SPSD II

INTERACTION BETWEEN MYCOTOXINS AND OTHER FOOD CONTAMINANTS: A NEW SAFETY CONCERN

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PART 1

SUSTAINABLE PRODUCTION AND CONSUMPTION PATTERNS

GENERAL ISSUES

AGRO-FOOD

ENERGY

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

Part 1:
Sustainable production and consumption patterns

FINAL REPORT

INTERACTION BETWEEN MYCOTOXINS AND OTHER FOOD CONTAMINANTS: A NEW SAFETY CONCERN

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1 INTRODUCTION

1.1 General context

The intestinal layer is the first barrier preventing the entry of foreign antigens into the underlying tissues. It becomes increasingly evident that enterocytes are much more than a simple 'passive filter' and, through highly complex interactions, play a major role in the regulation of the bioavailability of nutrients, drugs, contaminants, etc. They finely tune the expression and/or activity of proteins involved in the absorption process (also called phase 0), of numerous phase I and II enzymes, which transform absorbed compounds, as well as of efflux pumps (phase III) that excrete substances back to the lumen of the small intestine. Altogether, these processes modulate the total amount of the compounds and of their metabolites, which finally gain access to the systemic circulation. Probably even more important, it is now clear that nutrients and xenobiotics present in the gastro-intestinal tract influence the expression and/or activity of some key proteins involved in the absorption processes. Therefore, they can strongly affect the bioavailability of a substance, which may be totally unrelated. Thus, modulation of a contaminant bioavailability by other substances present in the intestine should be taken into account. Such food interactions, as it is now well established in the case of drug interactions (Murray, 2006), would be strongly affected by the nutritional habits and by the mode of production of the foodstuffs, which influence both the presence and concentrations of the contaminants.

Food safety can be adversely affected by the presence of various kinds of chemical contaminants such as pesticides, heavy metals, persistent organic pollutants (POPs), hormones, antibiotics and mycotoxins. The fact that the Maximal Residue Limits set by the authorities are not exceeded in the controlled foodstuffs may not be considered as a sufficient guarantee for food safety because there are still a lot of uncertainties linked to the toxicological assessment of the contaminants. Among these, it is frequently argued that the possible additive and, worst, synergistic effects of different contaminants are not taken into consideration. Therefore, there is a need to improve our knowledge on the possible interactions between all kinds of contaminants that can be present simultaneously in our diet.

1.2 Aims of the project

The project aims at studying the possible interactions of different contaminants or nutrients in a complex biological system, namely the intestinal barrier, the absorption site of most oral pollutants. The combined use of a cell culture system based on the Caco-2 cells, already validated as an *in vitro* model of the intestinal epithelium, and of appropriate analytical tools, allowed us to focus on two mycotoxins of major concern: deoxynivalenol (DON) and ochratoxin A (OTA). On the basis of existing databases, the major co-contaminants, which are likely to be present in our diet, were selected and realistic concentration ranges of chemicals were established. The effects of the selected contaminants on the bioavailability of these mycotoxins were tested in the Caco-2 cell model with the analytical methodologies already existing or developed within the consortium.

1.3 Structure of the report

Chapter 2 of the report deals with the description of the scientific methodology used in this project. Part 2.1 describes the strategy applied for the selection of relevant food contaminants and dietary components for their possible interactions with OTA and/or DON, the two major mycotoxins of the project. An approach used for the estimation of realistic dietary concentrations for these compounds is presented in part 2.2. Part 2.3 describes the biological methods based on the use of the Caco-2 cell culture system, a well-established and validated *in vitro* model of the intestinal epithelium. Part 2.4 is devoted to the description of analytical methods used to detect OTA, DON and their possible metabolites. These methods are based on HPLC and LC-MS/MS techniques. They were developed and validated in the laboratories of the partners for the specific purposes of this project.

A detailed description of the results is reported in chapter 3. The setting up of a database for the dietary contaminants and plant constituents, and a selection of relevant chemical mixtures, as well as the estimation of daily intakes and plausible intestinal concentrations for the selected chemicals, are presented in parts 3.1-3.4. Part 3.5 deals with OTA bioavailability studies including results of the experiments with the radio-labelled mycotoxin, detection of OTA and its metabolites by HPLC-FLD and LC-MS/MS methods and effects of food contaminants/constituents on OTA absorption. Results of DON bioavailability studies are given in part 3.6. Part 3.7 describes the investigation of DON effects on the intestinal function.

The main conclusions and recommendations, which can be put forward at the end of this project, are presented in chapter 4.

References, detailed tables and publications in the scope of the project are given in annexes (chapter 5).

2 DETAILED DESCRIPTION OF THE SCIENTIFIC METHODOLOGY

A project based on the study of interactions between different kinds of contaminants and food constituents needs a team of partners working in an efficient network. Our network fully meets this requirement, with three partners who already participated in related projects in close collaboration. During this project, the database analysis, contaminant and plant constituent selection have been performed by partners 2 and 3 with continuous exchange between partners. The cytotoxicity assays and transport studies based on the Caco-2 cell culture system were performed by partner 1, while DON and OTA analyses were carried out by partners 2 and 3, respectively (Fig. 1).

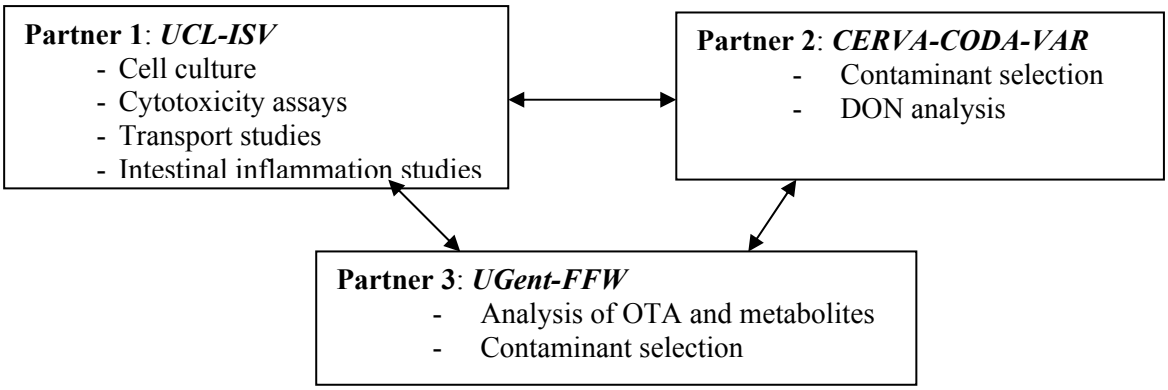


Figure 1. *Collaboration between the partners and tasks realized during this project*

2.1 Selection of relevant contaminants, plant constituents, and of plausible interactions

2.1.1 General objective

The study of chemical mixtures is an increasingly important research area because of the possible impact on risk assessment regarding human exposure to various types of chemicals. The classical approach to risk assessment is based on the surveys of a single compound, which represent only an over-simplification of the real (complex) exposure. Considering that chemical mixtures are the rule rather than the exception, they should be taken into account. The best approach to study chemical interactions from a toxicological perspective is to identify how chemicals can interfere at the cellular level and thus interact with the toxicity of other compounds. The use of simple *in vitro* biological models can provide a first rational

basis for predicting interactions between chemicals. Unfortunately, studies based on that approach are currently scarce, especially using realistic dietary concentrations.

This project is principally focused on two mycotoxins (OTA and DON), but other types of food contaminants and components of the human diet were considered for possible interactions with OTA and/or DON. Environmental contaminants, natural toxins, drugs, hormones and plant constituents, such as polyphenols, were screened for their capacity to enhance or limit the transport and/or metabolism of OTA or DON when passing through the *in vitro* model of the human intestinal barrier. Owing to the large number of contaminants and plant components occurring in foodstuffs, the selection was essential.

2.1.2 Strategy

Three major steps leading to the hypothesis of interactions between the mycotoxins OTA or DON and co-contaminants are described as follows:

- The general strategy started with **the first pre-selection**, favouring contaminants and plant constituents found in the main foodstuffs (relevance in food).

- **The second selection** was performed in order to refine the first rough pre-selection. The bibliographic strategy used to search and select interesting chemicals is presented in Figure 2. If only very limited relevant information was available or if this information showed evidence of absence of either relevant toxicity (for contaminant) or of the interaction with the intestinal barrier (for non-toxic constituents), the compound was rejected. Thus, we performed the selection of single contaminants.

- The chemicals, which were not rejected at that point, were submitted to a literature survey on the biochemical mechanisms of action, in order to identify possible **interactions** with bioavailability parameters and detoxification systems. Another selection criterion for the interactions was the co-occurrence in food. Hypotheses of interactions at the intestinal level were then formulated.

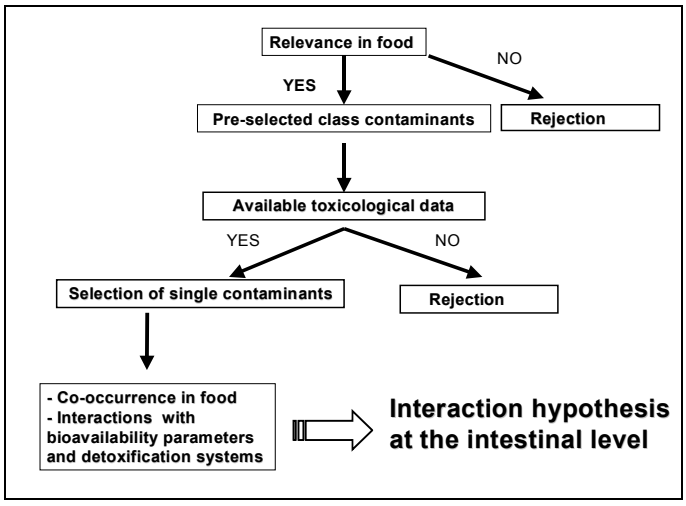


Figure 2 *Strategy applied to select relevant contaminants in terms of potential interactions*

2.2 Exposure assessment

The literature reports on some classical toxicological studies usually aim at revealing effects on specific targets. The relevance of such studies can be debated because the concentrations used are markedly higher than those found in the digestive tract (up to three orders of magnitude higher for some studies with OTA). Hence, it is important to design experimental studies using a range of contaminant concentrations, which are in line with the realistic human exposure. The scope of this project was the toxicological assessment of chemicals at plausible concentrations occurring in the gastro-intestinal tract. For that, realistic concentrations, which would reflect human physiological and pathological conditions, were needed and they were derived from the exposure assessment.

In this project, the only contamination route taken into account was the presence in food. Other routes of exposure (airways, skin, etc.) were not considered.

2.2.1 Estimation of a daily intake for the selected chemicals

Plausible concentrations to be applied to the *in vitro* intestinal system were calculated. For that, estimated daily intake (EDI) values were generated with different approaches and sources for each selected single contaminant. The method used was deterministic and called "point estimates".

The EDI values were calculated as follows:

$$EDI\ [ng/kg\ bw/day] = \Sigma_i\ (C_i \times L_i)$$

C_i: consumed quantity of foodstuff *i* (consumption data) [g/person/day]

L_i: concentration of a chemical in foodstuff *i* (contamination data) [µg/kg]

EDI of one contaminant in foodstuff *i* is obtained by multiplying consumed quantity of foodstuff *i* by the concentration of this chemical in foodstuff *i*. EDIs obtained for each foodstuff *i*, which can contain the chemical, are then summed up giving total EDI for the contaminant. This EDI is expressed in mass unit of a contaminant ingested by a person in one day. This unit can be converted in mass unit per kg of body weight per day using the average body weight of 70 kg**.**

Different sources providing consumption (C_i) and contamination (L_i) data were used.

- Consumption data:
    - SCOOP: these European reports, gathering results from different Member States, give the information on population and consumer exposure. They provide consumption and contamination data and estimates of dietary intake.
    - GEMS/FOOD Regional Diet (WHO, 2003): this consumption survey, realised by WHO for each continent, gives the quantity of a foodstuff consumed by one person per day.
- Contamination data:
    - SCOOP
    - Publication, opinions, other reports…
    - Norms: maximum (residue) levels [M(R)L]. These norms have been found in the European and Belgian legislation.

Depending on the combination of sources used, different types of daily intake can be calculated. Two major cases can be specified:

- Real-case: the combination of contamination data from monitoring analysis results (SCOOP, publications) with consumption data (SCOOP, GEMS) gives an EDI theoretically close to the real situation.

- Theoretical maximum daily intake (TMDI) (WHO, 1997): this "worst-case" situation can be obtained by multiplying M(R)L and consumption data from GEMS.

The relevance of the calculated EDIs or TMDIs can be reinforced by comparison with the Tolerable Daily Intake (TDI) or Acceptable Daily Intake (ADI, for pesticides). This parameter is fixed by the authorities and based on toxicological studies. It corresponds to a threshold level (No Adverse Effect Level) mainly derived from long-term animal studies multiplied by a safety factor.

2.2.2 Estimation of experimental concentrations of selected chemicals

Experimental concentrations were generated on the basis of calculated EDIs or TMDIs. To obtain a range of plausible concentrations corresponding to the real conditions of a "normal" person, two extreme situations were taken into consideration. Minimal and maximal concentrations were calculated on the basis of the following hypothesis:

- Maximal concentration (ppb or µg/l): the daily intake of a chemical (µg/person/day) is diluted during a meal in 1 l of body fluid, with the entire daily dose ingested in one meal.

[Maximal] = EDI or TMDI (µg/person/day)

- Minimal concentration (ppb or µg/l): the daily intake of a chemical (µg/person/day) is diluted during a meal in 3 L of body fluid, with 3 equal meals per day.

[Minimal] = [Maximal]/9

Those two models do not take into account other parameters, which can influence the quantity of chemicals really present in the intestinal lumen before the intestinal transport, such as bioaccessibility and inter-individual variations (gastric and intestinal lumen volume, rate of saliva secretion, rate of pancreatic or biliary secretions, composition of these secretions, etc.). Bioaccessibility is an important parameter which nowadays can be studied in the digestive-like conditions. For instance, aflatoxin B_1 (AFB_1) in peanuts and OTA in buckwheat show a bioaccessibility of 91 % and 63 %, respectively, in a digestive *in vitro* model developed at the National Institute of Public Health and the Environment (RIVM) of the Netherlands (Versantvoort et al., 2005).

2.3 Description of the biological methods

2.3.1 Biological model

We used a cell culture system based on Caco-2 cells, as a well-known *in vitro* model of the intestinal barrier. When Caco-2 cells are cultivated on microporous filters in bicameral inserts (Fig. 3), they fully differentiate and polarize, with tight junctions, microvillosities, functional brush border enzymes and transport systems. They express most intestinal biotransformation enzymes and efflux pumps. In addition, such a culture system provides a separate access to the basolateral and apical sides of the enterocytes and is adapted to evaluate the bioavailability of mycotoxins, food pollutants and/or food constituents. We used this model for studying OTA and DON absorption and the influence of contaminants: the mycotoxin, alone or in combination with selected contaminants or plant constituents, was introduced in the upper compartment (intestinal lumen). The appearance of mycotoxin in the basolateral compartment (blood pole) was quantified by analytical or radiochemical methods.

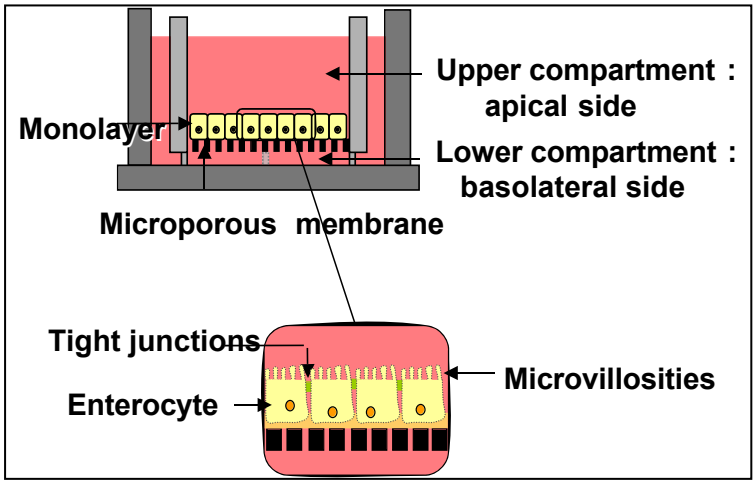


Figure 3. *Schematic illustration of the cell culture system as in vitro model of the intestinal barrier.*

2.3.2 Experimental methods

2.3.2.1 Cell culture

Caco-2 cells were routinely grown, 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 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 (Halleux & Schneider, 1991; Sergent-Engelen et al., 1993).

