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Development and application of analytical methods adapted to the determination of mycotoxins in organic and conventional wheat

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Development and application of analytical methods for the determination of mycotoxins in organic and conventional wheat

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Running Head : Mycotoxins determination in wheat

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Abstract

The aim of this study was to develop a multi-component analytical method for the determination of deoxynivalenol (DON), ochratoxin A (OTA) and zearalenone (ZEN), nivalenol (NIV), 3-acetyl-DON (3-acDON), 15-acetyl-DON (15-acDON), zearalenol (ZOL) and citrinin (CIT) in wheat. It also aimed to survey the presence and amounts of DON, OTA and ZEN in Belgian conventionally and organically produced wheat grain and wholemeal wheat flours. After solvent extraction, an anion-exchange column (SAX) was used to fix the acidic mycotoxins (OTA, CIT) whilst the neutral mycotoxins flowing through the SAX column were further purified by filtration on a MycoSep cartridge. OTA and CIT were then analysed by HPLC using an isocratic flow and the fluorescence detection while the neutral mycotoxins were separated by a linear gradient and detected by double mode (UV-Fluorescence) detection. The average DON, ZEN and OTA recoveries from spiked blank wheat flour were 92, 83 and 73 % (RSD_R = 12, 10 and 9 %), respectively. Moreover, this method offered the respective detection limits of 50, 1.5 and 0.05 µg kg^{-1} and good agreement with reference methods and inter-laboratory comparison exercises.

Organic and conventional wheat samples harvested in 2002 and 2003 in Belgium were analysed for DON, OTA and ZEN while wholemeal wheat flour samples were taken from Belgian retail shops and analysed for OTA and DON. Conventional wheat tended to be more frequently contaminated with DON and ZEN than organic samples, the difference being more significant for ZEN in samples harvested in 2002. The mean OTA, DON and ZEA concentrations were 0.067, 675 and 75 µg kg^{-1} in conventional samples against 0.063, 285 and 19 µg kg^{-1} in organically produced wheat in 2002. Wheat samples collected in 2003 were less affected by DON and ZEN than the 2002 harvest. Organic wholemeal wheat flours were more frequently contaminated by OTA than conventional samples ($p<0.10$). The opposite pattern was shown for DON, organic samples being more frequently contaminated than conventional flours ($p < 0.10$).

Keywords: *Mycotoxins, Deoxynivalenol, Ochratoxin A, Zearalenone, Analytical method, wheat, Organic, Conventional, Belgium*

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Introduction

Organic farming does not permit the use of synthetic pesticides and relies on the use of resistant varieties of seeds as well as the use of some agricultural techniques including crop rotation, manure and compost fertilization. In contrast to this mode of cultivation, conventional farms use mineral fertilization and pesticides in the vegetation period. 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 increasing fungal contamination and consequently in increased levels of mycotoxins in the final products. So, there is evidence that organic food often contains relatively high amounts of natural toxic compounds produced by fungi or plants, whereas corresponding conventional food tends to contain more synthetic toxins such as pesticide residues (Finamore et al. 2004). Moreover, the use of pesticides or preservatives in insufficient amounts could lead to an even worse situation since the stress imposed on the moulds is thought to stimulate mycotoxin production.

In principle, although the foodstuffs resulting from conventional production are not free of mycotoxins, the organic foodstuffs may present in some circumstances a greater risk of contamination (Pussemier et al. 2006). Moreover, as there is a greater number of micro-organism species able to develop during the organic production (in contrast to the conventional counterpart), a wider spectrum of mycotoxins may potentially be detected in the organic foodstuffs than in the conventional counterparts. So, there is a need for information related to food safety, to inform consumers of the health benefits and/or

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hazards of food products of both origins, in order to optimize the impact on health and minimize the risks. Identification and quantitative assessment of mycotoxins present in specific raw materials, feed and foodstuffs as well as in manufactured food deserves thus further safety evaluation for public health concerns.

So far, most mycotoxin analyses of foodstuffs have usually been conducted using single toxin methods validated according to the criteria established by the European Union. These methods although sensitive, are time-consuming and thus not applicable for monitoring a large panel of mycotoxins. In fact, it is well known that some fungi species are able to produce several mycotoxins and that the co-existence of two or more toxins in cereals has frequently been documented in the literature, e.g. barley, malt, maize and wheat contaminated with deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEN) (Lee et al. 1985, 1986, Park et al. 1992, Tanaka et al. 1990, 2000), maize contaminated with fumonisin B_1 (FB1), DON, T-2 toxin, NIV and ZEN (Ali et al. 1998), wheat contaminated with DON and ochratoxin A (OTA) (Birzele et al. 2000), barley contaminated with OTA and DON (Olsson et al. 2002), wheat, rye, barley and several cereal-based commodities contaminated with DON and T-2 toxin (Biselli and Hummert 2005). Consequently, if contaminated raw materials are used for their production, manufactured products (such as cornflakes, meal flour, beer) can also be contaminated by all key cereal mycotoxins, except perhaps citrinin (CIT), which does not seem to survive the mashing step (Galvano et al. 2005).

The wide extent of mycotoxin co-occurrence requires an analytical method for their simultaneous detection and quantification in food and feed. Most common methods for simultaneous determination of A and B-trichothecenes require GC-ECD, GC-MS or LC

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with post-column derivatization and fluorescence detection (Krska 2001). Recently, LC/MS/MS has been increasingly applied for such purposes (Biselli and Hummert 2005).

