SPSD II

MYCOTOXIN CONTAMINATION OF REGULAR AND "ORGANIC" FOODSTUFFS

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PART 1

SUSTAINABLE PRODUCTION AND CONSUMPTION PATTERNS

GENERAL ISSUES

AGRO-FOOD

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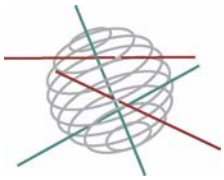
SCIENTIFIC SUPPORT PLAN FOR A SUSTAINABLE DEVELOPMENT POLICY
(SPSD II)



Part 1:
Sustainable production and consumption patterns

FINAL REPORT

Mycotoxin contamination of regular and "organic" foodstuffs

CP/30

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I. INTRODUCTION

1. General context

Mycotoxins are natural toxic compounds synthesized and secreted by different genera of filamentous fungi (belonging mainly to the genera *Aspergillus, Fusarium, Claviceps and Alternaria*). They present various types of toxicity, i.e. they can be carcinogenic, mutagenic, immuno-depressive, teratogenic, oestrogenic-like, … Contamination can occur all along the food chain and in a large set of products, from cereals to cheese and salted meat. Moreover, most mycotoxins present such a high physicochemical stability that they can be found in processed foodstuffs, even if microbiological contaminations occurred long before, during the production or storage of the raw materials. More than 200 different mycotoxins have been identified but the list is not exhaustive. Currently, only a few mycotoxins are being sporadically or systematically under investigation in a limited number of foodstuffs.

The use of pesticides to reduce microbial contamination during cereal production and storage, as well as the addition of preservatives at different stages of foodstuffs production and distribution, may lead to the presence of residues in the final products. In contrast, the non-use of pesticides or preservatives may result in an increased fungal contamination and in turn in an elevated amount of mycotoxins in the final products. Moreover, the use of pesticides or preservatives in insufficient amounts could lead to an even worse situation since the stress imposed on the moulds is thought to stimulate mycotoxin production.

In principle, although the foodstuffs resulting from conventional production are not free of mycotoxins, the organic foodstuffs present a greater risk of contamination. Moreover, as there is a greater number of microorganisms species able to develop during the organic production (in opposition to conventional production), a wider spectrum of mycotoxins may potentially be detected in organic foodstuffs than in conventional foodstuffs.

Considering these hypotheses, it seems essential to study the influence of the production mode (conventional or organic) on the mycotoxins content of foodstuffs.

2. Aims of the project

The aim of this project is to provide the public authorities with contamination data and analytical tools allowing evaluation and long-term management of the risks generated by mycotoxins.

Mycotoxins analysis in foodstuffs can be conducted using methods validated by the European Union. These methods are sensitive but time-consuming and thus not applicable on a large scale. We focused on developing powerful, rapid and complementary tools allowing effective foodstuffs controls at conventional intervals : 1) "multi-mycotoxins" chromatographic methods for simultaneous quantification of several toxins, 2) field semi-quantitative immuno-enzymatic methods, 3) biological methods based on direct and indirect cytotoxicity induced by contaminated food extracts on human and animal cultured cells.

In order to evaluate the occurrence of mycotoxins in cereal-based foods marketed in Belgium, a representative number of samples was collected with, as far as possible, a balance between conventional and "organic" modes of production.

Another objective of the initial project was to create a permanent Reference Centre in one of the federal State research institutes in order to ensure the maintenance of newly developed

analytical methods, as well as the management of a data base and a collection of reference samples.

3. Overview of the mycotoxins and commodities under investigation

The project focused on five mycotoxins or groups of mycotoxins : ochratoxin A (OTA), deoxynivalenol (DON), fumonisins (FB1, FB2 and FB3) and zearalenone (ZEN).

Several commodities deriving directly or indirectly from cereals likely to be contaminated by mycotoxins have been investigated : wheat cereals and wholewheat flour for bread-making, wholewheat pasta, beers, cornflakes, polenta (maize flour) and sweet corn. Not all samples were analysed for all mycotoxins since unlike other contaminants, mycotoxins are present only in certain types of products. Samples were thus selected for analysis for particular mycotoxins based on several factors including bibliographic knowledge, logical expectation and previous results (Table 1).

For example, in the case of fumonisins, we intended to focus our efforts on polenta and cornflakes because corn is the main target of fumonisin producing fungi (*Fusarium verticillioides* and *F. proliferatum*).

Table 1. Overview of the foodstuffs collected during the first and the second sampling periods and mycotoxins analysed.

	Wheat cereals	Wholemeal wheat flour	Beer	Wholemeal pasta	Cornflakes	Polenta	Sweet corn
Sampling period	*1 2*	*1 2*	*1 2*	*1 2*	*1 2*	*1 2*	*1 2*
OTA	× ×	×	× ×				
FB1			× ×		×	× ×	×
FB2			× ×		×	× ×	×
FB3					×		
DON	× ×	× ×	× ×	×			
ZEN	× ×						

Sampling period 1 : 2002-2003
Sampling period 2 : 2003-2004

4. Structure of the report

Chapter II of the report is devoted to the description of the analytical methods used. These methods are based on well-known and previously described principles such as purification with immunoaffinity columns, HPLC, LC-MS/MS, … Some of these methods were already validated in the laboratories of the partners and were sometimes adapted for new matrices, some have been specifically developed within the scope of this project.

The results of the analyses are reported in Chapter III. For each foodstuff, a description of the sampling plan is followed by the occurrence data of the mycotoxins investigated, with a comparison between conventional and organic foods.

The development of new original methods is presented in Chapter IV. A mutli-mycotoxin chromatographic method, which allows simultaneous quantification of several mycotoxins and a fast immunological method are described.

Chapter 0 deals with bioassays based on mammalian cells in culture. Those methods were evaluated for their ability to screen and to determine the presence and the toxicity of mycotoxins.

Chapter VI contains a description of the way we have approached the creation of the Reference Centre.

Chapter VII describes to what extend the objectives of the project have been achieved

The main conclusions and recommendations that can be put forward at the end of this 3 years project are presented in Chapter VII in the final stage of the 3 years of research. The lasts points of the report list papers published or in preparation in the scope of the project.

References, detailed results and publications are presented in annexes.

II. DESCRIPTION OF THE METHODS USED

The analytical methods used to determine the occurrence of mycotoxins in food matrices under investigation as well as to validate new methods described in Chapter IV are presented in the following pages. This part ends with a description of the statistical tests used.

1. Determination of ochratoxin A

1.1. Ochratoxin A in wheat cereals

OTA was extracted by using an adaptation of the extraction procedure of the Technical Committee for Standardisation (CEN/TC 275 1998). The method was previously described by Thirumala-Devi *et al.* (2001).

Whole wheat flour samples (50 g) were mixed with 200 ml chloroform and 20 ml of H_3PO_4 0.1 M. This mixture was triturated for 3 minutes with an Ultra-Turrax CAT × 620 shaft at 13 500 rpm to produce a slurry. After centrifugation for 10 minutes under 820*g* at 5-10°C, the chloroform phase was transferred into a beaker. The remaining part was extracted again with 200 ml chloroform and 20 ml of H_3PO_4 0.1 M. 300 ml of the chloroform combined phases were evaporated to dryness using a rotary evaporator at 35-40°C. The residue was dissolved in 100 ml of 0.5 M $NaHCO_3$. After 10 minutes centrifugation under 820g at 5-10°C, 20 ml of the sample were passed through an Ochratest™ immunoaffinity column (Vicam, Watertown, MA, USA).

Purification of OTA extracts was performed with an immunoaffinity column fitted with a 20-ml syringe reservoir and placed on a vacuum manifold.
Before loading the extract, the Ochratest™ column was conditioned with 20 ml of Phosphate Buffered Saline (PBS) solution, pH 7.4. The OTA extract was passed through the column at about 1-2 ml min^{-1}. 20 ml of de-ionized water were used to wash the loaded immunoaffinity column and OTA was eluted with 2 ml methanol and 2 ml de-ionized water. Atmospheric air (*ca* 20 ml) was passed through the column to collect all the eluate. This solution was stored at –20°C. Prior to HPLC analysis (fluorescence spectrophotometer detector; $\lambda_{excitation}$=332 nm, $\lambda_{emission}$=462 nm), it was brought to room temperature and then filtered with a 0.45 µm microfilter.

1.2. Ochratoxin A in wheat flour

OTA was extracted using a multi-mycotoxin extraction procedure developed in our laboratories and optimized for wheat and wheat products by Pierard *et al*. (2004). This procedure is described in the chapter IV.1 and was validated for OTA.

1.3. Ochratoxin A in beer

The samples were extracted according to the method described by Tangni *et al.* (2002).

Briefly, 40 ml of degassed beer were diluted with 5 ml water containing 4% (w/v) sodium bicarbonate and with 15 ml Phosphate Buffer Saline (PBS) solution (Sigma, St. Louis, MO, USA). Solid particles were separated by centrifugation of the mixture at 820*g* during 10 minutes at 5-10°C. 45 ml of the supernatant were used for Ochratest™ immunoaffinity column (Vicam, Watertown, MA, USA) clean-up.

The clean-up step and the HPLC analysis were similar to the ones used for OTA determination in cereals.

OTA confirmation by methylation was performed according to the method used by Zimmerli *et al.* (1995).

2. Determination of deoxynivalenol

2.1. Deoxynivalenol in wheat cereals

Samples were analysed by a method adapted from the extraction procedure proposed by Romer (1986) and described by Tangni (2003).

Twenty five grams of the milled and well mixed sample were transferred into a 500-ml centrifuge beaker and added with 125 ml of extraction solvent (acetonitrile : water, 84 : 16). The mixture was triturated for 3 minutes with the Ulta-Turrax device CAT x 620 shaft at 13 500 rpm to produce a slurry. The solvent phase was isolated by centrifugation at 1835 g during 10 minutes at 5-10°C. 10 ml of the resulting supernatant was placed in the MycoSep® test tube (Romer Labs, purchased from Coring system Diagnostix GmbH, Gernsheim, Germany) for purification. Four ml of the cleaned extract were evaporated to dryness, using a rotary evaporator at 40-50°C. The residue was dissolved in 2 ml of methanol : water (10 : 90) and stored at was stored at –20°C. Prior to HPLC analysis (UV detection at 218 nm), it was brought to room temperature and then filtered with a 0.45 µm microfilter.

The proficiency of this method was checked by the Food Analysis Performance Assessment Scheme (FAPAS), with very good results.

Samples were also analysed for the presence of DON by the multi-mycotoxin method described in point IV.1.

2.2. Deoxynivalenol in wheat flour

DON was extracted using a multi-mycotoxin extraction procedure developed in our laboratories and optimized for wheat and wheat products by Pierard *et al*. (2004). This procedure is described in the chapter IV.1 and was validated for DON.

2.3. Deoxynivalenol in beer

Bottles of beer were vigorously shaken and left for 10 minutes before opening. A sample of beer was taken and decarbonated by shaking again for 10 minutes. This degassed sample was then transferred into a 120-ml container and solid particles were separated by centrifugation at 820 *g* during 10 minutes at 10-20°C. A 10 ml sample was transferred into a pear-shaped flask and thoroughly mixed with acetonitrile (52-62 ml). Alcohol was then evaporated using a rotary evaporator at 40-50°C. The residue was solubilized in water (4 ml containing 1% (w/v) polyethyleneglycol and 5% (w/v) sodium bicarbonate). All recovered solution was poured on the DONTest™ for immunoaffinity column clean-up.

The immunity column was equipped on the top with an adapter and a 5-ml syringe reservoir and at the bottom with a stopcock placed on a vacuum manifold, to adjust the flow rate at 0.5 - 1 ml/min. 5 ml of water were then used to wash the loaded immunoaffinity colum and the toxin was eluate by passing 2 ml of methanol and collecting this eluate in a glass tube. Atmospheric air (*c.a.* 20 ml) was passed through the column to collect all the eluate and the single use column was then discarded.

The eluate was then evaporated to dryness in a SpeedVac system. The residue was dissolved with 2 ml of methanol:water (10:90, v/v) and stored at -20°C. Prior to HPLC analysis (UV detection at 218 nm), the eluate was left to room temperature and filtered through a 0.45 µm microfilter.

Samples collected during the second sampling period in 2003-04 were analysed with a method based on the one described above with the following modifications.

Hundred and ten ml of acetonitrile were added to 20 ml of degassed and centrifuged beer. The mixture was evaporated using a rotary evaporator at 40-50°C. The residue was solubilized in water (4 ml containing 1% (w/v) polyethyleneglycol and 5% (w/v) sodium bicarbonate). All recovered solution was applied to a DONPREP (R-Biopharm Rhône Ltd) immunoaffinity column and was allowed to pass through the column by gravity.

The column was washed with 5 ml water and was then dried. DON was eluted with 2 ml of methanol by backflush. The eluted extract was evaporated to dryness with a rotary evaporator, reconstituted in 1 ml of methanol:water (10:90) and stored at -20°C prior HPLC analysis.

Recovery experiments were performed in triplicate on three types of beer spiked at 20, 50 and 100 ng/ml. The overall average recovery was 93.7%. The limit of detection (LOD, at a signal to noise ratio=3) and the limit of quantification (LOQ, at a signal to noise ratio=10) were 2 and 6 ng/ml, respectively.

Care was taken in order to avoid saturation of the immuno-affinity colomn.

2.4. Deoxynivalenol in pasta

The extraction, clean-up and quantification procedures were the same as described for DON in wheat cereals.

Recovery was determined using pasta samples experimentally contaminated with 0.25, 0.51 and 0.76 µg/g DON. The overall average recovery was 108.2% (n=9). The LOD was 32 µg/kg and the LOQ 108 µg/kg.

3. Determination of zearalenone

3.1. Zearalenone in wheat cereals

ZEN was extracted using a multi-mycotoxin extraction procedure developed in our laboratories and optimized for wheat and wheat products by Pierard *et al*. (2004). This procedure is described in the chapter IV.1.

4. Determination of fumonisins

4.1. Fumonisins in sweet corn

Concerning the analysis of fumonisins in sweet corn, sweet corn from one container (can or glass jar) was first drained and grinded, then 20 g were extracted with 100 ml of methanol:water (3:1, v/v) by blending at high speed for 3 min. The solution was centrifuged (500 *g* for 10 min) and the supernatant was filtrated. If necessary, the pH was adjusted between 5.8 and 6.5 with NaOH (1 M). For the purification step, 10 ml of the filtrated supernatant were passed through a Strong Anion eXchange (SAX) column at a rate of less than 2 ml/min. After washing the column with 5 ml of methanol:water (3:1, v/v) and 3 ml of methanol, the column was eluted with 10 ml of methanol:acetic acid (99:1, v/v) at a rate of less than 1 ml/min. Finally, the collected eluate was evaporated to dryness and redissolved in 1 ml methanol and filtrated through a 0.45 µm filter. Redissolved sample (100 µl) was derivatized with 300 µl OPA for 4 min and 40 µl of this mix were injected into the column.

The HPLC apparatus consisted of an isocratic pump equipped with a degassing system, an automatic injector and of a fluorometer detector. The analytical column was a C18 reversed

phase (6.4 nm, 4 µm, 3.9 mm × 150 mm). The mobile phase was a mixture of methanol-0.1 M sodium dihydrogen phosphate (15.6 g $NaH_2PO_4 \cdot 2H_2O$ in 1 l Milli-Q water) (77:23), adjusted to pH 3.3 with *o*-phosphoric acid, and filtered through a 0.22 µm filter membrane. The flow rate was 1.0 ml/min. The fluorescence detector excitation and emission wavelengths were set at 335 and 440 nm, respectively. Measurements were made by peak height. The LOD (signal/noise=3) obtained by this procedure for FB1 and FB2 was 6 and 13 µg/kg, respectively. The LOQ (signal/noise=10) was 18 µg/kg for FB1 and 42 µg/kg for FB2.

4.2. Fumonisins in polenta

The method we developed was adapted from different published methods (Duncan et al., 1998; Thiel *et al.*, 1993; Visconti *et al.*, 2001). Our method is described in a paper published in collaboration with our partners (Paepens *et al.* 2004).

Sodium dihydrogen phosphate, o-phosphoric acid 87.5%, sodium chloride and PBS were of analytical reagent grade. The solution of derivatisation, o-phthaldialdehyde (OPA), was prepared by dissolving 0.2 g OPA in 5 ml methanol, adding 25 ml sodium tetraborate (0.1 M) and 250 µl 2-mercaptoethanol, followed by a final filtration through a 0.45 µm filter.

The HPLC apparatus consisted of an isocratic pump equipped with a degassing system, an automatic injector and of a fluorometer detector. The analytical column was a C18 reversed phase (6.4 nm, 4 µm, 3.9 mm x 150 mm). The mobile phase was a mixture of methanol-0.1 M sodium dihydrogen phosphate (15.6 g $NaH_2PO_4 \cdot 2H_2O$ in 1 l Milli-Q water) (77:23), adjusted to pH 3.3 with o-phosphoric acid, and filtered through a 0.22 µm filter membrane. The flow rate was 1.0 ml/min. The fluorescence detector excitation and emission wavelengths were set at 335 and 440 nm, respectively. Measurements were made by peak height. The LOD (signal/noise=3) obtained by this procedure for FB1 and FB2 was 30 and 65 µg/kg, respectively. The LOQ (signal/noise=10) was 97 µg/kg for FB1 and 212 µg/kg for FB2.

Maize 50 g (finely ground at 1 mm) was mixed with 5 g NaCl and extracted with a solution of 100 ml methanol:water (80:20, v/v) by blending at high speed for 3 min. After centrifugation (10 min at 500 × g) and filtration, 10 ml of the filtrate were diluted with 40 ml PBS and centrifuged again. Ten millilitres of this dilution were passed on an immunoaffinity column (FumoniTest, Vicam, Watertown, MA, USA). After washing the column with 10 ml PBS at the same rate, the fumonisins were eluted with 1.5 ml methanol by gravity. The eluate was evaporated to dryness and redissolved in 1 ml methanol and filtered through a 0.45 µm filter prior to analysis. The redissolved sample (100 µl) was derivatized with 300 µl OPA for 4 min and 40 µl of this mix were injected into the column.

In order to check our methods, we regularly participated to FAPAS (Food Analysis Performance Assessment Scheme) proficiency tests organized by the Central Science Laboratory, Sand Hutton, York, UK.

4.3. Fumonisins in beer

Concerning the fumonisins analysis in beers, as it is an aqueous solution, the extraction step was unnecessary. The purification step was adapted from the VICAM method for determination of fumonisins in beers (Fumonistest™ Instruction Manual). First, 5 ml of degassed beer were passed through an immunoaffinity column (FumoniTest, Vicam, Watertown, MA, USA) at a steady slow flow rate (gravity flow) until air came through. The column was washed with 1 ml solution of 2.5% NaCl and 0.5% sodium bicarbonate at the same rate until air came through, then with 1 ml of water (HPLC grade) at a rate of 2-3 drops per second. Finally, the column was eluted by passing 1 ml HPLC grade methanol at gravity flow rate and the collected eluate was evaporated to dryness. The dried sample was mixed

with 250 µl of *o*-phthaldialdehyde (OPA) derivatization solution and 50 µl were injected into the HPLC column.

The HPLC apparatus consisted of an isocratic pump equipped with a degassing system, an automatic injector and of a fluorometer detector. The analytical column was a C18 reversed phase (6.4 nm, 4 µm, 3.9 mm × 150 mm). The mobile phase was a mixture of methanol-0.1 M sodium dihydrogen phosphate (15.6 g $NaH_2PO_4 \cdot 2H_2O$ in 1 l Milli-Q water) (77:23), adjusted to pH 3.3 with *o*-phosphoric acid, and filtered through a 0.22 µm filter membrane. The flow rate was 1.0 ml/min. The fluorescence detector excitation and emission wavelengths were set at 335 and 440 nm, respectively. Measurements were made by peak height. Based on standards analyses, the LOD (signal/noise=3) obtained by this procedure for FB1 and FB2 was 0.44 and 1.17 ng/ml, respectively. The LOQ (signal/noise=10) was 1.46 ng/ml for FB1 and 3.88 ng/ml for FB2.

4.4. Fumonisins in cornflakes

The presence of FB_1, FB_2 and FB_3 was determined with a method derived from that of Visconti *et al.* (2001) with modifications. A publication of the method described below is submitted to a peer reviewed journal.

Cornflakes were ground using a grinder (GmbH & Co.KG, Germany), thoroughly mixed and stored at room temperature prior to analysis. Twenty g of sample were extracted with 50 ml methanol:water (70:30, v/v) adjusted to pH 4 with 0.1 M HCl, by shaking for 30 min using an orbital shaker. The mixture was then centrifuged for 10 min at 2217 *g* and filtered. The remaining solid material was extracted again with 50 ml extraction solvent, centrifuged and filtered as above. Ten ml of the combined extracts were mixed with 40 ml 0.01 M PBS buffer solution, pH 7.4.

A 15-ml volume was passed through a FumoniTest® immunoaffinity column (VICAM, Watertown, MA, USA). After washing the column with 15 ml H_2O, fumonisins were eluted with 2 ml methanol. The eluted extract was evaporated to dryness under nitrogen stream at 60°C and redissolved in 450 µl of mobile phase made of acetonitrile:water (60:40) containing 0.3% formic acid. A volume of 50 µl (2S,3R)-2-aminododecane-1,3 diol was added after sample clean-up and functioned as an internal standard. This synthetic compound was chosen because of its structural similarity to the fumonisin backbone and its chromatographic elution time between the target analytes.

Liquid chromatography analysis was carried out with a Waters Alliance 2695 XE HPLC system coupled to a Micromass Quatro micro triple quadrupole mass spectrometer (Waters, Milford, MA, USA). The analytical column was an Alltima C_{18}, 5 µm, 150 3.2 mm (Alltech Associates, Deerfield, IL, USA), while the protecting guard column was an Alltima C_{18}, 5 µm, 7.5 × 3.2 mm (Alltech Associates). A volume of 20 µl was injected into the Alltima C_{18} column. The chromatography was performed under isocratic conditions at a flow of 0.3 ml min^{-1} with a mobile phase consisting of acetonitrile/water (60/40, v/v) containing 0.3% formic acid. The mass spectrometer was operated in the positive electrospray ionization (ESI+) mode using multiple reaction monitoring (MRM). High purity nitrogen was used as the drying and electrospray ionization (ESI) nebulizing gas. Argon was used as the collision gas for collision induced dissociation. The ideal ionization conditions for each analyte were determined by continuous infusion of the tuning solution and varying the cone voltage between 40 and 90 eV. The capillary voltage was set at 3.60 eV and the source block and desolvation temperatures were 140 and 350 °C, respectively. Ideal fragmentation conditions were sought by varying the collision energy from 30 to 50 eV. For the detection of FB_1, $[MH]^+$ was chosen as the parent ion at *m/z* 722.30. The product ions followed were at *m/z* 352.8 and 334.8 with

the first one used for quantification. For FB_2, the parent ion was *m/z* 706.8. The product ions followed for the detection of FB_2 were *m/z* 354.8 and *m/z* 336.8 with the first transition used for quantification. For the detection of FB_3, $[MH]^+$ was chosen as the parent ion at *m/z* 706.8. The product ions *m/z* 688.8 and 354.8 were followed and *m/z* 354.8 was used for quantification. For the detection of the internal standard (2S,3R)-2-aminododecane-1,3 diol, $[MH]^+$ was chosen as the parent ion at *m/z* 218.73. The product ions followed were at *m/z* 170.50 and *m/z* 94.80 with the last transition used for quantification. Quantitative determinations were based on the peak area ratios of the selected fragment ions of respectively FB_1, FB_2 and FB_3 in comparison to that of the internal standard.

An intra-laboratory validation was conducted with fortified samples. Cornflakes with no detectable fumonisin levels were fortified with FB_1 at concentrations ranging from 25 to 500 µg/kg and with FB_2 and FB_3 at contamination levels between 15 and 250 µg/kg. For each component, calibration curves (n=4) were established in order to calculate the LOD and the LOQ. The corresponding concentration at 3 times the residual standard deviation of the y-intercept divided through the slope of the regression line equals the LOD. LOQ is equal to 6 times the residual standard deviation of the y-intercept divided through the slope of the regression line. Precision was expressed as coefficients of variation for analyses carried out under repeatability conditions (at 1, 1.5 and 2 times the LOQ concentration of the respective components, n=5) and for analyses carried out under within-laboratory reproducibility conditions (at 1, 1.5 and 2 times the LOQ concentration of the respective components, n=3). Fortified samples were also used to assess the FB_1, FB_2 and FB_3 recoveries. Specificity was checked by analysing blank cornflakes samples and looking for interfering peaks.

Because no interfering peaks could be detected when analysing blank samples, it could be concluded that the method was specific. Average determination coefficients (R^2) of the calibration curves (n = 4) were 0.991 for FB_1 and FB_3, while the R^2 for FB2 was 0.995. The LOD concentrations for FB_1, FB_2 and FB_3 were 20, 7.5 and 12.5 µg/kg, respectively. The LOQ was calculated as 40 µg/kg for FB_1, 15 µg/kg for FB_2 and 25 µg/kg for FB_3. The coefficients of variation under repeatability conditions varied from 7.1 to 13 %. Under within-laboratory reproducibility conditions, the coefficients of variation did not exceed 17 %. Mean recoveries (n = 6) for FB_1, FB_2 and FB_3 were respectively 84 ± 10 %, 78 ± 7 %, 87 ± 9 %.

5. Statistical analysis

The SAS Enterprise Guide software (SAS Institute, Inc., Cary, USA, version 3.0.0.369) was used for statistical studies.

Differences in contamination frequency at level over the limit of quantification between conventional and organic samples were evaluated using Chi-square test performed by the procedure "PROC FREQ" of SAS. Variance analyses were performed on quantifiable concentrations using the GLM procedure of SAS.

A probability value of 0.05 has been used in order to determine the statistical significance.

III. LEVEL OF CONTAMINATION OF CEREAL BASED FOODSTUFFS OF THE BELGIAN MARKET
ORGANIC VS CONVENTIONAL MODE OF PRODUCTION

1. Mycotoxins in wheat cereals

1.1. Sampling

Wheat cereals samples were harvested in August 2002 and in August 2003.

Cereals were obtained directly from the organic and conventional producers equally distributed in Belgium (half from Wallonia and half from Flanders), in order to reduce the variability (geographical, climatic, pedological factors) between the samples. The sampling was performed by the farmers themselves according to our recommendations. We asked them to take one kilogram of cereals in 5 different buckets or at 5 different places in the heap of grains, in order to obtain a 5-kg sample representative of the parcel of land.

Thanks to these collaborations, the origin of the cereals is assured and allows to overview the contamination of Belgian cereals and to compare organic and conventional samples.

Each year, 20 samples were collected in Wallonia and 20 others in Flanders, half coming from conventional farming, half from organic farming. Some additional samples were obtained.

Samples were ground in a Retsch centrifugal mill to pass a 1 mm sieve and stored at room temperature.

1.2. Results

1.2.1. Ochratoxin A in wheat cereals

The results of the analysis of 40 samples of cereals harvested in 2002 are summarized in Table 2.

OTA was quantified in four conventional samples and in 10 organic samples. This ratio is statistically significant ($\chi_1^2 = 3.956$, p=0.0467).

Mean concentration of conventional and organic samples were 0.067 and 0.063 µg/kg, respectively. The maximum level of contamination was recorded in a conventional sample, with 1.097 µg/kg.

OTA concentration never exceeded 5 µg/kg, the maximum contamination level fixed by the European Commission Regulation ((EC) N° 123/2005).

The low contamination is probably due to the reduced period of storage of the cereals tested since OTA is mainly produced during storage.

Table 2. Occurrence and levels of ochratoxin A in wheat cereals harvested in 2002.

Occurrence	**Conventional**	**Organic**	**Total**
Number of samples	20	20	40
n.d.[a]	15 (75%)	9 (45%)	24 (60)
trace[b]	1 (5%)	1 (5%)	2 (5)
LOQ-2.5 µg/kg	4 (20%)	10 (50%)	14 (35)
2.5-5 µg/kg	0	0	0
> 5 µg/kg	0	0	0
Incidence	**25%**	**55%**	**40%**
Levels of contamination (µg/kg)			
Mean[c] ± SD	0.067 ± 0.243	0.063 ± 0.082	0.065 ± 0.179
Median[c]	0.005	0.030	0.005
Range[d]	0.045 – 1.097	0.037 – 0.0303	0.037 – 1.097

[a]: n.d., not detected (OTA concentration in sample <0.01 µg/kg)
[b]: traces means that the concentration of OTA is between the limit of detection (LOD = 0.01 µg/kg) and the limit of quantification (LOQ = 0.035 µg/kg).
[c]: mean and median are computed assuming that non-detected samples contain half the LOD while samples with traces levels of OTA contain half the LOD+LOQ.
[d]: range of samples with OTA contents ≥ LOQ.

1.2.2. Deoxynivalenol in wheat cereals

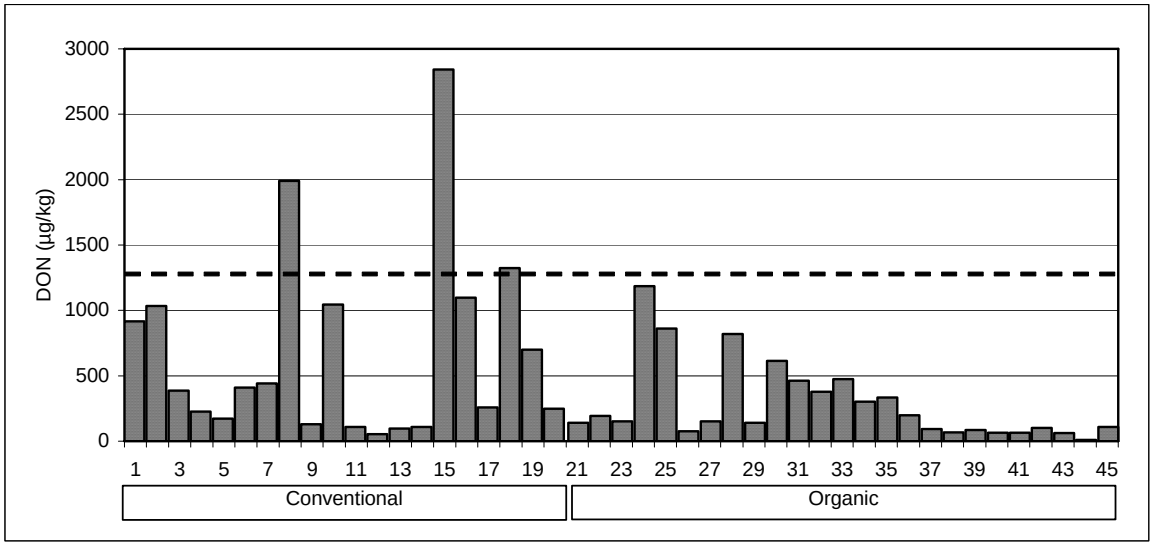

Figure 1. Deoxynivalenol content (µg/kg) in wheat cereals collected in Belgium during harvest 2002.

DON was quantified in 68% of organic samples and in 90% of conventional cereals. Some indication of a more frequent contamination over LOQ in conventionally produced cereals was found by Chi-square test ($\chi_1^2 = 3.1114$, p=0.0777).

As shown in Figure 1, the different levels of DON concentrations on grains collected at harvest during 2002 were found to be high because of the rainy weather during June 2002. The climatic conditions were indeed favourable to the growth of *Fusarium*. The average contamination levels for conventional and organic cereals were 675 µg/kg and 285 µg/kg, respectively. Among positive samples (>LOQ), analysis of variance indicates a tendency to levels of DON significantly higher in conventional samples (p=0.0738).

According to the last European proposal concerning mycotoxins in cereals and cereal derived products (Table 3), most of the samples had an acceptable level of contamination (<1250 µg/kg) (Figure 1, Table 4).

None of the organic samples was found to have a contamination above 1250 µg/kg while 3 conventional samples were found above this limit. Two conventional samples were found to have a very severe contamination level so (2842 and 1991 µg/kg).

Seven samples had a contamination level between 750 and 1250 µg/kg whereas 33 samples were contaminated at a level lower than 500 µg/kg (Table 4).

Table 3. New proposed maximum limits for Fusarium toxin : the DON concentration limits.

Product	Maximum DON level (µg/kg)
Unprocessed durum wheat	1750
Unprocessed wheat cereals	1250
Cereal flour	750
Dry pasta	750
Bread, pastries, biscuits, breakfast cereals	500
Cereal based baby food	250

Table 4. Occurrence and levels of the deoxynivalenol in wheat cereals collected during harvest 2002.

Occurrence	**Conventional**	**Organic**	**Total samples**
Number of samples	20	25	45
n.d.[a]	0 -	1(4%)	1 (2%)
traces [b]	2 (10%)	7 (28%)	10 (22%)
LOQ – 250 µg/kg	6 (30%)	8 (32%)	13 (29%)
250 – 500 µg/kg	4 (20%)	5 (20%)	9 (20%)
500 – 750 µg/kg	1 (5%))	1 (4%)	2 (5%)
750 – 1250 µg/kg	4 (20%)	3 (12%)	7 (16%)
1250 – 1750 µg/kg	1 (5%)	0	1 (2%)
> 1750 µg/kg	2 (10%)	0	2 (4%)
Incidence	**20 (100%)**	**24 (96%)**	**44 (98%)**
Levels of contamination (µg/kg)			
Mean[c]± SD	675 ± 579	285 ± 305	460 ± 562
Median[c]	425	151	226
Range[d]	100 - 2842	100 - 1184	100 - 2842

[a] : n.d., not detected (DON concentration in sample <30 µg/kg)
[b] : traces means that the concentration of DON is between the limit of detection (LOD = 30 µg/kg) and the limit of quantification (LOQ = 100 µg/kg).
[c] : mean and median are computed assuming that non-detected samples contain half the LOD while samples with traces levels of DON contain half the LOD+LOQ.
[d] : range of samples with DON contents ≥ LOQ.