Cells were seeded on a type I collagen (Sigma-Aldrich, St. Louis, MO) pre-coated poly(ethylene terephtalate) microporous membrane (1 µm pore diameter, Whatman SA, Louvain-la-Neuve, Belgium) in the bicameral insert (24 mm diameter, 5 cm^2 growing area) at 120,000 cells/cm^2. Cells were cultivated from 21 to 28 days to allow complete differentiation into enterocytes. The integrity of the monolayers was checked by measurement of the transepithelial electrical resistance (TEER) (Endohm-24, World Precision Instruments, Sarasota, FL). Only monolayers exhibiting TEER values of *ca*. 500 Ω.cm^2 were used.

2.3.2.2 [^{3}H]-radio-labelled ochratoxin A transport

The 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 contained 2.8 ml of the transport medium supplemented with 1% (w/v) bovine serum albumin (BSA). Transepithelial passage was assayed by the addition to the upper compartment of 1.8 ml of transport medium supplemented with [^{3}H]OTA (with or without non-radioactive OTA) as well as with [^{14}C]mannitol as the internal control for transport. In some experiments, selected contaminants, plant constituents or MK571 were further added to the upper compartment at the same time as OTA. After 3 h, the media from the upper and lower compartments were collected separately. After the dispersion in 2 ml of Aqualuma® (Lumac Lsc, Groningen, the Netherlands), an aliquot was analyzed by liquid scintillation spectrometry (Packard Tri-Carb 1600 TR, Packard, Meriden, CT).

The absence of toxicity was checked at the end of the incubations by optical phase contrast microscopy examination, TEER measurement and lactate dehydrogenase (LDH) release. LDH is a cytosolic enzyme released in the cell culture medium by necrosed cells.

2.3.2.3 Unlabeled ochratoxin A transport

Non-labeled OTA transport experiments were performed in the same conditions as for [^{3}H]OTA. The transepithelial passage was assayed by adding different concentrations of OTA to the upper compartment. After 3 hours, media from the upper and lower compartments were collected separately and centrifuged at 12,000 g. The supernatants were stored at –20°C until HPLC or LC-MS/MS analysis (section 2.4.1).

2.3.2.4 Deoxynivalenol transport

DON transport experiments were performed using the same protocol as for OTA transport. Transepithelial passage was assayed in the two opposite directions by the addition of various concentrations of DON to the upper or lower compartment. At each sampling time, an aliquot of transport medium was withdrawn from the acceptor compartment and replaced by fresh transport medium. The samples were analyzed for DON content by HPLC (section 2.4.3). To detect the possible appearance of DON-conjugated metabolites, some samples were treated with a mixture of β-glucuronidase and arylsulfatase, for 24 h at 37°C in 0.2 M acetate buffer, pH 5, before the analysis by HPLC.

To evaluate the effect of plant constituents as well as the implication of an efflux pump, either 100 µM verapamil, 50 µM MK571, 50 µM quercetin, 50 µM resveratrol, 200 µM 1-chloro-2,4-dinitrobenzene (CDNB) or 2 mM sodium taurocholate were added to the upper and lower compartments at the same time as DON. To assess the importance of the paracellular passage, EGTA was used at 2.5 mM.

The absence of toxicity was routinely checked at the end of transport experiments by optical phase contrast microscopy examination, TEER measurement and LDH release.

2.3.2.5 Assessment of the phosphorylation of mitogen-activated protein kinases (MAPKs)

The cells were cultivated on a microporous membrane for 21–27 days, as described for transport studies. They were then incubated overnight in the same medium without EGF and insulin. DON at 2 µg/ml was then added to the upper compartment for specified durations or at various concentrations during 24 h. The cells were analyzed by Western blotting for the activation of MAPKs. They were washed in ice-cold phosphate buffer and suspended in lysis buffer (phosphate buffer containing 1%, w/v, Igepal CA630, 0.5%, w/v, sodium deoxycholate and 0.1%, w/v, sodium dodecylsulfate, supplemented with 0.2 mM sodium ortho-vanadate, 50mM sodium fluoride and 1%, v/v, of a protease inhibitor cocktail for use in tissue culture

media (Sigma–Aldrich)). The lysate was centrifuged at 12,000 g for 10 min at 4°C. The protein content was determined in the resultant supernatant by the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Twenty micrograms of total cellular proteins was resolved by SDS-PAGE in an 11% (w/v) acrylamide gel and transferred to a Hybond-P polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Little Chalfont, UK). After blocking with Tris buffered saline solution (0.02 M, pH 7.6) containing 0.05% (v/v) Tween 20 (TBST) and 5% (w/v) non-fat milk powder, the membrane was incubated overnight at 4°C with an antibody raised against either phospho-p44/p42 Erk, phospho-p38 MAPK or phospho-SAPK/JNK (rabbit IgG) at 1:1000 dilution in TBST containing 0.5% (w/v) milk powder. The membrane was washed three times with TBST and incubated for 1 h with HRP-conjugated goat anti-rabbit IgG at a 1:2000 dilution in TBST containing 0.5% (w/v) milk powder. After thrice washing with TBST, the bound HRP-conjugated antibody was detected with an enhanced chemiluminescence detection kit (ECL-plus, Amersham Biosciences) according to the manufacturer's instructions. The membrane was then stripped and re-probed as described above with a specific antibody that recognized both the phosphorylated and unphosphorylated forms of the MAPK.

2.3.2.6 Transient transfection and luciferase assays

These assays were performed to analyze if exposure of Caco-2 cells to DON interferes with the activity of the NF-κB transcription factor, a downstream target of MAPKs implicated in inflammatory cascades.

The NF-κB luciferase reporter plasmid was kindly provided by Dr. B. Sugden (University of Wisconsin, Madison, WI). Caco-2 cells, used between passages 20 and 30, were seeded onto 24-wells culture dishes (Greiner Bio-one, Frickenhausen, Germany) pre-coated with type I collagen (Sigma-Aldrich) at a density of 22,000 cells/cm^2 and grown to 40 % confluence before transfection. JetPEI^{TM} (Promega, Madison, WI) was used as transfection reagent, according to the Polyplus Transfection Easy Protocol. Briefly, a 150 mM NaCl solution supplied with 1% (w/v) of NF-κB reporter plasmid and 1 % (v/v) jetPEI^{TM} 7.5 mM was incubated at room temperature for 30 min. It was then applied on each well containing 1 ml of culture medium. After incubation for 24 h at 37°C, cells were washed with PBS (pH 7.4) and incubated again for 24 h at 37°C with DON at 0, 50, 250, 500, 750, 1000, 5000 or 10,000 ng/ml. For the experiments with polyphenols, cells were pre-incubated for 4 h (no pre-incubation for the control) in the presence of 50 µM polyphenols before incubation with (or without) 1000 ng/ml DON for 24 h.

To perform luciferase assays, cells were washed twice with PBS, and 110 µl of Luciferase Assay Reporter Lysis Buffer (Promega) was added to each well, followed by 20 min incubation at -20°C. Cells were then harvested and centrifuged at 16,000 g for 4 min. The supernatant (10 µl) was combined with 50 µl of Luciferase Assay Substrate (Promega) in a microcentrifuge tube, and luminescence was read for 1 second with a GLOMAX^{TM} 20/20 luminometer (Promega). An expression vector containing the pCMV-βGal plasmid, kindly provided by Dr. R. Rezsohazy (Université catholique de Louvain, Louvain-la-Neuve, Belgium), was used as a control for the transfection efficiency.

2.3.2.7 Data analysis

Results were expressed as means ± S.E.M. Statistical analysis was performed using one-way analysis of variance (ANOVA). The computer program was Systat 5.2.1 (Systat Inc., Evanston, IL).

2.4 Analytical methodology to detect ochratoxin A and deoxynivalenol

2.4.1 Analytical methodology to detect ochratoxin A

The first *in vitro* experiments were carried out under the following conditions: OTA was added to 1.8 ml HBSS, pH 6.0, at the apical side of the Caco-2 cells. The basolateral compartment was filled with 2.8 ml HBSS, pH 7.4, + 1 % (w/v) of BSA. After 3 h incubation, samples from the apical and basolateral compartments were collected. Under these conditions, OTA transport was possible only from the apical to the basolateral side, due to the strong binding of OTA to BSA in the basolateral compartment. The basolateral samples were pre-treated with ortho-phosphoric acid to make this complex dissociated.

The developed analytical methodology to detect OTA consisted of high-performance liquid chromatography coupled to fluorescence detection (HPLC-FLD) and of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

2.4.1.1 Development of the HPLC-FLD method

An HPLC-FLD method was used for the analysis of OTA in the apical and basolateral samples from biological experiments with Caco-2 cells. The samples were cleaned up by solid phase extraction (SPE). The C_{18}-cartridges (Varian, Sint-Katelijne-Waver, Belgium) were

activated with methanol (3 ml). Conditioning of the column was carried out with water (3 ml), followed by HBSS (3 ml, pH 6.0 or 7.4, depending on the samples' origin). Then the sample was poured on the column. Afterwards, the column was washed with water (2 x 3 ml) and dried for 15 min under vacuum. OTA was finally eluted with methanol (3 ml). The solvent was evaporated to dryness under nitrogen. The residue was dissolved in 60 µl methanol, and 50 µl were injected into the HPLC system. This system was equipped with C_{18} column (25 cm x 4.6 mm, 5 µm, Supelco, Bellefonte, USA). Excitation and emission wavelengths of the fluorescence detector were 330 and 470 nm, respectively. The mobile phase used was acetonitrile/water/acetic acid (54:44:2, v/v/v) with a flow rate of 1 ml/min.

2.4.1.2 Validation of the HPLC-FLD method

The validation was carried out for OTA in HBSS, pH 6.0 and pH 7.4. Parallelism, limit of detection (LOD), limit of quantification (LOQ), precision (within-day and between-day) and recovery were calculated. Parallelism was determined to establish what calibration curve must be used. Thus two different calibration curves were obtained. The first one was made by including standards (HBSS fortified with known amounts of OTA) in SPE, and the second one by direct injection of known amounts of OTA in methanol into the HPLC system. No parallelism was found between these two calibration curves. Therefore, further validation was performed with the calibration curve obtained by including standards in the SPE. Precision was determined by analysing four calibration standards in HBSS, four times each, on the same day (within-day precision) and on different days (between-day precision). Different samples with known OTA concentration were analysed for the recovery experiments. The peak area was used to calculate OTA concentration.

Validation of the HPLC method for the detection of OTA in HBSS, pH 6.0, gave LOD and LOQ values of 0.047 ng/ml and 0.079 ng/ml, respectively. Coefficients of variation obtained in the precision experiments were between 3 and 11 % (within-day) and between 6 and 12 % (between-day). The recovery ranged from 86 to 100 %.

Validation of the HPLC method for the detection of OTA in HBSS, pH 7.4, gave LOD and LOQ values of 0.051 ng/ml and 0.084 ng/ml, respectively. Coefficients of variation were 2-10 % (within-day) and 4-12 % (between-day). The recovery ranged from 86 to 100 %.

2.4.1.3 Development of the LC-MS/MS method

The LC-MS/MS method was developed for the detection of OTA in the biological samples to confirm the results obtained with the HPLC-FLD method. Sample clean-up was almost the

same as for the HPLC-FLD method, except that after evaporation of the solvent the residue was dissolved in 40 µl acetonitrile/water (50:50, v/v) + 0.3 % formic acid. The injection volume was 20 µl. The LC-MS/MS system was also equipped with a C_{18} column (150 mm x 3.2 mm, 5 µm, Alltech, Deerfield, IL). The mobile phase consisted of solvent A (acetonitrile + 0.3 % formic acid) and solvent B (water + 0.3 % formic acid). The operation mode was Electro Spray Positive (ESI+). A gradient with a flow rate of 0.3 ml/min was used for the elution (Fig. 4).

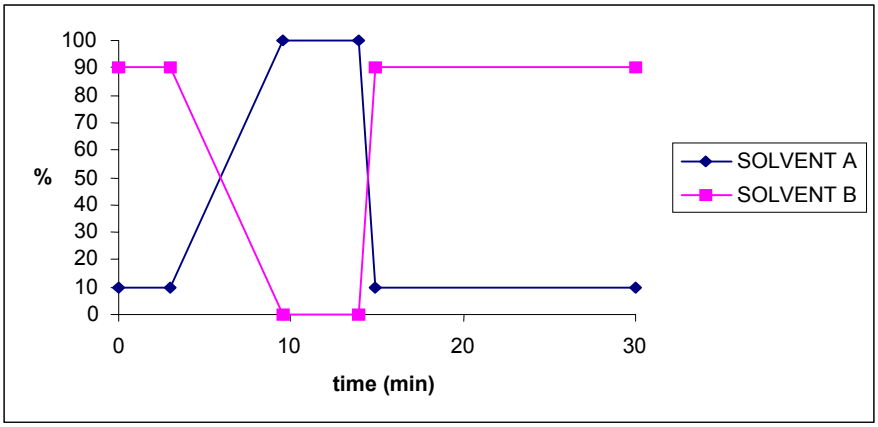


Figure 4. *LC-MS/MS gradient used for the elution of OTA*

2.4.1.4 Validation of the LC-MS/MS method

The following parameters were determined for the validation of the LC-MS/MS method: parallelism, LOD, LOQ, precision (within-day and between-day) and recovery.

No parallelism was found between the calibration curves. Validation of the LC-MS/MS method for the detection of OTA in HBSS, pH 6.0, gave LOD and LOQ values of 0.012 ng/ml and 0.020 ng/ml, respectively. Coefficients of variation were 6-11 % and 3-13 % for within-day and between-day precision, respectively. Recovery ranged from 84 to 105 %.

Validation of the LC-MS/MS method for the detection of OTA in HBSS, pH 7.4, gave LOD and LOQ values of 0.010 ng/ml and 0.017 ng/ml, respectively. Coefficients of variation were 11-16 % and 8-12 % for within-day and between-day precision, respectively. The recovery values ranged from 84 to 105 %.

2.4.2 Analytical methodology to detect ochratoxin A and its metabolites

The *in vitro* experiments were carried out as described in section 2.4.1, except that OTA was added to the apical compartment in the presence of certain inducers of cytochromes P450 (CYP). These enzymes are supposed to catalyze OTA processing *in vivo*. The analytical

methodology developed to detect OTA simultaneously with possible metabolites combined the HPLC-FLD and LC-MS/MS methods.

2.4.2.1 Development of the HPLC-FLD method

The HPLC-FLD method was used for the analysis of OTA, 4(R)-hydroxyochratoxin A (4(R)-OH OTA), 10-OH OTA, OTα and OTC in the biological samples. Sample clean-up was the same as in section 2.4.1.1. The HPLC-FLD system was equipped with a C_{18} column (150 x 3.9 mm, 5 µm, Waters Corp., Milford, MA). Excitation and emission wavelengths were 330 and 450 nm, respectively. The mobile phase consisted of the solvent A (methanol/isopropanol; 9:1; v/v) and solvent B (acidified water, pH 2.1). A gradient with a flow rate of 1 ml/min was used for the elution (Fig. 5). LOD and LOQ values for this method are given in Table 1.

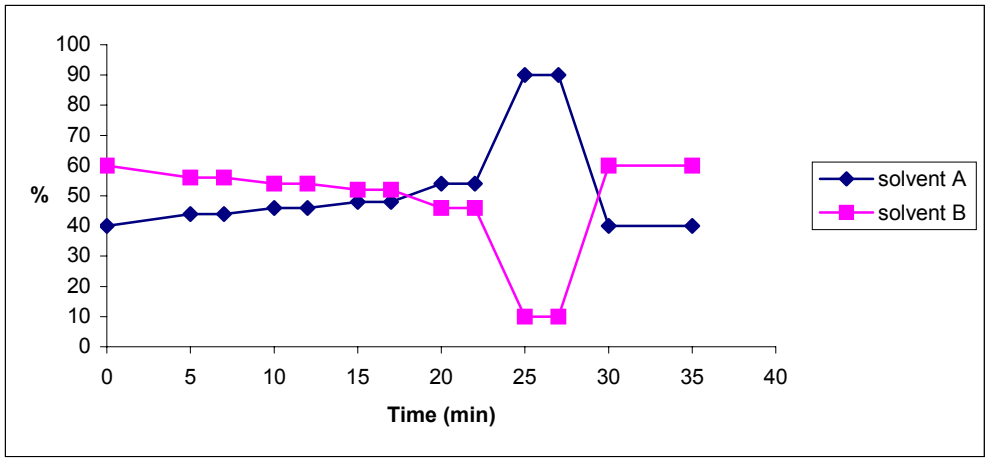


Figure 5. *HPLC-FLD gradient used for the elution of OTA and metabolites*

Table 1. LOD and LOQ of the HPLC-FLD method for the detection of OTA metabolites

Compound	LOD (S/N=6), ng/ml	LOQ (S/N=10), ng/ml
OTA	1.672	2.78
OTα	1.918	3.19
4R-OHOTA	1.398	2.33
10-OHOTA	1.312	2.18
OTC	1.608	2.683

2.4.2.2 Development of the LC-MS/MS method

The LC-MS/MS method was also developed for the detection of OTA and the metabolites to confirm the results obtained with the HPLC-FLD method. The sample clean-up was the same

as in section 2.4.1.3. The LC-MS/MS system was equipped with a C_{18} column (150 mm x 3.2 mm, 5 µm, Alltech). The Electron Spray Negative (ESI-) operation mode was used for the detection of OTA, 4R-OH OTA, 10-OH OTA and OTα, and the Electrospray Positive (ESI+) mode for the detection of OTC. The mobile phase consisted of water (solvent A) and acetonitrile (solvent B). A gradient with a flow rate of 0.3 ml/min was used for the elution.