The present contribution describes methods for the simultaneous determination of several mycotoxins in wheat using high performance liquid chromatography (HPLC) coupled with photodiode array-UV and fluorescence detectors. Emphasis is put on the clean-up procedures that combine in series an anion exchange (SAX) column and a MycoSep 226 column for the determination of NIV, DON, 15- and 3-acetyl deoxynivalenol (15-AcDON and 3-AcDON), zearalenol (ZOL), ZEN, CIT and OTA in wheat grains and wheat derived products. The validation of the method to determine the major mycotoxins DON, ZEN and OTA in wheat is first reported whilst further work is in progress in order to improve the analytical performances regarding the other toxins. To date, there is not enough information available regarding possible co-occurrence of these toxins taking into account the various contamination sources. A survey was therefore performed on wheat samples taken from organic and conventional farms in Belgium as well as on organic and conventional whole meal flour samples taken from the retail market.

Materials and methods

Reagents and standards

Pure crystalline form of NIV, DON, 3-AcDON, 15-AcDON, ZEN, ZOL, CIT and OTA were purchased from Sigma Chemical Co. (St Louis, MO, USA). Single standards were prepared in acetonitrile and composite working standard solutions were prepared mixing aliquots of stock solution in methanol:water (50:50).

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HPLC grade acetonitrile (AcN), methanol (MeOH) and toluene were obtained from Biosolve (Valkenswaard, The Netherlands). Acetic acid (AcOH), trifluoroacetic acid (TFA) and benzene were provided by Acros (New Jersey, NY, USA). Phosphoric acid was supplied by Sigma (St Louis, MO, USA). HPLC grade water was obtained using a ELIX3-Milli-Q combined system from Millipore (Molsheim, France).

SPE-SAX Extract Clean up columns (500 mg/4ml) were obtained from Alltech (Deerfield, IL, USA). Romer-MycoSep® cartridges 226 were provided by Coring Systems Diagnostix GmbH (Gernsheim, Germany). OTA immunoaffinity columns (OchraTest®) were obtained from Vicam (Watertown, MA, USA)

Sample collection and handling

Wheat grains and wholemeal wheat flour samples were tested in this study.

Wheat grains, harvested in August 2002 and in August 2003, were obtained directly from the organic and conventional producers. Taking into account the geographical (climatic and pedological factors) variability, samples were taken from different parts of Belgium with the minimum target sample size of 10 per mode of production and per region : Wallonia (20 samples) and Flanders (20 samples). Based on this size, volunteer farmers who were available to participate adhered to the study group. Thus, 20 conventional and 25 organic wheat producers were registered in 2002. In 2003, an additional 2 and 1 other farmers were enrolled in the conventional and organic farming groups, respectively. The sampling was performed by the farmers themselves according to our recommendations. They were asked to sample one kilogram of grains in 5 different buckets or at 5 different places in the heap of grains, in order to obtain a 5-kg sample representative of the parcel of land. The organic route of production was assured thanks to these collaborations. In the case of OTA

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analyses, all 20 conventional and 20 organic wheat samples were chosen at random from the 2002 sample set.

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

As for flour, eighty samples of wholemeal wheat flours were obtained from retail shops in Belgium. Forty conventionally produced samples (representing 10 brands) and forty organically produced flours (representing 10 brands) were assayed. To check the variation in batches and brands of the sampled materials, two batches per brand and two samples per batch were used in this study. All samples were stored at room temperature before analyses.

Sample preparation

A 25 g-portion of flour (ground and homogenized) sample was transferred into a 300 ml PTFE centrifuge vessel and mixed with 125 ml of extraction solvent (acetonitrile:water 84:16, v:v). The mixture was triturated for 3 min with the IKA Ultra-Turrax mixer (Stauffen, Germany) fitted with a T17−V shaft at 13500 rpm to produce slurry and then centrifuged for 10 min at 15300xg. An aliquot (10 ml) of the supernatant was then submitted to the following purification steps.

Because acidic and neutral mycotoxins could not be simultaneously purified on the MycoSep column, an anion exchange column (SAX) was used prior to the MycoSep column in order to fix the acidic mycotoxins on the SAX column. Before loading the extract, the SAX column was conditioned with methanol (5 ml) and water (5 ml). The centrifuged extract (10 ml) were poured on the column. After flowing through the SAX column, the neutral mycotoxins were further purified by filtration on the MycoSep cartridge. The SAX column was successively washed thereafter with water (5 ml),

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methanol (5 ml) and mixture (4 ml) of methanol:acetic acid (99:1, v:v). OTA and CIT were eluted with mixture (10 ml) of methanol:trifluoroacetic acid (99:1, v:v). Atmospheric air (about 10 ml) was passed through the column to collect all the acidic eluate which was then evaporated to dryness at 45 ± 5°C using a rotavapor. The residue was dissolved with 1 ml of methanol:water (50:50).

The eluate (9 ml) from SAX column were filled into Mycosep tube and then pushed through the Mycosep column. An aliquot of the purified eluate (4 ml) was taken from the flow-through and then evaporated to dryness at 45 ± 5°C using a rotavapor. The dry residue containing the neutral mycotoxins (DON, NIV, 3-AcDON, 15-AcDON, ZOL and ZEN) was dissolved with 1ml of methanol:water (50:50, v:v).

HPLC analysis

Identification and quantification of mycotoxins were performed by HPLC analysis. The chromatographic system consisted of a system of two Waters 510 pumps (Milford, MA, USA) equipped with an automatic WISP model 712 injector (Waters, Milford, MA, USA), a 2996 Diode array UV detector (DAD) and a 2474 fluorescence detector, all from Waters (Milford, MA, USA). Computer using the Millennium Empower software was used to process the signals of the detector and for data management. The reversed-phase analytical column, Alltima RP-C18 (100 x 4.6 mm id, 3 µm particles) obtained from Alltech (Deerfield, IL, USA) was thermostatically maintained at 35°C by a temperature controller Model 7990 (Jones Chromatography, Hengoed, UK) during HPLC analyses.

