DETERMINATION OF MASKED FUSARIUM MYCOTOXINS IN CEREALS AND CEREAL-BASED FOOD

Report Number:	FD 09/04
Authors:	F Berthiller, O Vendl and C Crews
Date:	September 2009
Sponsor:	Food Standards Agency Aviation House London
Sponsor's Project Number:	C03060
Fera Contract Number:	S6OU
Fera File Reference:	FLN 8968
Principal Workers:	O Vendl, F Berthiller, and C Crews
Team leader:	C Crews

The Food and Environment Research Agency
Sand Hutton

This page is intentionally left blank

Table of Contents

TABLES

GLOSSARY

$[M+H]^+$ The protonated molecular ion. In HPLC-MS and HPLC-MS/MS the ion produced by addition of H^+ to the unfragmented analyte molecule. This can give an indication of the analyte's molecular weight and to confirm the identity of a response (peak) in the chromatogram. Fragmentation of the protonated molecular ion is commonly used in HPLC-MS/MS.

$[M-H]^-$ The deprotonated molecular ion.

15AcDON, 15-Acetyldeoxynivalenol, a *Fusarium* mycotoxin.

3AcDON 3-Acetyldeoxynivalenol, a *Fusarium* mycotoxin.

D3G Deoxynivalenol-3-β-D-glucopyranoside, a bound *Fusarium* mycotoxin

ACN Acetonitrile

amu Atomic mass unit. The weight of a compound or part of a compound as measured by a mass spectrometer.

APCI Atmospheric Pressure Chemical Ionization, a means of producing ions for mass spectrometry.

Background. The non-analyte 'baseline' signal seen in a chromatogram that is due to small responses derived from minor unidentified compounds extracted from a sample and/or from impurities in the solvents used and/or from the column material and contaminants from prior samples.

C18 A material for chromatography based on the 18 carbon compound octadecylsilane

Chromatogram. A picture showing the signal produced by an LC-MS or LC-MS/MS system over a period of time. When a compound elutes from an HPLC column it produces a response in the detector that is plotted against time.

DON Deoxynivalenol, a *Fusarium* mycotoxin

EFSA European Food Safety Authority

ESI Electrospray ionisation. An operating mode in LC-MS in which ionised particles are produced.

ESI-MS Electrospray ionisation mass spectrometry. Usually operated in the positive mode ESI(+).

FB_1 fumonisin B_1 a *Fusarium* mycotoxin.

HPLC (LC) High performance liquid chromatography. An instrumental technique for separating relatively non-volatile compounds with high efficiency.

HPLC-MS/MS HPLC-MS/MS (HP)LC coupled to a mass spectrometer having a second fragmentation stage in tandem. An ion produced in the first stage can selected and fragmented by collision with gas molecules to produce a mass spectrum derived solely from that ion. Selected monitoring of the production of one or more ions from that spectrum (SRM) provides very high selectivity which in turn gives a low background signal and hence high sensitivity.

LOD The limit of detection. Defined as the lowest concentration that will be detected with a defined probability. In simple terms the lowest amount that can be distinguished from the background signal.

LOQ The limit of quantification. The smallest amount of analyte that can be quantitatively determined with suitable precision and accuracy. In simple terms the lowest amount that can be quantified with an acceptable degree of certainty and hence normally used as a reporting limit.

m/z The mass to charge ratio of an ion produced in a mass spectrometer, usually the same as amu.

Mass spectrometer. An instrument for measuring and identifying the output from and HPLC system (LC-MS). It fragments molecules emerging from the HPLC column into charged particles (ions) that are separated and measured in terms of mass (=identity) and intensity (=concentration).

MRM - multiple reaction monitoring, a method involving measurement of the ions produced during MS/MS.

MS Mass spectrometry. Detection technique where analytes (usually separated by chromatography) are ionised to produce fragments that can be separated and characterised as a mass spectrum and quantified.

MS/MS, a tandem mass spectrometry method for producing a secondary spectrum from selected single ions.

PSA Primary secondary amine. A sorbent material with properties of separating impurities from sample extracts

RSU The relative standard uncertainty associated with results of a measurement.

S/N The signal-to-noise-ratio. A measure of the intensity of a signal derived from LC-MS compared to the background signal.

SPE Solid-Phase Extraction

SRM Selected Reaction Monitoring

TDI tolerably daily intake, the maximum acceptable exposure to a toxin

TOF Time-Of-Flight. An ion separation system in mass spectrometry that provides highly accurate molecular weight information.

Z4G ZON-4-*β*-D-glucopyranoside, a bound form of the mycotoxin zearalenone.

Z4S Zearalenone-4-sulfate, a bound form of the mycotoxin zearalenone.

β-ZOL β-zearalenol, a *Fusarium* mycotoxin.

α-ZOL α-zearalenol, a *Fusarium* mycotoxin.

ZON zearalenone

Summary

- An HPLC-MS/MS method for reliable detection and quantification of free and bound *Fusarium* mycotoxins has been developed and validated in-house.
- The method can determine deoxynivalenol, acetyl-deoxynivalenol, deoxynivalenol-3-glucoside, zearalenone-4-glucoside, zearalenone-4-sulfate, α-zearalenol, β-zearalenol, α-zearalenol-4-glucoside and β-zearalenol-4-glucoside in cereals and cereal-based food.
- Strong matrix effects that altered signal response during HPLC-MS detection of the target analytes were observed and procedures were adopted to diminish these, however, no single cleanup procedure was entirely satisfactory.
- Method validation was accomplished for 10 cereal-based matrices analysed in both the UK and Austria. The method is suitable and stable for the determination of the target *Fusarium* toxins in cereals and cereal based food.
- Application of the method to 31 composites of cereal grain and cereal-based food showed deoxynivalenol in four samples, zearalenol in two samples and zearalenone-4-sulfate in 12 samples. None of the detected values exceeded European Commission regulatory levels, and no high risk of mycotoxin intoxication was indicated.
- Enzymatic pre-treatment of cereal samples gave heightened concentrations of deoxynivalenol and especially deoxynivalenol-3-glucoside.
- Simple pretreatment without enzyme also heightened deoxynivalenol-3-glucoside, possibly through simple aqueous extraction.

0. Project aims

0.1. Conjugated mycotoxins

Mycotoxins, as most other xenobiotics, are partly metabolised by living plants, including food crops. Humans consuming parts of the contaminated plants, especially cereals or their processed products, are therefore not just exposed to the native (free) mycotoxins, but also to conjugated forms. Little is known about the occurrence, bioavailability and further metabolism of some of these bound compounds, which also escape the usual analytical detection techniques used for routine control. This leads to an potential underestimation of the total consumers' exposure to mycotoxins.

Fusaria are among the most prevalent fungi in the UK. The main infections with *Fusaria* occur on storage or in the field, where crops are still alive, growing and able to metabolise mycotoxins. Therefore, research should focus on mycotoxins from field fungi, especially from *Fusarium*. Examples for microbial transformation and conjugation products can be found with the estrogenic mycotoxin zearalenone (ZON). Phase I metabolism products of ZON - α-zearalenol (α-ZOL) and β-zearalenol (β-ZOL) - can be formed by a variety of microorganisms (Böswald et al., 1995). Zearalenone-4-sulfate (Z4S) was identified after microbial transformation of ZON by *Rhizopus arrhizus* (El-Sharkawy et al. 1991). The same substance also was found to be a natural *Fusarium* metabolite, which is produced in a molar ratio from 8% to 50% of ZON, depending on the used strain and growth conditions (Plasencia and Mirocha 1991). Similarly, to Z4S, also ZON-4-*β*-D-glucopyranoside (Z4G) was originally found to be formed from ZON by *Rhizopus sp.* (Kaminura 1986), *Thamnidium elegans* and *Mucor bainieri* (El-Sharkawy and Abul-Hajj 1987). The major significance of Z4G, however, is that of a plant metabolite, which is discussed in the next section. *Thamnidium elegans* is also able to convert ZON to its 2,4-diglucoside (El-Sharkawy 1989).

In 1988, Z4G was shown to be a metabolite of plants, after ZON was transformed by maize cell suspension cultures to its glucoside (Engelhardt et al. 1988). A mini-survey of 24 wheat samples - 22 of them were contaminated with ZON above the limit of quantification - resulted in 10 samples (42%) being found also positive for Z4G (Schneweis et al. 2002). Approximately 10-20% of the total ZON content of these samples was detected as Z4G. Several papers describe the metabolism of mycotoxins in plants, especially the phase II transformation of ZON to Z4G (Gareis 1994, Wallnöfer et al. 1996, Engelhardt et al. 1999). The biotransformation products of ZON were previously elucidated using the model plant *Arabidopsis thaliana* by our group (Berthiller et al. 2006). After treatment of plant seedlings with 50µM ZON both the liquid media and the plant extracts were analysed with HPLC-MS/MS. An array of 17 different metabolites, most prominently glucosides, malonylglucosides, diglucosides and hexose-pentose-disaccharides of ZON, α-zearalenol

and β-zearalenol were detected in the samples. Two of those substances α-zearalenol-4-*β*-D-glucopyranoside (α-ZG) and β-zearalenol-4-*β*-D-glucopyranoside (β-ZG) were recently synthesised from Z4G by the co-proposers group (Krenn et al. 2007, Berthiller et al. 2009a).

There has been a lot of speculation about possible conjugation products of deoxynivalenol (DON). In 1983 it was shown that the DON concentration of *Fusarium graminearum* infected wheat reached a maximum and then declined up to harvest (Miller et al. 1983). A year later it was reported that the DON content of yeast fermented food products was higher than that of the contaminated flour used for their production (Young et al. 1984). Without knowing the exact structures of possible plant metabolites, glucose and fatty acids conjugates of DON were chemically synthesised (Savard 1991). Deoxynivalenol-3-*β*-D-glucopyranoside (D3G) was identified as the main DON metabolite after treatment of maize cell suspension cultures with DON (Sewald et al. 1992). In 2005 the first report on the natural occurrence of D3G was published by the co-proposers group (Berthiller et al. 2005). Recent, yet unpublished, results showed that D3G occurred with DON in 23 out of 23 naturally contaminated wheat and in 54 out of 54 naturally contaminated maize samples. The D3G concentration in the positive samples was in the range of about 8% to 70% of the DON concentration.

In addition to metabolism, processing of food (e.g. cooking, baking, brewing) is another source of mycotoxin conjugates. Fumonisins are the best studied mycotoxins in this regard. *N*-(carboxymethyl)-fumonisin B_1 (Howard et al. 1998) and *N*-(1-deoxy-D-fructos-1-yl)-fumonisin B_1 (Poling et al. 2002) have been identified as conversion products of fumonisin B_1 (FB_1) after heating with reducing sugars. Model experiments yielded evidence that FB_1 might be bound to other saccharides, amino acids (Seefelder et al. 2003) and proteins (Kim et al. 2003), (Park et al. 2004). A review of the effects of thermal food processing on the chemical structure and toxicity of fumonisin mycotoxins was published (Humpf and Voss 2004). Apart from the occurrence of D3G in beer, arising from the brewing process, (J. Hajslova, personal communication), little is known about conjugates of DON or ZON due to food processing.

The formation, determination and significance of conjugated mycotoxins was recently reviewed (Berthiller et al. 2009b).

Analytical methods for conjugated mycotoxins

Both direct and indirect methods exist for the qualitative and quantitative determination of mycotoxins and their conjugates. With indirect methods the precursor toxin is quantified before and after acidic or basic or enzymatic hydrolysis of the conjugate. Z4G is one of the best studied substances in this regard. β-Glucosidase sample treatment was used to indirectly determine Z4G concentrations (Gareis et al. 1990). Direct techniques employed HPLC separation with fluorescence (Zill et al. 1990) or MS (Schneweis et al. 2002) detection. Multimycotoxin LC-MS/MS methods able to quantify DON and its conjugates D3G, 3-

acetyldeoxynivalenol (3ADON), 15-acetyldeoxynivalenol (15ADON) as well as ZON and its metabolites α-ZOL, β-ZOL, Z4S, Z4G were developed by the co-proposers group using a 4000QTrap LC-MS/MS instrument (Sulyok et al. 2006), 2007).

In general, LC-MS/MS is fast becoming the most powerful analytical tool for the determination of mycotoxins and their metabolites. The main advantages include low detection limits, the ability to generate structural information, the requirement of minimal sample treatment and the possibility to cover a wide range of analytes differing in their polarities. Polar compounds are quickly accessible without the need of derivatisation. Matrix effects, however, limit the potential of LC-MS. Ion suppression (or enhancement) might be encountered due to matrix components that co-elute with the analyte of interest. Thorough validation of developed LC-MS/MS methods therefore is a necessity.

Fate of xenobiotic conjugates in animals

Gastrointestinal microorganisms are particularly adept at catalyzing hydrolytic reactions. Metabolism of conjugates by gut microflora may result in cleavage of the conjugate, releasing the less polar precursor which, as a result of increased lipophilicity, may be reabsorbed through the intestine (Ioannides 2002). After transport of the xenobiotic into the liver, where it is further metabolised, it can re-enter the gut via the bile. Partial hydrolysis by microorganism might then occur again, which prolongs the time of exposure of a given compound in the body. This phenomenon is known as enterohepatic circulation and works for all xenobiotics and their conjugates. As a consequence, plant derived mycotoxin conjugates can be reactivated in the digestion tract of animals, before final elimination can be achieved by renal excretion and loss via the intestine.

The only mycotoxin metabolite produced by plants that has been subject to a thorough animal study is Z4G. This conjugate is completely cleaved to ZON during digestion in swine (Gareis et al. 1990). In that same study the term “masked mycotoxin” was introduced to emphasise a substance that is usually not detected in routine analysis of feed but contributes to the total mycotoxin content. Initial results of hydrolysis studies for D3G proved that D3G is at least partly cleaved to DON both by human gut bacteria and in the digestive tract of rats. The situation gets even more complicated when bound residues are taken into account. Being non-extractable by definition, radioactive isotopes or (to some extent antibodies) seem to be the only ways to monitor such residues. A recent review on bound pesticidal plant residues reports bioavailability rates from as low as 2% to over 80% (Sandermann 2004).

Legislation

The Scientific Committee for Food (SCF) established a tolerably daily intake (TDI) of 1 µg/kg body weight/day for DON and a temporary TDI of 0.2 µg/kg body weight/day for ZON (EC Regulation 856/2005). While the SCF considered that the data available up to 2002 did not

support the establishing of a group TDI for trichothecenes, recent findings might render that conclusion incomplete. For instance, total trichothecene (DON+D3G+ADONs) levels of up to 90 µg/L have been found in beer from the European market (J. Hajslova, personal communication), meaning that the consumption of two pints of beer would already exceed the TDI levels of DON for an average human. Maximum levels for DON and ZON in cereals, flour and some cereal-products (e.g. bread, pasta, breakfast cereals) are in force, but conjugated forms of these mycotoxins are not regulated.

0.2. Scientific objectives

- Task 1: To develop and validate suitable analytical methods based on LC-MS/MS detection to quantify DON, 3AcDON + 15AcDON, D3G, ZON, Z4S, Z4G, α-ZOL, β-ZOL, α-ZG and β-ZG in standard solutions.

- Task 2: To develop novel and reliable sample preparation strategies, involving enzymatic treatments, to detect both soluble and otherwise insoluble forms of the listed substances.

- Task 3: To develop various clean-up strategies to purify and enrich the conjugated toxins.

- Task 4: To validate the method developed by comparison of its performance at both the Fera and IFA-Tulln laboratories.

- Task 5: To demonstrate application of the methods to a wide range of cereal products.

- Task 6: To present the results of the study to the FSA in the form of a written report (this document), and to prepare two publications for appropriate peer-reviewed scientific journals.

1. Method development and optimisation

Scope of task 01 - Optimisation of HPLC-MS/MS parameters

To develop and validate suitable analytical methods based on LC-MS/MS detection to quantify DON, 3- and 15-acetyl-DON, D3G, ZON, Z4S, Z4G, α-ZOL, β-ZOL, α-ZG and β-ZG in standard solutions. All substances were available as purified (>98%), NMR characterised, solid crystals at the IFA-Tulln.

Optimisation of the MS/MS parameters for the masked mycotoxins will include the optimisation of the LC-flow and the source temperature to obtain optimum ionisation efficiency. Different LC-MS-interfaces (ESI and APCI) will be tested, but the ESI interface will be used for the ZON metabolites, as ZON-4-sulfate cannot by ionised by APCI as it is already a charged compound in solution. Moreover, the selection of appropriate collision energy and of characteristic fragment ions will be used for structural identification. 2 MRM transitions will be incorporated into the method for each analyte (one quantifier, one qualifier). Both methods will use the negative ionization mode. Preliminary validation of this end-determination step will provide information on the linearity of the calibration function, sensitivity and precision of the LC-MS/MS method for the determination of calibrants (=standard solutions). MS/MS parameters will be optimised using flow injection of all of the available standards in solvent. HPLC parameters will be optimised for all of the available standards using methanol/water mobile phases with an appropriate column such as the Phenomenex Gemini RP-C18. If possible, the two methods will be combined into a single one.

1.2. Optimisation of MS/MS parameters

Standard solutions (100 ng/ml analyte in methanol) of all target compounds were injected into the mass spectrometer via a syringe pump. Ammonium acetate (NH_4Ac, 5mM) was added to standard solutions as acetate adducts frequently improve ionisation. Electrospray ionisation (ESI) in negative polarity was used as ion source. For each analyte, the full scan mode was initially applied (mass range: 50 – 500 amu, D3G: 50 – 600 amu), with fragmentation monitored in product ion scan mode (see Figure. 1). MRM (multiple reaction monitoring) methods were established and parameters optimised for each of the target compounds. Out of the five most intensive transitions, the three most distinctive were chosen for further analysis. The compound-specific declustering potential, as well as transition-specific parameters (collision energy, cell exit potential) were individually ramped for determination of optimum values, as demonstrated in Figure. 2. Table 1 gives an overview on the three most intensive MRM transitions (descending from transition 1 to transition 3) recorded for each analyte.

Table 1. MRM transitions for qualitative and quantitative mycotoxin analysis

Analyte	Precursor ion	trans. 1	trans. 2	trans. 3
DON	355 $[M+CH_3COO]^-$	355/59	355/295	355/265
3-acetyl-DON, 15-acetyl-DON	397 $[M+CH_3COO]^-$	397/59	397/337	397/307
DON-3-glucoside	517 $[M+CH_3COO]^-$	517/457	517/427	517/59
ZON	317 $[M-H]^-$	317/131	317/175	317/160
ZON-4-glucoside	479 $[M-H]^-$	497/317	497/175	497/131
ZON-4-sulfate	397 $[M-H]^-$	397/317	397/175	317/131
α-zearalenol	319 $[M-H]^-$	319/275	319/160	319/130
β-zearalenol	319 $[M-H]^-$	319/275	319/130	319/160
α-zearalenol-4-glucoside	481 $[M-H]^-$	481/319	481/275	481/130
β-zearalenol-4-glucoside	481 $[M-H]^-$	481/319	481/275	481/130

1.3. Establishment of an HPLC procedure

Initial retention test

In order to monitor relative retention of analytes within a wide polarity range, HPLC was performed in gradient elution from 10% aqueous methanol (eluent A) to 98% aqueous methanol (eluent B), both including 5mM NH_4Ac.

Conditions:

HPLC column: Phenomenex Gemini C_{18} (150 mm x 4.6 mm; particle size 5µm)

eluent A: 10% methanol (aqu.) + 5 mM NH_4Ac

eluent B: 98% methanol (aqu.) + 5 mM NH_4Ac

flow rate: 0.5 ml/min

injection: 5 µl

temperature: 25℃

detection: MS/MS (MRM mode)

Table 2. Elution gradient (left) and retention times (right) in the initial retention test

time (min)	eluent A (%)	eluent B (%)
0	100	0
2	100	0
12	0	100
15	0	100
15.5	100	0
23	100	0

analyte	tR (min)
DON	7.81
DON-3-glucoside	7.85
acetyl-DON	10.10
β-ZOL-glucoside	11.06
ZON-4-glucoside	11.87
α-ZOL-glucoside	11.97
ZON-4-sulfate	12.08
β-ZOL	12.74
α-ZOL	13.24
ZON	13.31

Because of insufficient separation of DON from DON-3-glucoside and of ZON from α-ZOL, (Table 2), it was decided to use a different separation column for further examinations. For shorter run duration, the methanol content of eluent A was increased from 10% to 25%, as the two most polar compounds (DON, DON-3-glucoside) eluted only after 7.8 min. The gradient was matched for retained slope to 100% eluent B (Table 3). In order to monitor the effect of temperature on the separation between DON and DON-3-glucoside, HPLC was performed at 25℃ and 35℃.