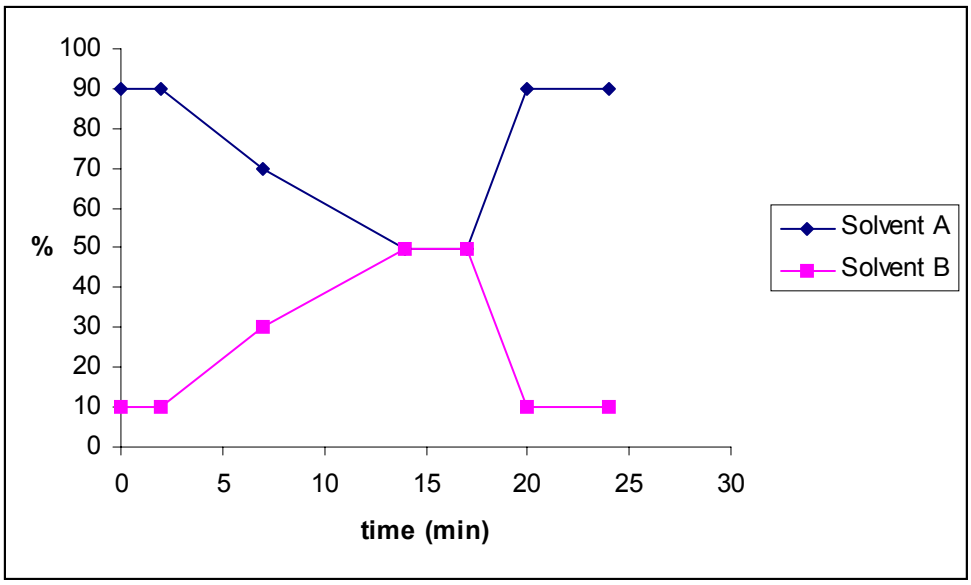


Figure 6. *LC-MS/MS gradient used for the elution of ochratoxin A and its metabolites.*

The parent and most abundant daughter ions (Table 2) were used for the detection of the metabolites. These ions are indeed characteristic for each compound.

Table 2. Parent and daughter ions of the components for the detection of OTA and its metabolites by LC-MS/MS method

Compound	Parent Ion	Daughter Ion (1)	Daughter Ion (2)
OTα	254.90	211.51	167.44
10-OHOTA	418.04	374.43	273.59
4R-OHOTA	417.97	270	330.51
OTA	401.96	358.44	167.47
OTC	432.5	239.51	193.46

2.4.3 Analytical methodology to detect deoxynivalenol

An HPLC-UV method was used for the analysis of DON in HBSS, pH 6.0 (apical), and HBSS, pH 7.4, + 1 % BSA (p/v) (basolateral). Samples of DON from the transport experiments (section 2.3.2.4) were homogenized and analysed without any extraction or

clean-up. The HPLC system consisted of a Waters 515 pump (Waters Corp.), an HPLC column oven system set at 25°C, a diode array detector (UV6000LP, Thermo Electron Corp., Boston, MA) set at a wavelength of 220 nm, an automatic injector (712 WISP, Waters Corp.) and a C_{18} Chromsep SS 100 column (10 cm×4.6 mm, 5 µm particle size, Varian) combined with a C_{18} guard column (2 cm×4.6 mm, Varian). The mobile phase consisted of water/acetonitrile (90:10, v/v) used in an isocratic mode with a flow-rate of 0.6 ml/min. The retention time of DON was 4.6±0.3 min. The injection volume was 50 µl. LOD (S/N = 3) and LOQ (S/N = 10) were 3 and 10 ng/ml for HBSS, pH 6.0, and 6 and 20 ng/ml for HBSS, pH 7.4, + 1 % BSA (p/v), respectively. The analytical methodology was not adapted to detect DON metabolites.

3 DETAILED DESCRIPTION OF THE RESULTS

3.1 Setting up the database

A general database for the dietary contaminants and plant polyphenols has been constructed. This database covers the main families of chemical contaminants and polyphenols and deals with toxicology, presence in food and feed, availability of risk assessments by national or international organizations. Moreover, a special focus is put on the studies with intestinal models. Analytical information was not collected because another database has already been compiled on contaminant analysis at VAR and this specific database is frequently updated. Peer review articles were the major database items, but abstracts, books, national or international organization press, monographs (e.g. pesticides), etc., have also been classified. The bibliographic investigation was performed mainly using two research tools, OVID and PubMed. The data were collected in a single database (using citation manager software Endnotes) with clear entries and classification. The research has been performed mainly for the two target mycotoxins of the project (DON and OTA) as well as for other mycotoxins, heavy metals, pesticides, polychlorinated biphenyls (PCBs), dioxins, food processing contaminants, antibiotics and hormones. A literature overview has been done for each class. The status of this database on July 1, 2006, is presented in Table 3.

Table 3. Status of the database set up for this project

Chemical class	N° Number of Items	Number of full-text format
Mycotoxins	652	353
Heavy metals	108	59
Pesticides	181	78
PCBs - dioxins	106	62
Plant constituents	380	291
Food processing contaminants	60	57
Risk assessment	295	276
Intestinal model	359	209
Other topics	40	23
Total	**2181**	**1408**

3.2 Selection of relevant contaminants and plant constituents

Based on the different selection criteria described previously (section 2.1.2), the following chemicals have been selected to be experimentally tested in combination with target mycotoxins OTA and DON (Table 4).

As far as plant constituents are concerned, some polyphenols have been described as possible modulators of the intestinal absorption or metabolism, what offers the first qualitative selection for OTA transport experiments.

Table 4. Chemicals selected in order to be experimentally tested in combination with target mycotoxins (OTA and DON).

Classes	Chemicals	Classes	Chemicals
Target contaminants	Ochratoxin A (OTA) Deoxynivalenol (DON)	**Dioxins**	2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)
		Polycyclic Aromatic Hydrocarbons (PAHs)	Benzo(a)pyrene
Other mycotoxins	Fumonisin B_1 (FB_1) Patuline (PAT) Penicillic acid (PA) Zearalenone (ZEA)	**Food processing contaminants**	Acrylamide
Pesticides	Atrazine Captan Chlorpyrifos Glyphosate Imidacloprid Thiabendazole Thiram	**Plant constituents**	Resveratrol Gallic acid Quercetin Chrysin (+)-catechin (-)-epigallocatechingallate Genistein Biochanin A
Heavy metals	Cadmium (Cd) Lead (Pb) Mercury (Hg)		

3.3 Estimation of a daily intake for the selected chemicals

This section reports the daily intakes (EDI) calculated for every selected contaminant using the previously described methodology (section 2.2.1). Different approaches were applied, depending on the contamination and consumption data sources available.

3.3.1 European SCOOP reports

These reports provide both consumption and contamination data collected in different Member States participating in the study. They are available for mycotoxins OTA (EC, 2002a), DON, FB_1, nivalenol (NIV), T-2 and HT-2 and ZEA (EC, 2003), PAT (EC, 2002b), as well as for heavy metals (EC, 2004) and dioxins (EC, 2000).

Different types of EDI can be specified in these reports:

- EDI mean: this intake is a mean value for a given Member State. It is calculated using the mean consumption and mean contamination levels.
- EDI 95^{th}: this intake is calculated for a given Member State using 95^{th} percentile of consumption values and mean contamination levels.

Table 5 shows these two types of EDI for the Member States with the lowest and the highest intake for a given contaminant. The medium between different Member States was also calculated for each contaminant. Values for children were not taken into account. TDIs are also reported in terms of fixed point, permitting relevant comparison with EDIs.

Table 5. Mean and 95th percentile EDI from SCOOP reports for mycotoxins, heavy metals and dioxins.

Contaminant	TDI (ng/kg bw/d)	EDI mean (ng/kg bw/d)[4]			EDI 95th (ng/kg bw/d)		
		Lowest M.S.	Highest M.S.	Medium	Lowest M.S.	Highest M.S.	Medium
OTA	5	0.1	3.5	1.0	/	/	/
DON	1 000	78.0	480.0	271.3	155.0	1806.0	887.8
FB1	2 000[1]	0.1	226.8	92.9	/	/	/
NIV	700	6.0	78.0	40.2	13.0	274.0	136.6
PAT	400	0.2	59.0	11.3	1.2	148.0	32.8
HT-2	60[2]	6.0	107.0	25.8	59.0	377.0	103.5
T-2	60[2]	5.0	104.0	29.7	28.0	159.0	109.0
ZEA	200	0.8	29.0	15.9	1.9	116.3	39.6
Cd	1 000[3]	5.6	358.6	205.7	/	/	/
Pb	3 570[3]	15.7	1900.0	485.8	/	/	/
Hg	715[3]	19.6	205.7	79.0	/	/	/
	TDI (pg /kg bw/d)	EDI mean (pg TEQ/kg bw/d)[5]					
2,3,7,8-TCDD	2.0	0.4	4.5	1.5	/	/	/

M.S. - Member State
[1] TDI for FB1+FB2+FB3
[2] Temporary TDI for T-2 + HT-2 toxins
[3] Values corresponding to the Provisional Tolerable Weekly Intake (PTWI) [ng/kg bw/week] divided by 7
[4] Body weight varies with Member States for OTA, ZEA and FB1. For other contaminants, body weight of 70 kg is used
[5] In SCOOP report, values are presented for the chemical group PCDD/F. They are extrapolated here to 2,3,7,8-TCDD only (TEF = 1). TEQ = Toxic Equivalent Quantity; TEF = Toxic Equivalent Factor.

Some EDI values are quite high in comparison with TDI values. This is especially the case for DON, OTA, T-2 and HT-2. High levels obtained for T-2 and HT-2 might be an artefact, because LOD/2 values were used for the calculations when the toxins were not detected. This approximation resulted in a significant increase in EDIs because of the high level of LOD compared to the contamination levels. In the case of DON and OTA, EDIs were more accurate (LOD << mean contamination levels). Thus, it appears from those data that daily intakes are of general concern. Considering that all the values presented in Table 5 have been calculated on the basis of mean contamination levels, the situation must be even worse in some cases.

The lack of harmonization in the SCOOP reports, regarding both the sampling procedures and the analytical methods could in some cases influence the soundness of the results.

Furthermore, a lack of information on many susceptible food commodities still persists in many countries, strongly influencing the evaluation of the overall intake both at European and national level. As far as consumption data are concerned, information on single food products is generally not available for participating Member States. Furthermore, it was impossible to handle the provided data homogeneously, since in some cases they were referred to all the population and in other cases - to specific groups of population.

As a consequence of the limiting factors, total dietary intake can be over or underestimated:

- One person is unlikely to be a consumer of all the considered food groups (overestimation).
- Calculations were made on the basis of data on occurrence in raw materials, whereas for several contaminants, the contamination can be reduced by the technological procedures used (overestimation).
- For each country, calculations were performed with only a certain number of tested foodstuffs (underestimation).
- Occurrence data were not corrected for recovery factors (underestimation).

3.3.2 Norm-based daily intake

Norms or MRLs were also used as contamination data and they were multiplied by European consumption data from GEMS. This approach was used for pesticides and benzo(a)pyrene. Norms for pesticide residues in fruits and vegetables in force in Belgium (February 2004) have been found in the Belgian legislation (Royal decree of March 13, 2000, amended by the royal decree of April 5, 2001). The Commission regulation (EC) 466/2001 of March 8, 2001, provided maximum levels in foodstuffs for benzo(a)pyrene. TMDI values are presented in Table 6, and details of calculations are reported in Annexes (section 5.2).

TMDI values are compared to ADI and presented as percentage of these ADI. No ADI is allocated to atrazine, and no TDI is allocated to benzo(a)pyrene since this PAH has been classified by the International Agency for Research on Cancer (IARC) as a potential carcinogen for humans (group 2A).

Table 6. TMDI for pesticides and benzo(a)pyrene and comparison with ADI or TDI.

Contaminant	TMDI (ng/kg bw/d)[1]	ADI / TDI (ng/kg bw/d)[1]	%ADI
Atrazine	1 438	/	/
Captan	7 434	100 000	7.4
Chlorpyrifos	1 347	10 000	13.5
Glyphosate	19 050	300 000	6.3
Imidacloprid	805	60 000	1.3
Thiabendazole	55 457	100 000	55.4
Thiram	11 833	10 000	118.3
Benzo(a)pyrene	10	/	/

[1] Body weight of 70 kg.

TMDI is an overestimation of the real daily intake. For example regarding pesticides, the following factors should also be considered: only a portion of a specific crop is treated with a pesticide; most treated crops contain residues far below MRL at the time of harvest; the residues are usually reduced during the storage, preparation, commercial processing and cooking; and each food, for which MRL is proposed, is unlikely to be treated with a pesticide during the whole lifetime of a consumer. Therefore, it should not be concluded that proposed MRL for a pesticide is unacceptable when TMDI exceeds ADI. However, if TMDI does not exceed ADI, ADI is very unlikely to be exceeded, even for high consumer intakes, provided that the main use of a pesticide is covered by Codex MRL (WHO, 1997).

3.3.3 Estimation of acrylamide intake

For acrylamide, no legislation specifies the maximum level in foodstuffs. However, this chemical was considered at the 64th meeting of the Joint FAO/WHO Expert Committee on Food Additives (Rome, February 8-17, 2005). The report (JECFA, 2005) of this meeting gives the distribution of acrylamide (µg/kg) in several commodities in 2002-2004. To obtain EDI, these values were multiplied by the consumption data from GEMS (Table 7). The details of calculation can be found in Annexes (section 5.2). Acrylamide has been classified by IARC as a potential carcinogen (group 2A), so no relevant comparison with TDI is possible in this case.

Table 7. EDI for acrylamide.

	EDI (ng/kg bw/d)[1]
Acrylamide	3 114.4

[1] Body weight of 70 kg.

3.3.4 No sufficient information available

In comparison with the studies of contaminants, investigations on human exposure to polyphenols are very limited. Therefore, the theoretical strategy for the determination of realistic concentrations (section 2.2) has not been applied to polyphenols. The experiments on the influence of polyphenols on OTA transport (section 3.5.3.1) were performed with 50 µM of these antioxidants, which has been estimated as an average concentration easily reached in the gastrointestinal tract of people with a normal diet.

As far as the mycotoxin penicillic acid is concerned, no information about human exposure has been found. In addition, data on the occurrence in foodstuffs are very limited. Therefore, the theoretical strategy for the determination of realistic concentrations (section 2.2) has neither been applied to penicillic acid.

3.4 Estimation of plausible concentrations for the selected chemicals

Using two assumptions for the dilution described in the section 2.2.2, the following ranges of plausible concentrations have been deduced for each contaminant on the basis of previously calculated EDI or TMDI (Table 8). For SCOOP reports, EDIs of the Member State with the lowest and highest intake value were used to calculate the minimal and maximal concentrations, respectively (Table 5).

Table 8. Plausible concentration ranges for different food chemicals.

Chemical	Minimal concentration (ppb or µg/l)	Maximal concentration (ppb or µg/l)	Chemical	Minimal concentration (ppb or µg/l)	Maximal concentration (ppb or µg/l)
OTA[1]	0.001	0.240	Atrazine	11.186	100.680
DON[1]	0.600	33.600	Captan	57.822	520.400
FB1[2]	0.001	36.170	Chlorpyrifos	10.478	94.300
NIV[1]	0.046	5.460	Glyphosate	148.167	1333.500
PA[1]	N.D.[4]	N.D.	Imidacloprid	6.260	56.340
PAT[1]	0.001	4.130	Thiabendazole	431.334	3882.010
HT-2[1]	0.046	7.490	Thiram	92.038	828.340
T-2[1]	0.038	7.280	2,3,7,8-TCDD (pg TEQ/l)	3.200	313.800
ZEA[3]	0.006	2.140			
Cd[1]	0.043	25.100	Benzo(a)pyrene	0.070	0.700
Pb[1]	0.122	133.000	Acrylamide	24.200	218.000
Hg[1]	0.152	14.400	Polyphenols	N.D.	N.D.

[1] Body weight of 70 kg for the Member State with the highest and the lowest intake values
[2] Body weight of 70 kg for the Member State with the highest intake value and of 81 kg for the Member State with the lowest intake value
[3] Body weight of 73.9 kg for the Member State with the highest intake value and of 70 kg for the Member State with the lowest intake value
[4] Not determined

The experimental concentrations used in the biological tests are as close as possible to those indicated in Table 8 but in some cases they are restricted by technical limitations.