The analytical method used a linear gradient of two mobile phases eluted at 1 ml min^{-1}. The mobile phase A was a mixture of acetonitrile:water (2:98, v:v) whereas the mobile phase B was a mixture of acetonitrile:water (25:75, v:v). Water of both mobile phases contained 1

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mM phosphoric acid. The mobile phases were filtered and degassed on a 0.22 µm Durapore filter (Millipore, Molsheim, France).

Neutral mycotoxins were separated by a linear gradient, starting from 5 % to 100 % of phase B in 20 min. UV detector wavelengths were set at 225 and 236 nm whilst the fluorescence detection was performed at $\lambda_{excitation}$ = 280 nm and $\lambda_{emission}$ = 465 nm for ZEN. OTA and CIT were analysed using an isocratic flow of 48 % phase A and 52 % phase B. The detection was performed with the fluorescence detector set at $\lambda_{excitation}$ = 332 nm and $\lambda_{emission}$ = 462 nm). The injection volume was 25 µl of the eluates.

Validation

Analytical method for multi-mycotoxin determination in wheat was validated in-house and within inter-laboratory ring tests.

A set of nine wheat flour was spiked by adding mixture of standards at 4 different levels ranged from 200 to 2000 µg kg^{-1} for DON, 20 to 200 µg kg^{-1} for ZEN and 0.5 to 10 µg kg^{-1} for OTA. The initial concentration of each toxin in the wheat samples before spiking was determined and taken into account in the recovery calculations. Recovery and repeatability experiments were performed with these spiked samples. The detection and quantification limits (limit of detection (LOD) and limit of quantification (LOQ)) of the chromatographic procedure were determined by the signal-to-noise ratio of 3:1 and 10:1, respectively.

Inter-laboratory (FAPAS® CSL, UK) proficiency tests (series 17 round 32 of May - July 2004 and series 22 round 14 of April - June 2004) were also used to evaluate the method for the determination of OTA (FAPAS 2004a) and DON (FAPAS 2004b).

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Qualitative and quantitative confirmation of DON and OTA was done by comparing the concentrations obtained with the multi-mycotoxin method with those obtained with reference methods.

Analytical methods targeting OTA or DON (single mycotoxin) determination

OTA was extracted by using an adaptation of the extraction procedure of the Technical Committee for Standardisation (CEN/TC 275 1998). The method was described by Thirumala-Devi et al. (2001). Whole wheat flour samples (50 g) were mixed with chloroform (200 ml) and 0.1 M H_3PO_4 (20 ml). This mixture was triturated for 3 min with the Ultra-Turrax CAT x 620 shaft at 13500 rpm to produce a slurry. After centrifugation for 10 min under 820xg at 8 ± 2°C, the chloroform phase was transferred into a beaker. The remaining part was again extracted with chloroform (200 ml) and 0.1 M H_3PO_4 (20 ml). The combined chloroform phases (300 ml) were evaporated to dryness using a rotary evaporator at 35 ± 5°C. The residue was dissolved in 0.5 M $NaHCO_3$ (100 ml). After 10 min centrifugation under 820xg at 8 ± 2°C, 20 ml of the sample were passed through an Ochratest® immunoaffinity column. This column was fitted with a 20 ml-syringe reservoir and placed on a vacuum manifold. Before loading the extract, the Ochratest® column was conditioned with Phosphate Buffered Saline (PBS, pH 7.4) solution (20 ml). The OTA extract was passed through the column at about 1-2 ml min^{-1}. De-ionized water (20 ml) were used to wash the loaded Ochratest® and OTA was eluted with methanol (2 ml) and de-ionized water (2 ml). Atmospheric air (*ca* 20 ml) was passed through the column to collect all the eluate. This solution was stored at –20°C. Prior to HPLC analysis (fluorescence spectrophotometer detector; $\lambda_{excitation}$=332 nm, $\lambda_{emission}$=462 nm), it was brought to room temperature and then filtered with a 0.45 µm microfilter prior to HPLC analysis.

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Samples were analysed for DON by a method adapted from the extraction procedure proposed by Romer (1986). Twenty five grams of the milled and well mixed sample were transferred into a 300-ml centrifuge beaker and added with 125 ml of extraction solvent (acetonitrile:water, 84:16). The mixture was triturated for 3 min with the Ultra-Turrax device CAT x 620 shaft at 13500 rpm to produce a slurry. The solvent phase was isolated by centrifugation at 1835xg during 10 min at 8 ± 2°C. The resulting supernatant (10 ml) was placed in the MycoSep® test tube (Romer Labs, Coring system Diagnostix GmbH, Gernsheim, Germany) for purification. Four ml of the cleaned extract were evaporated to dryness, using a rotary evaporator at 45 ± 5°C. The residue was dissolved in 2 ml of methanol:water (10:90) and was stored at –20°C. Prior to HPLC analysis (UV detection at 218 nm), it was brought to room temperature and then filtered with a 0.45 µm microfilter.

Data handling and statistical analyses

The SAS Enterprise Guide software (SAS Institute, Inc., Cary, USA, version 3.0.0.369) was used for statistical studies. Differences in contamination frequency at level over the limit of quantification between conventional and organic samples were evaluated using Chi-square test performed by the procedure "PROC FREQ" of SAS. Variance analyses were performed on quantifiable concentrations using the GLM procedure of SAS. Statements of significance were based on probability of $p < 0.10$.