Table 3. Altered eluent conditions

Time (min)	eluant A (%)	eluant B (%)
0	100	0
2	100	0
10.5	0	100
12.5	0	100
13	100	0
19.5	100	0

Conditions:

HPLC column: Phenomenex Synergi C_{18} (150 mm x 3 mm; particle size 4µm)

eluent A: 25% methanol (aqu.) + 5 mM NH_4Ac

eluent B: 98% methanol (aqu.) + 5 mM NH_4Ac

flow rate: 0.5 ml/min, injection: 5 µl, temperature: 25℃, 35℃

detection: MS/MS (MRM mode)

Separation between DON and DON-3-glucoside was improved by changing the analytical column and the relative retention behaviour of these respective compounds was reversed, hence DON-3-glucoside was eluted earlier than DON. Additionally, a significant improvement was achieved by ramping the temperature from 25℃ t o 35℃.

Optimisation of solvents

A comparison was made on the suitability of methanol and acetonitrile as the organic phase of HPLC solvents under the conditions shown above. Acetonitrile was used at concentrations with polarities that were comparable to methanol elution. The concentration of analytes in the multi-standard solution was 50 ng/ml. Tables 4 and 5 and Fig. 4, respectively, show the results.

Table 4. Comparison of retention (min): Methanol vs. acetonitrile

	D3G	DON	acDON	β-ZG	Z4S	α-ZG	Z4G	β-ZOL	α-ZOL	ZON
MeOH	4.55	5.11	10.05	10.57	10.64	11.31	11.77	12.05	12.42	12.94
ACN	2.09	2.76	7.88	8.14	8.33	8.59	9.07	10.05	10.44	11.12

Table 5. Comparison of resolution: Methanol vs. acetonitrile

	D3G/DON	β-ZG/α-ZG	β-ZOL/α-ZOL
MeOH	0.93	1.41	0.64
ACN	1.68	1.03	0.78

HPLC elution with acetonitrile brought earlier elution of all analytes without loss of separation. In addition, the separation of DON and DON-3-glucoside was considerably improved, and the intensities of the analyte peaks were comparable. For these reasons, acetonitrile was chosen as the organic component of solvents for further analyses.

Increased HPLC flow

Since solvent mixtures of acetonitrile/H_2O have lower viscosity than methanol/H_2O, HPLC can be performed at higher flow rates without exceeding critical pressure limits. The flow rate was increased to 1 ml/min which shortened the analysis time by a factor of 2 (see Figure. 5 and Table 6).

Table 6. Retention times of analytes (min), HPLC flow rate: 1 ml/min

D3G	DON	acDON	β-ZG	Z4S	α-ZG	Z4G	β-ZOL	α-ZOL	ZON
1.09	1.41	3.85	4.00	4.07	4.24	4.48	5.00	5.22	5.55

Final adjustments

Because of the early elutio time of DON-3-glucoside, it was decided to lower the acetonitrile concentration in eluent A from 18% to10%. The elution gradient was matched for retained separation. See Box 1 below for the technical parameters of the established method, Figure. 6 for a chromatogram and Table 7 for retention times.

Box1: Summary of technical parameters of the established HPLC-MS/MS method

HPLC conditions:

HPLC column: Phenomenex Synergi C_{18} (150 x 3 mm i. d.; 4µm particle size)

eluent A: 10 % acetonitrile (aq.) + 5µM NH_4COO^-

eluent B: 96 % acetonitrile (aq.) + 5µM NH_4COO^-

flow rate: 1 ml/min

temperature: 35℃

injection volume: 5 µl

HPLC gradient :

min	% A	% B
0	100	0
1	100	0
6	0	100
7.5	0	100
7.7	100	0
11	100	0

MS conditions:

ion source: Electrospray ionisation (ESI); mode: Multiple reaction monitoring (MRM)

polarity: negative; source temperature: 550℃

curtain gas: 10 psi; sheath gas: 50 psi; drying gas: 50 psi; collision gas: high

ion spray voltage: -4000V; dwell time: 50 ms

cycle time: 1.305 sec; pause between mass ranges: 5 ms

Table 7. Retention times, finalised HPLC-MS/MS method

D3G	DON	acDON	β-ZG	Z4S	α-ZG	Z4G	β-ZOL	α-ZOL	ZON
2.18	2.61	4.63	4.61	4.44	4.81	5.03	5.55	5.74	6.07

1.4. Quantification and Calibration

For monitoring linearity of quantification, the target analytes were dissolved as mixed multi-standards. A solution of 10% acetonitrile containing 1 µg/ml of each compound was prepared and diluted to obtain multi-standards at concentrations of 500 ng/ml, 300 ng/ml, 100 ng/ml, 50 ng/ml, 30 ng/ml, 10 ng/ml and 5 ng/ml.

Definition of qualifying and quantifying mass transitions

The selection of transitions as “quantifier” or “qualifier”, respectively, was made on basis of signal-to-noise (S/N) ratios in spectra recorded at 100 ng/ml analyte concentration. S/N ratios were calculated from the height ratio between analyte peak and baseline in a time width of 2 min before the peak slope. For each analyte, the transition with the highest S/N ratio was chosen as quantifier (Figure. 7), unless intensity was below a feasible level for quantification. Table 8 summarizes the chosen transitions for all quantifiers and qualifiers.

Table 8. Signal-to-noise ratios of quantifying and qualifying MRM transitions

Analyte	Quantifier	S/N	Qualifier	S/N
deoxynivalenol (DON)	355/265	69	355/59	58
acetyl-DON	397/59	90	397/337	36
DON-3-glucoside	517/457	52	517/427	28
zearalenone (ZON)	317/131	310	317/175	103
ZON-4-glucoside	479/317	417	497/175	51
ZON-4-sulfate	397/317	2192	397/131	774
α-zearalenol (ZOL)	319/160	133	319/130	75
β-zearalenol (ZOL)	319/160	102	319/130	50
α-ZOL-glucoside	481/319	127	481/130	n/a
β-ZOL-glucoside	481/319	173	481/130	n/a

A calibration curve was plotted for each analyte depicting the linearity of quantification within a concentration range from 10 to 1000 ng/ml. Quantification was accomplished by integration of peak areas. Regression was calculated with to the formula y=kx+d, unweighted. In Table 9, linearities are listed for the whole concentration range analysed (10–1000 ng/ml), as well as for the lower range (10-100 ng/ml) and for the higher range (100 –1000 ng/ml). Since ionisation is highly efficient for ZON-4-sulfate, quantification of this compound was only linear at concentrations up to 100 ng/ml. For this reason, and because of its great sensitivity (see

Table 10), it was decided to incorporate ZON-4-sulfate into multi-standard solutions at 10 ng/ml for future tests.

Calibration curves for all compounds measured are provided in the Annexe (section 8.2). The concentrations range from 10 to 1000 ng/ml for all analytes except for ZON-4-sulfate (5 to 100 ng/ml).

Table 9. Linearity of quantification depending on concentration

conc. (ppb)	DON	D3G	acDON	ZON	α-ZOL	β-ZOL	Z4G	α-ZG	β-ZG	Z4S
10-1000	0.9968	0.9995	0.9988	0.9999	0.9997	1.0000	0.9995	0.9999	0.9999	0.9889
10-100	0.9969	0.9992	0.9946	0.9997	0.9999	0.9984	0.9996	0.9994	0.9999	0.9998
100-1000	0.9975	0.9995	0.9990	0.9999	0.9994	0.9999	0.9995	0.9998	0.9999	0.9909

Limits of quantification (LOQ), limits of detection (LOD)

The lower limit of quantification was defined for each analyte as a S/N ratio below 10 with the limit of detection defined by a S/N ratio of less than 3.

Table 10. Limits of quantification (LOQ) and limits of detection (LOD), stated in ng/ml

	DON	D3G	acDON	ZON	α-ZOL	β-ZOL	Z4G	α-ZG	β-ZG	Z4S
LOQ	10	10	10	5	5	5	5	5	5	< 1
LOD	5	5	5	2	1	1	1	1	1	< 0.5

1.5. Ion source comparison: ESI vs. APCI

Up to this point of the project, all MS studies had been carried out using electrospray ionisation (ESI) as the ion source. Chemical ionisation (APCI) had not been applied because ZON-4-sulfate would probably not be ionised with this method, as its molecule is already charged in solution. Nevertheless, APCI and ESI were compared for other analytes. In Table 11, quantitative results from analyses of multi-standard solutions at 10 ppb and 100 ppb are shown. With the only exception of DON, all of the other analytes were ionised better with ESI. ZON-4-sulfate was only quantifiable at 100 ng/ml, hence APCI was not considered to be suitable for this study.

Table 11. Peak areas (counts) obtained from ESI or APCI, respectively

ng/ml		DON	D3G	acDON	ZON	a-ZOL	b-ZOL	Z4G	a-ZG	b-ZG	Z4S
10	ESI	586	2820	12200	23300	6400	2030	14800	11800	8360	158000
	APCI	1300	2750	6400	13800	5090	1880	5780	3780	4040	-
100	ESI	6170	28800	149000	269000	65900	18700	147000	131000	75400	1500000
	APCI	10500	28100	77900	140000	49400	16400	61900	33200	31400	4380

1.6. Conclusion

An HPLC-MS/MS method for reliable detection and quantification of all target analytes (DON, acetyl-DON, DON-3-glucoside, ZON, ZON-4-glucoside, ZON-4-sulfate, α-zearalenol, β-zearalenol, α-zearalenol-4-glucoside and β-zearalenol-4-glucoside) in pure organic solvents was successfully established. Electrospray ionisation (ESI) in negative polarity proved to be suitable for these analytes. Peak intensity and separation as well as overall retention were optimised by variations of column, eluent composition, flow rate and temperature. Limits of detection (LOD), limits of quantification (LOQ) and quantification linearity were evaluated for each analyte and plotted in calibration curves. The HPLC-MS/MS method established is expected to be well suited for the further development of an analysis procedure for masked *Fusarium* mycotoxins in cereal samples.

2. Sample preparation

2.1. Scope of task 02 - Optimization of extraction procedures

Novel and reliable sample preparation strategies, involving enzymatic treatments would be developed to detect both soluble and otherwise insoluble forms of the listed substances.

Spiked and naturally contaminated cereals (maize, wheat) as well as processed foods (bread, beer) would be extracted using different solvents (water/methanol/acetonitrile mixtures) at three pH-values (acidic, neutral, alkaline). The composition and pH of the extraction solvent, the extraction time and the sample/solvent ratio would be optimised. Furthermore, enzymatic pre-treatments would be performed prior to extraction, using amylase, cellulase and proteinases (Pronase®, papain).

Masked mycotoxins can either be stored in soluble form in plant vacuoles, or be incorporated in an insoluble form in various plant organelles (mainly the cell wall). To see the whole picture, it was intended break down the plant cell walls, so that any toxins present (either in free or still in conjugated from) would be released. The aim was to find conditions which allow to extract not only the soluble mycotoxins but also matrix-bound forms.

2.2. Determination of analyte recovery in spiked sample matrices

2.2.1. Liquid sample matrix: Beer

The purpose of this initial experiment was to monitor to what extent masked mycotoxins could be recovered from a matrix into which they had been supplemented (spiked) at defined concentrations. Lager beer was chosen for the first experiment, as this was simpler matrix requiring no complex optimisation of the extraction procedure. The beer was degassed to remove CO_2 using an ultrasonic bath. Spiking was done by adding 10 µl mycotoxin multistandard mixture per ml of sample. The composition of the multistandard solution that was used for spiking and as quantification reference throughout the current report is given below.

Appropriate volumes of spiked beer were transferred directly into HPLC vials for analysis. The results of this experiment are given in Table 12.

Composition of mycotoxin multistandard solution

Spiking of matrix samples was performed by adding 10 µl multistandard solution (in 10% ACN) per gram of sample (beer: fresh wgt.; bread, cereal samples: dry wgt.)

Standard	multistandard conc. (µg/l)	concn. in spiked samples (µg/kg)
DON	100000	1000
D3G	100000	1000
acDON	100000	1000
ZON	10000	100
α-ZOL	10000	100
β-ZOL	10000	100
Z4G	10000	100
α-ZG	10000	100
β-ZG	10000	100
Z4S	1000	10

Table 12. Quantities of mycotoxins recovered from spiked beer (µg/kg)

	D3G	DON	acDON	Z4S	β-ZG	α-ZG	Z4G	β-ZOL	α-ZOL	ZON
added	1000	1000	1000	10	100	100	100	100	100	100
spike	1150	980	845	0.6	58	68	73	168	129	110
control	0	0	0	0	0	0	0	0	0	0

100 - 199	90 - 99	80 - 89	70 - 79	60 - 69	50 - 59

Colour code for recovery of analytes (%) in respect to the spiked quantities

The results in Table 12 reveal that recovery was very high for compounds either at the most polar or at the least polar ends of the measured polarity range. Analytes with medium polarity, like β-ZG, seemed to be negatively influenced by matrix effects. Recovery of Z4S, at a rate of only 6%, was strongly suppressed. For optimising overall recovery, several clean-up methods were tested for beer samples in the next phase of the project.

2.2.2. Solid sample matrix: Wholemeal bread

Samples of wholemeal bread were spiked with the target compounds. Breadcrumbs were weighed, dried for 48h at 55 ℃, weighed a second t ime and finally ground in an electric mill. Portions of 5g of sample were spiked with 50 µl mycotoxin multistandard solution. For this experiment, extraction was performed at room temperature on a rotary shaker (180 rpm/90 min), under both neutral and acidic conditions, with 79% aqueous ACN solution. For acidic extraction, the extraction solution also contained 1% acetic acid. This composition had been successfully applied for extracting various mycotoxins (Sulyok et al. 2006) and was therefore

chosen for initial evaluation, before optimisation. After extraction, the filtered extract was evaporated to dryness and re-suspended in 10% ACN for HPLC-MS analysis.

Semiquantitative results from the initial mycotoxin extraction revealed natural contamination of the bread sample with four of the target compounds. For this reason, it was decided to choose a different sample matrix for monitoring recovery of spiked analytes under altered extraction conditions. Extraction under acidic conditions yielded better recoveries of all compounds, with recoveries between 50 and 127% depending on the analyte.

2.3. Optimisation of extraction

2.3.1. Variation of solvent composition

For evaluating how solutions of different composition would affect the mycotoxin extraction efficiency it was decided to investigate both spiked and naturally contaminated sample materials. Retail maize semolina was ground in an electric mill and spiked with mycotoxins at quantities as described. Ground wheat samples that were known to be naturally contaminated with (at least) DON and ZON were available at the IFA-Tulln. Spiked maize and naturally contaminated wheat were each extracted with solutions of different composition, which are listed in Box 2 below. The organic portion of every composition was ACN. The reason for not testing methanol was that, in prior investigations, methanol-based extraction had yielded significantly lower quantities of DON and ZON metabolites in comparison to ACN (Sulyok et al. 2006). The filtered extracts were evaporated and redissolved in 10% ACN for HPLC-MS quantification. Table 13 show the results from using different extraction solvent compositions for spiked maize and naturally contaminated wheat.

Box 2: Extraction solvent compositions tested with spiked maize and naturally contaminated wheat:

1.) Neutral: ACN/H_2O 80/20
2.) Acidic: ACN/H_2O/HAc 79/20/1
3.) Alkaline: ACN/H_2O 79/21, containing 1% NaOH (0.25 mol.l^{-1}). After extraction, the solution was neutralised equimolar (0.25mol.l^{-1}) with HCl.
4.) Polar neutral: ACN/H_2O 40/60
5.) Polar acidic: ACN/H_2O/HAc 40/60/1

Extraction time: 90 min; Rotary speed: 180 rpm; Temperature: 25 ℃

Table 13. Mycotoxins (µg/kg) measured in spiked maize semolina (top) and in naturally contaminated wheat (bottom)

	D3G	DON	acDON	Z4S	β-ZG	α-ZG	Z4G	β-ZOL	α-ZOL	ZON
neut. control	0	+	0	0	0	0	0	0	0	0
acid. control	0	+	0	0	0	0	0	0	0	0
alkal. control	193	0	0	0	0	0	0	0	0	0
polar neut. cont.	0	0	0	0	0	0	0	0	0	0
polar acid. cont.	0	0	0	0	0	0	0	0	0	0
neut. spike	924	831	1040	7.2	97.6	87.9	91.3	104	89.3	89.2
acid. spike	876	945	1070	6.3	85.2	87.7	105	78.2	80.3	62.8
alkal. spike	2450	7500	298	16.4	55.5	108	121	109	126	128
polar neut. spk.	1080	939	1050	4.9	77.3	69.4	79.6	67.8	48.6	45.1
polar acid. spk.	970	886	1030	0.5	76.9	88.5	83.7	39.5	26.9	28.5
neut.	53	2770	0	2000	0	0	0	238	49.8	4200
acid.	84	2860	0	1870	0	0	0	255	62.3	5040
alkal.	1040	2890	0	2140	0	0	0	265	54.7	5480
polar neut.	94	2920	0	1050	0	0	0	121	34.3	2220
polar acid	127	2910	0	960	0	0	0	370	93.8	4800

>150	100 - 120	90 – 99	80 – 89	70 – 79	60 – 69	40 – 59	< 40

Colour code for recovery of analytes (%) in respect to the spiked quantities:

Both in spiked maize and in naturally contaminated wheat, alkaline extraction gave the highest recoveries for the majority of analytes. Nevertheless, the recovery of acDON (from spiked maize) was considerably weaker under alkaline conditions compared to neutral or acidic extraction. Together with an obviously excessive recovery of DON, this might suggest that acDON is partially converted into DON during alkaline hydrolysis. For these reasons, alkaline extraction has to be considered unsuitable for the current approach. However, inexplicable high amounts of D3G were found in the “blank” maize and also in wheat after alkaline extraction. One possible explanation of that effect might be the hydrolysis of di- and higher glucosides of DON to D3G. A similar phenomenon after enzymatic treatment was observed later (chapter 2.4) and is discussed in detail in the project outcome (chapter 6.1).

Extraction with more polar solvent compositions (40% ACN) gave high recoveries of the more polar compounds (D3G, DON, acDON), whereas a notable decrease of recoveries was

observed with decreased polarity of analytes (α-ZOL, β-ZOL, ZON). Therefore, 40% ACN was not well suited for efficient extraction of all 10 target compounds.

For the exception of D3G, differences in yields after neutral, acidic or alkaline extraction were less pronounced in the naturally contaminated wheat. In consideration of the results that had been obtained for extraction of spiked bread, and of spiked maize and contaminated wheat, acidic extraction with 79% ACN was considered most suitable as a standard procedure for further experiments.

2.3.2. Extraction time

As the duration for the extraction of mycotoxins from natural samples has been optimised at 90 minutes (Sulyok et al., 2006), we decided to investigate whether and to what extent shorter or longer extraction times would alter the yield of extracted target compounds. For this experiment, the use of naturally contaminated grain samples was considered most feasible, as the release of certain (especially conjugated) mycotoxins that are naturally incorporated into the matrix was expected to depend more on extraction duration than in spiked material, in which the toxins are present only on the surface of the matrix. For each extraction, 2 g of sample material were added to 8 ml 79% ACN aq., containing 1% acetic acid. Extractions were performed at room temperature for 60, 90 and 120 minutes, respectively, on a rotary shaker rotating at 180 rpm. Before analysis, extracts were filtered, evaporated and redissolved in equal volumes of 10% ACN.