3.5 Ochratoxin A bioavailability studies

Caco-2 cells, as the *in vitro* model of human intestinal barrier, were used to investigate the OTA bioavailability, *i.e.* OTA transport rate from the apical to the basolateral side of the cells. The effect of different OTA concentrations, added to the apical compartment, on the OTA transport was studied on the basis of OTA concentrations selected from EDIs. Experiments were performed with radio-labelled OTA, which can be easily and rapidly quantified. The results were confirmed by HPLC-FLD and LC-MS/MS methods developed for the detection of unlabelled OTA in the biological samples. The influence of food contaminants and of plant constituents (polyphenols) on OTA bioavailability has been determined with the use of the radio-labelled approach.

3.5.1 Radio-labelled ochratoxin A absorption experiments

The apical to basolateral transport (*i.e.* absorption) of [^{3}H]OTA was determined after 3 h incubation with a large range of OTA apical concentrations, from 0.75 nM to 10 µM (Fig. 7). OTA absorption increased proportionally to OTA concentration at the apical pole of differentiated cells. This confirms our previous data suggesting OTA absorption by passive diffusion (Berger et al., 2003). The mean rate after 3 h was 18.7 ± 3.5 % of the initial apical concentration.

Accumulation of [^{3}H]OTA by Caco-2 cells was low, as compared to the OTA transport-rate, but also proportional to the OTA concentration in the apical compartment, representing, after 3 h, 2.7 ± 0.7 % of the initial concentration (results not shown). No cytotoxicity determined by TEER measurement and LDH release was observed.

From databases and from these experimental results, two OTA concentrations were selected and used for the next experiments: 0.75 nM (0.3 ng/ml) and 7.5 nM (3 ng/ml). They represent plausible physiological concentrations, which may be found in the human intestinal tract after the ingestion of moderately and highly OTA contaminated food, respectively. OTA concentration of 10 µM (4 µg/ml) was also selected as a concentration usually used in OTA toxicological studies.

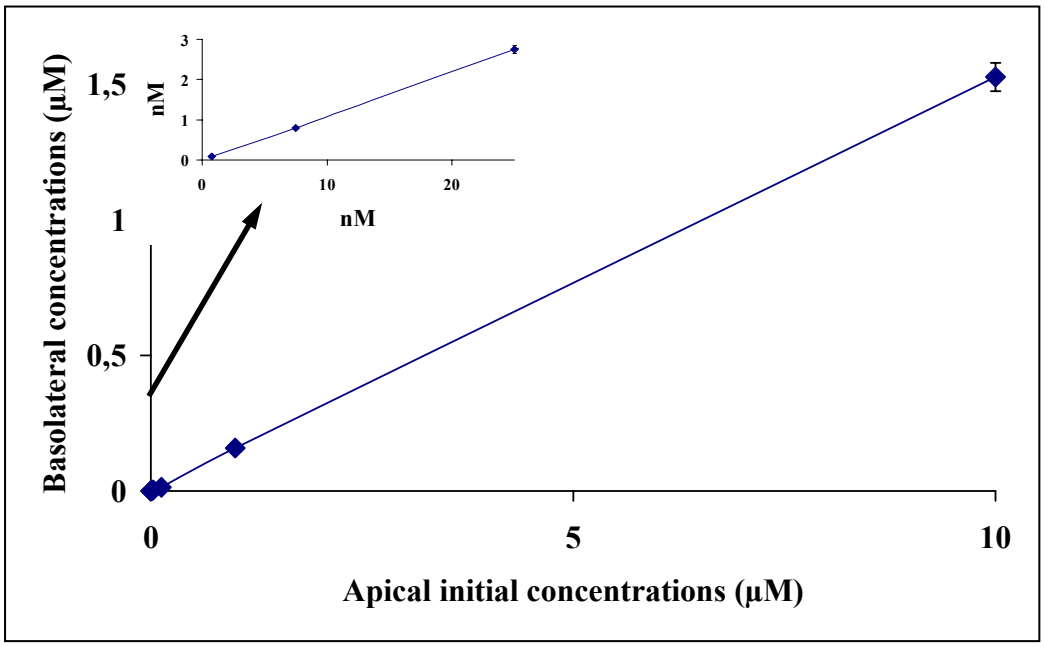


Figure 7. *Transport of OTA from the apical to basolateral pole of Caco-2 cells. Cells were incubated for 3 h with different concentrations of OTA: 0.75 nM [^{3}H]OTA or 7.50 nM [^{3}H]OTA in the presence of 0-10 µM of non-radioactive OTA. Results are expressed as OTA concentrations recovered in the basolateral compartment. Means ± S.E.M. (n = 6-12) are given.*

3.5.2 Unlabelled ochratoxin A absorption experiments

Transport experiments with non-labelled OTA were performed, on one hand, to confirm OTA transport results obtained with the radio-labelled approach, and on the other hand, to determine if OTA is metabolized by the cells.

Biotransformation of OTA has not been elucidated in details. Data on OTA metabolism are controversial. Several metabolites have been characterized in vitro and/or in vivo, while other metabolites remain to be characterized (for the review, see Ringot *et al*., 2006). The contribution of the metabolites in OTA toxicity is still unclear.

Hydrolysis of OTA into a much less toxic compound, OTα, is the major metabolic pathway of OTA. This metabolite is formed by cleavage of the peptide bond. Carboxypeptidase A, trypsin, α-chymotrypsin and cathespin C have been shown to be able to hydrolyse OTA in vitro (Pitout, 1969; Doster & Sinnhuber, 1972).

Phase I reactions include detoxification as well as bioactivation reactions. A small percentage of absorbed OTA is hydroxylated into hydroxyochratoxin A, principally in the liver (Marquardt & Frohlich, 1992). Human, pig and rat liver microsomes have been shown to

metabolize OTA into several compounds, among which two epimers of 4-hydroxyochratoxin A (4-OH OTA), 4(R)- and 4(S)-OH OTA (Stormer & Pederson, 1980). Data on the cytochrome P450 isoforms involved in OTA metabolism are contradictory. Bioactivation reactions are still not well characterized. A number of studies have established that an oxidative mechanism is involved in OTA-mediated DNA damage and lipid peroxidation.

Phase II reactions include conjugation reactions resulting in the formation of glucurono-, sulphate-, gluthatione conjugates, as well as other *O*-conjugates (identified as pentose and hexose conjugates). Classically, conjugation is a detoxification step, but in some cases it can be a bioactivation process.

3.5.2.1 Detection of OTA

In the first biological transport experiments OTA was added to the apical compartment of Caco-2 cells. After 3 h incubation, samples from apical and basolateral compartments were collected. According to the experimental results obtained with the radio-labelled approach, OTA concentration should be 80-90 % and 10-20 % of the added amount of OTA for the apical and basolateral samples, respectively.

HPLC-FLD method

HPLC-FLD (section 2.4.1.1) was used to determine OTA concentration in the apical and basolateral samples. Calibration curves were set up for both apical and basolateral samples. The following experimental parameters were established:

- Measured OTA concentration initially added to the cells: 2.578 µg/ml
- Calibration curve for the apical samples:
  - $Y = 7058.7x + 6E+06$ $R^2 = 0.9987$
- Calibration curve for the basolateral samples:
  - $Y = 654.7x + 75025$ $R^2 = 0.995$

OTA percentage values for the apical and basolateral samples (Table 9) were in agreement with the results of the experiments with [^{3}H]OTA. Similar results were obtained with other amounts of OTA added to the cells.

Table 9. OTA amount determined in the apical and basolateral samples by the HPLC-FLD method.

	OTA Conc Apical (µg/ml)	%	OTA Conc Bas (µg/ml)	%
OTA (1)	2.283	88.6	0.309	12
OTA (2)	2.289	88.7	0.283	11
OTA (3)	2.301	89.3	0.361	14

Bas – basolateral
(1), (2), (3): indicates that the 3 samples came from 3 different inserts, inoculated with the same cellular suspension, and constitute 3 independent points of 1experiment.

LC-MS/MS method

To confirm the results obtained by the HPLC-FLD method, the analysis was also performed with the developed LC-MS/MS method (section 2.4.1.3). Calibration curves were set up for the analysis of both apical and basolateral samples. Experimental parameters were as follows:

- Measured OTA concentration initially added to CaCo-2 cells: 3.609 µg/ml
- Calibration curves for the apical samples:
  - $Y = 426.41x + 54852$ $R^2 = 0.9987$
- Calibration curve for the basolateral samples:
  - $Y = 654.7x + 75025$ $R^2 = 0.995$

Table 10. OTA amount determined in the apical and basolateral samples by the LC-MS/MS method.

	OTA Conc Apical (µg/ml)	%	OTA Conc Bas (µg/ml)	%
OTA (1)	2.872	79.6	0.505	14
OTA (2)	3.179	88.1	0.487	13.5
OTA (3)	2.746	76.1	0.685	19

Bas – basolateral
(1), (2), (3): indicates that the 3 samples came from 3 different inserts, inoculated with the same cellular suspension, and constitute 3 independent points of 1experiment.

OTA percentage values for the apical and basolateral samples (Table 10) were also in agreement with the values determined by the radio-labelled approach. Therefore, both methods, HPLC-FLD and LC-MS/MS, could be used to determine OTA concentration in the apical and basolateral samples. Similar results were obtained with other amounts of OTA added to the cells.

3.5.2.2 Detection of OTA and metabolites

For these experiments, OTA was added to the Caco-2 cell culture in the presence of certain CYP inducers. The inducer should promote possible metabolism of OTA in the cells. Inducers of CYP 1A1, such as 3-methylcolanthrene (3MC) and 2,3,7,8-tetrachlorodibenzo-p-dioxin

(TCDD), and inducers of CYP3A4, such as vitamin D (vit D), could stimulate the hydroxylation of OTA into hydroxyl metabolites. Apart from that, it should not be forgotten that the hydrolysis of OTA to OTα and L-phenylalanine is a very important metabolic pathway *in vivo*. Different OTA concentrations were added to the cells in order to take the higher LOD and LOQ values of the methods developed into account. Apical and basolateral samples were collected after the incubation during 3 h.

HPLC-FLD method

The developed HPLC-FLD method (section 2.4.2.1) was used to determine the presence of hydroxylated metabolites and the hydrolysis product of OTA in the samples. For each metabolite, a calibration curve was set up for the apical and basolateral samples. Several experiments were performed with different OTA concentrations added to the cell culture.

Experiment A

- Measured OTA concentration initially added to the cells: 203.9 ng/ml
- Calibration curves for the analysis of the apical samples:
  - OTα: y = 25748x – 6877.7 $R^2 = 0.9973$
  - 10-OH OTA: y = 14384x – 5673.3 $R^2 = 0.9979$
  - 4R-OH OTA: y = 4641.8x + 5024.3 $R^2 = 0.9983$
  - OTA: y = 17846x + 2466.3 $R^2 = 0.9967$
  - OTC: y = 9894.8x + 6827.4 $R^2 = 0.9965$
- Calibration curves for the analysis of the basolateral samples:
  - OTα: y = 13403x – 370.16 $R^2 = 0.9942$
  - 10-OH OTA: y = 9712.2x – 666.94 $R^2 = 0.9996$
  - 4R-OH OTA: y = 3367.5x – 395.02 $R^2 = 0.9977$
  - OTA: y = 16152x – 4246.1 $R^2 = 0.9981$
  - OTC: y = 11680x + 5144.6 $R^2 = 0.998$

OTA was added to Caco-2 cells alone or together with 3MC or with TCDD. The chromatograms of both apical and basolateral samples revealed no peaks of metabolites. Only OTA peak was present. The results of this experiment are shown in Table 11.

When only OTA was added to the cell culture, the percentage values determined for the apical and basolateral samples were in agreement with the results of radio-labelled experiments.

Upon addition of 3MC or TCDD, the OTA transport appeared to be somewhat slowed down but this tendency needs confirmation.

Table 11. Results of OTA determination in the apical and basolateral samples by the HPLC-FLD method (experiment A).

	OTA Conc Apical (ng/ml)	%	OTA Conc Bas (ng/ml)	%
OTA (1)	150.78	74.9	15.90	7.8
OTA (2)	132.8	65.1	17.13	8.4
OTA (3)	178.16	87.4	13.46	6.6
OTA + 3MC (1)	116.61	57.2	16.72	8.2
OTA + 3MC (2)	135.97	66.7	3.87	1.9
OTA + 3MC (3)	153.75	75.4	15.08	7.4
OTA + TCDD	127.89	62.7	6.52	3.2

Bas – basolateral
(1), (2), (3): indicates that the 3 samples came from 3 different inserts, inoculated with the same cellular suspension, and constitute 3 independent points of 1experiment.

Experiment B

- Measured OTA concentration initially added to the cells: 85.5 ng/ml
- Calibration curves for the analysis of the apical samples:
  - OTα: y = 22068x – 5346.7 $R^2 = 0.995$
  - 10-OH OTA: y = 13992x – 2536.9 $R^2 = 0.9961$
  - 4R-OH OTA: y = 3355.3x - 815.67 $R^2 = 0.9935$
  - OTA: y = 12796x – 202.51 $R^2 = 0.9965$
- Calibration curves for the analysis of the basolateral samples:
  - OTα: y = 19708x – 285.9 $R^2 = 0.9961$
  - 10-OH OTA: y = 11348x – 249.19 $R^2 = 0.9957$
  - 4R-OH OTA: y = 2824.1x – 793.79 $R^2 = 0.9935$
  - OTA: y = 21265x – 4486.9 $R^2 = 0.9963$

OTA was added to Caco-2 cells alone or together with 3MC. The chromatograms of the apical and basolateral samples did not indicate the presence of metabolites, only the peak of OTA was present. The results of this experiment are shown in Table 12. A great dispersion appears in the percentages of recovery, making it difficult to estimate an effect of 3MC on the transport efficiency.

Table 12. Results of OTA determination in the apical and basolateral samples by the HPLC-FLD method (experiment B).

	OTA Conc Apical (ng/ml)	%	OTA Conc Bas (ng/ml)	%
OTA (1)	59.84	69.9	4.07	4.76
OTA (2)	5.86	6.85	9.83	11.5
OTA (3)	64.83	75.8	5.22	6.11
OTA + 3MC (1)	22.66	26.5	6.43	7.52
OTA + 3MC (2)	85.18	99.6	4.09	4.79
OTA + 3MC (3)	22.63	26.46	4.81	5.62

Bas – basolateral

(1), (2), (3): indicates that the 3 samples came from 3 different inserts, inoculated with the same cellular suspension, and constitute 3 independent points of 1experiment.

Experiment C

- Measured OTA concentration initially added to the cells: 2.84 µg/ml
- Calibration curves for the analysis of the apical samples :
  - OTα: y = 11872x + 52156 $R^2 = 0.9943$
  - 10-OH OTA : y = 13135x + 71507 $R^2 = 0.9936$
  - 4R-OH OTA : y = 2833.8x + 15525 $R^2 = 0.9919$
  - OTA : y = 14744x + 46419 $R^2 = 0.9973$
- Calibration curves for the analysis of the basolateral samples:
  - OTα: 14827x – 2717.6 $R^2 = 0.9931$
  - 10-OH OTA: y = 12906x + 1832.4 $R^2 = 0.9993$
  - 4R-OH OTA: y = 3486.9x – 1047.8 $R^2 = 0.9927$
  - OTA: y = 15887x + 907.53 $R^2 = 0.9969$

OTA was added to Caco-2 cells alone or together with 3MC, or with TCDD, or with vitamin D. The chromatograms of the apical and basolateral samples revealed only the peak of OTA, and no metabolites were detected. OTA percentages determined for the apical and basolateral samples were generally in agreement with the results of radio-labelled experiments (Table 13). Research is in progress to confirm the results of HPLC-FLD for the detection of OTA metabolites with the LC-MS/MS method.

Table 13. Results of OTA determination in the apical and basolateral samples by the HPLC-FLD method (experiment C).

	OTA Conc Apical (µg/ml)	%	OTA Conc Bas (µg/ml)	%
OTA (1)	1.87	65.8	0.138	4.85
OTA (2)	2.62	92.2	0.243	8.55
OTA (3)	2.64	92.9	0.205	7.25
OTA + 3MC (1)	2.58	90.8	0.162	5.72
OTA + 3MC (2)	1.37	48.2	0.142	5.0
OTA + 3MC (3)	2.45	86.3	0.086	3.04
OTA + TCDD	2.64	92.9	0.359	12.6
OTA + Vit D	2.29	80.6	0.304	10.7

Bas – basolateral

(1), (2), (3): indicates that the 3 samples came from 3 different inserts, inoculated with the same cellular suspension, and constitute 3 independent points of 1experiment.

