samples in which the residue was not detected (ND) is an important factor. Indeed, if residue concentrations in samples < LOQ are estimated to be half of the LOQ, or at LOQ, the intake automatically rises even though residues are not found. Therefore, to avoid an underestimation of the intake, different hypotheses were made for the cases where residues were sought for but not found in tested samples (the fact that a residue is not found in a sample means that the residue is not present at a concentration above the analytical LOQ). Three scenarios were set out by three boundaries for all the cases of non-detection. The lower boundary presumed that the residue was absent in the non-detected samples. The second boundary considered that the residue was present at a concentration equal to LOQ/2, whereas the upper bound estimated the residue concentration reached the LOQ.

**Table 4. Data from the national sampling campaign for the 10 residues with the higher intakes in terms of % of ADI**

| A.s. | # of samples | % of detection | % of MRLs exceedings | Commodities |
|---|---|---|---|---|
| Chlorpropham | 104 | 41 | 2,9 | Potatoes, carrots, parsnip |
| Imazalil | 323 | 26 | 0,3 | Orange, mandarine, potatoes |
| Thiabendazole | 331 | 19 | 0,6 | Oranges, mandarine, banana |
| Dithiocarbamates | 857 | 17 | 0,6 | Potatoes, apples, tomatoes |
| Bromide ion | 376 | 16 | 1,1 | Tomatoes, lettuces, cos |
| Iprodione | 865 | 16 | 0,2 | Lettuces, apples, tomatoes |
| Dimethoat | 197 | 10 | 0,5 | Cherries, lettuces, onions |
| Procymidone | 651 | 9 | 0 | Tomatoes, kiwis, cos |
| Chlorpyriphos | 509 | 3 | 0 | Apples, tomatoes, oranges |
| Lambda-cyhalothrin | 826 | 2 | 0,1 | Apples, tomatoes, oranges |

The example given in Figure 6 shows that the intake of lambda-cyhalothrin exceeds the intake of chlorpropham for the high-bound scenario even though lambda-cyhalothrin was only found twice in the 826 samples in which it was authorized. On the contrary, when using the middle bound and the lower bound scenarios, the intake of lambda-cyhalothrin is, respectively, lower and much lower than that of chlorpropham. This “grey zone” (left censored data) is encountered by many risk assessors and some guidance exist in order to help the scientists in the data handling of such databases [7].

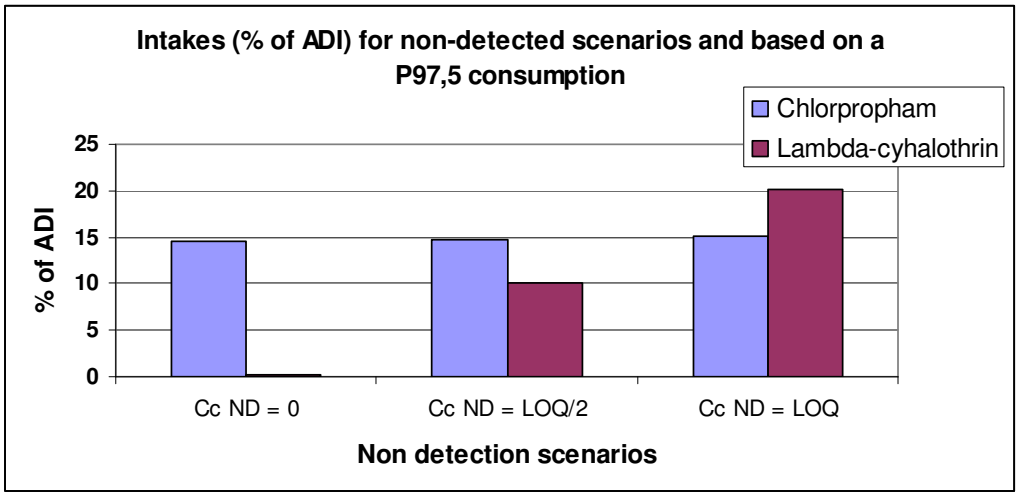


**Figure 6. Intakes based on a P97,5 consumption (% of ADI) for non-detected scenarios: lower bound (ND=0); middle bound (ND=LOQ/2) and upper bound (ND=LOQ)**

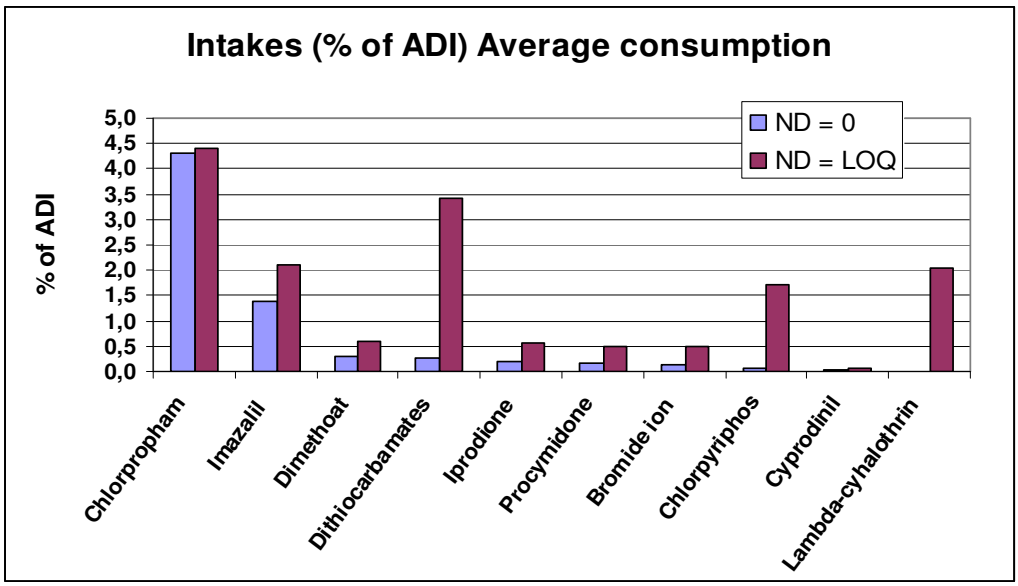


**Figure 7. Intakes for the low- and high- bound scenario expressed in % of ADI for 10 pesticides with the highest intake and according to an average dietary consumption (Deterministic approach; lower bound (ND=0) and upper bound (ND=LOQ))**

Calculations of intake were made on the 25 most often found residues in the sampling campaign achieved by the FASFC and on the 49 most consumed commodities (fresh fruits and vegetables) from the IPH database. From the results gathered, the 10 higher intakes in terms of % of ADI are given based on an average dietary consumption in Figure 7 and in Figure 8 based on a P97,5 consumption. Only authorized uses were taken into account for calculations. In order to be able to tackle the problems linked to the low detections, the results are shown either for the lower bound and for the upper bound scenario in figure 7 and figure 8.

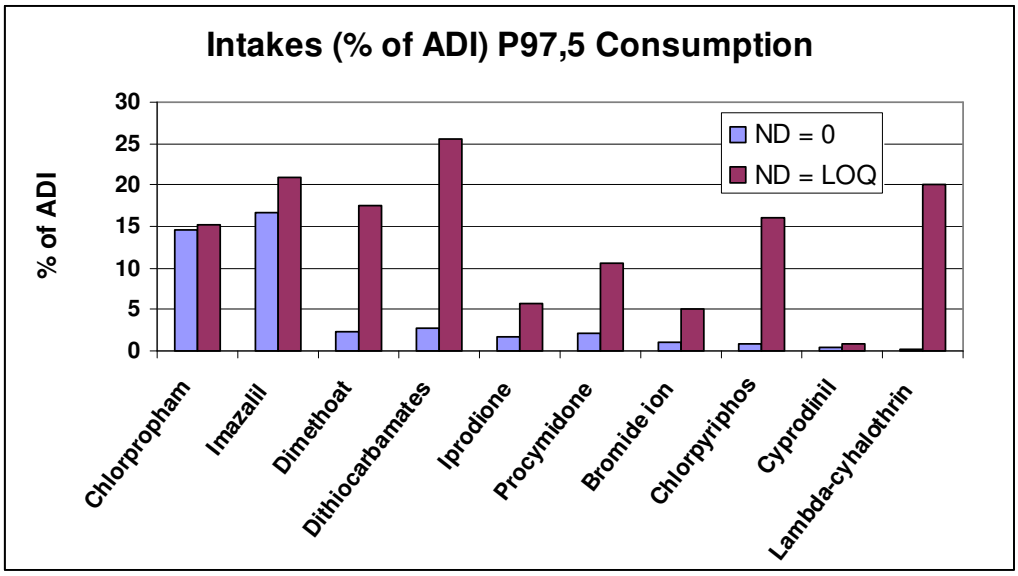


**Figure 8. Intakes for the low- and high- bound scenario expressed in % of ADI for 10 pesticides with the highest intake and according to a P97,5 dietary consumption (Deterministic approach; lower bound (ND=0) and upper bound (ND=LOQ))**

From all the results obtained it can be deduced that chronic intakes are rather low compared to the ADI (mostly < 1 % of ADI). In other words, intakes for the majority of the 25 residues analyzed in this project are 100 times lower than their ADI.
Public health in Belgium, based on the residue campaign of 2005 led by the FASFC, seems to be under control in terms of pesticide intakes. However, some residue should retain our attention. For a high consumer (P 97,5) the intake can reach 19% of the ADI for imazalil, 15% for chlorpropham, 14% for dithiocarbamates and 10% for lambda-cyhalothrin according to a scenario where the residue concentration of non-detected samples is equal to half the LOQ of the analytical method used (results not shown).

It is thus clear that paying attention only to MRLs exceedings (i.e. compliance of the foodstuffs with legal constraints) is providing only poor information in terms of food safety (see Table 4). Indeed, consumers' intakes of pesticide residues and the risk assessment linked to consumers' safety cannot rely only on MRLs exceedings. The approach followed in this project is useful to assess risk of the consumer on the basis of real exposure data. This is proving that MRLs exceedings are giving hints on residues and commodities to prospect but are not very useful when dealing about food safety.

## Comparison of The two indicators

The differences between the two indicators are summarized in Figure 1 and in Table 5. Since the two indicators are not grounded on the same approach and since calculations were performed using completely different databases, it is quite difficult to lead a comparison based on the results obtained. Nevertheless, it is important here to compare the type of results we can obtain with both indicators, the accuracy we can expect from them, and the behavior in terms of data handling.

**Table 5. Differences between PRIBEL and EXPO**

| | PRIBEL | EXPO |
|---|---|---|
| Formula | Exposure/endpointX Frequency of use | Exposure/endpoint |
| Input data | | |
| - *Dietary consumption* | GEMS/Food, Europe | Belgian data, IPH 2004 |
| - *Residue concentration* | MRL, worst-case approach | Annual sampling campaign, FASFC |
| Scope | | |
| - *Situation in food chain* | Only pre-harvest pesticide applications | Pre-harvest and post-harvest pesticide applications |
| - *Type of information provided* | Residue production potential for agricultural food and feed products produced in Belgium | Residue intake potential for foodstuffs consumed in Belgium (local production + imports) |
| Comparison in time | Need of yearly national sales figures | Need of yearly sampling campaign |
| Updates | Endpoints (ADI) and regulation (MRL) | Endpoints (ADI), performance of analytical methods (LOQ) and regulation (MRL) |

With PRIBEL*consumers*, the risk obtained through calculations is the amount of risk that is prevalent ? in Belgium at a given moment. The fact that the frequency of use is taken into account leads to high PRIBEL values for crops widely produced. This is the case with winter barley which encompassed 33% of the risk for consumer in Belgium in 2001. Although PRIBEL values are high for application cases involving winter barley, it is not appropriate to consider that consumers will be exposed to such a risk after crop harvesting. Indeed, the link between risk production regarding consumers and the real exposure is far from being evident. The frequency of use is relevant to estimate the amount of risk produced, but less relevant to estimate the real exposure for consumers.

With EXPO*consumers*, national data concerning residue concentration and dietary consumption are used for calculations. Exposure is obtained from residue concentrations as they are found in foodstuffs on the Belgian market. Dietary consumption is given for a representative sample of the Belgian population and for a wide range of commodities. Therefore, it is also possible to calculate consumption percentiles. The indicator did not target other foodstuffs than fresh fruits and vegetables. Results are underestimating the total exposure but nonetheless it is covering all the most relevant commodities in Belgium regarding pesticide residues.

Looking further to the results obtained with the 2 indicators, the following observations can be made about the pesticide residues present specifically in fruits and vegetables

(including potatoes). It seems that the pesticides identified as more risky are quite different when one or the other indicator is selected. Indeed, with PRIBEL, sulphur and copper hydrochloride in apples as well as thiram in fruit and greenhouse vegetables are by far the most risky pesticides in terms of residues produced in Belgium while in terms of exposure via consumption of fruits and vegetables, chlorpropham, imazalil, dithiocarbamates, dimethoate, lambda-cyhalothrin and chlorpyriphos bear the greatest risk. Chlorpropham and imazalil are post-harvest pesticides and, hence, are not taken into consideration with the PRIBEL approach. On the other hand, thiram belongs to the group of dithiocarbamates and, as such, is identified as more risky by the two approaches. The same is true for mancozeb and ziram that are also dithiocarbamates. As far as sulphur and copper are concerned, it is worth to mention that they are not mentioned in the results of monitoring programmes.

## CONCLUSIONS AND RECOMMENDATIONS

It has been seen in this chapter that indicators can provide us different type of information. According to each indicator, results concerning either exposure or risk production can be obtained. For an appropriate risk assessment, it is important to choose an indicator which allows to asses if consumers are at risk. In terms of data handling, both indicators need a yearly update of toxicological endpoints and legal applications.

For the Belgian situation, the indicator EXPO*consumers* seems appropriate to tackle the issue of pesticide residue exposure. Input data used in calculations are close to real national data that consumers encounter in the everyday life. Dietary consumption data are not supposed to change for the following years as dietary habits should not differ drastically among the population. Besides, data from pesticide residue campaign could be used every year in order to monitor trends followed by the indicator. The impact of a governmental policy could be measured by this mean.

According to the results obtained with this indicator, consumers' exposure has been found under the toxicological endpoints even at high consumption percentiles. Nevertheless, the dietary consumption provided by the IPH did not include data for children under 15 years old. A special attention could be brought on this sensitive group in further research.

For both indicators, improvement can be done to gain more accuracy. For example, processing factors that are known to reduce the residue concentration in foodstuffs are not taken into account in our calculations. Another improvement concerns the addition of adverse effects induced by pesticides. Indeed, different pesticide groups have the same adverse effects on human health. It would be interesting to take account of this property into the risk assessment.

*Acknowledgement*
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Belgian agriculture, consumption patterns of the Belgian population and toxicological endpoints.

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# Chemical contaminants, sustainable agriculture and food safety

# Food Contaminants and Sustainable Farming Systems: A review for Belgium

**L. Pussemier[1] and Y. Larondelle[2]**

*[1] CODA-CERVA, Leuvensesteenweg 17, B 3080 Tervuren, Belgium*
*[2] Institut des Sciences de la Vie & Université catholique de Louvain, Croix du Sud 2/8, B 1348 Louvain-la-Neuve, Belgium*
*e-mail: Lupus@var.fgov.be*

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Sustainable farming; Organic agriculture; Pesticides; Mycotoxins; Environmental contaminants

## SUMMARY

This paper presents an overview of the main results gathered on the food contamination issues in the framework of sustainable farming systems. Special attention has been paid on the results obtained within several projects carried out in Belgium. First, various sustainable (certified) production systems are presented with special emphasis on their importance in the Belgian context. Next, the results of some studies related to food contamination by pesticides and fertilizers, by natural toxins and by environmental contaminants are summarized. Finally, a comparative risk assessment exercise has been carried out on cereals produced in Belgium in order to compare, on the one hand, the conventional and the organic farming systems, and, on the other hand, several chemicals belonging to different classes of food contaminants (pesticides, mycotoxins and environmental contaminants).

## INTRODUCTION

The concept of sustainable development has grown steadily and significantly during the last years in Belgium and elsewhere [1]. In some parts of Belgium, agriculture is a very intensive sector. In Flanders, for example, very intensive techniques were developed during the last decades for the growing of horticultural and fruit crops as well as for animal husbandry (large facilities for the fattening of pigs and chickens, for laying hens and dairy cows,…). The effects of such intensive farming systems on the quality of the environment and on the quality and safety of the produced foodstuffs is regularly questioned not only by environmental and consumers' organizations but also by scientists and policy makers. In particular, the issues of chemical contamination of the environment and of the food chain by several types of chemical residues such as pesticides and fertilizers are regularly put forward.

Actually, several classes of food contaminants can be distinguished according to the nature of the process leading to their introduction in the food chain. Some classes of contaminants are linked to the crop management practices (pesticides and fertilizers), to the environmental exposure of the crop (dioxins, heavy metals), to stress factors and

diseases affecting the crop (plant and fungal toxins), to bad storage conditions (fungal toxins), to the use of additives, flavors and dyes (miscellaneous chemical compounds), to food processing (furans, acrylamide, benzene), to the release from packaging and cooking materials (heavy metals, phtalates, bisphenol A) (see [2] for a complete review of food contaminants in conventional and organic agriculture and [3] for a more specific analysis under Belgian conditions). In this paper, we will focus on the food contaminants that are more closely associated to the farming systems, i.e. the group of pesticides and fertilizers, the group of plant and fungal secondary metabolites (phytotoxins and mycotoxins) and the group of environmental contaminants. The main objective of this paper is to have an insight on the impact of the farming systems (and particularly the so-called "sustainable" production systems such as organic agriculture) on food contamination by chemicals.

## ORGANIC AGRICULTURE, LABELLED PRODUCTION SYSTEMS AND CONVENTIONAL AGRICULTURE

Organic agriculture is one of the strongest reactions to intensive agriculture. By putting forwards the need to respect natural equilibrium and by imposing a total ban on the use of synthetic fertilizers and pesticides, this farming system aims at presenting a rather drastic alternative to "conventional" farming. Therefore, despite a significant growth during the nineties, the market for organic agriculture showed some stagnation afterwards [4] and remains marginal with respect to the number of farmers involved and the percentage of foodstuffs consumed by the Belgian population (Table 1). For all the country, there was less than 2% of total arable land devoted to organic agriculture in 2003 (up to 2.7% in the Walloon Region and only 0.5% in the Flanders Region) [4]. The share of consumed organic products was as high as 2.3% for Belgium in 2003. Globally, the Belgian demand is higher than what can be produced in the country and, hence, import is necessary to respond to the needs.

**Table 1. Importance and recent trends in organic agriculture in Belgium (source: www.bioforum.be)**

| | 1997 | 2000 | 2001 | 2002 | 2004 |
|---|---|---|---|---|---|
| Organic arable land (ha) | | | | | |
| Belgium | 6818 | 20205 | 22410 | 24874 | 23563 |
| Walloon Region | 5998 | 16872 | 18384 | 20995 | 20344 |
| Flanders Region | 820 | 3392 | 4026 | 3879 | 3219 |
| Number of organic farmers | | | | | |
| Belgium | 291 | 666 | 694 | 710 | 712 |
| Walloon Region | 184 | 435 | 441 | 459 | 481 |
| Flanders Region | 107 | 231 | 253 | 251 | 231 |
| Sales of organic products (million €) | | | | | |
| Belgium | 62 | 149 | nd | 311 | nd |

nd, no data

The price of organic products put on the market is significantly higher, as compared to the corresponding conventional products. This price premium is often as high as 50 % [5] and can even rise to 175 % [2]. This economic constraint is an important challenge for the further extension of the organic market [6]. Hence, besides organic farming, many other initiatives exist in Belgium in the field of sustainable farming systems. They are promoted

either by the producers and the auctions (e.g. “Charte Perfect” for vegetables, “Terra Nostra” for potatoes, “Fruitnet” for fruits) or by the retail sector (e.g. Flandria and EurepGap for fruits and vegetables), or even by the government (example IFP - Integrated Fruit Production, which is regulated by law). Many of these other initiatives towards more sustainable production methods arose quite recently. As for organic agriculture, they rely on certification systems and labeling strategies and, here also, the aim is to put forward more sustainable production methods, better quality and increased safety of their products, and less environmental burden. But, with these initiatives, the sector is not willing to radically modify the production methods by completely banning pesticides and fertilizers. The aim is rather to offer to the consumers some differentiation from the conventional products, and this is done by using improved farming systems compared to the legal standards. Economically, these farming systems are, by contrast to organic farming, very profitable because the price offered is competitive and, hence, the number of producers involved is much higher than in organic agriculture.

## PESTICIDES AND FERTILIZERS

Pesticides and fertilizers are chemical inputs that are typically applied in large amounts in intensive agriculture. Therefore, the standards of organic agriculture and other certified production systems have focused on these items in order to reduce their use as much as possible. Two kinds of studies were carried out in Belgium in order to analyze more in depth the effects of such measures on food safety. The first one is a theoretical approach and is based on the analysis of the rules set in the standards (technical specifications) of the certified production systems. The second approach is the experimental analysis and is devoted to the identification and quantification of pesticide residues in the foodstuffs produced.

### *Food safety scores for certified production systems.*

Garreyn and Steurbaut [7] have applied a method enabling the comparison of the technical specifications set for various certified production systems used in Belgium. Different aspects of sustainable development were taken into account including food safety. From the analysis of the various rules mentioned in the standards it appears that food safety plays a predominant role since 307 rules related to food safety have been identified. This is much more than for other aspects that have been considered in the study (water quality: 254 rules; biodiversity: 216 rules; worker safety: 168 rules, etc). The results of this comparison exercise are summarized in Table 2 for what concerns the food safety aspects. For more details on this study, we refer to the chapter written by Mondelaers et al. in this issue and to the full report [7] in which the adopted methodology, the results obtained and the meaning of the scores are documented with more details. From this study it appears that organic agriculture is not the sole farming system that could contribute to the improvement of food safety. The score for the organic system is obviously not the highest. This is due to the fact that the standards (technical specifications) of organic agriculture are not as detailed as in other certified systems such as for example “Charte Perfect”.

**Table 2. Food safety scores obtained for different certified production systems according to the study of Garreyn and Steurbaut [7]**

| Charte Perfect | Terra Nostra | EurepGAP | Flandria | FlandriaGAP | IFP* | Fruitnet | Organic |
|---|---|---|---|---|---|---|---|
| 71.03 | 40.58 | 52.6 | 42.53 | 55.53 | 39.59 | 54.13 | 42.69 |

* Integrated Fruit Production

*Analysis of databases for pesticide residues in food*.

A study has been carried out by de Voghel and Pussemier [8] using the database for pesticide residues in fruits and vegetables measured during the year 2005 by the Federal Agency for the Safety of the Food Chain (FASFC). In short, it can be said that 66 % of the samples contained pesticide residues but only 7.9 % of the samples were above the Maximum Residue Limits (MRL) set by the EU. It is also noteworthy that out of a total of more than 200 pesticides sought for in 50 different food items (or food groups), only 2 were found in the foodstuffs in amounts higher than 1 % of the Acceptable daily Intake (ADI) when average consumption of the concerned foodstuffs was assumed (deterministic approach). Thus only two post-harvest pesticides (chlorpropham, a sprouting inhibitor for ware potatoes, and imazalil, a fungicide for citrus protection) were found in significant amounts while the pesticides used under field conditions for plant protection were much less worrying in terms of residues left in foodstuffs. Such a picture is quite reassuring and is in accordance with what is found in other EU countries in the framework of the coordinated programme for the monitoring of pesticide residues (for more details, see [8]) and the chaper written by de Voghel and Pussemier in this issue). As to the cereal products, Harcz et al. [9] have shown from the data of FASFC that most of the pesticides used to control field pests (weeds, aphids, fungal diseases) were hardly found during the monitoring programmes carried out in 2003, 2004 and 2005. On the other hand, the post-harvest insecticides chlopyrifos-methyl, dichlorvos and pirimiphos-methyl could be found quite regularly with an average concentration of 37.7, 70.8 and 84.2 µg/kg of fresh material, respectively. Taking into account the diet habits of the Belgian population, it could be derived that the consumption of cereals (with the worst assumption that there were no losses of residues during processing) could lead to an average daily intake of 0.11 µg/kg bw for chlorpyriphos-methyl, 0.20 µg/kg bw for dichlorvos and 0.24 µg/kg bw for pirimiphos-methyl. In all cases this is largely less than 1 % of the TDI (see section Risk assessment). Some reviews about the presence of pesticide residues in organic and conventional agriculture have been recently published [3], [10-11]. From the data summarized in Table 3, the situation observed in Belgium (retail sector) can be compared to the results published by a number of US organizations. Overall, it can be said that the rate of residue detection is 3 to 4 times higher in conventional foodstuffs. It is noteworthy to mention that the residues found in organic foodstuffs were mostly natural pesticides that are allowed in organic agriculture (sulfur, pyrethrum) as well as the synthetic synergist piperonyl butoxide [3]. In the US studies, a large number of positive organic samples (nearly half of them) were found to be contaminated by organochlorine insecticides. The latter were banned several decades ago, and can thus be considered as environmental contaminants.

**Table 3. Detection of pesticide residues according to various monitoring programmes [3, 10]**

| Monitoring program | Samples with residues detected (%) | |
|---|---|---|
| | Conventional | Organic |
| USDA | 73 | 23 |
| California dept Pesticide Regulation | 31 | 6.5 |
| Consumers Union (USA) | 79 | 27 |
| Belgium (retail stores) | 49 | 12 |

Concerning the other certified production systems, available data are very scarce. According to Baker et al. [10], it seems that less pesticide residues are also found in foodstuffs with market claims other than organic (example, Integrated Pest Management). Unfortunately, in the Belgian databases, no information is available on the farming system (conventional, organic or other certified production systems). Therefore, it should be very useful to further study the effect of the most important certified production systems (Charte Perfect, Flandria, Fruitnet, IFP and Organic) on the actual presence of pesticide residues in the concerned foodstuffs.

*Analysis of databases for nitrates in food.*

As far as nitrates are considered in conventional and organic foodstuffs, one can mention the review studies from Worthington [12] and from Woese et al. [13] from which can be derived that organic foodstuffs contain generally less nitrates. In Belgium, it has been reported that broad leaved vegetables sold in large retail stores showed a mean nitrate value of 1703 mg/kg for organic products versus 2637 mg/kg for their conventional counterparts whilst, for potatoes, there were no significant differences [3].

## NATURAL TOXINS

*Mycotoxins in organic and conventional foodstuffs*.

The presence of mycotoxins in organic food has been debated in many studies. Mycotoxins are fungal secondary metabolites that can be produced either under field conditions (example, the Fusarium toxins deoxynivalenol (DON), zearalenone (ZEA), fumonisins (FB1, FB2, FB3) and T-2 and HT-2 toxins) or during the storage of the harvested products (example, ochratoxin A (OTA) and patulin (PAT)). They can exhibit a large array of acute and, more worrying, chronic effects (carcinogenicity, nephrotoxicity, immunosuppression, hormonal disorders, etc). Many agricultural practices (including the use of pesticides) can influence the mycotoxin contents in foodstuffs and, hence, it is very important to have a clear picture on the mycotoxin contamination potential of both farming systems. A large scale study has been carried out in Belgium on cereals and cereal derived products [14]. From that study, it appears that a foodstuff like beer is more prone to OTA contamination when produced according to the organic system [15]. For other foodstuffs such as wheat grains and polenta, there is a trend towards more severe contaminations of conventional products by DON and ZEA [16]. Finally, whole wheat flour can be severely contaminated by DON when produced according to the conventional system, and by OTA when produced according to the organic standards. Table 4 gives a summary of the main results gathered during this study.

