SCIENTIFIC SUPPORT PLAN FOR A SUSTAINABLE DEVELOPMENT POLICY

Intermediary report - January 2003

MYCOTOXIN CONTAMINATION OF REGULAR AND «ORGANIC» FOODSTUFFS
CP-30

UCL – CERVA/CODA - RUG

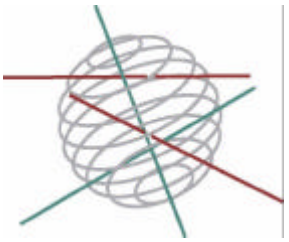
SPSD II

PART 1
SUSTAINABLE PRODUCTION AND CONSUMPTION PATTERNS

- GENERAL ISSUES
- AGRO-FOOD
- ENERGY
- TRANSPORT

This research project is realised within the framework of the Scientific support plan for a sustainable developmentpolicy (SPSD II)

Part I "Sustainable production and consumption patterns"



The appendixes to this report are available at :
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Published in 2003 by the
Belgian Public Planning Service Science Policy
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Scientific Support Plan for a Sustainable Development Policy – SPSD II

PART I - SUSTAINABLE CONSUMPTION AND PRODUCTION PATTERNS

INTERMEDIARY SCIENTIFIC REPORT - 2002

Mycotoxin contamination of regular and "organic" foodstuffs

1. PROJECT TITLE

Mycotoxin contamination of regular and “organic” foodstuffs.

2. INTRODUCTION

2.1. Context and summary

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

2.2. Objectives

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

Three mycotoxins will be the focus of this project : ochratoxin A (OTA), deoxynivalenol (DON), fumonisin B1 (FB1). In the project, we plan to focus on bread, pasta, beers and corn flakes. This restricted set of foodstuffs was selected because deriving directly or indirectly from cereals promed to be contaminated by mycotoxins. In the case of FB1, we intended to focus our efforts on maize because this cereal is the main target of fumonisin producing fungi (*Fusarium verticillioides* and *F. proliferatum*).

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

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

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

During the three years of the project, a representative number of samples from foodstuffs under investigation will be collected, with a balance between regular and “organic” food chain.

Mycotoxins analysis in foodstuffs can be conducted using methods validated by the European Union. These methods are sensitive but time-consuming and thus not applicable on a large scale. We focus on developing powerful, rapid and complementary tools allowing effective

foodstuffs controls at regular intervals : 1) “multi-mycotoxins” chromatographic methods for simultaneous quantification of several toxins, 2) field semi-quantitative immuno-enzymatic methods, 3) biological detection methods based on direct and indirect cytotoxicity induced by contaminated food extracts on human and animal cultured cells.

2.3. Expected outcomes

In the end, the project must allow the creation of a permanent Reference Center in one of the federal State research institutes. This Centre will ensure the maintenance of newly developed analytical methods, as well as the management of a data base and a collection of reference samples.

The project must also offer the accessibility to newly developed methods allowing a rapid, efficient, economic and more systematic control of the mycotoxin load of a large set of foodstuffs.

3. DETAILED DESCRIPTION OF THE SCIENTIFIC METHODOLOGY

A. Partner 1 : UCL-BNUT, Louvain-la-Neuve

A.1. Sampling plans for regular and organic foodstuffs

The sampling plan that was applied for each foodstuff (cereals of wheat suitable for bread-making, beer and sweet corn) was set up according to the origin of the food consumed in Belgium and the homogeneity of the matrix. This task of samples search and gathering, especially for cereals, was one of the main parts of our job during this first year.

A.1.1. Origin

Cereals were obtained directly from the organic and regular producers equally distributed between Wallonia and Flanders. It was indeed the best way to reduce the variability (geographical, climatic, pedological factors) between the samples. These farmers committed themselves to provide us with samples during the three years of the project. Thanks to these collaborations, the origin of the cereals is assured and allows a comparison between organic and regular cereals.

Five to ten kilograms of cereals were collected by the farmers themselves before storage (and thus before mixing with other cereals) according to our recommendations. Harvest was late this year, beginning during August.

After labelling, cereals were milled and stored in hermetic buckets kept in darkness at ambient temperature.

In the case of beer and sweet corn, we did not find a way to identify the origin of the raw materials (produced in Belgium or imported). For these commodities, we thus decided, in agreement with the committee of users, to base the sampling on their availability at the retail level, without taking their origin into account. In addition, a few organic beers were obtained directly from the producers.

A.1.2. Numbers of samples tested, according to the homogeneity of the matrix

Wheat cereals

To overview the contamination of Belgian cereals, 20 samples were collected in Wallonia and 20 others in Flanders, half coming from regular farming, half from organic farming.

Beers

A pilot scale test was set up in order to assess contamination levels of beers coming from the same lot. A lot is produced in one process with the same raw materials, and thus the contamination level should be the same in each bottle of the lot.

Determination of the OTA concentration in beer was performed using the method described at point 3.A.2. Five bottles of the same lot were tested and four brands of beers were analysed. Point 4.A.1 describes the results of this test.

Sweet corn

In order to choose the best sampling strategy for the analysis of sweet corn samples, we measured the FB1 concentration of five samples in different production lots. Preliminary results are presented at point 4.A.2 .

A.2. Determination of ochratoxin A in wheat flour and beer

A.2.1. Extraction of OTA in wheat flour

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

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

A.2.2. Extraction of OTA in beer

OTA extraction in beer was preformed according to the method described by Tangni *et al*. (2002).

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

A.2.3. Purification of OTA extract

Purification of OTA extracts was performed with an immunoaffinity column fitted with a 20-ml syringe reservoir and placed on a vacuum manifold.

Before loading the extract, the Ochratest^{TM} column was conditioned with 20 ml of PBS, pH 7.4. The OTA extract was passed through the column at about 1-2 ml min^{-1}. 20 ml of de-ionized water were used to wash the loaded immunoaffinity column and OTA was eluted with 2 ml methanol and 2 ml de-ionized water. Atmospheric air (*ca* 20 ml) was passed through the column to collect all the eluate. This solution was stored at –20°C. Prior to HPLC analysis (fluorescence spectrophotometer detector), it was brought to room temperature and then filtered with a 0.45 µm microfilter.

A.3. Determination of Fumonisin B in sweet corn

Before analysing selected foodstuffs, we decided to begin with validating, in our laboratory, the official (AOAC-IUPAC) HPLC method for determination of fumonisins in corn (Sydenham *et al.*, 1996). This validation requires the evaluation of some parameters like limit of detection (LOD) and quantification (LOQ), recovery, repeatability, reproducibility and accuracy. Moreover, we decided to participate to a FAPAS (Food Analysis Performance Assessment Scheme) proficiency test organized by the Central Science Laboratory, Sand Hutton, York, UK. A proficiency test is a determination of laboratory testing performance by means of interlaboratory comparisons (ISO GUIDE 43-1 :1997(E)). During the proficiency test, our results of FB1 and FB2 analyses were compared with the best estimate of the true value of the analytes.

Simultaneously to this validation, we decided to adapt the methodology for sweet corn. We took this decision mainly because of the similarity of the sweet corn matrix with the corn matrix used in the AOAC-IUPAC method. But we also selected this matrix because sweet corn samples from organic and regular origin are available in Belgian stores (even if they are not produced in Belgium). So we guess that the measurement of fumonisins in sweet corn will help in the evaluation of the influence of the production mode on the mycotoxins content of foodstuffs.

The AOAC-IUPAC method is based on a liquid chromatographic determination of fumonisins by HPLC. This method involves three main steps : extraction, purification and analysis. During the extraction step, the maize is finely ground and then extracted with a solution of methanol/water (3/1, v/v). After filtration of the extraction mixture, the purification step is initiated. The filtrate is purified on a Strong Anion eXchange (SAX) Solid Phase Extraction (SPE) column (Vicam, EA Middelburg, The Netherlands). After washing the column, the fumonisins are eluted with acetic acid/methanol (1/99, v/v), then eluates are evaporated to dryness in a SpeedVac system and redissolved in methanol prior to analysis. In the last step, fumonisins are first derivatized with *o*-phthaldialdehyde (OPA) and then measured with a fluorescence detector after an isocratic HPLC separation on a C18 reversed-phase column.

Concerning the analysis of sweet corn, the first step of the method is slightly modified because of specificity of the packaging. As the corn are packaged in an aqueous solution we have to drain the corn before the grinding and extraction step. In the near future, besides the drained corn analysis, we will perform the analysis of the aqueous solution and compare the results. If a correlation exists, it will be faster to analyse the aqueous solution because we would escape the grinding and extraction step.

B. Partner 2 : CERVA-CODA, Tervuren

Methods to determinate low levels of mycotoxins in cereals and animal feedstuffs are complex, lengthy and costly to carry out. Recent advances in technology such as the ability to raise antibodies against a larger range of mycotoxins is being increasingly used to develop simple and sensitive methods for individual compounds. However, while the inherent specificity of those immunoassays substantially overcomes problems of interference in the detection of co-extracted compounds, this specificity precludes their use to screen foods and feedstuffs for a range of mycotoxins.