As shown in Figure 2 and Table 5, the different levels of DON in grains collected at harvest in 2003 were found to be very low. Indeed, the dry weather during June 2003 over the flowering period for wheat was less favourable to the growth of *Fusarium*. The mean (median) contamination levels for conventional and organic samples were 311 µg/kg (248 µg/kg) and 123 µg/kg (129 µg/kg).

DON was quantified in 73% of organic samples and in 86% of conventional cereals. Proportions of contaminated cereals (>LOQ) of conventional and organic origin are statistically equivalent ($\chi_1^2 = 1.2755$, p=0.2587). Among samples contaminated at a level over LOQ, conventional wheat cereals were statistically more contaminated compared to organic cereals (p=0.0081).

According to the last European proposal (Table 3), all the collected and analysed wheat samples were acceptable. All the samples but sample 3 were found to have a contamination lower than 750 µg/kg.

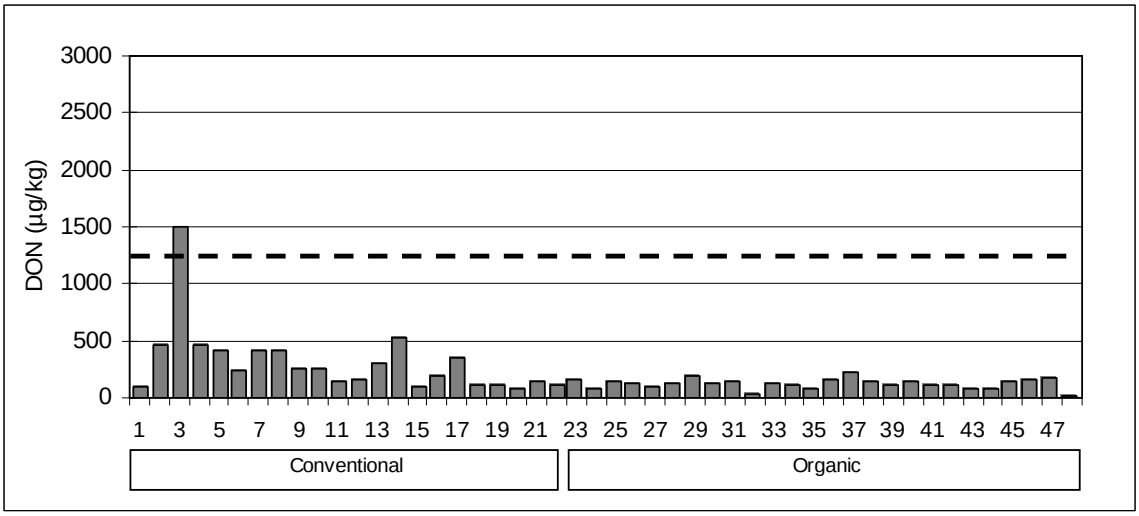

Figure 2. Deoxynivalenol content (µg/kg) in wheat cereals collected in Belgium during harvest 2003.

Table 5. Occurrence and levels of deoxynivalenol in wheat cereals collected during harvest 2003.

Occurrence	**Conventional**	**Organic**	**Total samples**
Number of samples	22	26	48
n.d.[a]	0 -	1 (4%)	1 (2%)
traces[b]	3 (14%)	6 (23%)	9 (19%)
LOQ – 250 µg/kg	8 (36%)	19 73%)	27 (56%)
250 – 500 µg/kg	9 (40%)	0	9 (19%)
500 – 750 µg/kg	1 (5%)	0	1 (2%)
750 – 1250 µg/kg	0	0	0
1250 – 1750 µg/kg	1 (5%)	0	1 (2%)
> 1750 µg/kg	0	0	0
Incidence	**22 (100%)**	**25 (96%)**	**47 (98%)**
Levels of contamination (µg/kg)			
Mean[c]± SD	311 ± 303	123 ± 47	210 ± 226
Median[c]	248	129	138
Range[d]	79 - 1503	10 - 226	10 - 1503

[a]: n.d., not detected (DON concentration in sample <30 µg/kg)
[b]: traces means that the concentration of DON is between the limit of detection (LOD = 30 µg/kg) and the limit of quantification (LOQ = 100 µg/kg)
[c]: mean and median are computed assuming that non-detected samples contain half the LOD while samples with traces levels of DON contain half the LOD+LOQ
[d]: range of samples with DON contents ≥ LOQ

1.2.3. Zearalenone in wheat cereals

Figure 3 and Table 7 present the results of ZEN levels in the organic and conventional samples collected in 2002.

Occurrence of ZEN at levels exceeding the LOQ was statistically higher in conventional samples (85%) than in organic samples (52%) ($\chi_1^2 = 5.4454$, p=0.0196). A statistical comparison between quantified conventional and organic samples indicated that the mode of production influenced the level of contamination (p=0.0128).

None of the organic sample showed a contamination level above the proposed limit of 100 µg/kg set by the European Commission for unprocessed cereals (Table 6). By contrast, 6 out of 20 conventional samples had a ZEN value above that limit. Nine samples had a ZEN value between 50 and 100 µg/kg and have therefore to be considered as not acceptable as bread and breakfast cereals (limit of 50 µg/kg). The samples included in the category (50 to 100 µg/kg) were from both production type (conventional and regular).

Table 6. New proposed maximum limits for Fusarium toxin : the ZEN concentration limits.

Product	Maximum ZEN level (µg/kg)
Unprocessed cereals other than maize	100
Cereal flour except maize flour	75
Bread, pastries, biscuits, breakfast cereals	50
Cereal based baby food	20

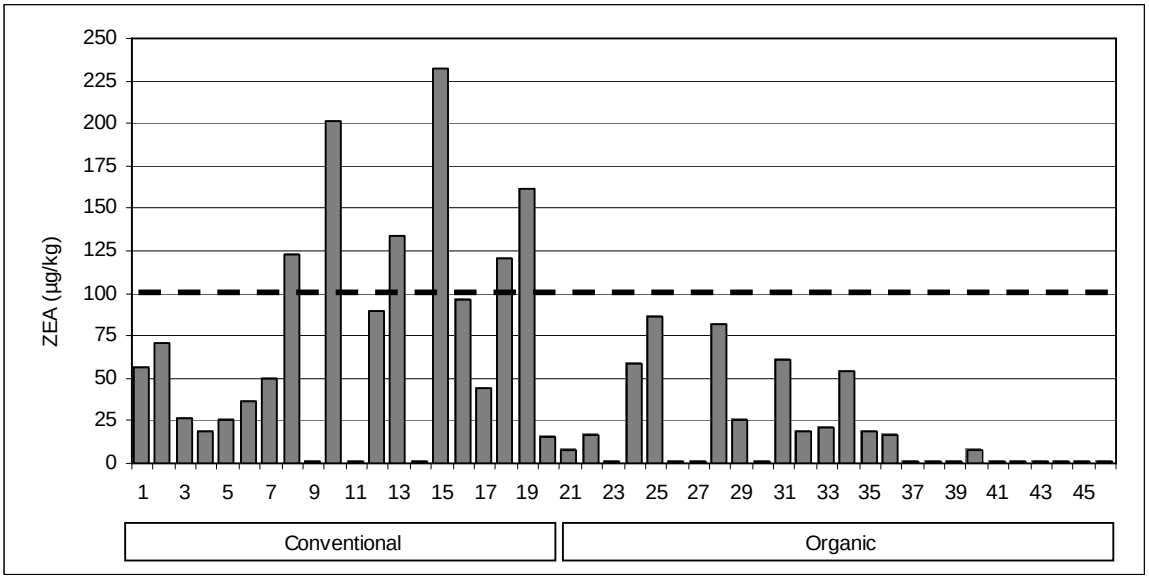

Figure 3. Zearalenone content (µg/kg) in wheat cereals collected in Belgium during harvest 2002.

Table 7. Occurrence and levels of zearalenone in wheat cereals collected during harvest 2002.

Occurrence	Conventional	Organic	Total samples
Number of samples	20	25	45
n.d.[a]	3 (15%)	12 (48%)	15 (33%)
traces[b]	0 -	0 -	0 -
LOQ – 50 µg/kg	7 (35%)	8 (32%)	15 (33%)
50 – 75 µg/kg	2 (10%)	3 (12%)	5 (11%)
75 – 100 µg/kg	2 (10%)	2 (8%)	4 (9%)
> 100 µg/kg	6 (30%)	0 -	6 (13%)
Incidence	**17 (85%)**	**13 (52%)**	**30 (67%)**
Levels of contamination (µg/kg)			
Mean[c]± SD	75 ± 68	19 ± 27	44 ± 67
Median[c]	53	8	19
Ranged	4 - 86	4 - 232	4 - 232

[a]: n.d., not detected (ZEN concentration in sample <1.5 µg/kg)
[b]: traces means that the concentration of ZEN is between the limit of detection (LOD = 1.5 µg/kg) and the limit of quantification (LOQ = 4 µg/kg)
[c]: mean and median are computed assuming that non-detected samples contain half the LOD while samples with traces levels of ZEN contain half the LOD+LOQ
[d]: range of samples with ZEN contents ≥ LOQ

The levels of ZEN concentrations in the samples collected at harvest in 2003 were found to be mostly below the detection or the quantification limit (LOD = 1.5 µg/kg; LOQ = 4 µg/kg) (Figure 4 and Table 8), which is in deep contrast with the results obtained in 2002. For 2003, the incidence of contamination was found to be as low as 4% for organic samples and 9% for the conventional ones. The dry weather during June 2003 (flowering period for wheat) most likely explains these differences since a dry period at flowering is not favourable to the growth of *Fusarium.* As shown in Figure 4 and Table 8, only 3 samples were found to contain a quantifiable level of ZEN. Because of the high number of non-detected samples, the mean, SD and median calculations are not representative.

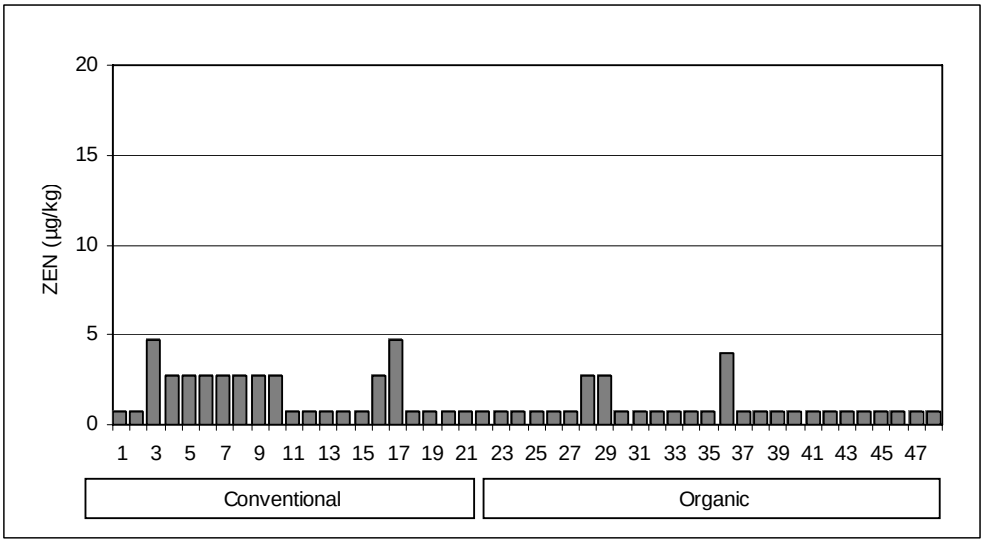

Figure 4. Zearalenone content (µg/kg) in wheat cereals collected in Belgium during harvest 2003.

Table 8. Occurrence and levels of zearalenone in wheat cereals collected during harvest 2003.

Occurrence	Conventional	Organic	Total samples
Number of samples	22	26	48
n.d.[a]	20 (91%)	25 (96%)	45 (94%)
traces[b]	2 (9%)	1 (4%)	3 (6%)
LOQ – 50 µg/kg	0	0	0
50 – 75 µg/kg	0	0	0
75 – 100 µg/kg	0	0	0
> 100 µg/kg	0	0	0
Incidence	**2 (9%)**	**1 (4%)**	**3 (6%)**
Levels of contamination (µg/kg)			
Mean[c]± SD	2 ± 1	1 ± 1	1 ± 1
Median[c]	1	1	1
Range[d]	0.75 - 4	0.75 - 4	0.75 - 4

[a]: n.d., not detected (ZEN concentration in sample <1.5 µg/kg)
[b]: traces means that the concentration of ZEN is between the limit of detection (LOD = 1.5 µg/kg) and the limit of quantification (LOQ = 4 µg/kg)
[c]: mean and median are computed assuming that non-detected samples contain half the LOD while samples with traces levels of ZEN contain half the LOD+LOQ
[d]: range of samples with ZEN contents ≥LOQ

Taking advantage of the fact that the same samples were used for the determination of DON and ZEN, we tested the possible correlation between these two mycotoxins (Figure 5). Since they are produced by the same *Fusarium* species, a significant correlation was found between DON and ZEN levels in 2002.

The nice correlation obtained between the DON and the ZEN results indicates that a multi-contamination is very likely to occur in many cases. A multi-mycotoxin determination method would thus allow a better evaluation of the effective toxic content of the commodities under investigation.

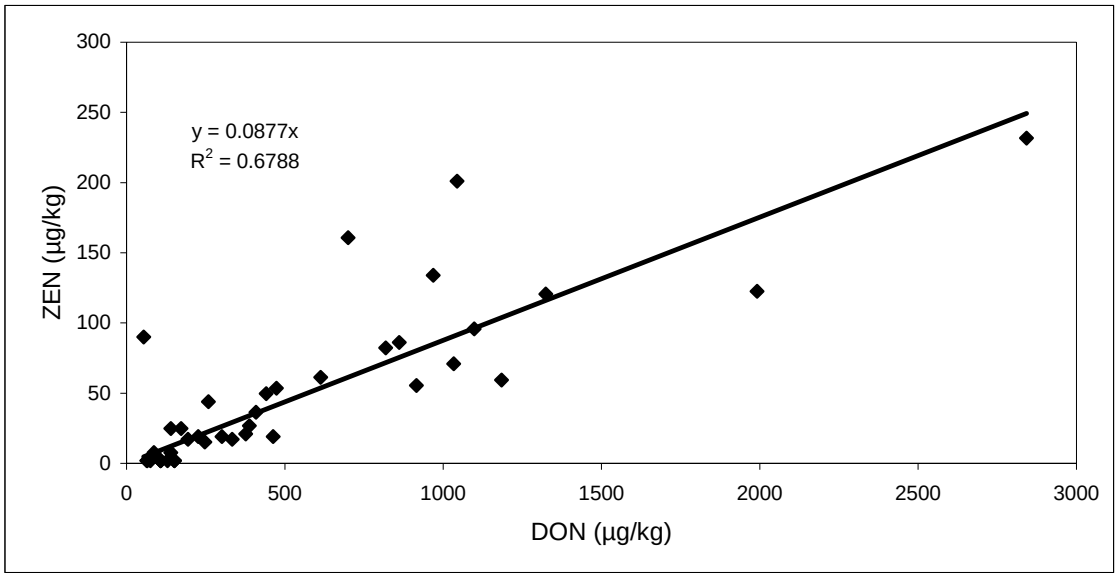

Figure 5. Correlation between DON and ZEN content in cereals.

1.3. Discussion and comparison with the literature

During harvest 2002, 20 samples of conventionally produced wheat cereals and 20 samples of organically produced wheat cereals have been collected prior to storage. The same sampling was performed during summer 2003.

Cereals from the first set of samples (2002) were analysed for OTA and DON by a validated method and for DON and ZEN by the multi-mycotoxin method. Results are summarized in Table 9.

Table 9. Ochratoxin A, deoxynivalenol and zearalenone contents in wheat cereals harvested in 2002 and 2003.

		OTA in positive samples[b]				DON in positive samples[b]				ZEN in positive samples[b]			
Type	n[a]	> LOQ (%)	Range (ng/kg)	Mean (ng/kg)	n[a]	>LOQ (%)	Range (µg/kg)	Mean (µg/kg)	n[a]	>LOQ (%)	Range (µg/kg)	Mean (µg/kg)	
2002													
Conventional	20	20a	45-1097	313a	20	90a	107-2842	746a	20	85a	15-232	88a	
Organic	20	50b	37-303	120a	25	68a	101-1184	389a	25	52b	8-86	37b	
2003													
Conventional	0	-	-	-	22	86a	108-1503	346a	22	9	-	5	
Organic	0	-	-	-	26	73a	106-226	145b	26	4	-	4	

Values of with different letters in the same column (conventional *vs* organic) are significantly different ($p<0.05$)
[a]: number of samples analysed
[b]: mycotoxin concentration > LOQ

Incidence and levels of contamination of OTA were quite low since the samples were not stored. Contamination at a level over the LOQ was statistically more frequent in organic samples (10 organic samples *vs* 4 conventional samples; $\chi^2_1 = 3.956$, p=0.0467).

A study carried out in 1997 on wheat samples collected in Poland after harvest detected OTA in none of the 32 samples from conventional farms (LOD=200 ng/kg) while wheat samples from ecological farming showed contamination of almost 8% within the range 480-1200 ng/kg (mean 830 ng/kg) (Czerwiecki *et al*., 2002a). These results are in the same line as ours in terms of frequency of contamination but not in terms of level.

Conversely, results of the same study performed one year later are contrasting with our results in terms of frequency. In wheat from conventional farms, OTA concentrations reached extremely high levels since the range stretched from 600 to 1024000 ng/kg (mean of 267 µg/kg) and the frequency of contamination was about 48%. By contrast, in wheat from ecological farming, OTA was detected in only 23% of samples in a range from 800 to 1600 ng/kg (mean of about 1200 ng/kg). These completely different results may have been due to varying weather conditions in the 2 years (Czerwiecki *et al*., 2002b).

Jorgensen *et al*. (2002) analysed conventionally (n=405) and organically (n=14) grown wheat kernels from 1992 to 1999. There was no difference in multiyear means between the different methods of production. OTA mean concentration (3000 ng/kg) was much higher than the one we found in Belgian 2002 samples.

Within the framework of his study, analysis of OTA on samples from 2003 was not repeated since it is a storage mycotoxin and our samples were collected prior to storage.

DON was detected in almost all the conventional and organic samples of 2002. The incidence at a level over the LOQ was 90 and 68%, respectively. Statistical analysis indicates a tendency to more frequent quantifiable amounts of DON in conventional samples than in organic ones ($\chi_1^2 = 3.1114$, p=0.0738). Mean levels of contamination in positive samples were 746 µg/kg for conventional products and 389 µg/kg for organic products. When contamination over LOQ occurred, it tended to be higher in conventionally produced cereals (p=0.0777). Three conventional samples were contaminated at a level over the proposed limit of 1250 µg/kg.

The same samples were analysed for ZEN and a significant correlation was found between DON and ZEN levels in 2002. Thus, ZEN was found in 85% of conventional samples (mean of 75±68 µg/kg) and in 52% of organic samples (mean of 19±27 µg/kg). Contamination at a level over the LOQ was more frequent in conventional samples ($\chi_1^2 = 5.4454$, p=0.0196) and among these contaminated samples, the conventional ones contained higher levels of ZEN (p=0.0128).

Cereals collected in 2003 were less affected by DON and ZEN than samples of 2002, due to a less favourable weather to contamination. In 2003, contamination by DON occurred as frequently as in 2002 but at much lower levels. Mean contamination was 210±226 µg/kg in conventional samples and 123±47 µg/kg in organic samples. Levels of DON higher than the LOQ occurred as frequently in organic cereals than in conventional ones ($\chi_1^2 = 1.2755$, p=0.2587) but were higher in conventionally produced samples (p=0.0081). In 2003, none of the samples contained ZEN at a level over the LOQ.

These results confirmed that the contamination by *Fusarium* mycotoxins is weather dependent. Similar variations in DON contamination in samples from two consecutive years of harvest (1997 and 1998) were previously attributed to different weather conditions during and following the flowering time were previously reported (Birzele, 2000).

2. Mycotoxins in wholemeal wheat flour

2.1. Sampling plan

Wholemeal wheat samples were obtained from retail shops. The total number of samples was 80 (10 conventional and 10 organic brands, 2 batches per brand and 2 samples per batch). Samples were stored at room temperature before analyses.

2.2. Results

Since the wheat cereals used for wholemeal wheat production are generally stored before use, storage mycotoxins are likely to be present in our samples. For this reason, both DON, representative of the trichothecenes produced in the field, and OTA, representative of storage mycotoxins, were evaluated in wholemeal wheat flour.

2.2.1. Ochratoxin A in wholemeal wheat flour

All data on the occurrence of OTA in wholemeal flour are presented in Figure 6 and in Table 10. Organic wheat flour samples were statistically more frequently contaminated (90%) at a quantifiable level than conventional samples (45%) ($\chi^2_1 = 18.4615$, $p<0.0001$), with concentrations ranging from 141 to 2199 ng/kg and from 64 to 1035 ng/kg, respectively.

Among positive samples, analysis of variance did not point out an effect of the mode of production (p=0.3753) and revealed homogeneous contamination between different brands (p=0.6779). Some indication of significant contamination differences between batches of a same brand was found (p=0.0811).

As shown in Figure 6, only one of the studied samples exceeded the European regulations set at 3000 ng/kg. The results presented result from duplicated analyses of each sample of flour. It clearly appears that the homogeneity of some samples was very low.

The mean and median values are given in Table 10. These values can be considered as low for both organic and conventional samples, which is a sign of good storage practices. However, the standard deviation (SD) remains high, illustrating the large range of OTA concentrations found in the analysed samples.

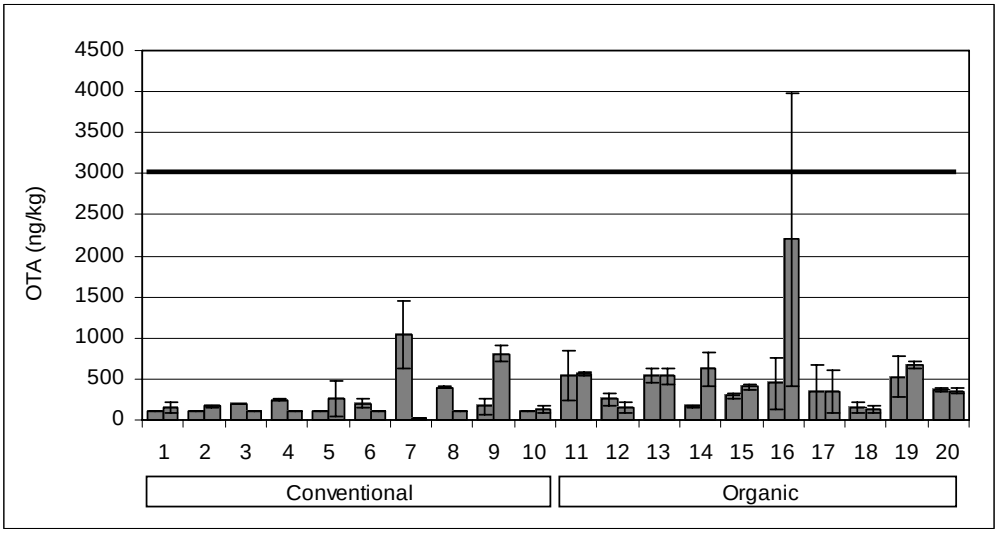

Figure 6. Ochratoxin A content (ng/kg) in wholemeal wheat flour. Each brand (n=20) is represented by 2 batches. Values are the average of two samples for each batch.

Table 10. Occurrence and levels of ochratoxin A in wholemeal wheat flour.

Occurrence	Conventional	Organic	Total
Number of samples	40	40	80
n.d.[a]	2 (5%)	0 -	2 (2.5%)
traces[b]	21 (52.5%)	5 (12.5%)	26 (32.5%)
LOQ-250 ng/kg	9 (22.5%	7 (17.5%)	16 (20%)
250 ng/l – 500 ng/kg	13 (34%)	14 (35%)	18 (22.5%)
500 ng/l – 1000 ng/kg	4 (10%)	13 (32.5%)	16 (20%)
>1000 ng/kg	1 (2.5%)	1 (2.5%)	2 (2.5%)
Incidence	38 (95%)	40 (100%)	39 (97.5)
Levels of contamination (ng/kg)			
Mean[c]± SD	228± 262	480±530	354±434
Median[c]	100	378	214
Range[d]	153-873	154 - 3460	153 - 3460

[a]: n.d., not detected (DON concentration in sample <50 ng/kg)
[b]: traces means that the concentration of OTA is between the limit of detection (LOD = 50 ng/kg) and the limit of quantification (LOQ = 150 ng/kg)
[c]: mean and median are computed assuming that non-detected samples contain half the LOD while samples with traces levels of OTA contain half the LOD+LOQ
[d]: range of samples with OTA contents ≥ LOQ

2.2.2. Deoxynivalenol in wholemeal wheat flour

Occurrence data and contamination levels of DON in wholemeal wheat flour are shown in Figure 6 and Table 11. In opposition to what was observed regarding the OTA concentrations found in the wheat flour samples, the frequency of contamination (>LOQ) was significantly higher in conventional flours (75%) than in the organic ones (17.5%) ($\chi_1^2 = 26.5996$, $p<0.0001$).

Among positive samples (>LOQ), conventional flours were more contaminated by DON than organic ones, but the difference was not significant (p=0.2325). There are neither significant variations between contamination of different brand (p=0.2064). By contrast, contamination among batches of a same brand is not homogeneous (p=0.0001).

The median for organic flour was found to be below the quantification limit and the observed range was very narrow, standing far below the limit of 750 µg/kg.

None of the organic samples (50% total samples) exceeded 250 µg/kg while two conventional sample (10 % total conventional samples) were found above 500 µg/kg but remained below 750 µg/kg, the future maximum level in wheat flour set by European regulations.

As also shown in Figure 7, the reproducibility (SD) of the replicate is better than the one found for the OTA concentrations in the same samples. So far the OTA is developed during storage, this could indicate non-optimised storage conditions of the wheat cereals before grinding or of the different meal brands after grinding.

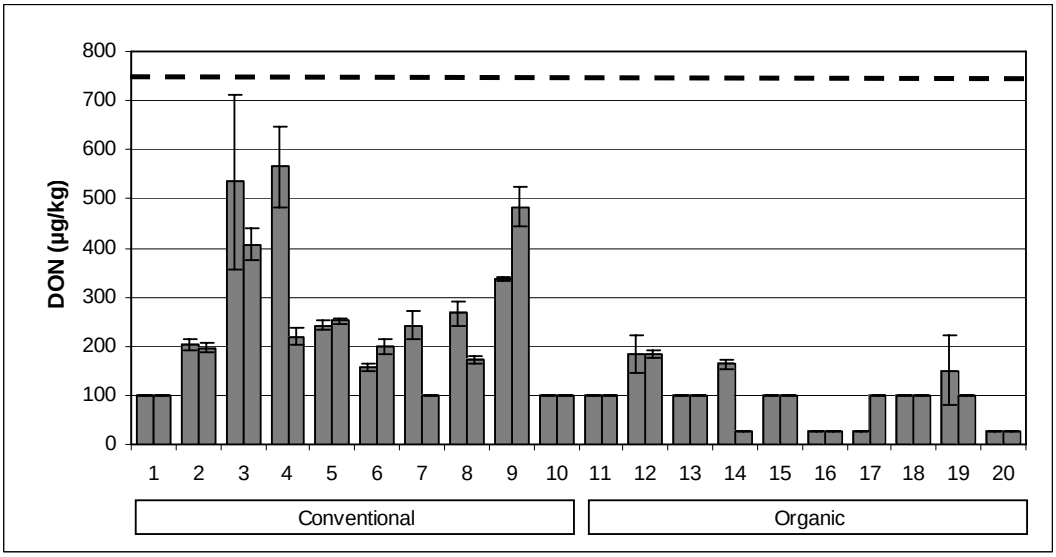

Figure 7. Deoxynivalenol content (µg/kg) in wholemeal wheat flour. Each brand (n=20) is represented by 2 batches. Values are the average of two samples for each batch.

Table 11. Occurrence and levels of deoxynivalenol in wholemeal wheat flour.

Occurrence	Conventional	Organic	Total samples
Number of samples	40	40	80
n.d. (< 30 µg/kg) [a]	0 -	12 (30%)	12 (15%)
Traces (< 100 µg/kg) [b]	5 (12.5%)	16 (40%)	21 (26%)
LOQ – 250 µg/kg	22 (55%)	12 (30%)	34 (43%)
250 – 500 µg/kg	9 (22.5%)	0	9 (11%)
500 – 750 µg/kg	4 (10%)	0	4 (5%)
750 – 1250 µg/kg	0 -	0	0
1250 – 1750 µg/kg	0 -	0	0
> 1750 µg/kg	0 -	0	0
Incidence	40 (100%)	28 (70%)	68 (85%)
Level of contamination			
Mean[c]± SD	249 ± 148	92 ± 54	170 ± 136
Median[c]	211	100	100
Range[d]	153 - 661	157 - 210	153 - 661

[a]: n.d., not detected (DON concentration in sample <50 µg/kg)
[b]: traces means that the concentration of DON is between the limit of detection (LOD = 50 µg/kg) and the limit of quantification (LOQ = 150 µg/kg).
[c]: mean and median are computed assuming that non-detected samples contain half the LOD while samples with traces levels of DON contain half the LOD+LOQ.
[d]: range of samples with DON contents ≥ LOQ.

2.3. Discussion and comparison with the literature

Wholemeal wheat flours for bread making studied by the multi-mycotoxin method showed that both OTA and DON contents were very low. Results are summarized in Table 12.

Table 12. Ochratoxin A and deoxynivalenol contents in wholemeal wheat flour.

Type	n[a]	OTA in positive samples[b]			DON in positive samples[b]		
		% samples	Range (ng/kg)	Mean (ng/kg)	% samples	Range (µg/kg)	Mean (µg/kg)
Conventional	40	45a	153-1329	394a	75a	153-661	299a
Organic	40	90b	154-3460	522a	17.5b	157-210	181a

Values of with different letters in the same column (conventional *vs* organic) are significantly different ($p<0.05$)

[a]: number of samples analysed

[b]: mycotoxin concentration > LOQ

OTA was detected in most of the samples and occurred at levels over the LOQ in 45% of the conventional flours and in 90% of the organic flours. Mean contamination in conventional and organic contaminated flours amounted to 394 ng/kg and 522 ng/kg, respectively. Organic wheat flour samples were statistically more frequently contaminated (90%) at a quantifiable level than conventional samples (45%) ($\chi_1^2 = 18.4615$, $p<0.0001$), but the mode of production did not influence the level of contamination ($p=0.3753$). Some indication of contamination differences between batches of a same brand was found ($p=0.0811$).

Biffi *et al*. (2004) found OTA in wheat flour from conventional and organic production. Conventional flours were on average more contaminated (134 ng/kg, n=14) than organic flours (53 ng/kg, n=11), but the difference was not significant. In terms of levels of contamination, OTA concentrations we found are much more higher and conclusions are in opposition.

One hundred and fifty six samples of conventional wheat flour and 120 samples of organic wheat flour purchased in Denmark between 1993 and 1999 were analysed by Jorgensen *et al.* (2002). Their results are similar to ours since little differences were reported in multiyear means of OTA between conventional samples (mean concentration of about 300 ng/kg) and organic ones (mean concentration of 500 ng/kg).

Cereals-based baby foods from Italy were analysed for OTA by Beretta *et al*. (2002). OTA occurred in 6 of 10 batches of semolina from conventional agriculture with concentrations ranging from 140 to 650 ng/kg. Only one of ten semolina batches from organically produced cereals contained a detectable amount of OTA (180 ng/kg; LOD=60 ng/kg). Once again, in terms of frequency of contamination, conclusions are conflicting.

Among the 80 samples analysed in the present study, contamination with DON (>LOQ) occurred more frequently in conventional flours than in organically produced flours ($\chi_1^2 = 26.5996$, $p=0.0001$), but levels of contamination were equivalent in the two types of samples ($p=0.2325$).

Schollenberger *et al*. (2002) analysed *Fusarium* toxins in wheat flour collected in Germany in 1999. The 36 conventional samples were contaminated with DON at a mean level of 394 µg/kg (incidence=100%) whereas the organic samples (n=24) contained on average 131 µg DON/kg (incidence=96%). DON content in conventional samples was statistically higher than that in organic products. Globally, these results are in line with our findings.

The same authors analysed the DON content in wheat bread conventionally and organically produced and showed some indication of a significant effect of the mode of production. Median DON levels in bread were higher for the conventional production mode.

3. Mycotoxins in beer

Beers produced with mould-contaminated grains can be contaminated with mycotoxins. A substantial loss of OTA occurs during mashing, but Baxter *et al.* (2001) reported that 13 to 32% of OTA contained in the original grist survive into the beer. The situation is even worse with DON since 80 to 93% of DON present in the malt grist are recovered in the beer (Schwartz *et al.*, 1995).

3.1. Sampling

In order to set up the best sampling procedure, we performed a preliminary pilote-scale test to determine if OTA contamination of beers from the same batch was homogeneous.