**Table 4. Summary of the mycotoxin contamination study carried out in Belgium (2002-2004) [14]**

| Myco-toxin | Wheat cereals | Whole-wheat flour | Beer | Whole-wheat pasta | Corn-flakes | Polenta | Sweet corn |
|---|---|---|---|---|---|---|---|
| OTA | nd | CP<br>OR > CV | CP<br>OR > CV | nd | nd | nd | nd |
| FB1 | nd | nd | No<br>OR = CV | nd | PA<br>OR > CV | CP<br>OR < CV | No<br>OR = CV |
| FB2 | nd | nd | No<br>OR = CV | nd | PA<br>OR > CV | CP<br>OR < CV | No<br>OR = CV |
| FB3 | nd | nd | nd | nd | PA<br>OR = CV | nd | nd |
| DON | CP<br>OR < CV | CP<br>OR < CV | No<br>OR = CV | PA<br>? | nd | nd | nd |
| ZEA | CP<br>OR < CV | nd | nd | nd | nd | nd | nd |

*nd, no data;*
*CP, the foodstuff presents a Critical Point for contamination by the specified toxin;*
*PA, the foodstuff presents a Point of Attention for contamination by the specified toxin;*
*No, No problems awaited in this foodstuff for the studied mycotoxin;*
*OR > CV, organic tends to be more contaminated;*
*OR < CV, conventional tends to be more contaminated;*
*OR = CV, organic and conventional show similar contamination potential.*

In the case of beer, a more thorough study on the intake of DON and OTA by the Belgian beer consumers has been carried out using a deterministic approach [17] (Harcz et al., 2007). The results are presented in Figure 1. Taking into account the new TDI set by EFSA for OTA, which is more than 3 times higher than the previous one (17.1 ng/kg bw instead of 5 ng/kg bw), it appears that only heavy consumers of organic beer are exposed to significant OTA intakes (example 11.23% of the TDI at percentile 99). This might be less worrying but, still, one should not forget that beer is not the sole source of intake for this toxin. Hence, it is important to continue controls of OTA in beer (especially organic) in order to lower as much as possible the levels of this contaminant.

Concerning foods other than cereal derived products, it is worth mentioning the studies carried out on PAT in apple juice [18-19] showing that the organic mode of production leads to higher contamination levels with, occasionally, non-compliant samples.

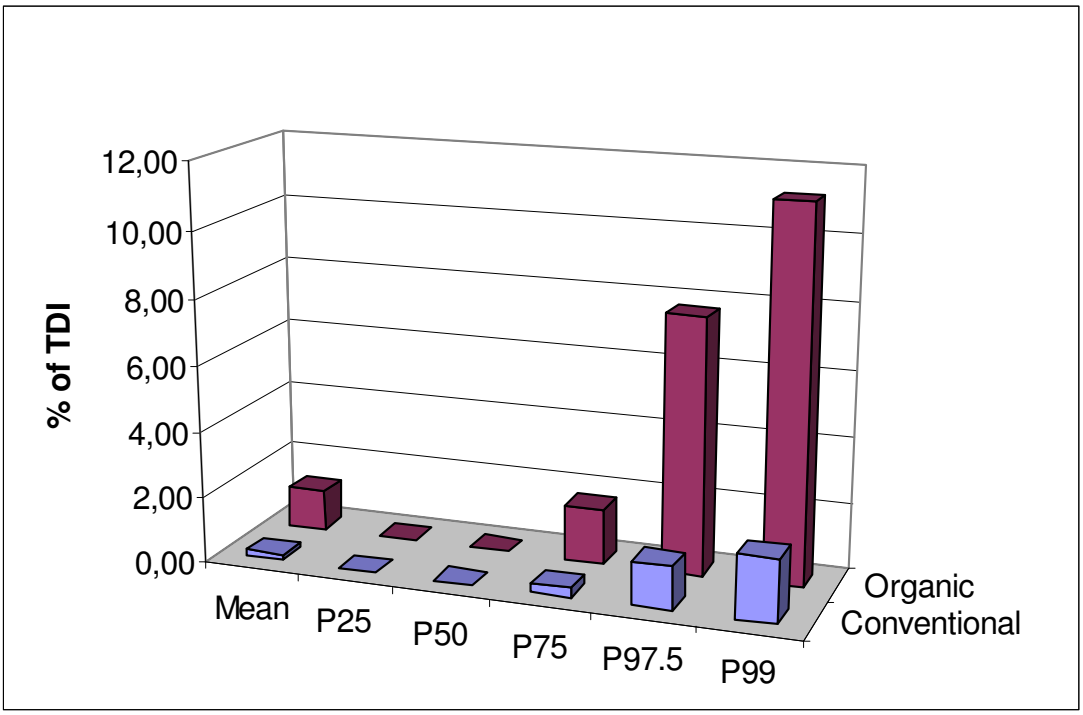


**Figure 1. Intake of OTA (in % of TDI – 17.1 ng/kg bw) via consumption of organic and conventional beer in Belgium [16]**

*Plant toxins*.

Regarding natural toxins or secondary plant metabolites, very little is known to date specifically for Belgium (see [2-3] for general reviews). It has to be reminded that potatoes can contain high amounts of toxic glycoalkaloids when damaged or exposed to light. In addition, selection for insecticide resistance has also led to higher glycoalkaloid contents. The same has also been reported for celery plants that may present high levels of linear furanocoumarins (with dermatitis and carcinogenic properties). It seems, thus, that agricultural practices should avoid any stress to the plants by keeping a balanced hydric and nutrient status and by providing protection against insect attacks and diseases [11]. Moreover, new cultivars arising from the breeding programmes should be carefully examined (even under stress conditions) as far as their toxin production potential is concerned. In addition, it has been shown, with experiments led in Belgium and elsewhere, that atmospheric pollutants such as $O_3$, $CO_2$ and $SO_2$ are able to influence the quality of several plant species such as, for example potatoes and brassicas, with important modifications in the profile of defense chemicals, vitamins, antioxidants, etc [20-22].

## ENVIRONMENTAL CONTAMINANTS

Regarding the environmental contaminants, it has to be pointed out that too little information is currently available. Few studies have mentioned the possible higher contaminant levels in extensive farming systems (for a review on this topic, see [3]) but,

until now, the available studies are still very scarce and limited to some issues such as eggs produced by free range hens and chemical pollution nearby industrial sites.

*Free range eggs*.

It has been shown that eggs from free range hens owned by private breeders close to the city of Antwerp contained much higher dioxin levels (on average 10 pg TEQ/g fat) than those produced in either organic or conventional commercial systems (see figure 2) [23]. Actually, all the samples coming from private owners were above the maximum level set by European Commission Regulation N° 1881/2006 for hen eggs and egg products. Qualitatively, there is a remarkable similarity between the dioxin profiles of egg and soil samples, indicating that the environment might be the main source of contamination. Daily intake of eggs with high contamination levels will increase the risk of exceeding the proposed maximum weekly intake for dioxins and furans of 14 pg TEQ per kg body weight. A quick risk assessment carried out under Belgian conditions partly confirmed these conclusions [23]. A deterministic approach, indeed, led to the conclusion that the mean exposure of consumers of home-produced eggs increases by 30%, as compared to the general population (1.31 versus 1.02 pg TEQ / kg bw). In addition, it was shown using a probabilistic approach that the contribution to the body burden of such home-produced eggs might be as high as that of milk or fish, for heavy consumers of those foodstuffs, and thus the risks of exceeding the proposed tolerable intake will be very high. The situation might even be worse, when assuming a doubling of the TEQ value due to the expected presence of dioxin-like PCBs.

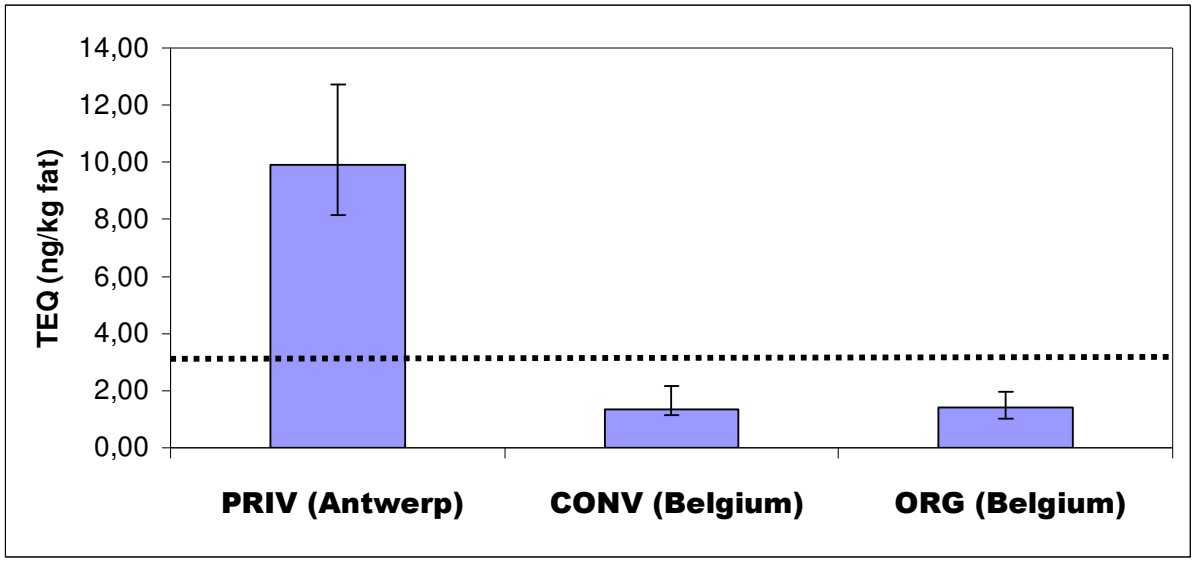


**Figure 2. Dioxin levels in eggs from private owners (PRIV) from the province of Antwerp compared to eggs from conventional (CONV) and organic (ORG) commercial farms in Belgium (the doted line represents the maximum level according to EC N° 1881/2006)**

Further studies have shown that the problem was not limited to the area of Antwerp neither to the group of dioxin contaminants because other environmental contaminants (namely, other POPs (Persistent Organic Pollutants) such as PCBs and pesticides as well as heavy metals) could also be found in higher concentrations in eggs from private owners all over Belgium. This was especially the case for elements like Pb, Tl and Hg (figure 3) and for some POPs like the group of DDT and its metabolites, the group of dioxins and furans, and the group of PCBs (either markers or dioxin-like) (figure 4) [24].

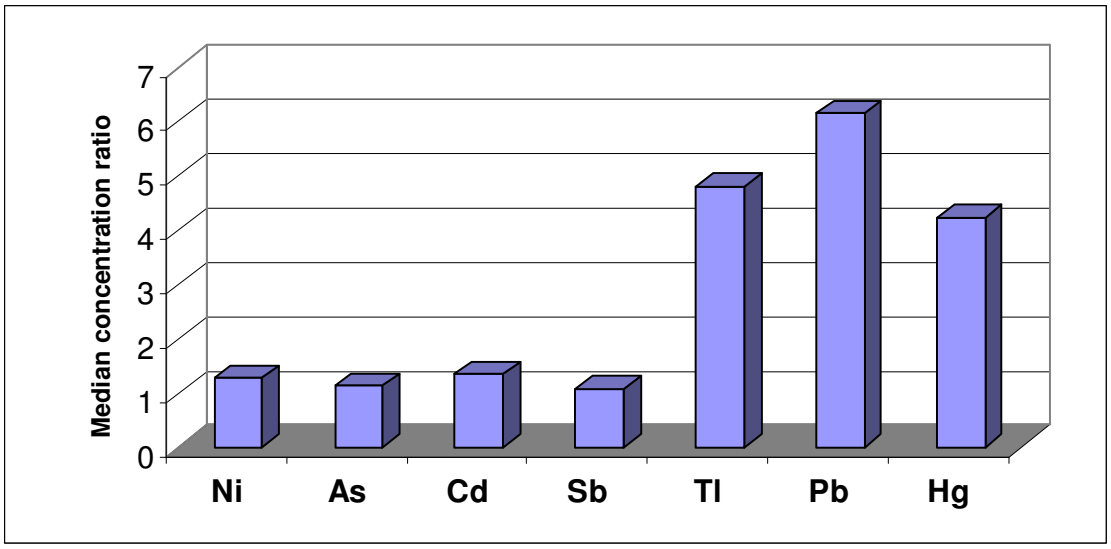


**Figure 3. Median concentration ratios (Private Owners : Commercial Farms) for toxic elements in free range eggs**

The origin of enhanced concentrations in free range hens is not obvious. Very probably non-compliant levels refer to a set of factors such as inappropriate feeding (intake of large amounts of contaminated soil, soil-borne organisms, spoiled feed,…) and poor layer breeding management (aged birds, decreased egg production when compared to farm hens). Further studies are needed in order to assess the real impact of the intake of such home-produced foodstuffs on the consumers' body burden for POPs and heavy metals.

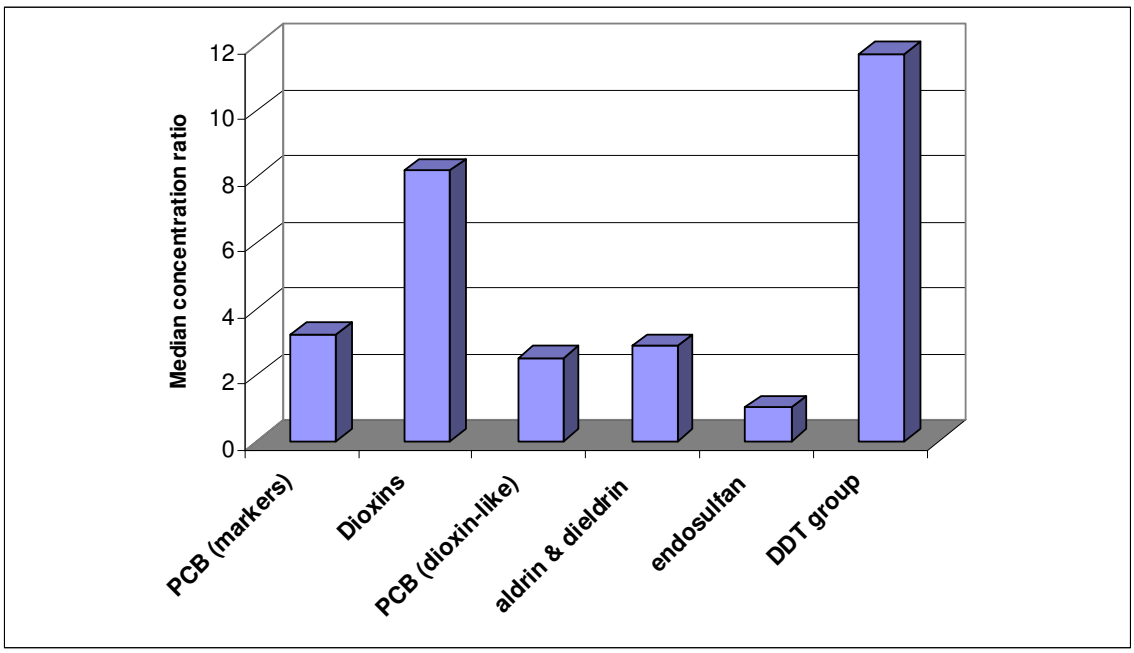


**Figure 4. Median concentration ratios (Private Owners : Commercial Farms) for Persistent Organic Pollutants (POPs) in free range eggs**

*Polluted sites.*

The local environment is also known to influence the heavy metal levels in several kinds of vegetables (especially Cd in carrots and Pb in cabbage) and in some animal products (Cd and Pb in offal and, in a lesser extent, in meat from cattle reared under outdoor conditions). This has been demonstrated clearly in Belgium for foodstuffs (potatoes, vegetables, and animal products) produced in historically polluted industrial areas [25]. POPs are also to be taken into account since PCBs and dioxins were found in animal products reared in sites close to hot spots of contamination [26-27]. Here again, more studies are needed in order to better assess the impact of locally produced food on human health.

## AN EXAMPLE OF COMPARATIVE RISK ASSESSMENT: CONTAMINANTS IN ORGANIC AND CONVENTIONAL CEREALS

In every farming system, contaminants such as pesticides, fertilizers, toxins, environmental pollutants may find their way in the food chain. Pesticides and fertilizers are more frequently present in conventionally produced foodstuffs but naturally occurring toxins and environmental pollutants can also be present in certified production systems including organic farming.

Even when there is evidence that a hazard is present it is still necessary to examine whether this hazard can present a risk or a threat, in other words if the measured levels are high enough to produce any harmful effect. Moreover, if different hazards are present

together, it is important to be able to make prioritizations and to identify those which are really threatening the health of the consumer.

In this section we will present an example of risk assessment for some specified contaminants (pesticides, mycotoxins and heavy metals) in a given set of foodstuffs (cereals and cereal derived products). The aim is to compare the risks due to several kinds of contaminants that can be present in both conventional and organic farming systems.

**Table 5. Contaminant intakes (in % of TDI/ADI) for contaminants of cereal products according to consumers dietary habits (mean, median and percentile 97.5) for cereal products and assuming that the levels found in unprocessed cereals remain unchanged in consumed foodstuffs [9]**

| Contaminant group | Chemical species | Organic products | | | Conventional products | | |
|---|---|---|---|---|---|---|---|
| | | mean | median | p97.5 | mean | median | p97.5 |
| Mycotoxins | DON | 56 | 53 | 111 | 99 | 94 | 197 |
| | ZEA | 16 | 15 | 31 | 33 | 31 | 64 |
| Environmental contaminants | Cd | 19 | 18 | 37 | 17 | 16 | 34 |
| | Pb | 7,9 | 7,4 | 16 | 3,3 | 3,1 | 6,6 |
| | Hg | 0,1 | 0,1 | 0,2 | 0,1 | 0,1 | 0,2 |
| Pesticides | Chlorpyriphos | ND* | ND | ND | 1,1 | 1 | 2,1 |
| | Dichlorvos | ND | ND | ND | 5 | 4,8 | 9,8 |
| | Pirimiphos | ND | ND | ND | 6 | 5,5 | 11,8 |

* ND, No Data

Mycotoxins that are not perceived as worrying by consumers can be present in both organic and conventional unprocessed cereals at levels that could potentially reach a significant portion or even exceed the TDI for average and high consumers, respectively. Environmental contaminants such as Cd and Pb can be present in unprocessed cereals whatever the farming system and the exposure via cereal product consumption can reach, respectively, one third or one sixth of the TDI for 97.5th percentile consumers.

In contrast to the risk perception by consumers, pesticides seem not to be the class of contaminants bearing the highest risk, as far as the potential exposure can be compared to the ADI (see Table 5). It is noteworthy, however, that post-harvest treatments in cereals, such as insecticide applications, may potentially induce a significant risk (up to 11.8 % of the ADI for the 97.5$^{th}$ percentile consumers, not considering the effect of cereal processing) while field treatments seem to play a negligible role (very few residues detected).

When comparing organic and conventional products, care should be taken when analysing the results. On the one hand, the most common pesticides (post-harvest insecticides) are regularly detected in conventional unprocessed cereals while they are not allowed in organic products. On the other hand, mycotoxins such as DON and ZEA are present in important amounts in both farming systems and Cd as well as Pb can be present in significant amounts too. Of course, this assessment is based on contaminant levels in unprocessed cereals and it is known that, after processing, the cereals (e.g. flour) will contain lower amounts of them. Still, one must be aware that more and more people give their preference to cereal products that are not or less processed (whole meal bread, for example) and, hence, it should be recommended to further assess the exposure towards

these contaminants by considering cereal and cereal based products as they are found in the shelves of the stores by the consumers of conventional and organic foodstuffs.

## CONCLUSIONS AND RECOMMENDATIONS

Belgium, like other countries, has seen the development of several farming systems with organic farming at one end and conventional farming at the other. The safety issues regarding the products generated by these different farming systems are not sufficiently documented. The few studies carried out during the last decade in Belgium have however allowed showing a great difference between the perceived risk and the actual risk in terms of food consumption. Consumers are very much worried about chemical residues resulting from some farming systems but are much less aware of the dangers originating from the environment (heavy metals, POPs) or from the biological material itself (plant toxins, mycotoxins). This is in deep contradiction with the results of risk assessment procedures, which indicate, in most cases, a much greater risk associated to the environmental contaminants and to natural toxins. Starting from that, it clearly appears that the safety issues should be investigated separately for each type of product. This paper has indeed indicated that the appropriate level of concern regarding the safety of foodstuffs largely depends on the type of matrix and on the type of contaminant and not primarily on the farming procedure chosen.

There is a need for more accurate food safety assessments for most of the available foodstuffs, regardless of the production system concerned. More numerous evaluations of the contamination levels are requested. Additional investigations are also needed to adjust the safety levels on the basis of mechanistic data with contaminants alone or in combination. It is indeed of paramount importance to assess food safety taking into account the whole panel of contaminants that may be present (either xenobiotic and natural substances) and the multiple effects that can occur when these contaminants are simultaneously present in the diet.

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# Emerging methods for mycotoxin analysis in food and feed

**S. Monbaliu[1], M. Lobeau[1], I.Y. Goryacheva[1,2], C. Van Peteghem[1] and S. De Saeger[1]**

*[1]Ghent University, Laboratory of Food Analysis, Harelbekestraat 72, B-9000 Gent, Belgium.*
*[2]Department of Common and Inorganic Chemistry, Chemistry Faculty, Saratov State University, Astrakhanskaya 83, 410012 Saratov, Russia.*
*e-mail: sarah.desaeger@ugent.be*

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## INTRODUCTION

Mycotoxins are a class of highly toxic compounds, secondary metabolites, produced under particular environmental conditions by several heterotrophic micro-organisms, fungi or moulds, developing in many foodstuffs. Their presence depends on several factors, such as: fungal strain, climate and geographical conditions, cultivation technique and foodstuff conservation. Mycotoxins may occur in various vegetal products, such as cereals, dried fruits, coffee beans, cocoa and beverages, such as beer and wine. Ingestion of these toxins can cause acute or chronic toxic effects (a.o. carcinogenic, teratogenic) in animals and humans. Because of these health risks, monitoring of mycotoxins in feed and food is necessary [1]. Today approximately 400 secondary metabolites with toxigenic potential produced by more than 100 moulds have been reported. Trichothecenes, fumonisins, aflatoxins, *Alternaria* toxins, zearalenone (ZEA), patulin and ochratoxin A (OTA) are the main representatives [2].
For mycotoxin analysis, two main groups of methods exist: laborious methods for determination of mycotoxins with high sensitivity and precision, and screening methods for rapid detection in a non-laboratory environment. The first group of methods is particularly represented by liquid chromatography in combination with mass spectrometry or fluorescent detection. Absolute leaders in the second group of analytical methods for mycotoxins are immunochemical methods with high sensitivity and selectivity provided by specific antibodies [3-14]. Immunomethods for rapid detection can be either instrumental or non-instrumental. Examples of instrumental immunomethods are immunosensors, fluorescence polarization immunoassays (FPIAs), capillary electrophoretic immunoassays (CEIAs). Immunosensors are devices based on the detection of analyte-antibody interactions. When biological molecules specifically interact, changes in physicochemical parameters are generated and are electronically sensed. To transform this interaction to an analytical signal suitable for analyte concentration measurement, three main groups of sensors have been developed: luminescent/colorimetric sensors, surface plasmon resonance sensors and electrochemical sensors. FPIA is a homogeneous technique, based on differences in polarization of the fluorescence-labeled compounds (tracers) in the free and bound fractions. It involves the competition between free analyte and tracer for binding to a specific antibody. CEIA is also a homogeneous method and allows

combining separation of analytes from each other and from sample matrix, with high specificity of antibodies and sensitive detection based on laser induced fluorescence.
In this paper we focalize on the emerging analytical technologies such as liquid chromatography tandem mass spectrometry and rapid non-instrumental immunochemical methods.

## LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

The development of new technologies for mycotoxin analysis has been directed to simplifying the analytical procedure and increasing the sensitivity and specificity. Chromatographic methods are powerful laboratory techniques to separate mixtures of chemical compounds. The separation of the target molecule(s) in the sample is based on the affinity difference between the mobile phase and the stationary phase. Gas chromatography (GC) and high performance liquid chromatography (HPLC) became increasingly popular, because they are sensitive, selective, precise and accurate [7]. HPLC, however, has acquired a role of growing importance in food analysis overcoming the traditional drawbacks of GC regarding volatility and thermal stability. There are a large number of different instruments for identifying the compounds separated by HPLC including the ultra-violet (UV), the diode array and the fluorescence detector. Although frequently used they all have limitations caused by the necessity of the presence of a chromophore or a fluorophore. Derivatisation of the target component can solve this problem. Recent advances have led to the coupling of mass spectrometric (MS) instrumentation with liquid chromatography (LC). LC-MS is now commonly accepted by authorities as a highly reliable analyte confirmation tool and has become a routine technique in food analysis. This technical and instrumental progress had also an increasing impact on the expanding field of mycotoxin analysis owing to their robustness, easy handling, high sensitivity and their compatibility with almost the whole range of compound polarities [2].
Recently, tandem mass spectrometry (MS/MS) has been proposed for surveillance purposes. LC-MS/MS consists of a HPLC system with two stages of mass analysis. This detection system allows obtaining a mass spectrum resulting from the decomposition of an ion selected in the mass analyzer. Mass spectrometry is a very useful technique for identification and quantification. Molecules have distinctive fragmentation patterns which provide structural information to identify chemical components. In the mass spectrometric process different steps can be distinguished. The first step takes place in the ion source where the target molecule(s) of the sample enter(s) and ions are formed. Then the formed ions are transferred into the mass filter. Mass spectrometers use the difference in mass-to-charge ratio (m/z) of the ions to separate them from each other. Different mass analyzers exist but for mycotoxin analysis the most important ones are the triple quadrupole and the ion trap.
A schematic overview of the triple quadrupole is shown in Figure 1. The first mass analyzer acts as a mass filter. It filters the formed ions according to their mass to charge ratio (m/z) by a quadrupole. In the next step the mass separated ions pass into the hexapole collision cell where they undergo collision induced decomposition. The fragmented ions are filtered in the second mass analyzer. Finally the selected fragment ions pass into the detection system where the signal is amplified, digitised and presented to the data system.

An ion trap can be considered as a 'three dimensional quadrupole' in which the ions of all masses are trapped on a three-dimensional trajectory. In quadrupole instruments, as described above, the potentials are adjusted so that only ions of a selected mass go through the rods. Here a different principle is used: ions with different masses are present together inside the trap. A schematic presentation of an ion trap is shown in Figure 2. The general sequence to perform tandem mass spectrometry in an ion trap is as follows. In a first step ions are selected of one mass-to-charge ratio by expelling all the others from the ion trap. Then energy provided by collisions causes fragmentation of the selected ions. And finally the fragmented ions are analyzed by expelling these of a selected mass-to-charge ratio. A special feature of an ion trap is the possibility to provide $MS^n$ (n times MS) spectra by selecting a fragment ion in the ion trap and let it further fragment [15].