To investigate cases of suspected mycotoxicosis, studies on the natural occurrence of mycotoxins in materials or for multi-toxin surveillance programmes, reliable, sensitive and proven multi-methods are required.

CERVA-CODA is therefore developing a multi-mycotoxin procedure able to determine most of the mycotoxins of interest. The mycotoxin nature, the chromatographic technique as well as the detection mode used can all influence the developed analytical approach. Taking into account the chemical diversity of the different mycotoxins, the preparation of samples has to be non-specific. Three stages play an important role in this system :

- the extraction : we must use a single solvent mixture which allows the extraction of a maximum of mycotoxins within the best possible extraction yields ;
- the clean-up : owing to the chemical diversity of mycotoxins, any purification column does not allow a purification in a single step. Thus, the purification must be done, either in series, or in parallel, according to the mycotoxins family suspected to be found in the samples ;
- the chromatographic analysis methods : aiming to identify and to quantify the greatest number of mycotoxins in a single run.

B.1. Extraction step

The mixture acetonitrile (ACN) – water (H_2O) is often used for the extraction of a lot of mycotoxins but in different proportions (Entwisle, 2000; Trucksess, 1996). According to the literature, OTA could be better extracted by using a mixture of 60-40 % of ACN-H_2O. On the opposite, trichothecenes are extracted using a higher content in acetonitrile (84 %). The most appropriate mixture, for the best extraction of all the mycotoxins of interest, has to be determined.

Therefore, the recoveries performances were determined using three mixtures of ACN-H_2O. The investigated ratios were : 60/40, 70/30 and 84/16. So, the investigated area would cover all the range of the mycotoxin polarity and hydrophobicity.

Whole wheat flour spiked with 9 mycotoxins (OTA, NIV, DON, 15-acDON, 3-acDON, T-2 toxin, HT-2 toxin, ZEL and ZEN) was used in order to determine the recoveries of these extraction mixtures. Fumonisin FB1 was not added to these mycotoxins because of its low recovery rate when extracted by ACN-H_2O mixture, as most authors say. The results of this research were used to determine mycotoxins concentration in real samples. Point 4.B.1 describes the results obtained in detail.

B.2. Purification step

This very important step has to be non-specific in order to maintain the recoveries for all the extracted toxins. Moreover, given the chemical diversity of mycotoxins, it would be very difficult to find a purification step for all the toxins at once. The first general purification step would be to separate the toxins according to their chemical properties : e.g. their neutral or acidic character. This goal could be reached using solid phase extraction (SPE) columns. Following this procedure, OTA, an acidic mycotoxin, could be isolated from the matrix using

an anion-exchange column (SPE-S/WAX). On the other hand, neutral mycotoxins (trichothecenes, ZEN, ZEL) could flow through the column and were quantitatively recovered from the ionic matrix.

OTA could also be isolated and purified in its neutral form using a C-18 (RP) SPE column. Both purification ways have still to be tested.

An immuno-affinity purification column (OchraPrep, Rhône-Diagnostics, Glasgow, Scotland) was used as an alternative way for the purification and the quantification of OTA alone. This expensive way was used in order to get rapid results on the global recoveries at the extraction-purification stage. In this method, used until now, a portion of the extract is purified for OTA determination with immunoaffinity columns (IAC) while a second portion of the same extract is purified to recover the 8 other mycotoxins using SPE columns (MycoSep ZEN columns, Romer Labs, Washington, MO, USA). Note that the OTA has to be separated from these eight toxins because of the use of the MycoSep columns. Indeed, polar molecules as OTA are trapped in an irreversible way in these purification columns.

B.3. Analytical methods

As seen in the purification step, OTA, produced during storage, has an acidic function and is strongly fixed on the MycoSep column. As far as OTA can be isolated from the matrix using ion exchange SPE columns, it is easier to develop a specific analytical method for OTA and other acidic mycotoxins.

OTA could be quantified using the multi-mycotoxin analytical method but its retention time is too long. Thus, two methods have to be developed : the first one for OTA determination and the second one, for the other mycotoxins.

For OTA determination, a specific HPLC method with an isocratic elution and a detection by fluorescence is used. For the other mycotoxins, a multi-linear gradient using a double mode of detection (UV-FLUO), depending on mycotoxins to be determined, is necessary for the analysis. Once the elution order is given (NIV, DON, 15-acDON, 3-acDON, ZEL, ZEN), it would even be possible to use only a part of the total gradient for the determination of only the first 4 mycotoxins (NIV, DON, 15-acDON and 3-acDON). The validation of this method is in progress.

The global analytical method used for the determination of the four selected trichothecenes, for ZEN and for OTA is presented in Table 1. Both methodologies are currently under development.

Table 1. Methods for mycotoxins analysis.

	OTA	**DON & metabolites**	**ZEN**
Extraction	ACN-H_2O (84-16, v/v)		
Purification	IAC – OchraPrep	MycoSep DON (ZEN)	MycoSep ZEN
Evaporation			
Solubilisation	MeOH-H_2O (50-50, v/v)	ACN-H_2O (10-90, v/v)	MeOH-H_2O (50-50, v/v)
HPLC analysis	Fluorescence	UV-DAD at 225 nm	Fluorescence

The methods for DON and ZEN determination were applied to the analysis of 20 wheat samples (10 from organic agriculture and 10 from regular agriculture) collected by partner 1 (see point A.1.). The results will be discussed in point 4.B.3.

ZEN is not included in the mycotoxins investigated in the present study. Nevertheless, this mycotoxin is often associated with DON and found in the cereals since both are produced by *Fusarium graminearum*.

C. Partner 3 : RUG –LFA, (Universiteit Gent, Lab. for Food Analysis)

C.1. Description of the assay components

C.1.1. Membrane

The Immunodyne ABC membrane (pore size 0.45 µm; from Pall Biosupport, Portsmouth, UK) is a microporous nylon membrane which has been surface modified with reactive groups to covalently bind proteins and other compounds containing amino groups. The coating procedure was similar to that used by Sibanda *et al*. (2000). First the membrane was cut into squares (2x2 cm). On each membrane, 1 µL undiluted rabbit anti-mouse (from Dako, Glostrup, Denmark, No Z 259; protein conc. 3.2 mg/mL) and 1 µL goat anti-HRP (from Sigma, Belgium, No P-5774, protein conc. 52.7 mg/mL), diluted 1/1500 in assay buffer, were spotted side-by-side. The membranes were dried at 37°C for 30 min. The remaining covalent binding sites were blocked by immersing in blocking buffer. After being dried at 37 °C for 30 min, the prepared membranes were stored at room temperature in a vacuum-sealed plastic bag.

C.1.2. Flow-through device and absorbent material

The flow-through device (obtained from Frosley Equipment, Dover, UK) consists of a plastic bottom and a top lid, which contains one central hole. Between the bottom and the top lid, an absorbent layer, and on top of it, the Immunodyne membrane are pressed tightly together. The absorbent layer is a cotton containing material, which actively draws liquid reagents through the membrane (European patent application N° 97870132).

C.1.3. Immunoreagents and buffers

Monoclonal antibodies against ochratoxin A (anti-OTA Mab) and fumonisin B1 (anti-FB1 Mab), as well as the HRP conjugates, respectively OTA-HRP and FB1-HRP were prepared by the Institute for Animal Sciences, Agricultural Biotechnology Center, Gödöllö, Hungary.

Phosphate buffered saline, PBS, pH 7.4 (0.2 g KH_2PO_4, 1.2 g Na_2HPO_4, 8.0 g NaCl, 0.2 g KCl per litre of water) was used to make the wash solution (PBS- Tween 0.05 %), the blocking solution (PBS – 2 % casein) and the assay buffer (PBS – 0.1 % casein). Proclin was added to the buffers as an antibacterial preservative.

C.2. Flow-through immunoassay procedure

In this project, the Laboratory of Food Analysis of Ghent University aims to develop rapid and simple qualitative screening methods for the detection of mycotoxins in food. The approach consists of a flow-through enzyme immunoassay that allows quick evaluation of contamination levels in the field. The term 'flow-through' refers to the test format where the plastic solid phase for the immobilization of the antibodies has been replaced by a membrane. The flow-through assay system consists of two layers, the first being the membrane coated with anti-mouse antibodies and anti-HRP antibodies. The second layer is an absorbent material. Due to the flow-through characteristics of the membrane and because of its contact with the absorbent layer, added reactants are drawn through the pores of the membrane. This brings the immunoreagents rapidly into close contact with the reactive sites on the membrane. The kinetics of the reaction between immobilized and soluble reactants closely approach the kinetics of binding in solution. As a consequence, assay time is significantly shorter. The flow-through is a sequential assay where each reagent is applied in sequence to the membrane. Between each step it is important to allow the added liquid to flow through the membrane. The anti-mycotoxin specific antibodies are first applied on the membrane, followed by a washing step. Then, the mycotoxin standard solution or the sample are added to

the membrane. Analyte, if present, will bind to the anti-mycotoxin antibody. After a washing step, a mycotoxin-HRP conjugate is added and is bound by any remaining free anti-mycotoxin antibodies and anti-HRP antibodies which acts as an internal control. The last washing step removes residual conjugate solution from the membrane. Finally, the colour substrate is added. The immunochemically bound enzyme-conjugate will convert the substrate to a coloured product. Less colour is produced with increasing concentrations of analyte in the sample. The smallest mycotoxin concentration that results in no colour development is defined as the visual detection limit. The colour development is visually evaluated and measured with a portable colorimeter (Chroma-meter CR-321, Minolta) which expresses the colour intensity as a single numerical value (ΔE*ab). In this report the optimization of the flow-through enzyme immunoassay for the detection of ochratoxin A (OTA) in wheat and wholemeal bread is described. For the detection of fumonisin B1 (FB1) in maize, a suitable extraction is evaluated and a provisional assay procedure is established.