Table 14. Yields of mycotoxins extracted from wheat grain with different extraction times (µg/kg)

time (min)	D3G	DON	acDON	Z4S	β-ZG	α-ZG	Z4G	β-ZOL	α-ZOL	ZON
60	84	2860	0	2090	0	0	0	256	62	5040
90	84	3270	0	1910	0	0	0	306	69	5750
120	77	2990	0	1910	0	0	0	287	63	5520

This experiment showed that the wheat analysed did not only contain DON and ZON, but also D3G, α-ZOL, β-ZOL and Z4S. As yields of the target compounds did not vary significantly with the duration of extraction, the time for further extractions was kept at 90 min.

2.3.3. Sample/solvent ratio

The most suitable relationship between sample and extraction solvent for maximum mycotoxin yield was investigated in the next experiment. Naturally contaminated wheat and spiked maize semolina were extracted. 79% ACN (containing 1% acetic acid) was added to 2 g of sample at quantities of 4, 8, 12 or 16 ml, thus obtaining sample/solvent ratios of ½, ¼,

1/6 and 1/8, respectively. After extraction (90 min on a rotary shaker at 180 rpm), the filtered extracts were evaporated and re-suspended in 10% ACN for analysis.

Table 15. Yield/recovery of mycotoxins at different sample/solvent ratios

	sol./ sam	D3G	DON	acDON	Z4S	β-ZG	α-ZG	Z4G	β-ZOL	α-ZOL	ZON
Maize (SP)	2	66	92	98	57	73	78	85	80	83	70
	4	80	85	98	48	88	79	75	73	80	67
	6	90	83	91	21	70	71	77	74	69	61
	8	89	91	98	20	79	75	83	73	77	66
Wheat (con)	2	56	2700	0	1430	0	0	0	253	58	5100
	4	80	3000	0	1440	0	0	0	211	58	4560
	6	86	2990	0	1510	0	0	0	276	64	5200
	8	85	2850	0	1390	0	0	0	286	66	5580

Recovery (%) from spiked maize (yellow) and quantities (µg/kg) in naturally contaminated wheat (brown).

Extraction yields of the target compounds were not increased by higher proportions of extraction solvent (i.e. lower sample/solvent ratio). In the case of Z4S, lower ratios even seemed to decrease recovery from spiked maize. A high sample/solvent ratio of ½, on the other hand, is likely to reduce diffusion by increased viscosity and was therefore not considered to be applicable in a wide range of different matrices. For any further extractions, sample/solvent ratio was kept at ¼.

2.4. Enzyme treatment

Besides detoxifying mycotoxins by conjugation, infected plants can also incorporate them (and their conjugates) into cell walls or storage structures, thus keeping them physically apart from necessary physiological processes (Engelhardt et al. 1999). Enzymatic degradation of such cellular structures (e.g. cellulose, starch, proteins), might be expected to release these incorporated mycotoxins, thus increasing the extraction yield. For this purpose, it was decided to investigate the potential of mycotoxin release by the action of five different enzymes, which are listed in Table 16. For determination of required enzyme concentration for each treatment, two considerations had to be made: At first, the amount of substrate was (approximately) estimated per gram of sample. Secondly, the enzyme concentration (in excess of the expected substrate quantity) was calculated on basis on given activities in respect to an incubation time of 20 hours.

One gram of naturally contaminated and ground maize and wheat, respectively, was incubated with 4 ml buffer solution with each of the enzymes. The buffer solutions were made from 0.1 M citric acid and 0.2 M $NaHPO_4 \cdot 2H_2O$ adjusted to pH 5.0, 6.0 and 7.0. For

cellulase, glucuronidase pH 5.0 buffer was used, for α-amylase, papain pH 6.0 buffer was used, and for Pronase pH 6.0 buffer was used

In order to monitor whether the degree of enzyme excess would alter mycotoxin release, different enzyme concentrations were tested for α-amylase, papain and Pronase (see Tables 17 and 18). Incubation was performed on a rotary shaker (90 rpm) at room temperature for reactions with α-amylase and papain for 20h. Incubation with all other enzymes was done in a shaker at 37 ℃, again for 20h. An incubated blin d control was included, for which 1 g of each sample matrix was incubated in buffer solution without enzyme.

After incubation, ACN and acetic acid (HAc) were added to the aqueous buffer solution at quantities to finally give the ratio ACN/H2O/HAc 79:20:1. Extraction was performed according to the optimised conditions, except for the different sample/solvent ratio of 1/20. The extract was centrifuged, evaporated and the residue redissolved in 10% ACN for HPLC-MS analysis. In addition to the incubated samples, a control extraction was performed with 1 g of each matrix.

Table 16. Enzymes used for treatment of mycotoxin-contaminated maize and wheat

Enzyme	Unit definition/ substrate reaction per unit	Temperature Optimum	pH optimum	Applied Units /g sample (20h)
α-amylase *(Aspergillus oryzae)*	1.0 mg maltose from starch in 3 min	20 ℃	6	10, 100, 1000
cellulase *(Aspergillus niger)*	1 µmol glucose from carboxymethylcellulose per minute	37 ℃	5	100
glucuronidase *(Helix pomatia)*	1 µg phenolphthalein from phenolphthalein-glucuronide per hour	37 ℃	4.5 - 5	1000
papain *(Carica papaya)*	hydrolyses 1 µmol N-benzoyl-L-arginine ethyl ester per min	25 ℃	6.2	10, 100
Pronase *(Streptomyces griseus)*	release of folin-positive aminoacids, equivalent to 1 µmol tyrosine, from casein per minute	40 ℃	7 - 8	10, 100

Table 17. Quantities of mycotoxins (µg/kg) in naturally contaminated maize, measured after enzyme treatment and extraction

	D3G	DON	acDON	Z4S	β-ZG	α-ZG	Z4G	β-ZOL	α-ZOL	ZON
control extract	475	3070	121	2.8	-	-	-	-	-	72.3
control incubation	1910	5470	142	1.9	-	-	-	-	-	82.2
α-amylase 10U	1910	3300	165	5.3	-	-	-	-	-	100
α-amylase 100U	1470	2440	134	4	-	-	-	-	-	62.1
α-amylase 1000U	1720	3560	116	3.2	-	-	-	-	-	79.1
cellulase 100U	-	3300	121	-	-	-	-	-	-	65.7
glucuronidase 1000U	1480	3820	88	4.7	-	-	-	-	-	87.5
papain 10U	2120	5030	143	8.5	-	-	-	-	-	150
papain 100U	1070	2800	127	4.8	-	-	-	-	-	79.8
Pronase 10U	2040	5740	153	4.0	-	-	-	-	-	66.6
Pronase 100U	1040	5350	128	2.7	-	-	-	-	-	60.3

- not detected

Table 18. Quantities of mycotoxins (µg/kg) in naturally contaminated wheat, measured after enzyme treatment and extraction

	D3G	DON	acDON	Z4S	β-ZG	α-ZG	Z4G	β-ZOL	α-ZOL	ZON
control extract	211	3320	-	1060	-	-	-	302	+	6640
control incubation	480	4680	-	792	-	-	+	72	+	8120
Amylase 10U	486	5400	-	1050	-	-	-	108	+	7150
Amylase 100U	590	4700	-	1060	-	-	-	148	+	6800
Amylase 1000U	567	4380	-	894	-	-	-	163	+	5990
Cellulase 100U	-	3550	-	-	-	-	-	197	+	9460
glucuronidase 1000U	409	4760	-	n.a.	-	-	-	151	+	9070
Papain 10U	604	5760	-	872	-	-	-	141	+	7120
Papain 100U	580	6050	-	976	-	-	-	72	+	7370
Pronase 10U	607	11900	-	671	-	-	-	79	+	8430
Pronase 100U	601	7450	-	847	-	-	-	115	+	9170

- not detected

Enzymatic treatment before extraction seemed to have a significant effect on the release of D3G in both matrices, as can be seen by comparing incubated samples with the control extract. The enzyme treated samples contained considerably higher amounts of this particular compound, but surprisingly the control incubation sample (in buffer solution without enzyme) also did. This observation might suggest that, in some cases, an important effect of sample pre-treatment is the preliminary aqueous extraction rather than an enzymatic

reaction. For DON and ZON, increased yields from incubation or enzymatic treatment were not observed to this extent, although Pronase treated samples seemed to show some enhancement. The use of a large excesses of enzymes did not seem to have any positive effects. This could be related to increased matrix effects induced by a greater amount of products released. However, to gain more confidence regarding the effects of enzyme action on mycotoxin yields, these treatments were repeated. Application of a large excess of enzymes was omitted this time, but a combination of α-amylase and papain was tested, in addition to solutions with single enzymes. The two-enzyme combination was easily applicable, since both enzymes require similar incubation conditions. Results from these repeated experiments are compiled in Table 19 (maize) and Table 20 (wheat).

Table 19. Quantities of mycotoxins (µg/kg) in naturally contaminated maize, measured after enzyme treatment and extraction. Repeated experiment

	D3G	DON	acDON	Z4S	β-ZG	α-ZG	Z4G	β-ZOL	α-ZOL	ZON
control extract	425	2960	128	2.7	-	-	-	-	-	27.8
control incubation	3310	2790	112	2.5	-	-	-	-	-	56.9
α-amylase 10U	4460	2400	166	4.0	-	-	-	-	-	37.4
Cellulase 100U	381	3050	132	-	-	-	-	-	-	54.2
glucuronidase 1000U	3340	2820	94	-	-	-	-	-	-	36.5
papain 10U	4310	2910	122	2.6	-	-	-	-	-	29.9
Pronase 10U	2980	4490	110	4.5	-	-	-	-	-	44.9
α-amylase + papain	3290	2990	148	3.2	-	-	-	-	-	87.7

- not detected

Table 20. Quantities of mycotoxins (µg/kg) in naturally contaminated wheat, measured after enzyme treatment and extraction. Repeated experiment

	D3G	DON	acDON	Z4S	β-ZG	α-ZG	Z4G	β-ZOL	α-ZOL	ZON
control extract	130	3000	-	1960	-	-	-	224	67	5460
control incubation	992	3160	-	1950	-	-	-	22	28	4760
α-amylase 10U	1120	3280	-	2120	-	-	-	126	54	4810
cellulase 100U	0	3430	-	27	-	-	-	136	45	3520
glucuronidase 1000U	1080	2940	-	32	-	-	-	168	116	11700
papain 10U	1180	3370	-	1870	-	-	-	72	51	5150
Pronase 10U	1070	4560	-	1410	-	-	-	+	11	5330
α-amylase + papain	1140	3180	-	1720	-	-	-	96	57	6110

- not detected

As in the first onset of enzymatic treatments, a significant incubation-induced increase in the quantity of D3G was also observed in the repeated experiments. Also, a slightly promoting effect of Pronase treatment for DON yields was observed again. In the case of β-ZOL, incubation or enzyme treatment seemed to have a rather negative effect, also supporting the results of the first experiment.

2.5. Conclusion

Optimisation of extraction solvent composition, extraction time, sample/solvent ratio, as well as experiments on enzymatic pre-treatment led to the establishment of following sample extraction procedure:

Samples were pre-treated by incubation with Pronase (10 U per g sample) in buffer solution (citric acid/NaHPO4, pH 7.0, 4 ml per g sample) at 37 ℃ for 20hours. they were then extracted with 4 volumes of ACN/HAc (79:1) per 1 volume incubated solution for 90 min. Extractions were performed at room temperature on a rotary shaker at 180rpm. Extracts were filtered or centrifuged, evaporated and redissolved in 0.2 or 0.25 vol. 10% ACN for HPLC-MS analysis.

Pre-treatment of samples with Pronase was expected to enhance extraction efficiencies for D3G and DON. According to repeated experiments, extraction of D3G notably profits from preliminary incubation. DON seems to be released at higher quantities when pre-treated with Pronase.

Differences in recoveries of target analytes obviously did occur in different matrices (bread, maize, beer, wheat), hence the requirement of method validation for every matrix group in task 04 of the project is evident.

3. Sample cleanup

3.1. Scope of task 03 - Optimization of cleanup procedures

Various clean-up strategies would be developed to purify and enrich the conjugated toxins. The effect of typical co-extracted compounds on the MS/MS response for each toxin would be studied. Calibrants over the expected working range would be analysed without and with presence of the extracted matrix from ten sample types (4 raw cereals and six processed foods). The study on possible matrix effects would be evaluated by direct infusion of a mixture of masked mycotoxins after HPLC-separation of blank matrix extracts by checking whether a decrease in the signal intensity of the analyte occurs or not. In addition, the linearity of the detector response (slope of the calibration curve) with a series of diluted matrix extracts would be investigated. Moreover, the suitability of internal standards, such as $^{13}C_{15}$-deoxynivalenol and $^{13}C_{18}$-zearalenone would be investigated. A prior cleanup step would be used, if required, due to the large number/range of samples and requirements of the HPLC-MS/MS instruments. In case of severe matrix effects, we would verify and optimise SPE (C18 based, especially for ZON derivatives), PSA (primary, secondary amines) and commercially available (immuno affinity columns, Mycosep® columns) cleanup procedures. Antibodies used in commercial immuno affinity columns (IACs) for DON and ZON may or may not recognise conjugated forms. This part of the project overlapped somewhat with the extraction procedure part, as both tasks are dealing with sample preparation.

3.2. Evaluation of matrix effects

3.2.1. Multi-level spiking series with 10 different matrices

In this experiment 10 different matrices were spiked each at 5 levels with a multi-standard solution of the target analytes prior to extraction. By allowing the multi-compound mixture to undergo extraction together with the sample matrix itself, deviant signal response caused by co-extracted substances could be monitored as well as alterations arising from exposure to the chemical conditions in the extraction solution. Sample types chosen for this experiment were beer, cornflakes, grissini, muesli, spaghetti, wholemeal bread, barley, spelt, maize, rye and wheat. In the case of solid and dry products (cornflakes, grissini, spaghetti), sample material was ground prior to weighting and spiking. Bread was oven-dried for 48h at 50 ℃ before grinding. Muesli was spiked unprocessed and then homogenised with an Ultraturrax laboratory homogeniser in an appropriate volume of the extraction mixture (10 g muesli in 40 ml solvent). The muesli homogenate was then extracted at usual conditions. Volumes of 1 ml lager beer were each spiked directly, subsequently evaporated to dryness and redissolved in equal volumes of 10% ACN for analysis.

Composition of mycotoxin multistandard solution:

	concn. of standard solution (µg/l)	concn. in spiked samples (µg/kg)
DON	100000	1000
D3G	100000	1000
AcDON	100000	1000
ZON	10000	100
α-ZOL	10000	100
β-ZOL	10000	100
Z4G	10000	100
α-ZG	10000	100
β-ZG	10000	100
Z4S	1000	10

The data from the multi-level spiking series are compiled in Excel sheet provided in the annexe (chapter 8.3). Strong matrix effects were present in several samples (especially for Z4S), hence a requirement to test several cleanup procedures or application of internal standards was evident.

3.2.2. Parallel direct infusion of blank matrix

This experiment was set out in order to monitor signal altering effects of matrix inherent compounds in direct comparison of three matrices and over the retention range that covers all target analytes. To achieve this, blank matrix samples were injected into the HPLC-MS/MS system while at the same time a multi standard solution of the target analytes was infused directly into the MS through a syringe pump. MRM spectra collected from this procedure showed continuous signal lines of each transition, whose courses follow effects of matrix compounds eluting at certain retention times. In contrast to spiking experiments, this test reveals the sole effect of co-eluted matrix compounds to the analytes' signal response without regard to putative analyte-matrix interchanges in the extract solution.

Figure 8 shows examples of direct comparison between signal alteration effects of different matrices. The dotted line in each chromatogram shows the retention time of the respective analyte. In most cases, no significant differences between the tested matrices were observed (e.g. DON, ZON). However, remarkably different signal alterations of Z4S were detected. This might indicate this compound's susceptibility to co-eluted substances which, alongside with possible interactions within the extraction solution, leads to its low recovery rates that have been observed throughout the study.

3.2.3. Signal response in diluted spiked matrices

Extracts of blank sample matrix were spiked in the sample vials, immediately before HPLC-MS measurement, hence the target analytes had not undergone extraction process in this experiment. In order to evaluate a correlation between matrix-altered signal response and concentration of matrix compounds, spiking was made at two levels and in pure as well as in diluted extracts. Details of the spiking of diluted matrix extracts are given in Box 3 below.

Box 3: Signal response of pure and diluted extracts:

Spiking levels (ng/ml extract) :

analyte	Spike Level 1	Spike Level 0.1
DON	1000	100
D3G	1000	100
Ac-DON	1000	100
ZON	100	10
α-ZOL	100	10
β-ZOL	100	10
Z4G	100	10
α-ZG	100	10
β-ZG	100	10
Z4S	10	1

Matrix samples: barley grain; maize grain; rye grain; wheat grain

Matrix dilution levels :

Dilution level 1	Dilution level 0.1
undiluted extract	extract diluted 10-fold

The results of the diluted spiked matrix experiment are compiled in Table 21. Peak response of compounds of lower polarities was significantly decreased by higher extract concentration, hence a direct correlation between the presence of co-eluting matrix compounds and signal decrease was evident. However, in the case of D3G, the opposite effect was observed in three out of four tested matrices, signal response seemed to be weakened by higher extract dilution. An explanation for this phenomenon could be that solubility of certain matrix inherent compounds is improved by higher dilution. The tested wheat grain was revealed to be naturally contaminated with ZON and, remarkably, similar amounts were found in the pure as well as in the diluted unspiked extracts. This might also be related to signal decrease by higher concentrations of co-eluing compounds.

Table 21. Signal response in diluted (lvl 0.1) and pure (lvl 1) spiked matrix extracts

	ng/ml	barley		maize		rye		wheat	
		0.1	1	0.1	1	0.1	1	0.1	1
D3G	0	0	0	0	0	0	0	0	0
	100	95.2	101	55.3	105	45.6	101	43.2	112
	1000	915	909	328	968	371	947	303	987
DON	0	0	0	0	51.5	0	68.8	0	49.2
	100	88.2	222	104	138	106	159	103	148
	1000	1060	1220	1080	1120	1070	1170	1010	1120
AcDON	0	0	0	0	0	0	0	0	0
	10	10.2	10.3	11.9	12.8	21.1	3.34	11.8	10
	100	96.3	121	118	104	114	94.6	89.3	103
Z4S	0	0	0	0	0	0	0	0	0
	1	0.9	0.9	0.1	0.7	1	6.9	1	0.6
	10	8.9	9.2	12	8.8	9.7	8	9.3	7.9
β-ZG	0	0	0	0	0	0	0	0	0
	10	8.9	12.1	9.3	10.8	9.2	10.7	10	8.3
	100	104	100	100	114	83.8	101	76.9	84.8
α-ZG	0	0	0	0	0	0	0	0	0
	10	8.6	9.3	10.4	9.3	9.4	8.4	11.1	9.5
	100	98.8	102	97.3	108	95.7	93.5	85.9	92.3
Z4G	0	0	0	0	0	0	0	0	0
	10	8.5	9.4	10	9.6	10.7	8.3	11.4	9.1
	100	109	108	114	113	107	86.6	109	93
β-ZOL	0	0	0	0	0	0	0	0	0
	10	5	6.2	8.8	6.8	11.9	4.5	14.3	7.4
	100	88.6	63	110	72.1	109	68.2	121	81.2
α-ZOL	0	0	0	0	0	0	0	0	0
	10	8.9	5	19.2	5.4	13	5.2	15.9	2.2
	100	117	59.7	167	74.6	153	55	150	65.2
ZON	0	0	0	0	0	0	1	1.9	2
	10	10.2	4.8	17.9	3.9	17.2	4.1	12.5	5.2
	100	94.2	69	182	51.3	157	41.6	132	52.5

3.3. Cleanup strategies

3.3.1. C-18 based SPE

Among the cleanup procedures tested in this study, C-18 SPE is the most generally applicable without being particularly targeted at *Fusarium* mycotoxins. The conditions applied are listed in Box 4 below. Results are listed in Table 22. Application of C-18 SPE cleanup lead to considerable improvement of the signal response of Z4S, which had been observed to exhibit the lowest recovery rates among the analytes included in this study. However, SPE cleanup lead to a slight loss of yield of all other ZON conjugates and to considerable loss of D3G and DON.

Box 4: C-18 SPE cleanup

Matrix samples: cornflakes
lager beer
wholemeal bread

Samples were spiked with multi standard solution at same quantities as above.