The results of this analysis are presented in Table 13 and show a homogeneous contamination level in each batch. Therefore, it is not necessary to take many bottles of beer coming from the same batch. In the future, we will thus evaluate the OTA contamination of beer by analysing only two bottles for each batch being controlled.

Table 13. Ochratoxin A contamination levels (ng/l) of four beer production batches from various origins. 5 bottles of each batch were analysed; the batches correspond to different brands.

Sample	Batch 1	Batch 2	Batch 3	Batch 4
1	Traces	17	126	14
2	Traces	17	124	13
3	Traces	19	121	13
4	Traces	18	85	12
5	Traces	16	117	16
Mean[a] ± SD	6.5	17 ± 1	115 ± 17	14 ± 1

[a] : mean is computed assuming that samples with traces levels of OTA contain half the LOD+LOQ

During the first sampling period (November 2002 to Janurary 2003), 70 beers (35 brands, 2 bottles from the same batch for each brand) were purchased randomly in retail shops. Among these samples, 44 were from conventional production. Twenty-six beers were brewed with organically grown raw material and labelled "biogarantie®".

Between September 2003 and September 2004, a total of 80 beers were collected; half of them being from organic grade and half from conventional grade. These samples represented 10 brands for each mode of production, two batches per brand and two samples per batch. The increased number of samples during the second sampling period was intended to improve the statistical analysis.

3.2. Results

3.2.1. Ochratoxin A in beer

All data on the occurrence of OTA in beers collected during the first sampling period are gathered in Table 14 and in Figure 8.

OTA was found in all organic samples at levels exceeding the LOQ and the contamination ranged from 10 to 520 ng/l. Seventy-two percents of conventional beers also contained quantifiable amounts of OTA, from 10 to 149 ng/l. OTA occurrence was statistically more frequent in organic beers ($\chi_1^2 = 8.6$, p=0.0034).

All samples of conventional beers were below the limit of 200 ng/l drafted by EU guidance limit (Scientific Committee on Food/EU 1999) and fixed by the Italian Ministry of Health in 1999 (Visconti *et al.*, 2000) while four samples (2 batches) from organic agriculture were above this limit. Levels of contamination above 200 ng/l were confirmed by methylation.

The average contamination levels for conventional and organic beers were 24 ng/l and 94 ng/l with medians of 16 and 22 ng/l, respectively.

Among positive samples, contamination by OTA was higher in beers from organic production ($p<0.0001$) due to the high content in some samples of this group (four samples were contaminated from 233 to 520 ng/l).

Analysis of variance performed on quantifiable samples also revealed variations between brands inside a mode of production ($p<0.0001$).

Table 14. Occurrence and levels of ochratoxin A in conventional and organic beer collected in 2002.

Occurrence	**Conventional**	**Organic**	**Total**
Number of samples	44	26	70
n.d.[a]	6 (14%)	0	6 (9%)
traces[b]	6 (14%)	0	6 (9%)
LOQ-100 ng/l	30 (68%)	20 (77%)	50 (71%)
101 ng/l – 200 ng/l	2 (5%)	2 (8%)	4 (6%)
> 200 ng/l	0	4 (15%)	4 (6%)
Incidence	**86%**	**100%**	**91%**
Levels of contamination (ng/l)			
Mean[c]± SD	24±31	89±137	48±92
Median[c]	16	22	19
Range[d]	10 - 149	12 - 520	10-520

[a]:n.d., not detected (OTA concentration in sample <3 ng/l)
[b]: traces means that the concentration of OTA is between the limit of detection (LOD = 3 ng/l) and the limit of quantification (LOQ = 10 ng/l)
[c]: mean and median are computed assuming that non-detected samples contain half the LOD while samples with traces levels of OTA contain half the LOD+LOQ
[d]: range of samples with OTA contents ≥ LOQ

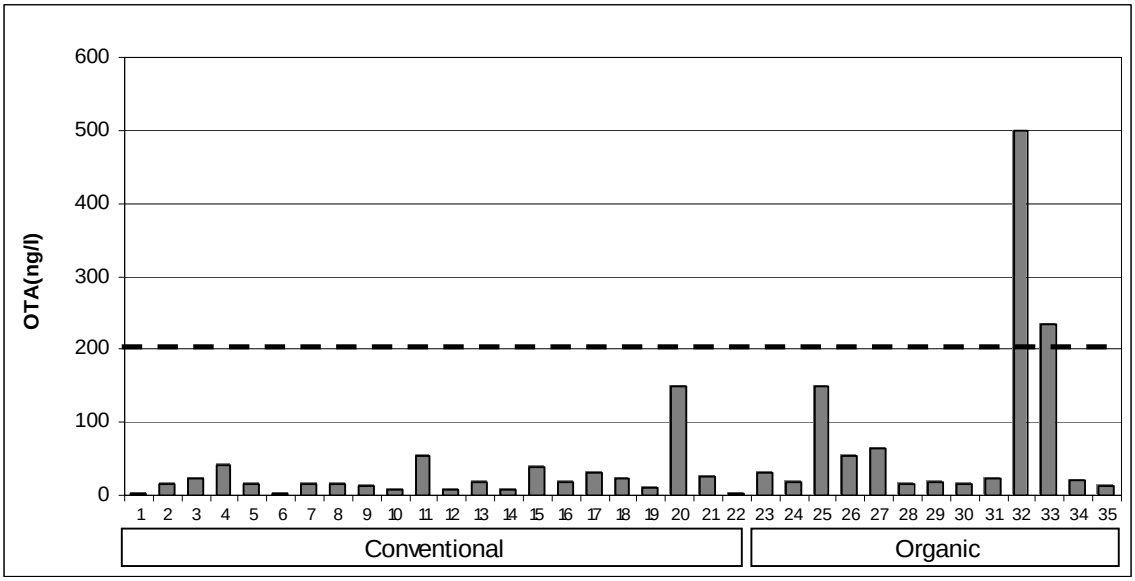

Figure 8. Ochratoxin A content (ng/l) in conventional and organic beers found on the Belgian market (2002-2003). For each brand (n=35), values are the average of two samples from the same batch.

Occurrence data of OTA in beers collected during the second sampling period are shown in Table 15 and in Figure 9. Comparing conventional and organic beers, organic beers were statistically more frequently contaminated (95%) at a quantifiable level than conventional beers (50%) with concentrations ranging from 18 to 1134 ng/l and from 18 to 198 ng/l, respectively ($\chi_1^2 = 20.3$, $p<0.0001$).

Among the total set of samples, 10 organic beers contained OTA above 200 ng/l.

The average contamination levels for conventional and organic beers were 25 ng/l and 182 ng/l with medians of 15 and 45 ng/l, respectively.

This time, the sampling was more extensive and thus allows a precise statistical analysis based on a more complete model including three classifications variables (the mode of production, the brand and the batch) with nested effects.
Among beers contaminated at a level over LOQ, organic beers were more contaminated by OTA than conventional ones, but the difference is not significant ($p=0.1758$). This result is due to a great dispersion of contamination levels of brands among organic beers and among conventional beers comparatively to the dispersion of global means of contamination of organic and conventional beers. There are neither significant variations between contamination of different brand ($p=0.2093$). By contrast, contamination among batches of a same brand is not homogeneous ($p<0.0001$).

Table 15. Occurrence and levels of OTA in beers collected on the Belgian market (2003-2004).

Occurrence	Conventional	Organic	Total
Number of samples	40	40	80
n.d.[a]	9 (22.5%)	0	9 (11%)
trace[b]	11 (27.5%)	2 (5%)	13 (16%)
LOQ-100 ng/l	18 (45%)	22 (55%)	40 (50%)
101-200 ng/l	2 (5%)	6 (15%)	8 (10%)
> 200 ng>l	0	10 (25%)	10 (13%)
Incidence	**77.5%**	**100%**	**89 %**
Levels of contamination (ng/l)			
Mean[c] ± SD	25 ± 38	182 ± 275	103 ± 162
Median[c]	15	45	29
Range[d]	19 - 198	18 - 1134	18 - 1134

[a]: n.d., not detected (OTA concentration in sample <5 ng/l)
[b]: traces means that the concentration of OTA is between the limit of detection (LOD = 5 ng/l) and the limit of quantification (LOQ = 18 ng/l)
[c]: mean and median are computed assuming that non-detected samples contain half the LOD while samples with traces levels of OTA contain half the LOD+LOQ
[d]: range of samples with OTA contents ≥ LOQ

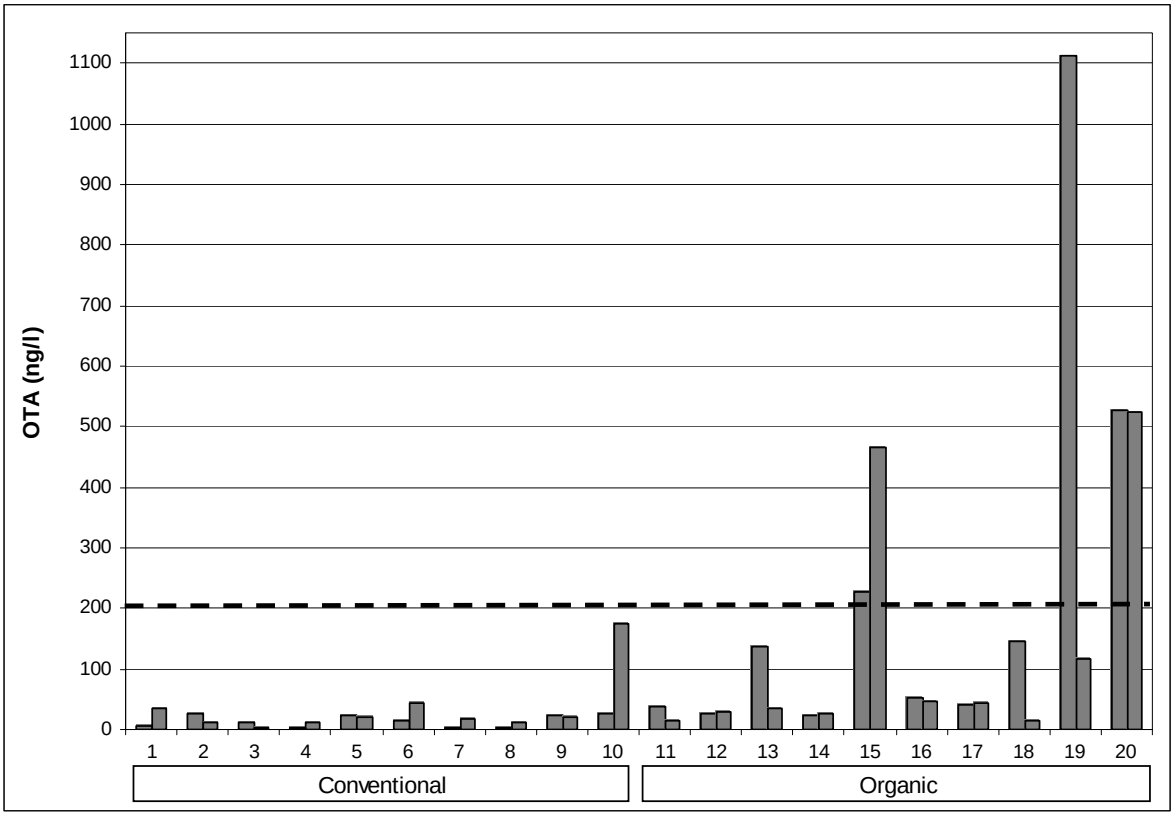

Figure 9. Ochratoxin A content (ng/l) in conventional and organic beers found on the Belgian market (2003-2004). Each brand (n=20) is represented by 2 batches. Values are the average of two samples for each batch.

3.2.2. Deoxynivalenol in beer

The level of DON in all beers sampled in 2002-2003 was below the LOD (35 µg/l), as shown in Table 16. This result prompted us to improve the method of analysis in order to lower the limit of detection.

Table 16. Occurrence and levels of deoxynivalelenol in beers collected on the Belgian market in 2002-2003.

Occurrence	**Conventional**	**Organic**	**Total**
Number of samples	44	26	70
n.d.[a]	37 (84%)	26 (100%)	63 (90%)
trace[b]	7 (16%)	0	7 (10%)
Incidence	**16%**	**0%**	**10 %**
Levels of contamination (µg/l)			
Mean[c] ± SD	7.8 ± 6.5	5.0 ± 0	6.8 ± 53
Median[c]	5.0	5.0	5.0
Range[d]	-	-	-

[a]:n.d., not detected (DON concentration in sample <10 µg/l)
[b]: traces means that the concentration of DON is between the limit of detection (LOD = 10 µg/l) and the limit of quantification (LOQ = 35 µg/l)
[c]: mean and median are computed assuming that non-detected samples contain half the LOD while samples with traces levels of DON contain half the LOD+LOQ
[d]: range of samples with DON contents ≥ LOQ

The analysis of samples collected during the second sampling period was thus performed on 20 ml of beer rather than on 10 ml and the final residue was dissolved in 1 ml of methanol:water (10:90) rather than in 2 ml. The final eluate was thus 4 times more concentrated compared to the one obtained with the initial method.

The occurrence of DON in conventional and organic beer samples collected in 2003 and 2004 is shown in Table 17 and in Figure 10.

A total incidence of positive samples (>LOQ) of 32.5% was observed for conventional samples with concentrations ranging from 6.0 to 22.1 µg/l. Twenty-five percents of organic beers contained quantifiable amounts of DON, from 6.0 to 14.0 µg/l. Proportions of contaminated beers (>LOQ) of conventional and organic origin are statistically equivalent ($\chi_1^2 = 0.5492$, p=0.4586).

The mean contamination levels for conventional and organic beers were 5.8 µg/l and 4.4 µg/l, respectively.

Among positive samples, some indication of a higher contamination of conventional beers compared to organic beers was found by statistical evaluation (p=0.0768).
Analysis of variance revealed homogeneous contamination between different brands of a mode of production (p=0.8639).). By contrast, contamination differences between batches of a same brand are significant (p<0.0001).

Table 17. Occurrence and levels of deoxynivalenol in beers collected on the Belgian market in 2003-2004.

Occurrence	**Conventional**	**Organic**	**Total**
Number of samples	40	40	80
n.d.[a]	13 (32.5%)	8 (20%)	21 (26%)
trace[b]	14 (35%)	22 (55%)	36 (45%)
LOQ - 10 µg/l	5 (12.5%)	8 (20%)	13 (16%)
10 - 20 µg/l	6 (15%)	2 (5%)	8 (10%)
20 - 30 µg/l	2 (5%)	0	2 (3%)
Incidence	**67.5%**	**80%**	**74%**
Levels of contamination (µg/l)			
Mean[c] ± SD	5.8 ± 5.7	4.4 ± 2.7	5.1 ± 4.5
Median[c]	4.0	4.0	4.0
Range[d]	6.0 – 22.1	6.0 – 14.0	6.0 – 22.1

[a]:n.d., not detected (DON concentration in sample <2 µg/l)
[b]: traces means that the concentration of DON is between the limit of detection (LOD=2 µg/l) and the limit of quantification (LOQ=6 µg/l)
[c]: mean and median are computed assuming that non-detected samples contain half the LOD while samples with traces levels of DON contain half the LOD+LOQ
[d]: range of samples with DON contents ≥ LOQ

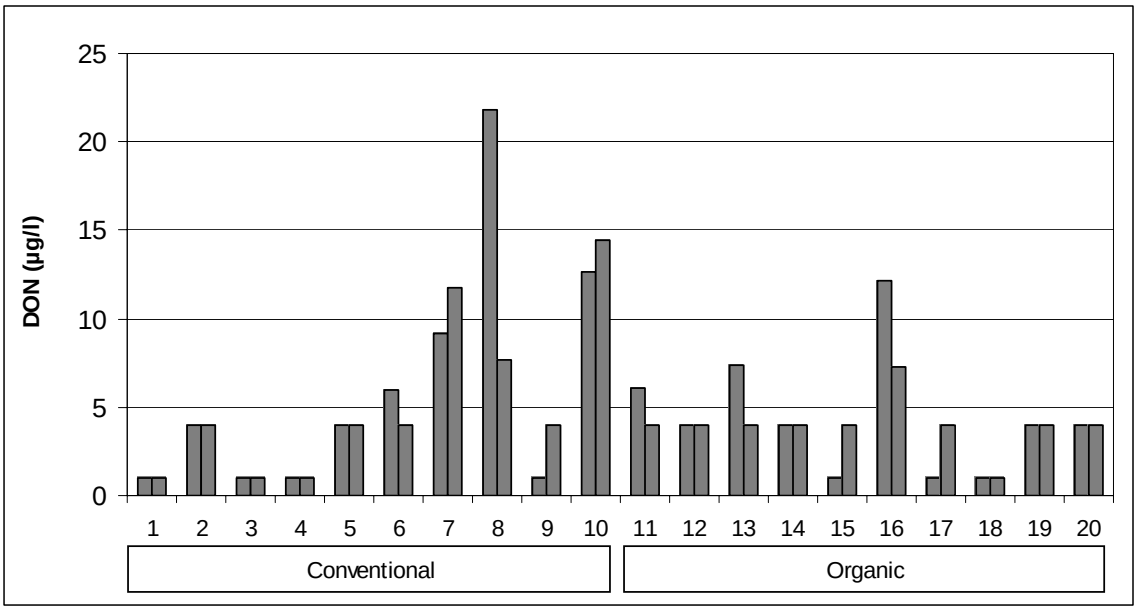

Figure 10. Deoxynivalenol content (µg/l) in conventional and organic beers collected on the Belgian market in 2003-2004. Each brand (n=20) is represented by 2 batches. Values are the average of two samples for each batch.

3.2.3. Fumonisins in beer

In a preliminary study, a total of twenty beer samples collected during the second sampling period were analyzed for FB1 and FB2. Samples were representatives of organic and conventional beers, ten organic and ten conventional.

Although the LOD and LOQ determined with FB1 standards were satisfactory (0.44 and 1.46 ng/ml, respectively), the detection of FB1 was impeded by the presence of a huge matrix peak in samples. However, the FB2 LOD and LOQ (1.17 and 3.88 ng/ml) was not affected by this matrix peak and the results showed that the contamination by FB2 in the samples was always below the LOD value.

These preliminary results showed at least that FB2 contamination was not a problem neither in organic nor in conventional beer samples. As reported in the literature, the level of FB1 in maize products is always around three times the level of FB2. Thus, although it was not possible to quantify FB1 in beers, we can speculate that the maximum FB1 concentration in these samples should not be much higher than 3.51 ng/ml (three times the LOD value of FB2), which can also be considered as not problematic.

3.3. Discussion and comparison with the literature

OTA and DON were analysed in conventional and organic beers collected during two sampling periods. Overall results are summarised in Table 18.

Table 18. Ochratoxin A and deoxynivalenol contents in beer.

Type	n[a]	OTA in positive samples[b]			DON in positive samples[b]		
		% samples	Range (ng/l)	Mean (ng/l)	% samples	Range (µg/l)	Mean (µg/l)
2002-2003							
Conventional	44	72a	10-149	32a	0	-	-
Organic	26	100b	12-520	89b	0	-	-
2003-2004							
Conventional	40	50a	19-197	41a	32.5a	6.0-22.1	12.6a
Organic	40	95b	18-1134	191a	25a	6.0-14.0	7.9a

Values of with different letters in the same column (conventional *vs* organic) are significantly different ($p<0.05$)

[a]: number of samples analysed

[b]: mycotoxin concentration > LOQ

In the present study, organic samples from the first sampling period as well as from the second sampling period were more frequently contaminated with OTA than conventional samples. Levels of contaminations of contaminated samples of the first sampling period were influenced by the mode of production ($p<0.0001$). Such a significant effect was not at all pointed out for samples from the second sampling period although mean levels were different. Thanks to our sampling plan, we showed that contamination variations take place at the level of batches more than at the level of the mode of production.

A few batches of organic beers analysed in the present study are much more contaminated than most of the samples.

Mycotoxins are usually distributed heterogeneously in stored-grain bulks and "hot spots" of OTA may occur even if the average conditions of the bulk are not favourable to moulds development. Exceptionally high concentrations of OTA in a batch of beer may occur if such contaminated barley is employed. Our study illustrates quite well the heterogeneity between different batches since we found a highly significant effect ($p<0.0001$) of the batch on OTA contamination. The same observation could be made regarding DON contamination.

The sampling methods for control analysis must respect the Commission Directive 2002/26/EC. On the other hand, malt should not contain more than 3 µg OTA/kg (Commission Regulation (EC) No 123/2005). Nowadays, beer producers must follow these regulations. This should guarantee the production of beers with low and acceptable levels of OTA.

Mean OTA contamination levels registered for conventional beers in the present study are similar to those reported in the literature.

Nakajima *et al.* (1999) analysed OTA in 46 European beers. The mycotoxin was detected in 43 samples (LOD=1 ng/l) in a range from 1.7 to 66.2 ng/l and with a mean concentration of 11.4 ng/l. The sample with the highest OTA level happened to be from a Belgian beer. Contamination by OTA was less frequent in the European beers analysed by Visconti *et al*. (2000) since 6 beers out of 15 were contaminated (LOD=10 ng/l). However, the mean concentration in positive samples was around 30 ng/l. The maximal level of OTA registered was 135 ng/l, in a Belgian beer too. Beers purchased in Danemark were investigated for OTA by Jorgensen (1998). All beers (n=21) contained traces of OTA (>1 ng/kg) with a mean content of 49 ng/l and the highest value was 160 ng/l. A study performed by Araguás *et al*. (2004) on 21 Spanish beers showed mean contamination levels of 38 ng/l (incidence = 67%) with a maximum level of 205 ng/l.

A comparison between conventionally and organically produced beers was performed by Tangni *et al*. (2002) and our results compare well with this study. The mean of OTA in organic beers sampled in 1998-1999 (81 ng/l) was higher than the mean contamination in conventional beers (22 ng/l) (p=0.007). By contrast, no statistical difference (p=0.233) was showed for beers collected in 2000-2001 although conventional beers contained higher levels of OTA (49 ng/l) than organic ones (29 ng/l).
We observed that bad cases of contamination occur occasionally in organic beers, with concentrations that can be much higher than the proposed limit of 200 ng/l. Tangni *et al.* (2002) also showed that the highest level of contamination (185 ng/l) was obtained in an organically produced beer.

Our results show that occurrence and levels of DON in beers should definitely not be a major cause for concern. DON was detected in a small number of beers collected during the second sampling plan thanks to the very low detection limit of the improved analytical method. The same conclusions could be drawn looking at the results reported in the literature.
Schothorst and Jekel (2003) analysed 51 beer samples collected in the Netherlands. They found DON at a concentration over 25 µg/l (LOQ) in only 3 samples, with quantities ranging from 26 to 41 µg/l. More recently, Papadopoulou-Bouraoui et al. (2004) detected DON in 87% of the analysed beers (n=313) with concentrations ranging from 4.0 to 56.7 µg/l. Most samples (73%) had contamination levels lower than 20 µg/l. Our results are in agreement with those studies since contamination is very low.

Our analytical method does not allow isolation of the FB1 peak from the matrix peak and should therefore be adapted by improving the composition of the mobile phase and the HPLC conditions. However, the results of the analysis of FB2 in beer samples revealed that the mycotoxin was never present at a level over the LOD (1.17 ng/ml). These data allow us to conclude that fumonisins in beers should not be problematic. Low levels of fumonisins in beer were previously reported by Hlywla and Bullerman (1999) whose results were in accordance with those of Scott and Lawrence (1995).

In March 2005, an additional set of OTA analyses were performed on newly sampled beers. Most of these beers were from the same brands as those analysed in 2003-2004. One bottle of each brand was investigated (one batch).

OTA was detected in 9 out of 10 conventional beers at levels ranging from 2.7 to 67.2 ng/l and in all organic beers at levels ranging from 19.3 to 158.0 ng/l. Mean (and median) in samples conventionally and organically produced were 21.1 ng/l (11.6 ng/l) and 74.9 ng/l (54.3 ng/l), respectively.

Interestingly, none of the beers sampled in 2005 exceeded the level of 200 ng OTA/l, suggesting a better control of the malts, which is in line with the recent regulation banning the use of malts with an OTA content above 3 µg/kg (Regulation EC 466/2001).

4. Mycotoxins in wholemeal pasta

4.1. Sampling

For the sampling of wholemeal pastas, we decided to follow the same sampling plan as for polenta.

As the availability of wholemeal pasta of conventional origin is quite limited on the Belgian market, it was not possible to find more than one brand. So, we collected five samples in four different batches of that brand, leading to a total of 20 conventional samples.

Five brands of organically produced pasta were available. During the sampling period (June 2003 - March 2004), we collected a total of 65 organic samples representing 5 brands with 1 to 5 batches per brand (depending on the restocking) and 5 samples per batch.

Samples were ground in a Retsch centrifugal mill to pass a 1 mm sieve and stored at room temperature.

4.2. Results

Table 19 and Figure 11 present the results of DON analyses in samples of wholemeal pasta. 35% of conventionally produced samples and 48% of organic samples presented DON levels higher than the LOQ. The incidence of DON in the two groups was statistically equivalent ($\chi_1^2 = 0.9967$; p=0.3181).

DON was detected in conventional and organic samples at levels ranging from 117 to 181 µg/kg and from 114 to 523 µg/kg, respectively. Mean concentrations were 68 µg/kg (conventional samples) and 169 µg/kg (organic samples).

Among positive samples (>LOQ), the contamination by DON of organic pasta was significantly higher than that for conventional pasta (p=0.0367).

Analysis of variance revealed homogeneous contamination between different brands of a mode of production (p=0.8763). By contrast, contamination differences between batches of a same brand were significant (p<0.0001).

Table 19. Occurrence and levels of deoxynivalenol in organic and conventional samples of wholemeal pasta collected on the Belgian market.

Occurrence	**Conventional**	**Organic**	**Total**
Number of samples	20	65	85
n.d.[a]	10 (50%)	22 (34%)	32 (38%)
trace[b]	3 (15%)	12 (19%)	15 (18%)
107-400 µg/kg	7 (35%)	24 (37%)	31 (36%)
401-750 µg/kg	0	7 (11)	7 (8%)
> 750 µg/kg	0	0	0
Incidence	**50%**	**66%**	**62%**
Levels of contamination (µg/kg)			
Mean[c] ± SD	68±60	169±108	145±147
Median[c]	43	70	70
Range[d]	117-181	114-523	114-523

[a]: n.d., not detected (DON concentration in sample <107 µg/kg)
[b]: traces means that the concentration of DON is between the limit of detection (LOD = 32 µg/kg) and the limit of quantification (LOQ = 107 µg/kg)
[c]: mean and median are computed assuming that non-detected samples contain half the LOD while samples with traces levels of OTA contain half the LOD+LOQ
[d]: range of samples with OTA concentrations ≥ LOQ

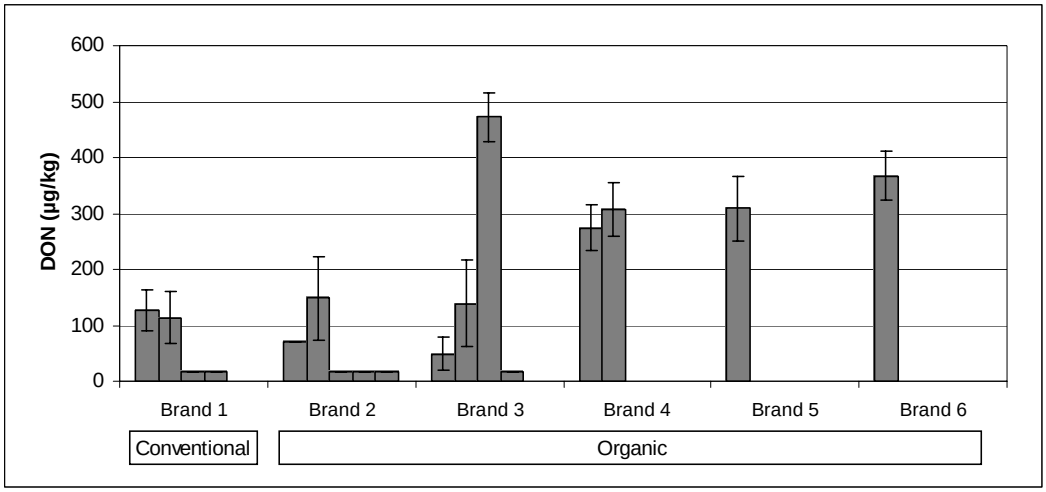

Figure 11. Deoxynivalenol content (µg/kg) in wholemeal pasta collected on the Belgian market. Each brand (n=6) is represented by 1 to 5 batches. Values are the average of five samples for each batch.

4.3. Discussion and comparison with the literature

Although the consumption of wholemeal pasta is less important than the consumption of pasta made without bran, this foodstuff was chosen for DON investigation since it represents a "worse case" situation. There is indeed an effective distribution of DON through the milling fractions. DON concentration is higher in the bran fraction, in a proportion depending on the degree of penetration of the mould into the kernel (Nowicki *et al.*, 1988).

Results of DON analysis in conventional and organic samples are summarized in Table 20.

Table 20. Occurrence of deoxynivalenol in wholemeal pasta.

Type	n[a]	DON in positive samples[b]		
		% samples	Range (µg/kg)	Mean (µg/kg)
Conventional	20	35a	117-181	142a
Organic	65	48a	114-523	316b

Values of with different letters in the same column (conventional *vs* organic) are significantly different ($p<0.05$)
[a]: number of samples analysed
[b]: mycotoxin concentration > LOQ

Thirty-five % of conventionally produced samples and 48% of organic samples presented DON levels higher than the LOQ. The incidence of DON in the two groups was statistically equivalent ($\chi_1^2 = 0.9967$; p=0.3181). Mean concentrations of positive samples were 142 µg/kg (conventional samples) and 316 µg/kg (organic samples). Among these samples (>LOQ), the contamination by DON of organic pasta was significantly higher than the contamination of conventional pasta (p=0.0367).

Once again, the batch effect was much greater than the effect of the brand or of the mode of production. All values were however lower than the recommended limit of 500 µg/kg.

Brera and Miraglia (2003) included in their report 29 samples of pasta from Belgium, Germany and the Netherlands. Mean concentrations of DON were 126 µg/kg (n=29), 110 µg/kg (n=219) and 163 µg/kg (n=92).

Noodles of conventional (n=50) and organic (n=16) origin were analysed by Schollenberger *et al.* (2003). Ninety-eight percent of conventional samples were contaminated at a mean level of 146 µg/kg, which is quite close to our results. By contrast, data of DON contamination are not in agreement since the mycotoxin occurred in 71% of ecological noodles at a mean level of 44 µg/kg. Median DON-content were lower in ecological than in conventional products but the difference was not significant.

5. Mycotoxins in sweet corn

5.1. Sampling

A total of 30 sweet corn samples from three brands (one organic and two conventional) were collected in retail stores during the first sampling period. Ten samples were collected for each brand.

5.2. Results

In a preliminary study, FB1 and FB2 were analysed in the 30 samples.

As all results were below the LOD value for fumonisins B1 and B2 (6 µg/kg for FB1 and 13 µg/kg for FB2) and as these LOD values were far below the unofficial limit for FB1 in corn flour (1000 µg/kg), it seemed that fumonisins in this food matrix did not represent an actual problem. That is why we decided not to carry on with the analysis of this matrix.

5.3. Discussion and comparison with the literature

Analysis of FB1 and FB2 in sweet corn samples do not reveal a safety problem at all since FB1 and FB2 were lower than their respective LOD. Similar results were reported by de Nijs *et al.* (1998) who found no positive samples among the 6 samples of canned maize analysed (LOD=8µg/kg).

6. Mycotoxins in corn flour for polenta

6.1. Sampling

Since the number of different organic and conventional brands of polenta available on the Belgian market is quite restricted, the best way to have enough samples was to multiply the number of batches per brand and the number of samples per batch, as much as possible. The sampling plan chosen was thus to collect four lots for five brands and to analyse five samples of each lot.

Sampling was made between June 2003 and April 2004. During that period, we found 3 brands of conventionally produced polenta (3 or 4 batches per brand) and 3 brands of organically produced maize flour (1 to 4 batches per brand), leading to a total of 52 conventional samples and 40 organic samples.

6.2. Results

Eighty-nine % and 70% of the 92 samples were contaminated with FB1 and FB2 over their respective LOD. As usual, FB1 concentrations exceeded FB2 concentrations. This follows the general pattern of fumonisin contamination in maize and maize-based food. Table 24 and Table 22 give an overview of the occurrence of FB1 and FB2, respectively, in the analysed corn flour for polenta samples.

Table 21. Occurrence and levels of fumonisin B1 in conventional and organic corn flour for polenta.

Occurrence	Conventional	Organic	Total
Number of samples	52	40	92
n.d.[a]	6 (12%)	4 (10%)	10 (11%)
traces[b]	7 (13%)	6 (15%)	13 (14%)
LOQ-1000 µg/kg	25 (48%)	27 (67.5%)	52 (57%)
>1000 µg/kg	14 (27%)	3 (7.5%)	17 (18%)
Incidence[c]	**88%**	**90%**	**89%**
Levels of contamination (µg/kg)			
Mean[d]± SD	997±1164	436±336	753±941
Median[d]	577	351	470
Range[e]	207-4353	215-1329	207-4353

[a]:n.d., not detected (FB1 concentration in sample <59 µg/kg)
[b]: traces means that the concentration of FB1 is between the limit of detection (LOD = 59 µg/kg) and the limit of quantification (LOQ = 197 µg/kg)
[c]: incidence means proportion of samples with at least detectable level of FB1
[d]: mean and median are computed assuming that non-detected samples contain half the LOD while samples with traces levels of FB1 contain half the LOD+LOQ
[e]: range of samples with FB1 contents ≥ LOQ

Table 22. Occurrence and levels of fumonisin B2 in conventional and organic corn flour for polenta.