C.3. Sample preparation

C.3.1. Detection of OTA in wheat and wholemeal bread

The extraction method described by De Saeger *et al*. (2002) was adapted and further simplified. Wheat and wholemeal bread were first ground in a household coffee grinder. A 5 g portion was mixed with 15 ml 80 % MeOH / H_2O (v/v) and shacked by hand for 6 min. 1000 µL of the supernatant was diluted with 1600 µL PBS-buffer and filtered through a 0.45 µm CHROMAFIL® Einmalfilter (Macherey-Nagel).

Wheat and wholemeal bread samples spiked at a level of 0, 2, 3, 4 and 5 ng OTA/g were prepared by adding the appropriate volume of an OTA standard solution (0.1 ng/µL; in methanol) to 5 g of ground wheat.

C.3.2. Detection of FB1 in maize

The extraction method was based on that for OTA. Maize was first ground in a household coffee grinder. A 5 g portion was mixed with 15 ml extraction solvent and shacked by hand for 6 min.

1000 µL of the supernatants were then diluted with 600 µL PBS-buffer and filtered through a 0.45 µm CHROMAFIL® Einmalfilter (Macherey-Nagel).

Maize samples spiked with FB1 at a level of 0, 500, 700, 800, 900, 1000 and 1500 ng FB1/ g were prepared by adding the appropriate volume of an FB1 standard solution (1 ng/µL; in methanol) to 5 g of ground maize.

D. Partner 4 : UCL-BIOC, Louvain-la-Neuve

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

In this context, bioassays based on mammalian cells cultures were evaluated for their ability to screen and to determine the presence and the toxicity of mycotoxins. Purified standards of mycotoxins were used to set up the methods. The MTT assay was the most sensitive direct detection method and would allow to detect mycotoxin concentrations frequently found in cereals. The mycotoxin detection limit was lowered with an indirect detection method based on the intestinal permeability functionality. Moreover, preliminary results on the intestinal model have revealed a repression of expression of the detoxification system genes. All these methods will be applied to food extracts.

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

D.1. The cell lines

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

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

Those cells are now routinely maintained in culture in our laboratory.

D.2. The direct detection methods

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

The direct effect of various substances on the cellular viability may be measured by determining different endpoints such as metabolic activities, plasma membrane damage, protein synthesis. We have chosen three colorimetric bioassays that could provide a rapid and sensitive screening of low concentrations of mycotoxins : the MTT assay, the neutral red assay and the LDH assay.

To validate the methods, commercially available mycotoxins were used : DON, OTA and FB1.

D.2.1. The MTT assay

Principle

This test measures the metabolic activity of the mitochondrial function. The assay is based on the reduction of MTT, a yellow tetrazolium salt, to dark purple formazan crystals by the mitochondrial enzyme, succinate dehydrogenase. This activity takes place only in living cells and the formed formazan quantity is proportional to the number of living cells. Cells should be maintained in an exponential growth phase during the assay.

Method

Cells were inoculated in 96-wells microplates. After 24 hours, the cell culture media were replaced by media containing different concentrations of one of the mycotoxins. Stock solutions of mycotoxins were prepared in appropriate solvents before their dilutions in cell culture medium : in 10 % ethanol for DON, in DMSO for OTA and in 70 % methanol for FB1. After 48 hours, media were removed and a MTT solution was added for 2 hours. Formazan crystals were then dissolved in DMSO. Absorbance was measured at 500 nm with a microplate reader.

D.2.2. The Neutral Red assay

Principle

The test measures the cell membrane integrity. The assay is based on the accumulation of neutral red (NR) taken up by viable cells and stored in their lysosomes and other acidic vesicules. The accumulated neutral red quantity is proportional to the living cells number.

Method

Cells were inoculated in 96-wells microplates. After 24 hours, the cell culture media were replaced by media containing different concentrations of one of the mycotoxins. After 48 hours, media were removed and a neutral red solution was added for 3 hours. The dye stored in the cells was then extracted in a 1 % acetic acid in 50 % (v/v) ethanol solution. Absorbance was measured at 500 nm with a microplate reader.

D.2.3. The LDH assay

Principle.

This test evaluates the plasma membrane damage. The assay measures the activity of a cytosolic enzyme, the lactate deshydrogenase (LDH), released in the cell culture medium by damaged cells. This activity is proportional to the number of necrosed cells.

Method.

Cells were inoculated in 96-wells microplates. After 24 hours, the cell culture media were replaced by media containing different concentrations of one of the mycotoxins. After 48 hours, media were collected and the LDH activity was measured with a kit from *Roche Molecular Biochemicals*. Absorbance was measured at 500 nm with a microplate reader.

D.3. The indirect detection methods

The Caco-2 cells were cultivated on microporous PET membrane for 14 days; in those conditions, the Caco-2 cells differentiate into enterocytes, to form the intestinal barrier cells. Cells were then cultivated for 7 days in the presence of low concentrations of mycotoxins. At 21 days, the transepithelial electric resistance (TEER) was measured in an Endohm chamber and cells were collected and analyzed by RT-PCR (Reverse Transcription-Polymerase Chain Reaction).

4. DETAILED DESCRIPTION OF THE INTERMEDIARY RESULTS, PRELIMINARY RESULTS, PRELIMINARY CONCLUSIONS AND RECOMMENDATIONS

A. Partner 1 : UCL/BNUT

A.1. Pilot-scale test to determine de best sampling procedure regarding OTA in beers

In order to set up the best sampling procedure, we had to determine if OTA contamination of beers from the same lot was homogeneous.

The results of this test presented in Table 2 show an homogeneous contamination level in each lot. Therefore, it is not necessary to take many bottles of beer coming from the same lot. In the future, we will thus evaluate the OTA contamination of beer by analysing only two bottles for each lot being controlled.

Table 2. Ochratoxin A contamination level of four beer production lots from various origin. 5 bottles of each lot were analysed; the lots correspond to different brands.

OTA concentration (ng/l)	Lot 1	Lot 2	Lot 3	Lot 4
Sample 1	Traces➔6.7	17.3	126.2	14.2
Sample 2	Traces➔6.7	16.8	123.6	12.9
Sample 3	Traces➔6.7	19.3	121.3	13.2
Sample 4	Traces➔6.7	17.8	84.9	12.4
Sample 5	Traces➔6.7	16.0	117.3	15.5
Mean ± SD	5.35 ± 1.02	17.44 ± 1.25	114.7 ± 16.97	13.6 ± 1.24

A.2. Evaluation of the homogeneity of FB contamination in sweet corn

The preliminary results (see Table 3) of these measures show great variations in the fumonisin B1 concentration between samples from the same lot. This variation may be explained by the heterogeneity of the sweet corn matrix ; only one heavily contaminated kernel in a sample will increase the contamination level. These results demonstrate that it is statistically necessary to analyse a high number of samples within a lot in order to obtain an accurate view of its contamination level. For the survey planned in the present project, we will analyse ten samples per lot.

Table 3. Fumonisin B1 contamination of five sweet corn production lots from various origin. 5 containers of each lot were analysed.

FB1 (ppm)	*Organic maize*		*Regular maize*		
Trademark	GB	Delhaize	Delhaize	Delhaize	Bonduelle
Packaging	Glass jar	Glass jar	Can	Glass jar	Can
Sample 1	0.08	0.06	0.21	0.75	0.32
Sample 2	**0.04**	**0.86**	**0.32**	0.14	0.60
Sample 3	**0.20**	0.06	0.13	**0.08**	0.65
Sample 4	0.07	0.08	**0.04**	0.17	**0.23**
Sample 5	0.07	**0.05**	0.27	**1.52**	**1.13**
Mean ± SD	0.09 ± 0.06	0.22 ± 0.34	0.19 ± 0.16	0.52 ± 0.57	0.54 ± 0.25

A.3. OTA Contamination of wheat flour

36 samples of wheat produced through regular farming or organic farming during 2002 have already been analysed. OTA was detected in one regular sample and in 9 organic samples. OTA concentration never exceeded 5 ppb, the maximum contamination level fixed by the European Commission Regulation ((EC) N° 472/2002). Multiple comparison test (Tukey test) shows that organic wheat from Flanders have a significantly higher OTA level than the three other types of wheat flour. Detailed results are presented in Table 4.

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

Table 4. Occurrence of Ochratoxin A in regular and organic wheat flour.