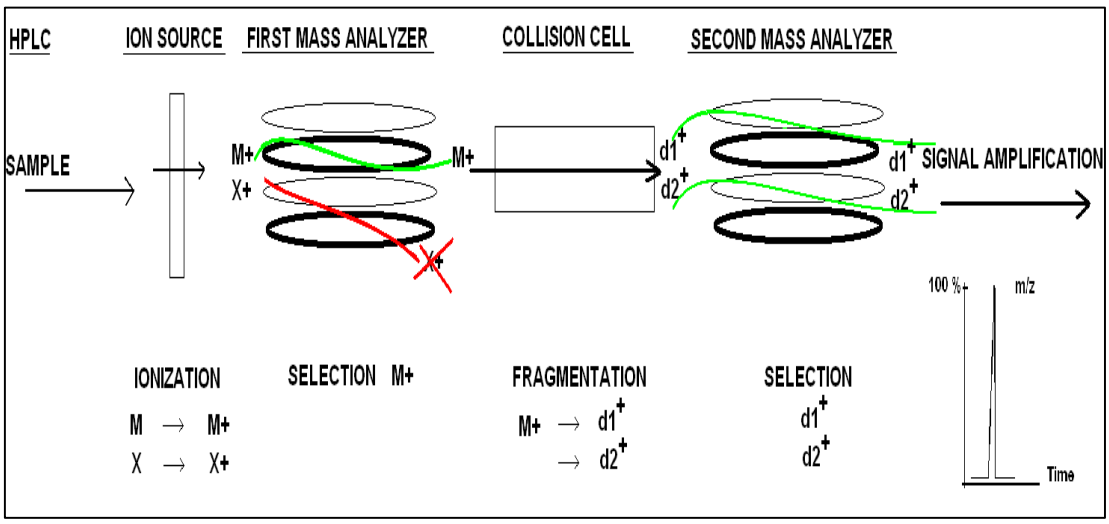


**Figure 1. Schematic presentation of the principle of triple quadrupole tandem mass spectrometry**

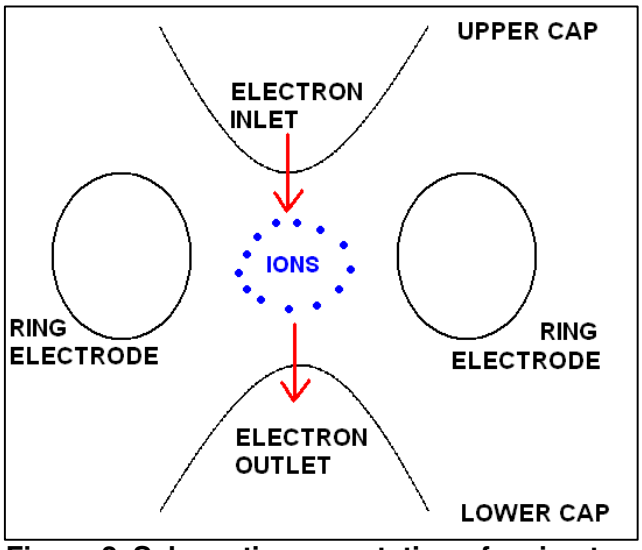


**Figure 2. Schematic presentation of an ion trap**

*Single-analyte determination*

To determine the most occurring mycotoxins, single-analyte methods have been developed. The European Union has established regulatory limits for mycotoxins, published in Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Therefore it is essential that validated methods are available with performance characteristics that meet certain criteria. Validated methods have been adopted as official international methods or as European standards by bodies such as AOAC (Association of Official Analytical Chemists) International or the European Committee for Standardization (CEN). An overview of official methods has been published by Gilbert and Anklam in 2002 [16].

Various LC-MS/MS methods were developed for the quantitative determination of mycotoxins in different food matrices. For example quantitative determination of OTA in kidneys, beer and spices has been described [17-19]. The sample clean-up procedure for the first two matrices was similar using anion exchange columns, whereas immunoaffinity columns were used for the sample clean-up of spices. A LC-MS/MS method for the determination of ZEA in grains using zearalanone as internal standard was published by Zöllner et al. [20]. The determination of aflatoxin $M_1$ ($AFM_1$) in milk and milk powder using high-flow solid-phase extraction was also described [21]. In addition a LC-MS/MS method for fumonisins B1, B2, and B3 (FB1, FB2, FB3) in corn-flakes has been developed [22]. This list of single-analyte LC-MS/MS methods is not exhaustive.

*Multi-analyte determination*

Several mycotoxins co-occur and can simultaneously contaminate foodstuffs. Therefore it is interesting to develop and validate methods for the simultaneous determination of various mycotoxins. The chemical structure of mycotoxins varies considerably which leads to differences in their polarity. This requires compromises in order to establish optimal conditions during extraction and clean-up procedures. It is also difficult to choose a suitable internal standard in the development of a multi-mycotoxin method.

The first step in mycotoxin analysis is the extraction of the toxins from the sample matrix. The composition of the solvent applied for extraction is a crucial parameter during the development of a multi-mycotoxin method. Polar organic solvents such as methanol or acetonitrile in combination with water are frequently used, sometimes acidified with acetic acid or sulphuric acid to recover the fumonisins [23-25].

Solid phase extraction (SPE) is commonly used as clean-up step to remove interferences. A recent development in clean-up methods is the very simple and rapid multifunctional Mycosep clean-up column (Coring System Diagnostics GmbH, Gernsheim, Germany). Mycosep columns contain a variety of adsorbents including charcoal, celite, ion-exchange resins and others. The clean-up procedure is reduced to 1-step extract purification and does not require time-consuming rinsing steps. It is designed especially for the analysis of numerous mycotoxins; nearly all analytical interfering substances are retained on the column whereas the mycotoxins are not adsorbed on the packing material. Mainly trichothecenes and aflatoxins are analyzed using the Mycosep clean-up columns [24, 26-34]. With Bond Elut Mycotoxin clean-up columns (Varian, St.-Katelijne-Waver, Belgium), the toxins pass through the cartridge, a silica-based ion exchange sorbent, while the food matrix components are retained. Twelve type A and type B trichothecenes are analyzed using the Bond Elut Mycotoxin clean-up column [35]. Other approaches are the use of Graphitized Carbon Black (Carbograph) clean-up columns (Grace Davison Discovery Sciences, Lokeren, Belgium) and OASIS HLB SPE columns (Waters, Zellik, Belgium).

Carbograph can behave as both reversed-phase and ion-exchanger sorbent and has a particular affinity for aromatic compounds with respect to aliphatics. Cavaliere *et al.* have published several scientific papers in which the Carbograph column is used. Major type B trichothecenes and macrocyclic lactones in field and contaminated maize were analyzed [36]. Adaptation of the previous method led to the analysis of major *Fusarium* mycotoxins in corn meal [37]. Recently a confirmatory method for aflatoxins in maize was published [38]. OASIS HLB is a hydrophilic-lipophilic-balanced sorbent made from a specific ratio of two monomers, hydrophilic N-vinylpyrrolidone and lipophilic divinylbenzene. This SPE clean-up column is frequently used for the analysis of polar components. Today a multi-mycotoxin method has been published for the determination of 18 mycotoxins in bovine milk [25].

A new trend in the development of multi-mycotoxin methods is the direct injection of crude extracts. Sulyok *et al.* developed a LC-MS/MS method for the determination of 39 mycotoxins in wheat and maize [39]. Also no SPE clean-up is used for the simultaneous determination of 16 mycotoxins on cellulose filters and in fungal cultures [40].

An improvement in chromatographic performance has been achieved by the introduction of ultra performance liquid chromatography (UPLC). This progression probably offers new opportunities for future developments in multi-mycotoxin analysis. The van Deemter equation indicates that as the particle size of the stationary phase decreases to less than 2.5 µm, there is a significant gain in efficiency which does not diminish at increased flow rates or linear velocities. Thus, UPLC takes full advantage of chromatographic principles to perform separations using columns packed with smaller particles (1.7 µm) and/or at higher flow rates resulting in a shorter analysis time, with superior peak capacity (number of peaks resolved per unit time in gradient separations) and sensitivity. The simultaneous analysis of aflatoxins $B_1$, $B_2$, $G_1$, $G_2$ and OTA in beer by UPLC-MS/MS was published [41]. More recently the simultaneous quantification of 17 *Aspergillus*, *Fusarium* and *Penicillium* mycotoxin contaminants in foods and feeds by UPLC was performed [24].

## NON-INSTRUMENTAL IMMUNOCHEMICAL METHODS

### *Dipstick enzyme immunoassay*

Dipsticks were probably the first potential ex-laboratory tests involving enzyme immunoassay technology. The dipsticks were made of plastic or a higher capacity matrix such as nitrocellulose or CNBr-activated paper attached to a plastic stick. They were limited by the capacity of the strip or by the diffusion of the samples and reagents through the matrix [42]. The dipstick, precoated with secondary antibodies, was immersed consecutively in solutions of primary antibodies, sample extract, analyte-enzyme conjugate and enzyme substrate solution, in this way performing a direct competitive enzyme immunoassay using a secondary antibody [43].

A two-step dipstick was established for 15-acetyldeoxynivalenol (15-acDON) with a limit of detection (LOD) in buffer solution of 5 ng/mL and a cut-off level (defined as total colour suppression) between 20-25 ng/mL [44]. A similar dipstick was established for T-2 toxin in wheat. In optimal conditions a visible LOD was at 0.25 ng/mL in buffer solution. Colour development was completely suppressed at 3 ng/mL T-2 toxin. It was possible to make visually a clear distinction between the negative control and a wheat extract spiked with 12 ng/g T-2 toxin [43]. To integrate the negative control into the dipstick, anti-horse radish peroxidase (HRP) was used. A dipstick for FB1 determination contained two lines: one with anti-FB1 antibodies and a control line with anti-HRP antibodies. The anti-HRP

antibodies bound FB1-HRP in a non-competitive format. After substrate application, colour developed on two lines [45].

*Flow-through enzyme immunoassay*

Valkirs and Barton (1985) [46] have established this rapid technique in clinical chemistry in which a monoclonal antibody coated membrane, incorporated into a cylindrical, disposable device, regulates sample and reagent delivery. In 1999, De Saeger and Van Peteghem [47-48] described a flow-through enzyme immunoassay carried out in a device format consisting of a plastic bottom and top member. In the device, cotton wool was acting as an adsorbent which actively drew liquid reagents through the membrane. The membrane with coated rabbit anti-mouse antibodies and anti-HRP antibodies (control) was held above the cotton wool, directed at the center of the top member for reagent access. The principle of the assay (direct competitive enzyme immunoassay using a secondary antibody) is schematically presented in Figure 3: a negative sample gives two coloured spots, a positive sample only one (the control spot). The flow-through assay is rapid, easy-to-use and is suitable for testing mycotoxins in the field. The method does not require any equipment.

A flow-through immunoassay for OTA detection in wheat was established using this format. An OTA concentration of 4 ng/g in spiked wheat completely suppressed the colour development [48]. For T-2 toxin the detection limit was 50 ng/g. A collaborative study of five laboratories showed that these flow-through kits could be used for the screening of wheat, rye, maize and barley for the presence of OTA and T-2 toxin [49]. Application of this format to $AFM_1$ detection in both liquid and powdered milk was also described. However, immunoaffinity columns had to be used to clean-up the milk samples [50]. Flow-through with internal control spots (anti-enzyme antibody) were applied for T-2 toxin detection with LOD at 50 ng/g in rye, wheat, barley and maize [51], for OTA with LOD at 4 ng/g in roasted coffee [52], at 8 ng/g in green coffee [53], for fumonisins with LOD at 1000 ng/g in maize [54], with LODs determined as complete colour suppression. An immunofiltration assay with LOD, determined as intensity reduction, was developed for detection of FB1 at 40-60 ng/g in corn-based food [45], and also for sporidesmin A with LOD at 1 ng/mL [55].

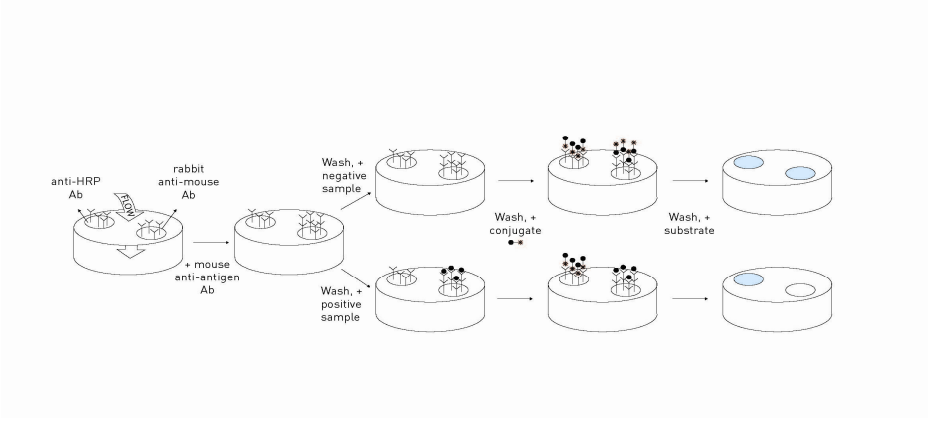


**Figure 3. Flow-through immunoassay principle**

*Lateral flow immunoassay*

There are many commercially available *in vitro* diagnostic test kits utilizing the principles of immunochromatography. The first major target analyte for this test format was (human) chorionic gonadotropin (HCG) for the detection of pregnancy.

The technology has been used for many years. However, its application in food analysis, especially mycotoxin testing, is quite recent [3]. A typical immunochromatography test strip is composed of a sample pad, a conjugate pad, a membrane, an absorbent pad and an adhesive backing.

The detector reagent, typically an antibody coupled to latex or a colloidal particle (most commonly gold), is deposited (but remains unbound) into the conjugate pad. When the sample is added to the conjugate pad, the detector reagent is solubilized and begins to move with the sample flow front up the membrane strip. Analyte present in the sample is bound by the antibody of the detector reagent. As the mixture passes over the zone to which the capture reagent (= antigen-protein conjugate) has been immobilized, the free antibody of the detector reagent is trapped (indirect competitive immunoassay). The colour of this test line is inversely proportional to the amount of analyte present in the sample (Figure 4). The strip may also contain a control line (secondary antibodies which bind the antibodies of the detector reagent) to indicate completion of the reaction. In this example, a negative sample gives two coloured lines while a positive sample gives only one. Movement of the sample by capillary action is maintained by the adsorbent pad at the far end of the strip.

A one-step lateral flow dipstick was developed for FB1 using polyclonal antibodies. Matrix effects were tested for corn, barley, peanuts, oats, rice and sorghum, and interference was completely eliminated by a 15-fold dilution of the sample extract with buffer solution [56]. For OTA detection a lateral flow dipstick based on monoclonal antibodies was developed with a detection limit of 500 ng/mL in buffer solutions [57]. The same format was used for aflatoxin $B_1$ ($AFB_1$) detection in pig feed with colloidal gold labelled monoclonal anti-$AFB_1$ antibodies. In optimal conditions the LOD, resulting in no colour on the test line, was at 5 ng/g $AFB_1$ [58]. Membrane-based immunoassays with colloidal gold labels were compared to a rapid variant (20 min) of a microtiter ELISA with HRP labels for FB1 determination by Wang *et al.* (2006) [56], and this under similar conditions with the same immunoreagents. Lateral flow and flow-through formats had a visual LOD of 1.0 ng/mL, so only two times higher than the ELISA format (0.5 ± 0.2 ng/mL).

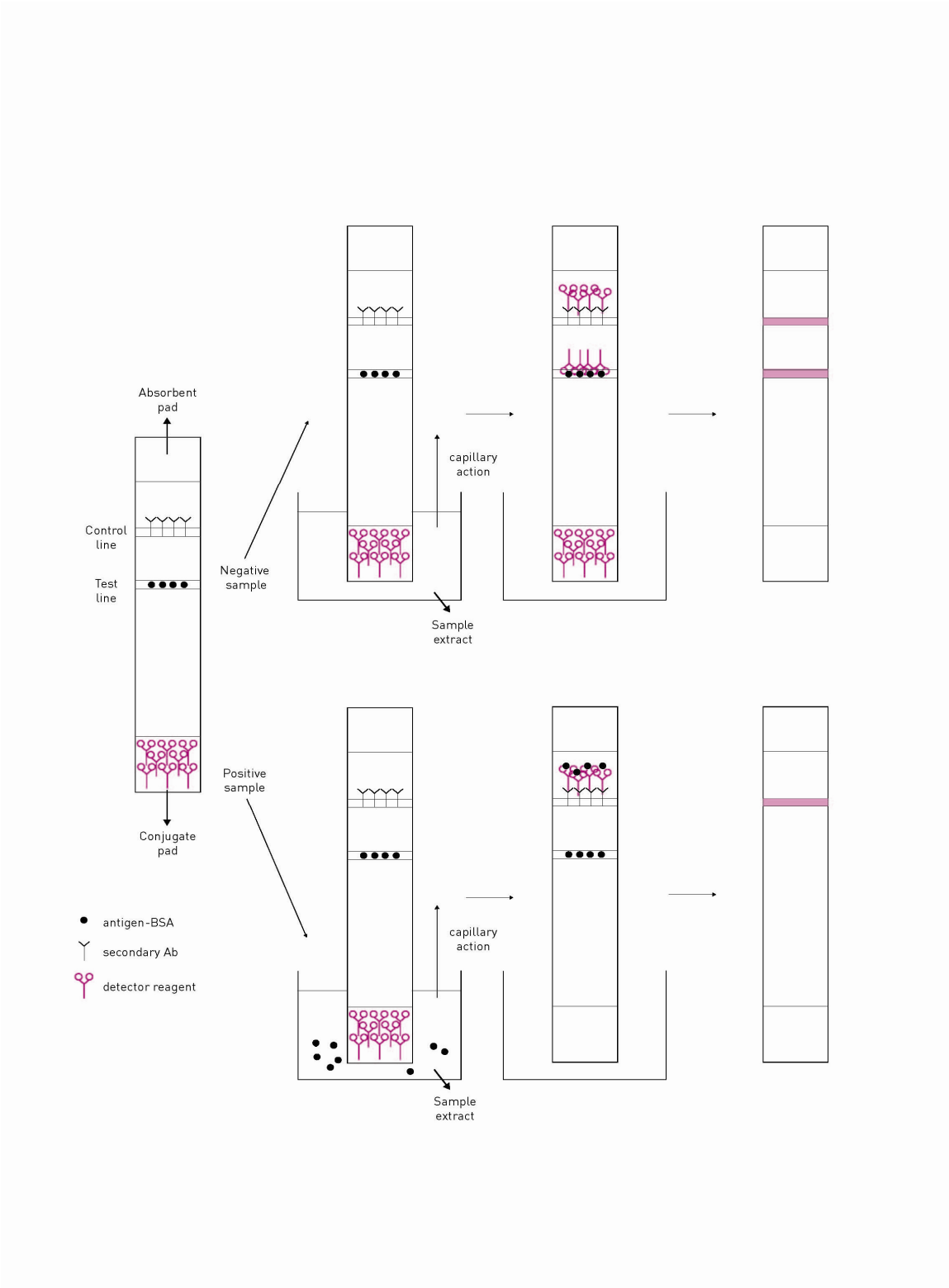


**Figure 4. Lateral flow immunoassay principle**

*Clean-up tandem immunoassay column*

To overcome the problem of coloured membranes for strongly coloured food matrices, in this way interfering with the visual detection, a new format for a rapid test was introduced. The clean-up tandem assay column comprises two superposed layers: a clean-up layer, capable of adsorbing at least part of the interfering fraction, and a detection layer where the direct immunoassay is performed. The clean-up tandem immunoassay column [59] has been applied for the development of rapid tests for the detection of OTA in roasted coffee [60], OTA in cocoa powder [61], OTA in spices [19,62] and also for the simultaneous detection of OTA and $AFB_1$ in spices [63]. The principle (direct competitive enzyme immunoassay using a secondary antibody) is explained in Figure 5: a negative sample gives a blue coloured detection layer, for a positive sample, the detection layer remains colourless.

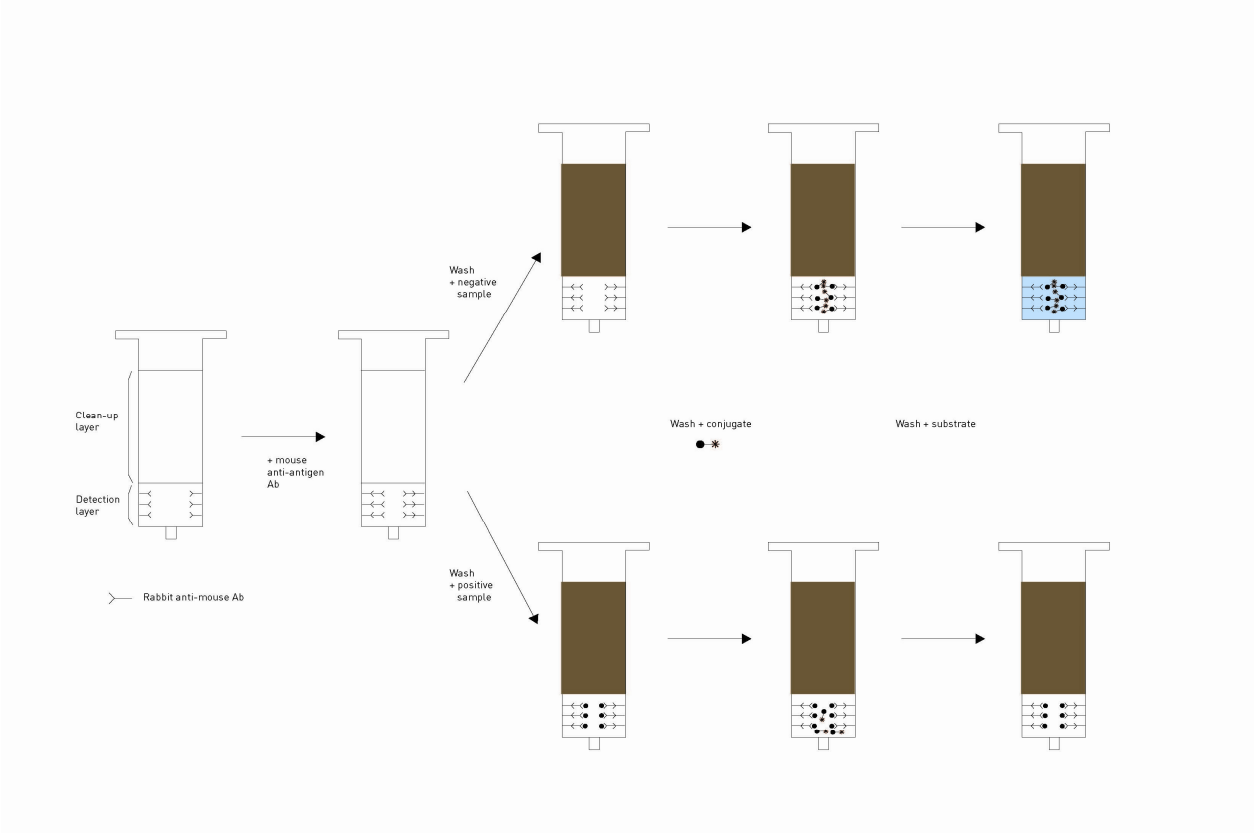


**Figure 5. Clean-up tandem immunoassay principle**

## CONCLUSIONS

For mycotoxin analysis, quantitative results can be obtained by chromatographic methods, the most emerging technique being LC-MS/MS. Methods for single-analyte determination are numerous. As several mycotoxins can co-occur in foodstuffs and feed, multi-analyte determination has won popularity.

However, these chromatographic methods yield results within hours or days. Competition within the food and feed industry forces them to reduce cost, employ cheaper labour and deliver goods more rapidly. Additionally, increased analytical complexity in the food industry requires a rapid report for each individual contaminant. Thus, rapid methods, such as the described immunochemical methods (flow-through enzyme immunoassays, lateral flow immunoassays, clean-up tandem immunoassay columns) have become increasingly important.
Main trends for research in the field of mycotoxin analysis are sensitivity improvement, matrix effect reduction, simplification, shorter time of analysis and evolution towards multi-analyte testing.

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# Determination of realistic concentrations for studying toxic effects of food chemical contaminants at the gastro-intestinal level

**L. Ribonnet[1,2], S. Garsou[1] and L. Pussemier[1]**

*[1] CODA-CERVA-VAR, Leuvensesteenweg 17, B-3080 Tervuren, Belgium.*
*[2] Université catholique de Louvain-la-Neuve, Institut des Sciences de la Vie, Croix du Sud 5/3, B-1348 Louvain-la-Neuve, Belgium.*
*e-mail: Lupus@var.fgov.be*

## Keywords
Food contaminants; daily intake; chronic exposure; intestinal concentrations

## SUMMARY

Risk assessment process for chemical contaminants in the diet stems from the hazard characterization at realistic exposure. In order to comply with this principle, *in vitro* chronic hazard characterization can be performed at the gastro-intestinal level by applying, on cultured cells, realistic contaminant concentrations. Therefore, we propose, in this chapter, an efficient methodology based on the dilution in the bolus of several food chemicals in order to estimate the intestinal concentrations that can be used to perform experimental investigations. Estimations of human exposure to food chemicals have been performed for various classes of contaminants: mycotoxins, pesticides, veterinary drugs and environmental contaminants, including dioxins, polycyclic aromatic hydrocarbon (PAH) and heavy metals. We compared theoretically maximum daily intakes obtained by multiplication of GEMS/FOOD Regional Diets consumption values and Maximum (Residue) Level (M(R)Ls) with intakes from European SCOOP tasks as well as with reference values such as the tolerable daily intake (TDI) or acceptable daily intake (ADI). This method slightly overestimates real concentrations occurring in gastro-intestinal tract but is still in accordance with the aim of our study since we expect to test theoretically maximal concentrations representative of chronic human exposure in order to cover a large range of consumers in chronic hazard characterization.