3.5.3 Effect of food contaminants/constituents on ochratoxin A absorption

Several mycotoxins and acrylamide were selected for the preliminary experiments on OTA absorption. Mycotoxins (*i.e.* deoxynivalenol, fumonisin B_1, zearalenone or penicillic acid) or acrylamide were introduced in the apical compartment of the Caco-2 cell culture system, at 25 or 100 µM, respectively, together with [^{3}H]OTA. Experiments were performed as described above (section 2.3.2.2). No effect of these contaminants on OTA transport from the apical to the basolateral compartment was detected (results not shown). No more investigations of OTA transport with these contaminants were performed, since many other food components had to be tested.

3.5.3.1 Effect of plant constituents on ochratoxin A absorption

Polyphenols (PPs) were considered for possible interactions with OTA transport. They are known to be powerful antioxidants. PPs receive increasing interest due to their incidence on the prevention of various diseases such as cancers, cardiovascular diseases, inflammation, etc. They are widely consumed, the main polyphenol dietary sources being fruits and beverages (fruit juices, wine, tea, coffee, chocolate and beer), as well as dietary supplements. PP representatives of the main classes were chosen: resveratrol (stilbene), gallic acid (phenolic acid) and flavonoids from major groups, *i.e.* quercetin (flavonol), chrysin (flavone), (+)-catechin, (-)-epigallocatechingallate (EGCG) (flavanol), genistein and biochanin A (isoflavone). Under the experimental conditions used, PPs did not alter the integrity of differentiated monolayers of Caco-2 cells, even though they affected metabolic activity (MTT assay) of proliferating cells. Experiments on OTA transport were performed with 50 µM PPs,

a non-toxic, average concentration easily reached in the gastrointestinal tract of people with a normal diet.

Figure 8 shows the effect of PPs on the absorption of OTA introduced apically at 0.75 nM, 7.5 nM or 10 µM, for 3 hours. Chrysin, quercetin, genistein, biochanin A and resveratrol increased significantly the transport of OTA to the basolateral pole: these PPs had a maximal effect at 7.5 nM OTA, increasing its absorption by 80 to 150 % above the control value. Used as a positive control, MK571, a well-known specific inhibitor of the multidrug resistance-associated proteins (MRPs), increased OTA absorption by 55.2 ± 16.2 %. Two flavanols, (+)-catechin and EGCG, as well as gallic acid, had no significative effect on OTA absorption**.**

To determine whether the effect of PPs on OTA absorption was dose-dependent, the apical to basolateral transport of OTA at 7.5 nM was investigated in the presence of PPs at different concentrations (Fig. 9). Used as negative control, (+)-Catechin, had no effect on OTA transport. In contrast, quercetin and resveratrol induced a concentration-dependent increase of OTA absorption that reached a plateau at ca. 50 µM, half-effect being attained at ca. 5 µM or 15 µM, respectively.

On the basis of the literature, we can assume that quercetin, chrysin, genistein and resveratrol are extensively taken up and metabolized by the Caco-2 cells. Their metabolites, being substrates of the MRP-2 efflux pump, could therefore inhibit the OTA efflux by competition with OTA or its metabolites for access to MRP-2. As additional evidence, the concentration-dependent effects observed for quercetin and resveratrol on OTA transport are consistent with a model of competitive inhibition of PPs or their metabolites, with saturation of MRP-2 efflux at higher concentrations.

According to a study initiated by the European Union, nearly everybody in Europe is exposed to OTA (EC, 1997). Recent studies (Galvano *et al*., 2001; Atroshi *et al*., 2002) have dealt with the development of dietary strategies to counteract the effects of mycotoxins and have reported on natural (vitamins, carotenoids, phenolics, etc.) and synthetic compounds with antioxidant properties that seem very efficient in protecting against the toxic effects of mycotoxins. Nevertheless, our study largely suggests that

some PPs, at concentrations that should be raised after ingestion of a normal diet, could strongly increase the intestinal absorption of OTA, in a range of concentrations that can again be encountered in usual contaminated food.

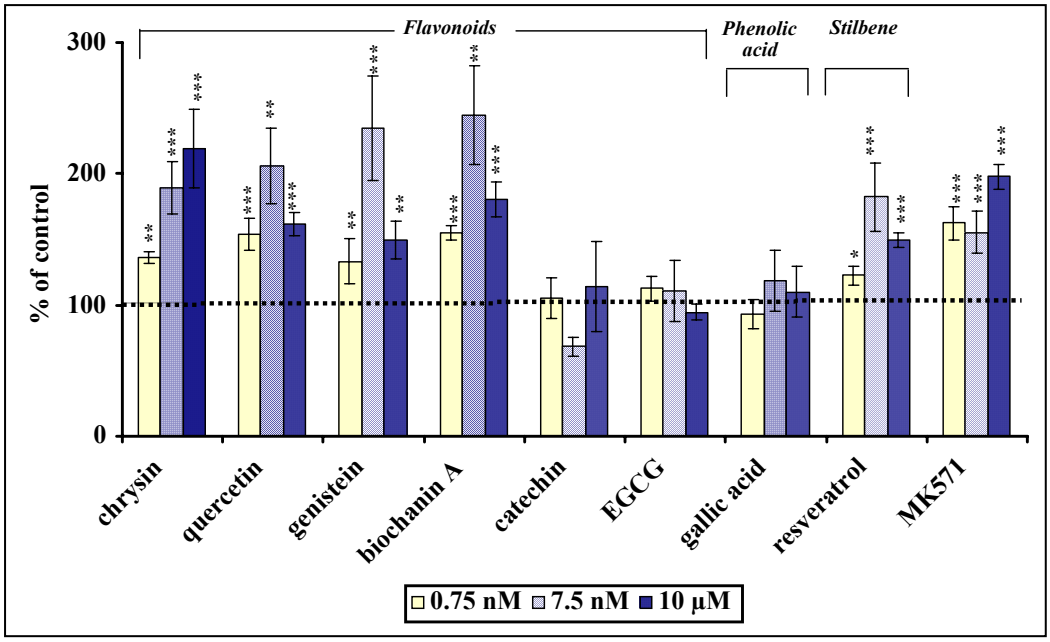


Figure 8. *Effect of different PPs on OTA absorption.*
*Caco-2 cells were incubated for 3 h apically with 0.75 nM [^{3}H]OTA, or 7.5 nM [^{3}H]OTA, or 7.5 nM [^{3}H]OTA with 10 µM non-radioactive OTA, in the presence or absence (control) of 50 µM PPs or 50 µM MK571. Results are expressed as a percentage of control of the OTA concentration recovered in the basolateral compartment. Means ± S.E.M. (n = 6-12) are given. ***, **, * indicate, respectively, P < 0.001, P < 0.01, P < 0.05, as compared with the control condition.*

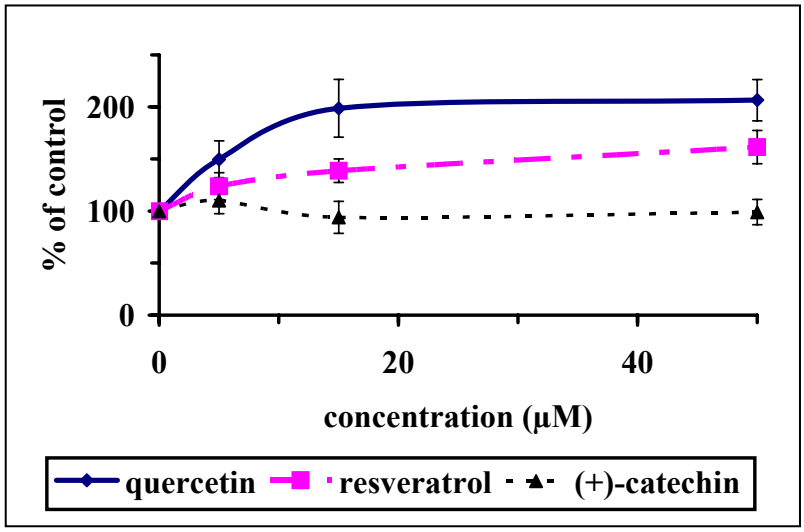


Figure 9. *Effects of PPs concentration on OTA absorption. Caco-2 cells were incubated apically with 7.5 nM [^{3}H]OTA for 3 h in the presence of 5, 15 or 50 µM of quercetin, resveratrol or (+)-catechin. Results are expressed as percentage of control of OTA transported to the basolateral compartment. Means ± S.E.M. (n = 6-11) are given.*

3.5.3.2 Effect of heavy metals on ochratoxin A absorption

Heavy metals represent one of the major groups of environmental contaminants. They naturally occur in rocks and soils. These metals can also originate from various industrial activities. They are absorbed by plants and thus may enter the food chain. Cadmium (Cd), mercury (Hg) and lead (Pb) are heavy metals of major concern (Vakharia *et al*., 2001). Therefore, they were considered for possible interactions with OTA transport.

Caco-2 cells were cultivated in bicameral inserts for 21 days. Afterwards, they were apically exposed to a high dose of one of three heavy metals during a short period of time (3 h) to mimic an acute contamination with the metals ($Pb(NO_3)_2$, 640 nM; $HgCl_2$, 70 nM; $CdCl_2$, 220 nM). The cells were then incubated for 3 h in the presence of 7.5 nM [^{3}H]OTA and the metals to determine if heavy metals affected OTA absorption. Our data indicated that the metals, at the concentrations tested, did not significantly modify OTA quantities transported from the apical to the basolateral side (data not shown).

Effects of heavy metals during longer periods of time were then studied at the same concentrations as described above. Caco-2 cells were cultivated in the inserts during 7 days and then during 14 days in the presence of high doses of the metals in order to mimic a chronic exposure. The cells were then incubated with [^{3}H]OTA (7.5 nM) in the presence of heavy metals or MK571 (50 µM), a specific inhibitor of MRP efflux pumps (Gekeler *et al*., 1995) (Fig. 10). MK571 and Cd^{2+} significantly increased OTA absorption, whereas Pb^{2+} and Hg^{2+} had only a slight tendency to increase it.

The effects of heavy metals on OTA cellular accumulation were also investigated through the collection and analysis of the cell layers at the end of the OTA transport experiments. The results demonstrated that MK571 triggered a significant OTA accumulation in Caco-2 cells (Fig. 11). By contrast, the accumulation of OTA in the cells treated with heavy metals was not significantly affected. The fact that both MK571 and Cd^{2+} significantly increased OTA transport, whereas only MK571 increased OTA accumulation in the cells suggests that Cd^{2+} probably affects the cellular permeability in our system.

It should be mentioned that under the experimental conditions described, no cytotoxicity was detected by the measurement of LDH activity.

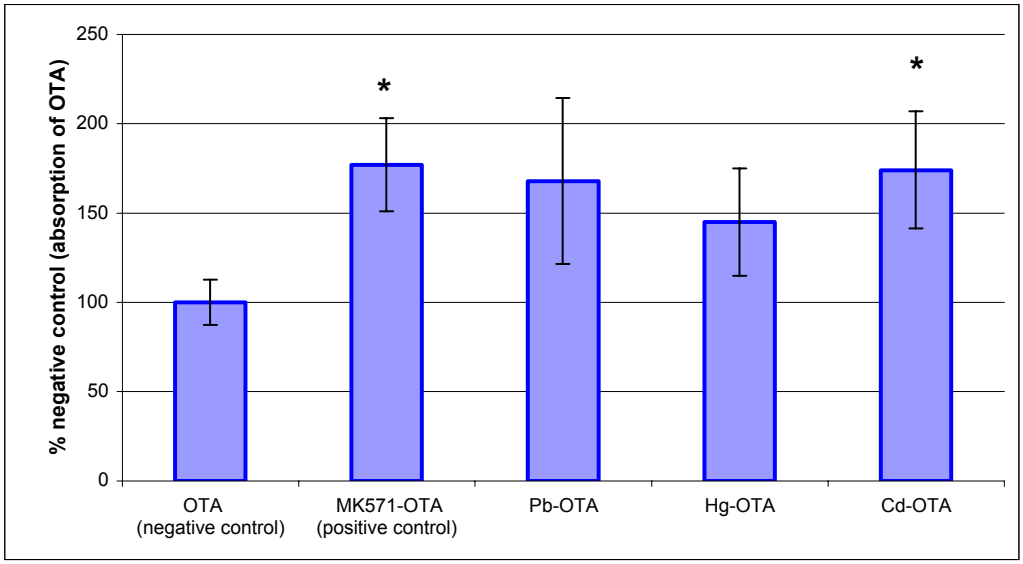


Figure 10. *Effect of heavy metals on OTA absorption. Caco-2 cells were cultivated in the inserts for 21 days and incubated during last 14 days in the presence of $Pb(NO_3)_2$ (640 nM), $HgCl_2$ (70 nM) or $CdCl_2$ (220 nM). They were then incubated for 3 h with 7.5 nM [^{3}H]OTA still in the presence of one of the above-mentionned metals. Results are expressed as a percentage of the amount of OTA transported to the basolateral compartment in the control conditions (absence of any treatment). The positive control was the absorption of OTA in the presence of MK571 (50 µM). Statistical analysis was performed with ANOVA 1 by the Sheffé method at the threshold of 5 %. Mean values ± SEM (three independent experiments, n = 9-15) are given. * indicates P<0.05, as compared with the absorption of OTA alone.*

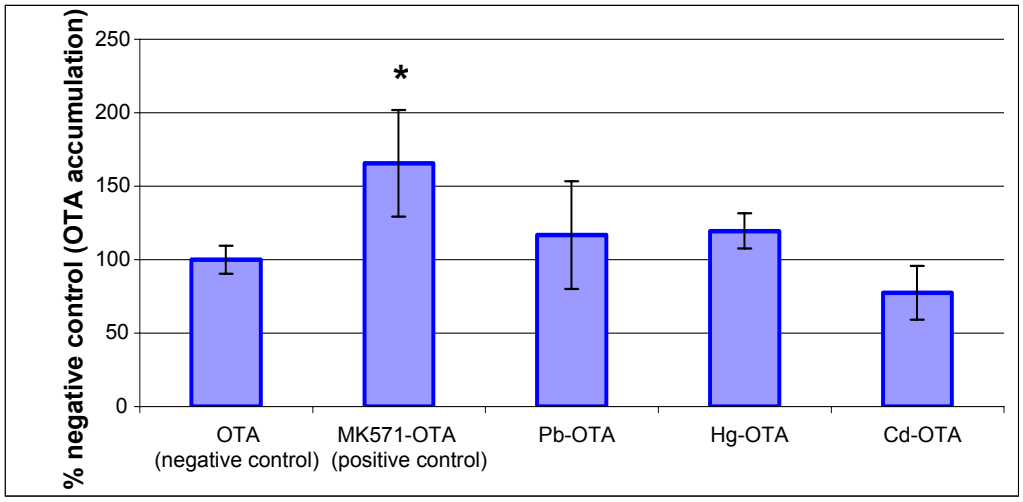


Figure 11. *Effect of heavy metals on OTA accumulation in Caco-2 cells. The same experimental protocol as for Fig. 9 was used. At the end of transport experiments, the cells were collected and lysed to measure the accumulation of OTA in the cells. Statistical analyses were performed with ANOVA 1 by the Sheffé method at the threshold of 5 %. Mean values ± SEM (three independent experiments, n = 9-15) are given. * indicates P<0.05, as compared with the accumulation of OTA in the cells without any treatment.*

3.5.3.3 Effect of pesticides on ochratoxin A absorption

Pesticides can be found as residues in the human food chain. Various relevant pesticides were selected and assayed for their potential effect on OTA absorption, as described above for other contaminants. The pesticides were introduced in the apical compartment of the Caco-2 cell culture system at their maximal plausible concentration, which can be found in the intestine after a meal, *i.e.* 500 ng/ml for imidacloprid and for thiram, 1000 ng/ml for chlorpyrifos, 2500 ng/ml for glyphosate, 1 ng/ml for atrazine, 5000 ng/ml for captan and for thiabendazole. They were incubated for 3 h together with 7.5 nM [^{3}H]OTA. The results presented in Figure 12 revealed that only captan increased OTA transport (± 62% above the control value). MK571, the positive control, increased it by ± 64%. At the end of the transport experiments, an increase in the cellular OTA content was also measured in the cells incubated in the presence of captan and MK571 (results not shown). Similar results have been observed in the experiments with higher concentrations of pesticides. No direct cytotoxicity determined by LDH assay was observed. However, a decrease of the TEER after 3 h incubation with captan was detected. TEER decrease usually corresponds to the disruption of intestinal barrier integrity. Thus, the increase in OTA transport in the presence of captan could result from two different effects: on one hand, an inhibitory effect on the MRP-2 efflux pump and, on the other hand, a modification of the cellular permeability. However, these results are preliminary and should be confirmed.