	Regular		Organic	
	Wallonia	Flanders	Wallonia	Flanders
Number of samples	8	10	8	10
Not Detected	7	8	7	2
Traces[(1)]	1	0	0	1
LOQ-5ppb	0	2	1	7
> 5 ppb	0	0	0	0
Incidence	12,5 %	20,0 %	12,5 %	80,0 %
Level of contamination (ppt)				
Mean[(2)]	22,50	50,21	37,38	123,68
Median[(2)]	22.50	50.21	37.38	100.14
Range[(3)]	-	44.96 - 55.47	-	43.18 - 303.39

(1) Traces means that the concentration of OTA is between the limit of detection (LOD = 10 ppt) and the limit of quantification (LOQ = 35 ppt).

(2) Mean and median are calculated on samples contaminated at a level over the LOD, assuming that samples with traces levels of OTA contain half LOD + LOQ.

(3) Range of samples with OTA contents ≥ 35 ppt.

A.4. OTA Contamination of beer

Among the 24 regular beers and 13 organic beers already collected, 7 beers from the first group and 9 beers from de second group have already been tested. For each beer, 2 samples from the same production lot have been analysed. Results are presented in Table 5.

Table 5. OTA contamination of regular and organic beer.

Conventional		Organic	
Number of beer	Mean* (ng/l)	Number of beer	Mean* (ng/l)
1	22.2	1	30.5
2	42.2	2	55.5
3	15.3	3	62.3
4	16.1	4	17.2
5	27.0	5	14.5
6	6.5	6	18.5
7	37.3	7	22.3
		8	19.7
		9	12.2

*Average value between 2 samples of the same lot.

OTA levels found in the samples analysed do not exceed the limit of 200 ng/l drafted by EC contaminants' legislation (Scientific Committee on Food/EU 1999) and fixed by the Italian Ministry of Health in 1999 (Visconti *et al.*, 2000).

A.5. Fumonisins in maize products

A.5.1. Results of the FAPAS proficiency test

At the time of the closing date (24th October 2002) of the FAPAS proficiency test on FB1 and FB2 in maize, we used a method which was not yet validated in our laboratory. Nevertheless, we sent the results and we received in November 2002 a statistical report on our performance.

In a FAPAS proficiency test the performance is expressed in the standardized form of a z-score. A z-score relates the error in a result to the designated standard deviation of the results for the analysis in question. In a normal distribution only about 1 in 20 results will be outside two standard deviations from the mean hence FAPAS z-scores of |z|=2 are considered satisfactory. Put another way, if a laboratory receives a z-score outside the range |z|=2 it is much more likely that this is due to poor performance rather than it being a "good" result that just happens to be at the extremes of the distribution.
Concerning FB1 our preliminary result was slightly outside the limits but for FB2 it is inside (see Figure 1 and Figure 2). Since these preliminary results we improved our method and we are on the way of validating it. To confirm, we will participate to the next FAPAS proficiency test organized in July 2003.

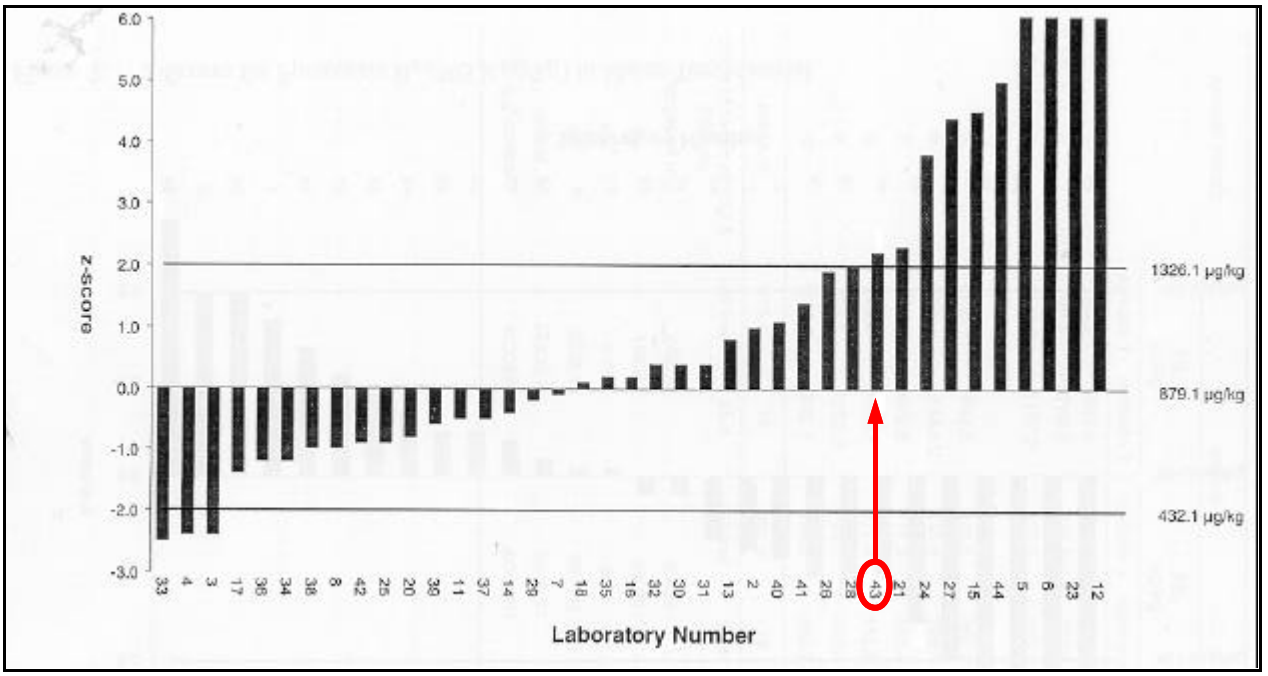


Figure 1. z-Scores for FB1 (879.1 µg/kg) in Maize Test Material. The number assigned to our laboratory is 43.

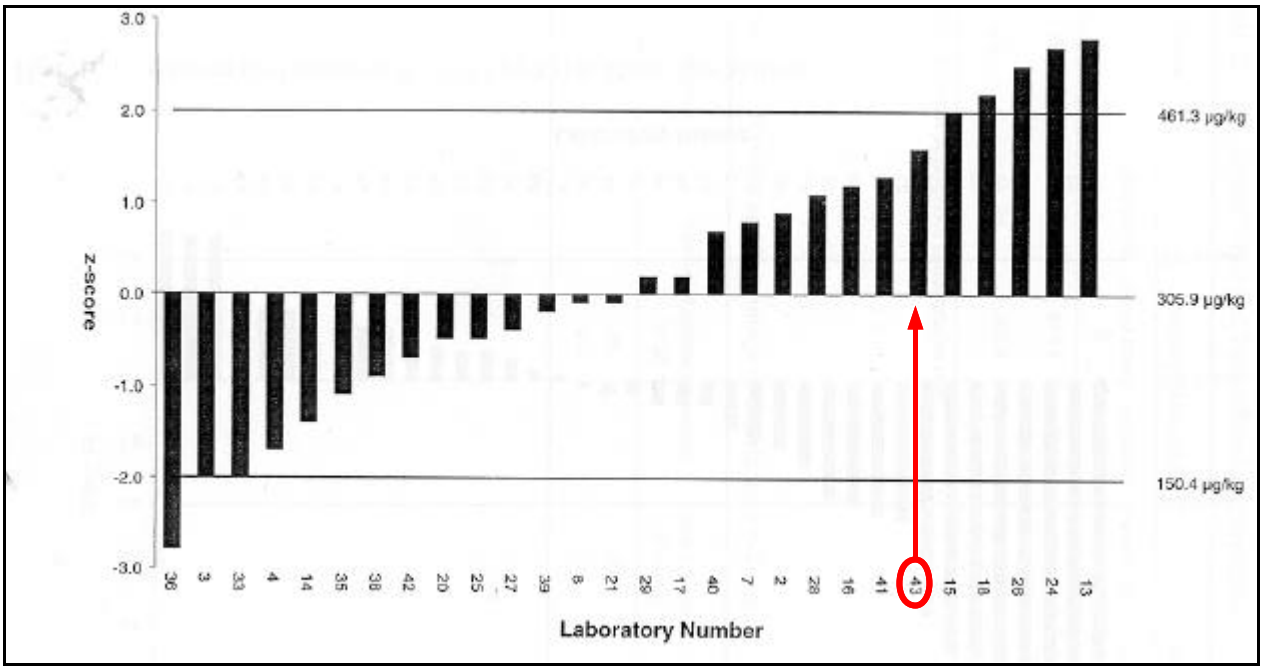
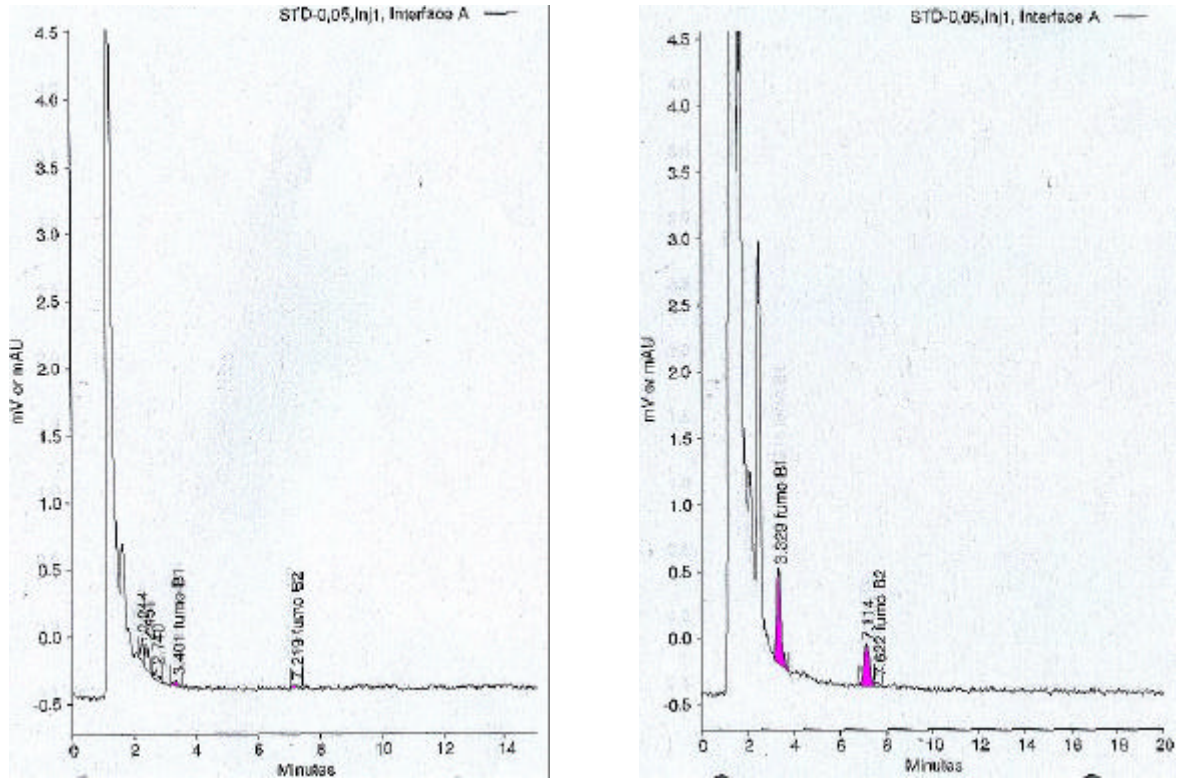