## Abbreviations
A, acaricide; ADI, acceptable daily intake; ATSDR, Agency for Toxic Substance and Disease Registry; B[a]P, benzo(a)pyrene; bw, body weight; $C_i$, consumption data; Cd, cadmium; DL-PCB, "dioxin-like" PCB; DON, deoxynivalenol; EDI, estimated daily intake; EFSA, European Food Safety Authority; EU, European Union; F, fungicide; FBS, food balance sheet; FB1, fumonisin B1; FDA, Food and Drug Administration; GEMS, GEMS/FOOD Regional Diets; H, herbicide; Hg, mercury; I, insecticide; IARC, International Agency for Research on Cancer; IPH, Belgian Scientific Institute of Public Health; JECFA, Joint FAO/WHO Expert Committee on Food Additives; JMPR, Joint FAO/WHO Meeting on Pesticide Residues; $L_i$, contamination data; MeHg, methylmercury; M(R)L, maximum (residue) level; MRPL, minimum required performance limit; M.S., Member State; NDL-PCB, non "dioxin-like" PCB; NIV, nivalenol; OC, organochlorine pesticide; OP, organophosphate pesticide, OTA, ochratoxin A; PAH, polycyclic aromatic hydrocarbon; PAT, patuline; Pb, lead; PCB, polychlorinated biphenyls; PCDD, polychlorodibenzo-p-dioxines; PCDF, polychlorodibenzofurane; POP, persistent organic pollutant; SC, SCOOP task; SCF, Scientific Committee on Food; TDI,

tolerable daily intake; TMDI, theoretical maximum daily intake; TWI, tolerable weekly intake; PTDI, provisional tolerable daily intake; PTMI, provisional tolerable monthly intake; PTWI, provisional tolerable weekly intake; TEQ, toxic equivalent quantity; WHO, World Health Organization; ZEA, zearalenone

## INTRODUCTION

Nowadays, food safety is of major concern since more and more chemicals are present in our environment. Diverse sources of food contamination can be expected. Among many other sources, chemicals are able to be released from consumer products and packaging (e.g. phthalates, brominated flame retardants), or deposited on soil after atmospheric transport from industrial sites (e.g. polychlorinated biphenyls (PCBs) and dioxins), or spread with sludge from sewage treatment plants (e.g. heavy metals) or applied as crop protection products. They consequently enter the food chain by bioaccumulation or biomagnification. Some of these contaminants are very persistent in the environment and are classified as “Persistent Organic Pollutants” (POPs). They are generally very lipophilic and accumulate in fat. In addition to environmental contaminants, mycotoxins may contaminate food in field or during storage, residues of veterinary drugs may occur in meat-based food and many other chemicals may be generated during food processing and cooking (e.g. benzo(a)pyrene (B[a]P), acrylamide). Consequently, food contains a wide range of non-desired substances, in addition to nutrients and micronutrients.

Therefore, it is necessary to assess risks linked to these already characterized or new emerging food chemicals. Risk assessment is defined as the process aiming to estimate the likelihood that a particular adverse effect will occur in a population following the exposure to a hazard [1]. Different steps are involved in the risk assessment process (Figure 1). First, the hazard must be identified. Secondly, in order to be relevant, the hazard must be characterized at realistic doses. This step needs information on consumer or whole population exposure. The third step of risk assessment results from the combination of hazard and exposure assessment. In the last step, risk is communicated to public authorities for risk management as well as to the large public.

In this study, we focused on chronic exposure assessment of various relevant chemicals from the diet. The purpose is to derive realistic intestinal concentrations. By this way, hazard characterization will be performed at realistic experimental concentrations, using an *in vitro* model of the human intestinal epithelium. Indeed, the intestine is the first barrier encountered by food chemicals before eventually entering the blood system and distributing through the body toward target organs where they can exert their toxicity. The passage through the intestine is thus a crucial step because the interactive relation between function of enterocytes and chemicals determine bioavailability. Until now, a lot of experimental studies, aiming *in vivo* or *in vitro* hazard characterization have been performed without previous exposure assessment. Consequently, results need to be interpreted with a lot of caution during the risk assessment process, since chemical concentrations are not representative of real exposure. The originality of our approach [2] is to perform a risk assessment at the intestinal level by combination of hazard characterization and realistic exposure estimates (see also the papers written by Sergent *et al.* and Schneider *et al.* in this issue).

Exposure assessment, as a component of the risk assessment framework, is defined as “the qualitative and/or quantitative evaluation of the likely intake of biological, chemical or

physical agents via food as well as exposure from other sources if relevant" [3]. In any

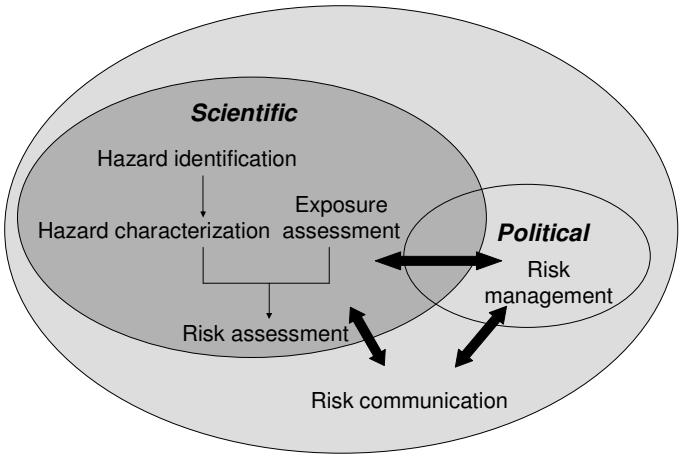


**Figure 1. Risk analysis framework**

food chemical risk assessment, exposure assessment if often a source of great uncertainty [4, 5]. It is generally formulated by the "Estimated Daily Intake" (EDI). For an accurate exposure assessment, three pieces of information are needed [4]:

- Which substances are present in which amount in a given food and/or the diet in general, and what affects their levels and characteristics, especially their biological activity?
- How much of the foods containing these substances are consumed and what is the consumption of potentially relevant risk groups, including high users?
- What are the conditions and the probabilities of consuming occasionally or regularly high amounts of such foods which at the same time contain high levels of the substance(s) in question?

Regarding these three questions, in its simplest form, the EDI for one contaminant in foodstuff *i* is obtained by multiplying consumed quantity of foodstuff *i* by the concentration of this chemical in foodstuff *i*. EDIs obtained for each foodstuff *i* susceptible to contain the chemical are then summed up giving total EDI for the chemical.

$$EDI\ [ng/person/day] = \Sigma_i\ (C_i \times L_i)$$

With - $C_i$: consumed quantity of foodstuff *i* per person per day (consumption data) [g/p/d]
- $L_i$: concentration of a chemical in foodstuff *i* (contamination data) [µg/kg]

Depending of the survey purpose, the way answering the three questions, and, consequently, the choice of consumption and contamination data in the calculation of EDI, can differ, leading to different exposure estimates. In this chapter, we have tried to expose and to compare different sources that can be useful in order to assess daily intake of various selected food contaminants. This exposure assessment enabled us to generate

realistic intestinal concentrations, after setting dilution hypotheses of contaminants in the bolus.

## Food consumption data

Food consumption data can be estimated in broad terms with food balance sheets (FBS) or in relative detail through country specific surveys.

In this study, we have mainly used consumption data from the GEMS/FOOD Regional Diets [6]. These diets give regional per capita consumption [g/person/day] of raw and semi-processed agricultural commodities. They are now used by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in order to estimate dietary exposure to pesticide residues, according to internationally accepted methodologies [3], and to contaminants in food, respectively.

Since consumption patterns vary greatly among countries, GEMS/FOOD Regional Diets are based on FBS data compiled by the FAO. These data provide statistics on a country's annual food production, imports and exports. Finally, five regional dietary patterns (Middle Easter, Far Easter, African, Latin American and European) are represented in term of average value per head of the population.

These diets are only convenient for chronic exposure assessment, since they greatly underestimate short-term high percentile intakes. Indeed, in predicting chronic exposure, long-term food consumption habits and not day-to-day variations should be used for intake to permit valid comparison with the "Acceptable Daily Intake" (ADI, for pesticides) or "Tolerable Daily Intake" (TDI, for other contaminants), which are based on intake over a lifetime. Thus, average daily consumption values are used in predicting exposure to contaminants for assessing long-term risks at the international level. This consumption evaluation method matches with the purpose of this survey since we investigated intestinal toxicity by mimicking long-term exposure to contaminants.

It should be noted that, because waste at the household or individual level is not taken into account, FBS data tend to slightly overestimate consumption (15% higher than actual average food consumption, in the worst case). In addition, refinement of consumption data for children which are known to have quantitative and qualitative differences in their consumption patterns has not been performed. This approach of food consumption is a first step in the risk assessment procedure and may be useful for comparisons among countries [4].

In 2006, the Belgian Scientific Institute of Public Health (IPH) published a national food consumption survey leading in 2004 [7]. In contrast to GEMS/FOOD Regional Diets which is based on data from FBSs, data have been collected from individual dietary surveys. They provide information on average food intake and their distribution over various well-defined groups of individuals. These data more closely reflect actual consumption. In order to collect data at the individual level, a 24-hour recall method has been used.

Table 1 compares consumption data from GEMS/FOOD Regional Diets (Europe) and from IPH survey (Belgium) for different food groups.

We can observe that GEMS/FOOD data (FBSs method) do not always overestimate consumption regarding to IPH data (individual dietary survey). Actually, data are often in the same order of magnitude. It should be noted that food elements considered in the different food groups can differ among studies. Furthermore, GEMS/FOOD data are based on long-term consumption assumptions, whereas IPH data which have been collected by a 24h-recall method would be more suitable for acute risk assessment.

**Table 1. Comparison between food consumption data from the GEMS/FOOD Regional Diets study (Europe) and data from the IPH survey (Belgium) for different food groups**

| Food groups | GEMS/FOOD Regional Diets (Europe) [g/person/day] | IPH (Belgium)[1] [g/person/day] |
|---|---|---|
| Cereals | 221.9 | 133 |
| Potatoes | 240.8 | 306.6 |
| Vegetables | 371.6 | 138.3 |
| Fruits | 212.4 | 118.2 |
| Dairy products | 336.1 | 158.6 + 30.2 (cheese) = 188.8 |
| Meat + fish + eggs | 217.3 + 46.8 + 37.6 = 301.7 | 161.1 |
| Spreadable fat | 17.3 (margarine) + 14 (butter) = 31.3 | 21.2 |

[1] Usual food consumption in the general population ≥ 15 years old

## Food contamination data

There are many different ways to integrate contaminant concentrations in food into the dietary exposure estimate.

First, the highest legal concentration value in the food of interest, such as Maximum (Residue) Level (M(R)L) can be used. However, the utilization of these legal limits in the exposure assessment can lead to a crude overestimate of exposure because actual concentrations are generally much lower than the M(R)Ls. For example, for a given crop, only a fraction is actually treated with a given pesticide. In addition, residue levels are usually reduced during storage, preparation, commercial processing, and cooking. It is unlikely that every food for which an M(R)L is proposed will have been contaminated at the higher permitted level over the lifetime of the consumer [3].

These M(R)Ls are set up by different organisms. Regarding pesticides, codex M(R)L are usually based on those recommended by the JMPR and are valuable at the international level. However, each country can have its own legislation and the European Union has set its own legal limits. Given the current movement of consumer goods and food around the world, it was chosen to use principally international and European M(R)Ls, instead of Belgian M(R)Ls in the exposure assessment calculation.

An alternative to the use of these legal limits is the use of monitoring data which might be a better representation of contaminant concentration in food. Indeed, these values are much lower than M(R)L. It should be noted that processing may lead to a decrease of contaminant concentration in the processed product relative to the raw agricultural commodity. Time between harvest and sale may also lead to contamination decrease.

## European SCOOP reports

European SCOOP tasks provide both consumption and contamination data collected in different European Member States participating in the study. They have been written for mycotoxins (ochratoxin A (OTA) [8], patuline (PAT) [9], *Fusarium* toxins such as deoxynivalenol (DON), fumonisin B1 (FB1), nivalenol (NIV), T-2 and HT-2 and zearalenone (ZEA) [10]), as well as for heavy metals [11], dioxins [12] and polycyclic aromatic hydrocarbons (PAHs) [13].

Depending on the used consumption data, two EDIs may be distinguished:

- EDI mean: this intake is a mean value for a given Member State. It is calculated using mean consumption and mean contamination level.

- EDI 95th: this intake is calculated for a given Member State using 95th percentile of consumption values and mean contamination levels.

## Assessment of the Daily intake of food contaminants

Depending on the way of combining consumption and contamination data, two kinds of daily intake can be distinguished (Figure 2):

- "Real-case" EDI: the combination of contamination data from monitoring analysis (SCOOP reports, national surveys, publications) with consumption data (SCOOP reports, GEMS/FOOD Regional Diets, national surveys) gives an EDI theoretically close to the real exposure.
- Theoretical maximum daily intake (TMDI) [3]: this "worst-case" approach results from the multiplication of M(R)Ls and consumption data from GEMS/FOOD Regional Diets or from other consumption surveys.

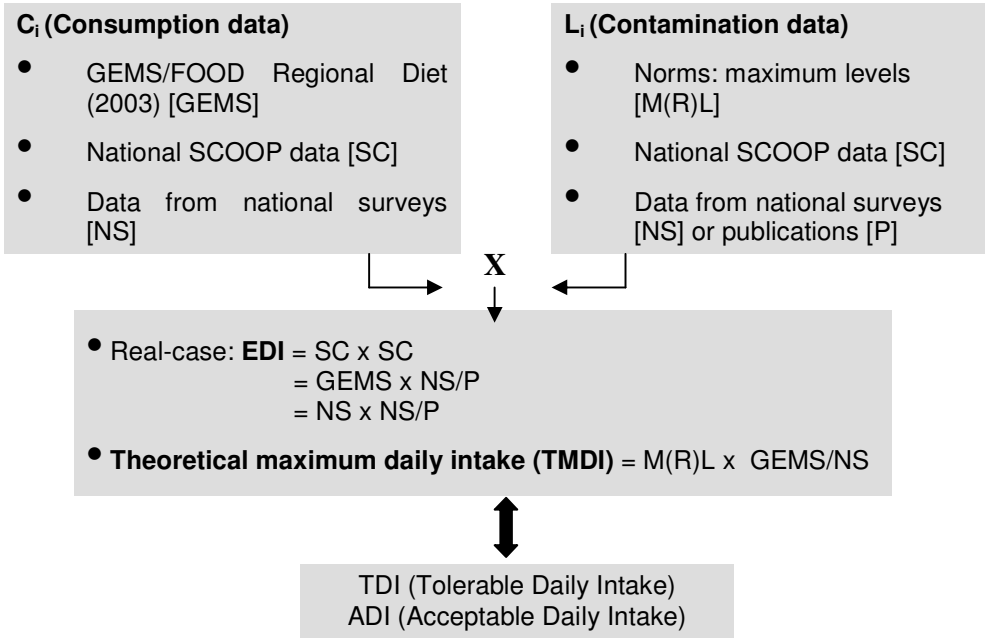


**Figure 2. The way combining diverse sources of consumption and contamination data leads to either "real-case" EDI or TMDI. Dietary intake is compared to safety values set by authorities**

When risk assessment is performed with chronic exposure data, the daily intake must be compared to safety reference values set by the authorities (ADI for pesticides and TDI for other contaminants). ADI and TDI correspond to the amount of a substance that can be ingested daily over an entire lifetime without harmful effect. Sometimes, a provisional tolerable weekly/daily intake (PTWI or PTDI) is used instead of a TDI for contaminants. However, a comparison of exposure estimates and acceptable intakes should be based on similar assumptions, e.g. using data, which reflect lifetime exposure for both values [14].
Theoretical maximum daily intake is a first crude overestimation of the actual consumer exposure, consisting in a first estimate. If the result is lower than threshold toxicological value (ADI or TDI), it is very likely that the chemical will not pose any problem and the risk

assessment can be suspended. On the contrary, if the theoretical maximum daily intake is higher than threshold toxicological value, a more precise assessment is needed and some kind of risk management action will be required.

### *Mycotoxins*

Maximum levels of mycotoxins (OTA, DON, PAT, aflatoxins and ZEA) in food have been set up by the European Commission in the Commission Regulation (EC) N°1881/2006 of 19 December 2006 (replacing Commission Regulation (EC) N°466/2001 of 8 March 2001). MLs specified in this regulation for fumonisins will be in application from 1 October 2007. Other mycotoxins, like T-2 and HT-2 have not yet their MLs, which have to be set in July 2007 (Commission Regulation (EC) 856/2005 amending the Commission Regulation (EC) N°466/2001 of 8 March 2001).
In a first tier approach, we multiplied these authorized limits with European consumption data from GEMS/FOOD Regional Diets. In a second tier, we reported more refined results of exposure assessment in different European countries from European SCOOP tasks.

*Detailed example: OTA*. MLs are fixed for OTA in cereals and dried vine fruits but also in coffee, spices, wine, cereal-based baby-food and special diet food for new-born babies. In SCOOP task 3.2.7, seven food categories were identified as potentially contaminated by OTA and taken into account in the exposure assessment: cereals and cereal products, wine, beer, grape juice, brewed coffee, cocoa and cocoa products as well as pork meat. Details of TMDI calculation are presented in Table 2.

*Overview mycotoxins.* The same methodology has been applied to other mycotoxins for which MLs are set in the Commission Regulation (EC) N°1881/2006 (AFB1, DON, FB1, PAT and ZEA) (Table 3). Details of TMDI calculations are not shown.

### *Pesticides residues*

The occurrence of pesticide residues in food is closely linked to the mode of agricultural production. They are found principally in fruits and vegetables. However, organochlorine pesticides are often considered as environmental contaminants since beside the fact that they have been banned, residues are still occurring in fruits and vegetables and in animal products with a high fat content (i.e. fish, milk, and meat) due to the lipophilic and persistent properties of these chemicals.
The most usually accepted methodology for pesticide residues exposure assessment has been proposed by the World Health Organization (WHO) [3] and recommends the use of Codex MRLs and consumption data from GEMS/FOOD Regional diets [6]. Despites slight overestimation, this methodology is very useful to provide a first exposure assessment, in order to point out pesticides with TMDI close to the ADI. In this study, we choose to follow this methodology, using Codex and Belgian MRLs. These legal limits can be found respectively on the frequently updated Codex Alimentarius web site (http://www.codexalimentarius.net) and on the "phytoweb" website (http://www.phytoweb.fgov.be). Another website centralizes MRLs in application in different countries as well as Codex and European MRLs (http://www.mrldatabase.com).

**Table 2. Example of calculation of dietary intake for the mycotoxin OTA. Comparison between a first tier approach based on European legal maximal limits in food (Commission Regulation (EC) N°1881/2006 of 19 December 2006) and a second tier approach based on results from the European SCOOP task 3.2.7 [8] (see the list for abbreviations)**

| TWI [1]: 120 ng/kg bw/d | | | | | | | |
|---|---|---|---|---|---|---|---|
| First tier approach: GEMS x MLs | | | | | Second tier approach: SC x SC | | |
| | | | | | EDI mean (ng/kg bw/d) [2] | | |
| Commodities | MLs (µg/kg) | $C_i$ (GEMS) (g/p/d) | GEMS code | TMDI (ng/p/d) | Lowest M.S. | Highest M.S. | Medium |
| cereal-based products | 3 | 221.9 | GC 80 | 665.7 | 0.1 | 3.5 | 1.0 |
| dried vine fruits | 10 | 2.3 | DF 269 | 23 | | | |
| | total | | (ng/p/d) | 688.7 | | | |
| | | | (ng/kg bw/d)[3] | **9.84** | | | |
| | | | % TDI [4] | 57% | 0.6 % | 20% | 5.8 % |

[1] Derived by EFSA in 2006 [15]
[2] Body weight varies among Member States (M.S.)
[3] Assuming a 70 kg body weight adult
[4] Assuming that the tolerable weekly intake is equally spread over the week and then that TDI = 17.14 ng/kg bw/d

**Table 3. Synopsis of the calculated dietary intakes for mycotoxins. Comparison between a first tier approach based on European legal maximal limits in food (Commission Regulation (EC) N°1881/2006 of 19 December 2006) and a second tier approach based on results from European SCOOP tasks (see the list for abbreviations)**

| | | First tier approach: GEMS x MLs | | Second tier approach: SC x SC [3] | | |
|---|---|---|---|---|---|---|
| | | | | EDI mean (ng/kg bw/d) [4] | | |
| mycotoxin | TDI [1] (ng/kg bw/d) | TMDI (ng/kg bw/d) [2] | %TDI | Lowest M.S. | Highest M.S. | Medium |
| AFB1 | / | 6.8 | / | / | / | / |
| DON | 1000 | 2 377 | 238% | 78 | 480 | 271.3 |
| FB1 | 2000 [(5)] | 194.6 [(6)] | 9.7% | 0.1 | 226.8 | 92.9 |
| OTA | 120 [(7)] | 9.84 | 57% [8] | 0.1 | 3.5 | 1.0 |
| PAT | 400 | 18 | 4.5% | 0.2 | 59 | 11.3 |
| ZEA | 200 | 237.75 | 119% | 0.8 | 29 | 15.9 |

[1] Derived by SCF

[2] Assuming a 70 kg body weight adult

[3] DON, FB1, ZEA: SCOOP, 2003 [10]; PAT: SCOOP, 2002 [9]

[4] Body weight varies among Member States (M.S.)

[5] TDI for FB1 + FB2 + FB3

[6] MLs are fixed for the sum FB1 + FB2

[7] TWI (ng/kg bw/week)

[8] Assuming that the tolerable weekly intake is equally spread over the week and then that TDI = TWI/7

**Table 4. Example of TMDI calculation for a fungicide (imazalil, group of imidazoles) using Codex MRLs and Belgian MRLs (see the list for abbreviations)**

| ADI [1]: 30 µg/kg bw/d | | | | | | |
|---|---|---|---|---|---|---|
| | | | Codex MRLs | | Belgian MRLs | |
| GEMS Code | Commodities | $C_i$ (GEMS) (g/p/d) | MRL (mg/kg) | TMDI (µg/p/d) | MRL (mg/kg) | TMDI (µg/p/d) |
| FI 327 | Banana | 22.8 | 2 | 45.6 | 2 | 45.6 |
| FC 1 | Citrus fruits | 44.6 | 5 | 223 | 5 | 223 |
| VC 424 | Cucumbers and gherkins | 9.0 | 0.5 | 4.5 | 0.2 | 1.8 |
| VC 46 | Melons | 18.3 | 2 | 36.6 | 2 | 36.6 |
| FP 9 | Pome fruits | 51.3 | 5 | 256.5 | 5 | 256.5 |
| VR 589 | Potatoes | 240.8 | 5 | 1204 | 5 | 1204 |
| FB 272 | Raspberries | 0.5 | 2 | 1 | / | / |
| FB 275 | Strawberries | 5.3 | 2 | 10.6 | / | / |
| GC 654 | Wheat | 178.0 | 0.01* | 1.78 | / | / |
| VO 448 | tomato | 66.6 | / | / | 0.5 | 33.3 |
| | | total | (µg/p/d) | 1783.58 | | 1800.80 |
| | | | (µg/kg bw/d) [2] | **25.48** | | **25.72** |
| | | | % ADI | 85% | | 86% |

* MRL is set at the limit of determination

[1] Set by JMPR

[2] Assuming a 70 kg body weight adult

**Table 5. Calculated TMDI of pesticides compared to ADI and EDI values from literature (see the list for abbreviations)**

| Pesticide names | Class | use | ADI [1] (µg/kg bw/d) | Calculated TMDI [2, 3] (µg/kg bw/d) | Calculated %ADI | Literature EDI (µg/kg bw/d) | Literature remark | Literature reference |
|---|---|---|---|---|---|---|---|---|
| Aldrine/dieldrine | OC | I | 0.1 | 0.73 | 730 | 0.001-0.01 | EFSA opinion | [16] |
| Benomyl [4] | benzimidazole | F | 100 | 1.56 | 1.5 | | | |
| Captan | phtalimide | F | 100 | 37.25 | 37 | 0.03856 | Canadian EDI | [17] |
| Chlorpyrifos | OP | I | 10 | 12.29 | 123 | 0.079-0.189 | Danish EDI [5] | [18] |
| | | | | | | 0.00264 | Canadian EDI | [17] |
| DDT | OC | I | 10 | 1.29 | 13 | 0.005-0.03 | EFSA opinion | [19] |
| | | | | | | 0.00244 | Canadian EDI | [17] |
| Dichlorvos | OP | I, A | 4 | 2.79 | 70 | | | |
| Dicofol | OC | A | 2 | 9.16 | 458 | 0.03636 | Canadian EDI | [17] |
| Endosulfan | OC | I, A | 6 | 5.85 | 97 | 0.0238 | Canadian EDI | [17] |
| Glyphosate | glycine derivative | H | 300 | 21.59 | 7 | 2.3 | U.K. EDI | [20] |
| Imazalil | imidazole | F | 30 | 25.48 | 85 | | | |
| Imidacloprid | neonicotinoid | I | 60 | 5.54 | 9 | | | |
| Lindane (γ-HCH) | OC | I | 1 | 0.09 | 9 | 0.00132 | Canadian EDI | [17] |
| Paraquat | bipyridylium | H | 4 | 2.93 | 73 | | | |
| Permethrin | pyrethroid | I | 50 | 10.75 | 21 | | | |
| Procymidone | dicarboximide | F | 100 | 12.07 | 12 | 0.00048 | Canadian EDI | [17] |
| Propiconazole | triazole | F | 40 | 0.85 | 2 | | | |
| Pyrethrins | natural pyrethrins | I, A | 40 | 1.32 | 3 | | | |
| Thiabendazole | benzimidazole | F | 100 | 66.45 | 66 | | | |
| Thiram | dimethyldithiocarbamate | F | 10 | 6.67 | 67 | | | |
| Tolylfluanid | sulfamide | F | 100 | 12.77 | 13 | | | |
| Vinclozolin | dicarboximide | F | 10 | 11.51 | 115 | 0.00033 | Canadian EDI | [17] |

[1] Set by JMPR
[2] Using Codex MRLs, excepting for benomyl
[3] Assuming a 70 kg body weight adult
[4] TMDI calculated using Belgian MRLs (No Codex MRLs or prior Codex MRLs have been revoked)
[5] Cumulative EDI for OPs and carbamates

European MRLs are also available on the UK government web site (http://www.pesticides.gov.uk/psd_databases.asp).

*Detailed example: imazalil*. Details of exposure calculation for imazalil, a well-known post-harvest fungicide, are presented in Table 4. The intake is estimated by using both Codex and Belgian MRLs, allowing comparison. This intake is also expressed in % of ADI.

*Overview pesticides.* This TMDI calculation methodology was applied to a list of pesticides, using Codex MRLs (details not shown) and compared to ADIs values and intake estimates from literature (Table 5Table).

### *Veterinary drug residues*

Currently, up to 80% of all animals bred for food purposes receive veterinary medicines (antibiotics and other drugs), in order to cure or prevent diseases. Animal drugs are also used as feed additives to promote growth, improve feed efficiency and breeding performance, and enhance feed acceptability [21]. In addition, contamination of feedingstuffs may occur in unmedicated feedingstuffs, due to carry over during feed production [22]. As a consequence of contamination and administration, veterinary drug residues occur in edible tissues and animal-derived foodstuffs.
Tetracyclines are a family of broad-spectrum bacteriostatics. In the European Union, MRLs in meat-based foodstuffs have been established in the Regulation (EC) N° 2377/90 of 26 June 1990 for tetracycline, chlortetracycline, doxycycline and oxytetracycline which are the only compounds authorized in the family of tetracyclines. These MRLs have been used to assess human exposure to tetracycline (Table 6). Afterwards, TMDI have been compared to the ADI proposed by the U.S. Food and Drug Administration (FDA).