Results

Analytical method performance

Importance of SAX column. Extract clarification with SAX column (figure 1B) allows to eliminate the impurities or undesirable substances which may interfere with mycotoxin

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peaks (figure 1 A). As for acidic mycotoxins, the clean up of the extract with SAX column allows the specificity of the peaks of OTA and CIT in the chromatograms.

[Insert figure 1 A and B about here]

Performance characteristics. Taking the whole analytical procedure of sample extraction, clean-up and HPLC analysis into account, the overall recoveries, RSD_R, LOD and LOQ determined with spiked wheat samples are shown in Table I.

[Insert table I about here]

For DON, OTA and ZEN, the current multi-mycotoxin method fulfills the guidelines of the European Committee of standardization (CEN, CR 13505, 1999).

Comparison with reference method. Figure 2 shows the agreement between the DON concentrations determined on wheat using the multi-mycotoxin and the reference methods.

[Insert figure 2 about here]

The fit between both methods is rather satisfying taking into account the quite drastic differences in the clean-up of the samples with both methods and the non-significant deviation between the regression line (r = +0.978) and the identity one (r = +1).

Inter-laboratory performances. The accuracy of the present multicomponent method was checked against the analysis of DON and OTA in test materials within the frame of the inter-laboratory ring-tests organized by Food Analysis Performance Assessment Scheme (FAPAS® CSL, UK). Proficiency testing gave the respective Z-scores of -0.3 and 0.8 for DON (assigned value = 898.5 µg. kg^{-1}) and OTA (assigned value = 5.6 µg. kg^{-1}) determination in naturally contaminated wheat samples..

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One must note that the results show that the developed method is reliable and exact in quantifying DON and OTA in wheat samples since Z-scores are ranged between -2 and 2.

Applications of the analytical methods to Belgian wheat

Wheat grains and whole meal wheat flour samples were tested in this study taking into account their organic route of production. OTA determination was carried out in the 2002-harvests whereas DON and ZEN were studied in wheat collected in 2002 and 2003.

OTA in wheat grains and wholemeal wheat flour. The results of the analysis of 40 samples of wheat harvested in 2002 are summarized in Table II.

[Insert table II about here]

OTA was quantified in 4 conventional and 10 organic samples. Moreover, trace level of OTA was found in one conventional and one organic sample, leading thus to 25 and 55 % as the overall incidence rates of OTA occurrence in conventional and organic wheat samples. Chi-square test revealed a significant difference between these two ratios ($p = 0.047$). In contrast, the mean OTA concentrations in conventional (0.067 µg kg^{-1}) and organic (0.063 µg kg^{-1}) samples were comparable. The highest OTA contamination (1.097 µg kg^{-1}) was recorded in a conventional sample. One must note that all contaminated samples had an OTA concentration of < 5 µg kg^{-1}.

As for the OTA occurrence in wholemeal wheat flour sampled in the Belgian retail market, data are summarized in Table III.

[Insert table III about here]

All organic wheat flour samples were contaminated against 95 % of the conventional ones, with concentrations ranging from 0.141 to 2.199 µg kg^{-1} and from 0.064 to 1.035 µg kg^{-1},

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respectively. Among positive samples, analysis of variance revealed homogeneous contamination levels between different brands (p = 0.678) whatever the mode of production (p = 0.375). In contrast, some indication of significant contamination differences between samples of the same batch were observed (p = 0.081), showing thus the heterogeneity (large range of OTA concentrations) in the collected samples.

DON in wheat grains. The spreads of DON contents in wheat harvested in 2002 (45 samples) and in 2003 (48 samples) are summarized in Table IV according to their mode of production**.**

[Insert table IV about here]

In 2002, DON contamination was most frequently (p = 0.078) quantified in conventional wheat (90 % of the samples), compared to their organic counterparts (68 %). Analysis of variance indicated that the mean concentration (675 µg kg^{-1}) in conventional samples was significantly higher (p = 0.074) than the one observed in organic wheat (285 µg kg^{-1}). Moreover, none of the organic samples was found to be contaminated above 1250 µg kg^{-1} while 3 conventional samples had DON contamination above this limit. One must note that two conventional samples were severely contaminated (1991 and 2842 µg DON kg^{-1}). In 2003, DON was quantified in 73 % of organic samples and in 86 % of conventional wheat. Proportions of contaminated wheat (>LOQ) of conventional and organic origin are statistically equivalent (p = 0.259). Among samples contaminated at a level over LOQ, conventional wheat were statistically more contaminated compared to organic wheat (p = 0.008). Note that only one of the tested wheat samples was contaminated at a concentration above 750 µg kg^{-1}.

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DON in wholemeal wheat flour. Occurrence data in wholemeal wheat flour are shown in Table V.

[Insert table V about here]

In contrast to the OTA occurrence in the wheat flour samples, the frequency of DON contamination (>LOQ) was significantly ($p < 0.0001$) higher in conventional flours (87.5%) than in the organic ones (30.0 %). Among the positive samples (concentrations > LOQ), conventional flours were more DON contaminated than the organic samples, but the magnitude of the difference was not statistically significant ($p = 0.232$). None of the organic samples exceeded 250 µg kg^{-1} while four conventional samples (10 % total conventional samples) were contaminated at a concentration above 500 µg kg^{-1} but remained below 750 µg kg^{-1}, the future maximum level in wheat flour to be set by European regulations.

ZEN in wheat grains. Table VI presents the results of ZEN contamination in the organic and conventional samples collected in 2002 and 2003.