Occurrence	Conventional	Organic	Total
Number of samples	52	40	92
n.d.[a]	15 (28.8%)	13 (32.5%)	28 (30.4%)
traces[b]	12 (23.1%)	26 (65%)	38 (41.3%)
>LOQ	25 (48.1%)	1 (2.5%)	26 (28.3%)
Incidence[c]	**71.2%**	**67.5%**	**69.6%**
Levels of contamination (µg/kg)			
Mean[d]± SD	386±418	137±69	278±339
Median[d]	180	180	180
Range[e]	284-1659	281-281	281-1659

[a]:n.d., not detected (FB2 concentration in sample<83 µg/kg)
[b]: traces means that the concentration of FB2 is between the limit of detection (LOD=83 µg/kg) and the limit of quantification (LOQ=276 µg/kg)
[c]: incidence means proportion of samples with at least detectable level of FB2
[d]: mean and median are computed assuming that non-detected samples contain half the LOD while samples with traces levels of FB2 contain half the LOD+LOQ
[e]: range of samples with FB2 contents ≥ LOQ

FB1 concentrations ranged from not detected (n.d.) to 4353 µg/kg with a mean and median concentration of respectively 753±941 and 470 µg/kg. For FB2, the concentration ranges varied from n.d. to 1659 µg/kg (mean=278±339 µg/kg; median = 180 µg/kg).

A statistical Chi-Square test was performed to evaluate whether the frequency of samples with FB1 contamination levels above 1000 µg/kg was equal between samples from organic and conventional production. There was a significant difference (χ^2_1=5.6627, p=0.0173) with 7.5% organic samples with contamination levels above 1000 µg/kg compared to 26.9% samples originating from conventional production.

Next, the quantified analytical results were subjected to analyses of variance using ANOVA III model. The agricultural practice did not have any statistically significant effect on the quantified FB1 and FB2 concentrations [p(FB1)=0.6209; p(FB2)=0.8478]. There was significant variation between FB1 contamination of different brands but no significant variation between FB2 contamination [p(FB1)=0.0380; p(FB2)=0.1045]. However, the variation in fumonisin concentrations between the different batches was significant [p(FB1)<0.0001; p(FB2)=0.0010]. This is visually demonstrated in Figure 12 and Figure 13 containing the mean concentrations of respectively FB1 and FB2 per batch (n=2 to 4) for each brand analysed (n=6). This could be due to a different level of fumonisin contamination of the raw materials employed in the production of each batch. These observations are in line with the heterogeneous distribution of mycotoxins in bulk commodities and emphasize the importance of a well defined sampling plan in the process of risk management and food safety control.

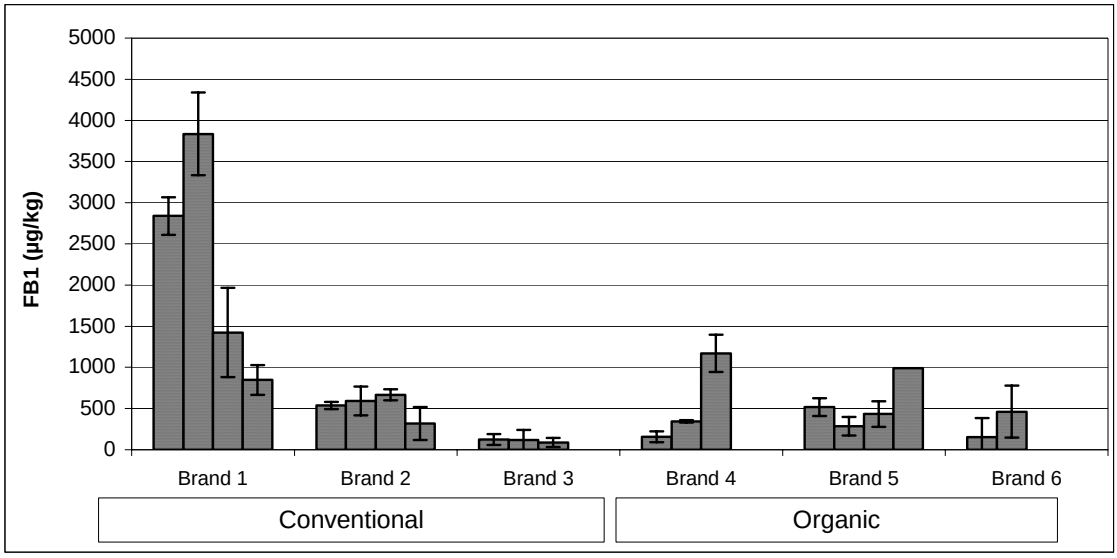

Figure 12. Fumonisin B1 content (µg/kg) in corn flour for polenta collected on the Belgian market. Each brand is represented by 2 to 4 batches. Values are the average of one to five samples for each batch.

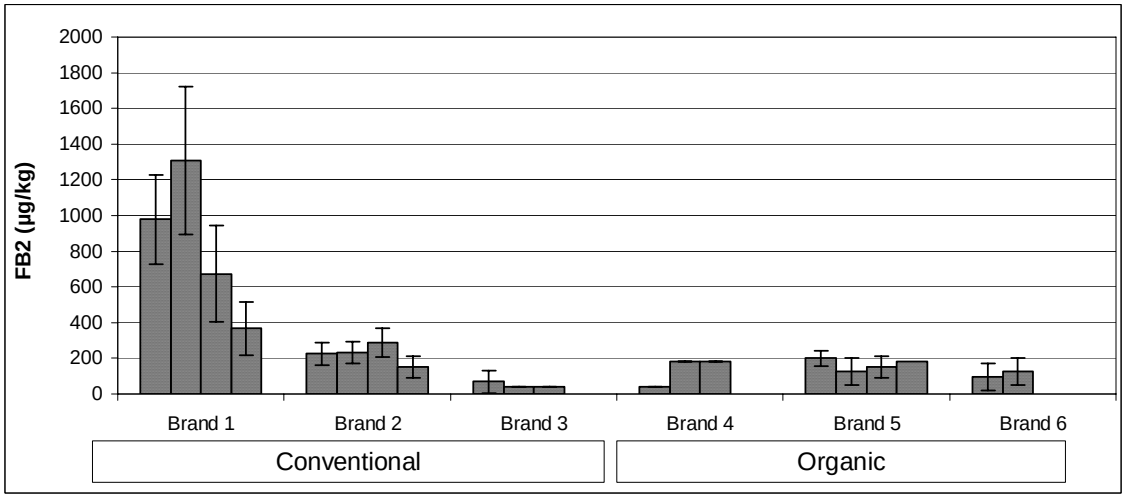

Figure 13. Fumonisin B2 content (µg/kg) in corn flour for polenta collected on the Belgian market. Each brand is represented by 2 to 4 batches. Values are the average of one to five samples for each batch.

6.3. Discussion and comparison with the literature

FB1 and FB2 contamination data of corn flour for polenta are summarized in Table 23.

Table 23. Occurrence of fumonisins B1 and B2 in corn flour for polenta.

Type	n[a]	FB1 in positive samples[b]			FB2 in positive samples[b]		
		% samples	Range (µg/kg)	Mean (µg/kg)	% samples	Range (µg/kg)	Mean (µg/kg)
Conventional	52	75a	207-4353	1301a	48.1a	284-1659	691a
Organic	40	75a	215-1329	552b	2.5b	281-281	281a

Values of with different letters in the same column (conventional *vs* organic) are significantly different ($p<0.05$)

[a]: number of samples analysed

[b]: mycotoxin concentration > LOQ

Contamination by FB1 occurred as frequently in conventional polenta as in organic polenta ($\chi_1^2 = 0$, p=1). By contrast, regarding samples with FB1 content above 1000 µg/kg, conventional samples were statistically more often contaminated (26.9%) than organically produced samples (7.5%) (χ^2_1=5.6627, p=0.0173). On the other hand, polenta originating from conventional production was more often contaminated with FB2 ($\chi_1^2 = 23.1646$, p<0.0001).

In a study purchased on corn meal traded in Sao Paulo, Bittencourt *et al.* (2005) detected FB1 and FB2 in all samples (n=30) at levels ranging from 1100 to 15300 µg/kg (FB1) and 200 to 3900 µg/kg (FB2). Mean concentrations for FB1 and FB2 were 5200 and 1000 µg/kg, respectively. These contaminations are higher that the results we report.

The nine Brazilian samples of corn meal analysed by Machinski *et al.* (2000) were also positive for FB1 and FB2. The mycotoxins were present at levels ranging from 560 to 4930 µg/kg (FB1) and from 210 to 1380 µg/kg (FB2). The average concentration of FB1 and FB2 were 2290 µg/kg and 600 µg/kg, respectively.

7. Mycotoxins in cornflakes

Although fumonisins are generally heat stable, physical parameters such as cooking time and temperature are critical factors that can affect fumonisin concentration during food processing. In general, the levels of fumonisin contamination found in cornflakes in different parts of the world are in the concentration range 0-300 µg/kg (Soriano and Dragacci, 2004) although occasional samples with concentrations of FB1 of more than 1000 µg/kg have been reported (Petersen and Thorup, 2001, Brera and Miraglia, 2003).

Information on the natural occurrence of fumonisins in cornflakes on the Belgian retail market is scarce. Twelve cornflakes samples originating from the Belgian market were included in the report of Brera and Miraglia, 2003. In none of these samples FB1 levels were found. This can be due to the high limit of detection (300 µg FB1/kg) of the method used.

7.1. Sampling

Cornflakes available in Belgian retail stores situated over the country were collected. The following sampling plan was chosen: 3 or 4 batches from 11 different brands (7 conventional and 4 organic) and 5 samples for each batch. This made a total of 205 samples analysed for fumonisin B1, B2 and B3 contamination with the LC-MS/MS method described in II.4.4.

7.2. Results

Sixty-five % of the 205 samples were contaminated with FB1, FB2 and FB3, over their respective limits of detection. FB1 concentrations always exceeded FB2 and FB3 concentrations. This follows the general pattern of fumonisin contamination in maize and maize-based food. There was an almost perfect correlation between the detection of these fumonisins with a correlation coefficient of respectively 0.98, 0.98 and 1 for FB1-FB2, FB1-FB3 and FB2-FB3. Table 24, Table 25 and Table 26 give an overview of the occurrence of respectively FB1, FB2 and FB3 in the analysed cornflakes samples.

Table 24. Occurrence and levels of fumonisin B1 in cornflakes collected on the Belgian market (2003-2004).

Occurrence of FB1	**Organic**	**Conventional**	**Total**
Number of samples	75	130	205
n.d.[a]	6 (8 %)	64 (49 %)	70 (34 %)
traces[b]	8 (11 %)	8 (6 %)	16 (8 %)
40-100 µg/kg	22 (29 %)	21 (16 %)	43 (21 %)
101-200 µg/kg	19 (25 %)	17 (13 %)	36 (18 %)
201-300 µg/kg	12 (16 %)	8 (6 %)	20 (10 %)
> 300 µg/kg	8 (11 %)	12 (9 %)	20 (10 %)
Overall incidence	**92 %**	**51 %**	**66 %**
Levels of contamination (µg/kg)			
Mean[c] ± SD	144 ± 114	82 ± 106	104 ± 113
Median[c]	116	30	54
Range[d]	40-464	40-393	40-464

[a]: n.d., not detected (FB1 concentration in sample < 20 µg/kg)
[b]: traces (20 µg/kg ≤ concentration of FB1 in sample < 40 µg/kg)
[c]: mean and median were computed assuming that non-detected samples contained half the LOD while samples with traces of FB1 contained half the LOD+LOQ
[d]: range of samples with FB1 concentrations ≥ 40 µg/kg

Table 25. Occurrence and levels of fumonisin B2 in cornflakes collected on the Belgian market (2003-2004).

Occurrence of FB2	Organic	Conventional	Total
Number of samples	75	130	205
n.d.[a]	5 (7 %)	66 (51 %)	71 (35 %)
traces[b]	35 (47 %)	23 (18 %)	58 (28 %)
15-30 µg/kg	32 (43 %)	41 (32 %)	73 (36 %)
31-50 µg/kg	3 (4 %)	0 (0 %)	3 (1 %)
> 50 µg/kg	0 (0 %)	0 (0%)	0 (0%)
Overall incidence	**93 %**	**49 %**	**65 %**
Levels of contamination (µg/kg)			
Mean[c] ± SD	16 ± 8	10 ± 8	12 ± 8
Median[c]	11	4	11
Range[d]	15-43	15-27	15-43

[a]: n.d., not detected (FB2 concentration in sample < 7.5 µg/kg)
[b]: traces (7.5 µg/kg ≤ concentration of FB2 in sample < 15 µg/kg)
[c]: mean and median were computed assuming that non-detected samples contained half the LOD while samples with traces of FB2 contained half the LOD+LOQ
[d]: range of samples with FB2 concentrations ≥ 15 µg/kg

Table 26 : Occurrence and level of fumonisin B3 in cornflakes collected on the Belgian market (2003-2004).

Occurrence of FB3	Organic	Conventional	Total
Number of samples	75	130	205
n.d.[a]	5 (7 %)	66 (51 %)	71 (35 %)
trace[b]	45 (60 %)	23 (18 %)	68 (33 %)
25-50 µg/kg	18 (24 %)	39 (30 %)	57 (28 %)
51-60 µg/kg	3 (4 %)	2 (2 %)	5 (2 %)
> 60 µg/kg	4 (5 %)	0 (0 %)	4 (2 %)
Overall incidence	**93 %**	**49 %**	**65 %**
Levels of contamination (µg/kg)			
Mean[c] ± SD	26 ± 17	18 ± 13	21 ± 15
Median[c]	19	19	19
Range[d]	25-90	25-50	25-90

[a]: n.d., not detected (FB3 concentration in sample < 12.5 µg/kg)
[b]: traces (12.5 µg/kg ≤ concentration of FB3 in sample < 25 µg/kg)
[c]: mean and median were computed assuming that non-detected samples contained half the LOD while samples with traces of FB3 contained half the LOD+LOQ
[d]: range of samples with FB3 concentrations ≥ 25 µg/kg

FB1 concentrations ranged from not detected (n.d.) to 464 µg/kg, with mean and median concentrations of 104±113 and 54 µg/kg, respectively. For FB2 and FB3 the concentration ranges varied respectively from n.d. to 43 µg/kg (mean = 12±8; median = 11) and from n.d. to 90 µg/kg (mean = 21±15; median = 19). The distributions of the FB1, FB2 and FB3 surveys were right asymmetric (skewness factors: 0.82-1.60) indicating the presence of outliers with high values. Indeed, 10% of the samples analysed had FB1 concentrations higher than 300 µg/kg. For FB2, only 1 % of the samples had a contamination level higher than 30 µg/kg, whereas for FB3 high concentrations (> 50 µg/kg) were found for 4% of the samples analysed.

Statistical Chi-Square tests (χ^2) were performed to evaluate whether the frequency of samples with quantified contamination levels was equal between organic and conventional production. There was an extremely significant difference for FB1 (χ^2_1=26.3312, p<0.0001) with 81% organic samples with contamination levels above the LOQ concentration compared to 45% samples originating from conventional production. Also for FB2, a significant difference was observed between the percentage of organic samples with FB2 concentrations above the LOQ (46.8%) and that for conventional samples (31.6%) (χ^2_1 = 4.6658, p = 0.0308). Proportions of FB3 contamination (> LOQ) of conventional and organic origin were statistically equivalent (χ^2_1 = 0.2849, p = 0.5935).

Next, the quantified analytical results were subjected to analyses of variance using ANOVA III model. The agricultural practice did not have any statistically significant effect on the quantified FB1, FB2 and FB3 concentrations [p(FB1)=0.6850; p(FB2)=0.9759; p(FB3)=0.6676]. There were no significant variations between contamination of different brands [p(FB1)=0.3917; p(FB2)=0.8178; p(FB3)=0.8318]. However, the variation in fumonisin concentrations between the different batches was significant [p(FB1) <0.0001; p(FB2)<0.0001; p(FB3) <0.0001]. This is visually demonstrated in Figure 14, Figure 15 and Figure 16 containing the mean concentrations of respectively FB1, FB2 and FB3 per batch (n=3 or 4) for each brand analysed (n=11). This could be due to a different level of fumonisin contamination of the raw materials employed in the production of each batch. These observations are in line with the heterogeneous distribution of mycotoxins in bulk commodities and emphasize the importance of a well defined sampling plan in the process of risk management and food safety control.

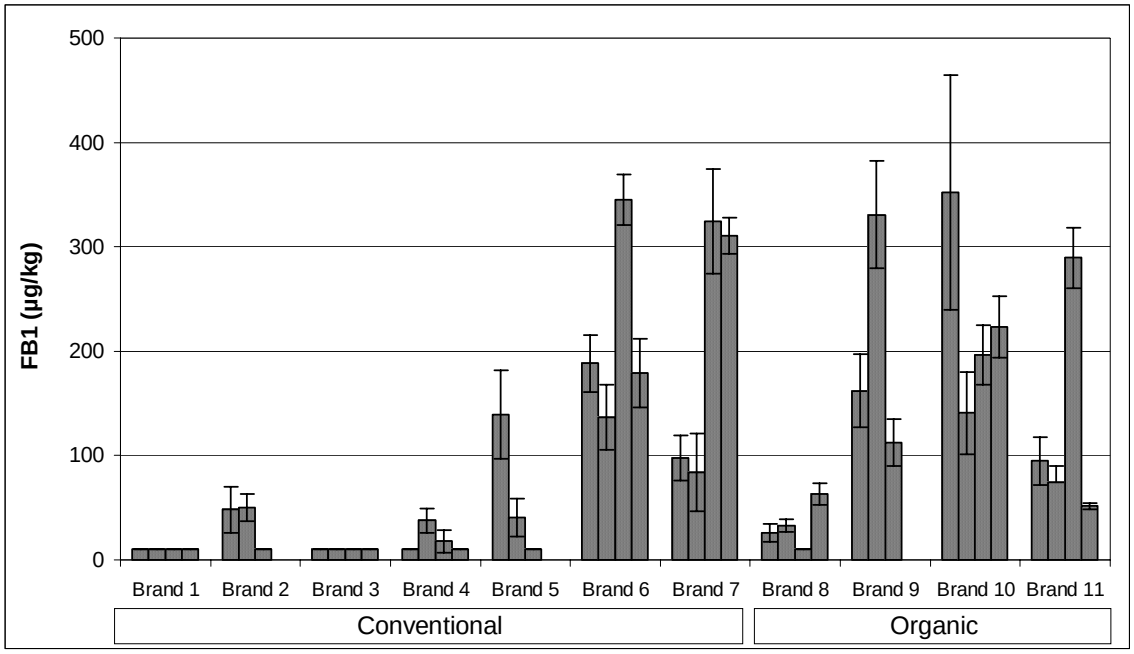

Figure 14 : Fumonisin B1 content (µg/kg) in cornflakes collected on the Belgian market. Each brand (n=11) is represented by 3 or 4 batches. Values are the average of five samples for each batch.

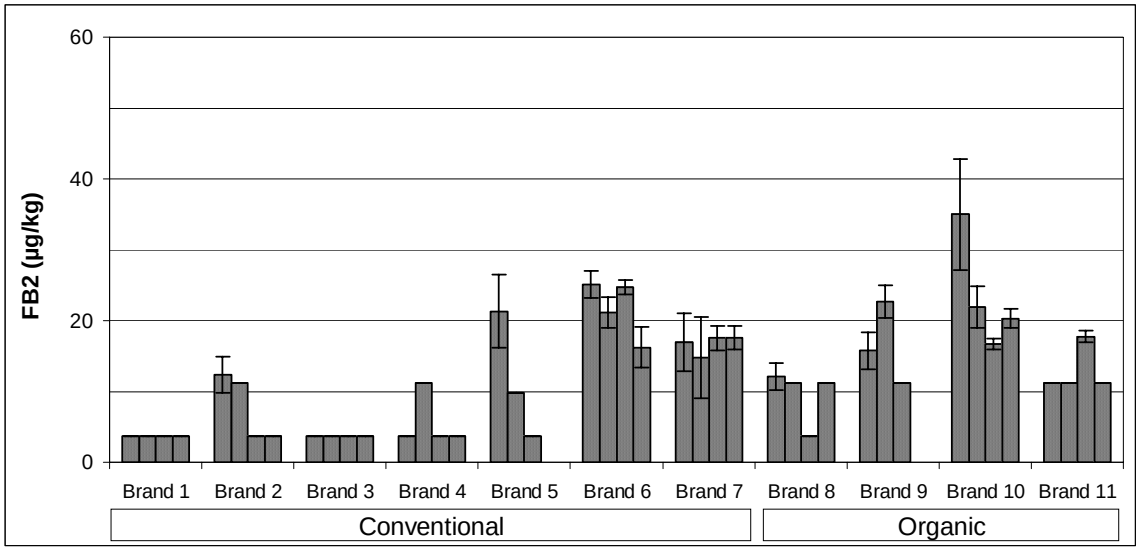

Figure 15 : Fumonisin B2 content (µg/kg) in cornflakes collected on the Belgian market. Each brand (n=11) is represented by 3 or 4 batches. Values are the average of five samples for each batch.

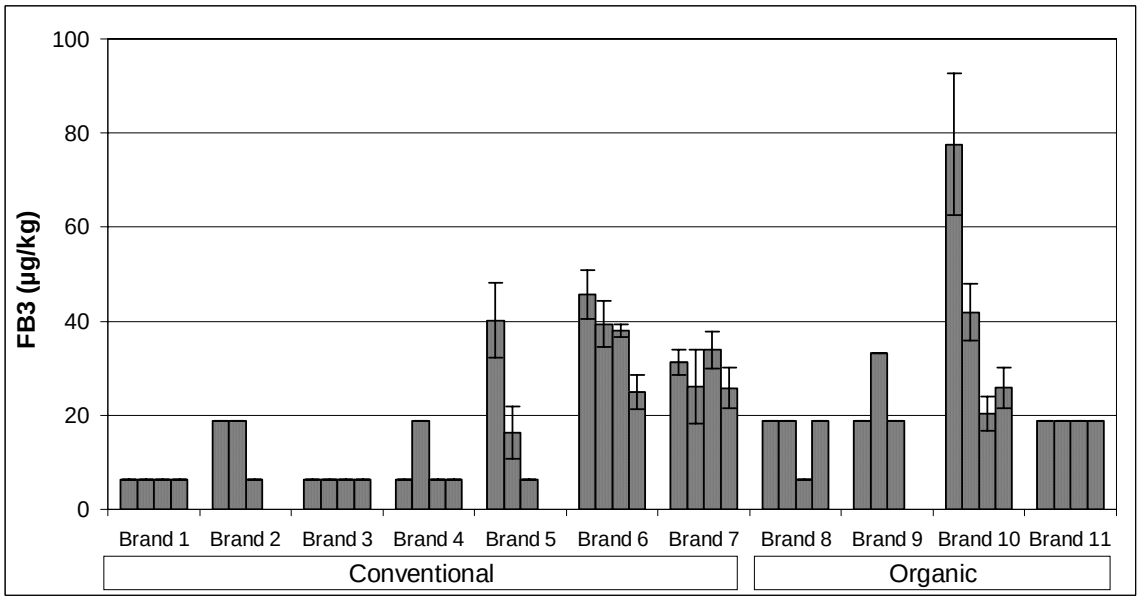

Figure 16 : Fumonisin B3 content (µg/kg) in cornflakes collected on the Belgian market. Each brand (n=11) is represented by 3 or 4 batches. Values are the average of five samples for each batch.

7.3. Discussion and comparison with the literature

A total of 205 cornflakes samples were analysed for FB1, FB2 and FB3 with the validated LC-MS/MS method developed during the project. Results are summarized in Table 27.

Table 27. Occurrence of fumonisins B1, B2 and B3 in cornflakes.

		FB1 in positive samples[b]			FB2 in positive samples[b]			FB3 in positive samples[b]		
Type	n[a]	> LOQ (%)	Range (µg/kg)	Mean (µg/kg)	>LOQ (%)	Range (µg/kg)	Mean (µg/kg)	>LOQ (%)	Range (µg/kg)	Mean (µg/kg)
Conventional	130	45a	41-393	168a	32a	16-27	20a	29a	25-54	35a
Organic	75	81b	44-464	172a	47a	16-43	22a	32a	26-92	42a

Values of with different letters in the same column (conventional *vs* organic) are significantly different ($p<0.05$)
[a]: number of samples analysed
[b]: mycotoxin concentration > LOQ

We can conclude that 65% of the cornflakes samples analysed in this survey were contaminated with FB_1, FB_2 and FB_3 concentrations above the respective detection limits [LOD (FB1) = 20 µg/kg; LOD (FB2) = 7.5 µg/kg; LOD (FB3) = 12.5 µg/kg]. FB2 and FB3 were not detected without FB1.

Fumonisin concentrations found in this survey were generally low, however batches with a considerable contamination (FB1 conc.>300 µg/kg) did occur. Therefore, conventional evaluation of fumonisins in cornflakes can be recommended. From the statistical tests (Chi-Square and ANOVA model III) performed, we can conclude that the agricultural practice did not have any statistically significant effect on the fumonisins concentrations, but that the variation between different batches is significant ($p<0.0001$). These observations are in line with the heterogeneous distribution of mycotoxins in bulk commodities and emphasis the importance of a well defined sampling plan in the process of risk management and food safety control.

Overall, the levels of FB1 and FB2 found in the cornflakes products in this survey were in accordance with contamination data reported in European studies. Brera and Miraglia (2003) observed that 46% of the samples had FB1 concentrations ranging from 5 to 1092 µg/kg, while the mean was 74 µg/kg. FB2 concentrations varied from 8 to 235 µg/kg with a mean value of 53 µg/kg. Twelve cornflakes samples originating from the Belgian market were included in the same report. In none of these samples FB1 levels were found. This can be due to the high limit of detection (300 FB_1 µg/kg) of the method used.

Petersen and Thorup (2001) reported that 6 of 10 cornflakes samples had detectable FB1 concentrations (LOD=5 µg/kg) with a mean value of 110 µg/kg. For FB2 detectable concentrations ranged from 4 to 243 µg/kg with a mean value of 25 µg/kg. Comparison of FB3 results can not be made as this is the first European survey that includes FB3 determinations.
Cirillo *et al*. (2003) analysed breakfast cereals (n=14) including corn flakes, muesli, etc, purchased in Italy for FB1 and FB2. Mean concentrations (and range) of FB1 and FB2 were 71 µg/kg (54-350 µg/kg) and 65 µg/kg (20-380 µg/kg), respectively.
The same authors (Cirillo *et al.*, 2003b) evaluate FB1 and FB2 in maize based foodstuffs of conventional (n=27) and organic origin (n=27). FB1 was detected in 30% of conventional samples and in 44% of organic foods. Medians and ranges were 345 µg/kg (27-2160 µg/kg) for conventional samples and 185 (10-600 µg/kg) for organic samples. Medians were not statistically different for the two types of samples. Occurrence of FB2 was estimated at 22% in conventional maize based foodstuffs and at 32% in organic samples. FB2 median levels were significantly higher ($p<0.05$) in organic foods (120 µg/kg; range: 30-150 µg/kg) than in conventional ones (median of 20 µg/kg; range: 10-400 µg/kg).

IV. DEVELOPED AND VALIDATED METHODS

1. Multi-mycotoxins method

A research project supported by a grant of the Federal Public Service of Public Health (DG4, sustained research), had allowed the development of a rapid screening method in order to analyse a maximum of mycotoxins in a single analytical run. The research was focused on the mycotoxins endemic in Western Europe, i.e. nivalenol (NIV), DON, 15- and 3-acetyl deoxynivalenol (15-AcDON and 3-AcDON), zearalenol (ZOL), ZEN and OTA. A Bio-engineer, supported by the DG4-Grant, conducted the development of this research.

A chemical technician, supported by this SSTC grant, was also involved into the development of this method. He concentrated his efforts into the adaptation of the method to the specific needs of the samples studied in the present project. He then used this method for mycotoxin determination in cereals and cereal flours focusing on DON, ZEN and OTA.

1.1. Material and methods

1.1.1. Reagents and standards

DON, ZEN and OTA were purchased by Sigma (St Louis, MO, USA).

HPLC grade acetonitrile (ACN), methanol (MeOH) and toluene were purchased by Biosolve (Valkenswaard, The Netherlands). Acetic acid (AcOH), trifluoroacetic acid (TFA) and benzene were purchased by Acros (New Jersey, NY, USA). Phosphoric acid was purchased by Sigma (St Louis, MO, USA). HPLC grade water was obtained using a Milli-Q system from Millipore (Molsheim, France).

SPE SAX Extract-Clean columns (500 mg/4ml) were purchased by Alltech (Deerfield, IL, USA). Römer MycoSep^{TM} 226 were purchased by Coring System Diagnostix GmbH (Gernsheim, Germany

1.1.2. Preparation of standard solutions

Stock solutions of the focused mycotoxins were prepared dissolving the solid crystals in HPLC grade acetonitrile for DON and in benzene for ZEN, respectively. The concentrations of the resulting solutions were determined using a spectrophotometer (AOAC International, 1995a; Pettersson, 1998).

The mix-solution of the mycotoxins (100 µg/ml for DON and 10 µg/ml for ZEN) was prepared by evaporating the appropriated volumes of stock solutions for each toxins and then dissolving the residue with methanol:water (50:50, v/v).

Calibration mix-solutions were obtained through dilution of the mix-solution with the appropriate methanol:water (50:50, v/v) volume to get concentrations of 0.1, 0.25, 0.5, 1.0, 2.5 and 5 µg/ml for DON and 0.01, 0.025, 0.05, 0.1, 0.25 and 0.5 µg/ml for ZEN, respectively.

The stock solution of OTA (10µg/ml) was prepared through dissolution of the crystals in a mixture of toluene:acetic acid (99:1, v/v) and the concentration was controlled using a spectrophotometer (Wood *et al.*, 1996).

The spiking solution (500 ng/ml) and the stock solution for calibration (50 ng/ml) were prepared by evaporating the appropriate amount of OTA and dissolving the residue with a mixture of methanol:acetic acid 2% in water (50:50, v/v). The standard solutions for

calibration were obtained dissolving the stock solution with the same mixture of methanol-acetic acid 2 % to obtain the concentrations of 0.25, 0.5, 1, 2.5 et 5 and 10 ng/ml.

All those solutions were stored at –20°C. The standard solutions were submitted to chromatography and the linearity of the response controlled according to Van Trijp and Roos (1991). Using the origin as fixed point, the peak surfaces of the different mycotoxins (Y) and the corresponding surfaces (X) were graphically analysed. Calibration curves were established from the HPLC analytical results.

1.2. Sample preparation from the selected food matrices

For the selected matrices, sample preparation included three steps: an extraction, a two stages purification and a final dissolution prior to the chromatography. An overview of the extraction and dilution conditions for DON, ZEN and OTA in food matrices is given in Table 28.

1.2.1. Extraction

Twenty-five grams of a ground and homogenised sample were weighed in a 500 ml PTFE centrifuge vessel. Extraction solvent (125 ml ACN:H_2O, 84:16, v/v) was added. The suspension was mixed for 3 minutes at high speed using an IKA Ultra-Turrax mixer (Stauffen, Germany) and then centrifuged for 15 minutes at 15300g. An aliquot (10ml) of the supernatant was then purified.

1.2.2. Purification

Because acidic and neutral mycotoxins could not be simultaneously purified on the MycoSep column, an anion exchange column (SAX) was added prior to the MycoSep column in order to fix the acidic mycotoxins on the SAX column. Flowing through the SAX column, the neutral mycotoxins could be further purified by filtration on the MycoSep cartridge.

1. The SAX column was conditioned using 5 ml of methanol and 5 ml of water. Ten ml of the centrifuged extract were poured on the column. The column was then washed with 5 ml methanol and 10 ml of a mixture of methanol:acetic acid (95:5, v/v). OTA was eluted by 10 ml a mixture of methanol:trifluoroacetic acid (99:1, v/v). The eluate was then evaporated to dryness.

2. Ten ml of the extraction solvent were used for the conditioning of the MycoSep column. Nine ml of the extract collected from the SAX column were then filtered through the dried MycoSep column. An aliquot of 4 ml was taken from the flow-through and evaporated to dryness.

1.2.3. Dissolution

The dry residue containing the acidic mycotoxins (OTA) was dissolved in 1 ml of methanol:acetic acid (2 %) in water (50:50, v/v) and 25 µl of the resulting solution were submitted to the HPLC chromatography.

The dry residue containing the neutral mycotoxins (DON and ZEN) was dissolved in 1ml of methanol:water (50:50, v/v) and 25 µl of the resulting solution were submitted to the HPLC chromatography.