**Table 6. Calculated TMDI of tetracycline (see the list for abbreviations)**

| ADI [1]: 25 000 ng/kg bw/d | | | | |
|---|---|---|---|---|
| Commodities | MRLs [2] (µg/kg) | $C_i$ (GEMS) (g/p/d) | GEMS code | TMDI (ng/p/d) |
| muscles | 100 | 217.3 | Total meat and offals | 21 730 |
| liver | 300 | / | | / |
| kidney | 600 | / | | / |
| milk | 100 | 289.3 | ML 106 | 28 930 |
| egg | 200 | 37.6 | PE 112 | 7 520 |
| | | total | (ng/p/d) | 58 180 |
| | | | (ng/kg bw/d)[3] | **831.14** |
| | | | % ADI | 3% |

[1] U.S. FDA (21CFR 556.720)
[2] Commission Regulation (EC) N° 2377/90 of 26 June 1990
[3] Assuming a 70 kg body weight adult

Chloramphenicol, another broad-spectrum antibiotic, is banned and listed in the Annex IV of the same regulation with veterinary medicines which do not have MRLs. As a consequence, an ADI has never been allocated. However, although the use of chloramphenicol in veterinary medicines have been restricted to non-food animals, residues have been found in foodstuffs originated from Southeast Asia [23]. In order to

check whether consignments imported from third countries fulfil the requirements of Community legislation, The Commission Decision (EC) N°181/ 2003 of 13 March 2003 (amending the Commission Decision (EC) N° 657/2002 of12 August 2002) set the minimum required performance limit (MRPL) above which the consignment is considered as non-compliant. This MRPL of 0.3 µg/kg, applying to meat, milk, eggs, honey and aquaculture products, was used in combination with GEMS/FOOD consumption data to assess human exposure to chloramphenicol (details not shown). We obtained a TMDI of 2.54 ng/kg bw/d.

### *Environmental contaminants*

PCBs, dioxins and PAHs such as B[a]P are very resistant to chemical and biological degradation, thus persisting in the environment and accumulating in the food chain. These environmentally stable organic contaminants as well as heavy metals, such as cadmium (Cd), Lead (Pb) and mercury (Hg) are released form a variety of industrial and natural sources. Among the huge chemical group of PCBs (209 congeners), only 12 congeners have toxicological properties similar to dioxins (dioxin-like PCB). The other PCBs are termed “non dioxin-like PCBs” (NDL-PCBs). NDL-PCBs circulate more easily than dioxin-like PCBs through muscles and blood and affect directly the nervous system and brain development. They could be several orders of magnitude more concentrated than dioxins in some feed and food matrices.

Commission Regulation (EC) N°1881/2006 of 19 December 2006, replacing Commission Regulation (EC) N°466/2001 sets, among other food contaminants, MLs for dioxins and dioxin-like PCBs, B[a]P and heavy metals (Cd, Hg, Pb) in various foodstuffs.

Regarding NDL-PCBs, no maximum levels in food have been set at Community levels. Belgium, however, established MLs for the sum of seven indicators PCB in animal products (Royal decree of 6 March 2002, modifying Royal decree of 19 May 2000).

Human exposure to these food contaminants was calculated on the base of the corresponding legal limits in foodstuffs and compared to SCOOP tasks existing for heavy metals [11], dioxins [12] and B[a]P [13].

*Detailed example: dioxins and dioxin-like PCBs*. It is well-established that the diet is the major source (>90%) of human exposure to dioxins, with animal-based foodstuffs being the predominant source. For this reason, the Commission Regulation N°1881/2006 fixes MLs for dioxins (PCDDs/Fs) and for the sum of dioxins and dioxin-like PCBs (PCDDs/Fs + DL-PCBs) mainly in animal products. Dioxins being very lipophilic compounds, MLs are expressed in pg/g fat. Table 7 shows results from TMDI calculation of the sum of dioxins (PCDDs/Fs) and dioxin-like PCBs and results from SCOOP task 3.2.5 related to dioxins (PCDDs/Fs).

*Overview environmental contaminants.* The same methodology has been applied to other environmental contaminants for which MLs are set in the Commission Regulation (EC) N°1881/2006 (B[a]P, Cd, Hg and Pb) and to NDL-PCBS, using Belgian legislation (Table 8). Details of TMDI calculations are not shown.

**Table 7. Example of calculation of dietary intake for the sum of dioxins (PCDDs/PCDFs) and dioxin-like PCBs. Comparison between a first tier approach based on European legal maximal limits in food (Commission Regulation (EC) N°1881/2006 of 19 December 2006) and a second tier approach based on results from the European SCOOP task 3.2.5 [12] (see the list for abbreviations)**

| Group-TWI [1]: 14 pg TEQ/kg bw/week | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | First approach: GEMS x MLs | | | | | Second approach: SC x SC [2] | | |
| Commodities | MLs (dioxins) (pg WHO-PCDD/F-PCB-TEQ/g fat) | $C_i$ (GEMS) (g/p/d) | GEMS code | % fat | TMDI (pg TEQ/p/d) | EDI mean (pg TEQ/kg bw/d) [3] Lowest M.S. | Highest M.S. | Medium |
| bovine/ovine fat | 4.5 | 0.1 | MF 822 | / | 0.45 | 0.4 | 4.5 | 1.5 |
| poultry fats | 4 | 5.3 | PF 111 | / | 21.2 | | | |
| pig fat | 1.5 | 7.3 | FA 818 | / | 10.95 | | | |
| fish and seafood | 8 pg TEQ/g fresh weight | 46.8 | total | / | 374.4 | | | |
| milk and milk products | 6 | 336.1 | AO 31 | 3 | 60.498 | | | |
| eggs | 6 | 37.6 | PE 112 | 10 | 22.56 | | | |
| vegetable oils and fats | 1.5 | 38.8 | total | / | 58.2 | | | |
| | | | total | (pg TEQ/p/d) | 548.258 | | | |
| | | | | (pg TEQ/kg bw/d) [3] | **7.83** | | | |
| | | | | % TDI [4] | 391% | 20 % | 225 % | 75 % |

[1] Derived by SCF (2001) [24] for the group PCDDs/Fs and dioxin-like PCBs
[2] Values are presented for the chemical group PCDDs/Fs
[3] Assuming a 70 kg body weight adult
[4] Assuming that the tolerable weekly intake is equally spread over the week and then that TDI = 2 pg TEQ/kg bw/d.

**Table 8. Synopsis of the calculated dietary intakes for dioxins & PCBs (in TEQ values), B[a]P, Cd, Hg and Pb. Comparison between a first tier approach based on legal maximal limits in food and a second tier approach based on results from European SCOOP tasks (see the list for abbreviations)**

| | | First tier approach: GEMS x MLs | | Second tier approach: SC x SC [5] | | |
|---|---|---|---|---|---|---|
| | | | | EDI mean (µg/kg bw/d) [3] | | |
| | PTWI [1] (µg/kg bw/week) | TMDI (µg/kg bw/d) [2, 3] | %TDI [4] | Lowest M.S. | Highest M.S. | Medium |
| PCDD/F & DL-PCBs | 14 $10^{-6}$ | 7.83 $10^{-6}$ | 391% | 0.4 $10^{-6}$ | 4.5 $10^{-6}$ | 1.5 $10^{-6}$ |
| NDL-PCBs | 0.02 [(6)] | 0.1157 | 578% | / | / | / |
| B[a]P | / [7] | 0.0039 | / | 0.0002 | 0.00457 | 0.00145 |
| Cd | 7 | 1.4 | 140% | 0.0056 | 0.36 | 0.2057 |
| Hg [8] | 5 (total Hg)<br>1.6 (MeHg) | 0.33 | 46.8%<br>146% | 0.0196 | 0.2057 | 0.079 |
| Pb | 25 | 2.84 | 79.5% | 0.0157 | 1.9 | 0.485 |

[1] Set by JECFA

[2] Calculated using MLs from Commission Regulation (EC) N°1881/2006 of 19 December 2006 for B[a]P and heavy metals and MLs from Belgian Royal Decree of 6 March 2002 (modifying Royal Decree of 19 May 2000) for the sum of seven indicator PCB (28, 52, 101, 118, 138, 153, 180)

[3] Assuming a 70 kg body weight adult

[4] Assuming that the tolerable weekly intake is equally spread over the week and then that TDI = PTWI/7

[5] B[a]P: SCOOP, 2004 [13] and heavy metals: SCOOP, 2004 [11]

[6] Oral minimum risk level (MRL) [µg/kg bw/day], equivalent to a TDI, derived by ATSDR for chronic exposure to PCBs (Aroclor 1254)

[7] B[a]P being classified by the International Agency for Research on Cancer (IARC) as a potential carcinogen for humans (group 2A), no TDI has been allocated.

[8] TMDI calculation and SCOOP exposure assessment are based respectively on MLs (in fish and seafood) and contamination data for total Hg. However, organic methylmercury (MeHg) is the most toxic chemical form of Hg and is mainly present in fish and seafood (more than 90% of the total Hg)

## Realistic intestinal concentrations of food contaminants

Experimental realistic intestinal concentrations were generated on the basis of calculated TMDIs. In order to obtain a range of plausible concentrations occurring in the intestine, two extreme situations of dilution of contaminants in the bolus have been taken into consideration. Minimal and maximal concentrations have been calculated on the basis of the following hypotheses:

- Maximal concentration (ppb or µg/L): the daily intake of a chemical (µg/person/day) is diluted during a meal in 1L of body fluid, with the entire daily dose ingested in one meal.
  [Max] = TMDI (µg/person/day)

- Minimal concentration (ppb or µg/L): the daily intake of a chemical (µg/person/day) is diluted during a meal in 3L of body fluid, with 3 equal meals per day.
  [Min] = [Max]/9

Since our interest is to investigate chronic hazard linked to chemical substances present in our diet at concentrations as high as plausible, it is relevant to use TMDIs in order to generate experimental concentrations. Therefore, a range of experimental concentrations have been generated for each contaminant on the basis of the calculated TMDI. They are presented in Table 9. In order to obtain working concentrations easily usable, we indicated between brackets the maximal concentrations rounded to the quarter of unit (0.25 - 0.5 - 0.75 - 1.00).

## CONCLUSIONS AND RECOMMENDATIONS

In this chapter, we have proposed a methodology allowing to estimate the real concentrations occurring in the gastro-intestinal tract. These concentrations can be used for *in vitro* cellular testing.
The deterministic approach was followed in the exposure assessment process. This method, called “point estimates” is very simple and refers to a method whereby a fixed value for food consumption is multiplied by a fixed value for residue or concentration level and the intakes from all sources are then summed up. However, it has the particularity to significantly overestimate actual exposure. In addition, no measurement of confidence is associated to the estimated daily intake [4].
The two dilution models used to generate intestinal concentrations from calculated TMDI do not take into account other parameters which can influence the amount of chemicals really present in the intestinal lumen before the intestinal transport, such as bioaccessibility and inter-individual variations (e.g. gastric and intestinal lumen volume, rate of saliva secretion, rate of pancreatic or biliary secretions, composition of these secretions). Bioaccessibility is an important parameter which nowadays can be studied in digestive-like conditions. For instance, aflatoxin B1 (AFB1) in peanuts and OTA in buckwheat show a bioaccessibility of 91% and 63%, respectively, in a digestive *in vitro* model developed at the National Institute of Public Health and the Environment (RIVM) of the Netherlands [25].
It is also well known that the quantity of residues effectively occurring in food can be lower than in raw materials after processing (e.g. removing of inedible parts, washing, cooking)

**Table 9. Range of realistic experimental concentrations (ppb or µg/L) with working concentrations between brackets (ppb or µg/L)**

| Contaminants | [Min] (ppb) | [Max] (ppb) | Contaminants | [Min] (ppb) | [Max] (ppb) |
|---|---|---|---|---|---|
| ***Mycotoxins*** | | | Paraquat | 22.8 | 205 (250) |
| AFB1 | 0.053 | 0.47 (0.5) | Permethrin | 83.6 | 753 (750) |
| DON | 18.5 | 166 (250) | Procymidone | 93.9 | 845 (1000) |
| FB1 + FB2 | 1.51 | 13.62 (25) | Propiconazole | 6.6 | 59.4 (75) |
| OTA | 0.077 | 0.69 (0.75) | Pyrethrins | 10.27 | 92.4 (100) |
| PAT | 0.14 | 1.26 (2.5) | Thiabendazole | 517 | 4651 (5000) |
| ZEA | 1.85 | 16.64 (25) | Thiram | 51.9 | 467 (500) |
| ***Pesticides*** [1] | | | Tolylfluanid | 99.3 | 894 (1000) |
| Aldrine/dieldrine | 5.72 | 51.5 (50) | Vinclozolin | 89.5 | 806 (1000) |
| Benomyl | 12.15 | 109.3 (250) | ***Veterinary drugs*** | | |
| Captan | 290 | 2607 (5000) | Tetracycline | 6.46 | 58.18 (75) |
| Chlorpyrifos | 95.6 | 860 (1000) | Chloramphenicol | 0.02 | 0.178 (0.25) |
| DDT | 10.03 | 90.3 (100) | ***Environ. contaminants*** | | |
| Dichlorvos | 21.7 | 195 (250) | Dioxins & DL- PCBs | $3.07\ 10^{-5}$ | $5.5\ 10^{-4}$ ($5\ 10^{-4}$) |
| Dicofol | 71.2 | 641 (750) | NDL-PCBs | 0.9 | 8.1 (10) |
| Endosulfan | 45.5 | 409 (500) | B[a]P | 0.03 | 0.27 (0.5) |
| Glyphosate | 168 | 1511 (2500) | Cd | 10.9 | 98 (100) |
| Imazalil | 198 | 1783 (2500) | Pb | 22.09 | 198.8 (250) |
| Imidacloprid | 43 | 388 (500) | Hg | 2.6 | 23.4 (25) |
| Lindane (γ-HCH) | 0.73 | 6.58 (7.5) | | | |

[1] Concentration calculated with TMDI generated from Codex MRLs

and storage (e.g. pesticides, mycotoxins), after addition of food acids or after dilution [4]. On the other hand if contaminants do not occur naturally, they can be formed during industrial processing (e.g. dioxins, PCBs) or domestic cooking (e.g. B[a]P). Sometimes, levels can increase due to water removal (e.g. in tea leaves) or due to accumulation of lipophilic materials in the fatty parts of foods [4].

Despite slight imprecision, this methodology was in accordance with the aim of the study since we expected to test theoretically maximal concentrations representative of chronic human exposure in order to cover a large range of consumers in chronic hazard characterization. Furthermore, sensitivity of experimental devices does not always permit the use of low concentrations. In order to obtain a more precise estimation of the daily intake, probabilistic assessment should be considered instead of point estimates. Probabilistic assessment is a very useful approach when the first deterministic estimation is at or above ADI/TDI, in order to refine the assessment and to consider if chemical exposure is a public health issue. Point estimates are commonly used as a first step in exposure assessment and are considered to be the most appropriate for screening purposes [26]. Inherent in the point estimate models are the assumptions that all individuals consume the specified food(s) at the same level, that the food component is always present in the food(s) and that it is always present at an average/high level [4].

Intake calculation (TMDI) has been performed with consumption data related to adults (GEMS/FOOD Regional Diets) and exposure estimates reported from SCOOP tasks refer to the adult population only. As a consequence children have not been taken into account in the exposure assessment and in the generation of experimental concentrations.

We have set up a very useful, rapid and easy methodology aiming the calculation of experimental realistic intestinal concentrations of contaminants, mimicking chronic human (adult) exposure, which can be directly applied on *in vitro* cultured cells. This

methodology can be applied to a large variety of chemicals for which maximum levels exist in legislation. Cell response permit to obtain a quick hazard characterization linked to these chemicals at realistic exposure and to assess more plausible risks for human health.

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# *In vitro* cellular and molecular tools for studying the effects of food contaminants at the gastrointestinal level

**T. Sergent, C. Jassogne, Y. Larondelle and Y.-J. Schneider**

*Institut des Sciences de la Vie & Université catholique de Louvain,*
*Biochimie cellulaire, nutritionnelle & toxicologique,*
*Croix du Sud 5/3, B-1348 Louvain-la-Neuve, Belgium.*
*e-mail: yjs@uclouvain.be*

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Food contaminants; Intestinal barrier; Caco-2 cells; Mycotoxins; Pesticides; PAHs;

## SUMMARY

Food is the major route of exposure to contaminants such as dioxins, mycotoxins, heavy metals, pesticides, polycyclic aromatic hydrocarbons, drugs or hormones. Adequate risk management relies on a better knowledge of the toxicological profile of these substances. As intestine is the first barrier encountered by food contaminants following their ingestion in the diet, the human epithelial intestinal Caco-2 cell culture system, a well-known *in vitro* model of the intestinal mucosa, was used to assess the biological effects of such toxicants. General cytotoxicity assays and specific endpoints measurements related to intestinal enzyme functions are described here with their application to particular food contaminants. The MTT and LDH assays provided indications of the mycotoxin and pesticide cytotoxicity. However, more specific bioassays permitted to detect a toxic effect of contaminants at lower, non-cytotoxic, concentrations corresponding to plausible intestinal concentrations that should be encountered in the gastrointestinal tract after ingestion of a normal diet. For instance, a micro-EROD-assay was set up as a rapid and sensitive screening tool for measuring the induction of cytochrome P4501A1 activity by xenobiotics. It allowed to detect an effect of some pesticides, mainly imazalil, benomyl and thiabendazole. Furthermore, some mycotoxins were shown to perturb various intestinal functions by affecting the integrity of the epithelium, by interacting with polyphenols, plant-food constituents, for the MRP-2 efflux pump, by activating inflammation related parameters. Overall, the use of molecular and cellular tools for detecting food contaminants by their biological effects constitutes a convenient and well-sensitive approach in human health risk assessment.

## INTRODUCTION

Food safety can be adversely affected by the presence of various kinds of chemical contaminants including natural toxins, e.g. mycotoxins, pesticides and environmental pollutants, e.g. heavy metals, persistent organic pollutants (POPs), *i.e.* dioxins, polycyclic aromatic hydrocarbons (PAHs) or polychlorinated biphenyls (PCBs), hormones or antibiotics. Beside economical loss considerations, health effects on humans and animals due to food contamination could be enormous and are generally neither fully characterized nor taken into account. Therefore, in addition to the importance of detecting the presence of contaminants in food and feed using (bio)chemical methods, it is of crucial importance to determine, in experimental

conditions as close as possible to the real conditions, the contaminant level above which adverse effects may be expected. *In vitro* methods could be the most appropriate tool. Furthermore, among all the substances that are present simultaneously in the gastrointestinal tract, contaminants may interact mutually, but also with nutrients as well as with natural bioactive molecules. This obviously complicates toxicology evaluations dramatically and therefore, mandatory requires high throughput *in vitro* screening methods.

To determine both biological and toxicological activities of food contaminants, cellular *in vitro* methods are good alternatives to *in vivo* methods, replacing and reducing the use of animals, avoiding species differences and giving more reproducible results [1]. In health risk assessment, relevant *in vitro* methods are largely based on mammalian cell cultures, primary cells or animal/human immortalized or continuous cell lines. *In vitro* methods allow detecting a direct cytotoxicity of chemicals that could correspond to an acute intoxication occurring rather at high doses of contaminants. They are usually rapid, applicable to screen a large number of samples, and often less expensive than chemical analysis. Such methods measure general cytotoxicity (dead/viable cells) or reduced cell multiplication and have been applied to mycotoxin's toxicity [1-3]. Other *in vitro* methods can be used to detect the indirect impact of contaminants on the cellular activity by measuring specific endpoints, depending on the cell type or on the contaminant. An example is the use of recombinant cell bioassay systems to detect and quantify dioxins and dioxin-like chemicals [4-5]. These *in vitro* methods should be applied to achieve lower detection limits and to increase insight in quantitative relationships between dose and response. Such an approach corresponds to what could happen in case of chronic intoxication occurring more likely at environmentally relevant concentrations. Finally, under appropriate culture conditions, some animal/human cells are also able to generate metabolites by phase I & II reactions and to decrease intracellular concentrations by active efflux (phase III), which dramatically affect the toxicological profiles of the substances to be tested.

Here we report on different *in vitro* methods based on the human intestinal Caco-2 cell culture system to detect the effects of various food contaminants. Indeed, the intestinal mucosa is the first barrier controlling the entry of foreign compounds into the underlying tissues and is recognized as playing a major role in the regulation of the bioavailability of nutrients, drugs, but also contaminants. Moreover, it is now clear that nutrients and xenobiotics present in the gastrointestinal tract influence the expression and/or activity of some key proteins involved in the absorption, metabolization and efflux processes [6-8]. Thereby, they can also modulate the bioavailability of unrelated substances with potential adverse effect for health. If the impact of intestinal metabolism on drug bioavailability is well studied, few reports have attempted to explore the effect of food contaminants at the intestinal level [9]. The human Caco-2 cell line is a well established and validated model of the human intestinal epithelium [8, 10-16] widely used to study drug absorption. Thanks to this model, we have already characterized the mechanisms of the intestinal absorption of two mycotoxins, ochratoxin A (OTA) and deoxynivalenol (DON) [17-19]. In this study, we used the Caco-2 cells to determine direct cytotoxicity of mycotoxins and pesticides, as well as to evaluate the effect of various food pollutants on intestinal functions related to intestinal permeability, xenobiotic-metabolizing enzyme and efflux pump activities, and inflammation parameters.

## MATERIALS AND METHODS

### *Chemicals*

Culture reagents were purchased from Invitrogen (Carlsbad, CA); biochemicals, mycotoxins, pesticides, polyphenols, 3-methylcholanthrene (3-MC), benzo(a)pyrene (B(a)P) were from Sigma-Aldrich ($S^t$ Louis, MO) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was from Wellington Laboratories Inc. (Guelph, Ontario, CA).

### *Cell culture*

Caco-2 cells (ATCC, Rockville, MD), used between passages 36 and 55, were routinely grown [12-13], in a serum-free medium (5:5:1 (v:v:v) mixture of Iscove's Modified Dubelcco's, Ham's F12 and NCTC 135 media, [20]) supplemented with glucose to 16 mM, 1 µg/ml insulin, 1 ng/ml EGF, 10 µg/ml albumin-linoleic acid, 2 nM T3 and 100 nM hydrocortisone, on type I collagen (Sigma-Aldrich) precoated flasks (Greiner, Frickenhausen, DE).

### *Cytotoxicity assays*

The cytotoxicity of mycotoxins and their carrier vehicle (dimethyl sulfoxide (DMSO) or ethanol) on Caco-2 cells was evaluated by the MTT assay. Cells, seeded in 96-wells plates (Nunc, Roskilde, DK) at a density of 3.5 $10^4$ cells/$cm^2$, were incubated, 24 h after seeding, with mycotoxins at concentrations between 0-100 µM for 48 h. MTT assay was carried out as in [21], using 100 µl of MTT (0.5 mg/ml in PBS), 2 h incubation at 37°C, solubilization in 100 µl DMSO and reading at 500 nm (Spectracount, Packard, Warrenville, IL). Cytotoxicity was calculated as percent relative to absorbance obtained from cells exposed to the medium containing the corresponding solvent.

At the end of the EROD assay experiments, the cytotoxicity of pesticides was determined on the cell culture supernatants by the lactate dehydrogenase (LDH) assay purchased as a kit (Cytotoxicity Detection Kit, Roche diagnostics, Mannheim, DE). Maximal LDH release was determined by exposing the cells to 1% (v/v) Triton X-100. The reduced formazan reaction product was measured as above.

### *Micro-EROD-assay*

Cells, seeded in 96-wells plates at 40,000 cells/well, were cultivated until 8-days post-confluence, to allow cell differentiation, with culture medium changing 3 times per week. Cells, treated in different conditions as described in the results, were rinsed with PBS and incubated for 1 h at 37°C with 5 µM 7-ethoxyresorufin (Sigma) in the phenol red-free basal Eagle's medium (Invitrogen). Fluorescence was measured, in the supernatants, with excitation and emission wavelengths of respectively 530 and 585 nm in a SFM25 fluorimeter (BioTek Instruments, Winooski, VT). The cell protein content was determined by the method of Lowry *et al.* [22].

### *Transient transfection and reporter gene analysis*

*The plasmids.* The pT81Luc/3 x DRE (Dioxin Responsive Element) was a gift from Dr. Schwarz (Department of Toxicology, University of Tubingen, DE) and has been described previously [23]: it was constructed by cloning three copies of a functional DRE motif and adjacent bases upstream of the luciferase reporter gene. The β–galactosidase expression vector CMV-*LacZ* was used as transfection efficiency control and was a gift of Dr. Reszohazy (ISV, UCL, Louvain-la-Neuve, BE).

*The co-transfection.* Caco-2 cells were seeded on 24-well plates at 20,000 cells/$cm^2$. Transient co-transfection was performed, 24 h later, with the jetPEI™ transfection Reagent (PolyPlus-transfection, Illkirch, FR), according to manufacturer instructions, by using 1 µg of pT81Luc/3 x DRE and 500 ng of CMV40-LacZ. After 24 h incubation,

cells were washed with PBS and fresh media containing the appropriate treatments were added for 24 h. Cells were then collected in 100 µl/well of reporter lysis buffer. Lysates were centrifuged at 13,000 g for 30 s and luciferase activity was determined on supernatant with the Luciferase Assay System kit from Promega. The β–galactosidase activity was determined with the β-gal Reporter Gene Assay from Boehringer (DE). The luciferase activity was normalized against the β–galactosidase activity.

*Transepithelial electrical resistance (TEER) measurement*

Cells were seeded on type I collagen precoated poly(ethylene terephtalate) microporous membrane (1 µm pore diameter, Whatman SA, Louvain-la-Neuve, BE) in bicameral insert (24 mm diameter, 5 $cm^2$ growing area) at 120,000 cells/$cm^2$ and cultivated for 21 days to allow complete differentiation. The integrity of the monolayers was checked by measurement of the TEER with an epithelial tissue voltohmmeter (Endohm 24, World Precision Instruments, Sarasota, FL).

*Multidrug resistance-associated protein-2 (MRP-2) efflux pump activity assay*

The assay was based on the transport of [$^3$H]OTA, a MRP-2 substrate [17], from the apical to the basolateral side of differentiated cells. Cells, cultivated until 21 days as described for TEER measurement, were first checked for monolayer integrity. The transport medium was Hank's balanced salt solution (HBSS) containing 5 mM glucose and 10 mM Hepes (pH 7.4) or 10 mM Mes (pH 6.0) for the lower and the upper compartments of the inserts, respectively. The lower compartment contained 2.8 ml of the transport medium supplemented with 1 % (w/v) bovine serum albumin. Transepithelial passage was assayed by adding, in the upper compartment, 1.8 ml of transport medium supplemented with 7.5 nM [$^3$H]OTA as well as [$^{14}$C]mannitol, as internal control. Polyphenols (PPs) or MK571, a specific MRPs inhibitor, were further added in the upper compartment at the same time as OTA. After 3 h, media from the upper and lower compartments were collected separately and an aliquot was analyzed by liquid scintillation spectrometry (Packard Tri-Carb 1600 TR, Packard, Meriden, CT) after dispersion in 2 ml of Aqualuma® (Lumac Lsc, Groningen, NL).

*Assessment of the mitogen-activated protein kinase (MAPK) phosphorylation*

The cells were cultivated on a microporous membrane for 21 to 27 days, as described for TEER measurement. Cells were then incubated overnight in the same medium without EGF and insulin. Thereafter, DON was added in the upper compartment at various concentrations for 24 h.