Extraction: 1g of sample extracted with 4 ml ACN/H_2O/HAc (79/20/1)
on a rotary shaker (190 rpm), at room temperature (25 ℃).

2 ml extract was diluted with 30 ml water in order to obtain a required maximum concentration of 5% ACN.

Columns used: J.T. Baker Bakerbond spe PolarPlus C18, 6 ml, 1000 mg

The columns were pre-washed with 2 x 4 ml 5 % ACN, then they were conditioned with 2 x 4 ml 5% ACN. The diluted sample extract was then applied to the column, succeeded by rinsing with 2 x 4 ml 5% ACN. For elution of compounds, 4 ml 100% ACN was applied.

The resulting volume of 4 ml cleaned extract was evaporated to dryness and re-dissolved in 1 ml 10% ACN for LC-MS/MS injection.

Table 22. Signal response of analytes after C-18 SPE cleanup (ng/g)

	cornflakes				lager beer				wholemeal bread			
	control		Spike		control		spike		control		spike	
	pure	SPE	pure	SPE	pure	SPE	pure	SPE	pure	SPE	pure	SPE
D3G	47.8	11.2	543	128	115	36.2	914	241	-	-	487	108
DON	257	62.4	3170	325	109	-	1030	267	220	22.2	1190	273
AcDON	-	-	980	930	-	-	964	824	-	-	889	890
Z4S	-	-	49	69.6	-	6.8	18.1	52	8.31	32.9	44.6	80.6
α-ZG	-	-	79.6	43.7	-	-	87.7	48.8	-	-	86.1	40.4
β-ZG	-	-	84.6	46.5	-	-	84.4	56.2	-	-	64.1	40.2
Z4G	-	-	79.7	75.9	-	-	93.2	78.9	-	-	73.4	62.6
β-ZOL	-	-	87.1	82.5	-	-	80.1	72.7	-	-	89.8	75.5
α-ZOL	-	-	69.1	76.3	-	-	88.5	73.7	-	-	61.4	62.2
ZON	12.8	12.3	95.9	90.9	13.6	10.3	97.7	79.2	6	5.5	76.2	75.3

- not detected

3.3.2. Comparison of straightforward cleanup methods: PSA and MycoSep®

Sample cleanup by applying primary and secondary amines (PSA) is very quickly and easily operated, which was the reason to test the efficiency of this procedure for the particular purpose of this study. Within the same experiment, ROMER MycoSep cleanup columns were also tested. These cleanup columns are specially designed for cleanup of *Fusarium* mycotoxins and are available for polar compounds like DON or Nivalenol (MycoSep® 230) as well as for apolar compounds as ZON (MycoSep® 226). For their straightforward applicability, they were compared to PSA in the same experiment. Respective conditions are listed in Box 5 below, results are given in Table 23. As the results reveal, no significant improvements in recovery were achieved for either matrix by the use of MycoSep 226, however, the signal response of medium-polarity compounds was considerably decreased for the ZOL glucosides and Z4S. MycoSep 230 did not prove to be appropriate for this application, nor did PSA treatment significantly improve detection.

Box 5: Conditions of Cleanup with PSA and MycoSep columns:

Sample matrices: Wholemeal bread
Maize grain

Samples were spiked with multi-standard solution at same quantities as stated above.

Extraction: 4 g of sample extracted with 16 ml ACN/H_2O/HAc (79/20/1) on a rotary shaker (190 rpm), at room temperature (25 ℃).

PSA: 50 mg of PSA was added to 1 ml extract and was thoroughly mixed for 1 min using a Vortex blender.

MycoSep® 226: 8 ml extract were manually pressed through the cartridge, 4 ml cleaned supernatant was withdrawn and transferred into another vial, evaporated to dryness and re-dissolved in 1 ml 10% ACN.

MycoSep® 230: 4 ml extract were manually pressed through the cartridge, 2 ml cleaned supernatant were withdrawn and transferred into another vial, evaporated to dryness and re-dissolved in 1 ml 10% ACN.

Table 23. Comparison of cleanup with PSA and MycoSep columns of wholemeal bread (WM) and maize grain (MG)

	D3G	DON	AcDON	Z4S	β-ZG	α-ZG	Z4G	β-ZOL	α-ZOL	ZON
WM no cleanup	26	96	96	57	67	64	79	106	78	64
WM PSA	26	99	93	69	74	59	73	90	88	83
WM MycoSep 226	0	90	88	8	0	0	30	49	66	75
WM MycoSep 230	0	61	38	0	0	0	0	0	0	0
MG no cleanup	97	93	87	43	75	80	73	89	89	91
MG PSA	87	95	95	39	76	73	78	82	88	88
MG MycoSep 226	24	89	93	36	38	65	67	73	94	87
MG MycoSep 230	11	63	39	0	0	0	0	0	0	0

RECOVERY (%)						
> 100	90 - 99	80 - 89	70 - 79	60 - 69	50 - 59	30 - 49

3.3.3. Immunoaffinity columns

In this experiment, cleanup columns designed for retaining DON or ZON, respectively, by interaction with specific antibodies were tested. For applicability in this study, particular

attention was given to whether only DON and ZON would be retained in the column or if the antibodies also recognised and binded the conjugated metabolites. Table 24 shows the results of the experiment. Apparently, none of the columns tested could be considered appropriate for this approach, since glucosides of both compound were not retained in the column and, furthermore, DON and ZON could not be fully recovered from multi-standard solutions.

Table 24. Recoveries of analytes (%) after treatment of Multi-standard solution and maize grain with IAC columns

		D3G	DON	AcDON	Z4S	β-ZG	α-ZG	Z4G	β-ZOL	α-ZOL	ZON
Multi Std.	DONprep	-	40	86	0.05	-	-	-	-	-	-
	DON Test	-	35	-	0.06	-	-	-	-	-	-
	Easi-Extract ZON	-	-	-	0.1	-	-	-	60	59	65
	ZearaStar	-	-	-	0.2	-	-	-	56	63	71
	ZearalaTest	-	-	-	0.1	-	-	-	55	68	69
Maize	DONprep	-	47	70	0.4	-	-	-	-	-	-
	DON Test	-	-	-	0.08	-	-	-	-	-	-
	Easi-Extract ZON	-	-	-	0.3	-	-	-	63	51	52
	ZearaStar	-	-	-	0.2	-	-	-	35	45.7	45
	ZearalaTest	-	-	-	0.1	-	-	-	62	66.7	57

- not detected

3.4. Use of internal standards

Application of fully ^{13}C labelled DON and ZON has already proven to yield more realistic recovery rates of their respective isotopes in matrix (Haeubl et al. 2006). In this study, $^{13}C_{15}$-DON and $^{13}C_{18}$-ZON were added to beer, maize grain and wheat grain samples in the experiment of multi-level spiking. Thus, a direct comparison could be made between linearity as well as recovery of DON and ZON calculated to external as well as to internal standards. Spiking of solid samples (maize, wheat) was done before extraction, at levels of 500 ng/g $^{13}C_{15}$-DON and 50 ng/g $^{13}C_{18}$-ZON, respectively. Spiking levels of beer and multi standard solutions were diluted by a factor of 4, thus providing equal levels of internal standards in every injection solution. Table 25 displays a comparison of recoveries between externally and internally calibrated spike series. In the case of DON in maize grain in particular, where calibration to external standards lead to obvious overestimation of the detected levels, internal standardisation brought a more realistic determination of the actual content. It was decided to use internal standards in further method validation as external standardisation could still be used if internal calibration was less suited to certain samples. If however, they

did not significantly improve analyte determination in the method validation matrices, internal standardisation could be dropped for following work.

Table 25. Comparison of external (white) and internal (blue) standardisation of DON and ZON spike levels

	spike level (ng/g sample)	Beer				Cornflakes				Wheat			
		external		internal		external		internal		external		internal	
			recov		recov		recov		recov		recov		recov
DON	0	0		0		1540		279		106		94	
	50	50	100	49	98	1720	3440	383	766	163	326	153	306
	100	99	99	88	88	2050	2050	398	398	172	172	172	172
	250	214	86	271	108	2830	1132	572	229	395	158	446	178
	500	464	93	480	96	4330	866	762	152	653	130.6	610	122
	1000	889	89	985	99	6340	634	1120	112	1130	113	1120	112
ZON	0	0				11		13		17		25	
	5	6	110	5	103	16	314	18	364	19	374	21	424
	10	11	108	12	116	21	212	26	263	31	307	34	344
	25	25	100	28	111	39	154	46	183	30	121.2	40	158
	50	51	101	51	102	57	114	63	126	62	124.2	78	155
	100	101	101	115	115	101	101	113	113	98	98.3	148	148

Unfortunately, only the beer was free from contamination with DON and ZON. The recovery values given have not been corrected for the basal contamination. With correction, the determination for e.g. ZON in cornflakes would provide excellent recoveries of 100% for the spiking levels 50 (63-13) or 100 (113-13) µg/L using ^{13}C standards.

3.5. Conclusion

Spiking various matrices and matrix extracts with multistandard solutions revealed strong matrix effects that altered signal response during HPLC-MS detection. To diminish those effects, several procedures of sample cleanup were tested, including C-18 based SPE, PSA, immunoaffinity columns and cleanup cartridges specially designed for mycotoxins (ROMER MycoSep®). However, due to the wide polarity range of the analytes, no single cleanup procedure was capable of improving the performance for all compounds. SPE cleanup improved performance for Z4S but yields of most other target compounds were decreased. PSA or MycoSep columns did not bring improvements that would justify the effort of

preparation and the requirement for higher sample volumes. Immunoaffinity columns proved to be inappropriate due to the loss of conjugated compounds.

The use of fully ^{13}C labelled DON was found to better display the actual quantity of DON in spiked and naturally contaminated samples.

4. Method validation

4.1. Scope of task 04 - Method transfer and validation

Following method development, the method would be transferred to Fera. The method would be validated in-house both in Tulln and in York by analysis of four cereals and six processed food samples spiked at four different concentration levels on 6 separate days. Validation studies would reveal recovery, LOD, LOQ, precision and robustness of the method developed. The method results obtained in the UK and in Austria are compared, as no certified reference materials are available.

4.2. Procedure of validation

4.2.1. Sample material

For the validation of the method developed, 10 different sample matrices were chosen, consisting of four different raw cereal types and six processed cereal products destined for direct consumption or further processing as food. The products covered a wide variety of grain types as well as matrix consistency, thus obtaining optimum estimation of signal deviation range in HPLC-MS/MS measurement. Samples chosen for method validation and extraction conditions are listed in Box 6 below, while further method details can be found in chapters 4.2.2. and 4.2.3.

The samples were purchased at local retail markets. Grain samples, grissini and raw spaghetti were milled in a laboratory mill. Baby oat porridge was purchased as a ground powder and was not further treated before spiking.

Box 6: Validated matrices:

Unprocessed samples:	Barley grain Maize grain Rye grain Wheat grain
Processed samples:	Cornflour Beer (Yorkshire ale) Bran flakes Baby porridge (oat based) Grissini Spaghetti
Extraction:	1g of sample extracted with 4 ml ACN/H_2O/HAc (79/20/1) on a rotary shaker (190 rpm), at room temperature (25 ℃).

4.2.2. Spiking and replication

Spiking of matrix samples was performed prior to extraction by adding 10 µl multistandard solution per gram of sample (beer: fresh wgt.; cereal samples, processed: dry wgt.) at four different concentrations, thus providing four different spike levels for each sample per validation series. For dry samples, extraction of 1.00 g was performed with 4 ml ACN/H2O/HAc (79/20/1) on a rotary shaker (190 rpm), at room temperature (25 ℃) for 90 min. 1 ml of extracts were evaporated to dryness and redissolved in equal volumes of 10% ACN for HPLC-MS/MS determination. Since beer was not further extracted, samples of 1 ml were spiked directly, evaporated to dryness and subsequently redissolved in 1 ml of 10% ACN. Mycotoxin concentrations and respective spike levels are listed in Box 7 below. Concentrations of AcDON in spike solutions were altered for validation at IFA-Tulln after results from validation at Fera had shown weak detectability of this analyte in spiked matrix at low levels. The whole procedure of spiking at four levels, extraction of spiked samples plus unspiked control and HPLC-MS/MS determination was repeated six times for each sample matrix. The whole validation process including repeated spiking, extraction and analytical determination was undergone at the Fera Laboratory (York, UK) and at IFA-Tulln (Tulln, Austria).

Box 7: Multistandard spike levels:
Solvent: 10% ACN (aq.)
spike levels (µg/g sample*): *beer: µg/l

analyte	Level 1	Level 0.5	Level 0.25	Level 0.1
DON	1000	500	250	100
D3G	1000	500	250	100
Ac-DON (Fera)	100	50	25	10
Ac-DON (IFA)	250	125	62.5	25
ZON	100	50	25	10
α-ZOL	100	50	25	10
β-ZOL	100	50	25	10
Z4G	100	50	25	10
α-ZG	100	50	25	10
β-ZG	100	50	25	10
Z4S	10	5	2.5	1

4.2.3. Instrumental conditions of HPLC-MS/MS determination

Both at Fera York and at IFA-Tulln, an Agilent 1100 HPLC connected to an Applied Biosystems 4000 triple quadrupole mass analyser were used for mycotoxin analysis.

Instrumental conditions used in the measurement method developed at IFA-Tulln were transferred and applied to the apparatus at Fera York.

The column used for HPLC separation at both institutes was a Phenomenex Synergi Polar-RP (150 x 3 mm; 4 µm). For validation at IFA Tulln, a new column of the same type was ordered. Injection sequences of measurement batches were arranged in a way that three series of external standard solutions were alternated by 25 samples each between first and second as well as second and third standardisation series.

4.2.4. Data calculation

Analyte concentrations were calculated on a peak area basis using Applied Biosystems Analyst® version 1.5 software. Lower limits of detection (LODs) were defined by signal-to-noise ratios of signal peaks of 3:1, while limits of quantification (LOQs) were defined at a signal-to-noise ratio of 10:1. Calibration curves were calculated on the basis of external standard solution by plotting signal intensities versus analyte concentration and weighted 1/x. Data were then further evaluated using unweighted linear regression in Validata® software (Wegscheider et al. 1999).

4.3. Validation results

Validation results are given in Tables for each sample matrix, in which average recovery, LOQ, LOD, number of quantifiable spike levels and repeatability are compiled for all analytes. The tables are provided as excel sheets attached to this report in separate files for the validation series at Fera York and IFA Tulln, respectively. Prior to the validation experiments, some matrices had been found to be naturally contaminated with some of the masked mycotoxins targeted in this study and have therefore not been included into the choice of test materials for validation. However, measurements for method validation have still revealed certain levels of DON, ZON and Z4S in bran flakes and wheat grain. Average contamination levels of DON, Z4S and ZON in bran flakes and wheat grain are given in Table 26.

Table 26. Natural contamination of bran flakes and wheat grain determined in control samples

	Bran flakes			Wheat grain		
	DON	Z4S	ZON	DON	Z4S	ZON
Fera	286	5.1	46.6	< LOQ	3.5	35.7
IFA	287	5.6	41.4	< LOQ	3.7	30.9

Numbers refer to average analyte concentrations in µg/kg

As no uncontaminated bran flakes and wheat grain were available, these matrices were nevertheless spiked as described above. Basis levels were subtracted before calculation of recoveries and repeatabilities.

For all 10 matrices and 10 analytes, repeatabilities were below 20% and considered acceptable. Around 70 out of 100 analyte/matrix combinations (depending on the place of validation) showed recoveries between 80 and 110%. Except for Z4S in beer, in which just about 25% was recovered, the method worked well in both locations. Reproducibility was not calculated, but visual comparison of the validation results gained in the UK and in Austria showed no major differences.

4.4. Conclusion

Method validation was accomplished for 10 cereal-based matrices which were spiked at four different levels in six replicates and performed at Fera York, UK as well as at IFA Tulln, Tulln, Austria. Repeatability was between 4.7 (α-ZOL, corn flour, York) and 19.3% (Z4S, wheat grain, York) for all analyzed matrices. The use of a new HPLC column of the same type brought lower LOD and LOQ values (due to decreased noise) and often also better repeatabilities. Comparison between the validation results from Fera York and from IFA Tulln suggest that the method developed is suitable and stable for determination of the target *Fusarium* toxins in cereals and cereal based food. Selected results from this study have been summarised and sent to Analytical and Bioanalytical Chemistry for publication as original paper. The manuscript has been accepted and published in a special issue about mycotoxins.

5. Determination of masked mycotoxins in cereal and cereal based food samples

5.1. Scope of task 05 - Sample collection and measurements

To demonstrate the applicability of the method to real world samples as many different retail matrices as possible, including flour, bread, pasta, breakfast cereals, beer, maize oil or cereal snacks would be purchased from local markets in the UK and in Austria over about 2 months. Cereal samples (maize, wheat, rye, barley, oats) would be obtained from local millers. Additionally, potatoes and rice would be purchased. Naturally contaminated cereals identified in Task 4 would also be included. A total of 100 samples was anticipated.

5.2. Procurement and collection of samples

Cereal products destined for food use (either for direct consumption or for further processing) were purchased in retail markets in the UK and in Austria. As the sample preparation and measurement was carried out at Fera York, the majority of cereal-based food samples was purchased from local supermarkets in or around York. In order to obtain more reliable information on the mycotoxin exposure of a particular product group to the customer, it was decided to combine 3 – 5 products of different manufacturers within the same product group to one composite. As a total number of 100 individual samples was expected a feasible amount, about 30 composites (depending on the availability) were estimated to be included into the mini-survey. Besides food, raw and unprocessed samples of several types of grain were procured from organic retailers in the UK and Austria. In addition, four lots of wheat grain were provided from Harper Adams University College, Shropshire, UK. Table 27 shows the complete list of composites screened in this study.

5.3. Sample processing

Dry samples like raw grains, corn flakes, wheat flakes and raw pasta were milled in a laboratory mill. Samples containing higher amounts of fat or sugar were milled under a supply of dry ice. Samples that were processed in the latter way belonged to the following composites: Extruded maize snacks, biscuits, muesli, crackers, popcorn, cereal snack bars, extruded oat snacks and some particular samples of the baby food composites. Bread samples were dried at 45 ℃ for 48 hours and subseq uently milled. Milling was performed for every sample separately, then equal amounts of 100 g per milled sample were combined and homogenised in a laboratory blender.

Canned maize was mashed in a blender together with an equal weight amount of water (1 ml water per 1 g sample), which corresponded to the water content in the extraction solution. Homogenised canned maize samples were extracted right after homogenisation. For the

beer composite, equal volumes (100 ml each) of beer samples were combined and degassed in a sonic bath for 10 min.

Extraction of all dry composites was performed as follows:

1.00 g sample was extracted with 4 ml ACN/H2O/HAc (79/20/1) on a rotary shaker (190 rpm), at room temperature (25 ℃) for 90 min. 1 ml of extracts was evaporated to dryness and redissolved in equal volume of 10% ACN for HPLC-MS/MS determination. Since beer was not extracted, samples of 1 ml were evaporated to dryness and subsequently redissolved in 1 ml 10% ACN for analysis. For the extraction of canned maize, 400 ml of a mixture of ACN/HAc (97:1) was added to 100 ml homogenate.