Figure 2. z-Scores for FB2 (305.9 µg/kg) in Maize Test Material. The number assigned to our laboratory is 43.

A.5.2. Optimization and validation of our fumonisin analysis method

By modification of the derivatization and injection parameters we improved the limits of detection (LOD) and quantification (LOQ) of our method (see Figure 3). The LOD expressed in ppm for FB1 and FB2 are 0.009 and 0.019, respectively, while the LOQ for FB1 and FB2 are 0.029 and 0.064. The FB1 limits are largely below the lowest proposed maximum contamination level for FB1 in food (0.1 ppm for infant food).

Using these optimised parameters we determined the repeatability and the recovery of our method with maize flour spiked at three different levels (see Table 6). We obtained a good repeatability (below 10 %) at each spiked level for FB1 as well as for FB2. In comparison, a Measurements & Testing intercomparison study for fumonisin analysis in maize generated repeatability levels of 10% and 11% for FB1 and FB2, respectively (Visconti *et al.*, 1995). Concerning the recovery, we observe that it is largely dependent on the spiking level for FB1 and on a lesser extent for FB2 but the results are in agreement with published studies (Visconti *et al.*, 1999).



	Noise (µg/ml)	LOD (µg/ml)	LOQ (µg/ml)	LOD (ppm)	LOQ (ppm)	Noise (µg/ml)	LOD (µg/ml)	LOQ (µg/ml)	LOD (ppm)	LOQ (ppm)
FB1	0,028	0,085	0,284	0,064	0,213	0,0038	0,012	0,039	0,009	0,029
FB2	0,045	0,135	0,450	0,101	0,337	0,0085	0,026	0,085	0,019	0,064

Figure 3. HPLC chromatograms before and after improvement of derivatization and injection parameters of our fumonisin analysis method.

Table 6. Determination of repeatability and recovery of FB1 and FB2 in spiked maize flour at 0.1, 0.5 and 2 ppm.

Spiked maize flour at	***FB1 ± SD# (ppm)***	***Repeatability***	***Recovery***
0.1 ppm	0.045 ± 0.002	5.1 %	45.5 %
0.5 ppm	0.274 ± 0.008	2.8 %	54.9 %
2 ppm	1.401 ± 0.034	2.4 %	70.0 %
	FB2 ± SD# (ppm)	***Repeatability***	***Recovery***
0.1 ppm	0.067 ± 0.001	1.7 %	67.4 %
0.5 ppm	0.338 ± 0.028	8.3 %	67.5 %
2 ppm	1.512 ± 0.060	3.9 %	75.6 %

Measured in triplicate.

B. PARTNER 2 : CERVA-CODA, Tervuren

B.1. Influence of the extraction solvent

As developed in point 3.B.1, an extraction solvent had to be found to get the best recoveries for the mycotoxins to be extracted. Table 7 and Figure 4 present the current recoveries obtained for all the mycotoxins except for T-2 toxin. These values are the average of three replicates (n=3).

Table 7. Recovery of mycotoxins from spiked samples of whole wheat flour, as a function of the extraction mixture.

ACN-H_2O (%)	OTA	NIV	DON	15-acDON	3-acDON	HT-2	ZEL	ZEN
60-40	87,1	45,2	66,1	77,0	71,0	72,6	54,0	33,0
70-30	86,5	29,5	64,7	72,2	65,0	73,2	35,0	36,0
84-16	77,0	59,5	80,2	86,6	86,8	82,4	67,0	65,0

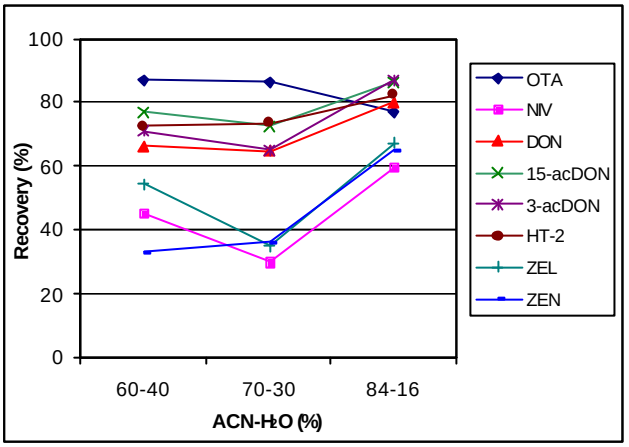


Figure 4. Recovery of mycotoxins from spiked samples of whole wheat flour, as a function of the extraction mixture.

For all the mycotoxins under investigation, excepting OTA, the results show that the best solvent mixture is ACN-H_2O (84-16, v/v). Indeed, this mixture gives the highest recoveries for all the trichothecenes with a minimum of 60 % for NIV and only about 65 % for ZEL and ZEN. This could be explained by the high polarity and the solubility in water of NIV and by the low polar character of ZEN and ZEL.

OTA could be extracted with better recoveries at 60-40 or 70-30. The recovery at 84-46 remains however close to 80 %, which is acceptable.

This determination was done on whole wheat flour spiked with pure standards following the general purification way described in point 3.B.2.

In the future, the solvent mixture (84-16%) will be used to extract samples for multi-mycotoxin determination.

B.2. Influence of the matrix

The influence of the matrix has been investigated. Two cereal samples (wheat and rye) have been extracted, purified with MycoSep ZEN and analysed. Figure 5 and Figure 6 present the chromatogram of wheat and rye samples, respectively.

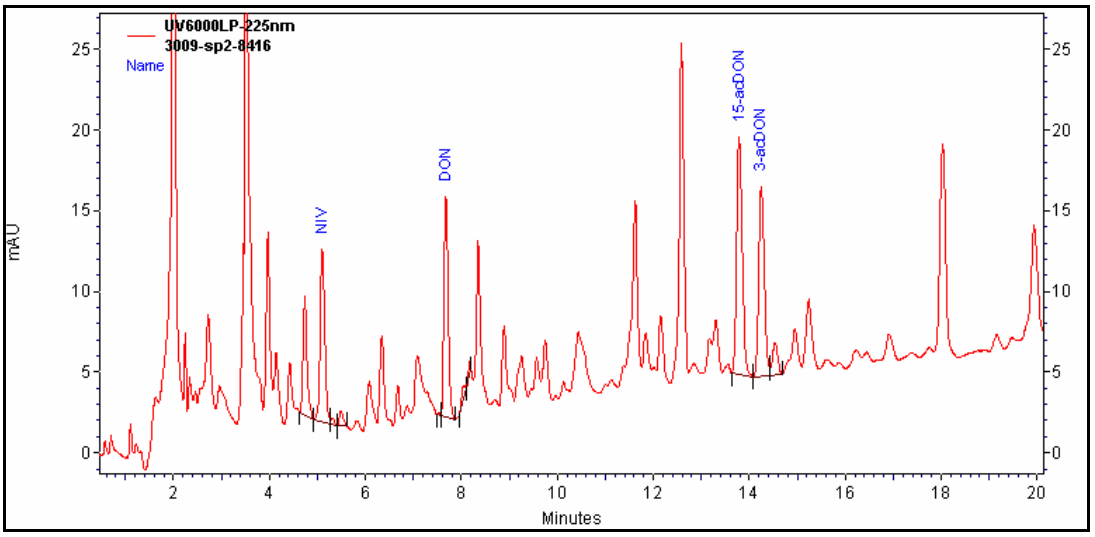


Figure 5. Chromatogram of a wheat sample.