Table 28. Overview of the procedure used for the extraction and purification of a panel of mycotoxins from wheat matrices.

step	Method	Comments	Used solvent v/v	Mycotoxins in eluate
1. Extraction	Blending	Dilution factor 5	MeOH-H²O, 84/16	DON, ZEN, OTA
2.1. SAX	Separation of acidic and neutral mycotoxins	Flowing through	MeOH-H²O, 84/16	DON, ZEN
		Eluted from the column	MeOH-TFA	OTA
3. MycoSep	Purification of neutral mycotoxins on MycoSep column	Flowing through	MeOH-H²O, 84/16	DON, ZEN

1.3. HPLC equipment and methods

1.3.1. HPLC equipment

The chromatographic system is composed by two 515 pumps, a 717 automatic injector, a 2996 DAD UV detector, a 2474 fluorescence detector, all from Waters (Milford, MA, USA). Millenium Empower was used as system and data management. The reversed-phase analytical column, Alltima RP-C18 (100 × 4.6 mm id, 3 µm particles), was purchased by Alltech (Deerfield, IL, USA). The column was thermostatically controlled at 35°C using an air circulating oven (Model 7990, Jones Chromatography, Hengoed, UK).

1.3.2. HPLC methods

The analytical method used a linear gradient of two mobile phases at 1 ml/min. The mobile phase A was a mixture of acetonitrile:phosphoric acid 1 mM in water (2:98, v/v) whereas the mobile phase B was a mixture of acetonitrile:phosphoric acid 1 mM in water (25:75, v/v). The mobile phases were filtered and degassed on a 0.22 µm Durapore filter (Millipore, Molsheim, France).

Neutral mycotoxins are separated by a linear gradient from 5% B to 100% B in 20 minutes. The used UV detector wavelengths were 225 and 236 nm. The fluorescence detection was performed with at $\lambda_{excitation}$=280 nm and $\lambda_{emission}$=465 nm for ZEN.

OTA was analysed using an isocratic flow of 48% A and 52% B. The detection was performed with the fluorescence detector set at $\lambda_{excitation}$=332 nm and $\lambda_{emission}$=462 nm.

1.3.3. Validation

The determination of the method performances was done using the recovery tests of samples spiked at 4 different levels (n=9) from 200 to 2000 µg/kg for the trichothecenes, from 20 to 200 µg/kg for ZEN and from 0.5 to 10 µg/kg for OTA. The initial concentration of each toxin was determined by means of a spectrometer and used for the recovery calculations.

LOD and LOQ were determined with a signal to noise ratio of 3:1 and 10:1, respectively.

Inter-laboratory proficiency tests were also used to evaluate the method for the determination of OTA and DON (FAPAS, UK).

1.4. Results

1.4.1. Performance of the analytical method

In the calibration range used, the HPLC responses (peak surfaces) were positively correlated with the concentrations of the different mycotoxins and a good linearity was observed with $R^2 \geq 0.995$ and an inter-day RSD ≤ 10 %. The recoveries were determined on wheat samples for DON, ZEN and OTA and the results are shown in Table 29.

Table 29. Validation parameters of the developed analytical method.

	Recovery (%)	**RSD_r[b] (%)**	**LOD[c] (ppb)**	**LOQ[d] (ppb)**
DON (50-1500 µg/kg)	92.3	12.3	50	150
ZEN[a] (2-500 µg/kg)	83.0	10.5	1.5	4
OTA[a] (0.5-10 µg/kg)	73.3	8.8	0.050	0.150

[a] : Detected by fluorescence
[b] : RSD_r, intra-laboratory reproducibility
[c] : LOD, limit of detection (signal/noise=3)
[d] : LOQ, limit of quantification (signal/noise=10)

For the three studied mycotoxins, the developed method fulfils the guidelines of the European Comity for standardization (CEN, CR 13505, 1999, #42) respecting the performances (RSD<20% and recoveries 70-110%).

1.4.2. Inter-laboratory performances

The developed multi-mycotoxin method was evaluated by inter-laboratory ring-tests organized by FAPAS for the analysis of DON and OTA in naturally contaminated wheat samples.
The results obtained are shown in Table 30. The accuracy was evaluated using the method of Z-scores

$$\text{Z-score} = X_{(\text{measured value})} - \hat{A}_{(\text{mean value})}/\sigma$$

where $\sigma = 0.22*c / mr$ if c<120 µg/kg

or $\sigma = 0.22*c^{0.8495} / mr$ if c>120 µg/kg where *mr* is a dimensionless value (e.g. : 1 ppm = 10^{-6}) according to Thompson, (2000).

Table 30. Inter-laboratories performance results.

	Calculated value (µg/kg)	**Mean value (µg/kg)**	**Z-score[a]**
DON	858.0	898.5	-0.3
OTA	6.5	5.6	0.8

[a] : Accepted Z-score scale: -2 < Z-scores < 2

The results show that the method developed is reliable and exact for DON and OTA analysis in wheat samples.

2. Flow-through enzyme immunoassay method

The flow-through enzyme immunoassay (Figure 17) uses simple equipment and limited operational steps, providing a yes/no response indicating whether the analyte is present or not above a certain threshold value, i.e. the visual detection limit or cut-off level. Coupled to a simple and fast sample preparation, this approach offers a cost-effective screening tool that can be conducted and interpreted by users that are as close to the source of contamination as possible. In this project, it was our objective to optimize the format of the flow-through enzyme immunoassay for the detection of OTA and fumonisins in the following selected matrices: wheat, wholemeal bread, maize and cornflakes and beer.

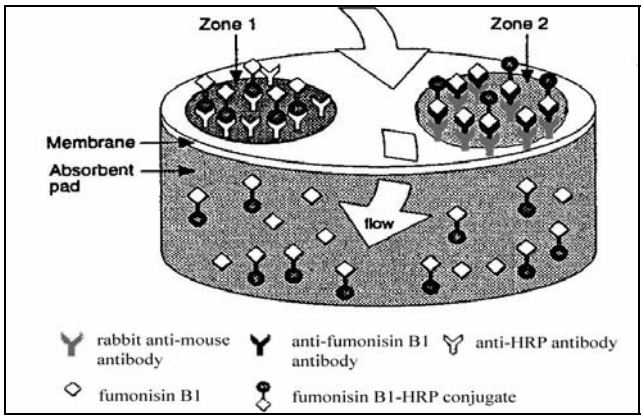

Figure 17. Principle of the flow-through enzyme immunoassay.

2.1. Sample preparation of ochratoxin A and fumonisins in the selected solid matrices

Sample preparation methods that emphasize speed and minimal steps are desirable in a field test approach. Therefore, a sample preparation procedure described by De Saeger *et al.* (2002) was adapted and further simplified. For solid matrices, sample preparation included three steps: extraction, dilution and filtration. An overview of the extraction and dilution conditions for OTA and fumonisins in solid matrices is given in Table 31. The solid samples were first ground in a household coffee grinder and directly submitted to extraction. Therefore a 5 g portion of sample was mixed with 15 ml extraction solvent and shaken by hand for 6 minutes. The supernatant was diluted with a PBS buffer solution 0.01 M, pH 7.4, and filtered through a 0.45 µm Chromafil® membrane filter. This filtrate was used in the flow-through assay.

Table 31. Overview of the sample preparation conditions for ochratoxin A and fumonisins in the selected matrices.

Compound and matrix	Extraction solvent	Dilution factor
Ochratoxin A (OTA)		
wheat	MeOH/H_2O [80/20, v/v]	2.6
wholemeal bread		1.3
Fumonisins (FB1,FB2 and FB3)		
maize	MeOH/H_2O [50/50, v/v]	1.6
cornflakes		1.6

2.2. Sample preparation of ochratoxin A in beer

Applying beer samples treated with a minimum of sample preparation (degassing, adapting the pH to neutral and membrane filtration) on the membrane in the flow-through assay resulted in a yellowish-brown background colour, especially in the case of dark beer. Blue

colour development of the control and sample spots was hindered, making visual evaluation impossible. This problem of background interferences could be circumvented by diluting the beer samples. This approach however, made it impossible to reach the postulated visual detection limit of 200 ng/l. Therefore, a solid phase extraction (SPE) step was incorporated in the sample pre-treatment procedure in order to eliminate matrix components and to concentrate OTA in the eluate. The flow-through assay preceded by clean-up with C18 columns still resulted in too much background. Further clean-up research was concentrated on strong anion exchange (SAX) columns (Varian) and on an Immunoaffinity column (Ochratest®, VICAM). For the SAX columns, different sizes of sorbent quantity (500 mg, 2 g and 5 g) in combination with different volumes of sample brought on to the column (from 5 to 25 ml) were evaluated. Based on these results, the following clean-up procedures were selected:

- **Clean-up on SAX-columns:**
  - Condition with 5 ml methanol and 5 ml 0.5 M TRIS/HCl buffer (pH 7.2),
  - Add 5 ml beer sample (adjust pH to 7 with 1 drop 2 M NaOH),
  - Wash with 5 ml 0.5 M TRIS/HCl buffer, 5 ml H_2O, 5 ml methanol and 2 ml methanol:acetic acid (99:1, v/v),
  - Elute with 2 ml methanol:acetic acid (95:5, v/v) and evaporate
  - For application in the flow-through assay: dissolve in 1 ml 30 % methanol/ 0.01 M PBS buffer,
  - For HPLC application: dissolve in 500 µl methanol.
- **Clean-up on OTA immunoaffinity columns:**
  - Add 15 ml beer + 10 ml 5% $NaHCO_3$/1% polyethyleneglycol 6000/H_2O,
  - Wash with 10 ml H_2O,
  - Elute with 2 ml methanol,
  - For application in the flow-through assay: dilute 1 ml eluate with 2 ml PBS buffer,
  - For HPLC application: evaporate 2 ml eluate and dissolve in 500 µl methanol.

In order to choose the most suitable clean-up procedure, recoveries of OTA with HPLC-fluorimetric detection [Column: Supelco Discovery® C18 (25 cm × 4.6 mm, 5 µm); mobile phase: ACN:H_2O: acetic acid (54:44:2, v/v/v); flow rate: 1 ml /min, injection volume: 50 µl] and visual results in the flow-through assay were evaluated. Pale and dark beer samples fortified with OTA at 0, 150, 200, 300, 400 and 500 ng/l were analysed 5 times at each fortifying level to determine OTA recoveries. Clean-up with SAX columns gave a recovery of 86 % at 500 ng OTA/l (CV=4.6%). Using immuno-affinity columns, recoveries at 150, 200, 300, 400 and 500 ng OTA/l were respectively 97% (CV=6.3%), 99% (CV=7.4%), 98 % (CV=5.5%), 92% (CV=6.2 %) and 98% (CV=1.8%). Experiments combining the clean-up procedures with the flow-through enzyme immunoassay were performed. Applying dark beer samples cleaned-up on a SAX column still resulted in some background on the membrane hindering visual interpretation. With immuno-affinity columns, no background problems were observed.

2.3. Principle of the flow-through enzyme immunoassay

The flow-through assay system (European patent No. 0893690) consisted of two layers, the first being the Immunodyne ABC membrane coated with rabbit anti-mouse antibodies and anti-HRP antibodies. The second layer was an absorbent material. In the flow-through assay, each reagent was applied in sequence on the membrane. Between each step, it was important to allow the liquid added to flow completely through the membrane. Anti-mycotoxin antibodies were first applied on the membrane, followed by a washing step. Then, the

extracted sample was added to the membrane. Analyte, if present, bound to the anti-mycotoxin antibody. After a washing step, a mycotoxin-HRP conjugate was added and was bound by any remaining free anti-mycotoxin antibodies and anti-HRP antibodies which acted as an internal control. The last washing step removed residual conjugate solution from the membrane. Finally, the colour substrate was added. Owing to the competitive enzyme immunoassay test principle, less colour was produced with increasing concentrations of analyte in the sample. Colour development was visually evaluated and also measured with a portable colorimeter, which expresses the colour intensity as a single numerical value (ΔE^*ab). Assay results were valid when a blue internal control spot (consisting of anti-HRP antibodies) developed. The colour intensity of the blank sample served as a control for immunoreagent stability. Performing this flow-through assay in series of maximal 6 samples took approximately 20 minutes, sample preparation included.

2.4. Optimization of the flow-through enzyme immunoassays

For each matrix, a sample with respectively no detectable OTA or fumonisin was selected for fortification with these mycotoxins. These blank samples were fortified with analyte concentrations in an appropriate range in order to optimize each flow-through assay. Experiments were carried out varying the working dilutions of the immunoreagents and amount of sample volume applied on the membrane in order to evaluate the following criteria: colour intensity at zero fortifying level; degree of colour reduction with increasing contamination concentrations; the analyte concentration resulting in complete colour inhibition (= visual detection limit or cut-off level). For OTA, it was our goal to reach a visual detection limit for each flow-through assay at concentrations set or proposed by the European Commission (Table 32). For the detection of fumonisins in maize, the cut-off level was based on the Swiss legislation. Regarding the cornflakes matrix, a cut-off level as low as possible was postulated. For samples contaminated with analyte concentrations equal to or above this visual cut-off, no colour appeared on the membrane and they were considered as "non-compliant". When a blue coloured spot appeared, even substantially lighter coloured than the blank control sample, the sample was considered to be "compliant". It should be emphasized that in the case of OTA in beer or fumonisins in maize/cornflakes, these quotations are only related to the visual detection limit and do not indicate containing a violative concentration, since currently the European Regulation (EC) No 123/2005 does not include them.

Table 32. Overview of the visual detection limits reached in the flow-through assays for ochratoxin A and fumonisins in the selected matrices.

Compound and matrix	Visual detection limit	Reference
Ochratoxin A (OTA)		
wheat	5 µg/kg	European Regulation (EC) No 123/2005
wholemeal bread	3 µg/kg	
beer	0.2 µg/L	Visconti *et al.*, 2000
Fumonisins (FB1 + FB2)		
maize	1000 µg/kg	Weidenbörner (2001)
Cornflakes	275 µg/kg	/

2.5. Validation of the flow-through enzyme immunoassays

For each flow-through assay optimized, an intra-laboratory validation was performed according to the AOAC test kit validation procedure for qualitative tests. The following performance characteristics were determined: dose-response curve, precision, false non-compliant and false compliant rates, specificity and accuracy. For the dose-response curves, the analyte concentration (µg/kg) was plotted against the values of colour intensity (ΔE^*ab). Precision was quantified using the coefficient of variation (CV %) of the ΔE^*ab-values for within-day and between-day experiments. A maximum CV of 20 % was postulated. However for a qualitative screening test it is more correct to express the precision as the number of false non-compliant and false compliant results. These rates were determined at several concentrations including the level of the visual detection limit. In view of assay specificity, two aspects were studied: the response to other structural analogues (cross-reactivity) and the effect of the sample matrix (matrix interferences). Monoclonal antibodies against OTA and FB_1 were produced and characterized by the Institute for Animal Sciences, Agricultural Biotechnology Center, Gödöllö, Hungary. Accuracy of the flow-through method was demonstrated by comparison of the results with a validated chromatographic method (HPLC or LC-MS/MS).

2.5.1. Flow-through assay for OTA in wheat and wholemeal bread

Based on previous research experience of S. De Saeger (2002), the flow-through assay for the detection of OTA in wheat was re-optimized and validated in order to obtain a visual detection limit of 5 µg/kg. This is in accordance with the EU Regulation No 123/2005. The ΔE*ab values of the colour intensities ranged from 10.08±0.95 (n=12) for a blank wheat sample to 6.35±0.14 (n=12) for wheat samples fortified at a level of 5 µg OTA/kg. At this level, no blue coloured spot was developed and the measured ΔE*ab value was completely due to a slightly coloured background. Coefficients of variation for within-day experiments and between-day experiments ranged respectively from 0.1 to 14.01% and from 2.14 to 13.76%. No false compliant results were observed. False non-compliant results at a level of 3 µg OTA/kg (n=12) and at 4 µg OTA/kg (n=12) were respectively 8 and 16%. The monoclonal OTA antibodies were very specific with only a cross-reactivity against ochratoxin B of 9.3%. In order to evaluate the accuracy of this flow-through assay method, a total of 36 wheat samples collected during the harvest of the year 2002 were screened. Results were visually evaluated and intensity of colour development was measured (ΔE*ab). For all samples, a blue sample spot appeared on the membrane, indicating that OTA contamination levels were below the visual detection limit of 5 µg/kg. Figure 18 gives an example of a visual result. All screening results were in agreement with those obtained by HPLC analysis performed by partner 1: UCL/BNUT (III.1.2.1. p.11).

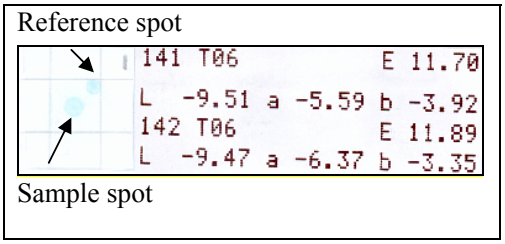

*Figure 18. Visual result for a wheat sample, originating from a Walloon conventional farmer. The ΔE*ab values for the control and sample spots were respectively 11.70 and 11.89.*

Then, the assay was applied to the matrix wholemeal bread, for which the European Commission has established a limit of 3 µg/kg. A dose response curve was established with fortified wholemeal bread samples at OTA concentration levels near the cut-off level of 3 µg/kg. The intensity of the blue colour (ΔE*ab) developed for a blank wholemeal bread sample was 9.32±0.03 (n=3). Whereas for samples fortified with 3 µg/kg or more, no blue colour development could be observed [ΔE*ab value of 6.1 ±0.34 (n =6)]. Because it was decided to include wheat flour for bread-making instead of the finished product in the sampling plan of the project, validation on the matrix wholemeal bread was not further proceeded.

2.5.2. Flow-through assay for OTA in beer

Beer samples fortified with OTA concentrations ranging from 100 to 300 ng/l were analysed several times (n=3) on different days (n=5). Concentrations of OTA in the fortified samples were plotted against the values of colour intensity and within-day and between-day coefficients of variation were calculated. We could observe an intense coloured blue spot for the blank (Δ*E**ab=13.79±1.07, n=15) in combination with no colour development at 200 ng/l (Δ*E**ab=6.08±0.27, n=15). CV's were all within acceptable ranges (Table 33). No false compliant results were obtained. The number of false non-compliants at the 100 (n=15), 150 (n=15) and 175 (n=15) ng OTA/l were respectively 7, 7 and 13%. Four beer samples collected from local market stores in Belgium (year 2002) were screened with the flow-through assay method coupled to immuno-affinity columns clean-up. For all the samples, a blue coloured spot was developed indicating that the OTA contamination in the screened beer samples did not exceed the visual cut-off limit of 200 ng/l. This was in agreement with HPLC results performed by partner 1: UCL/BNUT (II.1.3 p. 24).

Table 33. Summary of statistics of the within-day and between-day reproducibility of the flow-through assay for OTA in fortified beer samples.

Concentration of OTA fortified in beer (ng/l)						
	0	100	150	175	200	300
Within day (ΔE*ab) n=3	12.45±1.19	9.24±0.87	7.90±0.10	6.83±0.12	6.13±0.14	5.94±0.45
CV %	9.57	9.42	1.21	1.69	2.22	7.63
Between day (ΔE*ab) n=5	13.79±1.07	8.86±0.44	7.84±0.23	7.17±0.42	6.08±0.27	6.04±0.10
CV %	7.75	5.01	2.94	5.90	4.44	1.68

2.5.3. Flow-through assay for fumonisins in maize

Results of the optimization and validation of the flow-through assay for the detection of fumonisins in maize were published in a peer reviewed journal (Paepens *et al.*, 2004). Dose-response curves were established using maize samples fortified with FB1 in a range from 0 to 1200 µg/kg (spike series FB1) and FB1+FB2 mixture (ratio 1:1) at concentrations between 0 and 700 µg/kg for each component (spike series FB1+FB2) (Figure 19). The flow-through test was validated for the above mentioned parameters by analysing the fortified samples several times (n=3) on different days (n=5). According to our objectives, a cut-off level of 1000 µg FB1 equivalents/kg could be reached. For within-day experiments, coefficients of variation for spike series FB1 and spike series FB1+FB2 ranged respectively from 0.4 to 7.6% and 0.5 to 10.2%. For between-day experiments, the coefficients of variation varied respectively from 1.1 to 9.0% and 1.1 to 8.9%. These values were within the acceptable limit of 20%. No false compliant results were observed. The number of false non-compliants at the 500 (n=17), 700

(n=21) and 900 (n=22) µg FB1/kg levels were respectively 6, 9 and 13%. Comparable false non-compliant rates were obtained with spike series FB1+FB2: 6% at 575 (n=16), 12% at 767 (n=16) and 15% at 959 (n=19) µg FB1 equivalents /kg. Monoclonal antibodies against FB1 with cross-reactivity against FB2 and FB3 of respectively 91.8% and 209% were used in this assay. There was no cross-reaction with hydrolysed FB_1. Therefore, fumonisin concentrations were expressed as "FB1 equivalents". These were calculated using the corresponding cross-reactivity coefficient for FB2. At the time validation was performed, the FB3 standard was not commercially available. Regarding cross-reactivity, it is worth mentioning that in real environmental situations, contamination with a single component is not likely to be expected. This makes simultaneous detection of fumonisins of the B-series favourable.

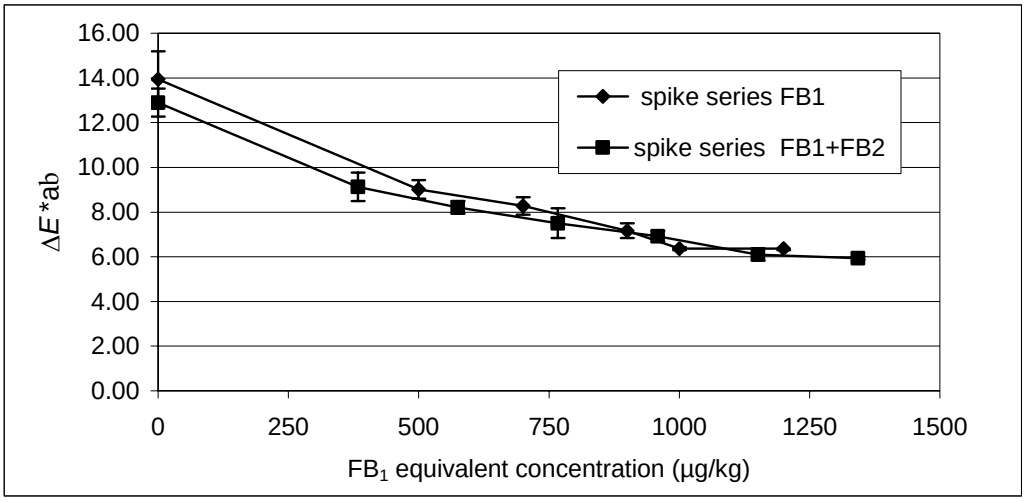

Figure 19. A dose-response curve plotting out the FB_1 equivalent concentration (µg/kg) against the intensity of colour development (ΔE^*ab) (n=15).

Naturally contaminated samples were analysed in triplicate with the flow-through enzyme immunoassay for fumonisin. To evaluate matrix interferences, three types of maize matrices were represented: ground maize grains, maize grits and maize flour (polenta). Table 34 gives an overview of the results obtained with the flow-through enzyme immunoassay compared to the contamination values determined by HPLC. The flow-through assay method has demonstrated its accuracy: results of visual evaluation were in agreement with the HPLC results.

Table 34. Comparison of results obtained by flow-through enzyme immunoassay and HPLC.

Sample (origin)	Visual results flow-through assay[a] (n=3)	Results HPLC confirmation[b]		
		Conc. FB1 (µg/kg)	Conc. FB2 (µg/kg)	Total FB1 equivalents (µg/kg)
Maize				
S1 (Italy)	±, ±, ±	780	203	966
S2 (Italy)	-, -, -	1414	558	1926
S3 (Italy)	±, ±, ±	780	203	966
B1 (Belgium)	+, +, +	<LOQ	<LOD	/
B2 (Belgium)	+, +, +	<LOQ	<LOD	/
B3 (Belgium)	+, +, +	<LOQ	<LOD	/
Maize flour				
P1 (Italy)	±, ±, -	729	119	838
P2 (The	±, ±, ±	342	41	383
P3 Netherlands)	-, -, -	950	145	1083
P4 (Italy)	-, -, -	2795	103	3165
Maize (Italy) grits				
P5 (Venezuela)	+, +, +	<LOQ	<LOD	/

[a] + spot, compliant; ± weak spot, compliant; - no spot, non-compliant.
[b] HPLC analysis of the samples S1-S3 was performed by M. Solfrizzo *et al.*
HPLC confirmation for all other samples was carried out according the method described in Paepens *et al.* (2004). Limit of detection (LOD) for FB1 = 30 µg/kg; for FB2 = 65 µg/kg. Limit of quantification (LOQ) for FB1 = 97 µg/kg; for FB2 = 212 µg/kg.

2.5.4. Flow-through assay for fumonisins in cornflakes

The validated flow-through assay for fumonisin detection in maize was adapted for the matrix cornflakes by lowering the visual detection limit. The working solutions of the immunoreagents were optimized using cornflakes fortified with a FB1+FB2 standard mixture (spikes FB1+FB2; 10 ng/µl in methanol, ratio 1:1). A good colour intensity (average ΔE^*ab=10.45 ± 1.23, n=12) for the blank in combination with complete colour inhibition (average ΔE^*ab=6.35±0.15, n=12) at 275 µg FB1 equivalents/kg was obtained. Therefore 275 µg FB1 equivalents/kg was selected as the visual detection limit. In order to evaluate the accuracy, 90 cornflakes samples collected by partner 1: UCL/BNUT were screened using the flow-through format. These included nine brands of cornflakes (7 conventional and 2 organic brands, two different batches/brand, 5 samples/batch). Visual results were compared with quantitative results obtained by LC-MS/MS (III.7.2 p.40) and are here presented in a contingency table (Table 35). Also for this flow-through test, it is important to underline that no false compliant results were observed. The rate of false non-compliant results was 18%.

Table 35 : Comparison of flow-through results with LC-MS/MS results regarding fumonisins in our cornflakes.

		Results LC-MS/MS		
		Total FUM conc. > 275 FB1 equivalents (µg/kg)	Total FUM conc. < 275 FB1 equivalents (µg/kg)	Total
Results flow-through assay	Non-compliant	12	14	26
	Compliant	0	64	64
	Total	12	78	90

V. CELLULAR BIOLOGY

1. Introduction

The possible health risks of exposure to mycotoxins present in food and feed have increased the interest for developing cellular systems for toxicity testing. Cellular *in vitro* systems will allow to estimate the combinatory effects of mixtures of mycotoxins and of uncharacterized metabolites.

In this context, our objective was to develop biological methods allowing the detection of mycotoxins, present as food contaminants, based on their direct or indirect toxicity towards human or animal cells in culture.

The direct cytotoxicity detection methods were based on the evaluation of living cell number and/or cell mortality, after incubation of cells in the presence of mycotoxins. They are rapid and allow a large screening of samples. The selection of the most sensitive method was realized with commercially available mycotoxins. Then this method was applied to food extracts.

The indirect cytotoxicity detection methods were based on the measurement of mycotoxins effects on specific cellular functions, mostly on intestinal functions, using an *in vitro* model of the human intestinal epithelium, based on the Caco-2 cells, as the intestine is the first absorption site of nutrients and contaminants after food ingestion.

2. Description of biological methods

2.1. Direct toxicity detection methods

The methods that we have applied to the mycotoxins are derived from methods routinely used in our laboratory for screening the effects of drugs or other xenobiotics, like PCBs.

The direct effect of various substances on the cellular viability may be measured by determining different endpoints such as metabolic activities, plasma membrane damage, protein synthesis. We have tested three colorimetric bioassays that could provide a rapid and sensitive screening of low mycotoxins concentrations: the methylthiazoletetrazolium (MTT) assay measuring the activity of a mitochondrial enzyme of living cells, the neutral red assay, which is a measure of the cell membrane integrity, and the lactate deshydrogenase (LDH) assay which evaluates the cell mortality.

Various cell lines are classically utilized in toxicological studies as *in vitro* model of intestinal, hepatic or renal functions. Based on the literature and on our previous expertise, we have selected three cell lines as representative of organs that may be implicated in animal or human mycotoxicosis :

- the human Caco-2 cell line for the intestine;
- the animal MDCK cell line for the kidney;
- the human Hep G2 cell line for the liver.

Those three cell lines were used to set up and select the most sensitive method.

The detailed description of the methods is limited to the MTT assay since the results that will be presented focus on this approach.

The MTT assay is based on the reduction of methylthiazoletetrazolium, a yellow tetrazolium salt, to dark purple formazan crystals by the mitochondrial enzyme succinate dehydrogenase. This activity takes place only in living cells and the quantity of formazan formed is

proportional to the number of living cells. Cells should be maintained in an exponential growth phase during the assay.

Caco-2 cells were routinely grown, as in Halleux & Schneider (1991) and Sergent *et al.* (1993), on collagen coated flasks 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 (Schneider, 1989) 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. MDCK and Hep G2 cells were routinely grown in 10% (v/v) fetal bovine serum-containing DMEM. Cells were inoculated, in their respective culture medium, in 96-wells microplates at 35 000 cells/cm^2. After 24 hours (h), the cell culture medium was replaced by medium containing different concentrations of one of the mycotoxins. Stock solutions of mycotoxins were prepared in appropriate solvents before their dilutions in cell culture medium: 10% (v/v) ethanol for DON, 10% (v/v) dimethylsufoxide (DMSO) for OTA and 70% (v/v) methanol for FB1 and further diluted in culture medium. Appropriate controls for the presence of these solvents were included. After 48 h incubation of the cells, the media were removed and a MTT solution at 0.5 mg/ml in PBS was added for 2 h. Formazan crystals were then dissolved in DMSO. Absorbance was measured at 500 nm with a microplate reader.

2.2. Indirect toxicity detection methods

The indirect toxicity detection methods were developed with Caco-2 cells. Regarding the toxicological effects of FB1 on cells in culture, specific methods, *i.e.* apoptosis, lipid peroxidation and *de novo* sphingolipid biosynthesis assays, were chosen on the basis of a focussed literature survey.

In a more general approach, the effects of OTA, DON and FB1 were evaluated on different intestinal functions and on the expression of particular genes, in relation with the intestinal detoxification systems.

2.2.1. Specific FB1 toxicity detection methods

– Apoptosis assay by the DNA ladder method

Caco-2 cells were inoculated in collagen-coated 6-wells TCPS plates at a density of 80 000 cells/cm^2, in the serum-free medium. Twenty-four our after inoculation, for experiments with proliferating cells, cells were incubated with FB1 from 1 to 100 µM for 3 h and then treated with lysis buffer. For experiments with cells at confluence, cells were cultivated for 48 h and then incubated with FB1 from 1 to 100 µM for different durations, from 3 h to 96 h. The Caco-2 cells were lysed with 500 µl of the lysis buffer (0.01 M tris-HCl, 0.1M NaOH, 0.025M EDTA, 10 % w/v SDS, pH 8). Ten µl of RNAse were added and the solution was incubated for 18 h at 37°C. Ten µl of proteinase K were added and the solution was incubated for 3 to 5 h at 37°C. The DNA was extracted with phenol:$CHCl_3$:isoamylic alcohol (25:24:1, v/v/v) and analysed after agarose gel electrophoresis in the presence of ethidium bromide.

– Lipid peroxidation assay

Caco-2 cells were inoculated in collagen-coated 6-wells TCPS plates at a density of 80 000 cells/cm^2, in the serum-free medium or in a 1% (v/v) fetal bovine serum-containing medium. For experiments with proliferating cells, the cells were incubated with FB1 24 h after inoculation at 7 or 70 µM for 3 h and then treated as below. For experiments with cells at confluence, cells, cultivated for 48 h, were incubated with 7 or 70 µM FB1 for different durations, from 3 h to 96 h. The cellular lipid peroxidation was determined by measuring the

thiobarbituric acid reactive substances (TBARS). After the mycotoxin stimulation, the Caco-2 cells pellets were suspended in 1 ml of 20 % (v/v) trichloroacetic acid and 1 ml thiobarbituric acid solution (0.67% v/v in 0.05 N NaOH). The tubes were incubated at 95°C for 30 min, cooled to room temperature and centrifuged (300g for 10 min). The absorbance was measured at 546 nm. Alternatively, the solution was treated with 1 ml n-butanol and the supernatant was measured spectrofluorimetrically, at wavelenghts of 515 and 550 nm for excitation and emission, respectively. Malonaldehyde was used as standard.

– De novo sphingolipid biosynthesis assay

Caco-2 cells were inoculated in collagen-coated 6-wells TCPS plates at a density of 45 000 cells/cm^2, in the serum-free medium. After 24 h, non-confluent cells were incubated in the presence of ^{3}H-L-serine for 24 or 48 h. The cells pellets were base-hydrolysed with 0.5 ml of 0.125 M methanolic KOH in $CHCl_3$ (4/1) for 2 h at 37°C. The free sphingoid bases were then extracted by adding 0.5 ml $CHCl_3$, 0.5 ml alkaline water and 100 µl 2N NaOH. After centrifugation (4 000g for 10 min), the $CHCl_3$ phase was dried. The lipids were separated on silica gel 60 thin-layer chromatography plates, developed with $CHCl_3$:methanol:formic acid:water (53:30:4:2, v/v/v/v). The plates were dried and cut. Each fraction was quantified for the radioactivity in a scintillation counter. The labeled sphingolipids were identified by comparison with known standards for sphingomyelin and sphinganine that were run on the same plate and visualized by spraying a 2'-7'-dichorofluorescein solution.

2.2.2. In vitro model of human intestine and evaluation of the indirect toxicity of mycotoxins

The *in vitro* model used to evaluate the indirect toxicity of mycotoxins is based on the Caco-2 cells culture. When Caco-2 cells are cultivated on microporous filters in bicameral inserts, they fully differentiate into enterocytes and polarize, with intercellular tight junctions, microvillosities, functional brush border enzymes, transport systems. They also express intestinal detoxification enzymatic systems and efflux pumps. Such culture system provides a separate access to the apical and basolateral sides of enterocytes, representing, respectively, the intestinal lumen and the blood pole.