Table 27. Composites of cereal grains and cereal-based food included in the present mini-survey, indicating numbers of samples procured from Austria or from the UK, respectively

Composite	sample type	# samples (UK)	# samples (Austria)
1	wheat grain	5	1
2	barley grain	3	1
3	rye grain and flour	3	1
4	maize grain	3	1
5	spelt grain	3	3
6	oat grain	3	2
7	wheat flour	3	0
8	wheat bread	4	2
9	rye bread	4	1
10	wheat bread wholemeal	3	0
11	wheat semolina	3	1
12	maize meal and flour	3	0
13	extruded maize snacks	3	0
14	biscuits	4	1
15	pasta	4	0
16	beer including wheat beer	4	3
17	canned maize	4	0
18	breakfast cereals - corn flakes	3	1
19	breakfast cereals - wheat flakes	4	0
20	breakfast cereals - bran	3	0
21	breakfast cereals - muesli	4	1
22	breakfast cereals - oats	3	0
23	crackers	3	0
24	baby food wheat based	3	1
25	baby food oat based	3	0
26	popcorn	3	0
27	cereal snack bars	3	0
28	buckwheat grain or flour	3	0
29	quinoa grain or flour	4	2
30	extruded oat snacks	3	0
31	polenta	3	0

5.4. Results

Results from HPLC-MS/MS determination of masked mycotoxins in all 31 composites are given in the Annexe (chapter 8.3). The only analytes found in quantifiable amounts were DON, Z4S and ZON. In wheat grain, wheat bread and wheat wholemeal bread, also D3G was detected at levels below LOQ. The highest quantities of DON (603 µg/kg), and Z4S (6.4 µg/kg) were found in unprocessed wheat grain. Bran flakes contained the highest level of ZON (44.2 µg/kg). The latter matrix has also been observed to exhibit contaminations of DON, Z4S and ZON in prior experiments on method validation (see chapter 4). Relatively high contents of DON were also been found in crackers (248 µg/kg) and wheat flour (237 µg/kg). Both latter matrices also showed Z4S contents of over 2 µg/kg), although neither of them displayed significantly high levels of ZON. Due to the its high ionisation efficiency, Z4S was detectable at very low levels and was therefore quantitatively determined in 12 composites, namely wheat grain, rye grain, wheat flour, wholemeal wheat bread, maize meal, biscuits, wheat flakes, bran flakes, muesli, crackers, cereal snack bars and polenta. Furthermore, Z4S was detected below the LOQ in wheat bread, rye bread and extruded maize snacks. ZON, which was quantifiable in wheat grain and bran flakes, was also detected in wholemeal wheat bread, extruded maize snacks, corn flakes, wheat flakes, crackers and cereal snack bars.

5.5. Conclusion

Thirty-one composites of cereal grain and cereal-based food were examined for natural occurrence of masked mycotoxins. DON was quantified in four samples, ZON in two samples and Z4S in 12 samples of the collection. None of the detected values exceeded European Commission regulatory levels, which suggests that no high risk of mycotoxin intoxication is imposed by any of the tested products and, furthermore, that the applied screening method is suitable for regulatory contamination levels of the target analytes.

6. Summary

6.1. Project Outcome

An HPLC-MS/MS method for reliable detection and quantification of all target analytes (DON, acetyl-DON, DON-3-glucoside, ZON, ZON-4-glucoside, ZON-4-sulfate, α-zearalenol, β-zearalenol, α-zearalenol-4-glucoside and β-zearalenol-4-glucoside) was successfully established. Electrospray ionisation (ESI) in negative polarity proved to be suitable for these analytes. Peak intensity and separation as well as overall retention were optimised by variations of column, eluent composition, flow rate and temperature.

Optimisation of extraction solvent composition, extraction time, sample/solvent ratio, as well as experiments on enzymatic pre-treatment led to the establishment of a sample extraction procedure. Enzymatic pre-treatment of cereal samples with Pronase resulted in heightened concentrations of DON and especially D3G. Surprisingly, not only did enzymatic treated samples exhibit higher amounts of D3G, but so did the control incubation sample, which was only kept in buffer solution without enzyme. This observation might suggest that, in some cases, an important effect of sample pre-treatment is the preliminary aqueous extraction rather than an enzymatic reaction. The cause for this effect is unknown but definitely worth further exploration. While alkaline extraction degraded acetylated forms of DON and was therefore deemed inappropriate, it also showed a significant increase of D3G from naturally contaminated samples. A possible explanation of the increase of D3G recovery in both cases could be the cleavage of multiple glucoside residues from higher conjugated compounds, e.g. DON-diglucoside or DON-triglucoside. During this project such conjugates were found in beer (J. Hajslova, personal communication), although currently standards for those substances are not available. As the ether bond between DON and glucose in D3G is very stable, it is conceivable that higher conjugates of DON might degrade to D3G but not further towards DON.

In order to simplify and speed up the overall method, though, we omitted a 20 hour steeping step and used the following procedure: Extraction of 1.00 g of milled food with 4 ml of ACN/HAc (79:1) is performed at room temperature on a rotary shaker at 180 rpm for 90 min. Extracts are filtered or centrifuged, 1 ml is evaporated and redissolved in 1 ml 10% ACN for HPLC-MS analysis. This approach was performed in the best intention to recover all the soluble analytes, including D3G. However, it cannot be ruled out that currently unknown higher conjugates of DON and D3G (presumably diglucosides, triglucosides, etc.) can also present in naturally contaminated samples.

Spiking various matrices as well as matrix extracts with mycotoxin multi standard solutions revealed strong matrix effects that alter signal response during HPLC-MS detection of the target analytes. To diminish those effects, several procedures of sample cleanup were

tested, including C-18 based SPE, PSA, Immunoaffinity columns and specially designed cleanup cartridges for mycotoxins (MycoSep®). However, due to the wide polarity range of the target analytes, no single cleanup procedure was capable of improving the performance of the entity of included compounds. SPE cleanup was capable of improving performance of Z4S while yields of most other target compounds were decreased. Application of PSA or MycoSep columns did not bring about improvements that would justify the effort of preparation and the requirement of higher sample volumes. Immunoaffinity columns proved to be inappropriate for this study due to the loss of conjugated compounds.

The use of fully ^{13}C labelled DON and ZON was found to better display the actual quantities spiked and naturally contaminated samples. Method validation was performed with internal and external calibration. The beneficial effect of slightly better repeatabilities with internal standards was set off with the increased price per sample. Overall, internal standards were not found to be necessary in this method.

Method validation was accomplished for 10 cereal-based matrices which were spiked at four different levels in six replicates and performed at Fera in York, UK as well as at IFA Tulln, Tulln, Austria. Repeatability was between 4.7 and 19.3% for all analyzed matrices. The use of a newly ordered HPLC column of the same type brought lower LOD and LOQ values (due to decreased noise) and often also better repeatabilities. Comparison between the validation results from Fera York and from IFA Tulln, respectively, suggest that the method developed is suitable and stable for determination of the target *Fusarium* toxins in cereals and cereal based food. Selected results from this study have been summarised and sent to Analytical and Bioanalytical Chemistry for publication as original paper. The manuscript has been accepted and will be published in a special issue about mycotoxins later this year.

Thirty-one composites of cereal grain and cereal-based food were examined for natural occurrence of masked mycotoxins. DON was quantified in four samples, ZON in two samples and Z4S in 12 samples of the collection. None of the detected values exceeded European Commission regulatory levels, which suggests that no high risk of mycotoxin intoxication is imposed by any of the tested products and, furthermore, that the applied screening method is suitable for regulatory contamination levels of the target analytes.

Two publications have been prepared from the results of this project:

Vendl O, Berthiller F, Crews C, Krska R, 2009. Simultaneous determination of deoxynivalenol, zearalenone and their known masked mycotoxins in cereal based food by HPLC-MS/MS. Analytical and Bioanalytical Chemistry 395, 1347-1354.

Vendl O, Berthiller F, MacDonald S, Crews C, Krska R. Occurrence of free and conjugated *Fusarium* mycotoxins in cereal based food. (submitted to Food Additives and Contaminants)

7. References

- Berthiller F, Dall'Asta C, Schuhmacher R, Lemmens M, Adam G, Krska R, 2005. Masked Mycotoxins: Determination of a Deoxynivalenol Glucoside in Artificially and Naturally Contaminated Wheat by LC-MS/MS. J. Agric. Food Chem. 53, 3421-3425.

- Berthiller F, Werner U, Sulyok M, Krska R, Hauser MT, Schuhmacher R, 2006. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) determination of phase II metabolites of the mycotoxin zearalenone in the model plant *Arabidopsis thaliana*. Food Addit. Contamin. 23(11), 1194-1200.

- Berthiller F, Hametner C, Krenn P, Schweiger W, Ludwig R, Adam G, Krska R, Schuhmacher R, 2009a. Preparation and characterization of the masked Fusarium mycotoxins zearalenone-4O-β-D-glucopyranoside, α-zearalenol-4O-β-D-glucopyranoside and β-zearalenol-4O-β-D-glucopyranoside by MS/MS and 2D-NMR. Food Additives and Contaminants 26, 207-213.

- Berthiller F, Schuhmacher R, Adam G, Krska R, 2009b. Formation, determination and significance of masked and other conjugated mycotoxins. Anal Bioanal Chem (in press) DOI 10.1007/s00216-009-2874-x.

- Böswald C, Engelhardt G, Vogel H, Wallnöfer PR, 1995. Metabolism of the *Fusarium* mycotoxins zearalenone and deoxynivalenol by yeast strains of technological relevance. Nat. Toxins 3, 138-144.

- El-Sharkawy S, Abul-Hajj Y, 1987. Microbial transformation of zearalenone, I. formation of zearalenone-4-0-beta-glucoside. J. Nat. Prod. 50(3), 520-521.

- El-Sharkawy S, 1989. Microbial transformation of zearalenone, III. Formation of 2,4-O-β-diglucoside. Acta Pharm. Jugosl. 39, 303-310.

- El-Sharkawy S, Selim MI, Afifi MS, Halaweish FT, 1991. Microbial transformation of zearalenone to a zearalenone sulfate. Appl. Environ. Microbiol. 57(2), 549-552.

- Engelhardt G, Zill G, Wohner B, Wallnöfer PR, 1988. Transformation of the *Fusarium* mycotoxin zearalenone in maize cell suspension cultures. Naturwissenschaften 75, 309-310.

- Engelhardt G, Ruhland M, Wallnöfer PR, 1999. Metabolism of mycotoxins in plants. Adv. Food Sci. 21(3/4), 71-78.

- European Commission Regulation 856/2005 of 6 June 2005 amending Regulation (EC) No 466/2001 as regards *Fusarium* toxins. Off. J. Europ. Union L143, 3-7.

- Gareis M, Bauer J, Thiem J, Plank G, Grabley S, Gedek B, 1990. Cleavage of zearalenone-glycoside, a "masked" mycotoxin, during digestion in swine. J. Vet. Med. 37, 236-240.
- Gareis M, 1994. Maskierte Mykotoxine. Übers. Tierernährung 22 (1), 104-113.
- Haeubl G, Berthiller F, Krska R, Schuhmacher R, 2006. Suitability of a fully 13C isotope labelled internal standard for the determination of the mycotoxin deoxynivalenol by LC-MS/MS without cleanup. Anal Bioanal Chem. 384: 692-696.
- Howard PC, Churchwell MI, Couch LH, Marques MM, Doerge DR, 1998. Formation of N-carboxymethyl-fumonisin-B_1, following the reaction of fumonisin B_1 with reducing sugars. J. Agric. Food Chem. 46, 3546-3557.
- Humpf HU, Voss KA, 2004. Effects of thermal food processing on the chemical structure and toxicity of fumonisin mycotoxins. Mol. Nutr. Food Res. 48(4), 255-269.
- Ioannides C, 2002. In: Enzyme Systems that Metabolise Drugs and Other Xenobiotics, John Wiley & Sons, Ltd, Chichester, UK, 1-32.
- Kamimura H, 1986. Conversion of zearalenone to zearalenone glycoside by *Rhizopus* sp. Appl. Environ. Microbiol. 52(3), 515-519.
- Kim EK, Scott PM, Lau BP, 2003. Hidden fumonisin in corn flakes. Food Addit. Contam. 20(2), 161-169.
- Krenn P, Berthiller F, Schweiger W, Hametner C, Ludwig R, Adam G, Krska R, Schuhmacher R, 2007. Production of zearalenone-4-glucoside, alpha-zeraralenol-4-glucoside and beta-zearalenol-4-glucoside. Mycotoxin Research 23(4), 180-184.
- Miller JD, Young JC, Trenholm HL, 1983. *Fusarium* toxins in field corn. I. Time course of fungal growth and production of deoxynivalenol and other mycotoxins. Can. J. Bot. 61, 3080-3087.
- Park JW, Scott PM, Lau BP, Lewis DA, 2004. Analysis of heat-processed corn foods for fumonisins and bound fumonisins. Food Addit. Contam. 21(12), 1168-1178.
- Plasencia J, Mirocha CJ, 1991. Isolation and characterization of zearalenone sulfate. Appl. Environ. Microbiol. 57(1), 146-150.
- Poling SM, Plattner RD, Weisleder D, 2002. *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1, the initial reaction product of fumonisin B_1 and D-glucose. J. Agric. Food Chem. 50(5), 1318-1324.
- Sandermann H, 2004. Bound and unextractable pesticidal plant residues: chemical characterization and consumer exposure. Pest Manag. Sci. 60, 613–623.

- Savard ME, 1991. Deoxynivalenol fatty acid and glucoside conjugates. J. Agric. Food Chem. 39, 570-574.
- Schneweis I, Meyer K, Engelhardt G, Bauer J, 2002. Occurrence of zearalenone-4-β-D-glucopyranoside in wheat. J. Agric. Food Chem. 50(6), 1736-1738.
- Seefelder W, Knecht A, Humpf HU, 2003. Bound fumonisin B1: analysis of fumonisin-B1 glyco and amino acid conjugates by liquid chromatography-electrospray ionization-tandem mass spectrometry. J. Agric. Food Chem. 51(18), 5567-5573.
- Sewald N, Lepschy von Gleissenthall J, Schuster M, Müller G, Aplin RT, 1992. Structure elucidation of a plant metabolite of 4-desoxynivalenol. Tetrahedron 3(7), 953-960.
- Sulyok M, Berthiller F, Krska R, Schuhmacher R, 2006. Development and validation of a liquid chromatography/tandem mass spectrometric method for the determination of 39 mycotoxins in wheat and maize. Rapid Commun. Mass Spectrom. 20, 2649-2659.
- Sulyok M, Krska R, Schuhmacher R, 2007. A liquid chromatography/tandem mass spectrometric multi mycotoxin method for the quantification of 87 analytes and its application to semiquantitative screening of moldy food samples. Anal. Bioanal. Chem. (epub ahead of print).
- Wallnöfer PR, Preiß U, Ziegler W, Engelhardt G, 1996. Konjugatbildung organischer Schadstoffe in Pflanzen. UWSF - Z. Umweltchem. Ökotox. 8(1), 43-46.
- Wegscheider W, Rohrer C, Neuböck R (1999) Validata (Excel-Makro zur Methodenvalidierung), Version 3.02.48
- Young CJ, Fulcher GR, Hayhoe JH, Scott PM, Dexter JE, 1984. Effect of milling and baking on deoxynivalenol (vomitoxin) content of eastern canadian wheats. J. Agric. Food Chem. 32(3), 659-664.
- Zill G, Ziegler W, Engelhardt G, Wallnöfer PR, 1990. Chemically and biologically synthesized zearalenone-4-β-D-glucopyranoside: comparison and convenient determination by gradient HPLC. Chemosphere 22(4-5), 435-442.

8. Annexe

8.1. Figures

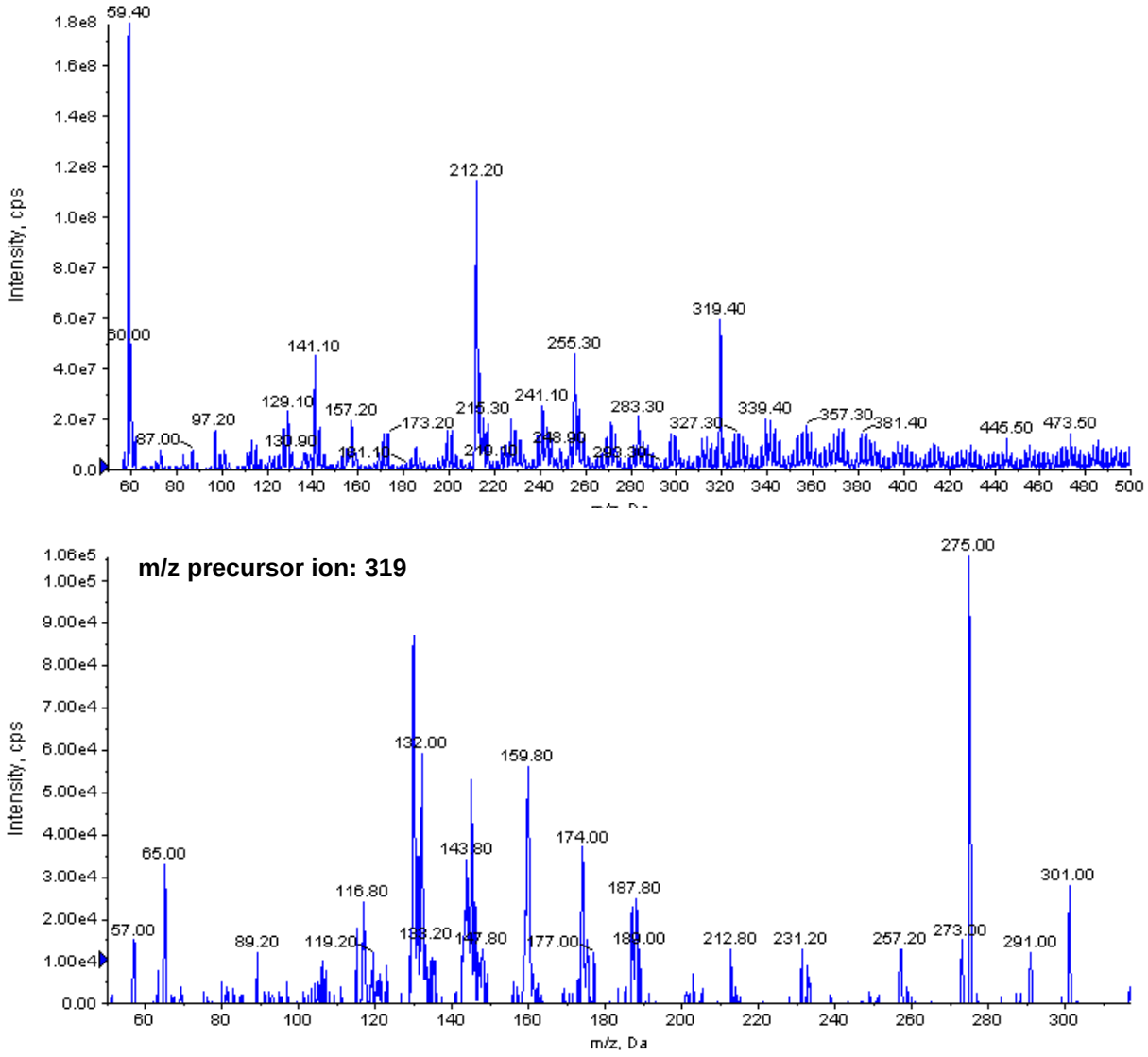


Fig. 1. MS spectra of α-ZOL.
top: Q1 full scan mode; bottom: MS/MS product ion scan mode.