3.5.3.4 Effect of polycyclic aromatic hydrocarbons and TCDD on ochratoxin A absorption

Polycyclic aromatic hydrocarbons (PAHs), such as benzo(a)pyrene (B(a)P), 3-methyl-cholanthrene (3-MC) and TCDD, are environmental pollutants, which can occur in the food chain. Their possible effect on OTA transport was also investigated as described above. None of them induced a significant increase in OTA transport (Fig. 13).

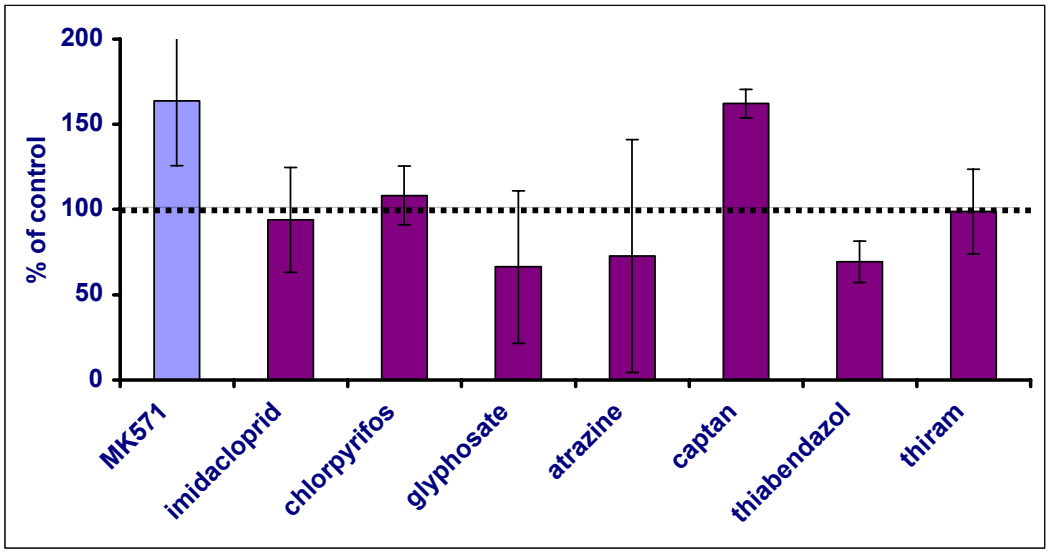


Figure 12. *Effect of pesticides on OTA absorption. Caco-2 cells were incubated for 3 h apically with 7.5 nM [^{3}H]OTA in the presence or absence (control) of 50 µM MK571, 500 ng/ml imidacloprid, 1000 ng/ml chlorpyrifos, 2500 ng/ml glyphosate, 1 ng/ml atrazine, 5000 ng/ml captan, 5000 ng/ml thiabendazol or 500 ng/ml thiram. Results are expressed as a percentage of the control of OTA recovered in the basolateral compartment. Means ± S.D. (n = 3) are given.*

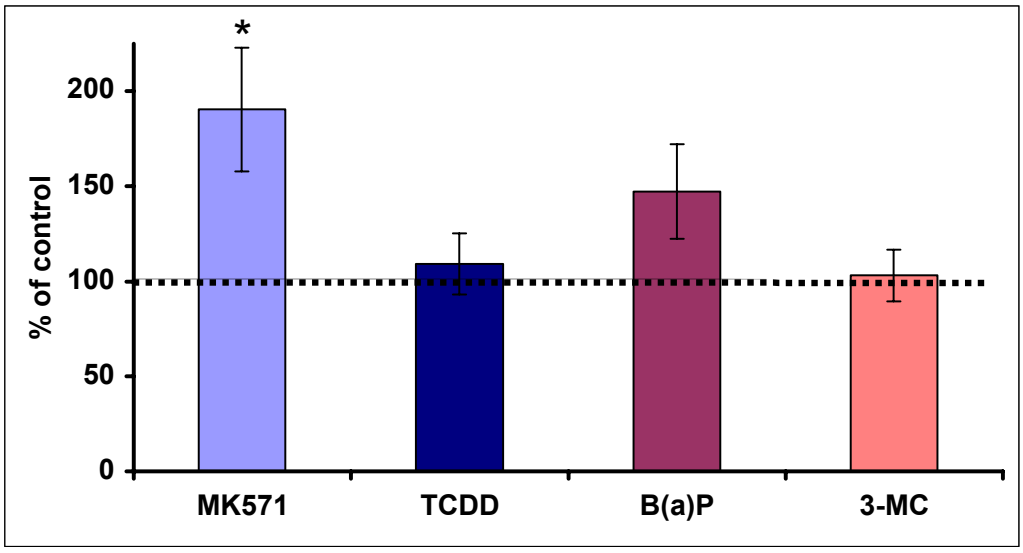


Figure 13. *Effect of TCDD and PAHs on OTA absorption. Caco-2 cells were incubated for 3 h apically with 7.5 nM [^{3}H]OTA in the presence or absence (control) of 50 µM MK571, 0.322 pg/ml TCDD, 1 ng/ml benzo(a)pyrene (B(a)P) or 1 ng/ml 3-methylcholanthrene (3-MC). Results are expressed as a percentage of the control of OTA recovered in the basolateral compartment. Means ± S.E.M. (n = 11-20) are given. * indicates P<0.05, as compared with the control condition.*

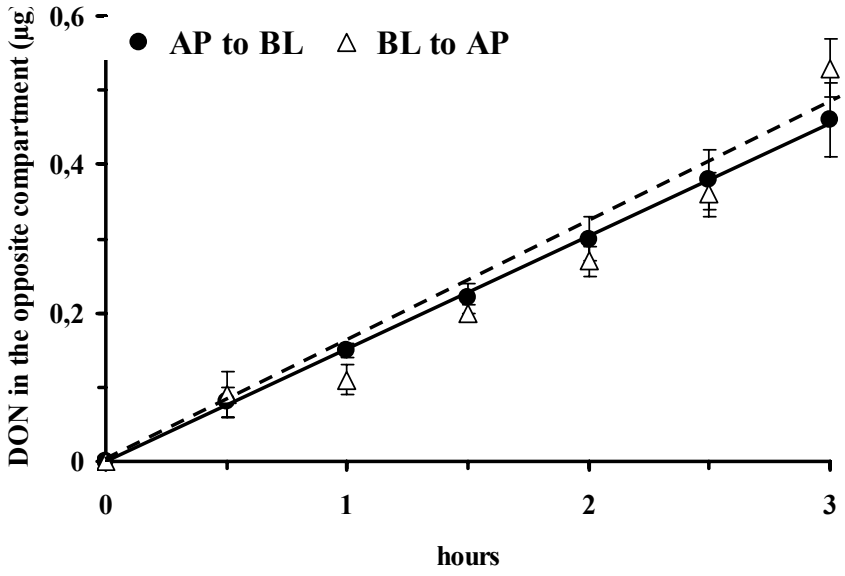
3.6 Deoxynivalenol bioavailability studies

Little is known about the metabolism and kinetics of DON when it passes through the human digestive tract. Transport rates of DON from the apical to the basolateral side and vice versa were used to investigate the mechanism involved in DON transport across Caco-2 cells. Concentrations from 0.16 to 2 µg/ml were used in the experiments. DON analysis was performed by HPLC-UV (section 2.4.3). The influence of plant constituents (polyphenols) and specific inhibitors of some proteins known to be involved in the transport of various contaminants was then determined.

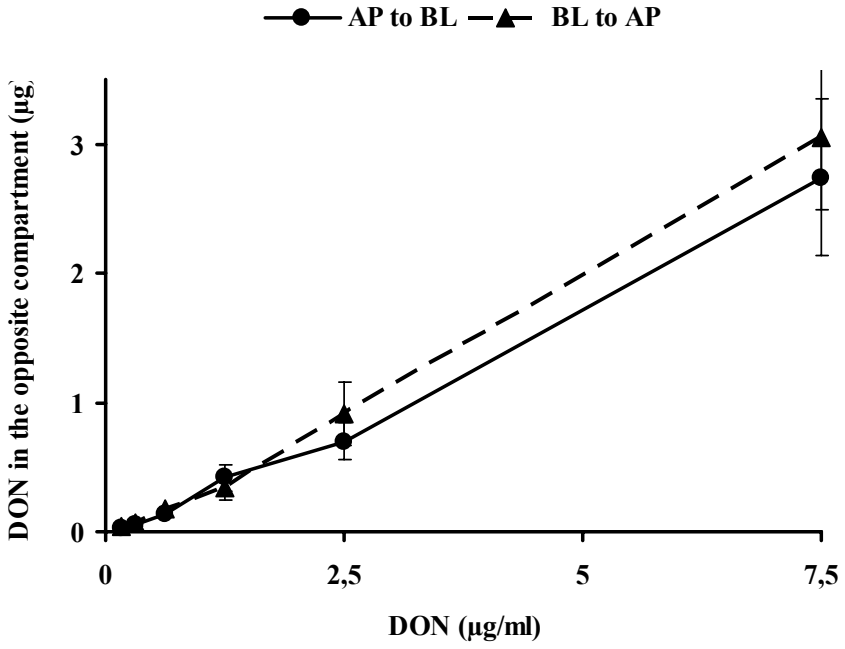
3.6.1 Deoxynivalenol transport experiments

The passage of DON was studied on differentiated Caco-2 cells cultivated for 21–27 days in bicameral inserts. DON at 2 µg/ml was added to either the apical (AP) or basolateral (BL) compartment and its appearance was determined in the opposite compartment after different durations (Fig. 14A). Results indicate that the passage of DON was proportional to the duration of the incubation. The transport rate of DON was similar in the AP to BL and BL to AP directions, reaching values of 0.152 and 0.153 µg h^{-1}, respectively.

The transport of DON was also investigated in both directions at different toxin concentrations ranging from 0.16 to 7.5 µg/ml. The duration of incubation was then set at 3 h (Fig. 14B). DON transport in both directions was strictly proportional to the concentration over the entire range. The mean absorption (passage from AP to BL) rate after 3 h was 13.8 ± 2.1 % of the initial dose, whereas the mean excretion (passage from BL to AP) rate was 10.5 ± 1.1 % of the initial concentration, with no significant difference. DON recovered in the apical plus basolateral compartments in each culture insert corresponded to 102 ± 2 % of the initially introduced DON. No differences in the HPLC profiles were seen after treatment of the samples with the deconjugation enzymes, β-glucuronidase and arylsulfatase, suggesting the absence of DON processing in the cells.



(A)



(B)

Figure 14. *DON transport across Caco-2 cells cultivated in bicameral inserts. (A) Kinetics of passage using 2 µg/ml DON added in either the apical (AP) or basolateral (BL) compartment of the inserts. The appearance of DON in the opposite compartment was monitored after various durations from 0.5 to 3 h. (B) Dose-effect of DON added in either the AP or BL compartment at concentrations ranging from 0.16 to 7.5 µg/ml. The duration of incubation was set at 3 h. Results are means ± S.E.M. (two independent experiments, n = 5 to 10).*

During our transport experiments limited to a maximum of 3 h, no toxic effects of DON, as determined by LDH release, cellular morphology and TEER measurements, was detected, indicating that a short exposure to low or high DON concentrations did not alter the epithelial

barrier integrity. By contrast, 24 h incubations led to falls in TEER values. However, this last observation did not seem to result from a cytotoxic effect since no LDH release was observed.

3.6.2 Effect of specific inhibitors on deoxynivalenol absorption

To investigate the possible involvement of transport systems, DON transport experiments were performed on the monolayers incubated in the absence or presence of inhibitors of the efflux pumps. EGTA, a calcium chelator known to open the intercellular tight junctions, was also tested for its effect on DON transport. As shown in Figure 15, neither verapamil, an inhibitor of P-glycoprotein (PgP), nor MK571, CDNB, quercetin, resveratrol and taurocholate, inhibitors of multidrug resistance-associated proteins (MRPs), did affect the transport of DON in both AP to BL and BL to AP directions. No significant decrease in TEER values was measured. In the presence of EGTA, a large increase of AP to BL and BL to AP passage of DON, with absorption greater than excretion, was measured, while a TEER fall was observed.

Taken together, our results suggest that DON is neither an efflux pump substrate, nor metabolized significantly, which would be in line with the hypothesis that DON does not penetrate in the Caco-2 cells to a level high enough to become a substrate of these systems. This could further argue in favour of the DON transport largely by the paracellular way.

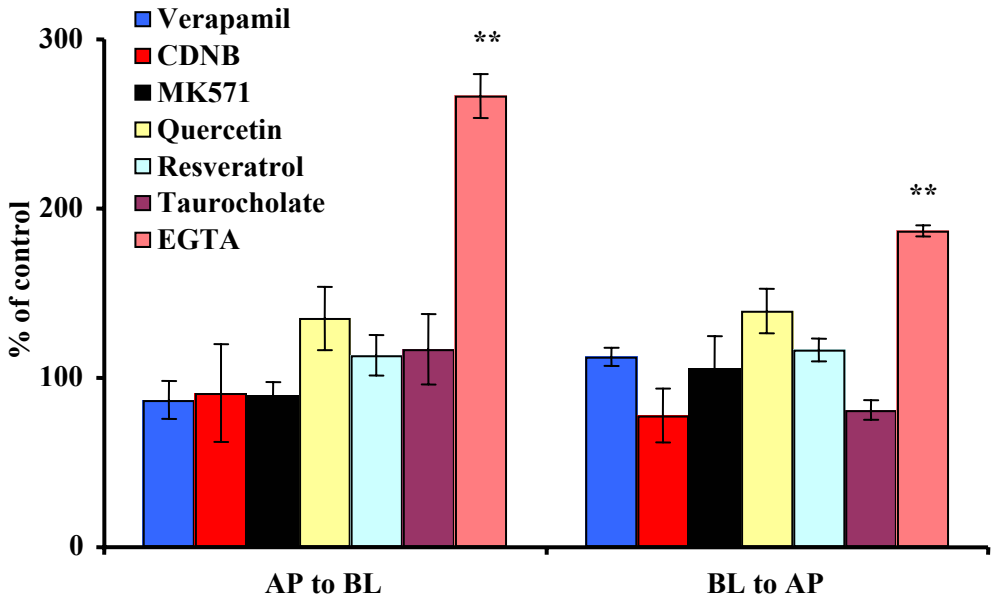


Figure 15. *Effect of efflux pump inhibitors or EGTA on the apical (AP) to basolateral (BL) and BL to AP transport of DON across Caco-2 cells. The cells were incubated for 3 h with DON at 2 µg/ml in the presence of 100 µM verapamil, 200 µM CDNB, 50 µM MK571, 50 µM quercetin, 50 µM resveratrol, 2mM sodium taurocholate or 2.5mM EGTA. Results are expressed as a percentage of the control of the DON concentration recovered in the opposite compartment. Means ± S.E.M. (two independent experiments, n = 6) are given. ** indicates P < 0.001, as compared to the control condition.*

3.7 Investigation of deoxynivalenol effect on the intestinal inflammatory parameters

Since our results suggest that DON is transported across the intestinal epithelium mostly by simple diffusion, the effect of other food contaminants on DON transport has not been checked. Then a shift has been made to investigate the possible effects of DON on the intestinal function.

3.7.1 Effect on mitogen-activated protein kinases (MAPKs) and intestinal permeability

The effect of DON on MAPK phosphorylation was assessed on differentiated Caco-2 cells incubated with 2 µg DON/ml at their apical side for 5–240 min (Fig. 16A). DON transiently increased the phosphorylation of Erk1/Erk2 during 5 min as well as the phosphorylation of p38, from 5 to 30 min. A slight and prolonged phosphorylation of SAPK/JNK (5–240 min) was also observed. Total Erk1/Erk2, p38 and SAPK/JNK remained unchanged in these conditions.

The phosphorylation of MAPKs was also investigated on Caco-2 cells apically exposed for 24 h to 0.5 µg DON/ml, 1 µg DON/ml or 2 µg DON/ml. The results (Fig. 16B) revealed that a prolonged exposure to DON induced the phosphorylation of MAPKs: Erk1/Erk2 phosphorylation was observed from 0.5 µg DON/ml and was maximal at 1 µg DON/ml. The phosphorylation of p38 and SAPK/JNK increased with increasing DON concentrations. Total MAPKs were unchanged.

A marked effect of DON on Caco-2 monolayer integrity was observed along the incubations with 2 µg DON/ml (Fig. 17A): after 24 h incubation, the TEER values decreased to 30% of the control. A dose-dependent decrease of TEER was also obtained after 24 h exposure to different DON concentrations (Fig. 17B): a significant reduction of TEER was reached at 0.5 µg DON/ml and the effect was maximal at 4 µg/ml.

No apoptosis increase, evaluated by DNA fragments ELISA assay, was observed in any of the tested conditions. No cytotoxicity was detected at the end of the experiments either through

morphological observation under phase contrast microscopy or through the measurement of the LDH activity in the cellular media.