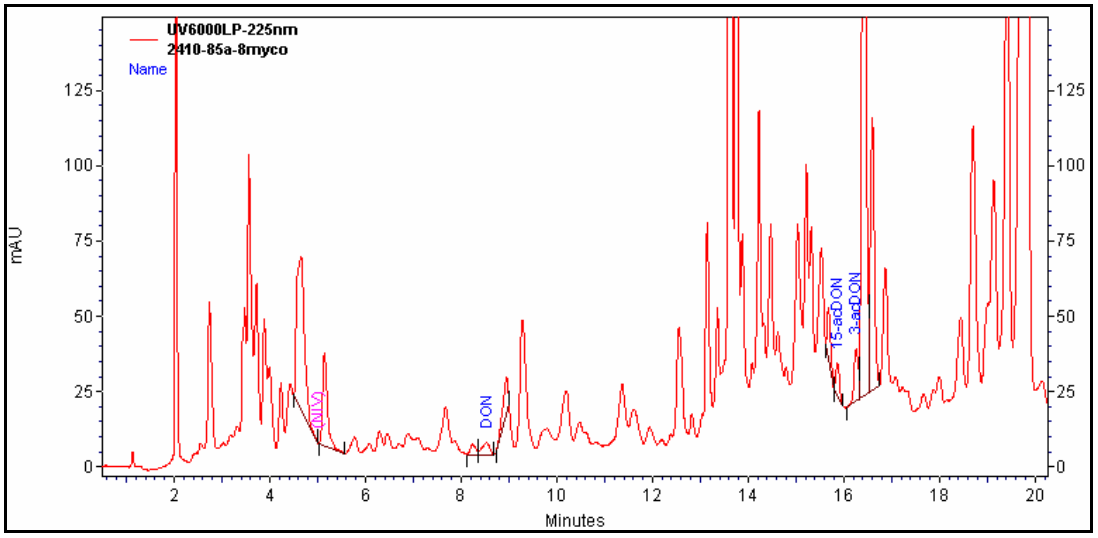


Figure 6. Chromatogram of a rye sample.

In spite of the purification, we can still see the presence of interfering peaks in the two matrices. Moreover, depending on the matrix, the high intensity of some interfering peaks precludes any good separation of the trichothecenes, especially in the rye sample. Thus, new and further developments of the purification step are essential in order to obtain cleaner chromatograms.

B.3. Common sample analysis

20 samples of wheat flour were analysed for DON and ZEN content. Table 8 and Figure 7 present the results for both DON and ZEN in a way to distinguish between organic and regular samples.

Table 8. DON and ZEN contamination of organic and regular samples.

	DON (ppm)		ZEN (ppb)	
	Organic n=10	**Regular n=10**	**Organic n=10**	**Regular n=10**
Min.	0.06	0.11	8	15
Max.	1.18	2.84	86	232
Mean*	0.38	0.92	29	84
SD	0.37	0.91	28	72
Median	0.25	0.66	18	62
Not detected	0/10	0/10	2/10	2/10

*Mean is calculated on positive samples.

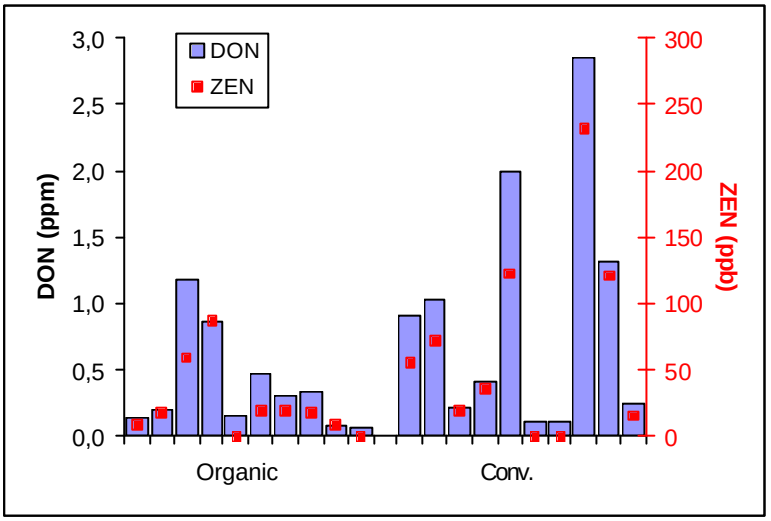


Figure 7. Comparison between organic and regular samples of wheat flour for DON and ZEN.

These preliminary results show that we can find great variations between samples of the same type. Moreover, the conventional samples seem more contaminated that the organic samples.

We can also see that the ZEN contents follow the DON ones. The higher the DON contents, the higher the ZEN contents, and vice-versa.

Concerning DON, 2 organic and 5 conventional samples are out of range (> 0.75 ppm). Until now, for ZEN, no legal limit exists but a limit of 50 ppb was proposed by Germany at the EC. Then, six samples should be excluded for human food on both DON and ZEN contents. One sample would be excluded on its DON content alone.

A second series of 20 ring-test samples are now under treatment for the analysis of their DON contents. The results of both serials will then be compared with the ones obtained by partner 1 using a validated AOAC method.

Our multi-mycotoxin methods allowed us to detect the presence of ZEL, 15-acDON and 3-acDON in very low amounts.

The limit of quantification (LOQ) for DON and ZEN are 50 ppb (UV) and 5ppb (Fluo), respectively.

C. PARTNER 3 : RUG –LFA, (Universiteit Gent, Lab. for Food Analysis)

Previous research at the laboratory in the field of rapid immunoenzymatic assay development for the detection of OTA focused mainly on the matrix cereals (S. De Saeger et al., 1999). In a first stage of the experimental work, the flow-through assay for the detection of OTA in wheat is optimised and applied to a food matrix directly derived from cereals : wholemeal bread. The EU has established maximum levels for OTA in cereals and derived cereal products (including processed cereal products and cereal grains intended for direct human consumption) of respectively 5 µg/kg and 3 µg/kg (Commission Regulation No 472/2002). It is our goal to optimise the flow-through enzyme immunoassay procedures so that no colour is developed at these levels.

The second mycotoxin we focus on is FB1, which has been found as a worldwide contaminant mainly in maize and maize-based products. In the EU there is still no legislation on FB1 levels. Switzerland is the only country in Europe with a maximum level for fumonisins (sum of FB1 and FB2) in maize produced for human consumption of 1000 µg/kg. It is our goal to develop a field test with a visual detection limit of 1000 µg/kg. In a first stage, we evaluated the extraction solvent and established a provisional assay procedure.

C.1. Evaluation of the flow-through enzyme immunoassay for the detection of OTA in wheat

The assay was carried out as follows :

- Anti-OTA Mab	1/200	100 µL
- Wash solution		300 µL
- Sample extract		800 µL
- Wash solution		300 µL
- OTA-HRP conjugate	1/250	100 µL
- Wash solution		600 µL
- Chromogen solution		100 µL

The visual detection limit of the test can be adjusted to a certain extent by adapting the amounts of antibodies and HRP-conjugate or by modification of the amount of sample added to the membrane. Dilutions of these immunoreagents are made in assay buffer. In a first experiment the sample volume was fixed on 600 µL, while the following dilutions of OTA antibody : 1/50; 1/100 and 1/200, were tested in combination with a range of OTA-HRP conjugate dilutions : 1/100; 1/200 and 1/250. Following trial and error, the dilution of anti-OTA Mab 1/200 and OTA-HRP 1/200 gave a well coloured spot for the blank (mean $\Delta E^*ab = 12,17$) and no visible colour development (mean $\Delta E^*ab = 7,4$) at 5 µg/kg in 50 % of the test cases. Increasing the sample volume to 800 µL resulted in a 100 % colour inhibition at 4 µg/kg. The dose-response curve is shown in Figure 8.

Washing steps are necessary to reduce background colour on the uncoated part of the membrane by non-specific reactions. In order to reduce the number of steps in the assay format, elimination of washing steps or reduction of the amount of wash volume applied on the membrane resulted in a background colour development which made it difficult to distinguish a sample spot from the background.

The chromogen substrate used is ColorBurst®Blue (Alercheck, Portland, USA), a ready-to-use single-component solution. Two alternative substrate solutions, TMB-Membrane single-component HRP substrate (Biognost, Wevelgem, Belgium) and TMB-membrane peroxidase substrate (KPL, Maryland, USA) were also evaluated for colour development. Comparing the

colour intensity at 0 µg/kg and the time needed for colour development, the best results were obtained using ColorBurst®Blue.