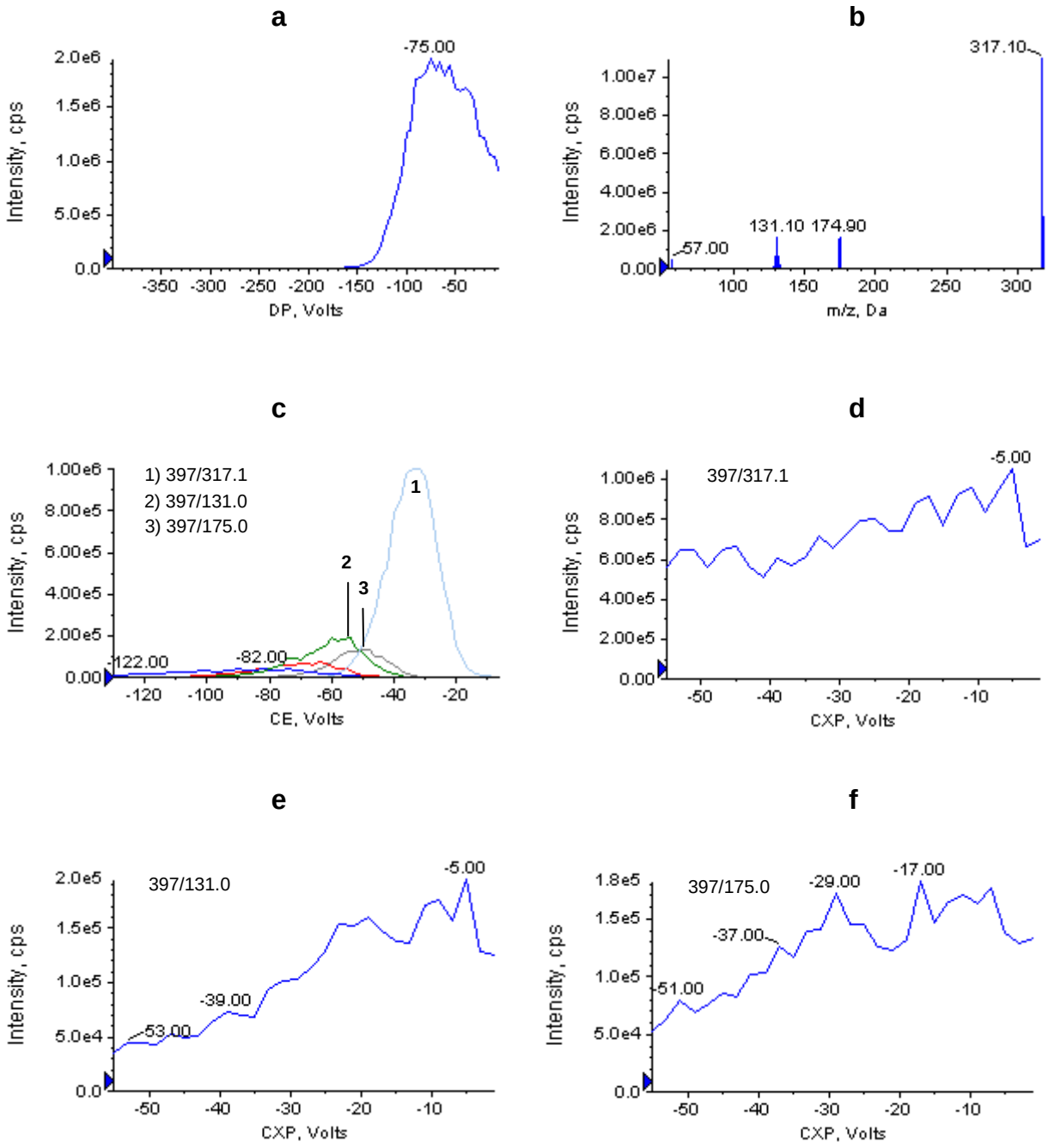


Fig. 2. Optimisation of MRM parameters for ZON-4-sulfate.
a, optimisation of declustering potential; **b,** obtained fragment masses; **c,** ramping of collision energies; **d – f,** optimisation of cell exit potential for each fragment.

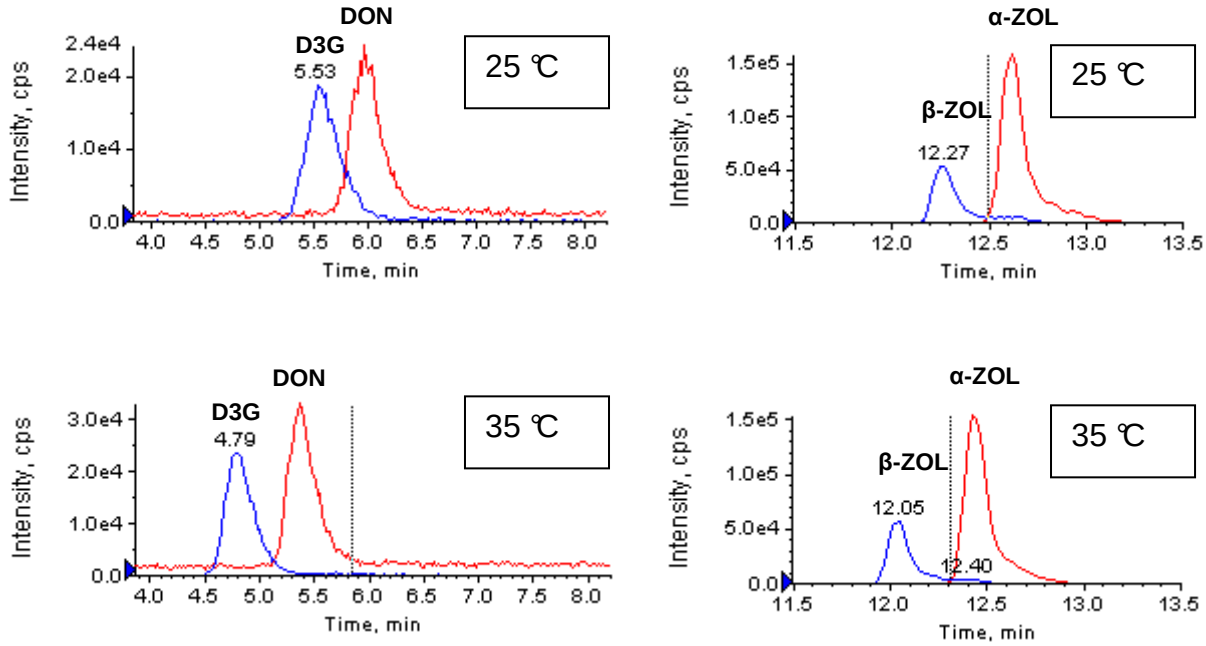


Fig. 3. Altered retention and separation between DON and DON-3-glucoside (left) and α-ZOL from β-ZOL (right), respectively.

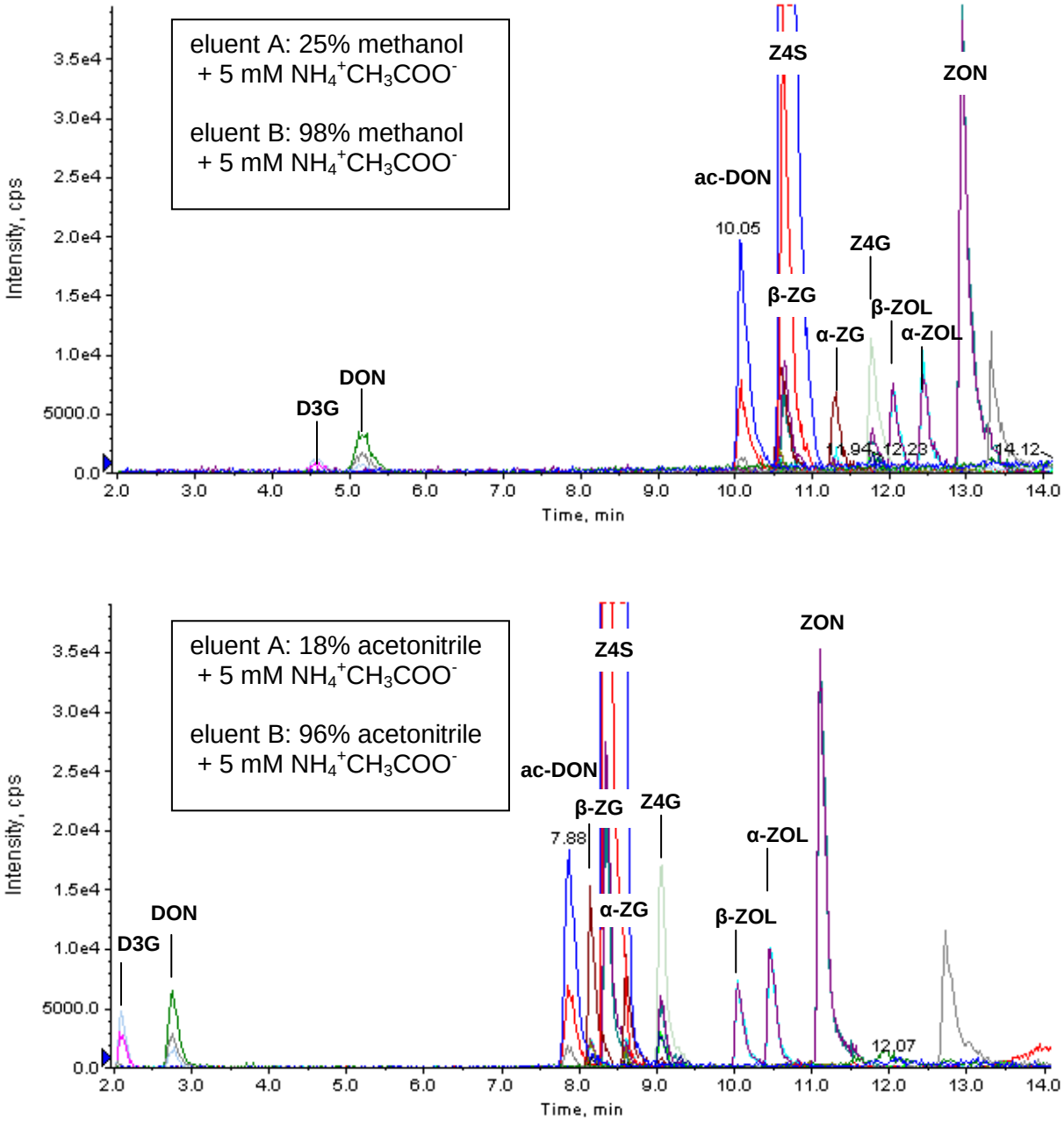


Fig. 4. Comparison of gradient elution with methanol (top) and acetonitrile (bottom).