[Insert table VI about here]

Occurrence of ZEN at levels exceeding the LOQ was statistically higher ($p = 0.020$). in conventional samples (85 %) than in organic samples (52 %). A statistical comparison between quantified ZEN concentrations in conventional and organic samples indicated that the organic mode of production influenced the level of contamination ($p = 0.013$). None of the organic samples showed a contamination level above the proposed limit of 100 µg kg^{-1} to be set by the European Commission for unprocessed cereals. In contrast, 6 out of 20 conventional samples had a ZEN value above that limit. In addition, nine samples (either organic or conventional) had a ZEN value between 50 and 100 µg kg^{-1} and have therefore

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to be considered as not acceptable as bread and breakfast cereals (proposed limit set at 50 µg kg^{-1}). As for the 2003 harvest, ZEN concentrations were found to be mostly below the detection limit (LOD = 1.5 µg kg^{-1}) or the quantification limit (LOQ = 4 µg kg^{-1}). Only 3 samples were found to contain trace levels of ZEN. Because of the high number of non-detected samples, the mean, SD and median calculations are not representative. This finding is in deep contrast with the results obtained in 2002. In fact, the incidence of contamination was found as low as of 4 and 9 % for organic and conventional samples, respectively. The dry weather during June 2003 (flowering period for wheat) may likely explain these differences since a dry period at flowering is not favourable to the growth of *Fusarium.*

Co-occurrence of DON and ZEN in wheat grains. A significant correlation coefficient (r = +0.8239) was found between DON and ZEN contents in wheat grains harvested in 2002 (Figure 3), showing that a linear relationship exists between the concentrations of these two toxins. ZEN and DON were thus simultaneously found in the wheat samples harvested in 2002.

[Insert figure 3 about here]

Discussion

Mycotoxins in harvested grains

Incidence and levels of OTA contamination in the 2002 harvest were quite low. So far, contamination at levels above the LOQ was frequently found in organic samples than in conventional ones (10 *versus* 4 samples). The highest OTA level (1.097 µg kg^{-1}) was recorded in a conventional sample. One must note that all contaminated samples had an OTA concentration < 5 µg kg^{-1}, the maximum contamination level fixed by the European

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Commission Regulation (EC N° 123/2005). Within the framework of this study, OTA analysis was not performed in wheat samples harvested in 2003, since it was observed in 2002 samples that low OTA levels can be associated with fungal (pre-harvest) contamination, while extremely high (but not often) occurring levels of OTA are thought to be associated with post-harvest (storage) contamination.

A study carried out in 1997 on wheat samples collected in Poland after harvest detected OTA in none of the 32 samples from conventional farms (LOD = 0.2 µg kg^{-1}) while wheat samples from ecological farming showed contamination of almost 8% within the range 0.48-1.2 µg kg^{-1} (mean = 0.83 µg kg^{-1}) (Czerwiecki et al. 2002a). These results are in accordance with the present study as far as the frequencies of contamination are concerned but not in terms of the concentration levels. Conversely, results of the same study performed one year later contrast with our results in terms of frequency. In wheat from conventional farms, OTA concentrations reached extremely high levels since the range stretched from 0.6 to 1024.0 µg kg^{-1} (mean = 267 µg kg^{-1}) and the frequency of contamination was of about 48 %. In contrast, in wheat from ecological farming, OTA was detected in only 23 % of samples (range: 0.8 to 1.6 µg kg^{-1} and mean = 1.2 µg kg^{-1}). This finding which is different from the previous one may be due to the change in weather conditions observed within these 2 years (Czerwiecki et al. 2002b). Jorgensen & Jacobsen (2002) analysed conventionally (n = 405) and organically (n=14) grown wheat kernels from 1992 to 1999 and found that there was no difference in multiyear means between these two modes of production. OTA mean concentration (3.0 µg kg^{-1}) was much higher than the level obtained in Belgian wheat grains harvested in 2002.

As far as DON contamination is concerned, this toxin was detected in almost all the conventional and organic samples harvested in 2002. The incidence at a level above LOQ

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was of 90 and 68 %, respectively. Statistical analysis indicates a tendency to more frequent quantifiable amounts of DON in conventional samples than in organic ones. When contamination over LOQ occurred, it tended to be higher in conventionally produced wheat ($p = 0.077$). According to the last European proposal concerning mycotoxins in unprocessed wheat cereals, most of the samples had an acceptable level of contamination (< 1250 µg kg^{-1}). Concentrations were below this proposed limit in organically produced wheat while 3 conventional samples were contaminated at levels above 1250 µg kg^{-1}. High levels of DON (up to 2842 µg kg^{-1}) in grains collected at harvest in 2002 were observed because of the rainy weather recorded in June 2002. The climatic conditions were indeed favourable to the growth of *Fusarium* species. At harvest in 2003, DON concentration was found to be very low and may be explained by the dry weather observed in June 2003 over the flowering period. The mean (median) contamination levels in conventional and organic samples were of 311 (248) and of 123 (129) µg kg^{-1}, respectively. Similar variations in DON contamination in samples from two consecutive years of harvests carried out in 1997 and 1998 were previously attributed to different weather conditions during and after the flowering time (Birzele et al. 2000).

ZEN Contamination at a level above LOQ was frequently observed in conventional than in organic samples. Comparison of contaminated samples showed that the conventional ones contained higher levels of ZEN ($p = 0.013$). Wheat grains collected in 2003 were less affected by DON and ZEN than those harvested in 2002, due to a less favourable weather to contamination. In 2003, DON contamination occurred as frequently as in 2002, but at much lower levels (210 against 460. µg kg^{-1}). DON contamination higher than the LOQ occurred nearly as frequently in organic as in conventional wheat but the levels were higher in conventionally produced samples ($p = 0.008$). In 2003, none of the samples contained

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ZEN at a level above the LOQ value. These results confirmed once more that the contamination by *Fusarium* mycotoxins is weather dependent.