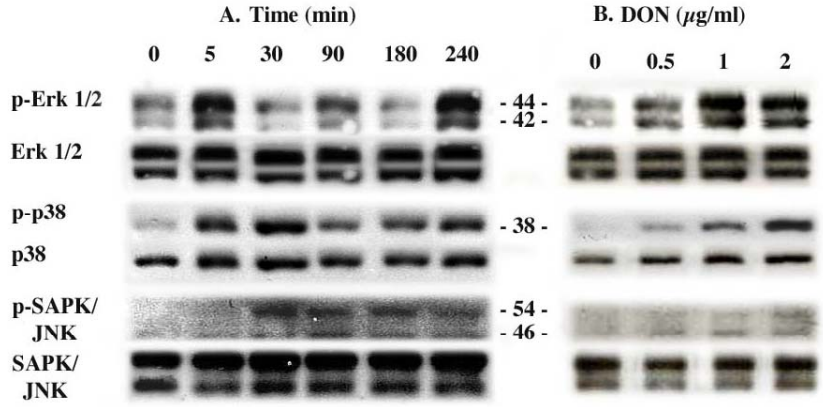
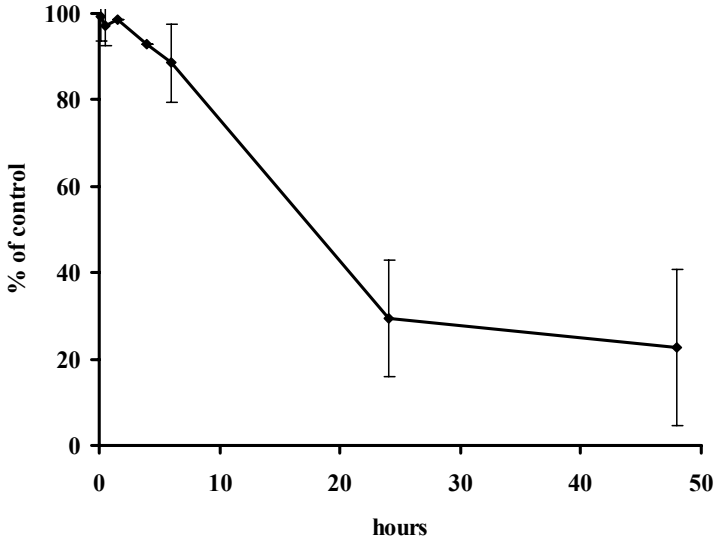
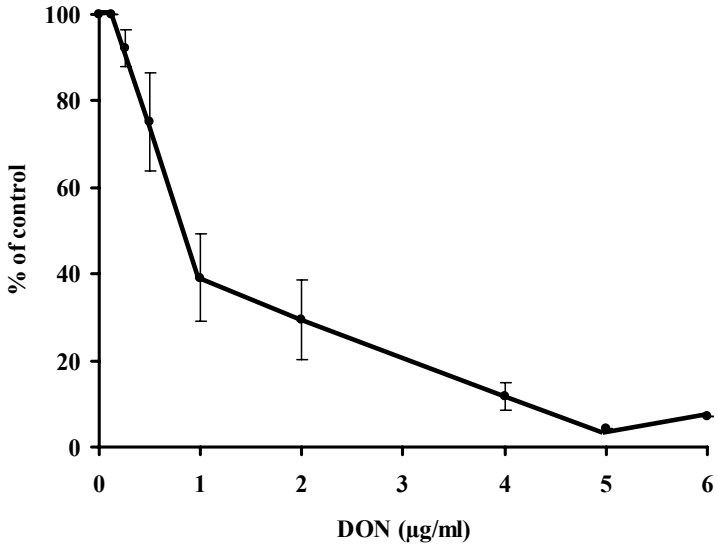


Figure 16. *Effects of DON on MAPK phosphorylation. (A) Time-course activation of Erk1/Erk2, p38 and SAPK/JNK in Caco-2 cells incubated, or not, apically with 2 μg/ml of DON. (B) Dose-dependent effect of DON on Erk1/Erk2, p38 and SAPK/JNK phosphorylation in Caco-2 cells incubated, or not, apically with 0.5, 1, 2 or 4 μg/ml of DON for 24 h. Cell lysates were resolved on SDS-PAGE and submitted to Western blot analysis with antibodies specific to phosphorylated Erks 1/2, p38 and SAPK/JNK. Bands were detected using the ECL-plus system. Afterwards, the blots were stripped and re-probed with specific antibodies that recognized both phosphorylated and unphosphorylated forms of each MAPK for the assessment of protein loading.*

DON has been reported to induce MAPK phosphorylation in macrophages, which contributes to enhanced cytokine gene expression, up-regulated cytokine production and apoptosis (Zhou *et al*., 2003; Pestka *et al*., 2005). The data presented here provide evidence of the activation by DON of the three well-known groups of MAPKs, i.e. Erk1/2, p38 MAPK and SAPK/JNK, in the intestinal Caco-2 cells. The MAPK phosphorylation was concentration-dependent and was maintained during prolonged exposures to DON, while a concomitant decrease in TEER was observed. No apoptosis was detected. A similar decrease in TEER has been described by Kasuga *et al*. (1998) after a chronic 2-weeks incubation of Caco-2 cells with DON at the concentrations from 0.05 to 0.2 μg/ml, introduced in both apical and basolateral compartments of the cell culture inserts. A toxic effect of DON on the intestinal permeability and on nutrient absorptive functions has also been reported by Maresca *et al*. (2002) after 48 h incubation of HT-29-D4 cells, a human intestinal cell line, with high concentrations of DON, i.e. 10 and 100 μM (29.8 μg/ml).



(A)



(B)

Figure 17. *Effects of DON on the intestinal permeability as assayed by TEER measurements. (A) Time-course effect of DON (2 µg/ml) on Caco-2 cells. (B) Dose-dependent effect of DON upon 24 h incubations with the CaCo-2 cells. Results are expressed as a percentage of control and are means ± S.E.M. (four to six independent experiments).*

3.7.2 Effect on the activation of transcription factor NF-κB in Caco-2 cells

Our studies indicate that a prolonged exposure of Caco-2 cells to DON provokes the phosphorylation of MAPKs Erk1/2, p38 and SAPK/JNK. A decrease of TEER was also observed in these cells, resulting in increased intestinal permeability. Inflammatory bowel diseases (IBD) are characterized by the alteration of intestinal barrier integrity and implication of signal transduction pathways including activation of NF-κB and MAPKs. DON has been suggested as a pro-inflammatory substance by a number of *in vivo* and *in vitro* studies (Wong *et al*., 2002; Zhou *et al*., 2003). On the basis of our observation with Caco-2 cells that DON disrupts the integrity of the intestinal barrier and activates MAPKs, it is plausible that DON could trigger intestinal inflammation.

Our previous results demonstrated a dose-dependent increase in MAPK phosphorylation in Caco-2 cells exposed for 24 h to DON at concentrations ranging from 0.5 to 4 µg/ml (section 3.7.1). In order to investigate whether downstream targets of MAPKs were also activated in these conditions, we analyzed the activity of the NF-κB transcription factor using a luciferase reporter plasmid driven by a promoter containing four response elements to NF-κB. Transfected Caco-2 cells were treated for 24 h with DON at concentrations from 50 ng/ml to 10 µg/ml. As shown in Figure 18, exposure of Caco-2 cells to DON concentrations higher than 1 µg/ml significantly increased NF-κB induced luciferase activity ($p \leq 0.05$).

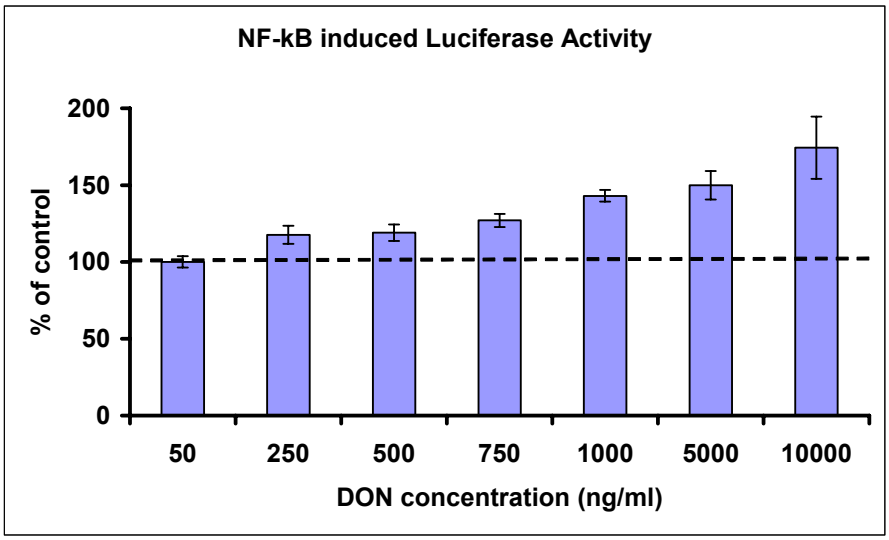


Figure 18. *Effect of DON concentration on the luciferase activity in Caco-2 cells transfected with a luciferase reporter plasmid containing a promoter with 4 response elements to NF-κB. Cells were treated for 24 h with different DON concentrations. Results are expressed as a percentage of the relative luminescence of the control consisting of untreated transfected Caco-2 cells. Means ± S.E.M. (n = 7-15) are given.*

Polyphenols have been reported to modulate parameters of the intestinal inflammation in Caco-2 cells (Liang *et al.*, 2001) as well as in other cell systems (Galvez *et al*., 2001; Manna *et al.*, 2000; Surh *et al*., 2001). Thus, we tested the effect of polyphenols on DON-induced NF-κB activity. Caco-2 cells transfected with the plasmid mentioned above were pre-incubated for 4 h with 50 µM polyphenols, *i.e.* ellagic acid, chrysin, epicatechin, genistein or resveratrol, before their exposition to DON at 1000 ng/ml for 24 h. Controls in the presence/absence of polyphenols but without DON were also analyzed. Exposure of Caco-2 cells to DON increased NF-κB induced luciferase activity but this increase was totally inhibited by some polyphenols, *i.e.* ellagic acid and chrysin (Fig. 19). The other polyphenols (epicatechin, genistein, resveratrol) did not have any effect on DON-induced NF-κB-luciferase activity.

Our observations indicate that DON at high concentrations induces the activation of the transcription factor NF-κB in Caco-2 cells. These data are consistent with the previous findings of *in vitro* studies performed on human immune cells (Wong *et al.,* 2002). Regarding our previous results (section 3.7.1) we can assume that activation of NF-κB in Caco-2 cells is linked to the phosphorylation of MAPKs. Some polyphenols (ellagic acid, chrysin) inhibit DON-induced NF-κB activity and thus protect cells against DON toxicity. Investigation is in progress to determine if other food chemicals (*i.e.* ω-3 fatty acids) known to have an effect on inflammation could diminish or even suppress the action of DON on intestinal epithelial cells.

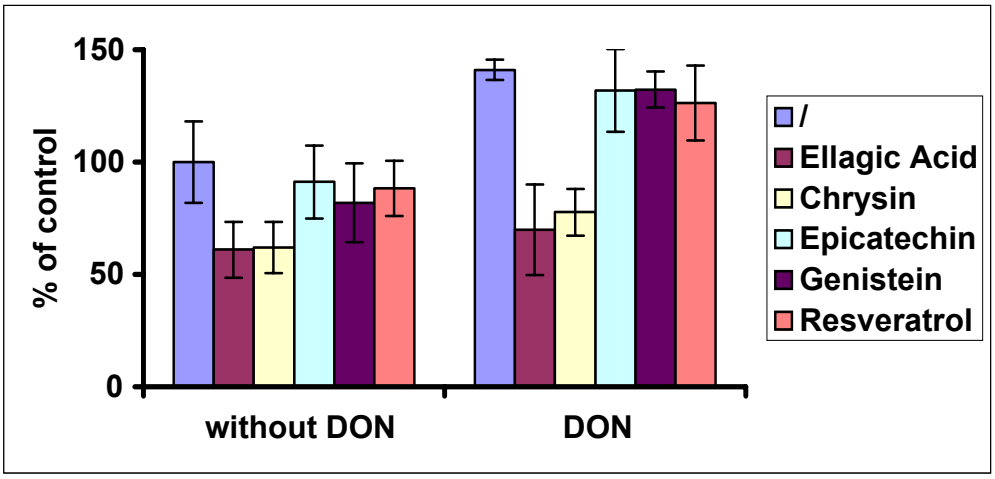


Figure 19. *Effect of polyphenols on the DON-induced luciferase activity in Caco-2 cells transfected with a luciferase reporter plasmid containing a promoter with 4 response elements to NF-κB. Cells were pre-treated for 4 h (no pre-incubation for the control) with 50 μM ellagic acid, chrysin, epicatechin, genistein or resveratrol before incubation with (or without) 1000 ng/ml DON for 24 h. Data are means ± SD (n = 3).*

4 CONCLUSIONS AND RECOMMENDATIONS

4.1 Analytical methodology

Conclusions

HPLC-FLD and LC-MS/MS methods were developed and validated for the detection of OTA in biological samples, i.e. cellular extracts and culture media after OTA transepithelial transport experiments. These methods and the classical radio-labelled approach led to similar results in terms of OTA transepithelial transport efficiency.

The HPLC-FLD method was successfully developed to detect OTA together with its possible metabolites, such as OTα, 4(R)-OH OTA and 10-OH OTA. In order to detect the presence of metabolites, OTA was added to the apical compartment of Caco-2 cells in the presence of inducers of cytochromes P450, such as 3MC, TCDD and vitamin D. No metabolites of OTA were detected in all the experiments. Only OTA was present in the samples. Possible explanations are:

- Non-physiological conditions of the *in vitro* system used, i.e. the use of buffer solutions instead of intestinal fluid, the fact that Caco-2 cells are derived from an adenocarcinoma, the absence of the other cell types, which are normally present in the intestinal mucosa, and the absence of neuroendocrine control.
- Weak expression of CYP 3A4 in Caco-2 cells. Treatment with a CYP inducer should increase the expression level, but maybe not up to the levels observed *in vivo*.
- Poor hydroxylation of OTA *in vivo*. It is possible that the concentration of formed metabolites is too low to be detected with the developed HPLC-FLD method.

Recommendations

New experiments should be performed. Increased incubation time should be checked to examine whether higher amounts of the metabolites can be formed. It would also be necessary to analyse the biological samples with a fully validated LC-MS/MS method to confirm the absence of metabolites. An effort should be made to determine other possible metabolites, such as glucurono-, sulphate-, gluthatione-, pentose- and hexose-conjugates. However, the standards are currently not available. Synthesis of the conjugates is a likely solution of this problem.

4.2 Biological effects

4.2.1 Modulation of OTA bioavailability by dietary compounds

Conclusions

At plausible intestinal concentrations, some polyphenols (PPs) and dietary contaminants, such as captan (pesticide) or cadmium (heavy metal), markedly increase OTA absorption.

The results on OTA interactions with PPs are of particular importance. Our experiments demonstrate that several PPs significantly increase the absorption of OTA *in vitro* at concentrations that should easily be reached in the gastrointestinal tract of people with a classical diet. This effect probably results from a competitive inhibition of the MRP2 efflux pump. Since the Caco-2 cell culture system is a widely accepted tool to investigate transport parameters in human intestine, one should expect that these PPs would also increase the bioavailability of OTA *in vivo*. Therefore, ingestion of a classical diet including OTA contaminated foodstuffs and PPs could lead to an increased OTA concentration in the bloodstream with possible adverse effects for human health. These results provide a proof of concept evidencing that the strategy used in the present project is appropriate to highlight the interactions between chemicals resulting in the modulation of mycotoxin bioavailability.

Recommendations

Since the beneficial effects of PPs appear increasingly evident for human health, it is of particular importance to improve the detection methods of OTA in food and feed, to increase controls of contamination and to develop prevention strategies to avoid excessive OTA contamination levels. In parallel, further research is required to better understand the effects of PPs on intestinal physiology and, in particular, on gene expression and protein activities.

4.2.2 DON transport and its effects on intestinal function

Conclusions

The results of DON transport experiments suggest that this mycotoxin is transported across the epithelial barrier of the intestine mainly by passive diffusion through the paracellular

route. Our data imply that DON is neither an efflux pump substrate, nor metabolized significantly by Caco-2 cells. No modulation of DON absorption by PPs has been observed.

Our results clearly indicate that a chronic exposure to DON at concentrations that could be reached in the gastrointestinal tract of people with a moderately to highly DON contaminated diet has a negative impact on the intestinal epithelium by disrupting the intestinal barrier integrity and activating MAPKs and NF-κB, implicated in intestinal inflammation. Some PPs (ellagic acid, chrysin) could prevent DON activation of NF-κB and may thus protect against inflammatory bowel diseases.

Although we cannot simply extrapolate the results obtained in this *in vitro* study to the *in vivo* situation of a chronic exposure of humans to DON, this study provides insight into how DON found in food might enter into the organism to attain the immune system and induce acute gastroenteritis by modulating the intestinal function and metabolism.