The cells were analyzed by Western blotting for the activation of the MAPKs. They were washed in ice-cold phosphate buffer and suspended in lysis buffer (phosphate buffer containing 1 % (w/v) Igepal CA630, 0.5 % (w/v) sodium deoxycholate and 0.1 % (w/v) sodium dodecylsulfate, supplemented with 0.2 mM sodium ortho-vanadate, 50 mM sodium fluoride and 1 % (v/v) of a protease inhibitor cocktail for use in tissue culture media (Sigma-Aldrich)). The lysate was centrifuged at 12,000 g for 10 min at 4°C. The protein content was determined in the resultant supernatant by the Bradford assay (Bio-Rad Laboratories, Hercules, CA). 20 µg of total cellular proteins were resolved by SDS-PAGE in an 11 % (w/v) acrylamide gel and transferred to a Hybond-P polyvinylidene difluoride (PVDF) membrane (AmershamBiosciences, Little Chalfont, UK). After blocking with a Tris buffered saline solution (pH 7.6, 0.02 M) containing 0.05 % (v/v) Tween 20 (TBST) and 5 % (w/v) non-fat milk powder, the membrane was incubated overnight at 4°C with an antibody raised against either phospho-p44/p42 Erk, phospho-p38 MAPK or phospho-SAPK/JNK (rabbit IgG) at a 1:1,000 dilution in TBST containing 0.5 % (w/v) milk powder. The membrane was washed three times with TBST and incubated with HRP-conjugated goat anti-rabbit IgG at a 1:2,000

dilution in TBST containing 0.5 % (w/v) milk powder for 1 h. After three washings with TBST, the bound HRP-conjugated antibody was detected with an enhanced chemiluminescence (ECL-plus) detection kit (Amersham Biosciences) according to the manufacturer's instructions. The membrane was then stripped and reprobed as described above with a specific antibody that recognizes both the phosphorylated and unphosphorylated forms of the MAPK.

*Data analysis*

Results were expressed as means ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Systat 5.2.1 (Systat Inc., Evanston, IL).

## RESULTS AND DISCUSSION

*General cytotoxicity assays*

Mycotoxins are contaminants that may be present in food and feed and have been associated with human and animal diseases. In order to possibly detect their presence in food by their cytotoxicity, we used the MTT assay to screen various mycotoxins on the intestinal cell growth. As presented in Figure 1, all the tested mycotoxins, except fumonisin $B_1$ ($FB_1$), showed a clear dose-dependent effect on the metabolic activity of intestinal Caco-2 cells. DON and OTA were the most cytotoxic with significant effect at very low concentrations, 0.2 and 0.12 µg/ml, respectively. Penicillic acid (PEN), citrinin (CIT) and zearalenone (ZEN) had a weak cytotoxic effect at low concentrations, but became as toxic as DON or OTA at higher concentrations. $FB_1$ had no cytotoxic effect in the whole range of concentrations.

In the case of DON, the minimal concentration detected as significantly cytotoxic corresponds to a concentration that could contaminate cereals and is considered to be as hazardous to animals as to humans. For the other mycotoxins, the MTT assay is not sensitive enough to allow their detection in food samples.

$IC_{50}$, defined as the toxin-dose to reduce cell viability by 50 %, can be calculated from these curves and should allow comparison with results obtained in other studies.

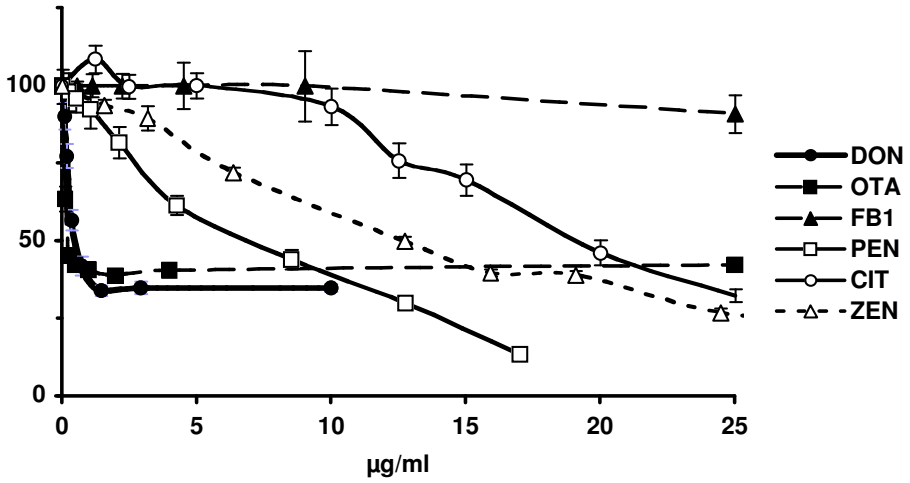


**Figure 1. Dose-effect of various mycotoxins on Caco-2 cells growth. Cellular metabolic activity was determined by the MTT assay after incubation of proliferating cells, for 48 h, in the presence of mycotoxins at different concentrations. Results are expressed as percentage of control response and are means of 2-3 independent experiments ± SD (n = 8-12)**

Another example of this *in vitro* approach is provided by a study on several pesticides of common use. Pesticides are environmental food contaminants. To evaluate their toxicities on intestinal cells, plausible concentrations of pesticides that could be reached in the intestine following ingestion of "normally" contaminated food were calculated as described in the precedent chapter by Ribonnet *et al.*: the values, presented in Table 1, were derived from daily estimated intakes, deterministically calculated assuming that the food contaminant is ingested in one meal, diluted in 1 liter of gastrointestinal fluid, and is totally bioaccessible.

**Table 1. Relevant pesticides and concentrations used for experimental studies**

| Pesticide | Working concentration (ng/ml) | Pesticide | Working concentration (ng/ml) |
|---|---|---|---|
| Aldrin | 25 | Propiconazole | 75 |
| Dieldrin | 25 | Benomyl | 2500 |
| Dicofol | 500 | Thiabendazole | 5000 |
| α-endosulfan | 250 | Captan | 5000 |
| β-endosulfan | 250 | Imidacloprid | 500 |
| Lindane | 7.5 | Paraquat | 250 |
| pp'-DDT | 250 | Procymidon | 2500 |
| pp'-DDE | 250 | Vinclozoline | 1000 |
| Chlorpyrifos | 1000 | Pyrethrum | 100 |
| Dichlorvos | 250 | Permethrin | 750 |
| Glyphosate | 2500 | Thiram | 500 |
| Atrazine | 0.25 | Tolylfluanide | 1000 |
| Imazalil | 2500 | | |

The effect of different pesticides, used at realistic intestinal concentrations, was determined on Caco-2 cells by the LDH assay, which measures the activity of LDH, a cytosolic enzyme released by the damaged or lyzed cells, in the cell culture media, and is therefore a cell mortality index.

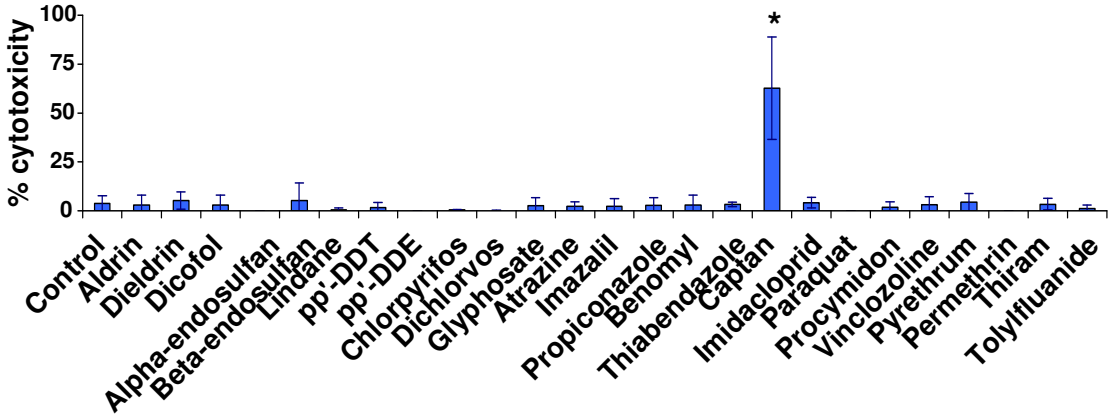


**Figure 2. Cytotoxicity of pesticides on Caco-2 cells, as determined by the LDH assay. Cells were incubated for 24 h in the presence of pesticides, used at realistic intestinal concentrations (Table 1), or their vehicle (DMSO or ethanol) as control. Media were then collected and assayed for LDH activity. Results, expressed as percentage of total LDH release, are means of 3 independent experiments ± SD (n = 9). * indicates P < 0.05, as compared with vehicle control**

Figure 2 shows that for all the pesticides, except for captan, the mortality level was of same magnitude than for control cells and remained lower than 5 % of cytotoxicity. Among the 25 tested pesticides, only captan provoked a strong and significant toxicity with ± 60 % of cell mortality. This result suggests that captan could induce an acute toxic effect on the intestine after ingestion of common contaminated food.
This simple method is sensitive enough to detect the final toxicity of food contaminants used at realistic concentration. Moreover, it is not a cell destructive method and it can systematically be applied as a complementary tool in more specific cellular assays.

As illustrated in the case of mycotoxins and pesticides, *in vitro* cytotoxicity tests on living cultured cells represent an alternative approach to *in vivo* classical evaluations. The MTT assay, on one hand, provides a good proportional relationship between the metabolic activity of cells and the cell number, but to be quantitative, this assay must be applied on cell cultures in proliferation state. LDH assay, on the other hand, is another approach to measure toxicity in confluent cell cultures. Nevertheless, it should be stressed that, although these *in vitro* methods appear accurate at evaluating a direct toxicity on a particular cell type, they totally lack specificity since they are unable to identify the molecule(s) responsible of the deleterious effect. Only (bio)chemical approaches may provide information on that particular point. Moreover, the biological methods, so far as relevant cell culture systems are used, allow taking into account the presence of metabolites that may be formed upon biotransformation of the parent molecule by cellular enzymes. Finally, the biological tests also integrate the actual intracellular concentration to which cells are exposed, since this may be modulated by cellular mechanisms, among them the activity of various efflux pumps.

## *Specific endpoints*

### *A gene recombinant assay to detect dioxin-like contaminants*

Halogenated aromatic hydrocarbons (HAHs), such as TCDD, PAHs, PCBs, are persistent environmental contaminants, known to exert their biological and toxicity effects by their ability to bind and activate the aromatic hydrocarbon receptor (AhR), a ligand-dependent transcription nuclear factor, and the AhR signal transduction pathway. After binding to the AhR, the complex AhR-TCDD, or related HAHs, migrates into the nucleus where it associates with a related nuclear protein. The formed complex binds to a specific DNA recognition site, the dioxin responsive element (DRE), stimulating the transcription of particular genes, such as the *CYP1A1* resulting in an increased expression of CYP1A1. Molecular bioassays measuring AhR-dependent gene expression using stable or transitory transfected recombinant mouse and human cell lines containing DRE and an AhR-responsive reporter genes have been developed that can detect TCDD and other AhR agonists, as review by Denison *et al.* [4]. Here we have used a CALUX (chemically activated luciferase expression) bioassay with transiently DRE-transfected Caco-2 cells to assess the effect of dioxin-like contaminants. As illustrated in Figure 3 A., TCDD and PAHs, *i.e.* 3-MC and B(a)P, activate this bioassay system inducing the luciferase activity from 2 to 3-fold over the control. Our results confirm that TCDD, 3-MC and B(a)P are AhR ligands and validate this gene recombinant system as a tool to detect dioxin-like contaminants.
Furthermore, this tool can be used to detect AhR antagonists on living cells: Figure 3 B. shows that genistein, quercetin and chrysin, three plant-food constituents, blocked the TCDD-induced luciferase response and therefore can be considered as potent AhR antagonists in Caco-2 cells. This result, in agreement with studies realized in different cell systems [24-26], indicates that certain compounds in fruits and vegetables could reduce AhR-dependent biological effects caused by dioxins, since these foodstuffs

abundantly contain various natural antagonists. In this latter application, AhR gene recombinant bioassays would allow the selection of AhR antagonists, as a promising tool for cancer prevention and chemotherapy or, generally speaking, to diminish the risks associated with unavoidable exposures to AhR inducers.

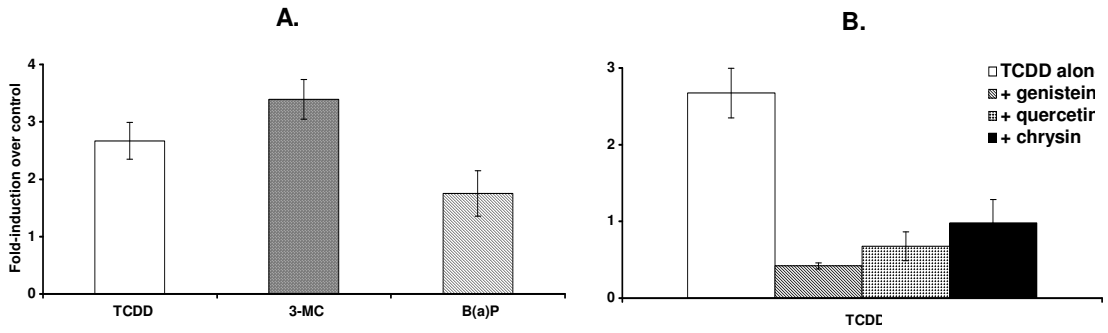


**Figure 3. A. Effect of TCDD, 3-MC and B(a)P on DRE-driven reporter gene expression in transiently transfected Caco-2 cells. After transfection, cells were incubated with 1 nM TCDD or 1 µM 3-MC or B(a)P for 24 h. B. Effect of genistein, quercetin and chrysin on the TCDD-induced luciferase activity in the DRE-driven reporter gene assay. After transfection, cells were incubated with 1 nM TCDD in the presence or absence of 50 µM genistein, quercetin or chrysin.**
**Data are shown as fold-induction over cells treated with vehicle alone and represent means ± S.E.M. from 3 independent experiments (n = 6 to 9)**

*The micro-EROD-bioassay to detect CYP1A1 metabolizing enzyme inducers*

Intestinal xenobiotic cytochrome P450 (CYP)-mediated biotransformations are of particular importance for human health. Most notably, the CYP1A1 isoform, a highly inducible enzyme mainly regulated by the AhR, is widely known as an activator of putative carcinogenic food contaminants such as PAHs [27]. Therefore its metabolic activation is generally considered as potentially harmful.

We have set up a micro-EROD-bioassay with differentiated living Caco-2 cells to screen food contaminants as CYP1A1 inducers: the assay, realized in 96-wells plates, is based on the ability of CYP1A1 to metabolize 7-ethoxyresorufin (EROD) to resorufin, a fluorescent product, in induced Caco-2 cells. This *in vitro* assay, firstly standardized with TCDD, the most potent CYP1A1 inducer, is very sensitive and allows to detect as low as 25 pg/ml (2.5 pg/well) TCDD.

This method has been used to study the effect of B(a)P, a PAH regarded as the most potent carcinogen, incubated for various durations, at different concentrations from 1 to 100 ng/ml: 1 ng/ml represents the realistic intestinal concentration, as defined above, whereas 100 ng/ml is a concentration widely used in toxicological studies. As shown by Figure 4, whatever the concentration, B(a)P increased the CYP1A1 activity in function of the incubation duration with maximal effect at 6 h. At the highest B(a)P concentration, the inducing effect on the CYP1A1 activity was observed until 24 h incubation, although its extent was decreased. At the other B(a)P concentrations, after the maxima at 6 h, the inducing effect diminished strongly and became negligible to nil. This time- and dose-dependent effect may probably be explained by the B(a)P metabolization in Caco-2 cells. B(a)P is a strong CYP1A1 inducer, but also a substrate of this phase I enzyme: different metabolites are formed with the B(a)P-7,8-diol-9,10-epoxide as the ultimate reactive carcinogenic molecule [27-29]. In Caco-2 cells, Buesen *et al.* [28] have shown that B(a)P was also metabolized, mainly to non-toxic sulfate

conjugates: after 8 h incubation, B(a)P and several metabolites were detected in the cells and media whereas after 24 h incubation, B(a)P was found at a very low level in the cells and was no more detected in the culture media. Our results are also in agreement with Wen *et al.* [30] who described, in the human hepatic HepG2 cells, a concomitant increase in B(a)P-DNA binding and CYP1A1 activity upon 6 h incubation in the presence of high B(a)P concentration (1 µM = 250 ng/ml), followed by a decrease at 24 h incubation.

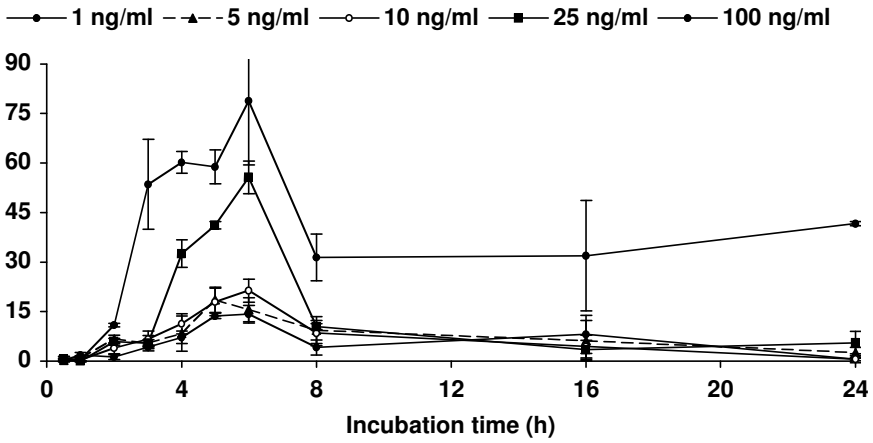


**Figure 4. Time-course effect of B(a)P at different concentrations on the CYP1A1 activity, as determined by the micro-EROD-bioassay. 8-days post-confluent Caco-2 cells were incubated with 1, 5, 10, 25 or 100 ng/ml B(a)P for various durations from 30 min to 24 h. Results are means ± SD of 3 independent experiments (n = 9)**

The micro-EROD-bioassay was further used to screen other food contaminants, as illustrated in Figure 5 for pesticides, with B(a)P as positive control. The pesticides were assayed at realistic intestinal concentrations. Results show that imazalil strongly induced the CYP1A1 activity to a level comparable to B(a)P, used here at a classical "toxicological" concentration. Other pesticides, *i.e.* benomyl, thiabendazole and chlorpyrifos, also increased significantly the CYP1A1 activity, but to a lesser extent. They are very few reports in the literature about the effects of pesticides on CYP enzymes and most studies reported on hepatic metabolism. One study reported an enhanced EROD activity in small intestinal microsomes of mices fed with an imazalil daily dose of 10 mg/kg that represents a dose 1000 times higher than that of acceptable daily intakes [31]. Other studies, in human hepatic or hepatoma cells, have shown that benomyl and thiabendazole were CYP1A1 inducers [22-33].

The CYP1A1 activity assay allowed us to point out imazalil as a strong CYP1A1 inducer, as potent as B(a)P. Studies are now in progress to understand the activation pathway of imazalil.

Another possible application of the micro-EROD-bioassay is the screening of food contaminant mixtures. EROD bioassays have already been used, to detect various environmental pollutants, in cell lines and primary cultures from a diverse range of species and cell type, mostly in primary cultures of hepatocytes, but not in intestinal cells, with good correlation between EROD induction and chemical analysis of contaminant levels [34-35].

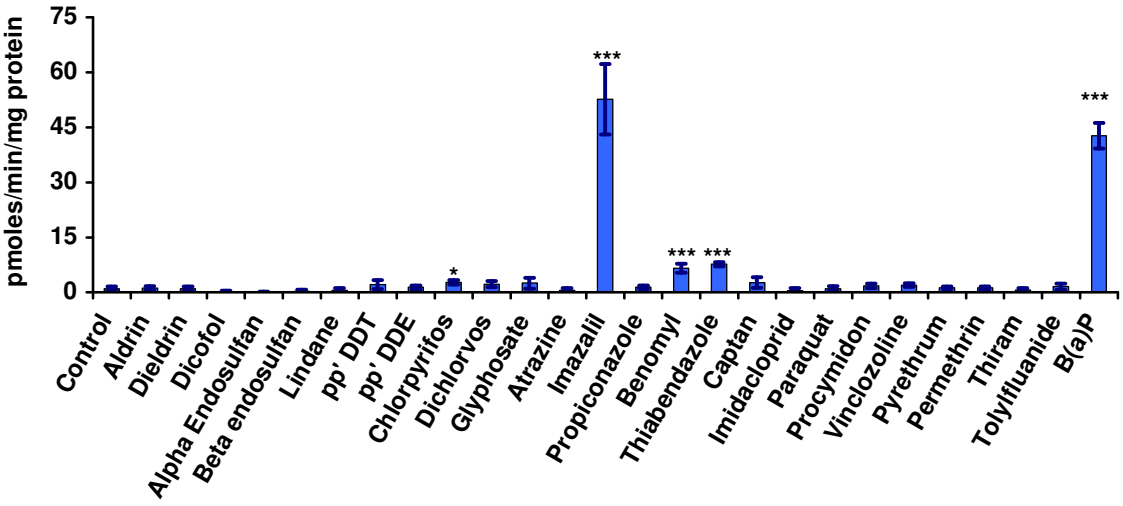


**Figure 5. Pesticide effect on CYP1A1 activity, as determined by the micro-EROD-bioassay. Differentiated Caco-2 cells were incubated with realistic intestinal concentrations of pesticides, as described Table 1, 1 µM B(a)P or their vehicle (DMSO or ethanol) as control for 24 h. Results are means ± SD of 3 independent experiments (n = 9). *, *** indicate P < 0.05 and P < 0.001, respectively, as compared to the appropriate control condition. (LDH assay, depicted Figure 2, and CYP1A1 assay, presented Figure 5, were realized on the same experiments)**

*The TEER measurement as an indicator of intestinal cell layer integrity*

The intestinal epithelium integrity is critical in maintaining a physical, but selective, barrier between external and internal environments. This barrier function is assumed by well-organized intercellular structures including tight junctions, adherent junctions and desmosomes surrounding the apical region of epithelial cells [36]. The transepithelial electric resistance (TEER) of cell monolayers can be considered as a good indicator of the epithelial integrity and of the degree of organization of the tight junctions over the cell monolayer [37]. Various chemical factors can alter the structure and function of tight junctions, such as hormones, cytokines, but also food-derived substances, bacterial substances and xenobiotics [38].

In that context, the Caco-2 cells were cultivated on microporous membrane in bicameral inserts during 18-21 days to allow full differentiation and polarization of cells into a tight monolayer, which can be followed by TEER measurement. This assay was then further used to determine a chronic effect of mycotoxins, incubated continuously during 7 days, at low, non-cytotoxic, concentrations. Figure 6 shows that OTA and DON had an important dose-effect on the cell monolayer integrity with significant TEER decrease from 4 and 50 ng/ml, respectively, while FB1 had no such an effect.

These results demonstrate that TEER assay is a very sensitive method: it allows the detection of OTA and DON indirect toxicity at concentrations 3 to 4 times lower than in the MTT assay (Figure 1). These OTA and DON concentrations represent realistic intestinal concentrations that could be reached in the human intestinal lumen after ingestion of moderately contaminated food.

TEER decrease by mycotoxins has already been reported [19, 39-41]. These intestinal permeability perturbations could explain the gastrointestinal disorders associated with mycotoxin ingestion in humans and animals [42].

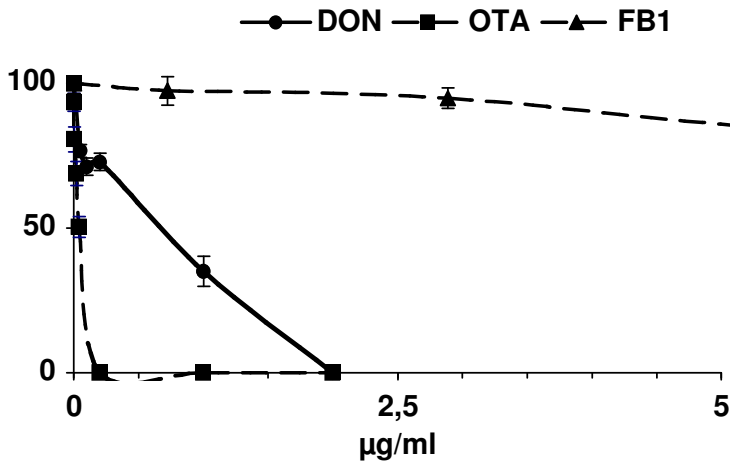


**Figure 6. Dose-effect of mycotoxins on the intestinal integrity as determined by TEER measurement. Differentiated Caco-2 cells, cultivated in bicameral inserts, were incubated, or not (control), with various concentrations of OTA, DON or FB1 during 7 days. Results are expressed as percent of control and are means of 3 independent experiments ± SD (n = 6-15)**

*The OTA intestinal transport to assess the polyphenol effect on the MRP-2 efflux pump*

Intestinal efflux pumps, such as P-glycoprotein (PgP), multidrug resistance-associated proteins (MRPs) and breast cancer resistance protein (BCRP), in combination with other transport proteins and biotransformation enzymes, play a key role in the bioavailability (absorption, distribution, metabolism and excretion) of many exogenous compounds (drugs, dietary compounds and various contaminants), but also of endogenous metabolites. As an example, the absorption of OTA, a food-borne mycotoxin, through intestinal cells towards the blood pole has been shown to be limited thanks to its efflux back to the intestinal lumen by the MRP-2, an efflux pump located at the apical side of cells [17-18]. Therefore, we used the [$^3$H]OTA apical-to-basolateral transport determination as a tool for measuring the MRP-2 activity in differentiated Caco-2 cells. Various polyphenols (PPs), plant constituents known as antioxidants and for their incidence on the prevention of various diseases such as cancers, inflammation… [43-44], were investigated on the OTA absorption through fully differentiated Caco-2 cells cultivated in bicameral inserts. Results (Figure 7) show that some PPs, *i.e.* chrysin, quercetin, genistein, biochanin A, resveratrol and naringenin, increased the transport of OTA from the apical to the basolateral pole, from 80 to 150 % as compared to control cells, whereas MK571, a well-known specific inhibitor of the MRPs [45], increased the OTA absorption by only ± 55 %. Catechin, EGCG and gallic acid had no effect. OTA and PPs, food-derived components that may be present simultaneously in the gastrointestinal tract, were used at plausible physiological concentrations, derived from total estimated daily intakes, which should be easily encountered in the gut after ingestion of a normal diet. Our results therefore imply, on one hand, that interactions between OTA and PPs could lead to a greater bioavailability of the mycotoxin in the bloodstream with possible adverse effects for human health. On the other hand, our results highlight the use of the [$^3$H]OTA transport assay as a valid tool to follow the intestinal MRP-2 activity and to study food contaminant interactions.