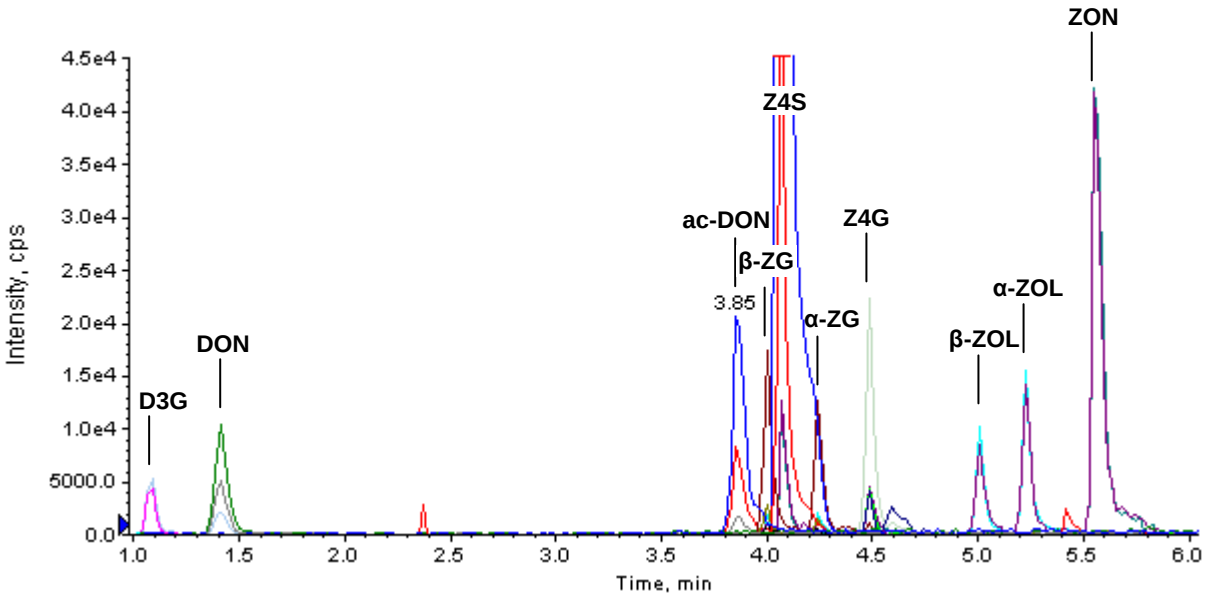


Fig. 5. MRM spectra of multi-standard analysis, HPLC flow rate: 1 ml/min

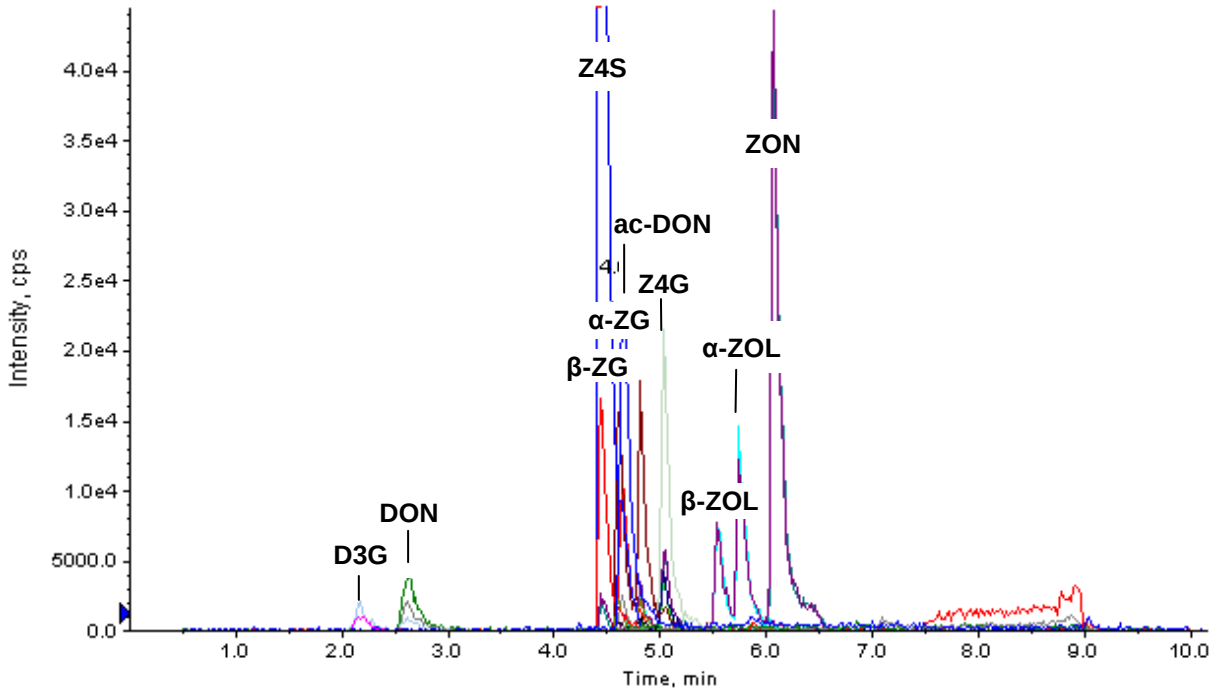


Fig. 6. MRM spectra of multi-standard analysis, finalised HPLC-MS/MS method

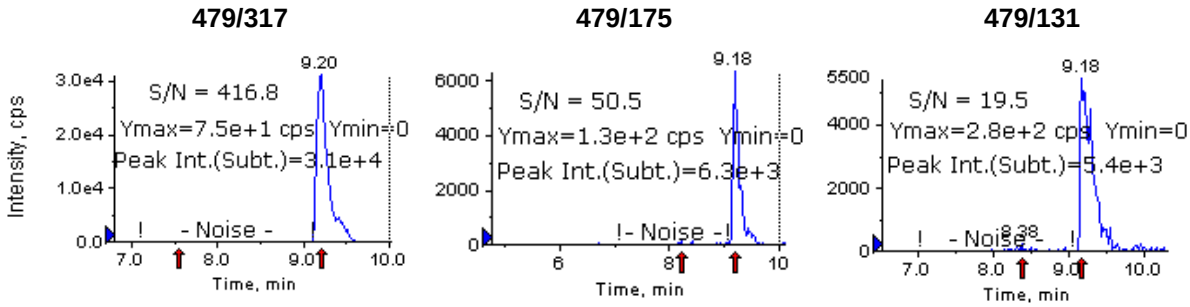


Fig. 7. Calculated signal-to-noise ratios of MRM transitions of ZON-4-glucoside

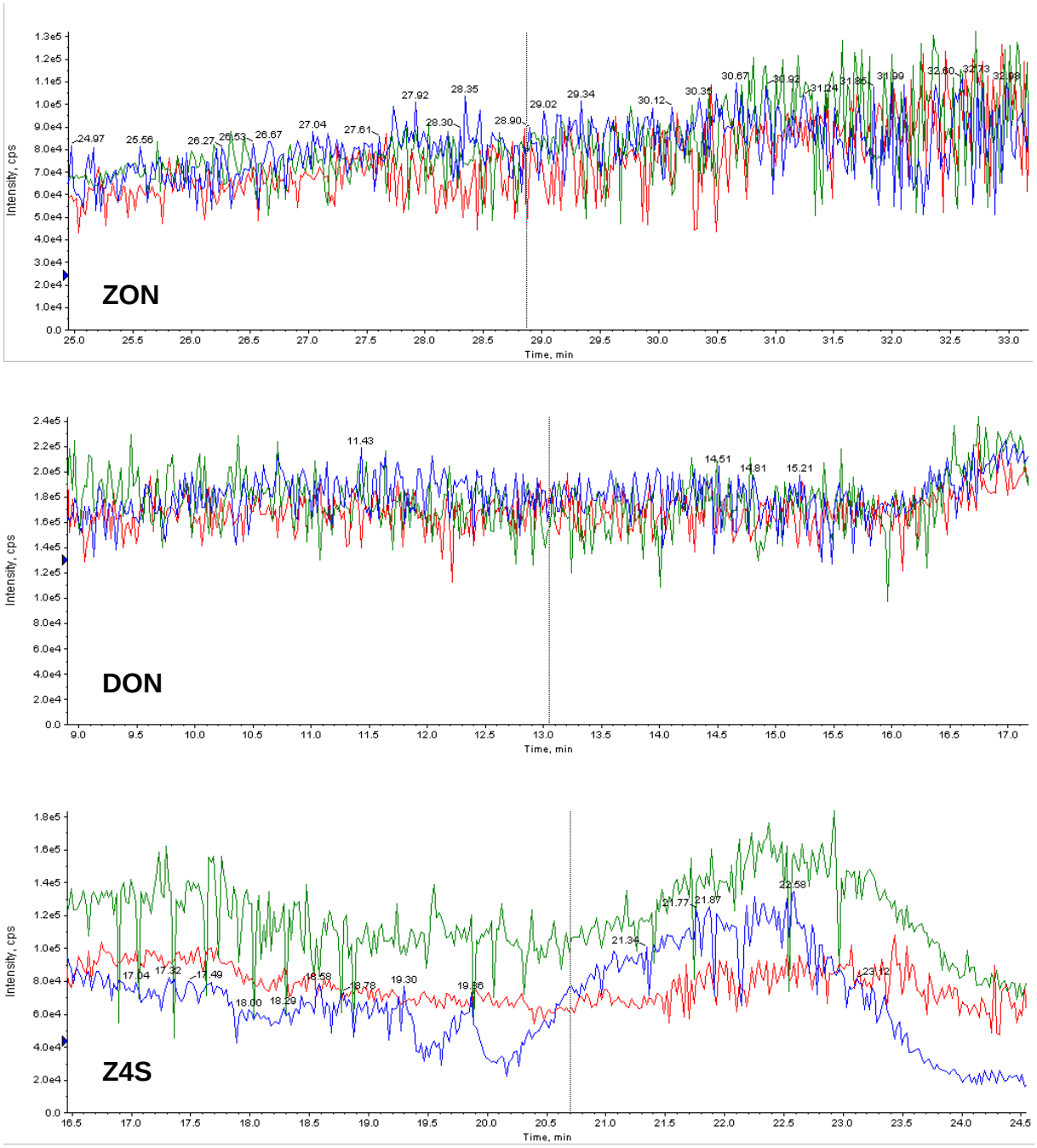
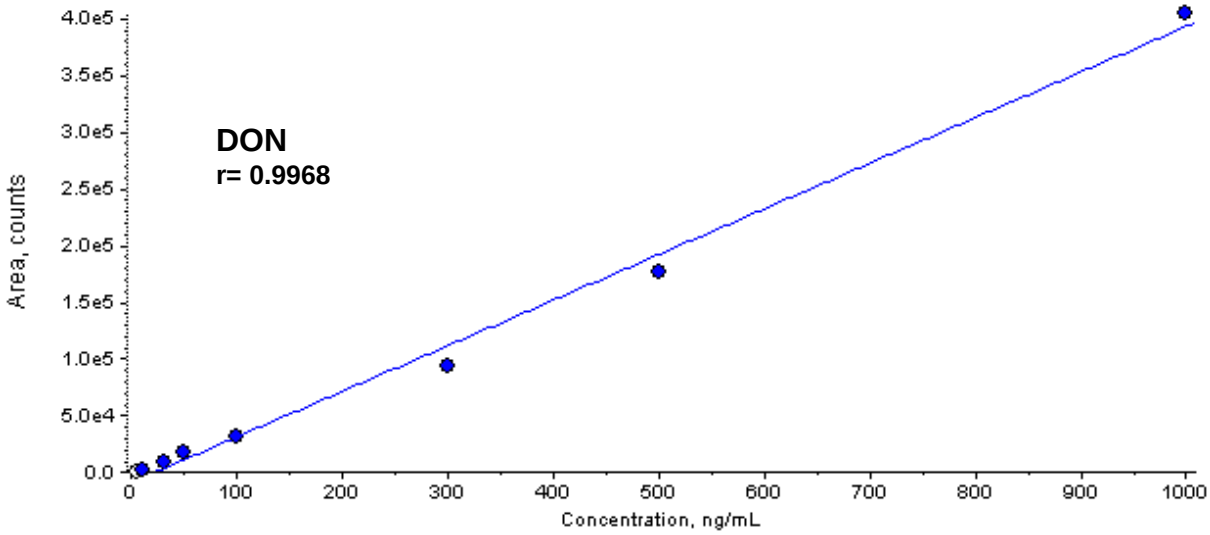
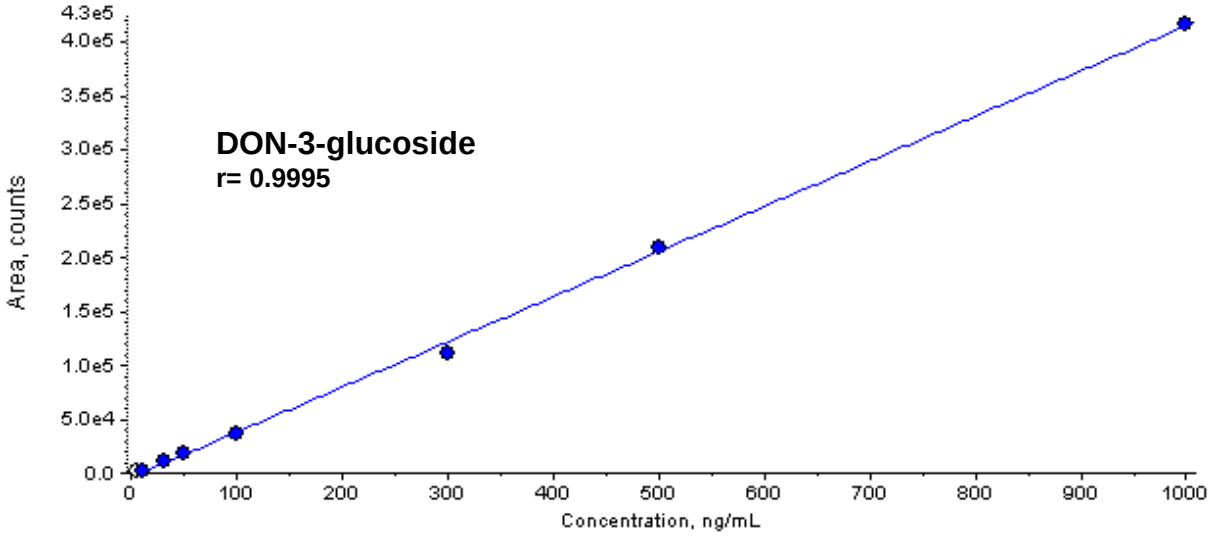
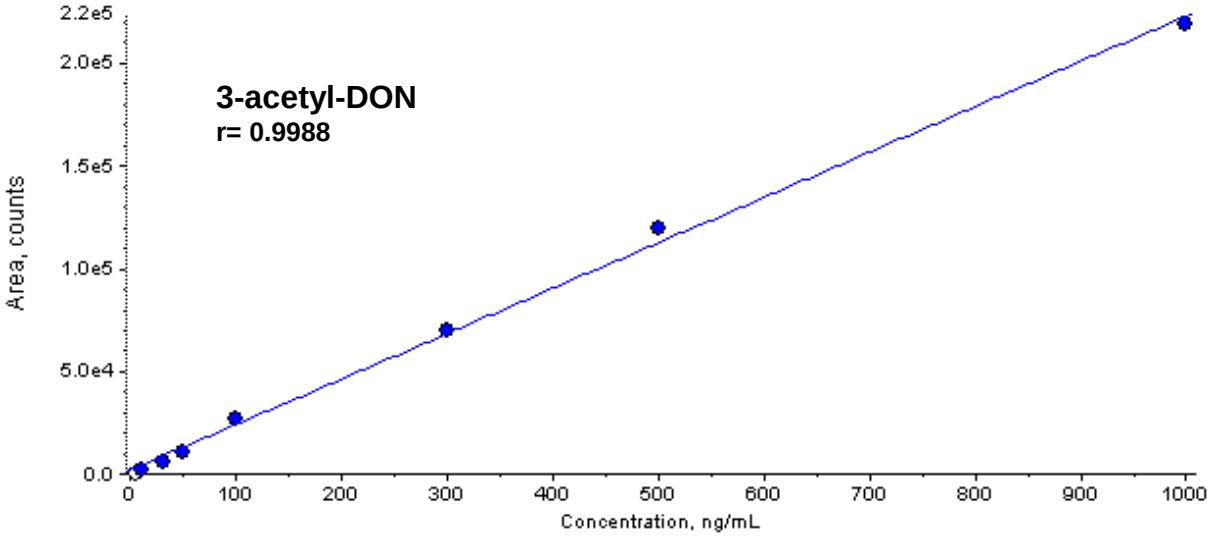


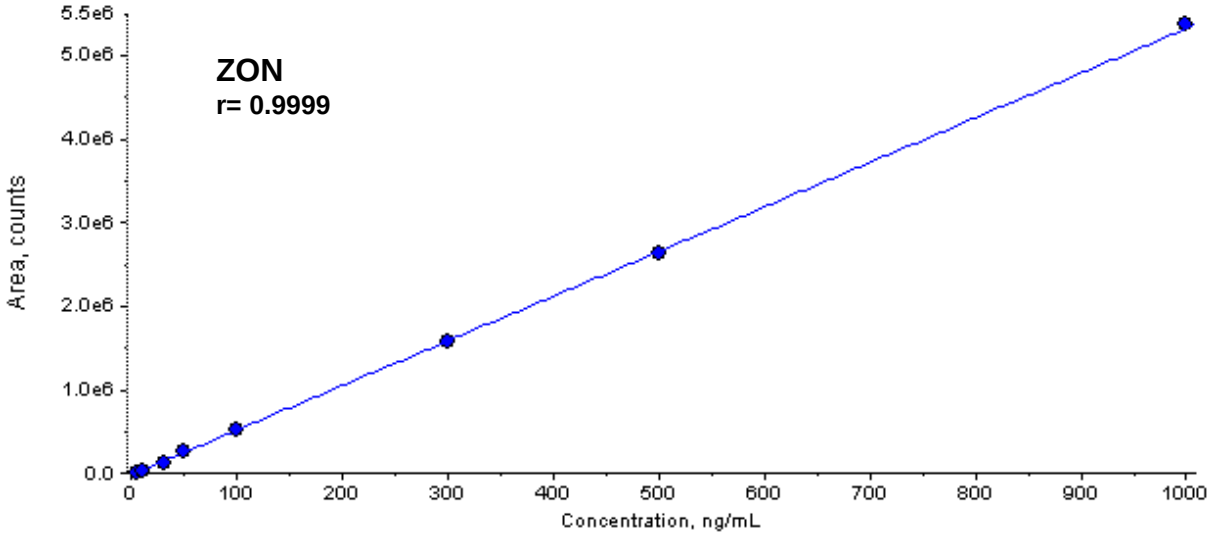
Fig. 8. Comparison of signal alteration by parallel infusion of mycotoxin standards and sample extracts. Tested matrices: Beer (blue), maize grain (red) and wholemeal bread (green).

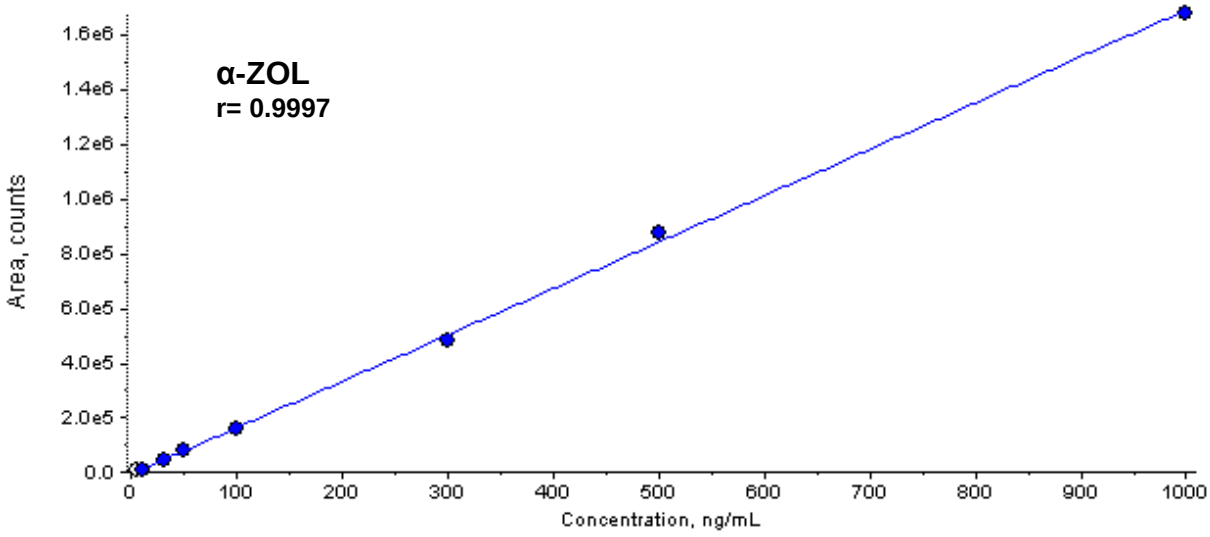
8.2. Calibration curves

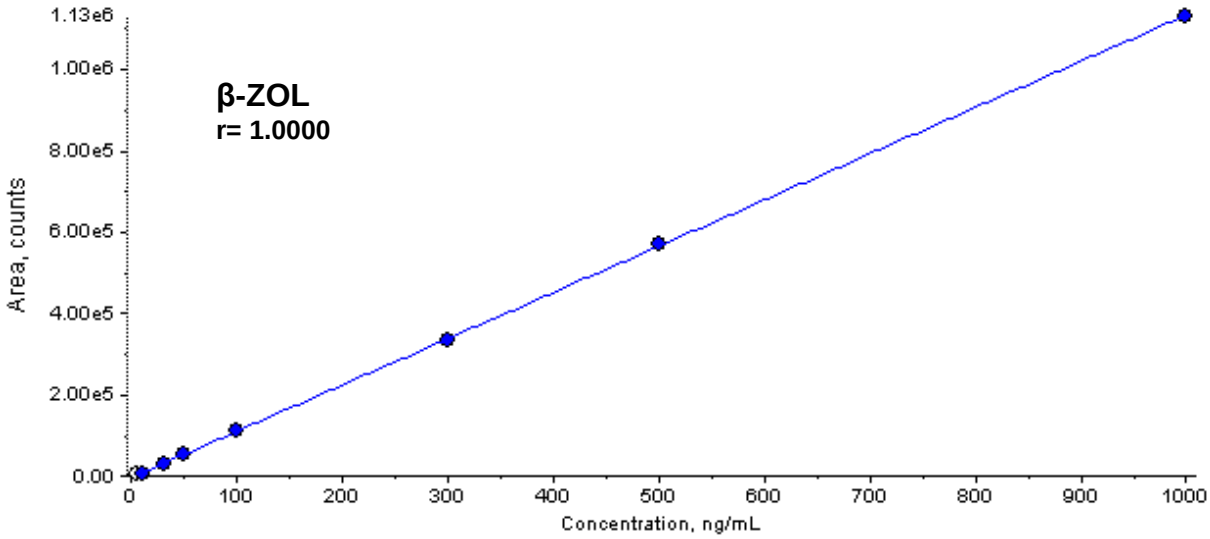


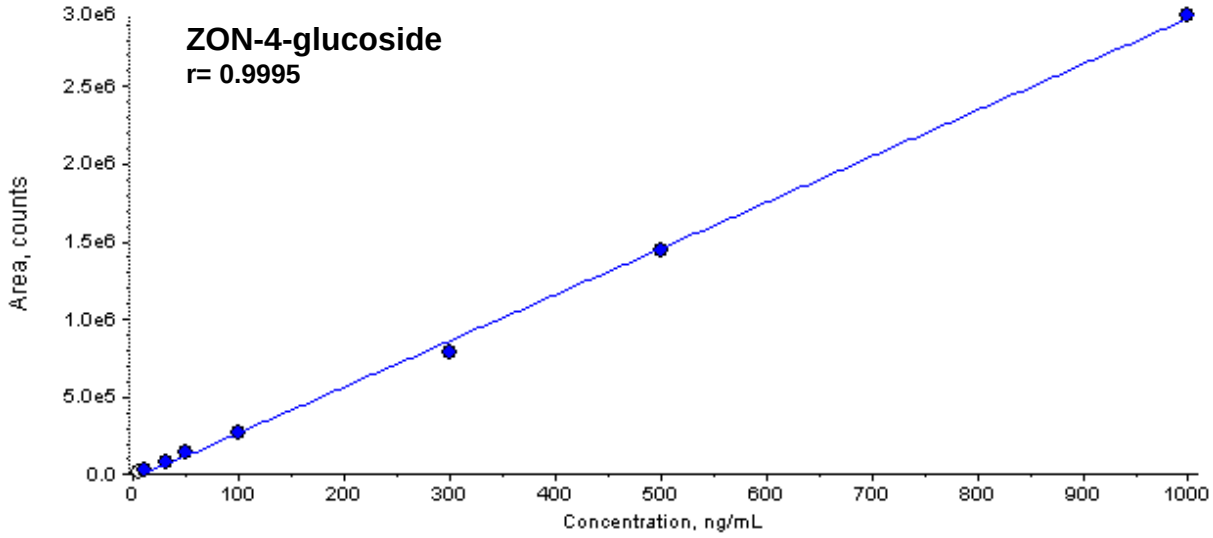


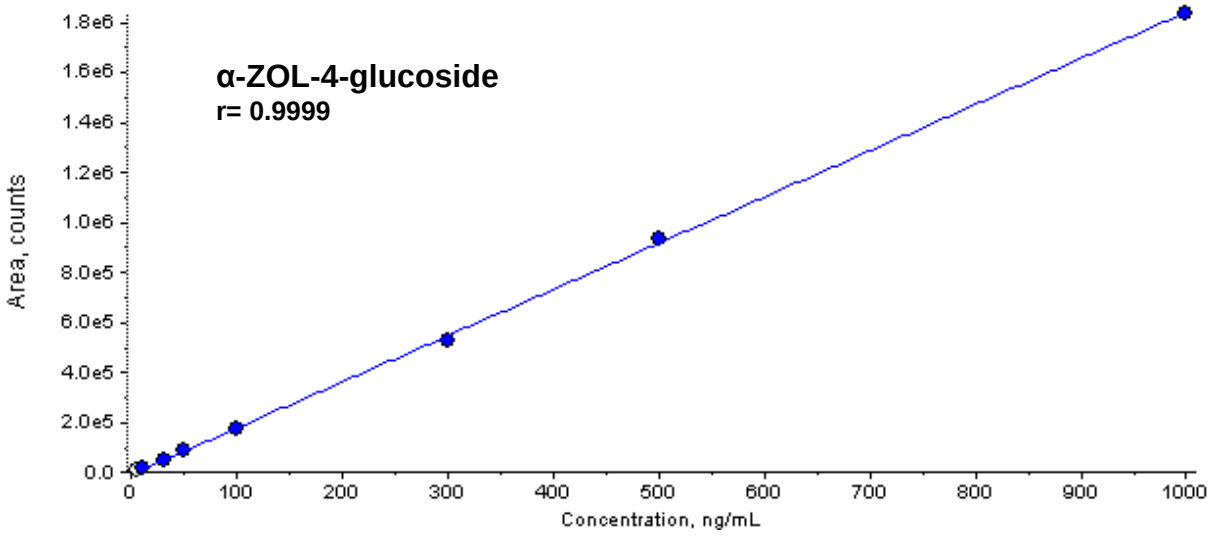


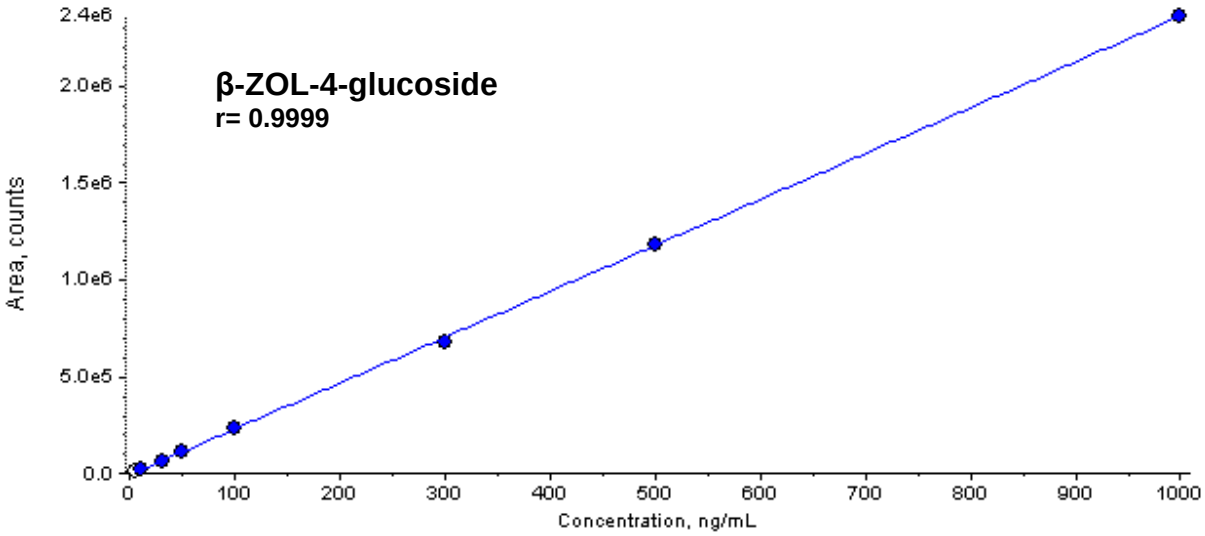

ZON
r= 0.9999
Area, counts
Concentration, ng/mL
5.5e6
5.0e6
4.0e6
3.0e6
2.0e6
1.0e6
0.0
0
100
200
300
400
500
600
700
800
900
1000

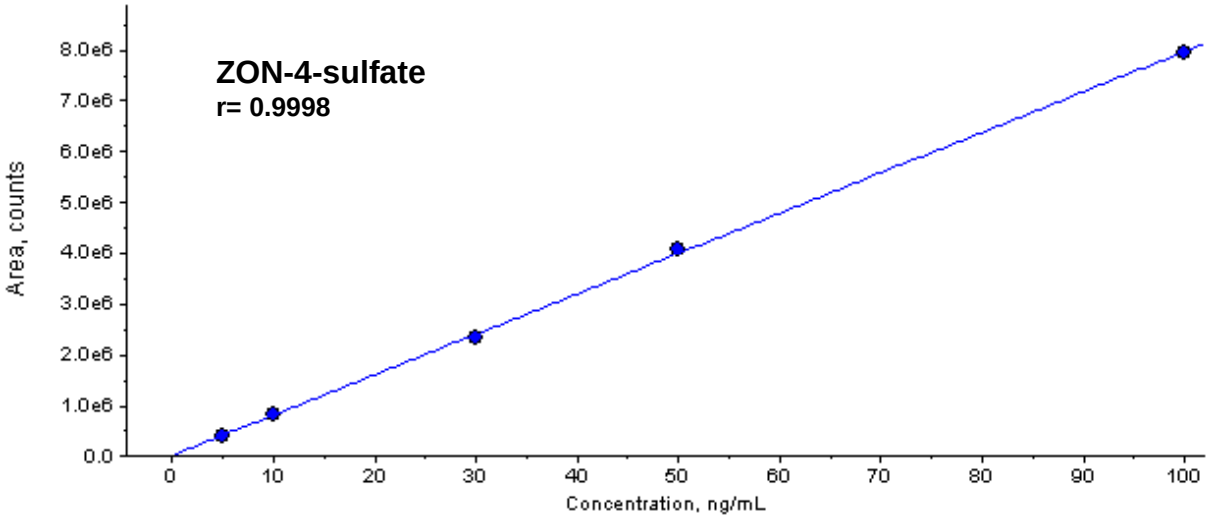

α-ZOL
r= 0.9997
Area, counts
Concentration, ng/mL
1.6e6
1.4e6
1.2e6
1.0e6
8.0e5
6.0e5
4.0e5
2.0e5
0.0
0
100
200
300
400
500
600
700
800
900
1000