Mycotoxins in wheat flour from the retail market

Using the developed multi-mycotoxin method, the tested wholemeal wheat flours for bread making showed that both OTA and DON contents were very low. OTA was quantified in 42.5 and 87.5 % of the conventionally and organically produced flours, respectively. The organic route of production did not influence the level of contamination. An accidental high contamination may be observed since some indication of contamination differences between batches of a same brand was noticed.

Of 156 samples of conventional wheat flour and 120 samples of organic wheat flour purchased in Denmark between 1993 and 1999, Jorgensen & Jacobsen. (2002) found little difference in multiyear means of OTA between conventional samples (mean concentration of about 0.3 µg kg^{-1}) and organic ones (mean concentration of 0.5 µg kg^{-1}). These findings are in accordance with the current study. Biffi et al. (2004) found that conventional wheat flours were more OTA contaminated (0.134 µg kg^{-1}, n = 14) than organic flours (0.053 µg kg^{-1}, n = 11), but the magnitude of the difference was not significant. OTA analyses were performed on cereal-based baby food from Italy by Beretta et al. (2002). OTA occurred in 6 out of 10 batches of semolina from conventional agriculture with concentrations ranging from 0.14 to 0.65 µg kg^{-1}. Only one out of ten semolina batches from organically produced cereals contained a detectable amount of OTA (0.18 µg kg^{-1}; LOD = 0.060 µg kg^{-1}). In terms of frequency of contamination, these conclusions are opposed to those obtained in this study.

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As for DON contamination, the 80 samples analysed in the present study showed that concentration above LOQ occurred more frequently in conventional flours than in organically produced ones, but levels of contamination were equivalent in the two types of samples.

Schollenberger et al. (2002) analyzed Fusariotoxins in wheat flour collected in Germany in 1999. The 36 conventional samples were DON contaminated at a mean level of 394 µg kg^{-1} (incidence = 100 %) whereas the organic samples (n = 24) contained on average 131 µg DON kg^{-1} (incidence = 96 %). DON content in conventional samples was statistically higher than that in organic products. Globally, these results match with our findings. The same authors analysed the DON content in conventionally and organically produced wheat bread and showed some indication of a significant influence of the organic mode of production. Median DON levels in bread were higher for the conventional production mode.

Acknowledgments

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Figure 1: Representative HPLC separations of neutral mycotoxins (NIV (1),DON (2); 15-acDON (3), 3-acDON (4), ZOL (5), ZEN (6) in a wheat flour purified without (A) and with (B) SAX column.

Figure 2: DON concentrations determined by the multi-mycotoxin against the results obtained with the reference method on the same wheat samples

Figure 3: Correlation between DON and ZEN contents in wheat grains.

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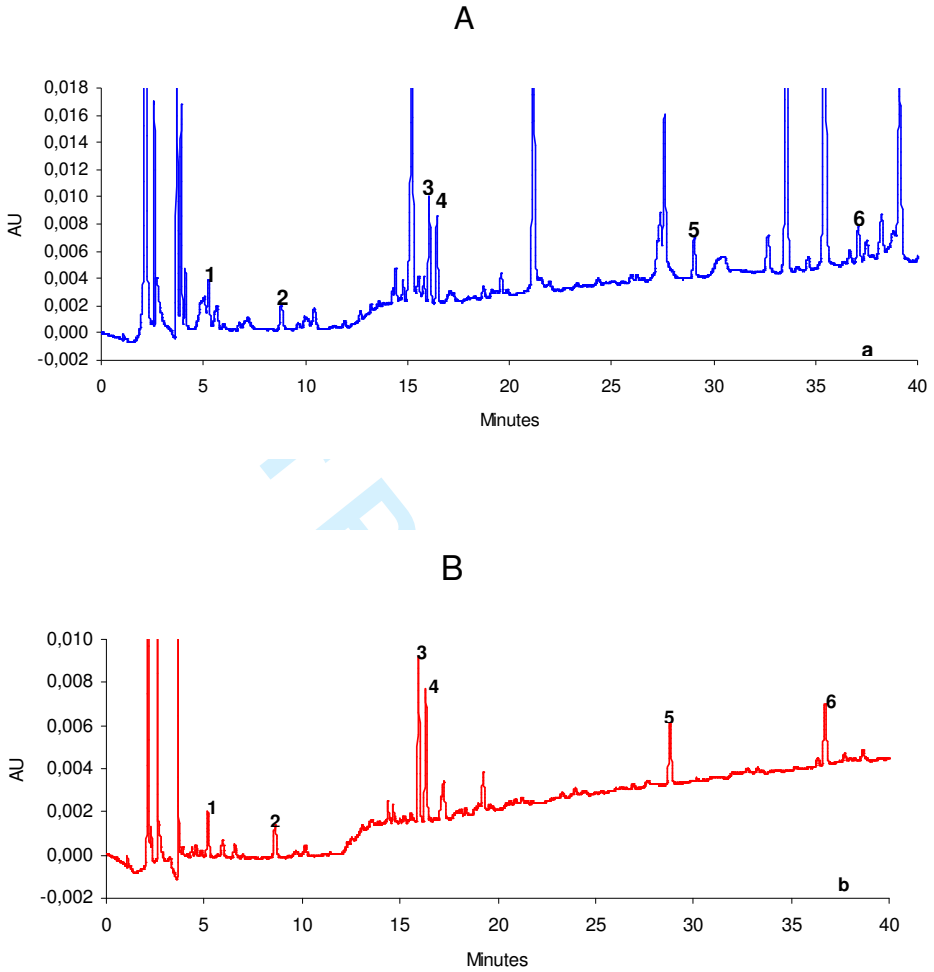