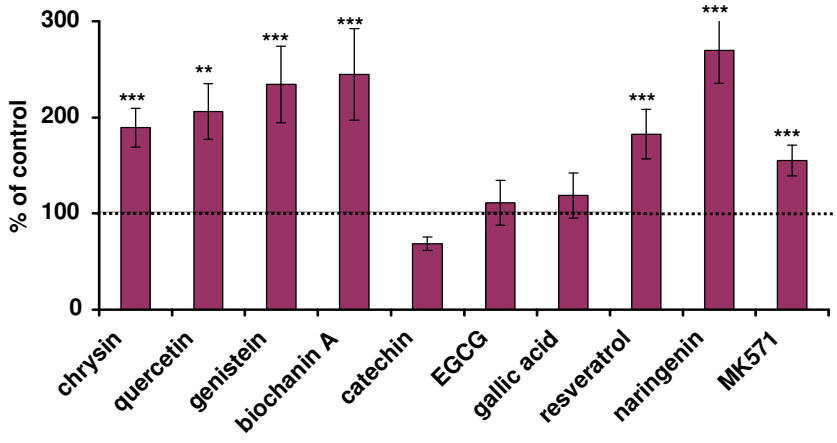


**Figure 7. Effect of PPs on the MRP-2 activity as determined by OTA transport measurement. Caco-2 cells cultivated on microporous membrane in bicameral inserts for 18-21 days (full differentiation) were incubated for 3 h apically with 3 ng/ml of [$^3$H]OTA in the presence, or absence (control), of 50 µM PPs or 50 µM MK571. Results were expressed as a percentage of control of the OTA concentration recovered in the basolateral compartment. Means of 3 independent experiments ± SD (n = 6-12) are given. ***, ** indicate, respectively, P < 0.001 and P < 0.01 as compared with the control condition**

*The MAPK phosphorylation as an indicator of inflammation*

Inflammatory bowel diseases (IBDs) occur following defects in the intestinal epithelial barrier and mucosal immune system, resulting in active inflammation and tissue destruction [46]. This disregulation is characterized by a succession of mechanisms implying two main signal transduction pathways, the NFκB/IκB and MAPK pathways [47]. The MAPK family makes up a group of important intracellular mediators from cell-surface to the nucleus in response to various stimuli, *i.e.* cytokines, hormones, growth factors, but also various xenobiotics like mycotoxins of the trichothecene family [48-49]. Here, the assessment by Western blot analysis of the phosphorylation of three major groups of MAPK (Erk 1/2, p38 and SAPK/JNK) was used as an indicator of the intestinal inflammation activation.

DON, a trichothecene, was shown to induce, in the intestinal Caco-2 cells, the phosphorylation of the 3 MAPK groups upon a prolonged exposure (Figure 8). These activations were dose-dependent, occurring from 0.5 µg DON/ml. These results, previously published [19], suggest that DON could trigger intestinal inflammation. This mechanism could explain epidemiological observations reporting DON associated human food poisoning causing vomiting and inflammatory diarrhea [50].

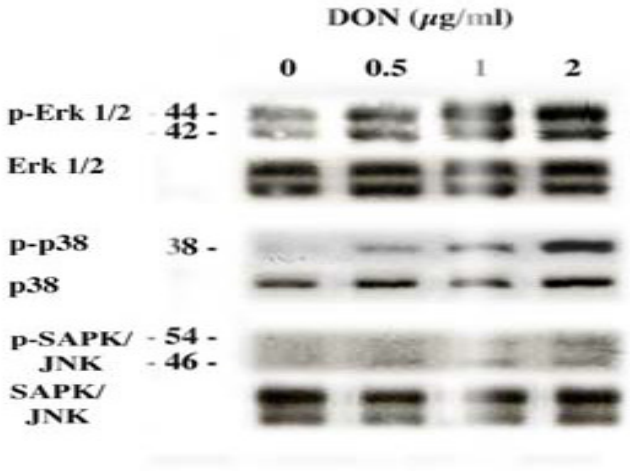


**Figure 8. Dose-dependent effect of DON on the MAPK phosphorylation. Caco-2 cells cultivated on microporous membrane in bicameral inserts, until full differentiation, were incubated, or not, apically with 0.5, 1 or 2 µg/ml of DON for 24 h. Cell lysates were resolved on SDS-PAGE and submitted to Western blot analysis with antibodies specific for phosphorylated Erks 1/2, p38 and SAPK/JNK. Bands were detected using ECL-plus sytem. Afterwards, the blots were stripped and reprobed with specific antibodies that recognized both phosphorylated and unphosphorylated forms of each MAPK for assessment of protein loading**

## CONCLUSIONS

The results of our current studies highlight the validity of methods assessing the general cytotoxicity as well as those related to specific endpoints to account for effects and possible interactions of food natural compounds and contaminants and to assess the risks for human health linked to the presence of toxins in the diet. In this case, the results illustrated here should further validate the use of the human intestinal Caco-2 cells as a particularly appropriate model of the human intestinal mucosa, the first barrier encountered by these contaminants.

As related, the bioassays evaluating the general cytotoxicity, like the MTT and LDH assays, are simple, rapid and reliable *in vitro* screening tests, but remain unspecific and do not contribute to the understanding of the specific molecular and cellular mode of action of food contaminants. Measurement of more specific endpoints has allowed detecting contaminant effects at non-cytotoxic concentrations. Such methods should therefore increase the predictive value of *in vitro* assays with regard to the toxic properties of food pollutants. Moreover, if some specific endpoints are expensive, time and labour consuming, such as the MRP-2 activity and MAPK phosphorylation assays, other tools are faster and cheaper methods, while providing pertinent information on the effect of toxins on the intestinal biotransformation activity, like the CYP1A1 activity. Therefore, such a method constitutes a more useful and very sensitive screening tool.
In the future, *in vitro* bioassays should be further applied on food matrix extracts to test their ability to detect contaminant combinations that could co-occur naturally in the diet and interact at the cellular level. A variety of organic and aqueous extraction and clean-up procedures have been developed that minimize background activity from sample extracts, but still allow quantitative extraction. Another more physiological approach may be performed consisting in an *in vitro* digestion procedure mimicking in a simplified manner the digestion processes in the mouth, the stomach and the small intestine in

order to enable investigation of the bioaccessibility of compounds from their matrix during the transit in the gastrointestinal tract.

Overall, the use of molecular and cellular tools allowing biological measurements of food contaminants will give us more information on risk assessment for the human health and on chemical safety.

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# XtraFood: An integrated model for the impact analysis of contaminants in the food chain

**K. De Brouwere[1], M. Van Holderbeke[1], C. Cornelis[1], I. Marrannes[1], P. Seuntjens[1], S. Claeys[2] and W. Steurbaut[2]**

*[1]VITO – Flemish Institute for Technological Research, Boeretang 200, B-2400 Mol, Belgium*
*[2]Ghent University, Laboratory for Crop Protection and Phytopharmacy, Coupure Links 653, B-9000 Gent Belgium.*
*e-mail: katleen.debrouwere@vito.be*

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risk assessment; integrated exposure assessment; contaminants; modelling; farm-to-fork; pesticides; cadmium; food chain

## SUMMARY

In view of recent food crises, quality control and risk assessment of the food chain from the farm to primary food products is an important issue and food safety is one of the major issues on the Agenda of the European Commission. In this paper, an integrated model, called XtraFood (Xenobiotics transfer in the Food chain) is presented and demonstrated. The model calculates transfer of contaminants in the agro-ecosystem and associated impacts on human health. The transfer model consists of modules calculating transfer to the farm (e.g., atmospheric deposition, import of manure, fodder, incidental transfers ...), transfer in and from soil (i.e., leaching, plant uptake, volatilization, degradation), and transfer to cattle (intake, transfer to organs, fat, milk, muscular tissue ...). Model outputs are contaminant levels in various primary food products such as crops, grains, milk, meat, and eggs. The transfer model is coupled to food consumption data. The XtraFood model provides as output the food intake data and resulting contaminant intake, segregated into age and gender categories. Exposure can be calculated as being representative for a population (group) or separately for local and background intake. All these intakes are linked to the output of the farm model. Additional inputs are provided to allow for concentration data in non-farm related foods (e.g., fruit juice, fish …). Human exposure is calculated and compared to the available toxicological levels to estimate impacts on human health. The model is demonstrated for two case studies: in the first case study, Cd levels in animal and plant tissues for the Cd polluted Campine region were modelled, and taken forward to predictions of total dietary intake of Cd for people living in the Campine region of Belgium. The second case study demonstrates the pesticide module of the XtraFood model. Finally, some possible future extensions of the model are discussed.

## INTRODUCTION

Food safety is one of the major issues on the Agenda of the European Commission and the Belgian government. Incidents like the dioxin- and BSE-crisis lead to important economic losses and to concern about the protection of public health through the food chain. At the European level, the White Paper on Food Safety [1] was published, including the organisation of a European Food Authority which defines priorities for research and regulation. At the Belgian level, the Federal Agency of Food Safety was

established. Following these initiatives, regulatory initiatives for setting limits on contaminants in human and animal food products are accelerated [2-4]. The Belgian Food Safety Agency is well aware that risk assessment strategies and models are essential for the construction of a food safety policy.

Existing instruments such as Hazard Analysis Critical Control Points (HACCP) are primarily concerned with quality control and risk assessment in the production and distribution part of the food chain, i.e., after the products left the farm (post-farm gate). Other instruments, such as life cycle assessment (LCA), are a compilation and evaluation of the inputs, outputs and the potential environmental impacts of a product system throughout its life cycle ('from cradle to grave'), but LCA typically does not address the health risk aspects of a product (ISO14040). Quality control and risk evaluation of the food chain starting from the farm to the primary food products (pre-farm gate) is gaining importance. This is clearly acknowledged by the food processing industries and the retailers (see e.g., EUREPGAP, GFSI initiatives).

An integrated instrument that calculates transfer of contaminants from the inlet of the farm to primary food products (crops, cereals, meat, eggs, milk), and that assesses impacts of contaminated primary food products on public health is lacking. Currently, no generic modelling tools are available that predict the impact of contaminants in the environment to the primary food chain.

On the one hand, separate tools to assess the transfer of contaminants from the environment to plants and animals do exist, but were up to now not coupled with human dietary patterns. For example, the model of Travis and Arms [5] is such a tool and allows the prediction of persistent organic compounds in meat and milk for given environmental and feeding conditions. Other environment-animal tissue models are described in the literature [6-7]. Also for plants, numerous models are available for the prediction of inorganic and organic contaminant concentrations from soil or air [8-10].

On the other hand, studies investigating human exposure to contaminants via the diet are also described in the literature [11-12]. However, these studies are limited to food concentrations for foods purchased at local supermarkets, with poorly defined relations to environmental conditions under which the food was produced. Such studies do not allow assessing the impact of changes in environmental conditions on changes in dietary intake of contaminants.

All this illustrates the need for a model coupling the transfer of contaminants with dietary intake assessments.

It is recognized that monitoring data or measured concentrations of contaminants in plants and animals tissues are often preferred over modelled concentrations. Analytical uncertainties of measured concentrations are normally minor to the larger uncertainties of model predictions; however spatial and temporal variability in plant concentrations can be substantial. In cases where measured data are unavailable or insufficient, modelling is a good alternative to get insight into expected concentrations in plants and animals grown at these locations. In addition, for scenario analysis and assessment of (future) impact of policy measures aiming at reducing the contaminants in the environment, modelling is the best way forward.

## METHODS/ MODEL DESIGN

### *Model components*

The integrated model is composed of two main modules: i) the transfer module, and ii) the exposure module. The transfer and the exposure module, and their linkage are described below.

*Transfer module for heavy metals and organic compounds*

Figure 1 shows the various possible transfers of contaminants in a model agro-ecosystem. Contaminants are transferred either directly or indirectly to the food products. Chemicals can enter the agro-ecosystem via the soil by irrigation, (wet and dry) atmospheric deposition or the application of fertilisers. Contaminants can be deposited either directly on the soil or on the aboveground parts of the crop. The application of plant protection products is a direct input term for pesticides to soil or crops. Contaminants can also enter the farm through the import of animal manure or through feed supplies. Contaminants can leave the agro-ecosystem via the soil by volatilisation to the atmosphere, run-off to surface water or leaching to groundwater, or they can be degraded in the soil. Contaminants can leave the agro-ecosystem by export of animal manure, and by exporting cattle and/or crops as food products. Internal flows are plant uptake (soil → plant) and intake by cattle (soil → plant → cattle or soil → cattle). Indirect transfer of contaminants to food products thus partly occurs via the soil system. Crops are closely connected to the soil by their root system, extracting water and nutrients (or contaminants). Cattle ingests plants growing in the soil, and soil particles. Modelling the transfer of contaminants in soils of agro-ecosystems therefore is indispensable for the impact analysis.

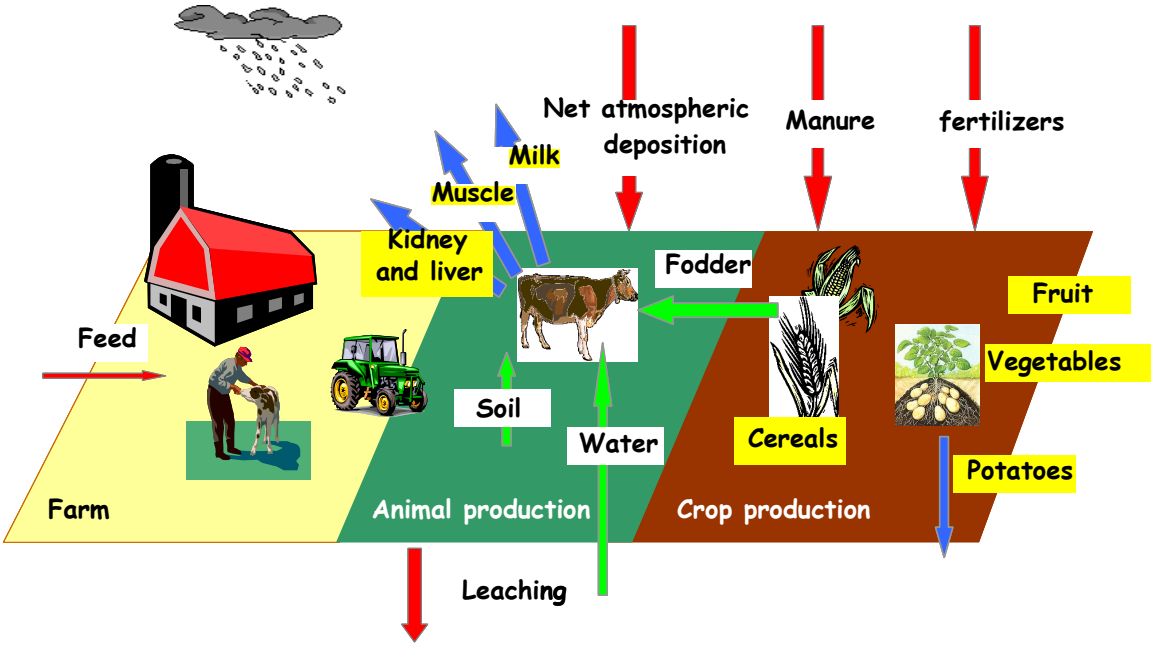


**Figure 1. Overview of contaminant flows in the agro-ecosystem and the food chain**

An inventory of available models for each of the above described transfer pathways was made based on a literature survey. We selected the most appropriate model for each pathway to incorporate it in the integrated XtraFood model.

The selected models for each transfer route are listed briefly in Table 1; the pesticide model is described briefly below the table. For a more extended version of the selection procedure and full mathematical description of the selected models, we refer to the report of the XtraFood project [13].

**Table 1. Ecosystem transfer models selected and used as cornerstones of the transfer module of the XtraFood model**

| Sub module | Description | Reference |
|---|---|---|
| soil concentration | dynamic simple first-order model | Vissenberg and van Grinsven, 1995 [14] |
| atmosphere-plant transfer | plant-X model: includes dry gas (kinetic) deposition of chemicals present in the gas phase, and dry and wet (kinetic) particle deposition | McLachlan, 1999 [15] |
| soil-plant transfer (organic chemicals) | plant-X model: equilibrium model, based on chemical properties (e.g., $K_{ow}$) of the contaminant, and plant properties (growth period, plant height, plant density …) | Trapp and Matthies, 1995 [8]; updated with Trapp 2002 [9] |
| soil-plant transfer (metals) | regression model based on crop species and soil properties (soil metal concentration and soil pH) | Jannson et al., 2006 [16] |
| cattle (milk, muscle tissue and egg fat) model (organic chemicals) | steady-state model accounting for chemical properties of the contaminant ($K_{ow}$), empirical constants (bioconcentration factors: BCF), and contaminant fluxes based on feed, soil and water ingestion and corresponding concentrations of these media (for plants as feed: coupled with output of the plant transfer modules) | model: Travis and Arms (1988) [5]; and for parameterization: Schuler et al., 1997 [17]; Olling et al., 1991 [18]; Fries et al., 1999 [19]; McLachlan et al., 1990 [20] … |

*Pesticide model*

The fate of pesticide residues on the plant after spray application depends upon different processes. A first-order kinetic model, based on the initial dose of active substance on the plant after application, has been selected.

$$\frac{dC}{dt} = -k * C + J_{in} \quad \text{or} \quad C(t) = C_0 * e^{-k*t} + \frac{J_{in}}{k} * (1 - e^{-k*t})$$

Where $C_0$ is the initial concentration in/on the plant at time 0 (mg $kg^{-1}$), $J_{in}$ is the input factor (mg $kg^{-1}$ $d^{-1}$), k is the degradation factor ($d^{-1}$) and t is the time between the last application and harvest (d).

Since the model only considers the residues on leafs/fruits by spray application and not by atmospheric deposition or by uptake from the soil, the input factor ($J_{in}$) can be set as zero and the equation can be written as:

$$C(t) = C_0 * e^{-k*t}$$

In several cases, more than one application is done during the growth season. Therefore, it is obvious to insert a “multiple application” factor (MAF) and to work with a “time weighted average” factor ($f_{twa}$), which takes into account the time between two applications [21].

The equation can then be rewritten as:

$$C(t) = C_0 * MAF * f_{twa}$$

The “multiple application” factor (MAF) is calculated by:

$$MAF = \frac{1 - e^{-nki}}{1 - e^{-ki}}$$

In which n is the number of applications, k is the degradation factor ($d^{-1}$) and i is the interval between two applications (d).
The “time weighted average” factor ($f_{twa}$) is given by:

$$f_{twa} = \frac{1 - e^{-kt}}{kt}$$

In which k is the degradation factor ($d^{-1}$) and t is the time between the last application and harvest (d).

*Dietary exposure module*

Food consumption data for the Belgian population are integrated in the XtraFood model. Hereto, a database of food consumption of > 300 food items for different age classes (3-5 y, 6-9 y, 10-14 y, 15-20 y, 21-30 y, 31-40 y, 41-50 y, 51-60 y and > 60 y) and gender classes (males and females) was constructed based on 3 earlier food consumption surveys performed in Belgium, namely a teenager survey (GASTON: Ghent Adolescent Study on Nutrition, [22]), a young children survey [23], and a women survey [24]. This database was amended with data of a Dutch study [25] for food items or age/gender categories not covered by one of the 3 Belgian studies. Parameters such as average, median, $5^{th}$, $25^{th}$, $75^{th}$ and $95^{th}$ percentile food consumption of food items are stored in the database.
The food classification system was developed in order to optimize the compatibility with the crop/animals tissue classification system of the transfer module.

*Linkage between transfer module and dietary exposure module*

A flow scheme of the integration of food consumption data and farm transfer models into human exposure assessment is presented in Figure 2.
The first aspect taken into account is that food items recorded in the human dietary surveys do not always match with animal or crop products of the transfer module database. Indeed, some food items are the result of a combination of different simple farm products. Hereto, the database was adapted to convert the food items of the dietary survey into basic farm products (primary plant products and primary animal products) and primary non-farm products. Concentrations of non-farm products such as fish, tropical fruits … are entered manually in the database because the farm transfer module is not relevant for these food items.
The second factor accounted for in the linkage between the farm module and the dietary exposure module is the change in weight and possible reduction in contaminant levels upon food preparation (washing, peeling, boiling, frying). These reduction factors were retrieved from studies providing experimental evidence [26-28]. If unavailable, no modification factors were applied as a conservative approach.
The third factor in the linkage between the transfer module and the dietary exposure module is the import and aggregation factor. The impact analysis of contaminants requires for each farm product the origin and distribution of that product. The origin of the food package is essential in the link between the farm model and contaminant exposure since the origin determines the environmental conditions, and thus the contamination level. Three levels of food allocation are applied in the model: local

(home-grown) products, regional products and imported products. For the first two types of products, concentrations are modeled using the transfer module, while for the import category, measured concentrations can be entered in the database.

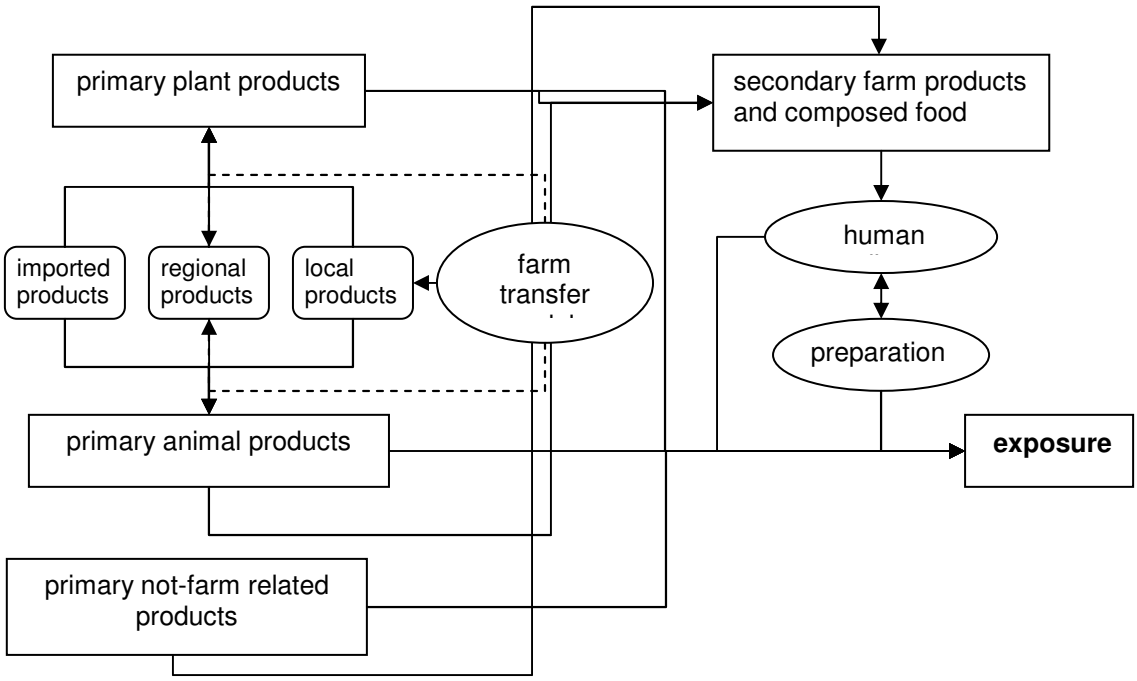


**Figure 2. Flow scheme of combination of biological transfer of contaminants and food consumption data for the assessment of human exposure to contaminants by diet**

*Database structure and program language*

XtraFood is programmed in an SQL environment, combining calculations in SQL language with databases constructed in MS-Access. The MS-Access database stores information on chemical properties, crop properties, crop yields, agricultural data, contaminant levels in air, deposition, food consumption data, composition of composed food items.

The information stored in the database is used to calculate the output of the various (sub)modules, and the output of the modules is linked to the input of the appropriate modules. For example, the output of the atmosphere module is imported in the soil-plant module for transfer calculations in the soil-plant continuum. Predicted plant concentrations are used for calculation of levels in animal products in case the plants are used for feeding cattle, or serve as output in case the plants or edible plant parts are used for human consumption. If data on contaminant levels in the environment are available, they can be entered in the model to bypass calculations.

*Model uncertainties*

The XtraFood model is subject to some uncertainties:

- uncertainty about measurements, parameters and variables, i.e., a measured environmental contaminant concentration, a value of food intake …;
- uncertainty about processes and models, i.e., a steady-state model for transfer of chemicals to animal or crop products is used at the moment (except for pesticides)…

We tried to assess model uncertainty by comparing predicted and observed variables or by comparing models. A detailed analysis of uncertainties is not shown here but elaborated in the full report of this project [13].

## RESULTS/ MODEL DEMONSTRATION

The model demonstration and validation presented in this paper is limited to 2 case studies: 1) transfer of cadmium and the linked dietary exposure assessment, and 2) transfer of pesticides. Another case study, namely the transfer of PCBs and PCDD/Fs to plants and animal products, is not shown here but elaborated in the full report of this project [13].

### *Case study 1: Cd in the Campine region of Belgium*

We applied XtraFood to calculate primary food product concentrations of cadmium close to the industrial sites of Balen, Lommel and Overpelt (= Campine region) and compared the predictions with results of an independent monitoring campaign held by the Belgian Federal Food Safety Agency (FAVV) in 2004 in the same region [29].

### *Model input data for the Campine environment*

The Campine region is a heavy metal-contaminated area of about 2700 $km^2$ situated at both sides of the Dutch-Belgian border. The area is contaminated by the emissions of former and operating zinc smelters during the past hundred years. As a result, the topsoil is contaminated with Cd and other trace metals to levels above intervention values. Due to the relatively high mobility of the metals in the sandy soil, this has resulted in increased levels of the metals in groundwater and food products.

The area is intensively investigated in terms of heavy metal concentrations, the various fluxes of metals to the soil and in the agro-ecosystem. Data on concentrations in air, soil and water close to the non-ferrous industrial sites were retrieved via different sources and are compiled in table 2. Elevated atmospheric deposition as compared to the background is largely due to resuspension of contaminated soil and dust and does not represent primary deposition. In addition to data for the Campine region, table 2 also contains data for background regions in Flanders. The background concentration data are used to calculate Cd levels of crops and animals for the regional scale of Flanders. Both background and local concentrations are taken into account for scenarios where the food package is partly composed of local food grown at the Campine region and partly composed of food originating outside the Campine region, i.e., at background Cd levels in Flanders.