– Caco-2 cells culture

Cells were seeded on a type I collagen precoated poly(ethylene terephtalate) (PET) microporous membrane (1 µm pore diameter) in a bicameral insert (24 mm diameter, 5 cm^2 growing area) at 120 000 cells/cm^2. They were cultivated for 21 to 28 days to allow complete differentiation into enterocytes.

– Transepithelial electrical resistance measurement

The integrity of 3 weeks-old cells layers was checked by measurement of the transepithelial electrical resistance (TEER) with an epithelial tissue voltohmmeter.

– Gene expression analysis

In order to measure the effect of mycotoxins on gene expression, Caco-2 cells were first cultivated on microporous PET membrane for 14 days and were then cultivated during 7 days in the presence of low concentrations of mycotoxins (see results for details). The cells were then collected and the expression of several genes was evaluated by RT-PCR (Reverse Transcription-Polymerase Chain Reaction).

Total RNA was isolated from *ca*. 15 10^6 Caco-2 cells scrapped and recovered in 1 ml TRI-Instapure. RNA was extracted with 200 µl chloroform and precipitated with 500 µl isopropanol. The precipitate was rinsed with 1 ml ethanol, dried and resuspended in 50 µl water. Total RNA (2 µg) was then reverse transcribed in 20 µl transcription buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM of each deoxynucleoside triphosphate, 150 ng of random primers) with 200 U of Superscript RT reverse transcriptase at 42°C for 1 h, followed by heating to 70°C for 15 min and immediate cooling on ice. The reverse transcription mixture (1 µl) was amplified by PCR in 40 µl of buffer (75 mM Tris-HCl, pH 9.0, 20 mM $(NH_4)_2SO_4$, 0.01 % (w/v) Tween 20, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate) with 0.2 µM of sense and anti-sense primers, specific of the targeted gene, and 0.8 U of GoldStar DNA polymerase. The PCR cycles started with a 4 min. period at 94°C. PCR was then run at 94°C for 1 min., at the primers optimum temperature for 1 min and at 72°C for 1 min. The cycles number was specific for each studied gene. Amplification was ended by an incubation at 72°C for 5 min. The PCR products (20 µl) were separated on 1% agarose gels and visualized by ethidium bromide staining. PCR amplification of RNA not reverse transcribed was used as negative control to exclude amplification of genomic DNA.

– CYP1A1 enzyme activity assay

The CYP1A1 is an isoform of the P450-cytochrome, the main intestinal phase I detoxification enzymatic system (phase I refers to oxidation reactions). The CYP1A1-dependent 7-ethoxyresorufin O-deethylase (EROD) activity was determined by a fluorimetric assay. The Caco-2 cells were cultivated on microporous PET membrane for 18 to 21 days. Cells were then incubated for 2 h, 16 h or 7 days in the presence of mycotoxins. Thereafter, they were exposed for 1 h to 5 µM ethoxyresorufin in a phenol red-free medium. The resorufin produced was monitored with a fluorimeter with wavelengths of 530 and 585 nm for excitation and emission, respectively.

– Glutathione-S-transferase activity assay

Glutathione-S-transferase (GST) is a phase II detoxification enzyme (phase II refers to conjugation reactions). Its activity was determined by a colorimetric assay. The Caco-2 cells were cultivated on microporous PET membrane for 18 to 21 days. Cells were then incubated for 2 h, 16 h or 7 days in the presence of mycotoxins. Thereafter, they were exposed for 1 h to 50 µM CDNB (1-chloro-2,4-dinitrobenzene) in a phenol red-free medium. The reaction was monitored photometrically by the increase in absorbance at 340 nm. The glutathione-CDNB conjugates formation was determined using the molar extinction coefficient of 10 $mM^{-1}.cm^{-1}$.

– MRP-2 efflux pump activity assay

MRP-2 is an isoform of the multidrug resistance-associated protein (MRP), an efflux pump located at the apical side of the Caco-2 cells. We have previously described that OTA is a MRP-2 substrate (Berger *et al.*, 2003). The MRP-2 activity assay is based on the [^{3}H]OTA transport from the apical to the basolateral side of differentiated Caco-2 cells. Transport medium was Hank's balanced salt solution (HBSS) containing 5 mM glucose and 10 mM Hepes (pH 7.4), for the lower compartment of the inserts, or 10 mM Mes (pH 6.0), for the upper compartment. The lower compartment was further supplemented with 1 % (w/v) bovine serum albumin. Transepithelial passage was assayed by adding 7.5 nM [^{3}H]OTA (with 10 µM non radioactive OTA) in the upper compartment as well as [^{14}C]mannitol, as internal control for transport. Contaminants or MK571, a specific MRPs inhibitor, were further added in the upper compartment at the same moment than OTA. After 3 h, media from the upper and

lower compartments were collected separately and an aliquot was analyzed by liquid scintillation spectrometry after dispersion in 2 ml of Aqualuma.

– P-glycoprotein activity assay

P-glycoprotein (PgP) is the other major efflux pump located at the apical membrane of the intestinal epithelium. Digoxin, a cardiotonic drug, is substrate of PgP. The PgP activity assay is based on the transport of [^{3}H]digoxin from the apical to the basolateral side and from the basolateral to the apical side of the Caco-2 cells. Transport media are the same as mentioned for MRP-2 assay. 15 nM [^{3}H]digoxin supplemented with 50 µM non-radioactive digoxin were added in the apical or basolateral compartment. Contaminants or 100 µM .verapamil, a PgP inhibitor, were further added in the apical compartment at the same moment than digoxin. After 3 h, media from the upper (for digoxin transepithelial passage from the basolateral to the apical side) or lower (for transepithelial passage from the apical to basolateral side) compartments were collected and analyzed by liquid scintillation spectrometry after dispersion in 2 ml of Aqualuma.

3. Results

3.1. Direct detection methods

Our objective was to develop bioassays based on mammalian cell cultures and evaluate their ability to screen and to determine the presence and the toxicity of mycotoxins in food extracts.

3.1.1. Bioassay selection

Three colorimetric bioassays that could provide a rapid and sensitive screening of low mycotoxins concentrations, the MTT assay, the neutral red assay and the LDH assay, were applied on three cell lines representative of organs that may be implicated in animal or human mycotoxicosis (see section 2.1.). Commercially available mycotoxins (DON, OTA, FB1) were used to set up the methods. For each cell line, the cellular density has been adapted to be optimal for the assay and for each mycotoxin, dose/response curves were established.

The MTT assay with the intestinal Caco-2 cells appeared to be the most sensitive assay.

DON and OTA have a clear concentration-dependent effect on the metabolic activity of the Caco-2 cells as determined by the MTT bioassay (Figure 20). DON induces a significant cytotoxicity (20 %) from 0.2 µg/ml and OTA from 0.1 µg/ml. FB1 does not inhibit cell proliferation at all the concentrations tested, from 0.6 to 72 µg/ml (not shown).

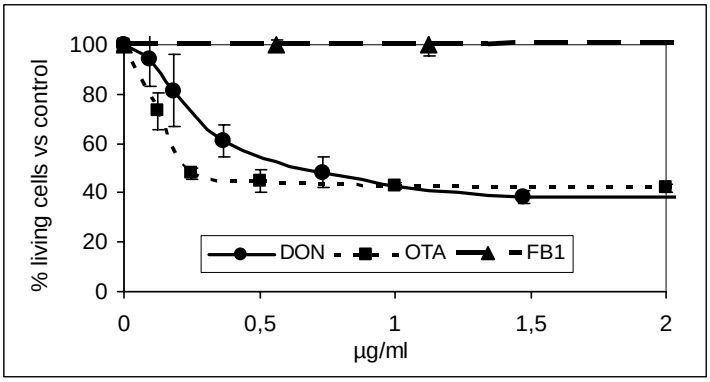

Figure 20. Effect of various concentrations of DON, OTA and FB1, on the metabolic activity of Caco-2 cells as determined by the MTT bioassay. The values are expressed as percent of control response and are means of 4 wells ± SD.

For the Hep G2 and MDCK cell lines, comparable curves were obtained (results not illustrated). The mean IC_{50}* values have been calculated and are presented in Table 36. The three cell lines do not have the same sensitivity towards the mycotoxins. The Hep G2 cells are the most resistant to the mycotoxins and the Caco-2 cells the most sensitive. FB1 had a minor effect on the metabolic activity of the cells.

Table 36. IC_{50} values for DON, OTA and FB1 exposed to Caco-2, MDCK and Hep G2 cells, determined by the MTT bioassay.*

	Caco-2 cells	MDCK cells	Hep G2 cells
DON	0,66±0,23	0,52±0,18	>30
OTA	0,19±0,06	27,91±0,57	>40
FB1	>70	>70	>70

* IC_{50} (µg mycotoxin/ml cell culture medium that reduces cell viability to 50 %). Data are means ± S.E.M. (N = 3, n = 9 to 12) of three independent experiments.

At the same concentrations as those used in the MTT assay, DON and OTA had only a minor effect on Caco-2, MDCK and Hep G2 cells, when the toxicity was evaluated by the Neutral Red assay (results not illustrated) and no cytotoxic effect of FB1, whatever the conditions tested, was observed.

The LDH assay has been performed on the cell culture medium collected after 48 h incubation with the mycotoxins. The LDH activity released is not significantly different from controls, except for the highest mycotoxin concentration (100 µM). This confirms that, at low concentrations, mycotoxins do not induce cell necrosis.

If we compare the three methods, the MTT assay appears to be the most sensitive and allows to detect the cytotoxicity at low mycotoxin concentrations. Table 37 indicates the minimal mycotoxin concentrations that provoke a significant toxicity, in relation with the cell lines. The Caco-2 and MDCK cells are sensitive to DON concentrations that may be frequently found in cereals and to concentrations considered hazardous to animals and humans.

Table 37. Minimal toxic values (µg/ml) for DON, OTA and FB1 on Caco-2, MDCK and Hep G2 cells, as determined by the MTT bioassay.

	Caco-2 cells	MDCK cells	Hep G2 cells
DON	0.20	0.10	0.40
OTA	0.12	4.00	2.00
FB1	70.00	70.00	70.00

The MTT method based on the Caco-2 cells culture has been selected for the next experiments. The MTT method is also rapid and allows the screening of a large number of samples at the same time. Nevertheless, this bioassay does not provide any information on the chemical nature of the molecules that are responsible of the cytotoxic effect.

3.1.2. Extension of the MTT assay to other contaminants and to contaminants mixtures

Three additional mycotoxins were assayed for their cytotoxicity with the MTT bioassay: penicillic acid (PEN), citrinin (CIT) and ZEN.

Figure 21 presents the dose/response curves established for the 3 new mycotoxins compared to DON, OTA and FB1. PEN, CIT and ZEN have a weak cytotoxic effect at low concentrations but become as toxic as DON and OTA at higher concentrations.

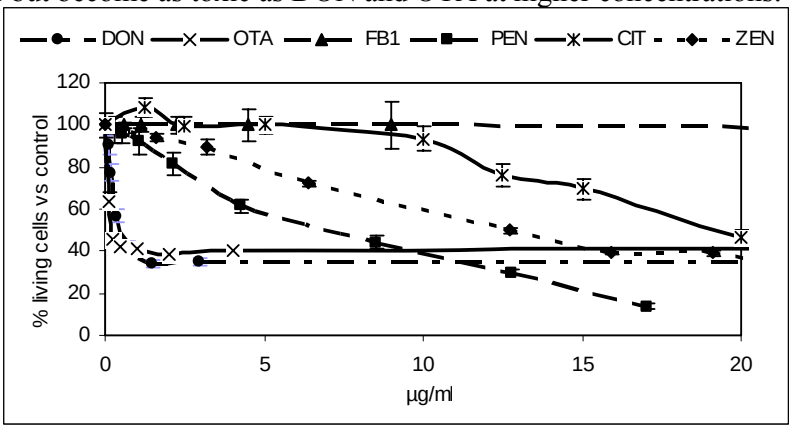

Figure 21. Effect of various concentrations of mycotoxins, on the metabolic activity of Caco-2 cells, as determined by the MTT bioassay. The values are expressed as percent of control response and are means of 4 wells ± SD.

PEN, CIT and ZEN have been described to co-contaminate food or feed, simultaneously with OTA, DON and/or FB1. The cytotoxicity of mycotoxins mixtures was evaluated with the MTT assay: for each mycotoxin, a non-toxic concentration and a concentration that induces 20 to 30% of cytotoxicity were chosen and combined with a non-toxic or a toxic concentration of another mycotoxin. The mycotoxin combinations tested were: DON/OTA, DON/OTA/FB1, OTA/FB1, OTA/CIT, OTA/PEN, DON/ZEN. No synergistic effect was observed, whatever the combination tested.

The toxicity of two other food contaminants was determined with the MTT assay: acrylamide and Aroclor 1260 (a PCBs mixture). Acrylamide was tested from 75 nM to 1 mM: 20% of cytoxicity was obtained with 1 mM, a non-relevant food concentration. Aroclor 1260, assayed from 1 ng/ml to 1 µg/ml of cell culture medium, did not shown any significant toxicity in that concentration range. Mixtures of those toxics with mycotoxins have not yet been assayed.

3.1.3. Inter-comparative study for DON in wheat flour

The suitability of the MTT assay for detection of mycotoxins in food extracts has been evaluated in an inter-comparative study performed on a flour sample spiked with DON. DON has been selected from Table 37, as it was shown that Caco-2 cells were sensitive enough to DON concentrations found in cereals.

The assayed spiked flour sample contained 26.7 ppm of DON, as quantified by partner 2. The flour was extracted with acetonitrile:water. The extract was then evaporated, resuspended in the cell culture medium and used in the MTT assay at different dilutions, knowing the initial DON concentration. Commercial DON was used as control, at the same concentrations. Figure 22 shows that the same cytotoxicity has been obtained: the DON concentration determined by the MTT assay thus corresponds to the DON concentration assayed by the HPLC method.

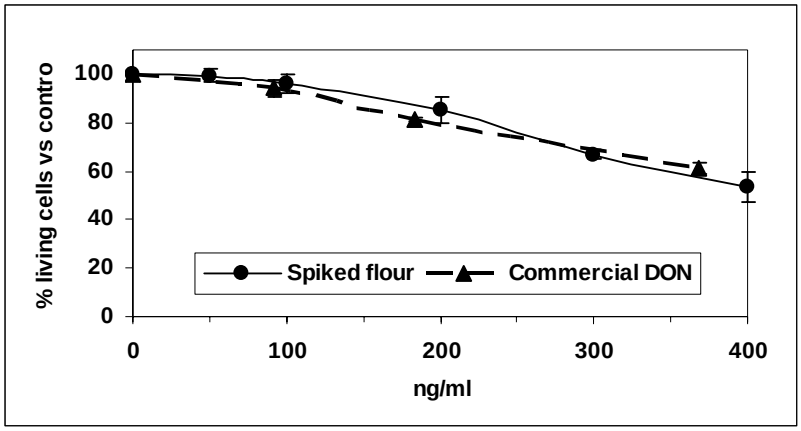

Figure 22. Comparison of the effect of a DON spiked-flour extract and commercial DON, at different concentrations, on the cell metabolic activity, as determined by the MTT bioassay. The values are expressed as percent of control response and are means of 4 wells ±SD.

The MTT assay applied to flour samples extracted with organic solvent, as it is done for the analytical methods, has been shown to be a suitable method to detect DON contamination.

3.1.4. Identification of interfering factors from the matrices

The organic extraction step of foods is rather specific of each particular mycotoxin and therefore does not account for all the contaminants that may induce cytotoxicity. Accordingly, to avoid this step, the MTT assay has been performed on food samples that were directly dissolved in the cell culture medium.

Two types of matrices were tested for the presence of interfering factors: flour and beer.

Conventional and organic flour samples were directly dissolved in the cell culture medium. The media were then centrifuged and the supernatants filtered. These samples were used at different dilutions in MTT bioassays. Results (not illustrated) have shown that the extracts were cytotoxic (independently from the mycotoxin load) and had to be diluted 20 times to maintain ± 80% of cellular viability. Considering the DON and OTA detection limits in the MTT bioassay, these results indicate that this type of test is not appropriate for the detection of these mycotoxins, or comparable contaminants, in naturally contaminated flours.

Three commercial beer samples were assayed : beer 1 (233.4 ppt OTA), beer 2 (519.6 ppt OTA) and beer 3 (OTA<LOD). The samples were degassed and the pH adjusted to ± 7.2

before filtration and MTT assay. Results, presented in Figure 23, show that the beers must be diluted 10 times to maintain ± 70% of cellular viability, independently from the mycotoxin load. Such a dilution precludes the use of the MTT assay for the analysis of naturally contaminated beers.

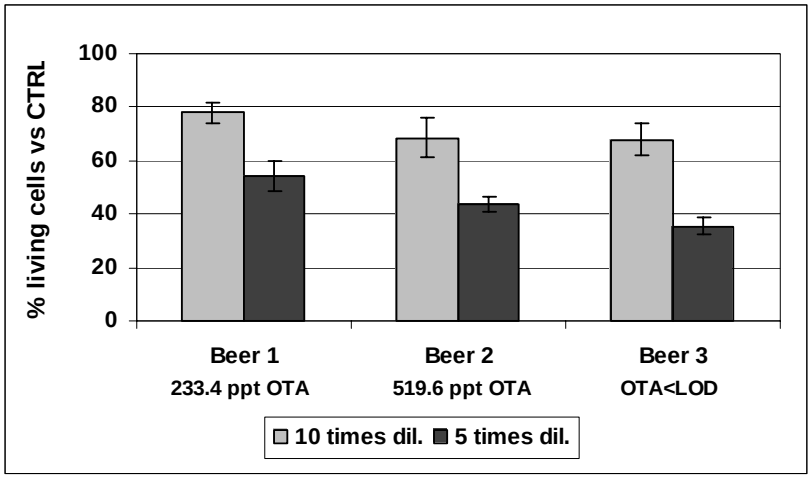

Figure 23. Effect of different dilutions of 3 beer samples on the metabolic activity of Caco-2 cells, determined by the MTT bioassay. The values are expressed as percent of control response and are means of 4 wells ±SD.

3.1.5. Conclusions

The MTT assay, based on the metabolic activity of Caco-2 cells has been revealed as the most sensitive assay to evaluate the direct mycotoxin toxicity.

The MTT assay has been used to screen various mycotoxins and mycotoxins mixtures.

OTA and DON appeared as the most cytotoxic mycotoxins. The MTT assay may detect DON at concentration that could contaminate cereals, whereas for OTA, the MTT assay is not sensitive enough to allow its detection in food samples. FB1 has shown no direct cytotoxicity at relevant food concentrations.

No synergic effect has been observed with mycotoxins mixtures. This type of test might however be useful to screen for other interactions between toxic compounds.

The suitability of the MTT assay has been shown with a DON spiked flour extracted with organic solvent.

Without organic extractions of the samples, flour and beer matrices had been revealed to interfere with the MTT assay and to mask the potential presence of mycotoxins in the samples. Chemical extraction is thus required. In that case, the MTT assay of food samples becomes too laborious to screen a lot of samples: since each mycotoxin needs a specific solvent, the MTT assay would need to be performed several times on the same food sample. Moreover, other contaminants potentially present in the food samples could be discarded and not detected. The direct solubilization of the food samples in the cell culture medium is more rapid and should allow to take into account all the contaminants present in the sample. Since its applicability to the MTT assay requires an important sample dilution to avoid matrix cytotoxicity, the contaminants concentrations are likely to fall below the sensitivity limit of the method. Indeed, no more cytotoxicity was detected.

As the MTT method was loosing its advantages on the analytical methods, the food samples analysis by the MTT bioassay was discarded, after a common agreement with the other partners of the project, and the users committee, for the benefit of the study of the indirect mycotoxins cytotoxicity.

3.2. Indirect detection methods

The second objective of the cellular biology methods was to select and set up assays that allow the evaluation of the mycotoxins indirect cytotoxicity. A specific effort was put on FB1 in order to show its cellular effects at plausible physiological concentrations. In a more global approach, the effects of OTA, DON and FB1 were also evaluated.

3.2.1. FB1 cytotoxicity determination

In the MTT assays, FB1 had a weak cytotoxic effect only at high concentrations. In ordeer to detect the influence of FB1 at lower concentrations, some more specific assays have been developed: from literature data, FB1 could provoke cell apoptosis and lipid peroxidation. FB1 could also inhibit the sphingolipids synthesis. These 3 cellular parameters were followed on Caco-2 cells in the presence of FB1.

– Apoptosis

The DNA ladder method for determining the apoptosis has firstly been set up. The effect of FB1 at concentrations from 1 to 100 µM was evaluated on the Caco-2 cells, proliferating or at confluence, incubated for different durations (3 h, 24 h, 48 h, 96 h), as described in the methodology section V.2.2.1. (p. 58). No DNA fragmentation was observed in the presence of FB1, whatever the experimental conditions used, as compared with control cells (not illustrated).

– Lipid peroxidation

The cellular lipid peroxidation was evaluated by the TBARS method on Caco-2 cells cultivated in different conditions as described in the methodology section V.2.2.1. (p. 58). The effect of FB1 was evaluated at low (7 µM) or high (70 µM) concentrations for different durations. Whatever the experimental conditions, no TBARS was detected neither by the colorimetric nor by the fluorimetric assay: in our culture conditions, this indicates that FB1 does not induce lipid peroxidation.

– *De novo* sphingolipid biosynthesis

FB1 is known to prevent the sphingolipid synthesis by inhibiting ceramide synthase, thus inducing sphinganine accumulation.

Figure 24A shows the chromatographic profiles obtained with Caco-2 cells incubated or not (control) with 25 µM FB1 for 48 h. In FB1 treated cells, the sphyngomyelin-corresponding peak was 3 times lower than in the control, while another peak, corresponding to sphinganine, appeared.

The accumulation of sphinganine (SA) *vs* sphingomyelin (SM) was determined in cells incubated for 24 or 48 h in the presence of various concentrations of FB1: results (Figure 24B) indicate a significant effect of FB1 from 12.5 µM in the case of a 48 h-incubation. This assay allows to detect an effect of FB1 at a concentration 10 times lower than in the MTT assay (see Table 37, section 3.1.1.). Nevertheless, this concentration (12.5 µM = 7.2 µg/ml) remains high in comparison with those found in cereals.

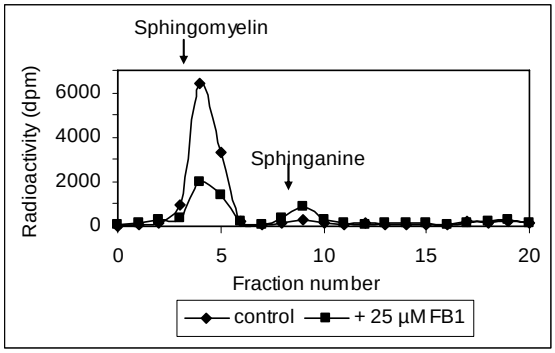

Figure 24 A.

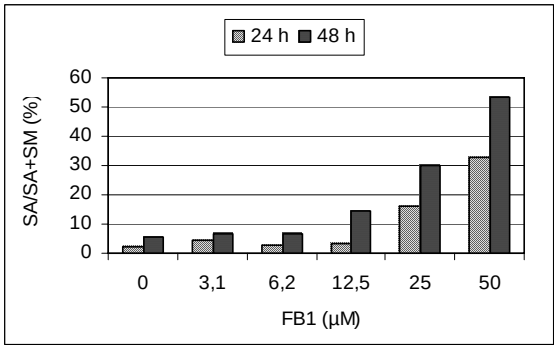

Figure 24 B.

Figure 24. A) Chromatograms of de novo sphingolipid biosynthesis in Caco-2 cells incubated or not (control) with 25 µM FB1 for 48 h. The radiolabeled sphingolipids were identified by comparison with known standards for sphingomyelin and sphinganine. B) Effect of various concentrations of FB1, incubated for 24 or 48 h with Caco-2 cells, on the sphingolipid biosynthesis. Results are expressed as percent of sphinganine (SA) versus total sphingolipids (sphinganine, SA, plus sphingomyelin, SM), calculated from chromatograms and are means of 2 wells.

3.2.2. Study of the effects of mycotoxins on intestinal functions

– Effect on the intestinal permeability and on gene expression

It has been demonstrated in the literature (Kasuga *et al.*, 1998; Maresca *et al.*, 2002) that mycotoxins at concentrations that might be reasonably expected to occur in our food, have no short-term toxicity on differentiated intestinal cells, but that a chronic exposure to lower doses affects the intestinal functions.

In order to detect the influence of lower mycotoxins concentrations during a chronic contamination (*i.e.* 7 days incubation), we have selected to follow the integrity of tight junctions by measuring the transepithelial electrical resistance (TEER) and the expression of specific genes by the RT-PCR technique.

Cells were cultivated on a microporous membrane in bicameral inserts during 21 days to allow complete differentiation into enterocytes. During the last 7 days, they were incubated in the presence, or not, of various concentrations of OTA, DON or FB1. At the end of the incubation, TEER was measured and cells were collected for RT-PCR analysis.

Figure 25 shows that DON and OTA have an important dose-dependent effect on the intestinal permeability to ions, as determined by the TEER measurements with a significant attenuation of the TEER from 50 ng/ml for DON and from 4 ng/ml for OTA. It should be noted that the alteration of the integrity of tight junctions in DON- or OTA-treated cells occurred at concentrations where there is no effect on the cell viability evaluated by microscopic examination. For FB1, its effect on the cellular permeability is significant from 7.2 µg/ml (10 µM).

These results demonstrate that DON and OTA have a clear toxic effect at very low concentrations on the intestinal permeability, measured by the TEER. This must be compared to the MTT assay (Table 37), for which the minimal toxic concentrations of OTA and DON on proliferating Caco-2 cells were 3 to 4 times higher.

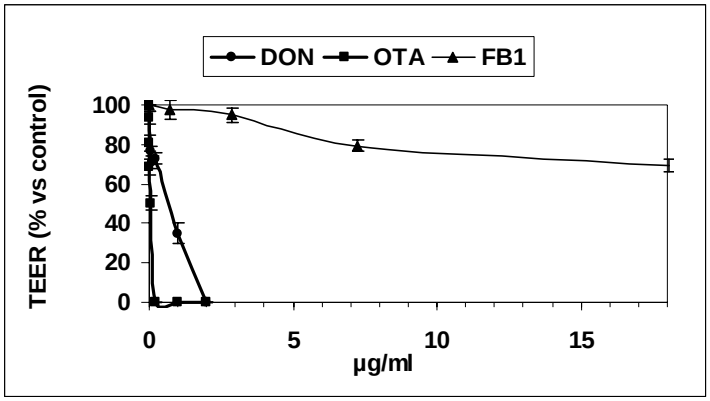

Figure 25. Effect of various concentrations of DON, OTA or FB1, on the intestinal permeability, as determined by the TEER measurements on differentiated Caco-2 cells incubated, or not (control), with mycotoxins during 7 days. Results are expressed as percent of control and are means of 3 independent experiences ± S.E.M. (n = 6-15).

The transcription of different genes in relation with phase I and II detoxification systems and with the efflux pumps was evaluated on the cDNA from DON-, OTA- or FB1-treated cells. Neither induction nor repression of the expression of the genes under unvesigation has been detected with OTA or FB1.

For DON-treated cells (Figure 26), a dose-dependent reduction of the cytochrome-P450 3A4 (CYP3A4) isoform expression has been observed from 500 ng/ml DON. There was also a decrease in the gene expression of phase II (conjugation) enzymes, i.e. the 1A6 and 2B7 UDP-glucuronyltransferase (UGT 1A6, UGT 2B7) isoforms, and for the phenolsulfotransferase (PST), from 500 ng/ml DON. No effect on efflux pumps expression has been observed (not shown).

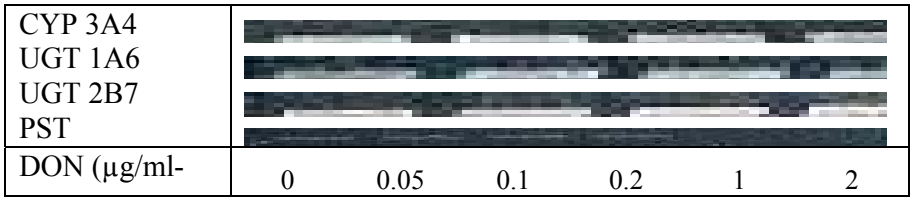

Figure 26. Effect of various DON concentrations in contact during 7 days with differentiated Caco-2 cells on the expression of different genes, as determined by RT-PCR.

As shown by RT-PCR, DON provokes a repression of the transcription of particular genes at low concentrations: these genes are implicated in the cellular metabolism of xenobiotics, linked to the detoxification system.

– Effect on CYP1A1 activity

Cytochrome P450 1A1 (CYP1A1) is a well-known drug-metabolizing enzyme expressed in intestinal cells. Its expression in response to mycotoxins was measured through the EROD activity in differentiated Caco-2 cells.

In a first set of experiments, Caco-2 cells were cultivated until complete differentiation (21 days). Mycotoxins (DON, FB1, OTA or ZEN) were then incubated for 2 h on the cells before the EROD activity was assayed. Figure 27 shows that DON and ZEN had an inhibitory effect on the EROD activity: DON reduced the EROD activity to ± 80% of the control value from 50 ng/ml and had a maximal effect (30% of reduction) at 1 µg/ml; ZEN inhibited the EROD activity by ± 30% at all the tested concentrations (from 10 ng/ml to 1 µg/ml). As DON

and ZEN can be present simultaneously in cereals, mixtures of these 2 mycotoxins have been assayed on the EROD activity: no synergic effects were detected. Neither FB1 (at 4, 10 or 25 µg/ml) nor OTA (assayed from 40 ng/ml to 1 µg/ml) showed any significant effects on the EROD activity.

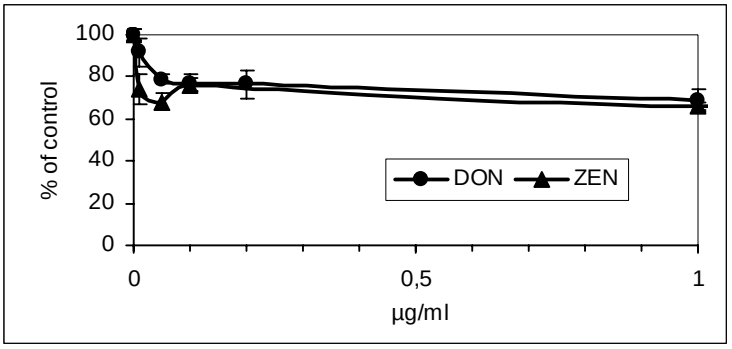

Figure 27. Effect of DON and ZEN on the CYP1A1 activity. Differentiated Caco-2 cells were incubated for 2 h with, or without (control), different concentrations of mycotoxins. The EROD activity was then determined. Results are expressed as percent of the activity (pmoles/min./mg cell protein) in control and are means ± SD.

In a second set of experiments, the mycotoxins were incubated for 16 h with Caco-2 cells. No effect on the EROD activity was detected at any concentrations.

In a third set of experiments, a chronic contamination has been realized by incubating cells during 7 days in the presence of low mycotoxins concentrations. Caco-2 cells were then assayed for the EROD activity. No effect on the CYP1A1 activity was established with OTA (at 10 or 40 ng/ml), DON (125, 250 or 500 ng/ml) or FB1 (4 or 10 µg/ml).

PCBs are known to induce the CYP1A1 activity. A preliminary experiment has been performed with Aroclor 1260, a commercial mixture of PCBs representative of that found in contaminated food. Aroclor 1260 at 1 or 5 µg/ml were incubated on differentiated Caco-2 cells for 2 h, 24 h, 48 h or 96 h. TEER was measured and EROD activity was determined. No effect was observed on the TEER.

An increase of the EROD activity, ± 70 % above the control value, has been detected in the Caco-2 cells incubated with Aroclor 1260 at 5 µg/ml for 48 h. The other conditions were without effects.

Mixtures of Aroclor 1260 and mycotoxins were assayed on the CYP1A1 activity (Figure 28). Only DON, at 250 ng/ml, significantly reduced the Aroclor 1260-induced EROD activity, from ± 194% to ± 150%. These results are preliminary and have therefore to be reproduced. But it seems that there could be an antagonism between DON and Aroclor 1260 on the CYP1A1 activity in the Caco-2 cells.

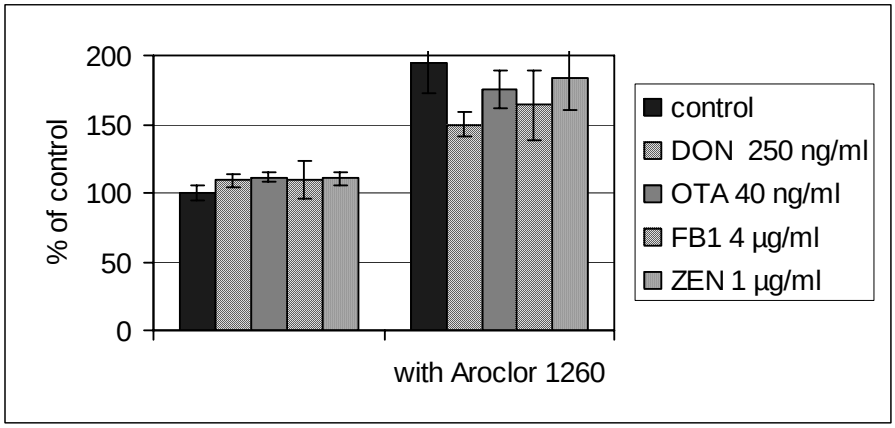

Figure 28. Effect of mycotoxins alone or in combination with Aroclor 1260 on the CYP1A1 activity. Differentiated Caco-2 cells were incubated for 48 h in the presence, or absence (control), of mycotoxins combined, or not, with 5 µg/ml Aroclor 1260. The EROD activity was then measured. Results are expressed as percent of the activity in the control (without mycotoxins nor Aroclor 1260) and are means of 3 inserts ± SD.