Figure 1: Representative HPLC separations of neutral mycotoxins (NIV (1),DON (2); 15-acDON (3), 3-acDON (4), ZOL (5), ZEN (6) in a wheat flour spiked at 500 µg NIV kg^{-1}, 500 µg DON kg^{-1}, 500 µg 15-acDON kg^{-1}, 500 µg 3-acDON kg^{-1}, 50 µg ZOL kg^{-1}, 50 µg ZEN kg^{-1} and purified without (A) and with (B) SAX column.

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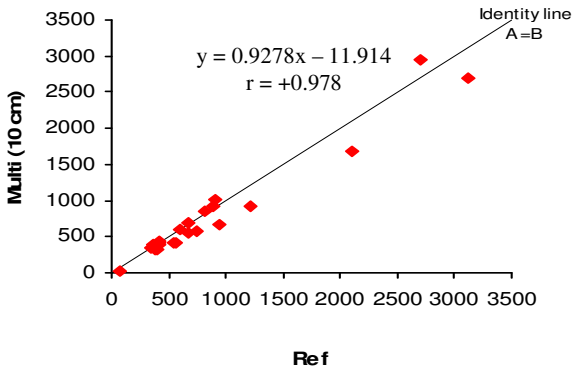

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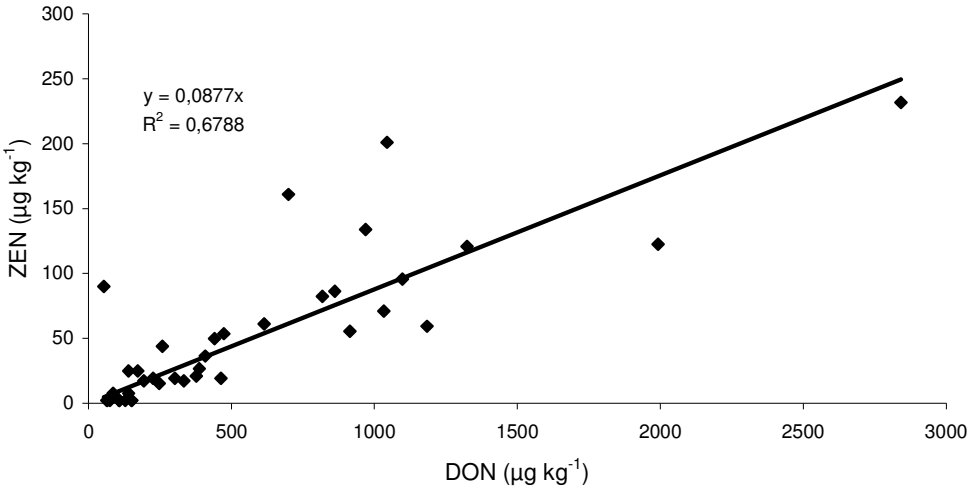

Figure 3: Correlation between DON and ZEN contents in wheat grains.

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Table I. Overall recoveries, reproducibility, detection limit and quantification limit of the multi-mycotoxin method.

Mycotoxins (spiking levels)	Recovery (%)	RSD_R[b] (%)	LOD[c] ($\mu g\ kg^{-1}$)	LOQ[d] ($\mu g\ kg^{-1}$)
DON (50-1500 $\mu g\ kg^{-1}$)	92	12	50	150
ZEN[a] (2-500 $\mu g\ kg^{-1}$)	83	10	1.5	4
OTA[a] (0.5-10 $\mu g\ kg^{-1}$)	73	9	0.050	0.150

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

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Table II. Occurrence of OTA in wheat grains harvested in 2002.

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

[a]: n.d., not detected (OTA concentration in sample <0.01 µg kg^{-1})
[b]: traces means that the concentration of OTA is between the limit of detection (LOD = 0.01 µg kg^{-1}) and the limit of quantification (LOQ = 0.035 µg kg^{-1}).
[c]: mean and median are computed assuming that non-detected samples contain half the LOD while samples with traces levels of OTA contain half the LOD+LOQ.
[d]: range of samples with OTA contents ≥ LOQ.

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Table III. Occurrence of OTA in wholemeal wheat flour.

Occurrence	Conventional	Organic	Total
Number of samples	40	40	80
n.d.[a]	2 (5%)	0	2 (2.5%)
traces[b]	21 (52.5%)	5 (12.5%)	26 (32.5%)
LOQ-1.0 µg kg^{-1}	16 (40.0%	34 (85.0%)	50 (62.5%)
> 1.0 µg kg^{-1}	1 (2.5%)	1 (2.5%)	2 (2.5%)
Incidence	38 (95%)	40 (100%)	78 (97.5 %)
Levels of contamination (µg kg^{-1})			
Mean[c]± SD	0.228 ± 0.262	0.480 ± 0.530	0.354 ± 0.434
Median[c]	0.100	0.378	0.214
Range[d]	0.064-1.035	0.141 – 2.199	0.064– 2.199

[a]: n.d., not detected (DON concentration in sample < 0.050 µg kg^{-1})
[b]: traces means that the concentration of OTA is between the limit of detection (LOD = 0.050 µg kg^{-1}) and the limit of quantification (LOQ = 0.150 µg kg^{-1})
[c]: mean and median are computed assuming that non-detected samples contain half the LOD while samples with traces levels of OTA contain half the LOD+LOQ
[d]: range of samples with OTA contents ≥ LOQ