**Table 2. Chemical concentrations in the environment close to non-ferrous industry in the Campine region**

| | Source data | Stat. parameter | Location measuring points | # data | Period | Data |
|---|---|---|---|---|---|---|
| deposition | | | | | | **mg $m^{-2}$ $y^{-1}$** |
| | Umicore (edge of sites) | geomean | Balen & Overpelt | 72 | 2000-2005 | 6.0 |
| | | median | Balen & Overpelt | 72 | 2000-2005 | 6.0 |
| | | P5 | Balen & Overpelt | 72 | 2000-2005 | 1.3 |
| | | P25 | Balen & Overpelt | 72 | 2000-2005 | 2.9 |
| | | P75 | Balen & Overpelt | 72 | 2000-2005 | 8.0 |
| | | P95 | Balen & Overpelt | 72 | 2000-2005 | 11.7 |
| | VMM, 2004 | geomean | background (nature) | 8 | 2004 | 0.07 |
| | | median | background (nature) | 8 | 2004 | 0.07 |
| | | P5 | background (nature) | 8 | 2004 | 0.05 |
| | | P25 | background (nature) | 8 | 2004 | 0.07 |
| | | P75 | background (nature) | 8 | 2004 | 0.07 |
| | | P95 | background (nature) | 8 | 2004 | 0.10 |
| air (PM 10) | | | | | | **µg $m^{-3}$** |
| | VMM, 2004 | average | Lommel & Overpelt | 227 | 2004 | 0,.001 |
| | | median | Lommel & Overpelt | 227 | 2004 | 0,.001 |
| | | P10 | Lommel & Overpelt | 227 | 2004 | 0,.001 |
| | | P25 | Lommel & Overpelt | 227 | 2004 | 0,.001 |
| | | P75 | Lommel & Overpelt | 227 | 2004 | 0,.003 |
| | | P95 | Lommel & Overpelt | 227 | 2004 | 0,.012 |
| | | background | Knokke | 300 | 2004 | 0,.001 |
| ground water | | | | | | **µg $l^{-1}$** |
| | IHE, 1985 | average | campine region | 2589 | 1983 | 10.2 |
| | | median | campine region | 2589 | 1983 | 4.6 |
| | | P5 | campine region | 2589 | 1983 | 0.6 |
| | | P25 | campine region | 2589 | 1983 | 2.1 |
| | | P75 | campine region | 2589 | 1983 | 11 |
| | | P95 | campine region | 2589 | 1983 | 37 |
| | | background | Flanders | | | 1 |
| Soil | | | | | | **mg $kg^{-1}$** |
| | VITO database, 2006 | average | campine region | 1912 | 1980-1998 | 3.1 |
| | | median | campine region | 1912 | 1980-1998 | 2.7 |
| | | P5 | campine region | 1912 | 1980-1998 | 0.5 |
| | | P25 | campine region | 1912 | 1980-1998 | 1.6 |
| | | P75 | campine region | 1912 | 1980-1998 | 4.4 |
| | | P95 | campine region | 1912 | 1980-1998 | 9.4 |
| | CODA, 2000 | background | Flanders, sandy region | | | 0.32 |

*Transfer model demonstration for Cd in the Campine region of Belgium*

Various scenarios using different percentiles of the statistical distribution (P5, P25, P50, P75 and P95) were used to calculate Cd concentrations in the primary food products. For example, the average scenario for the Campine region was calculated using average data for soil Cd, average deposition, average groundwater and average air data. Analogously, the P95 scenario is based on P95 data for these compartments. It is recognized that in reality P95 concentrations of compartment X do not always coincidence with P95 concentration of compartment Y. However, none of the databases contained linked Cd data in all of the environmental compartments, and therefore, it cannot be verified if P95 values for different media coincide.

The calculated concentrations were compared to measured data [29]. The results are displayed in figure 3 (crops) and figure 4 (animals). It is emphasized that data used for the model parameterization are independent of the validation data.

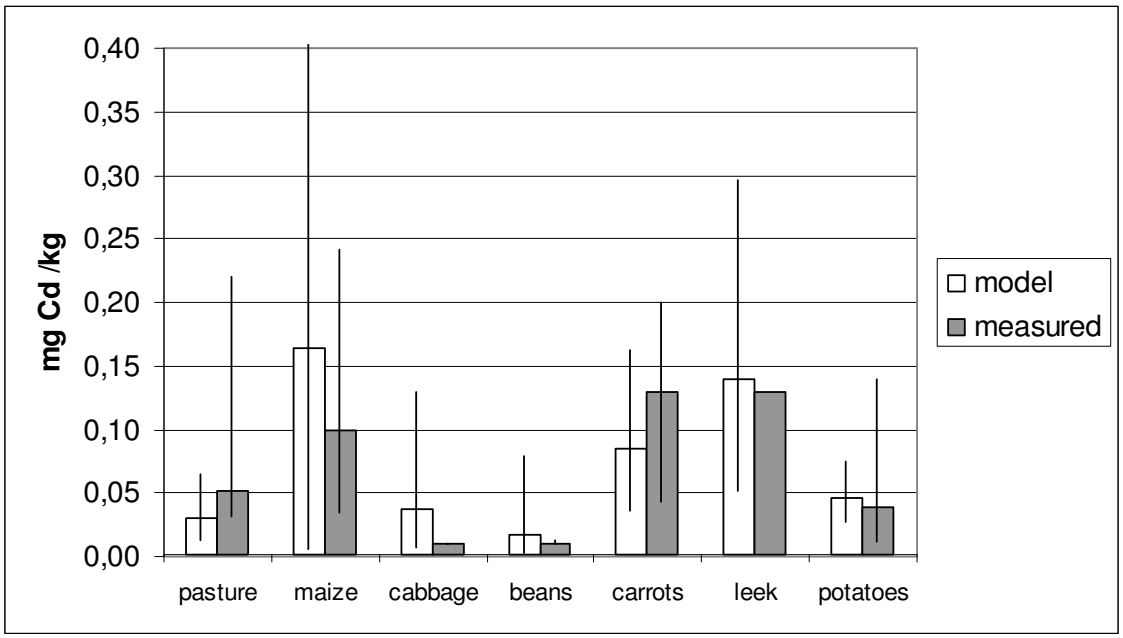


**Figure 3. Measured versus XtraFood predicted Cd concentrations in animal feed crops (pasture, maize, on dry weight basis) and food crops (cabbage, beans, carrots, leek, potatoes, on wet weight basis). Bars represent median concentrations. Error bars represent minimum and maximum for measured concentrations and P5 and P95 for calculated concentrations**

Among all crops, there was a wide range in measured Cd concentrations in the Campine region. The overlap of error bars of predicted Cd concentrations with error bars of measured concentrations suggests a realistic prediction of Cd crop concentrations. Concentrations for the median environmental contamination in the Campine region are below maximum levels in foodstuffs set by the European Commission for potatoes (0.10 mg Cd $kg^{-1}$ fw), cabbage and beans (0.05 mg Cd $kg^{-1}$ fw) [30]. In contrast, the Cd crop limits were exceeded for leek and carrots (0.10 mg Cd $kg^{-1}$ fw). Cadmium concentrations in animal feed crops were below maximum levels for animal feed (maize & pasture: 1 mg Cd $kg^{-1}$ feedstuff with a moisture content of 12 %)

Exceeding of the limit is predicted for beans and cabbage under a worst case scenario (P95 environmental contamination), though this is not confirmed by measured data. The reverse is true for potatoes: the model does not predict that the limit would be exceeded under a worst case scenario (P95), whereas it was observed in the measured dataset.

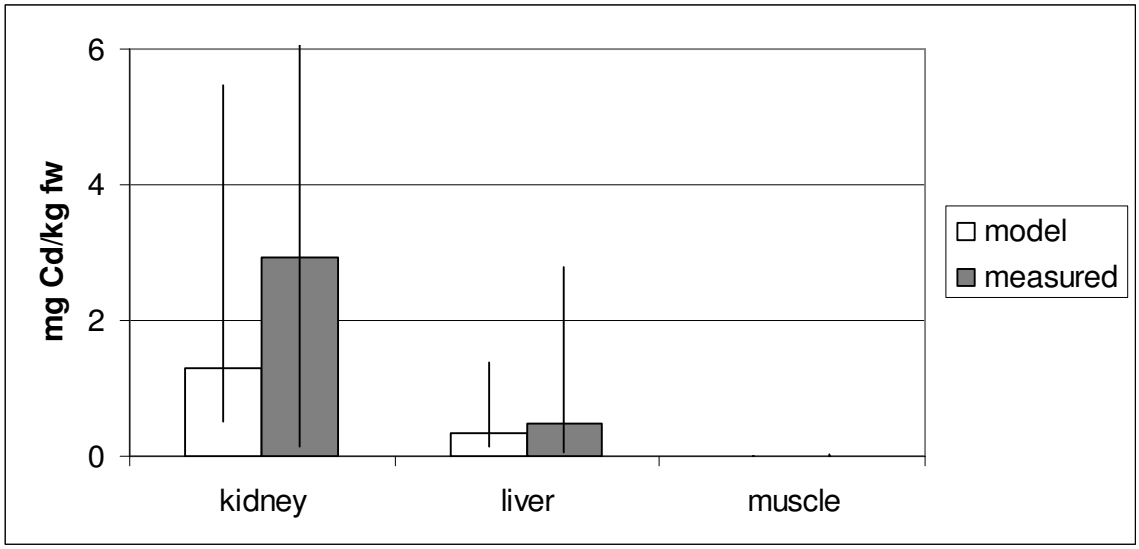


**Figure 4. Measured versus XtraFood predicted Cd concentrations in animal products. Bars represent average concentrations. Error bars represent minimum and maximum for measured concentrations and P5 and P95 for calculated concentrations**

Average Cd concentrations in muscle were below the detection limit for Cd (0.01 mg $kg^{-1}$). Model calculations were also below 0.01 mg Cd/kg (P95: 0.004 mg Cd $kg^{-1}$). There was a wide range in measured Cd concentrations in kidney. Predicted Cd concentrations for kidney fell in that range. Cadmium concentrations in liver were lower than in kidney; this trend was also predicted with the XtraFood model. A wide variety in measured concentrations was also observed for liver. The model predicts for average Cd environmental contamination in the Campine region concentrations above the EU limit of 1 mg Cd $kg^{-1}$ for kidney [30]. Maximum levels of Cd in kidney samples of the Campine region exceeded more than 10-fold this limit. Average predicted Cd in liver is below the limit of 0.5 mg Cd $kg^{-1}$, but might be exceeded in a worst case scenario. Liver Cd concentrations exceeding the limit were also observed in the samples from the Campine region (figure 4).

### *Integrated model demonstration: outcome of the dietary exposure assessment for the Campine region*

Exposure to Cd by food intake in the Campine region was assessed by combining the food consumption records for the Belgian population with Cd concentrations in farm-bound foods predicted with the XtraFood transfer module using environmental conditions specific for the Campine region. Cadmium concentrations in non-farm bound foods (e.g., fish) were derived from literature. In this exercise, Cd reduction factors related to peeling, washing, frying and boiling were ignored. This conservative approach was preferred since the reduction factors were considered unreliable for a number of food products. Also, the plant-soil relations used are generally based on washed vegetables.

Cadmium exposure by food was calculated for 5 scenarios as given in table 3.

**Table 3. Scenario assumptions for the Cd dietary exposure**

| Scenario | Environmental conditions | Ratio local (Campine) versus regional (Flanders) | Food consumption pattern | Age/gender category |
|---|---|---|---|---|
| 1 | Campine; average environmental Cd | 100 % local | average | men, 21-30y |
| 2 | Flanders; background environmental Cd | 100 % regional | average | men, 21-30y |
| 3 | Campine; average environmental Cd | 25 % local + 75 % regional | average | men, 21-30y |
| 4 | Campine; average environmental Cd | 25 % local + 75 % regional | average for all food items except P95 bread | men, 21-30y |
| 5 | Campine; average environmental Cd | 25 % local + 75 % regional | average | women, 21-30y |

In scenario 1, average environmental conditions of the Campine region, 100 % consumption of local Campine food and average food consumption is assumed. Scenario 2 differs from the first in the environmental conditions: background Flanders instead of Campine region. For scenario 3 it was assumed that 25 % of all farm-bound foods consumed were grown under Campine average environmental conditions, while 75 % originated from the remainder part of Flanders (background). This is a more realistic scenario than scenario 1. According to data from the Campine region (personal communication Tim Nawrot), the average fraction of home-grown food is around 25 % with large variation. This corresponds with values from France (CIBLEX). Scenario 4 differs from scenario 3 in the food consumption pattern: Cd dietary exposure by high bread eaters is assessed. Scenario 5 is similar to scenario 3 except for the target population (women instead of men).

The XtraFood output for these 5 scenarios is shown in figure 5.

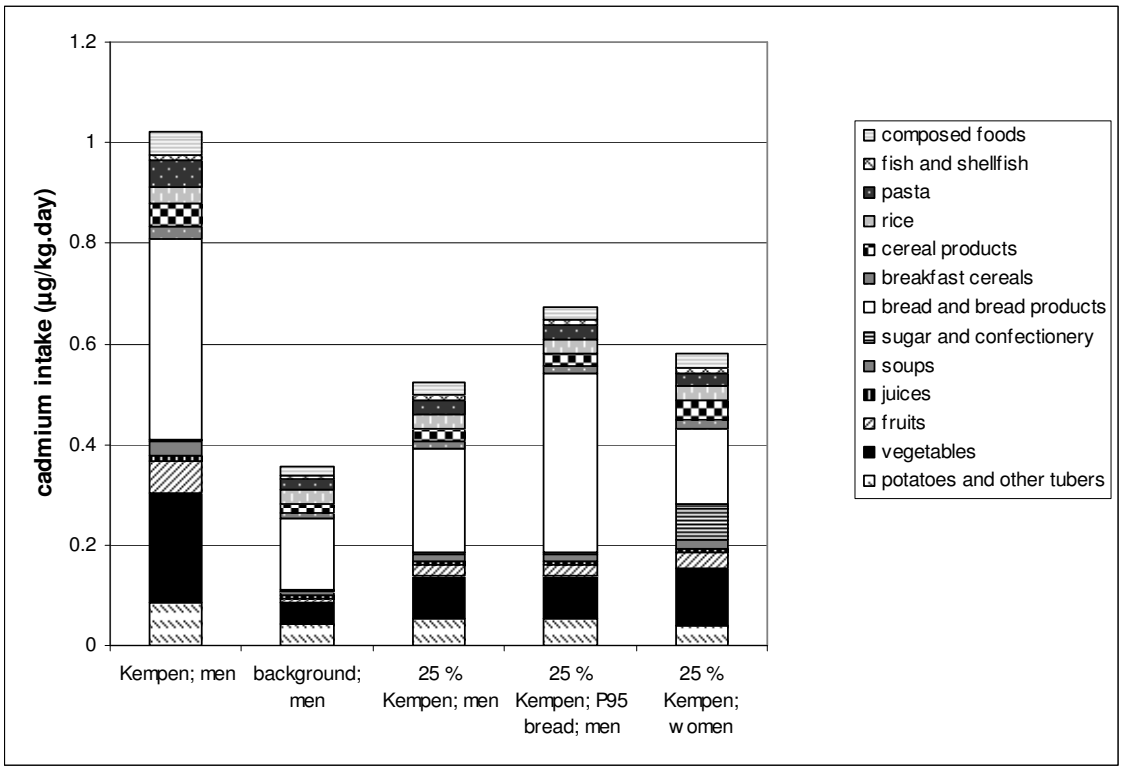


**Figure 5. Cd dietary exposure (in µg Cd $d^{-1}$ $kg^{-1}$ bodyweight) for inhabitants (21-30 years) of the Campine region predicted by the XtraFood model. Calculations were run for 5 scenarios defined in Table. Contribution of food items to total cadmium intake is depicted**

The main contribution to Cd dietary intake comes from bread and bread products in all scenarios, followed by vegetables and potatoes.
As it is assumed that cereals (bread and bread products), vegetables and potatoes are locally grown in the Campine region in scenarios 1, 3, 4 and 5, the contribution from the Campine area is mainly seen in these food items. The importance of the contribution of locally grown cereals should be nuanced, as cereal products are mainly made from imported cereals.
The XtraFood model predicts a nearly 3-fold higher Cd dietary intake for inhabitants in the Campine region who eat 100 % locally grown farm-related products, compared to background Cd exposure elsewhere in Flanders (scenario 1 versus scenario 2). This difference between scenario 1 and 2 (0,7 µg Cd $d^{-1}$ $kg^{-1}$ body weight) is levelled off to 0.17 µg Cd $d^{-1}$ $kg^{-1}$ body weight when shifting from scenario 1 (100 % local food) to the more realistic scenario 3 (25 % local food).
Scenario 3 (25 % local food, 75 % regional food, average food consumption pattern) was also modelled for other age and gender categories than the 21-30 years window. The dose per age group and the average dose calculated at 50 years of age (cumulative dose from 3 – 50 years divided by total number of years) are shown in figure 6.

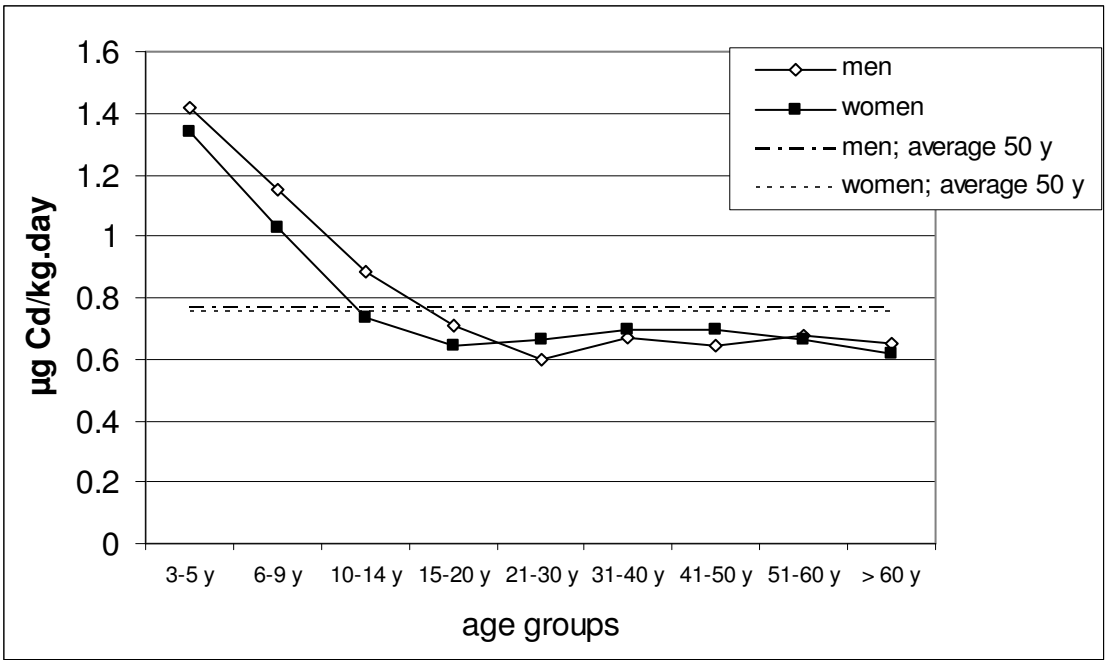


**Figure 6. Cadmium intake per age group and average cadmium intake from 3 to 50 years predicted for inhabitants of the Campine region according to the XtraFood model**

From the curves, it is clear that children show higher Cd exposure than adults due to their higher food consumption per unit body weight; body weight normalized Cd exposure levels decreases from young childhood to the age of 15 – 20 years and stays rather constant with age from then on. Differences between men and women are seen, mainly at younger age. On a cumulative basis (average dose at 50 years) the difference is very limited. The high exposure at young age is a point of attention; however for cadmium the risk for renal effects (critical endpoint) is related to the cumulative dose at about 50 years.

*Case study 2: transfer of pesticides*

The pesticide model results have been validated by comparing the results with literature data (results of supervised residue trials). Most of the data were obtained by the FAO (USA) [31]. As an example, some results for dimethoate are given in table 4.
The current model gives satisfying results. In most of the cases the model calculations are an overestimate of the real situation. A possible explanation is that in reality the degradation on fruits and leaves starts very quickly and then slows down the next days. In fact, the degradation can be described with two degradation factors: one for the first hours/days and one for the following days. In the model, only one degradation factor has been taken into account, resulting in a slight overestimation. Nevertheless, it is a useful tool in predicting the human intake of pesticide residues by food.

**Table 4. Validation exercise for dimethoate [31]**

| Crop | Form | Country | AR (kg ha$^{-1}$) | n | t (d) | TRIAL residue (mg kg$^{-1}$) | MODELLING residue (mg kg$^{-1}$) |
|---|---|---|---|---|---|---|---|
| cherries | EC | Germany | 0.72 | 3 | 14 | 0.14 | 0.48 |
| | | | | | 21 | < 0.05 | 0.34 |
| | | | | | 28 | 0.05 | 0.25 |
| | | | | | 35 | < 0.05 | 0.20 |
| cherries | EC | Germany | 0.8 | 1 | 4 | 1 | 0.96 |
| | | | | | 7 | 1 | 0.73 |
| | | | | | 10 | 0.58 | 0.57 |
| | | | | | 14 | 0.66 | 0.43 |
| | | | | | 21 | 0.19 | 0.30 |
| cherries | EC | Germany | 0.8 | 2 | 4 | 3.38 | 1.15 |
| | | | | | 10 | 3.84 | 0.69 |
| | | | | | 14 | 1.64 | 0.52 |
| | | | | | 21 | 1.48 | 0.36 |
| cherries | EC | Germany | 0.6 | 4 | 14 | 0.37 | 0.41 |
| | | | | | 21 | 0.06 | 0.28 |
| onion | EC | Germany | 0.04 | 2 | 7 | 0.31 | 0.59 |
| | | | | | 14 | 0.14 | 0.36 |
| | | | | | 21 | 0.04 | 0.25 |
| onion | EC | Germany | 0.24 | 1 | 7 | 0.2 | 0.69 |
| | | | | | 28 | 0.05 | 0.21 |
| | | | | | 7 | 0.1 | 0.69 |
| | | | | | 28 | < 0.01 | 0.21 |
| cauliflower | EC | UK | 0.32 | 2 | 7 | 0.1 | 0.44 |
| | | | | | 14 | 0.13 | 0.26 |
| | | | | | 21 | 0.05 | 0.18 |
| cauliflower | EC | UK | 0.4 | 3 | 7 | 0.04 | 0.56 |
| | | | | | 14 | < 0.02 | 0.34 |
| | | | | | 21 | < 0.02 | 0.23 |
| cauliflower | EC | UK | 0.3 | 1 | 7 | 0.3 | 0.34 |
| | | | | | 14 | 0.18 | 0.20 |
| | | | | | 21 | 0.1 | 0.14 |
| cauliflower | EC | UK | 0.4 | 6 | 3 | 0.44 | 0.82 |
| | | | | | 7 | 0.34 | 0.57 |
| | | | | | 14 | 0.21 | 0.34 |
| | | | | | 21 | 0.11 | 0.23 |
| Brussels sprouts | EC | Germany | 0.32 | 2 | 7 | 0.12 | 0.43 |
| | | | | | 14 | 0.06 | 0.26 |
| | | | | | 21 | 0.02 | 0.18 |
| | | | | | 28 | < 0.02 | 0.14 |
| Brussels sprouts | EC | Germany | 0.24 | 3 | 3 | 0.11 | 0.49 |
| | | | | | 7 | 0.08 | 0.34 |
| | | | | | 14 | < 0.05 | 0.20 |
| | | | | | 21 | < 0.05 | 0.14 |
| Brussels sprouts | EC | Germany | 0.24 | 3 | 3 | 0.14 | 0.49 |
| | | | | | 7 | < 0.05 | 0.34 |
| | | | | | 14 | < 0.05 | 0.20 |
| | | | | | 21 | < 0.05 | 0.14 |

## DISCUSSION

The XtraFood model is a complete model for integrated dietary exposure assessment, linking environmental transfer and human intake. The validation exercise of the model for cadmium en dioxins showed that acceptable results are obtained. However, experience learns that for each contaminant considered, critical analysis of the transfer concepts and parameter values is needed to account for the specificity in behaviour of the contaminants. As such, the model has been applied and adapted to be used for calculation of critical deposition values for PCBs and dioxins and to estimate exposure of the Flemish population to PAHs. In this, validation of model predictions is of high importance.

Ingestion of food is a primary pathway of human exposure to many chemicals. In actual practice, it is rare to be exposed to a chemical solely by the dietary pathway. Other possible exposure pathways are inhalation, ingestion of soil and dust (children) ... If the aim is to evaluate health risks due to exposure to a chemical, total exposure must be considered. Total exposure assessment is also necessary to evaluate the efficiency of risk reduction measures. Without knowledge of the baseline exposure in the absence of a pathway, risk managers do not know the extent to which mitigation of the risk associated with that pathway will lower the total risk [32]. Total exposure to a single chemical from multiple sources, pathways and routes is referred to as aggregate exposure. If necessary from a toxicological point of view, total exposure to multiple contaminants, i.e., cumulative exposure, must be considered. In order to allow for aggregate exposure in the XtraFood model, modules that calculate the non-food related exposures may be linked to the XtraFood model. The integrated model should look at the combined exposure from e.g. crops, meat, milk and fish, soil and dust ingestion, inhalation, dermal exposure to dust, smoking, drinking water.

Although validation of the transfer module is sometimes hampered by the lack of coupled data, some kind of validation is mostly possible. Validation of the exposure module however is, as with other exposure models, difficult. Comparison with internal human biomarker[6] measurements can contribute to this validation, since biomarkers represent real body burden. However, between external dose (model prediction) and internal biomarker lays the processes of absorption, distribution, metabolism and elimination. The relationship between external dose and internal biomarker may be nonlinear. Linking external dose to internal biomarker therefore requires pharmacokinetics, which describes the rate processes of absorption, distribution, metabolism and elimination of a chemical in the human body. Moreover, identical external doses may give different biomarker values in different persons, due to physiological differences between individuals,. Physiologically based pharmacokinetic (PBPK) models may be used to translate model predictions into internal biomarkers, and thus advance the validation. When using biomarkers for validation, it should be allowed for that biomarkers integrate exposures from all sources and pathways.

Application of the model is not limited to the area of food safety. It can be used to link sources to exposure by coupling it with more detailed fate and transfer models (such as atmospheric dispersion models), and has its application as a complementary instrument to measurements. Analysis and prediction of exposures, as well as

[6] BIOMARKERS OF EXPOSURE ARE EXOGENOUS CHEMICALS, THEIR METABOLITES, OR PRODUCTS OF INTERACTIONS BETWEEN A XENOBIOTIC CHEMICAL AND SOME TARGET MOLECULE OR CELL THAT ARE MEASURED IN A COMPARTMENT WITHIN AN ORGANISM [32].

development of environmental quality objectives are areas of application that are explored.

Further model development will focus on the above-mentioned aspects of aggregate exposure and coupling with PBPK models. In addition the construction of a model environment that allows for spatial differentiation within a GIS environment would strongly extend the applicability of the model, allowing for assessment of regional differences in input and output data. Given the uncertainties and variation on the input data, the addition of a probabilistic modelling approach is considered.

## CONCLUSIONS

XtraFood calculates contaminant transfer in the agro-ecosystem to primary food products and subsequent human exposure through ingestion of food. This allows quantifying the impact of changes in environmental contamination and farming practices on the quality of primary food products, and through this pathway, on human health.
The model was demonstrated and validated for 3 case studies: cadmium in the Campine area, dioxins (not discussed in this paper) and pesticides. Implicit and explicit methods were applied to deal with uncertainties. The added value is the development of a completely new integrated model that couples transfer models for the primary food chain to exposure models. Most research projects deal with either one of the themes, but fail on integrating them. Extending the model to the assessment of different exposure routes and to the coupling with a PBPK module are future challenges.

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BELGIAN SCIENCE POLICY

HEAD OF THE DEPARTMENT 'RESEARCH PROGRAMMES' : DOMINIQUE FONTEYN

CONTACT PERSON : CHRISTINE MATHIEU

**FOR MORE GENERAL INFORMATION :**

SECRETARIAT: MIREILLE LECOUTRE

WETENSCHAPSSTRAAT 8, RUE DE LA SCIENCE

B-1000 BRUSSELS

TEL : +32 (0)2 238 34 80

FAX : +32 (0)2 230 59 12

EMAIL : LECO@BELSPO.BE