– Effect on glutathione-S-transferase activity

The GST is a phase II drug-metabolizing enzyme expressed in intestinal cells. Its activity has been assayed after incubating differentiated Caco-2 cells for 2 or 16 h with 1 µg/ml OTA, DON or ZEN. No mycotoxins effect was detected after 2 h incubation. Figure 29 shows the differential mycotoxins effects on the GST activity after 16 h incubation: DON inhibits the GST activity to 70% of the control value, OTA has no effect and ZEN increases the GST activity to ± 30% above the control value. Mycotoxins mixtures have not been tested.

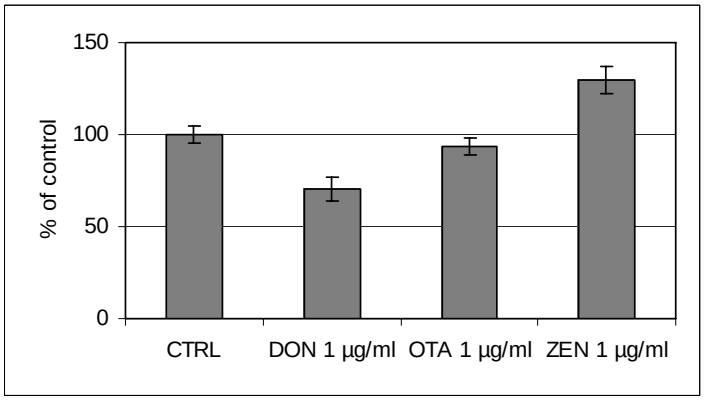

Figure 29. Effect of DON, OTA or ZEN on GST activity. Caco-2 cells were incubated for 16 h with, or without (control), 1 µg/ml of mycotoxins and assayed for GST activity. Results are expressed as percent of control and are means of 2 experiments ± SEM (n = 6).

– Effect on MRP-2 activity

MRP-2 is an isoform of the multidrug resistance-associated protein (MRP) located at the apical side of enterocytes and responsible of the efflux back to the intestinal lumen of various drugs or xenobiotics, limiting their bioavailability.

As shown in Figure 30, different contaminants (DON, FB1, ZEN, PEN or acrylamide) were assayed for their effect on the MRP-2 activity: no difference in the transport of OTA, used as MRP-2 substrate, was observed. Thus, these contaminants do not affect the MRP-2 activity. It

also means that the simultaneous presence of OTA and of these contaminants does not influence the OTA bioavailability. MK571, a specific inhibitor of the MRPs, was used as positive control: it increased the OTA absorption by ± 98% above the control value.

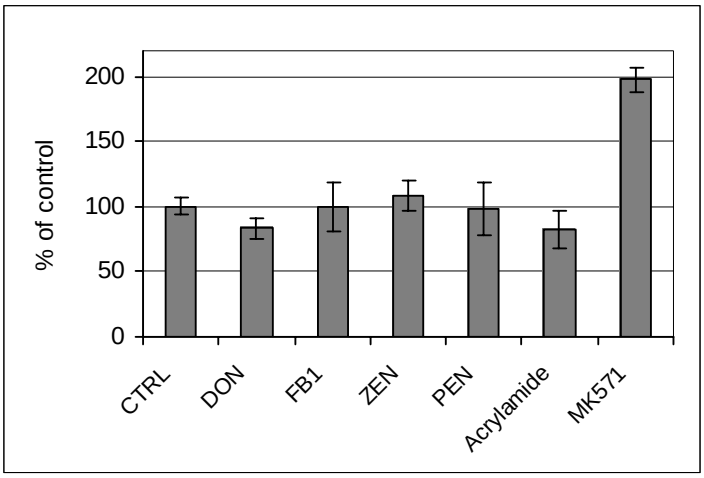

Figure 30. Effect of contaminants or MK571 on MRP-2 activity. Caco-2 cells were incubated for 3 h apically with 10 µM [^{3}H]OTA, the MRP-2 substrate, in the presence, or absence (control, CTRL) of 25 µM of mycotoxins (DON, FB1, ZEN or PEN) or 100 µM acrylamide or 50 µM MK571. Results are expressed as a percent of control of the OTA recovered in the basolateral compartment. Means ± SD (n = 3-9) are given.

As part of the CP57 SPSD II programme, which is the continuation of this project, we have clearly demonstrated, in the same Caco-2 system, a differential effect of polyphenols (dietary plant constituents) on the MRP-2 activity, *i.e.* on the OTA bioavailability. The consumption of an OTA-contaminated diet, which contains common polyphenols, may lead to a greater bioavailability of the mycotoxin in the bloodstream with possible adverse effects for human health.

– Effect on the P-glycoprotein activity

PgP is an apically located efflux pump of the intestinal epithelium, responsible of the cellular resistance to many drugs.

In a first set of experiments, Caco-2 cells were incubated with OTA (0.5 µg/ml), DON (0.5 µg/ml), FB1 (18 µg/ml) or ZEN (1 µg/ml) during 24 h before the PgP activity assay. No significant difference in the transport of digoxin, the P-gP substrate, was observed in any case (results not illustrated).

A second set of experiments (Figure 31) was realized by incubating Caco-2 cells during 7 days with OTA, at 10 or 40 ng/ml, or with DON, at 125, 250 or 500 ng/ml. The mycotoxins were also added during the PgP activity assay. The transport from the basolateral to the apical side was reduced by verapamil, the PgP inhibitor, to 40% of the control value. OTA and DON also decreased the digoxin efflux (b->a) but only by 20 to 25%. The digoxin transport from the apical to the basolateral compartment was too unstable to draw any conclusion.

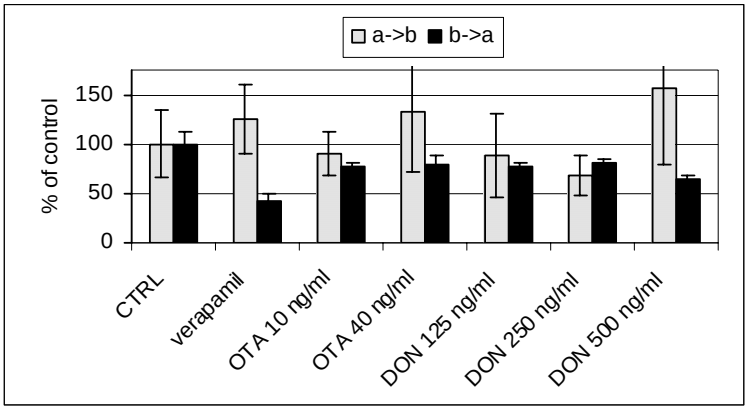

Figure 31. Effect of mycotoxins of the PgP activity. Caco-2 cells were incubated, or not (control, CTRL), for 7 days with various concentrations of OTA or DON. The PgP activity was then determined by following the digoxin transport, in the presence or not of mycotoxins or 100 µM verapamil, from the apical to the basolateral (a->b) and from the basolateral to the apical (b->a) sides of Caco-2 cells. Results are expressed as percent of control values and are means ± SD.

3.2.3. Conclusions

We have set up and applied a large battery of biological assays to detect the indirect cytotoxicity of mycotoxins.

- FB1 specific methods were developed: the *de novo* sphingolipid synthesis analysis showed that FB1 disturbs this function from 7.2 µg/ml. This concentration is 10 times lower than the FB1 concentration inducing a significant cytotoxicity by the MTT assay but it remains high as compared to those that could be admitted in cereals.
- The mycotoxins have various effects on the intestinal functions:
  - A very sensitive method is the TEER measurement: it can detect an effect of OTA and DON from 4 ng/ml and 50 ng/ml, respectively. This assay is 3 to 4 times more sensitive than the MTT assay. Those OTA and DON concentrations are plausible intestinal concentrations that could be present in the human intestinal lumen after ingestion of moderately contaminated food.
    By affecting the TEER, the mycotoxins increase the intestinal permeability. That means that the intestinal barrier function becomes altered and that the intestinal epithelium could become permeable to xenobiotics (drugs, dietary contaminants, microorganisms) in the blood stream, which could lead to adverse effects to human health.
  - In the gene expression analysis, DON has been shown to affect or inhibit the expression of genes related to detoxification systems, *i.e.* CYP3A4 and conjugation enzymes (UGT 1A6, UGT 2B7, PST). The CYP3A4 is the main intestinal drug-metabolizing enzyme and it will be very interesting to see whether the activity of this enzyme is also affected by DON, as this could imply toxicological problems.
  - The CYP1A1 assay is also a very sensitive detection method, mainly for DON and ZEN, which have inhibitory effects from 50 ng/ml and 10 ng/ml, respectively.
  - For CYP1A1, GST, MRP-2 and PgP assays, the experiments should be confirmed and completed. Nevertheless, preliminary results have shown that mycotoxins affect differentially the intestinal functions and it will be of great interest, after a complete screening of different mycotoxins or other contaminants with the different assays, to select and test mycotoxins/contaminants mixtures.

VI. REFERENCE CENTRE

Reference materials constitute an important tool for laboratory quality assurance and allow comparison of analytical methods.

The manufacturing of naturally contaminated certified reference material is a long and expensive process. So, the choice of matrix-mycotoxin combination covered by the Institute for Reference Materials and Measurements (IRMM, ex-BCR Bureau Communautaire de Reference) is very limited.

Providers of proficiency testing also supply to laboratories their remaining batches of materials, even if not considered "certified materials". In this case, the mycotoxins levels are derived from the overall mean obtained by participants in the proficiency tests.
FAPAS® - Food Analysis Performance Assessment Scheme - offers to analysts a variety of such materials which has increased in the last few years. This method to obtain reference material is convenient, economical and ensures sample-to-sample homogeneity within a batch.

Within the scope of the project, a set of reference samples was selected among the field samples collected and analysed with reference methods. For each foodstuff and mycotoxin investigated, and for at least four levels of contamination (uncontaminated, slightly contaminated, moderately contaminated and highly contaminated), two to three samples were chosen (Table 38 to Table 43). Samples are stored in a way which will ensure at the most their preservation and will allow to avoid mycotoxin content modifications. Samples prone to be infected by insects (cereals, wheat cereals) are stocked into watertight packaging at room temperature prior to one night in -18°C. Beers are frozen. Other samples are stored at room temperature.

Table 38. Wheat cereals samples selected as reference material.

Mycotoxin	Level of contamination	Sample identification			Real concentration
		Sampling period	Mode	Samples	
OTA (µg/kg)	n.d.	1	C	1	
		1	C	2	
		1	O	5	
	0.050	1	C	10	0.054
		1	C	19	0.055
		1	O	20	0.043
	0.100	1	O	10	0.106
		1	O	14	0.102
		1	O	19	0.098
	0.200	1	O	17	0.224
DON (µg/kg)	<100	1	C	12	traces
		2	C	15	traces
		2	O	5	traces
	140	1	O	1	140
		2	C	11	138
		2	O	18	137
	450	1	C	7	441
		1	O	13	474
		2	C	2	470
		2	C	4	457
	1000	1	C	1	915
		1	C	10	1044
		1	O	5	861
	> 1500	1	C	8	1991
		1	C	15	2842
		2	C	3	1503
ZEN (µg/kg)	n.d.	1	C	11	
		2	C	15	
		2	O	10	
	15	1	C	20	15
		1	O	11	19
		1	O	15	17
	85	1	C	12	90
		1	O	5	86
		1	O	8	82
	> 150	1	C	10	201
		1	C	15	232
		1	C	19	161

Table 39. Wholemeal wheat flour samples selected as reference material.

Mycotoxin	Level of contamination	Sample identification				Real concentration
		Mode	Brand	Batch	Samples	
OTA (ng/kg)	< 150	C	1	1	A	traces
		C	10	1	A	traces
		O	7	1	A	traces
	235	C	6	1	A	242
		C	9	1	B	237
		O	6	1	A	229
	750	C	9	2	A	742
		O	1	1	B	755
		O	4	2	A	770
	> 1000	C	7	1	B	1329
		O	6	1	B	3460
DON (µg/kg)	n.d.	O	4	2	B	
		O	7	1	B	
		O	10	1	A	
	200	C	2	1	B	210
		C	4	2	B	207
		O	9	1	A	200
	380	C	3	1	B	408
		C	3	2	B	383
		C	9	1	A	340
	> 500	C	3	1	A	661
		C	4	1	A	508
		C	9	2	B	511

Table 40. Cornflakes samples selected as reference material.

Mycotoxin	Level of contamination	Sample identification				Real concentration
		Mode	Brand	Batch	Samples	
FB1 (µg/kg)	n.d.	O	1	3	A	
		C	1	1	A	
		C	2	3	A	
	50 µg/kg	O	01	04	A	50.13
		O	04	04	C	50.72
		C	02	01	B	50.60
	150 µg/kg	O	2	1	B	149.09
		O	1	1	D	151.63
		C	5	1	A	154.86
	300 µg/kg	C	6	3	B	304.90
		C	7	3	D	295.07
		C	7	4	C	300.12
FB2 (µg/kg)	n.d.	O	1	3	A	
		C	1	1	A	
		C	2	3	A	
	16.5 µg/kg	O	2	1	D	16.53
		O	3	3	A	15.51
		C	7	3	A	16.57
	25 µg/kg	O	3	2	C	25.10
		C	6	3	A	25.28
		C	6	3	E	25.15
	40 µg/kg	O	3	1	A	38.02
		O	3	1	B	42.74
		O	3	1	D	40.69
FB3 (µg/kg)	n.d.	O	1	3	A	
		C	1	1	A	
		C	2	3	A	
	30 µg/kg	O	3	4	B	29.96
		C	7	1	A	30.27
		C	7	4	C	30.22
	50 µg/kg	O	3	2	C	51.82
		C	5	1	B	50.46
		C	6	1	B	50.53
	90 µg/kg	O	3		A	83.93
		O	3	1	B	90.34
		O	3	1	D	89.29

Table 41. Wholemeal pasta samples selected as reference material

Mycotoxin	Level of contamination	Sample identification				Real concentration
		Mode	Brand	Batch	Samples	
DON (µg/kg)	n.d.	O	1	3	A	
		O	2	4	A	
		C	1	3	A	
	115	O	2	2	A	114
		C	1	1	A	120
		C	1	2	E	117
	260	O	3	1	C	266
		O	3	1	D	252
		O	4	1	B	260
	400	O	2	3	E	407
		O	3	2	D	391
		O	5	1	D	404

Table 42. Beer samples selected as reference material

Mycotoxin	Level of contamination	Sample identification					Real concentration
		Sampling period	Mode	Brand	Batch	Samples	
OTA (ng/l)	n.d.	2	C	3	2	B	
		2	C	7	1	B	
		2	O	5	2	A	
	30	2	C	1	2	A	31
		2	O	1	1	B	30
		2	O	2	2	B	30
	45	2	O	1	1	A	43
		2	C	6	2	A	46
		2	O	7	2	B	45
	150	2	O	8	1	B	149
		2	C	10	2	B	151
	540	2	O	10	1	B	566
		2	O	10	2	B	533
	1100	2	O	9	1	A	1089
		2	O	9	1	B	1134
DON (µg/l)	n.d.	2	C	3	2	B	
		2	O	5	1	A	
		2	C	4	2	A	
	6.00	2	O	1	1	B	6.12
		2	O	9	2	B	6.06
	8.50	2	C	7	1	A	9.02
		2	C	8	2	B	8.07
	14.00	2	O	6	1	B	13.98
		2	C	10	2	A	14.44
	22.00	2	C	8	1	A	21.84
		2	C	8	1	B	22.05

Table 43. Polenta samples selected as reference material.

Mycotoxin	Level of contamination	Sample identification				Real concentration
		Mode	Brand	Batch	Samples	
FB1 (µg/kg)	n.d.	C	3	1	E	<59
		O	3	2	E	<59
		C	3	2	A	<59
	220	C	3	1	B	214
		C	2	4	B	224
		O	3	2	C	231
	620	O	2	1	A	615
		O	2	3	A	620
		C	2	4	D	637
	1200	O	1	3	B	1213
		C	1	3	A	1335
		C	1	4	A	1115
	> 3000	C	1	1	D	3057
		C	1	2	D	3372
		C	1	2	A	4118
FB2 (µg/kg)	n.d.	O	1	1	A	<83
		O	2	2	A	<83
		C	3	1	B	<83
	290	O	2	1	D	281
		C	2	1	B	294
		C	2	3	B	304
	650	C	1	4	A	582
		C	1	3	A	634
		C	1	2	D	780
	1150	C	1	1	D	1265
		C	1	2	C	1172
		C	1	3	E	1117

VII. ACHIEVEMENT OF THE OBJECTIVES

The two main objectives of the project were to develop analytical tools for mycotoxins detection and quantification and to obtain contamination data of mycotoxins in cereals produced in Belgium and in cereal-based foodstuffs of the Belgian market. Table 44 gives an overview of the methods developed and used for samples analysis.

Table 44. Overvieuw of analytical methods and number of samples analysed. Detail results of analytical methods in bold (chosen for the analysis of the whole of the samples analysis) are presented in the report. Methods in italic lead to no satisfactory results.

	Wheat cereals	Whole-wheat flour	Beer	Wholemeal pasta	Cornflakes	Polenta	Sweet corn
OTA	**VM n=40** FT n=36	**MM n=80**	**VM n=150** FT n=4				
FB1			*VM n=20*		**VM n=205** FT n=90	**VM n=92** FT n=12	**VM n=30** FT n=18
FB2			**VM n=20**		**VM n=205** FT n=90	**VM n=92** FT n=12	**VM n=30** FT n=18
FB3					**VM n=205**		
DON	**MM n=93**	**MM n=80**	**VM n=150**	**VM n=85**			
ZEN	**MM n=93**						

VM: validated methods (based on HPLC)
FT: flow-through method
MM: multi-mycotoxins method
n: number of samples analysed in order to validate de method.

The following paragraphs deal in detail with the way we have reached our objectives and in what extent.

1. Sampling and contamination data

Sampling was a huge and important task. The number of samples collected influences the validity of contamination data obtained. During the three years of the project, more than 700 samples have been collected among seven food matrices, with, as far as possible, a balance between organically and conventionally produced foodstuffs. Wheat cereals were collected during two harvest (2002 and 2003) and allowed to show the influence of climatic factors.

Contamination data acquired during the project are discussed at point I with a focus on differences in mycotoxins occurrence and level in organic and conventional foodstuffs.

2. Extension of official methods

In order to enlarge validated analytical tools already acquired (OTA and DON in cereals, OTA in beer, …) some of official methods were adapted to new matrices (DON in pasta, FB1 and FB2 in polenta), some were improved (DON in beer) leading to very good results.

A new analytical tool was also developed for the quantification of fumonisins in cornflakes, notably in order to confirm the flow-through screening results. In the literature, the analysis of commodities and foods for fumonisins encompasses a variety of methods, mostly HPLC based with fluorescence detection after derivatisation. Using mass spectrometric detection, the derivatisation step can however be eliminated. Moreover, the two stage mass spectrometric process (MS/MS) provides the highest degree of certainty in analyte identification. LC-MS methods have been described for the quantification of FB_1 and FB_2 but not for FB_3, although it is one of the three major fumonisins compounds produced in nature. Neither do these described methods use an internal standard for quantization, while its use has been demonstrated to compensate for matrix effects and other MS detector variations. Therefore, we aimed to provide a validated LC-MS/MS method for the quantitative analysis of FB1, FB2 and FB3 in cornflakes, allowing evaluation of the risks generated by these mycotoxins at the level of food safety. In respect of the benefits of applying an internal standard, a suitable compound with structural properties and LC behaviour similar to FB1, FB2 and FB3 was selected during LC-MS/MS development.

3. Development of new original methods

The project aimed to develop reliable and powerful tools for the conventional and effective control of mycotoxins in food products proposed to the consumer.

Because *Fusarium* species produce quite different mycotoxins, as DON and ZEN, the need for a multi-mycotoxin method was considered as important. The method developed in the framework of the present project allows to analyse DON and ZEN together in the same run, starting from the same extraction mixture. Moreover, this method allows the concomitant determination of a range of other mycotoxins.
This procedure does not involve the use of expensive immuno-columns and is less time consuming.
Different detectors must be used in series. These equipments would be advantageously replaced by the more universal MS-MS detection system allowing the determination of mycotoxins by the detection of three specific ions, even if the focused mycotoxins are not chromatographically separated.

The approach of the flow-through enzyme immunoassay developed proved to offer a useful screening tool for the detection of OTA and fumonisins in the selected matrices. Sample preparation could be kept simple and fast for the matrices wheat, wholemeal bread, maize and cornflakes. However for the matrix beer, an additional solid phase extraction step was needed to solve the background interferences on the membrane during the flow-through assay. A method using OTA immunoaffinity columns was selected because of good OTA recoveries and good visual results in the flow-through assay both for pale and dark beer. Incorporation of this additional SPE step in the sample pre-treatment slowed down the screening considerably and made it more complex. Regarding our goal to develop a field test, this was not an interesting evolution. The development of a lateral-flow based technique for this matrix would be a more useful approach. During the optimization of the flow-through assays for the

selected solid matrices, we were able to meet the criteria for colour development and to reach the postulated cut-off levels. Validation results showed reproducibility within acceptable ranges and a good precision with no false compliants and a percentage of false non-compliants lower than 20%. The format demonstrated its accurateness and its applicability to different types of matrices. Therefore we can conclude that the flow-through format offers a useful and cost-effective screening tool for mycotoxin assessment

Biological detection methods based on cytotoxicity induced by contaminated foodstuffs on human cultured cells turned out to be inappropriate for rapid detection of mycotoxins. In order to work, the method would require either an additional step of organic extraction of the samples to avoid interference with the matrix or a dilution of the sample. The first solution would let to a laborious method, inadequate for the screening of a lot of samples. The second perspective would undoubtedly cancel the influence of the matrix but also the toxicity of the mycotoxin since the contaminants concentration would fall below the sensitivity limit of the method.

4. Reference centre

A little bit more than 300 samples are now available in the reference centre. They have been chosen in order to include a wide range of mycotoxin concentrations.

VIII. CONCLUSION AND RECOMMENDATION

1. Levels of contamination of cereals-derived foodstuffs on the Belgian market

Seven food matrices marketed in Belgium and prone to be contaminated by mycotoxins have been investigated. Wheat cereals have been collected during two consecutive harvests (2002 and 2003). Wheat cereal-based foodstuffs including wholemeal wheat flour for bread making, beer, wholemeal pasta, as well as sweet corn, corn flour for polenta and cornflakes have been collected in retail stores.

Concerning wheat cereals at harvest, OTA, DON and ZEN have been measured.
OTA was detected in only 40% of the samples at very low levels (mean contamination of 0.065±0.179 µg/kg) which in not surprising since OTA is a storage mycotoxin.
By contrast, DON occurred in almost all samples at high levels, especially in 2002 (mean concentration of 460±562 µg/kg). Three samples contained DON at a level over the proposed limit of 1250 µg/kg. In 2003, DON content was on average two times lower than in 2002 (mean concentration of 210±226 µg/kg). These results illustrate quite well the weather-dependent contamination of cereals by DON. Rainfall during summer 2002 was indeed important while June 2003, corresponding to the flowering period of wheat, was characterized by a dry weather. Weather conditions also influenced ZEN contamination. In 2002, 67% of the samples contained detectable amounts of ZEN, including 6 samples with ZEN concentration above the proposed limit of 100 µg/kg. By contrast, in 2003, ZEN was found in only 6% of the samples, at trace levels.
Since DON and ZEN are produced by the same species of *Fusarium*, a nice correlation between DON and ZEN concentrations in cereals emerged from the results of 2002.
These data demonstrate once again that multi-contaminations may occur in many cases.

Wholemeal wheat flours, unlike wheat cereals samples have been collected after a storage period. Thus, OTA was found in almost all samples (98%) but at moderate levels. Mean concentration was evaluated at 354±434 ng/kg. Only one sample was contaminated at a level over 3 µg/kg.
Occurrence of DON in the same samples was a little less important (85%). Mean concentration was not high (170±136 µg/kg) and the proposed limit of 500 µg/kg was never exceeded.

Regarding OTA contamination of beer, the mycotoxin was detected in about 90% of the samples and levels were often far below the proposed limit of 200 ng/l. However, a few batches were much more contaminated, at levels exceeding this limit.
Mycotoxins are usually distributed heterogeneously in stored-grain bulks and "hot spots" of OTA may occur even if the average conditions of the bulk are not favourable to moulds development. Exceptionally high concentrations of OTA in a batch of beer may occur if such contaminated barley is employed.
Our study illustrates quite well the heterogeneity of contamination levels since we found highly significant differences ($p<0.0001$) in contamination between different batches of the same brand.
In order to avoid highly contaminated beers, the sampling on malt must be performed in a very efficient way. The sampling methods for control analysis must respect the Commission Directive 2002/26/EC. Malt should not contain more than 3 µg OTA/kg (Commission Regulation (EC) No 123/2005). Nowadays, malt and beer producers must follow these

regulations. This should guarantee the production of beers with low and acceptable levels of OTA. Results of OTA analysis on beers sampled in 2005 are in line with this hypothesis.
Our results show that occurrence and levels of DON, FB1 and FB2 in beers should definitely not be a major cause for concern since these mycotoxins were detected at very low levels and in a small number of beers.

Wholemeal wheat pasta were analysed for DON, which was detected in around 60% of the samples at a mean level of 145±147 µg/kg. Maximum levels were always below the proposed limit of 750 µg/kg. Contamination variations in samples from different batches of a same brand were very significant. Levels of DON do not seem to be worrying but the pasta foodstuff deserves to be followed.

Sweet corn samples were screened for FB1 and FB2. All results were below the LOD which lead to the conclusion that this matrix is not a matter of concern in terms of fumonisins.

FB1 and FB2 were analysed in corn flour for polenta. FB1 was detected in about 90% of the samples. Mean level of contamination was 753±941 µg FB1/kg. A considerable part of the samples (18%) contained FB1 at levels from 1000 to 4353 µg FB1/kg. Such alarming contaminations were also found for FB2 since 70% of the samples contained DON at a mean level of 278±339 µg/kg with a maximum level of 1659 µg/kg. Polenta may thus contribute to an important part to the daily intake of FB1 and FB2 and should therefore be taken into account when setting up monitoring programs. Here again, contamination variations in samples from different batches of a same brand were very significant.

A total of 205 cornflakes samples were analysed for FB1, FB2 and FB3 with the validated LC-MS/MS method. Sixty-five % of the cornflakes samples analysed in this survey were contaminated with FB1, FB2 and FB3 concentrations above the respective detection limits. FB2 and FB3 were not detected without FB1. FB1 concentrations always exceeded FB2 and FB3 concentrations.
Fumonisin concentrations found in this survey were generally low. However, batches with a considerable contamination (FB1 concentration > 300 µg/kg) did occur. Therefore, conventional evaluation of fumonisins in cornflakes can be recommended. The variation between different batches of a same brand was significant. These observations are in line with the heterogeneous distribution of mycotoxins in bulk commodities and emphasize the importance of a well defined sampling plan in the process of risk management and food safety control.

The sampling plan plays an important part and influences the interpretation and statistical analysis of the results. Our results show that market-oriented supply studies should include samples from a large number of different batches since this factor is determinant regarding the contamination of products from a same brand.

2. Contamination levels in conventional and organic foodstuffs

Among the different food matrices under investigation, the results of our study show that differences in mycotoxin occurrence and content between conventional and organic products depend on a large set of parameters including the mycotoxin investigated, the type of foodstuff and the year of production as demonstrated in the following paragraphs.

Wheat cereals grown under organic farming were more frequently contaminated by OTA than organic samples ($p=0.0467$) whereas DON and ZEN seem to be more often present in conventional cereals, difference being significant for ZEN in samples from 2002. Among the positive samples, levels of contamination were significantly higher in conventional samples for DON ($p<0.01$) in 2003 and ZEN in 2002 ($p<0.05$).

Organic wholemeal wheat flours were more contaminated by OTA than conventional samples ($p<0.05$) in terms of frequency. The opposite pattern was shown for DON, organic samples being more frequently contaminated than conventional flours ($p<0.05$).

Organic beers were more frequently contaminated with OTA than conventional beers ($p<0.001$) and tended to contain higher amounts of the mycotoxin. While OTA was in some samples present in relatively high amounts, DON was detected at low levels and in a small number of beers. No significant differences in DON content were noted between conventional and organic samples. In both cases, we found an extremely significant effect of the batch on the level of contamination with mycotoxins ($p<0.0001$).

Conventional and organic samples of wholemeal pasta were contaminated in the same proportions but levels of DON in positive samples were higher for pasta of organic grade ($p<0.05$).

Contamination by FB1 occurred as frequently in conventional polenta as in organic polenta. By contrast, conventional samples were statistically more often contaminated by FB1 at a level over 1000 µg/kg ($p<0.05$). On the other hand, the occurrence of FB2 was higher in polenta originating from conventional production ($p<0.0001$).

Regarding occurrence and contamination of cornflakes with fumonisins, no differences were noted between conventional and organic samples, except for the frequency of contamination with FB1, which was higher in organic samples ($p<0.0001$).

Consumption of organically grown products is most often motivated by the pursuit of healthier and safer food although the organic label refers more to the manufacturing process than to the characteristic and properties of the final product.
Foodstuffs of organic agriculture contain less nitrates and less pesticides residues but there is no strong evidence that organic and conventional products differ regarding their nutritional value, sensory quality or natural toxin content (Bourn *et al.*, 2002; Pussemier *et al.*, 2005).

The results of the present study indicate that the issue of the mycotoxin contamination is really complex. Our results are in agreement with those of Malmauret *et al.*(2002) or Magkos *et al.*(2003) who showed no conclusive evidence whether conventional products are more or less safe than organic ones. The general belief that mycotoxins are more present in organic food since fungicides are not allowed in this production is not confirmed by our study.

3. Development of new analytical tools

Our project has allowed the development of two different approaches to improve the food quality control in terms of mycotoxins: the flow-through enzyme immuno-assay, which is a very rapid screening method, and the multi-mycotoxin method, which allows the simultaneous detection of a set of mycotoxins in a single analytical run.

The flow-through enzyme immunoassay uses simple equipment and limited operational steps, providing a yes/no response indicating whether the analyte is present or not above a certain threshold value. The response appears in the form of a blue spot, the intensity of the developed colour decreasing with increasing concentrations of the mycotoxins. Coupled to a simple and fast sample preparation, this approach offers a cost-effective screening tool that can be conducted and interpreted by users that are as close to the source of contamination as possible.

The format of the flow-through enzyme immunoassay was optimized and validated on naturally contaminated samples for the presence of OTA in wheat (n=36) and wholemeal bread, fumonisins in maize (polenta: n=12; sweet corn: n=18) and cornflakes (n=90).
For the matrix beer, an additional solid phase extraction step using OTA immunoaffinity columns was needed to solve background interferences on the membrane due to beer pigments. Incorporation of this additional step in the sample pre-treatment slowed down the screening considerably, made it more complex and took us away from our goal of developing a field test.

Because of their quite different chemical structures, mycotoxins can be determined at low level in cereals and animal feedstuffs by complex, time-consuming or costly methods. Simple and sensitive methods for individual compounds were increasingly developed using specific antibodies. However, while the inherent specificity of those immunological tools substantially overcomes problems of interference in the detection of co-extracted compounds, this specificity precludes their use to screen foods and feedstuffs for a range of mycotoxins.

To investigate cases of suspected mycotoxicosis or for multi-toxin surveillance programmes, reliable and sensitive multi-methods are required. During the project, we have therefore developed a multi-mycotoxin procedure able to determine most of the mycotoxins of interest in a single run, focussing on the analysis of DON, ZEN and OTA in cereals and cereals flours. This analytical approach takes into account the chemical diversity of the different mycotoxins that directly influences the chromatographic technique as well as the detection mode. Even the sample preparation has to be non-specific.

Sample preparation included three steps: an extraction, a two stages purification and a final dissolution prior to the chromatography.
Extraction of the mycotoxins, which had to be non-specific, was performed with a solvent mixture of ACN:H_2O (84:16, v/v). The first step of the purification included an anion-exchange column (SAX) in order to fix the acidic mycotoxins (OTA). The neutral mycotoxins flowing through the SAX column were further purified by filtration on a MycoSep cartridge.
OTA was analysed using an isocratic flow and the detection was performed with a fluorescence detector. Neutral mycotoxins were separated by a linear gradient and detected using a double mode of detection (UV-FLUO).

The multi-mycotoxin method was evaluated by inter-laboratory ring-tests organized by FAPAS for the analysis of DON and OTA in naturally contaminated wheat samples. Results

showed that the method was reliable and exact.
In the framework of this project, the method was validated for DON and ZEN in cereal samples (n=93) and OTA and DON in cereal flour samples (n=80).