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Table IV. Occurrence of DON in wheat grains collected during harvest 2002 and 2003

Occurrence	Conventional		Organic		Total samples	
	2002	2003	2002	2003	2002	2003
Number of samples	**20**	**22**	**25**	**26**	**45**	**48**
n.d.[a]	0	0	1(4%)	1 (4%)	1 (2%)	1 (2%)
traces[b]	2 (10%)	3 (14%)	7 (28%)	6 (23%)	10 (22%)	9 (19%)
LOQ – 250 µg kg^{-1}	6 (30%)	8 (36%)	8 (32%)	19 73%)	13 (29%)	27 (56%)
250 – 500 µg kg^{-1}	4 (20%)	9 (40%)	5 (20%)	0	9 (20%)	9 (19%)
500 – 750 µg kg^{-1}	1 (5%))	1 (5%)	1 (4%)	0	2 (5%)	1 (2%)
750 – 1250 µg kg^{-1}	4 (20%)	0	3 (12%)	0	7 (16%)	0
1250 – 1750 µg kg^{-1}	1 (5%)	1 (5%)	0	0	1 (2%)	1 (2%)
> 1750 µg kg^{-1}	2 (10%)	0	0	0	2 (4%)	0
Incidence	**20 (100%)**	**22 (100%)**	**24 (96%)**	**25 (96%)**	**44 (98%)**	**47 (98%)**
Levels of contamination (µg kg^{-1})						
Mean[c]± SD	675 ± 579	311 ± 303	285 ± 305	123 ± 47	460 ± 562	210 ± 226
Median[c]	425	248	151	129	226	138
Range[d]	100 - 2842	79 - 1503	100 -1184	10- 226	100 - 2842	10 - 1503

[a] : n.d., not detected (DON concentration in sample <30 µg kg^{-1})
[b] : traces means that the concentration of DON is between the limit of detection (LOD = 30 µg kg^{-1}) and the limit of quantification (LOQ = 100 µg kg^{-1}).
[c] : mean and median are computed assuming that non-detected samples contain half the LOD while samples with traces levels of DON contain half the LOD+LOQ.
[d] : range of samples with DON contents ≥ LOQ.

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Table V: Occurrence and levels of DON in wholemeal wheat flour.

Occurrence	Conventional	Organic	Total samples
Number of samples	40	40	80
n.d. (< 30 µg kg^{-1}) [a]	0	12 (30%)	12 (15%)
Traces (< 100 µg kg^{-1}) [b]	5 (12.5%)	16 (40%)	21 (26%)
LOQ – 250 µg kg^{-1}	22 (55%)	12 (30%)	34 (43%)
250 – 500 µg kg^{-1}	9 (22.5%)	0	9 (11%)
500 – 750 µg kg^{-1}	4 (10%)	0	4 (5%)
> 750 µg kg^{-1}	0	0	0
Incidence	40 (100%)	28 (70%)	68 (85%)
Level of contamination (µg kg^{-1})			
Mean[c] ± SD	249 ± 148	92 ± 54	170 ± 136
Median[c]	211	100	100
Range[d]	153 - 661	157 - 210	153 - 661

[a]: n.d., not detected (DON concentration in sample <50 µg kg^{-1})
[b]: traces means that the concentration of DON is between the limit of detection (LOD = 50 µg kg^{-1}) and the limit of quantification (LOQ = 150 µg kg^{-1}).
[c]: mean and median are computed assuming that non-detected samples contain half the LOD while samples with traces levels of DON contain half the LOD+LOQ.
[d]: range of samples with DON contents ≥ LOQ.

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Table VI. Occurrence of ZEN in wheat grains collected in Belgium during harvest 2002 and 2003

Occurrence	Conventional		Organic		Total samples	
	2002	2003	2002	2003	2002	2003
Number of samples	20	22	25	26	45	48
n.d.[a]	3 (15%)	20 (91%)	12 (48%)	25 (96%)	15 (33%)	45 (94%)
traces[b]	0	2 (9%)	0	1 (4%)	0	3 (6%)
LOQ – 50 µg kg^{-1}	7 (35%)	0	8 (32%)	0	15 (33%)	0
50 – 75 µg kg	2 (10%)	0	3 (12%)	0	5 (11%)	0
75 – 100 µg kg^{-1}	2 (10%)	0	2 (8%)	0	4 (9%)	0
> 100 µg kg^{-1}	6 (30%)	0	0 -	0	6 (13%)	0
Incidence	**17 (85%)**	**2 (9%)**	**13 (52%)**	**1 (4%)**	**30 (67%)**	**3 (6%)**
Levels of contamination (µg kg^{-1})						
Mean[c]± SD	75 ± 68	2 ± 1	19 ± 27	1 ± 1	44 ± 67	1 ± 1
Median[c]	53	1	8	1	19	1
Range[d]	4 - 86	0.75 - 4	4 - 232	0.75 - 4	4 - 232	0.75 - 4

[a]: n.d., not detected (ZEN concentration in sample <1.5 µg kg^{-1})
[b]: traces means that the concentration of ZEN is between the limit of detection (LOD = 1.5 µg kg^{-1}) and the limit of quantification (LOQ = 4 µg kg^{-1})
[c]: mean and median are computed assuming that non-detected samples contain half the LOD while samples with traces levels of ZEN contain half the LOD+LOQ
[d]: range of samples with ZEN contents ≥ LOQ

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