Recommendations

Further studies are needed to understand the mechanisms of action of DON at the intestinal level, both regarding the monolayer permeability and the pro-inflammatory effects.

4.3 General conclusions

The present project aimed at studying the possible interactions of different contaminants or nutrients at the intestinal level. It is now clear that many compounds of our diet, including contaminants and bioactive components, influence the metabolism of the intestinal cells. Some of these compounds may interfere with each other at the level of the intestinal metabolism, modulating thus the toxicity of some food contaminants. Investigations on such interferences are still scarce but should be stimulated since they are necessary to fine tune the recommendations of the toxicologists. The present project addressed these questions regarding two major mycotoxins, potential interfering contaminants and plant polyphenols. One major originality of this project consisted in the use of a realistic range of food contaminant/constituent concentrations estimated from dietary exposure in Europe. In most cases, these ranges are far below the concentrations used in toxicological studies. Nevertheless, even at these low concentrations, interactions between food components and

influence on the intestinal functions have been demonstrated, underlining potential adverse effects on human health.

4.4 General recommendations

Our results on food interactions demonstrate the significance of the selection strategy, which should be applied in order to test the most relevant combinations of food contaminants and/or constituents. Theoretical considerations based on the modes of action and application of *in vitro* methods would be very useful as a starting point to screen certain combinations for their possible effects and for toxicity assessment. A higher priority for further research should then be given to the combinations of compounds, which can have particular hazardous or beneficial impacts.

Selection of the appropriate mixtures should be extended to other contaminant families, which can modulate the intestinal functions: PCBs, dioxins, pesticides, drugs, hormones, food processing contaminants, etc. It was possible to test only a limited number of mixtures during this project. However, the theoretical approach used here to select the appropriate combinations of contaminants could be used as a rational basis for other projects dealing with dietary contaminants and food constituents influencing the intestinal functions.

This project has already demonstrated that, with the coordinate use of adequate tools, it is possible to manage such a complex problem as the simultaneous effect of different substances present in food on the intestinal function. Only a few mixtures, compared to the huge possibilities, were tested to underline plausible interactions between chemicals at the level of the intestinal barrier. *In vivo* models, which are too expensive and ethically restrictive, cannot be used for this kind of studies. Thus, efforts should be made at the national level and coordination has to be strengthened at the international level to support the development of suitable high throughput screening (HTS) *in vitro* models coupled with rapid and sensitive analytical tools, which can handle a large number of samples and quantitatively detect metabolites.

This complex problem is not limited to the intestine, the use of validated *in vitro* cell culture systems should also be applied to other sites of interactions (liver, kidney, lung, etc). Furthermore, transcriptomic (microarrays), proteomic and bioassay (e.g. based on cells transfection with cytosolic or nuclear receptors coupled to reporter genes) methods, which can

highlight important interaction events, must also be developed in order to bring new information about molecular and cellular mechanisms. The development of well defined methodologies improving the planning and interpretation of chemical mixture studies, as well as biological *in silico* models, should also be supported in the near future.

5 ANNEXES

5.1 References

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5.2 Tables

Table 14. Calculation of EDIs for pesticides using Belgian MRLs (Royal decree of March 13, 2000, and April 5, 2001) for vegetables and fruits and European consumption data from GEMS report (Ci) (WHO, 2003).

		ATRAZINE		CAPTAN		CHLORPYRIFOS		GLYPHOSATE	
Commodities	**C_i: [g/p/d]**	**L_i: MRL [mg/kg]**	**EDI [µg/p/d]**	**L_i: MRL [mg/kg]**	**EDI [µg/p/d]**	**L_i: MRL [mg/kg]**	**EDI [µg/p/d]**	**L_i: MRL [mg/kg]**	**EDI [µg/p/d]**
Wheat	178	0.1	17.8					5	890
Early potatoes	240.8	0.1	24.08						
Stored potatoes	240.8	0.1	24.08						
Apple	40	0.1	4	3	120	0.5	20		
Onion	27.8	0.1	2.78			0.2	5.56		
Barley	19.8	0.1	1.98			0.2	3.96	20	396
Lettuce	22.5	0.1	2.25	2	45				
Pea	14	0.1	1.4	2	28				
Cauliflower	13	0.1	1.3						
Tomato	66.6	0.1	6.66	3	199.8	0.5	33.3		
Bean	12	0.1	1.2	2	24				
Pear	11.3	0.1	1.13	3	33.9	0.5	5.65		
Maize	8.8	0.1	0.88						
Leek	2	0.1	0.2	2	4				
Spinach	2	0.1	0.2						
Artichoke	5.5	0.1	0.55			1	5.5		
Pepper	10.4	0.1	1.04			0.5	5.2		
Gherkin	4.5	0.1	0.45						
Kiwi	1.5	0.1	0.15			2	3		
Plum	4.3	0.1	0.43	2	8.6	0.2	0.86		
Cherry	3	0.1	0.3	2	6	0.3	0.9		
Garlic	3	0.1	0.3						
Strawberry	5.3	0.1	0.53	3	15.9	0.2	1.06		
Broccoli	2.7	0.1	0.27						
Brussels sprouts	2.7	0.1	0.27						
Aubergine	2.3	0.1	0.23			0.5	1.15		
Carrot	22	0.1	0.22			0.1	2.2		
Celery leaves	2	0.1	0.2						
Kale	2	0.1	0.2			1	2		
Oat	2	0.1	0.2					20	40
Radish	2	0.1	0.2			0.2	0.4		
Rhubarb	2	0.1	0.2						
Turnip	2	0.1	0.2						
Asparagus	1.5	0.1	0.15						
Red currant	0.3	0.1	0.03	10	3	1	0.3		
Rye	1.5	0.1	0.15					5	7.5
Peach	12.5	0.1	1.25	2	25	0.2	2.5		
Endive	2	0.1	0.2	2	4				
Cucumber	9	0.1	0.9						
Parsley	0.1	0.1	0.01						
Gooseberry	0.5	0.1	0.05	3	1.5	1	0.5		
Chicory	0.1	0.1	0.01	2	0.2				
Raspberry	0.5	0.1	0.05	3	1.5	0.5	0.25		
Watercress	0.1	0.1	0.01						
Hop	0.1	0.1	0.01			0.1	0.01		
Total EDI [µg/p/d]			100.68		520.40		94.30		1333.50
Total EDI [ng/kg bw*/d]			1438.30		7434.30		1347.14		19050.00

* Body weight of 70 kg

Commodities	C_i: [g/p/d]	IMIDACLOPRID		THIABENDAZOLE		THIRAM	
		L_i: MRL [mg/kg]	EDI [µg/p/d]	L_i: MRL [mg/kg]	EDI [µg/p/d]	L_i: MRL [mg/kg]	EDI [µg/p/d]
Wheat	178	0.05	8.9				
Early potatoes	240.8	0.05	12.04				
Stored potatoes	240.8	0.05	12.04	15	3612		
Apple	40	0.2	8	5	200	3	120
Onion	27.8	0.05	1.39				
Barley	19.8	0.05	0.99				
Lettuce	22.5	0.05	1.125			5	112.5
Pea	14	0.05	0.7				
Cauliflower	13	0.05	0.65				
Tomato	66.6	0.05	3.33			5	333
Bean	12	0.05	0.6				
Pear	11.3	0.05	0.565	5	56.5	3	33.9
Maize	8.8	0.05	0.44				
Leek	2	0.05	0.1				
Spinach	2	0.05	0.1				
Artichoke	5.5	0.05	0.275				
Pepper	10.4	0.05	0.52			5	52
Gherkin	4.5	0.05	0.225			5	22.5
Kiwi	1.5	0.05	0.075			3	4.5
Plum	4.3	0.05	0.215			3	12.9
Cherry	3	0.05	0.15			3	9
Garlic	3	0.05	0.15				
Strawberry	5.3	0.05	0.265			3.8	20.14
Broccoli	2.7	0.05	0.135	5	13.5		
Brussels sprouts	2.7	0.05	0.135				
Aubergine	2.3	0.05	0.115			5	11.5
Carrot	22	0.05	1.1				
Celery leaves	2	0.05	0.1				
Kale	2	0.05	0.1				
Oat	2	0.05	0.1				
Radish	2	0.05	0.1				
Rhubarb	2	0.05	0.1				
Turnip	2	0.05	0.1				
Asparagus	1.5	0.05	0.075				
Red currant	0.3	0.05	0.015			3	0.9
Rye	1.5	0.05	0.075				
Peach	12.5	0.05	0.625			3	37.5
Endive	2	0.05	0.1			5	10
Cucumber	9	0.05	0.45			5	45
Parsley	0.1	0.05	0.005				
Gooseberry	0.5	0.05	0.025			3	1.5
Chicory	0.1	0.05	0.005				
Raspberry	0.5	0.05	0.025			3	1.5
Watercress	0.1	0.05	0.005				
Hop	0.1	0.05	0.005	0.1	0.01		
Total EDI [µg/p/d]			56.34		3882.00		828.34
Total EDI [ng/kg bw*/d]			804.85		55457.30		11833.40

* Body weight of 70 kg

Table 15. Calculation of EDI for benzo(a)pyrene using European ML (Li) (directive EC 466/2001) and European consumption data from GEMS report (Ci) (WHO, 2003).

Commodities	C_i [g/p/d]	L_i [µg/kg fresh weight]	EDI [ng/p/d]
Oil and fat	100	2	200
fish	250	2	500
Total EDI [ng/p/d]			700
Total EDI [ng/kg bw*/d]			10

* Body weight of 70 kg

Table 16. Calculation of EDI for acrylamide using monitoring data (Li) (JECFA, 2005) and European consumption data from GEMS report (Ci) (WHO, 2003).

Commodities	C_i [g/p/d]	L_i [µg/kg]	EDI [ng/p/d]
Cereals	221.9	343	76111.7
Fish – sea food	46.8	25	1170
Meat and offal	217.3	19	4128.7
Milk and milk products	336.1	5.8	1949.38
Nuts and oilseeds	29.9	84	2511.6
Pulses	9.4	51	479.4
Roots and tubers	242	477	115434
Stimulants (coffee, green tea, cocoa)	14.4	509	7329.6
Sugar and honey (mainly chocolate)	107.3	24	2575.2
Vegetables	371.6	17	6317.2
Total EDI [ng/p/d]			218006.78
Total EDI [ng/kg bw*/d]			3114.4

* Body weight of 70 kg

5.3 Publications

5.3.1 Papers

Sergent, T., Garsou, S., Schaut, A., De Saeger, S., Pussemier, L., Van Peteghem, C., Larondelle, Y., Schneider, Y.-J. 2005. Differential modulation of ochratoxin A absorption across Caco-2 cells by dietary polyphenols, used at realistic intestinal concentrations. *Toxicol. Lett.* 159, 60-70.

Sergent, T., Parys, M., Garsou, S., Pussemier, L., Schneider, Y.-J., Larondelle, Y. 2006. Deoxynivalenol transport across human intestinal Caco-2 cells and its effects on cellular metabolism at realistic intestinal concentrations. *Toxicol. Lett.* 164, 167-176.

Pussemier, L., Larondelle, Y., Van Peteghem C., Huyghebaert, A. 2006. Chemical safety of conventionally and organically produced foodstuffs: a tentative comparison under Belgian conditions. *Food Control*, 17, 14-21

Anselme, M., Tangni, E.K., Pussemier, L., Motte, J.-C., Van Hove, F., Schneider, Y.-J., Van Peteghem, C., Larondelle, Y. 2006. Comparison of ochratoxin A and deoxynivalenol in organically and conventionally produced beers sold on the Belgian market. *Food Addit. Contam.* 23(9): 910-918.

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Harcz, P., Wilmart, O., Moons, E., Tangni, E.K., Schneider, Y.-J., De Saeger, S., Larondelle, Y., Pussemier, L. Contribution of beer to OTA and DON exposure in Belgium. (submitted to *Food Addit. Contam.*).

Harcz, P., De Temmerman, L., Wilmart, O., Schmit, J.-F., Moons, E., De Voghel, S., Schneider, Y.-J., De Saeger, S., Larondelle, Y., Pussemier, L. Contaminants in organically and conventionally produced winter wheat (*Triticum aestivum*) in Belgium. (submitted to *Food Addit. Contam.*).

5.3.2 Oral presentations

Schneider, Y.-J., Sergent-Engelen, T., Anselme, M., Larondelle, Y., Garsou S., Pussemier, L., Paepens, C., De Saeger, S., Van Peteghem, C. Food ingredient interactions modulate intestinal bioavailability: use of a cell culture system (Caco-2 cells). Beltox meeting on bioavailability of toxicants, Liège, Belgium, April 2004.

Pussemier, L., Pierard, J.-Y., Anselme, M., Paepens, C., De Saeger, S., Hoenig, M., Van Overmeire, I., Hanot, V., Van Hove, F., De Temmerman, L., Motte, J.-C., Schneider, Y.-J., Goeyens, L., Van Peteghem, C., Larondelle, Y. Development and application of analytical methods adapted to the determination of mycotoxins and environmental pollutants in some organic and home-produced foodstuffs. The Third International Symposium on Recent Advances in Food Analysis, Prague, Check Republic, November 2-4, 2005.

Larondelle, Y., Sergent, T., Garsou, S., Schaut, A., Anselme, M., De Saeger, S., Pussemier, L., Van Peteghem, C., Schneider Y.-J. Mycotoxins, polyphenols and human intestinal epithelium: *in vitro* study of the interactions regarding absorption and metabolic processing. Symposium Euro-Maghrébin "Contaminants biologiques, chimiques et sécurité alimentaire", Fez, Morocco, 7-9 September 2005.

Pussemier, L., Anselme, M., Paepens, C., Pierard, J.-Y., De Saeger, S., Van Hove, F., Motte, J.-C., Schneider, Y.-J., Van Peteghem, C., Larondelle Y. Mycotoxins in organic and conventional foodstuffs in Belgium: a survey of cereal-based products and beer. International Satellite Congress: Platform for scientific concertation: Food safety, Antwerp, Belgium, May 16, 2006.

Larondelle, Y., Sergent, T., Garsou, S., Schaut, A., Anselme, M., De Saeger, S., Pussemier, L., Van Peteghem, C., Schneider Y.-J. Mycotoxins, polyphenols and human epithelial intestine: *in vitro* studies of the interactions in terms of absorption and metabolic processing. International Satellite Congress: Platform for scientific concertation: Food safety, Antwerp, Belgium, May 16, 2006.

Larondelle, Y., Schneider, Y-J.
Physiological interactions between food bioactive components : a new field of research regarding the relationship between food and health.
Xth BioForum, Liege, Belgium, May 17, 2006.

Pussemier, L. Contaminants chimiques en agriculture biologique et conventionnelle. Workshop "Contaminants chimiques et agriculture durable" -Platform for scientific concertation: Food safety, Tervuren, Belgium, June 15, 2006.

Larondelle, Y. Les mycotoxines et la sécurité alimentaire. Workshop "Contaminants chimiques et agriculture durable" Platform for scientific concertation: Food safety, Tervuren, Belgium, June 15, 2006.

De Saeger, S. Analyse van mycotoxines. Workshop "Chemische contaminanten en duurzame landbouw" Platform for scientific concertation: Food safety, Tervuren, Belgium, June 15, 2006.

Sergent, T. Outils moléculaires pour l'étude des effets des mycotoxines *in vitro*. Workshop "Contaminants chimiques et agriculture durable" Platform for scientific concertation: Food safety, Tervuren, Belgium, June 15, 2006.

Schneider, Y-J. Interactions entre contaminants chimiques au niveau gastro-intestinal. Workshop "Contaminants chimiques et agriculture durable" Platform for scientific concertation: Food safety, Tervuren, Belgium, June 15, 2006.

5.3.3 Poster presentations

Schaut, A., Sergent, T., De Saeger, S., Garsou, S., Paepens, C., Schneider, Y-J., Pussemier, L., Larondelle, Y., Van Peteghem, C.
Analytical tools for ochratoxin A bioavailability studies.
KVCV conference «Trends in food analysis», Ghent, Belgium May 26, 2005.

De Saeger, S., Pussemier, L., Paepens, C., Motte, J.-C., Pierard, J.-Y., Van Hove, F., Van Peteghem, C., Larondelle, Y. Rapid and multi-analyte detection techniques for mycotoxins in food. International Satellite Congress: Platform for scientific concertation: Food safety, Antwerp, Belgium, May 16, 2006.

Schneider, Y.-J., Sergent, T., Parys, M., Garsou, S., Pussemier, L., Larondelle, Y. Deoxynivalenol transport across human intestinal Caco-2 cells and its effects on cellular metabolism at realistic intestinal concentrations. International Satellite Congress: Platform for scientific concertation: Food safety, Antwerp, Belgium, May 16, 2006.