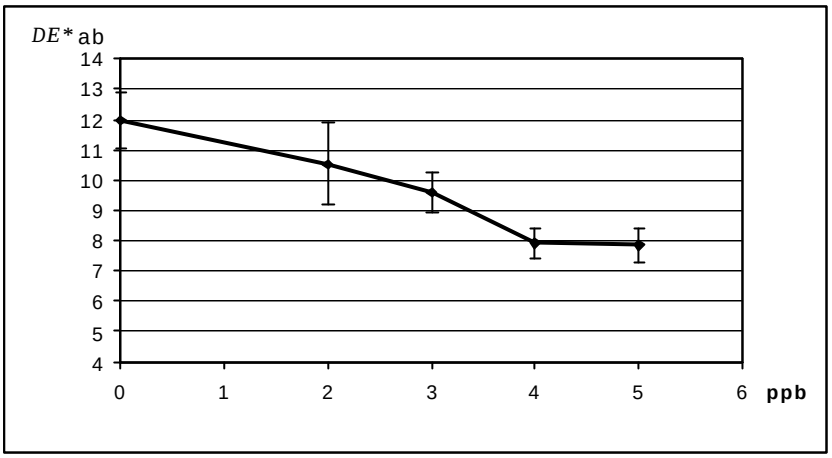


Figure 8. Dose-response curve of the flow-through membrane-based for the detection of ochratoxin A in spiked wheat samples (n=10).

C.2. Evaluation of the flow-through enzyme immunoassay for the detection of OTA in wholemeal bread

The first experiments carried out on wholemeal bread used the assay protocol as described above. In order to lower the visual detection limit to 3 µg/kg, as prescribed in the EU regulation, the amount of sample extract, added on the membrane, was increased to 1000 µL and 1500 µL. In theory the more analyte applied on the membrane, the more OTA antibody binding sites occupied, the less OTA-HRP conjugate is allowed to bind, resulting in a lower visual detection limit. But increasing the sample volume prolonged the exposure time of the analyte to the OTA antibody. Thus a higher volume of extracted sample added on the membrane did not result in an improvement of the sensitivity or a lower cut-off level.

C.3. Evaluation of the extraction procedure for the detection of FB1 in maize

Key point is the solubility of FB1 in polar solvents such as water and methanol. When applying the flow-through assay in the field, the use of an aqueous extraction solvent would be preferable to a methanol mixture. Based on the article of E. Kulisek *et al*. (2000), we evaluated spiked maize extracted with the following solvents : 100 % ultrapure water (MilliQ), 50 % MeOH / H_2O (v/v) and 100 % PBS-buffer (pH 7.4). Figure 9 shows the results when the different extraction samples were applied in a flow-through enzyme immunoassay using the assay protocol described in 3.C.2 (dilutions for the anti-FB1 Mab and FB1-HRP are both 1/200). The 100% ultrapure water (MilliQ) and 100% PBS-buffer (pH 7.4) gave to much background noise, making evaluation of the test results difficult. Sample preparation will be done for now on using 50 % MeOH / H_2O as the extraction solvent.

100% ultrapure water (milliQ) 100% PBS-buffer pH 7.4 50% MeOH / H_2O (v/v)

Figure 9. Comparison of extraction solvents applied in the flow-through assay for blanc maize samples.

C.4. Establishment of a provisional flow-through enzyme immunoassay procedure for the detection of FB1 in maize

A range of assay procedures were evaluated using FB1 standard solutions [27 % MeOH / PBS-buffer, pH 7.4 (v/v)]. An overview is given in Table 9.

Table 9. Overview of parameters evaluated to establish a provisional assay procedure.

	Assay procedure							
	I	II	III	IV	V	VI	VII	VIII
Coating membrane : amount (µL) rabbit anti-mouse used	3	1.5	3	1.5	1.5	1	1	1
Anti-FB_1 Mab dilution	1/200	1/200	1/300	1/300	1/300	1/300	1/200	1/200
FB_1-HRP conjugate dilution	1/200	1/200	1/200	1/200	1/1250	1/200	1/200	1/200
Sample volume (µL)	1000	1000	1000	1000	1000	1000	1000	600

The test results were evaluated for the following criteria : colour intensity at the zero standard, decreasing colour development with increasing FB1 concentrations and the FB1 concentration resulting in complete colour inhibition. Procedure VIII resulted in a visual cut-off limit of 200 ng FB1/ml and was selected for further use. Applying the assay procedure VIII to spiked maize resulted in a complete colour inhibition close to the targeted value of 1000 µg/kg.

C.5. Conclusions and recommendations

The flow-through enzyme immunoassay for the detection of OTA in spiked wheat samples resulted in a visual cut-off value of 4 µg/kg. It was our goal to reach a visual detection limit near 5 µg/kg. Adapting the assay protocol for the use of dropping bottles instead of micropipettes will have an influence on the visual detection limit. Therefore a final procedure with application of dropping bottles will be elaborated. Applying the immunoassay protocol for the detection of OTA in wheat to the matrix wholemeal bread revealed no significant matrix interferences. Experiments to establish a procedure with a cut-off of 3 µg/kg are still going.

During the optimisation of the flow-through enzyme immunoassay for the detection of FB1 in maize 50% MeOH / H_2O (v/v) was selected as an extraction solvent because of the minimal background development. Assay procedure VIII showed the most promising results using standard solutions and spiked maize, with a visual detection limit of 200 ng FB1/mL and close to 1000 µg/kg, respectively.

D. Partner 4 : UCL-BIOC, Louvain-la-Neuve

D.1. The direct detection methods

D.1.1. The MTT assay

For each cell line, the cellular density has been adapted to be optimal for the assay. For each mycotoxin, dose/response curves were established, as represented in Figure 10.

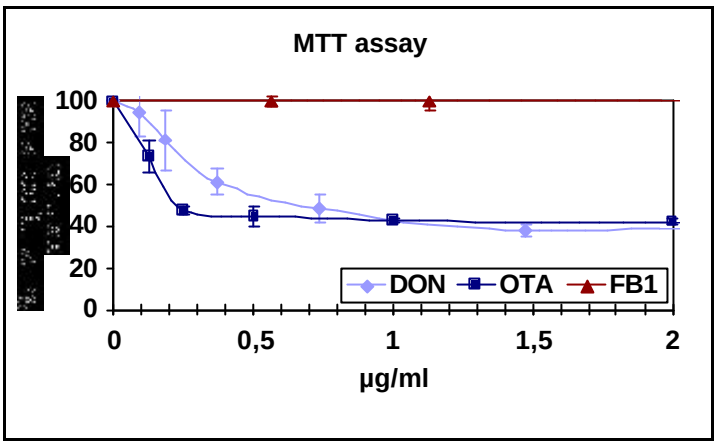


Figure 10. The effect of various concentrations of DON, OTA and FB1, on the viability of Caco-2 cells, was determined by the MTT bioassay. The values are expressed as percent of control response and are means of 4 wells ± SD.

DON and OTA have a clear concentration-dependent effect on the metabolic activity of the Caco-2 cells determined by the MTT bioassay (Figure 10). DON induces a significant cytotoxicity (20 %) from 0.2 µg/ml and OTA from 0.1 µg/ml. FB1 do not inhibit cell proliferation at all the concentrations tested, from 0.6 to 72 µg/ml (not shown).

For the Hep G2 and MDCK cells, comparable curves were obtained (results not illustrated).

The mean IC_{50}* values are calculated and presented in Table 10 : the three cell lines do not have the same sensitivity towards the mycotoxins. The Hep G2 cells are the most resistant to the mycotoxins and the Caco-2 cells the most sensitive. FB1 had a minor effect on the metabolic activity of the cells.

Table 10. IC_{50}* values for DON, OTA and FB1 exposed to Caco-2, MDCK and Hep G2 cells, determined by the MTT bioassay.

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

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

D.1.2. The Neutral Red assay

For each mycotoxin, dose/response curves were established, as represented in Figure 11. At the same concentrations used in the MTT assay (Figure 10), DON and OTA have only a minor effect on Caco-2 cells viability determined by the NR assay (Figure 11). Comparable results were obtained on the MDCK and Hep G2 cells. We were unable to observe a cytotoxic effect of FB1, whatever the condition tested.

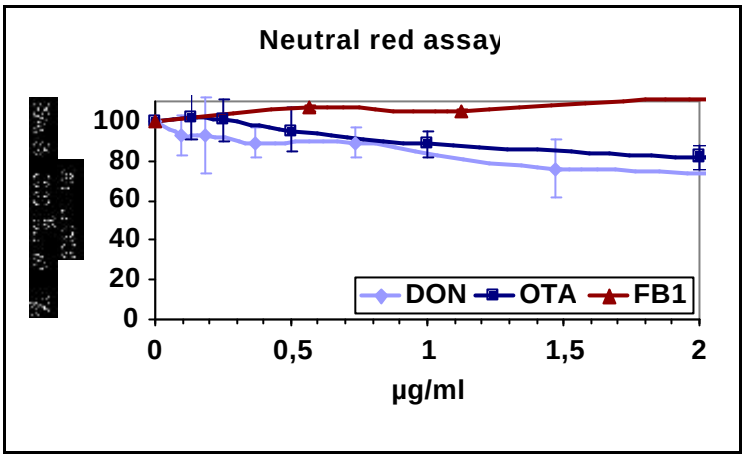


Figure 11. The effect on the viability of Caco-2 cells exposed to various concentrations of DON, OTA and FB1 was determined by the neutral red bioassay. The values are expressed as percent of control response and are means of 4 wells ± SD.