By contrast, the cellular biology approach allows to study the effect of mycotoxins on different biochemical functions at the cellular level. Several tools have been set up to perform these studies.
For FB1, effects on the *de novo* sphingolipid synthesis have been demonstrated. Several mycotoxins have been shown to interfere with the trans-epithelial resistance of the intestinal cells monolayer.
DON has been shown to affect the expression of genes related to detoxification systems or the activity of some of the enzymes produced.

Finally, this approach is a very promising tool to study the potential interactions between several mycotoxins or between mycotoxins and other contaminants of our diet. This is the main objective of another project of the Belgian Science Policy (CP-57).

IX. ANNEXES

1. References

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2. Tables

Table 45. Occurrence of OTA (µg/kg) in wheat cereals harvested in 2002.

	OTA (µg/kg)	
Sample	Conventional	Organic
1	n.d.	0.037
2	n.d.	n.d.
3	traces	n.d.
4	n.d.	n.d.
5	n.d.	n.d.
6	n.d.	n.d.
7	n.d.	n.d.
8	n.d.	n.d.
9	1.097	0.084
10	0.054	0.106
11	n.d.	0.303
12	n.d.	n.d.
13	n.d.	traces
14	n.d.	0.102
15	n.d.	0.063
16	n.d.	n.d.
17	n.d.	0.224
18	n.d.	0.133
19	0.055	0.098
20	0.045	0.043

n.d., not detected (DON concentration in sample <0.01 µg/kg)
traces means that the concentration of OTA is between the limit of detection (LOD = 0.01 µg/kg) and the limit of quantification (LOQ = 0.035 µg/kg).

Table 46. Occurrence of DON (µg/kg) and ZEN (µg/kg) in wheat cereals harvested 2002.

	Conventional		Organic	
Sample	DON (µg/kg)	ZEN (µg/kg)	DON (µg/kg)	ZEN (µg/kg)
1	915	56	140	8
2	1033	71	194	17
3	387	27	151	n.d.
4	226	19	1184	59
5	172	25	861	86
6	409	36	traces	n.d.
7	441	50	151	n.d.
8	1991	123	818	82
9	129	n.d.	140	25
10	1044	201	614	61
11	107	n.d.	463	19
12	traces	90	377	21
13	traces	134	474	54
14	107	n.d.	301	19
15	2842	232	334	17
16	1098	96	198	n.d.
17	258	44	traces	n.d.
18	1324	121	traces	n.d.
19	700	161	traces	8
20	248	15	traces	n.d.
21			traces	n.d.
22			101	n.d.
23			traces	n.d.
24			n.d.	n.d.
25			107	n.d.

n.d., not detected (DON concentration in sample <30 µg/kg; ZEN concentration <1.5 µg/kg)
traces means that the concentration of DON is between the limit of detection (LOD = 30 µg/kg) and the limit of quantification (LOQ = 100 µg/kg) and the concentration of ZEN in between the LOD (1.5 µg/kg) and the LOQ (4 µg/kg)

Table 47. Occurrence of DON (µg/kg) and ZEN (µg/kg) in wheat cereals harvested 2003.

	Conventional		Organic	
Sample	DON (µg/kg)	ZEN (µg/kg)	DON (µg/kg)	ZEN (µg/kg)
1	traces	n.d.	159	n.d.
2	470	n.d.	traces	n.d.
3	1503	5	138	n.d.
4	457	traces	126	n.d.
5	419	traces	traces	n.d.
6	242	traces	132	traces
7	412	traces	193	traces
8	418	traces	130	n.d.
9	261	traces	137	n.d.
10	253	traces	traces	n.d.
11	138	n.d.	128	n.d.
12	166	n.d.	108	n.d.
13	298	n.d.	traces	n.d.
14	527	n.d.	164	4
15	traces	n.d.	226	n.d.
16	188	traces	151	n.d.
17	354	5	113	n.d.
18	114	n.d.	137	n.d.
19	108	n.d.	106	n.d.
20	traces	n.d.	118	n.d.
21	144	n.d.	traces	n.d.
22	108	n.d.	traces	n.d.
23			138	n.d.
24			166	n.d.
25			180	n.d.
26			n.d.	n.d.

n.d., not detected (DON concentration in sample <30 µg/kg; ZEN concentration <1.5 µg/kg)
traces means that the concentration of DON is between the limit of detection (LOD = 30 µg/kg) and the limit of quantification (LOQ = 100 µg/kg) and the concentration of ZEN in between the LOD (1.5 µg/kg) and the LOQ (4 µg/kg)

Table 48. Occurrence of OTA (ng/kg) in wholemeal wheat flour.

			OTA (ng/kg)	
Sample			Conventional	Organic
Brand 1	Batch 1	A	traces	327
		B	traces	755
	Batch 2	A	194	543
		B	traces	578
Brand 2	Batch 1	A	traces	199
		B	traces	310
	Batch 2	A	158	traces
		B	168	194
Brand 3	Batch 1	A	198	472
		B	190	605
	Batch 2	A	traces	600
		B	traces	463
Brand 4	Batch 1	A	235	180
		B	251	162
	Batch 2	A	traces	770
		B	traces	475
Brand 5	Batch 1	A	traces	266
		B	traces	320
	Batch 2	A	traces	373
		B	411	430
Brand 6	Batch 1	A	242	229
		B	168	668
	Batch 2	A	traces	3460
		B	traces	937
Brand 7	Batch 1	A	741	traces
		B	1329	580
	Batch 2	A	ND	164
		B	ND	530
Brand 8	Batch 1	A	413	traces
		B	385	193
	Batch 2	A	traces	traces
		B	traces	154
Brand 9	Batch 1	A	traces	357
		B	237	701
	Batch 2	A	742	694
		B	873	631
Brand 10	Batch 1	A	traces	388
		B	traces	360
	Batch 2	A	traces	331
		B	153	382

n.d., not detected (OTA concentration in sample <50 ng/kg)
traces means that the concentration of OTA is between the limit of detection (LOD=50 ng/kg) and the limit of quantification (LOQ = 150 ng/kg)
permitted concentration level (E.C.): 5000 ng/kg

Table 49. Occurrence of DON (µg/kg) in wholemeal wheat flour.

			DON (µg/kg)	
Sample			Conventional	Organic
Brand 1	Batch 1	A	traces	traces
		B	traces	traces
	Batch 2	A	traces	traces
		B	traces	traces
Brand 2	Batch 1	A	195	157
		B	210	210
	Batch 2	A	190	190
		B	203	179
Brand 3	Batch 1	A	661	traces
		B	408	traces
	Batch 2	A	429	traces
		B	383	traces
Brand 4	Batch 1	A	508	171
		B	623	157
	Batch 2	A	233	n.d.
		B	207	n.d.
Brand 5	Batch 1	A	249	traces
		B	237	traces
	Batch 2	A	256	traces
		B	248	traces
Brand 6	Batch 1	A	153	n.d.
		B	163	n.d.
	Batch 2	A	211	n.d.
		B	188	n.d.
Brand 7	Batch 1	A	222	n.d.
		B	263	n.d.
	Batch 2	A	traces	traces
		B	traces	traces
Brand 8	Batch 1	A	249	traces
		B	284	traces
	Batch 2	A	179	traces
		B	168	traces
Brand 9	Batch 1	A	340	200
		B	335	traces
	Batch 2	A	455	traces
		B	511	traces
Brand 10	Batch 1	A	traces	n.d.
		B	traces	n.d.
	Batch 2	A	traces	n.d.
		B	traces	n.d.

n.d., not detected (DON concentration in sample <50 µg/kg)
traces means that the concentration of DON is between the limit of detection (LOD=50 µg/kg) and the limit of quantification (LOQ = 150 µg/kg)
proposed concentration level in wheat flour: 500 µg/kg

Table 50. Occurrence of OTA (ng/l) in beers collected during the first sampling period.

		OTA (ng/l)	
Sample		Conventional	Organic
Brand 1	Batch 1	n.d.	33
	Batch 2	n.d.	28
Brand 2	Batch 1	12	19
	Batch 2	18	18
Brand 3	Batch 1	21	148
	Batch 2	24	152
Brand 4	Batch 1	40	59
	Batch 2	45	51
Brand 5	Batch 1	15	58
	Batch 2	15	69
Brand 6	Batch 1	n.d.	17
	Batch 2	n.d.	15
Brand 7	Batch 1	16	16
	Batch 2	13	18
Brand 8	Batch 1	17	14
	Batch 2	15	15
Brand 9	Batch 1	14	22
	Batch 2	13	23
Brand 10	Batch 1	traces	520
	Batch 2	traces	481
Brand 11	Batch 1	55	233
	Batch 2	51	236
Brand 12	Batch 1	traces	19
	Batch 2	10	20
Brand 13	Batch 1	18	13
	Batch 2	19	12
Brand 14	Batch 1	traces	
	Batch 2	traces	
Brand 15	Batch 1	39	
	Batch 2	36	
Brand 16	Batch 1	15	
	Batch 2	19	
Brand 17	Batch 1	35	
	Batch 2	29	
Brand 18	Batch 1	24	
	Batch 2	20	
Brand 19	Batch 1	12	
	Batch 2	traces	
Brand 20	Batch 1	149	
	Batch 2	148	
Brand 21	Batch 1	27	
	Batch 2	27	
Brand 22	Batch 1	n.d.	
	Batch 2	n.d.	

n.d., not detected (OTA concentration in sample <3 ng/l)
traces means that the concentration of OTA is between the limit of detection (LOD = 3 ng/l) and the limit of quantification (LOQ = 10 ng/l)

Table 51. Occurrence of OTA (ng/l) in beers collected during the second sampling period.

			OTA (ng/l)	
Sample			Conventional	Organic
Brand 1	Batch 1	A	traces	43
		B	n.d.	30
	Batch 2	A	31	20
		B	38	traces
Brand 2	Batch 1	A	27	25
		B	27	29
	Batch 2	A	traces	28
		B	traces	30
Brand 3	Batch 1	A	traces	133
		B	traces	142
	Batch 2	A	n.d.	34
		B	n.d.	34
Brand 4	Batch 1	A	n.d.	23
		B	n.d.	23
	Batch 2	A	traces	27
		B	traces	26
Brand 5	Batch 1	A	20	223
		B	26	230
	Batch 2	A	21	n.d.
		B	20	n.d.
Brand 6	Batch 1	A	traces	50
		B	19	53
	Batch 2	A	46	45
		B	42	46
Brand 7	Batch 1	A	n.d.	41
		B	n.d.	42
	Batch 2	A	26	41
		B	traces	45
Brand 8	Batch 1	A	n.d.	141
		B	n.d.	149
	Batch 2	A	traces	traces
		B	traces	18
Brand 9	Batch 1	A	21	1089
		B	23	1134
	Batch 2	A	22	113
		B	19	120
Brand 10	Batch 1	A	26	487
		B	26	566
	Batch 2	A	197	517
		B	151	533

n.d., not detected (OTA concentration in sample <5 ng/l)
traces means that the concentration of OTA is between the limit of detection (LOD = 5 ng/l) and the limit of quantification (LOQ = 18 ng/l)

Table 52. Occurrence of DON (µg/l) in beers collected first the first sampling period

		DON (µg/l)	
Sample		Conventional	Organic
Brand 1	Batch 1	n.d.	n.d.
	Batch 2	n.d.	n.d.
Brand 2	Batch 1	n.d.	n.d.
	Batch 2	n.d.	n.d.
Brand 3	Batch 1	traces	n.d.
	Batch 2	traces	n.d.
Brand 4	Batch 1	n.d.	n.d.
	Batch 2	n.d.	n.d.
Brand 5	Batch 1	n.d.	n.d.
	Batch 2	n.d.	n.d.
Brand 6	Batch 1	n.d.	n.d.
	Batch 2	n.d.	n.d.
Brand 7	Batch 1	traces	n.d.
	Batch 2	traces	n.d.
Brand 8	Batch 1	n.d.	n.d.
	Batch 2	n.d.	n.d.
Brand 9	Batch 1	n.d.	n.d.
	Batch 2	n.d.	n.d.
Brand 10	Batch 1	n.d.	n.d.
	Batch 2	n.d.	n.d.
Brand 11	Batch 1	n.d.	n.d.
	Batch 2	n.d.	n.d.
Brand 12	Batch 1	traces	n.d.
	Batch 2	traces	n.d.
Brand 13	Batch 1	n.d.	n.d.
	Batch 2	traces	n.d.
Brand 14	Batch 1	n.d.	
	Batch 2	n.d.	
Brand 15	Batch 1	n.d.	
	Batch 2	n.d.	
Brand 16	Batch 1	n.d.	
	Batch 2	n.d.	
Brand 17	Batch 1	n.d.	
	Batch 2	n.d.	
Brand 18	Batch 1	n.d.	
	Batch 2	n.d.	
Brand 19	Batch 1	n.d.	
	Batch 2	n.d.	
Brand 20	Batch 1	n.d.	
	Batch 2	n.d.	
Brand 21	Batch 1	n.d.	
	Batch 2	n.d.	
Brand 22	Batch 1	n.d.	
	Batch 2	n.d.	

[a] :n.d., not detected (DON concentration in sample <10 µg/l)

[b] : traces means that the concentration of DON is between the limit of detection (LOD = 10 µg/l) and the limit of quantification (LOQ = 35 µg/l)

Table 53. Occurrence of FB1 and FB2 in beer samples (ng/ml).

	Occurrence of FB			FB_1	FB_2
Conventional	Brand 1	Batch 1	A	-	n.d.
	Brand 2	Batch 1	A	-	n.d.
	Brand 3	Batch 1	A	-	n.d.
	Brand 4	Batch 1	A	-	n.d.
	Brand 5	Batch 1	A	-	n.d.
	Brand 6	Batch 1	A	-	n.d.
	Brand 7	Batch 1	A	-	n.d.
	Brand 8	Batch 1	A	-	n.d.
	Brand 9	Batch 1	A	-	n.d.
	Brand 10	Batch 1	A	-	n.d.
Organic	Brand 11	Batch 1	A	-	n.d.
	Brand 12	Batch 1	A	-	n.d.
	Brand 13	Batch 1	A	-	n.d.
	Brand 14	Batch 1	A	-	n.d.
	Brand 15	Batch 1	A	-	n.d.
	Brand 16	Batch 1	A	-	n.d.
	Brand 17	Batch 1	A	-	n.d.
	Brand 18	Batch 1	A	-	n.d.
	Brand 19	Batch 1	A	-	n.d.
	Brand 20	Batch 1	A	-	n.d.

-, not determined because of the presence of a huge matrix peak
n.d., not detected: FB_2 concentration in sample <1.17 ng/ml

Table 54. Occurrence of DON (µg/l) in beer collected during the second sampling plan.

			DON (µg/l)	
Sample			Conventional	Organic
Brand 1	Batch 1	A	n.d.	6.12
		B	traces	6.94
	Batch 2	A	n.d.	traces
		B	n.d.	6.01
Brand 2	Batch 1	A	traces	traces
		B	n.d.	traces
	Batch 2	A	traces	traces
		B	n.d.	traces
Brand 3	Batch 1	A	n.d.	7.35
		B	traces	6.68
	Batch 2	A	n.d.	traces
		B	n.d.	traces
Brand 4	Batch 1	A	n.d.	traces
		B	n.d.	n.d.
	Batch 2	A	n.d.	traces
		B	n.d.	traces
Brand 5	Batch 1	A	traces	n.d.
		B	traces	n.d.
	Batch 2	A	traces	traces
		B	traces	traces
Brand 6	Batch 1	A	6.01	12.13
		B	traces	13.98
	Batch 2	A	traces	7.30
		B	traces	6.78
Brand 7	Batch 1	A	9.20	n.d.
		B	9.40	traces
	Batch 2	A	11.72	traces
		B	13.05	traces
Brand 8	Batch 1	A	21.84	n.d.
		B	22.05	n.d.
	Batch 2	A	7.71	n.d.
		B	8.07	n.d.
Brand 9	Batch 1	A	n.d.	traces
		B	traces	traces
	Batch 2	A	traces	traces
		B	traces	6.06
Brand 10	Batch 1	A	12.64	traces
		B	13.00	traces
	Batch 2	A	14.44	traces
		B	14.95	traces

[a] :n.d., not detected (DON concentration in sample <2 µg/l)
[b] : traces means that the concentration of DON is between the limit of detection (LOD = 2 µg/l) and the limit of quantification (LOQ = 6 µg/l)

Table 55. Occurrence of DON (µg/kg) in wholemeal pasta collected on the Belgian market.

Occurrence of DON (µg/kg)		Sample	Brand 1	Brand 2	Brand 3	Brand 4	Brand 5
Organic	Batch 1	A	traces	traces	238	334	306
		B	traces	n.d.	342	260	412
		C	traces	n.d.	266	311	345
		D	traces	traces	252	388	404
		E	traces	traces	272	250	367
	Batch 2	A	traces	114	280		
		B	206	206	277		
		C	231	traces	293		
		D	traces	235	391		
		E	164	traces	296		
	Batch 3	A	n.d.	523			
		B	n.d.	490			
		C	n.d.	463			
		D	n.d.	477			
		E	n.d.	407			
	Batch 4	A	n.d.	n.d.			
		B	n.d.	n.d.			
		C	n.d.	n.d.			
		D	n.d.	n.d.			
		E	n.d.	n.d.			
	Batch 5	A	n.d.				
		B	n.d.				
		C	n.d.				
		D	n.d.				
		E	n.d.				
Conventional	Batch 1	A	120				
		B	traces				
		C	135				
		D	135				
		E	173				
	Batch 2	A	traces				
		B	traces				
		C	129				
		D	181				
		E	117				
	Batch 3	A	n.d.				
		B	n.d.				
		C	n.d.				
		D	n.d.				
		E	n.d.				
	Batch 4	A	n.d.				
		B	n.d.				
		C	n.d.				
		D	n.d.				
		E	n.d.				

n.d., not detected (DON concentration in sample <32 µg/kg)
traces (32 µg/kg≤concentration of DON in sample<107 µg/kg)

Table 56. Occurrence of FB1 (LOD = 6 ppb, LOQ = 18 ppb) and FB2 (LOD = 13 ppb, LOQ = 42 ppb) (ppb, µg/kg) in sweet corn samples.

Occurrence of FB				FB_1	FB_2
Conventional	Brand 1	Batch 1	A	n.d.	n.d.
			B	n.d.	n.d.
			C	n.d.	n.d.
			D	n.d.	n.d.
			E	n.d.	n.d.
			F	n.d.	n.d.
			G	n.d.	n.d.
			H	n.d.	n.d.
			I	n.d.	n.d.
			J	n.d.	n.d.
	Brand 2	Batch 1	A	n.d.	n.d.
			B	n.d.	n.d.
			C	n.d.	n.d.
			D	n.d.	n.d.
			E	n.d.	n.d.
			F	n.d.	n.d.
			G	n.d.	n.d.
			H	n.d.	n.d.
			I	n.d.	n.d.
			J	n.d.	n.d.
Organic	Brand 3	Batch 1	A	n.d.	n.d.
			B	n.d.	n.d.
			C	n.d.	n.d.
			D	n.d.	n.d.
			E	n.d.	n.d.
			F	n.d.	n.d.
			G	n.d.	n.d.
			H	n.d.	n.d.
			I	n.d.	n.d.
			J	n.d.	n.d.

n.d., not detected (FB concentration in sample < LOD)

Table 57. Levels of FB1 (µg/kg) in polenta collected on the Belgian market.

Occurrence of FB1 (µg/kg)		Sample	Brand 1	Brand 2	Brand 3
Organic	Batch 1	A	273	615	n.d.
		B	traces	397	n.d.
		C	traces	579	traces
		D	traces	593	n.d.
		E	traces	403	557
	Batch 2	A	333	traces	599
		B	338	350	716
		C	370	243	231
		D	334	277	735
		E	339	431	n.d.
	Batch 3	A	1329	620	
		B	1213	351	
		C	836	490	
		D	1299	491	
		E		215	
	Batch 4	A		991	
		B			
		C			
		D			
		E			
Conventional	Batch 1	A	2782	547	traces
		B	2919	566	214
		C	2472	580	traces
		D	3057	470	traces
		E	2962	516	n.d.
	Batch 2	A	4180		n.d.
		B		469	n.d.
		C	3436	796	207
		D	3372	681	288
		E	4353	422	n.d.
	Batch 3	A	1335	668	traces
		B	882	738	n.d.
		C	1536	575	traces
		D	1074	682	traces
		E	2285		n.d.
	Batch 4	A	1115	216	
		B	808	224	
		C	699	traces	
		D	679	637	
		E	931	379	

n.d., not detected (FB1 concentration in sample <59 µg/kg)
traces (59 µg/kg≤concentration of FB1 in sample<197 µg/kg)

Table 58. Levels of FB2 (µg/kg) in polenta collected on the Belgian market.

Occurrence of FB2 (µg/kg)		Sample	Brand 1	Brand 2	Brand 3
Organic	Batch 1	A	n.d.	traces	traces
		B	n.d.	traces	n.d.
		C	n.d.	traces	n.d.
		D	n.d.	281	n.d.
		E	n.d.	traces	traces
	Batch 2	A	traces	n.d.	traces
		B	traces	traces	traces
		C	traces	traces	n.d.
		D	traces	n.d.	traces
		E	traces	traces	n.d.
	Batch 3	A	traces	traces	
		B	traces	n.d.	
		C	traces	traces	
		D	traces	traces	
		E		traces	
	Batch 4	A		traces	
		B			
		C			
		D			
		E			
Conventional	Batch 1	A	959	traces	traces
		B	986	294	n.d.
		C	854	291	n.d.
		D	1265	traces	n.d.
		E	829	traces	n.d.
	Batch 2	A	1659		n.d.
		B		traces	n.d.
		C	1172	284	n.d.
		D	780	288	n.d.
		E	1622	traces	n.d.
	Batch 3	A	634	371	n.d.
		B	411	304	n.d.
		C	681	traces	n.d.
		D	517	294	n.d.
		E	1117		n.d.
	Batch 4	A	582	traces	
		B	304	traces	
		C	348	n.d.	
		D	traces	traces	
		E	428	traces	

n.d., not detected (FB2 concentration in sample <83 µg/kg)
traces (276 µg/kg≤concentration of FB2 in sample<276 µg/kg)

Table 59. Levels of FB1 (µg/kg) in cornflakes collected on the Belgian market.

Occurrence of FB1 (µg/kg)		Sample	Brand 1	Brand 2	Brand 3	Brand 4	Brand 5	Brand 6	Brand 7
Organic	Batch 1	A	traces	116.33	401.14	75.56			
		B	traces	149.09	463.79	132.42			
		C	n.d.	185.91	186.91	90.90			
		D	traces	151.63	418.22	76.86			
		E	traces	206.60	290.11	98.83			
	Batch 2	A	traces	269.46	122.62	87.87			
		B	traces	350.66	98.27	58.82			
		C	traces	338.32	198.42	65.54			
		D	43.61	293.40	122.82	66.74			
		E	traces	401.25	160.98	93.29			
	Batch 3	A	n.d.	75.79	190.34	306.82			
		B	n.d.	122.43	236.17	269.72			
		C	n.d.	107.76	214.40	332.89			
		D	n.d.	128.82	173.83	267.49			
		E	n.d.	127.55	168.35	271.74			
	Batch 4	A	50.13		241.30	48.15			
		B	68.28		193.45	55.97			
		C	67.42		263.23	50.72			
		D	53.96		197.39	53.12			
		E	75.51		219.50	49.63			
Conventional	Batch 1	A	n.d.	84.28	n.d.	n.d.	154.86	183.87	70.82
		B	n.d.	50.60	n.d.	n.d.	205.81	212.77	96.99
		C	n.d.	45.65	n.d.	n.d.	99.26	212.08	85.49
		D	n.d.	traces	n.d.	n.d.	111.63	146.29	105.03
		E	n.d.	traces	n.d.	n.d.	125.63	186.11	128.53
	Batch 2	A	n.d.	66.11	n.d.	traces	40.56	138.99	145.53
		B	n.d.	51.26	n.d.	traces	57.38	187.80	66.27
		C	n.d.	traces	n.d.	traces	45.94	112.49	79.08
		D	n.d.	49.62	n.d.	43.17	49.57	132.70	81.59
		E	n.d.	52.76	n.d.	56.44	n.d.	111.32	47.73
	Batch 3	A	n.d.	n.d.	n.d.	n.d.	n.d.	365.12	351.66
		B	n.d.	n.d.	n.d.	n.d.	n.d.	304.90	319.18
		C	n.d.	n.d.	n.d.	traces	n.d.	340.38	393.21
		D	n.d.	n.d.	n.d.	n.d.	n.d.	361.14	295.07
		E	n.d.	n.d.	n.d.	traces	n.d.	353.55	263.79
	Batch 4	A	n.d.		n.d.	n.d.		134.61	314.11
		B	n.d.		n.d.	n.d.		160.53	292.01
		C	n.d.		n.d.	n.d.		179.49	300.12
		D	n.d.		n.d.	n.d.		205.49	309.84
		E	n.d.		n.d.	n.d.		215.14	336.66

n.d., not detected (FB1 concentration in sample < 20 µg/kg)
traces (20 µg/kg ≤ concentration of FB1 in sample < 40 µg/kg)

Table 60. Levels of FB2 (µg/kg) in cornflakes collected on the Belgian market.

Occurrence of FB2 (µg/kg)		Sample	Brand 1	Brand 2	Brand 3	Brand 4	Brand 5	Brand 6	Brand 7
Organic	Batch 1	A	traces	traces	38.02	traces			
		B	traces	15.98	42.74	traces			
		C	traces	17.23	25.10	traces			
		D	15.52	16.53	40.69	traces			
		E	traces	17.76	28.33	traces			
	Batch 2	A	traces	20.28	20.63	traces			
		B	traces	22.82	18.34	traces			
		C	traces	23.14	26.41	traces			
		D	traces	20.97	22.19	traces			
		E	traces	26.24	21.94	traces			
	Batch 3	A	n.d.	traces	16.51	18.77			
		B	n.d.	traces	17.38	17.45			
		C	n.d.	traces	17.49	18.53			
		D	n.d.	traces	16.60	16.86			
		E	n.d.	traces	15.64	17.17			
	Batch 4	A	traces		21.16	traces			
		B	traces		20.20	traces			
		C	traces		21.58	traces			
		D	traces		18.09	traces			
		E	traces		20.38	traces			
Conventional	Batch 1	A	n.d.	16.81	n.d.	n.d.	26.58	25.70	traces
		B	n.d.	traces	n.d.	n.d.	26.93	27.06	17.10
		C	n.d.	traces	n.d.	n.d.	15.61	26.05	15.90
		D	n.d.	traces	n.d.	n.d.	17.79	22.15	17.59
		E	n.d.	traces	n.d.	n.d.	19.70	24.49	22.69
	Batch 2	A	n.d.	traces	n.d.	traces	traces	21.15	24.44
		B	n.d.	traces	n.d.	traces	traces	24.67	traces
		C	n.d.	traces	n.d.	traces	traces	20.07	15.64
		D	n.d.	traces	n.d.	traces	traces	21.23	traces
		E	n.d.	traces	n.d.	traces	n.d.	18.80	traces
	Batch 3	A	n.d.	n.d.	n.d.	n.d.	n.d.	25.28	16.57
		B	n.d.	n.d.	n.d.	n.d.	n.d.	23.41	17.84
		C	n.d.	n.d.	n.d.	n.d.	n.d.	23.93	20.16
		D	n.d.	n.d.	n.d.	n.d.	n.d.	25.80	17.57
		E	n.d.	n.d.	n.d.	n.d.	n.d.	25.15	15.51
	Batch 4	A	n.d.		n.d.	n.d.		traces	16.59
		B	n.d.		n.d.	n.d.		16.39	17.77
		C	n.d.		n.d.	n.d.		17.25	17.04
		D	n.d.		n.d.	n.d.		18.43	16.16
		E	n.d.		n.d.	n.d.		17.66	20.31

n.d., not detected (FB2 concentration in sample < 7.5 µg/kg)
traces (7.5 µg/kg ≤ concentration of FB2 in sample < 15 µg/kg)

Table 61. Levels of FB3 (µg/kg) in cornflakes collected on the Belgian market.

Occurrence of FB3 (µg/kg)		Sample	Brand 1	Brand 2	Brand 3	Brand 4	Brand 5	Brand 6	Brand 7
Organic	Batch 1	A	traces	traces	83.93	traces			
		B	traces	traces	90.34	traces			
		C	traces	traces	55.39	traces			
		D	traces	traces	89.29	traces			
		E	traces	traces	69.16	traces			
	Batch 2	A	traces	31.32	40.35	traces			
		B	traces	37.55	35.51	traces			
		C	traces	36.84	51.82	traces			
		D	traces	30.38	39.65	traces			
		E	traces	30.23	42.25	traces			
	Batch 3	A	n.d.	traces	traces	38.33			
		B	n.d.	traces	26.87	36.70			
		C	n.d.	traces	traces	37.88			
		D	n.d.	traces	traces	37.76			
		E	n.d.	traces	traces	32.51			
	Batch 4	A	traces		26.52	traces			
		B	traces		29.96	traces			
		C	traces		28.57	traces			
		D	traces		traces	traces			
		E	traces		25.48	traces			
Conventional	Batch 1	A	n.d.	traces	n.d.	n.d.	43.63	45.04	30.27
		B	n.d.	traces	n.d.	n.d.	50.46	50.53	34.20
		C	n.d.	traces	n.d.	n.d.	28.55	46.80	28.84
		D	n.d.	traces	n.d.	n.d.	38.98	37.25	28.99
		E	n.d.	traces	n.d.	n.d.	39.19	48.87	34.24
	Batch 2	A	n.d.	traces	n.d.	traces	traces	46.37	37.61
		B	n.d.	traces	n.d.	traces	traces	42.13	traces
		C	n.d.	traces	n.d.	traces	traces	35.07	26.71
		D	n.d.	traces	n.d.	traces	traces	34.71	28.47
		E	n.d.	traces	n.d.	traces	traces	38.74	traces
	Batch 3	A	n.d.	n.d.	n.d.	n.d.	n.d.	36.25	38.30
		B	n.d.	n.d.	n.d.	n.d.	n.d.	37.65	31.32
		C	n.d.	n.d.	n.d.	n.d.	n.d.	39.95	36.85
		D	n.d.	n.d.	n.d.	n.d.	n.d.	38.24	34.22
		E	n.d.	n.d.	n.d.	n.d.	n.d.	37.73	28.73
	Batch 4	A	n.d.		n.d.	n.d.		traces	25.71
		B	n.d.		n.d.	n.d.		traces	28.46
		C	n.d.		n.d.	n.d.		26.28	30.22
		D	n.d.		n.d.	n.d.		28.16	traces
		E	n.d.		n.d.	n.d.		26.45	25.94

n.d., not detected (FB3 concentration in sample < 12.5 µg/kg)
traces (12.5 µg/kg ≤ concentration of FB3 in sample < 25 µg/kg)

3. Publications

3.1. Publications

Paepens C., De Saeger S., Sibanda L., Barna-Vetró I., Léglise I., Van Hove F. and Van Peteghem C. , 2004, A flow-through enzyme immunoassay for the screening of fumonisins in maize. *Analytica Chimica Acta*, 523, 229-235.

Pussemier L., Larondelle Y., Van Peteghem C., Huyghebaert A., 2005. Chemical safety of conventionally and organically produced foodstuffs: a tentative comparison under Belgian conditions. *Food control*, in press.

Pierard J.-Y., Depasse C., Delafortrie A., Motte J.-C., 2004. Multi-mycotoxin determination methodology. In "Meeting the mycotoxin menace" Edited by Barug D., van Egmond H.P., Lopez-Garcia R.,van Osenbruggen W.A. & Visconti A., Wageningen Academic Publishers, The Netherlands, pp. 253-266., ISBN 9076998280

3.2. Publications in preparation

Development of a confirmatory LC-MS/MS method for the quantification of fumonisin B_1, B_2 and B_3 in cornflakes. C. Paepens, S. De Saeger, and C. Van Peteghem. To be submitted to Rapid Communications in Mass Spectrometry.

Evaluation of fumonisin contamination in cornflakes on the Belgian market: "flow-through" assay screening and LC-MS/MS confirmation. C. Paepens, S. De Saeger, L. Sibanda, I. Barna-Vetró, M. Anselme, Y. Larondelle and C. Van Peteghem

3.3. Abstracts for poster presentations

Paepens C., De Saeger S., Sibanda L., Barna-Vetró I. and Van Peteghem C.. A flow-through enzyme immunoassay for the screening of fumonisin B_1 in maize. Poster presented at the second World Mycotoxin Forum, 17-18 February 2003, Noordwijk, The Netherlands.

Paepens C., De Saeger S., Sibanda L., Barna-Vetró I. and Van Peteghem C.. Development of a flow-through enzyme immunoassay screening method for ochratoxin A in beer. Poster presented at the XI International IUPAC Symposium on Mycotoxins and Phycotoxins, 17-21 May 2004, Bethesda, Maryland, USA.

Paepens C., De Saeger S., Sibanda L., Barna-Vetró I., Anselme M., Larondelle Y. and Van Peteghem C.. Application of the flow-through enzyme immunoassay in a market orientated supply study for the evaluation of fumonisins in cornflakes. Abstract submitted for Rapid Methods Europe 2005, 23-25 May 2005.

Pierard J.-Y., Depasse C., Dal Cero C., Etienne J., Declercq O., Motte J.-C., 2003. Optimization of the purification for multi-mycotoxin determination. HPLC 2003, Nice, France, 15-19 june, 2003.

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