D.1.3. The LDH assay

The LDH assay has been done on the cell culture medium discarded after 48 hours mycotoxin incubation in the neutral red experiments. The amount of LDH activity released by the cells incubated with the mycotoxin is not significantly different from controls, except for the highest mycotoxin concentration (100 µM). At low concentrations, mycotoxins do not induce cell necrosis.

D.1.4. Conclusion and recommendations

If we compare the three methods, the MTT assay appears as the most sensitive and allows to detect low mycotoxin concentration by their cytotoxicity.

Table 11 indicates the minimal mycotoxin concentration that provokes a significant toxicity in relation with the cell lines. The Caco-2 and MDCK cells are sensitive enough to DON concentrations frequently found in cereals and to concentrations considered hazardous to animals and humans.

In the future, the MTT method will be mainly applied. The MTT method is also rapid and allows the screening of large number of samples at the same time.

Table 11. Minimal toxic values (µg/ml) for DON, OTA and FB1 exposed to Caco-2, MDCK and Hep G2 cells determined by MTT bioassay.

	Caco-2 cells	MDCK cells	Hep G2 cells
DON	0.20	0.10	0.40
OTA	0.12	4.00	2.00
FB1	10.00	10.00	70.00

D.2. The indirect detection methods

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

To try to detect the influence of lower mycotoxins concentrations, we have selected to follow, on the intestinal model, the tight junctions integrity by measuring the transepithelial electrical resistance (TEER) and the specific genes expression by the RT-PCR technique.

D.2.1. Transepithelial electrical properties

DON and OTA have a dose dependent effect on the intestinal ion permeability determined by the TEER measurements (Figure 12). DON induces a significant attenuation of the TEER from 50 ng/ml and OTA from 4 ng/ml. This indicates an alteration of the integrity of tight junctions in DON- or OTA-treated cells at concentrations where there is no effect on the cell viability evaluated by microscopic examination.

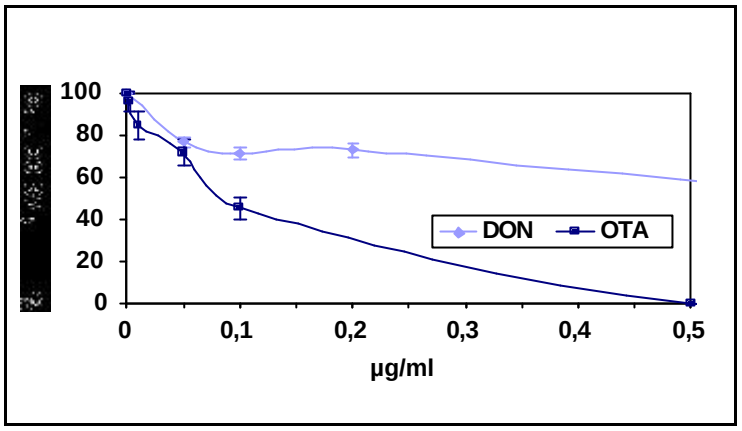


Figure 12. The effect on the permeability of Caco-2 cells exposed to various concentrations of DON and OTA was determined by TEER measurements. The values are expressed as percent of control response and are means of 2 independent experiments ± S.E.M (N = 2, n = 10 to 12).

D.2.2. Genes expression

We have screened the expression of different genes in relation with biotransformation systems on the cDNA from DON-treated cells. Preliminary results (Figure 13) show a dose-dependent reduction of the cytochrome P450 3A4 isoform expression in DON-treated cells from 500 ng/ml DON and an inhibition from 1 µg/ml. There is also a decrease in conjugation enzymes expression (UGT 1A6, UGT 2B7, PST) from 500 ng/ml DON. No effect on efflux pumps expression has been observed (not shown).

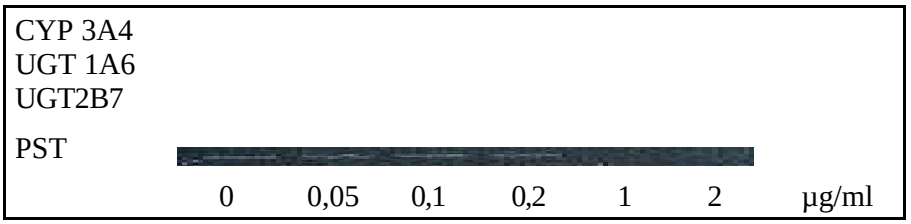


Figure 13. Genes expression, determined by RT-PCR, in DON-treated Caco-2 cells.

D.2.3. Conclusion

These preliminary results demonstrate that DON and OTA mycotoxins have a clear toxic effect at very low concentrations on the intestinal permeability functionality, measured by the TEER : if we compare with the MTT assay (Table 11), the minimal toxic concentrations of OTA and DON on Caco-2 cells TEER are 3 to 4 times lower. This indirect detection method allows to detect lower concentrations of mycotoxin.

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

5. FUTURE PROSPECTS AND FUTURE PLANNING

A. PARTNER 1 : UCL-BNUT, Louvain-la-Neuve

Analyses planned in the near future will complete OTA contamination data of the 40 cereals available. Results of OTA contamination in cereals lead us to find other contaminated samples to build up the sample bank (task 1.3). We are planning to perform artificial contamination of wheat grains since it is the best way to get highly contaminated samples quite rapidly (with the cooperation of partner 2, task 2.2).

Evaluation of DON contamination of the 40 cereal samples is in progress. Wheat samples with high DON content (50 ppm or more) are already available in the lab to build up the sample bank. The study will then consider the detection of DON in beers already collected. We will also continue the investigations to find more organic beers (task 1.3).

Once OTA and DON analyses of wheat flour and beer are finished, we will set up a sampling plan for other matrices (pasta, breakfast cereals) and adapt analysing methods for these kinds of foodstuffs (task 1.3).
New samples of cereals and other matrices deriving from cereals and produced during 2003 will be collected and distributed to other partners for analyses (task 1.4).

Concerning FB1, with the HPLC quantification method properly validated on maize kernels and sweet corn, we plan to analyse the samples that were already collected during the first year (task 1.2). Moreover we will supply others partners with reference samples in order to perform an intercomparison study (task 1.4). We will also adapt the quantification method to other maize based foodstuffs studied in this project (breakfast cereals, …) and determine the homogeneity of these foodstuffs in order to set up the best sampling procedure (task 1.3).

B. PARTNER 2 : CERVA-CODA, Tervuren

As seen, depending of the matrices, interfering peaks can be found on the chromatogram. To get the interfering peaks out of the purified samples, new purification techniques have to be developed using, e.g., multimode polymeric columns, ion exchange columns, other SPE columns based either on reversed phase or normal phase adsorption. Thus, the clean-up step has to be deeply investigated before any extension of the method for other matrices.
To separate interfering peaks from the peaks of interest, both the multi-linear elution gradient and the pH of the eluents have to be investigated. Even the determination method based on the results coming from the DAD detector should be developed in order to separate the mycotoxin peaks from the remaining interfering peaks. This last goal could be reached using an analytical program based on "Origin" and developed in our Centre.

Last goal to be reached, this multi-mycotoxin analytical method has to be validated by measures of linearity, repeatability, recovery, …

The extension of the method for the other matrices could then be developed (task 2.1).

Samples collected by partner 1 for the reference sample collection will be analysed for inter-comparison studies (task 2.3). Isolation and identification of unknown molecules by chromatography combined to mass spectrometry is planned for 2003 (task 2.4).

C. PARTNER 3 : RUG-LFA, (Universiteit Gent, Lab. for Food Analysis)

In the near future we plan to screen the samples supplied by the coordinator and coming from regular and “organic” Belgian wheat samples (task 3.5). The flow-through enzyme immunoassay for wholemeal bread will be further optimised to reach visual detection limit of 3 µg/kg. Assay protocols will be adapted for the use of dropping bottles. OTA field test will be applied to other matrices selected in the project (for example beer).

The screenings test for the detection of FB1 in maize will be further optimised and validated (task 3.1).

Participation to intercomparative studies (task 3.2) and elaboration of rapid field methods (task 3.4) are planned for 2003.

D. PARTNER 4 : UCL-BIOC, Louvain-la-Neuve

Up to now, we have only evaluated the direct cytotoxicity of pure mycotoxins. The suitability for detection of mycotoxin extracted from contaminated cereals is now in progress (task 4.1). That will permit to identify the matrix influence. Other experiments should also evaluate cytotoxicity of sample contaminated by more than one mycotoxin or by a mycotoxin, in the presence of other pollutants (task 4.2).

The indirect detection studies are now in progress with OTA and FB1 in an attempt to determine the lowest concentration that alters the intestinal function and to detect an alteration or an induction of genes that would be specific to each mycotoxin.

Participation to intercomparative studies will continue (task 4.2) and allow to determine the specificity and the limit of sensitivity of the methods studied.

New test to assess FB1 toxicity (measure of apoptosis) is under consideration (task 4.3).

6. ANNEXES

6.1. References

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