β-ZOL
r= 1.0000
Area, counts
Concentration, ng/mL
1.13e6
1.00e6
8.00e5
6.00e5
4.00e5
2.00e5
0.00
0
100
200
300
400
500
600
700
800
900
1000


ZON-4-glucoside
r= 0.9995
Area, counts
Concentration, ng/mL
3.0e6
2.5e6
2.0e6
1.5e6
1.0e6
5.0e5
0.0
0
100
200
300
400
500
600
700
800
900
1000


α-ZOL-4-glucoside
r= 0.9999
Area, counts
Concentration, ng/mL
1.8e6
1.6e6
1.4e6
1.2e6
1.0e6
8.0e5
6.0e5
4.0e5
2.0e5
0.0
0
100
200
300
400
500
600
700
800
900
1000


β-ZOL-4-glucoside
r= 0.9999
Area, counts
Concentration, ng/mL
2.4e6
2.0e6
1.5e6
1.0e6
5.0e5
0.0
0
100
200
300
400
500
600
700
800
900
1000


ZON-4-sulfate
r= 0.9998
Area, counts
8.0e6
7.0e6
6.0e6
5.0e6
4.0e6
3.0e6
2.0e6
1.0e6
0.0
0
10
20
30
40
50
60
70
80
90
100
Concentration, ng/mL

8.3. Excel tables

Excel table A: [Multilevel_ Spike_Series.xls]

Recoveries of all analytes at 6 spiking levels in 11 matrices:

	spike level	Beer		Cornflakes		Grissini		Muesli		Spaghetti		Wholemeal		Barley		Spelt		Maize		Rye		Wheat	
	(ng/g sample)		recov		recov		recov		recov		recov		recov		recov		recov		recov		recov		recov
	0	0		68,4		0		0		0		0		0		0		0		0		0	
	50	60	120	149	298	126	252	54,9	109,8	37,8	75,6	82	164	pos.		33,1	66,2	46,9	93,8	pos.		38,6	77,2
	100	107	107	179	179	169	169	89,8	89,8	60,6	60,6	122	122	79	79	67,5	67,5	90,8	90,8	57,9	57,9	72,7	72,7
	250	252	100,8	325	130	230	92	204	81,6	189	75,6	204	81,6	161	64,4	163	65,2	205	82	131	52,4	226	90,4
	500	549	109,8	592	118,4	499	99,8	441	88,2	415	83	325	65	329	65,8	315	63	383	76,6	359	71,8	366	73,2
D3G	1000	1100	110	976	97,6	904	90,4	923	92,3	731	73,1	595	59,5	654	65,4	697	69,7	804	80,4	793	79,3	751	75,1
	0	0		1540		273		pos.		0		607		0		0		pos.		0		106	
	50	50,2	100,4	1720	3440	589	1178	161	322	0		755	1510	0		pos.		102	204	0		163	326
	100	98,7	98,7	2050	2050	742	742	176	176	113	113	826	826	143	143	151	151	148	148	pos.		172	172
	250	214	85,6	2830	1132	1540	616	334	133,6	301	120,4	1460	584	268	107,2	299	119,6	281	112,4	212	84,8	395	158
	500	464	92,8	4330	866	3000	600	600	120	543	108,6	2170	334	549	109,8	563	112,6	537	107,4	497	99,4	653	130,6
DON	1000	889	88,9	6340	634	5350	535	1130	113	1010	101	3560	356	999	99,9	1060	106	1080	108	1190	119	1130	113
	0	0		0		0		0		0		0		0		0		0		0		0	
	50	38	76	25,8	51,6	36,6	73,2	n/a		24,6	49,2	n/a		0		pos.		29	58	n/a		n/a	
	100	73	73	86,9	86,9	67,8	67,8	73,1	73,1	84,1	84,1	n/a		55,5	55,5	pos.		76,1	76,1	n/a		81	81
	250	187	74,8	238	95,2	233	93,2	197	78,8	254	101,6	197	78,8	170	68	188	75,2	227	90,8	191	76,4	212	84,8
	500	388	77,6	551	110,2	494	98,8	446	89,2	521	104,2	486	97,2	329	65,8	373	74,6	444	88,8	407	81,4	409	81,8
ac-DON	1000	771	77,1	965	96,5	987	98,7	982	98,2	1060	106	917	91,7	751	75,1	826	82,6	903	90,3	859	85,9	943	94,3
	0	0		0,5		0		pos.		0		0,9		0		0		0		0,8		0,6	
	0,5	0		0,6	120	0,1	20	< 0.1	< 20	< 0.1	< 20	1	200	0	0	< 0.1	< 20	< 0.1	< 20	1,1	220	0,9	180
	1	< 0.1	< 10	0,8	80	0,2	20	0,2	20	0,2	20	1	100	< 0.1	< 10	0,3	30	0,1	10	1,2	120	0,9	90
	2,5	0,4	16	1,3	52	0,6	24	0,5	20	0,7	28	1,5	60	0,3	12	0,3	12	0,8	32	1,2	48	1	40
	5	1,4	28	2	40	1,5	30	1,3	26	1,5	30	2,5	50	2,1	42	0,7	14	2,4	48	2,6	52	2	40
Z4S	10	1,8	18	2,8	28	3	30	2,6	26	2,8	28	2,9	29	3,6	36	1,8	18	4,2	42	4,4	44	3,2	32
	0	0		0		0		0		0		0		0		0		0		0		0	
	5	2,9	58	3,5	70	pos.		pos.		5	100	0		n/a		pos.		0		0		0	
	10	4,6	46	6,1	61	7,5	75	10,2	102	8,8	88	9,9	99	n/a		7,2	72	9,2	92	pos.		5,4	54
	25	11,2	44,8	20	80	18,1	72,4	18,1	72,4	22,8	91,2	18,9	75,6	n/a		14,9	59,6	17,1	68,4	18,1	72,4	14	56
	50	24,1	48,2	35	70	37,5	75	38,3	76,6	36,1	72,2	35,6	71,2	23,3	46,6	34	68	36,5	73	34,7	69,4	32,7	65,4
b-ZG	100	47,5	47,5	65,8	65,8	76,8	76,8	76,6	76,6	72	72	69,5	69,5	47,9	47,9	58,2	58,2	69,7	69,7	61,9	61,9	68,2	68,2
	0	0		0		0		0		0		0		0		0		0		0		0	
	5	4,2	84	5,9	118	pos.		0		6,2	124	0		n/a		pos.		0		0		pos.	
	10	6,2	62	10,3	103	9,2	92	pos.		9,6	96	pos.		n/a		7,5	75	9,4	94	pos.		8	80
	25	11,6	46,4	18	72	21,9	87,6	18,4	73,6	23,4	93,6	19,8	79,2	n/a		16,8	67,2	20,4	81,6	16,2	64,8	19,7	78,8
	50	26,6	53,2	46,4	92,8	43,9	87,8	44	88	44,4	88,8	39,3	78,6	39,7	79,4	40,2	80,4	31,1	62,2	33,3	66,6	34,5	69
a-ZG	100	50,4	50,4	72,6	72,6	71,6	71,6	81,1	81,1	83,5	83,5	72,2	72,2	71	71	61,3	61,3	69,2	69,2	65,9	65,9	74,9	74,9
	0	0		0		0		0		0		0		0		0		0		0		0	
	5	pos.		pos.		0		0		pos.		0		0		pos.		pos.		0		0	
	10	6	60	12,6	126	10,3	103	9,1	91	9,3	93	pos.		pos.		9,9		7,9	79	pos.		pos.	
	25	13	52	24,3	97,2	23	92	19,2	76	20,2	80,8	27,3	109,2	15,9	63,6	19,5	78	15,9	63,6	19	76	17,2	68,8
	50	28,8	57,6	40,6	81,2	46	92	36,3	72,6	43,7	87,4	44,5	89	39,9	79,8	34,7	69,4	30,8	61,6	40,8	81,6	36,2	72,4
Z4G	100	60,4	60,4	79,9	79,9	76,8	76,8	80,1	80,1	81,9	81,9	79,5	79,5	66,5	66,5	67,1	67,1	65,2	65,2	76,6	76,6	69,3	69,3
	0	0		0		0		0		0		0		0		0		0		0		0	
	5	8	160	pos.		pos.		pos.		9,4		0		0		0		0		0		5,6	112
	10	13,5	130	16,4	164	15,2	152	9,5	95	13,9	139	pos.		10,1	101	6	60	pos.		pos.		15,6	156
	25	32,8	131,2	31	124	33,3	133	22,8	91,2	30,8	123,2	25,2	100,8	17,5	70	22,4	89,6	19,5	78	28,2	112,8	25,7	102,8
	50	63,9	127,8	57,6	115,2	53,6	107	41,9	83,8	61,1	122,2	50,5	101	50,1	100,2	55,4	110,8	45,7	91,4	57,3	114,6	42,9	85,8
bZOL	100	125	125	130	130	114	114	105	105	157	157	96,4	96,4	61,4	61,4	120	120	81,1	81,1	110	110	100	100
	0	0		0		0		0		0		0		0		0		0		0		0	
	5	6,4	128	pos.		0		pos.		pos.		0		4,6		0		0		pos.		5,7	114
	10	10,3	103	11,4	114	9,3	93	7,3	73	11,9	119	9,6	96	12,5	125	pos.		pos.		7,4	74	11,6	116
	25	26,1	104,4	24,7	98,8	19,3	77,2	15,2	60,8	26,5	106	19,7	78,8	16	64	17,2	68,8	16,6	66,4	18,1	72,4	23,4	93,6
	50	55	110	54,3	108,6	41	82	36,5	73	60,6	121,2	38	76	32,6	65,2	39,7	79,4	38,8	77,6	38,5	77	36,4	72,8
aZOL	100	109	109	90,6	90,6	89,5	89,5	78,8	78,8	103	103	87	87	69,7	69,7	79,6	79,6	77,1	77,1	72,7	72,7	86,4	86,4
	0	0		11,1		0		0		0		6,2		0		0		0		0		17,2	
	5	5,5	110	15,7	314	4,9	98	5,4	108	5,9	118	8,7	174	pos.		5,5	110	pos.		3,8	76	18,7	374
	10	10,8	108	21,2	212	7	70	8,3	83	11,5	115	14	140	12,2	122	8,1	81	6,5	65	9,7	97	30,7	307
	25	25	100	38,5	154	23,2	92,8	16,4	65,6	29,3	117,2	25,2	100,8	18,8	75,2	17,2	68,8	13,1	52,4	19,1	76,4	30,3	121,2
	50	50,6	101,2	56,8	113,6	42,6	85,2	32	64	54	108	45,1	90,2	34,3	68,6	39,1	78,2	31	62	39,2	78,4	62,1	124,2
ZON	100	101	101	101	101	87,3	87,3	70,4	70,4	116	116	77,1	77,1	65,8	65,8	80,2	80,2	74,9	74,9	83,6	83,6	98,3	98,3

Numbers refer to concentrations in ng/g sample calculated from standardised solutions. Abbreviations: pos.: below LOQ; n/a: detection/quantification not possible for the lack of baseline definition; recov.: recovery (% of spike quantity)

Excel Table B: [VALIDATION_CSL_YORK.xls]

Validation data (LOD, LOQ, recovery and repeatability) of 10 matrices, as validated on a LC-MS/MS system in York, UK.

CORN FLOUR

analyte	LOD [µg/kg]	LOQ [µg/kg]	# measurm.	recovery [%]	atability [RSDr, %]
D3G	40	100	24	92	9,3
DON	40	100	24	105	7,8
3ADON	10	25	18	109	8,9
Z4S	0,4	1	24	102	10,1
bZG	4	10	24	115	12,8
aZG	4	10	24	105	7,9
Z4G	4	10	24	106	7,9
b-ZOL	10	25	18	101	11,2
a-ZOL	20	50	12	97	4,7
ZON	4	10	24	97	6,2

SPAGHETTI

analyte	LOD [µg/kg]	LOQ [µg/kg]	# measurm.	recovery [%]	atability [RSDr, %]
D3G	40	100	24	74	11
DON	100	250	18	98	5,8
AcDON	20	50	12	97	5,1
Z4S	1	2,5	18	69	9,6
bZG	10	25	18	92	8,1
aZG	10	25	18	100	11,3
Z4G	4	10	24	105	7,9
bZOL	10	25	18	109	9,1
aZOL	10	25	18	104	7,7
ZON	4	10	24	95	8,6

BEER

analyte	LOD [µg/kg]	LOQ [µg/kg]	# measurm.	recovery [%]	atability [RSDr, %]
D3G	40	100	24	90	10,2
DON	40	100	24	92	9,2
3ADON	10	25	18	85	10,3
Z4S	0,4	1	24	24	11,1
bZG	4	10	24	84	5,5
aZG	4	10	24	80	9,6
Z4G	4	10	24	80	8,1
b-ZOL	4	10	24	99	11,3
a-ZOL	4	10	24	83	10,9
ZON	4	10	24	98	10,9

BARLEY GRAIN

analyte	LOD [µg/kg]	LOQ [µg/kg]	# measurm.	recovery [%]	atability [RSDr, %]
D3G	40	100	24	73	15,1
DON	40	100	24	94	11,7
AcDON	10	25	18	95	5,6
Z4S	0,4	1	24	65	11,5
bZG	4	10	24	78	13,9
aZG	4	10	24	88	11,5
Z4G	4	10	24	85	12,8
bZOL	10	25	18	69	11,1
aZOL	4	10	24	69	12,8
ZON	4	10	24	69	12,6

BRAN FLAKES

analyte	LOD [µg/kg]	LOQ [µg/kg]	# measurm.	recovery [%]	atability [RSDr, %]
D3G	40	100	24	50	11,7
DON	40	100	24	95	12,2
AcDON	10	25	18	93	11,6
Z4S	0,4	1	24	59	17,8
bZG	5	10	24	90	6
aZG	5	10	24	95	9,3
Z4G	5	10	24	93	6,7
bZOL	5	10	24	87	8,6
aZOL	5	10	24	75	12,4
ZON	5	10	24	68	12,8

MAIZE GRAIN

analyte	LOD [µg/kg]	LOQ [µg/kg]	# measurm.	recovery [%]	atability [RSDr, %]
D3G	40	100	24	97	9,5
DON	40	100	24	104	8,7
AcDON	10	25	18	83	7,7
Z4S	0,4	1	24	55	10,6
bZG	4	10	24	86	7,2
aZG	4	10	24	99	6,5
Z4G	4	10	24	89	11,8
bZOL	10	25	18	83	9,3
aZOL	10	25	18	58	9,5
ZON	4	10	24	62	12

PORRIDGE OATS

analyte	LOD [µg/kg]	LOQ [µg/kg]	# measurm.	recovery [%]	atability [RSDr, %]
D3G	100	250	18	85	6,7
DON	100	250	18	103	7
AcDON	10	25	18	80	9
Z4S	1	2,5	18	71	8
bZG	4	10	24	89	10,3
aZG	4	10	24	106	9,5
Z4G	4	10	24	101	13,2
bZOL	10	25	18	107	7,1
aZOL	10	25	18	87	6,4
ZON	4	10	24	93	9,4

RYE GRAIN

analyte	LOD [µg/kg]	LOQ [µg/kg]	# measurm.	recovery [%]	atability [RSDr, %]
D3G	40	100	24	91	5,7
DON	100	250	18	105	5,6
AcDON	10	25	18	85	9,5
Z4S	0,4	1	24	69	8,9
bZG	4	10	24	81	10,9
aZG	4	10	24	88	13,7
Z4G	10	25	18	90	7,6
bZOL	10	25	18	62	10,6
aZOL	10	25	18	69	13,6
ZON	10	25	18	56	9

GRISSINI

analyte	LOD [µg/kg]	LOQ [µg/kg]	# measurm.	recovery [%]	atability [RSDr, %]
D3G	100	250	18	48	10,6
DON	40	100	24	99	9,4
AcDON	100	250	18	105	9,7
Z4S	0,4	1	24	72	10,3
bZG	4	10	24	92	11,4
aZG	4	10	24	84	9,5
Z4G	4	10	24	96	6,5
bZOL	10	25	18	90	6,7
aZOL	4	10	24	70	13,6
ZON	4	10	24	71	11,5

WHEAT GRAIN

analyte	LOD [µg/kg]	LOQ [µg/kg]	# measurm.	recovery [%]	atability [RSDr, %]
D3G	40	100	24	85	13,5
DON	40	100	24	106	14,5
AcDON	10	25	18	95	7,4
Z4S	0,4	1	24	60	19,3
bZG	4	10	24	85	7,2
aZG	4	10	24	83	12,1
Z4G	4	10	24	90	9,4
bZOL	10	25	18	83	8,5
aZOL	10	25	18	57	10
ZON	4	10	24	81	11,9

Excel Table C: [VALIDATION_IFA_TULLN.xls]

Validation data (LOD, LOQ, recovery and repeatability) of 10 matrices, as validated on a LC-MS/MS system in Tulln, Austria.

CORN FLOUR					
analyte	LOD [µg/kg]	LOQ [µg/kg]	# measurm.	recovery [%]	atability [RSDr, %]
DON	40	100	24	105	6,8
D3G	40	100	24	96	7,7
3ADON	10	25	24	101	10,8
ZON	4	10	24	96	12,3
a-ZOL	4	10	24	95	9,7
b-ZOL	4	10	24	97	9,5
Z4G	4	10	24	102	9,4
aZG	4	10	24	102	7,8
bZG	4	10	24	107	9,2
Z4S	0,4	1	24	109	12,1

SPAGHETTI					
analyte	LOD [µg/kg]	LOQ [µg/kg]	# measurm.	recovery [%]	atability [RSDr, %]
D3G	40	100	24	74	13,4
DON	40	100	24	98	6,4
AcDON	10	25	24	100	7,6
Z4S	0,4	1	24	67	9,6
bZG	4	10	24	89	6,9
aZG	4	10	24	96	8,1
Z4G	4	10	24	102	5,3
bZOL	4	10	24	102	9
aZOL	10	25	18	91	8,9
ZON	4	10	24	90	9

BEER					
analyte	LOD [µg/kg]	LOQ [µg/kg]	# measurm.	recovery [%]	atability [RSDr, %]
DON	40	100	24	103	8,1
D3G	40	100	24	95	7,6
3ADON	10	25	24	77	9,3
ZON	4	10	24	90	9,1
a-ZOL	4	10	24	84	7,8
b-ZOL	4	10	24	78	9,1
Z4G	4	10	24	87	8,2
aZG	4	10	24	83	7,1
bZG	4	10	24	77	7,4
Z4S	0,4	1	24	25	11,1

BARLEY GRAIN					
analyte	LOD [µg/kg]	LOQ [µg/kg]	# measurm.	recovery [%]	atability [RSDr, %]
D3G	40	100	24	75	14,1
DON	40	100	24	92	10,9
AcDON	10	25	24	95	11
Z4S	0,4	1	24	65	14,2
bZG	4	10	24	86	10,6
aZG	4	10	24	91	10,1
Z4G	4	10	24	92	9,5
bZOL	4	10	24	74	12,3
aZOL	4	10	24	76	9,1
ZON	4	10	24	69	14,4

BRAN FLAKES					
analyte	LOD [µg/kg]	LOQ [µg/kg]	# measurm.	recovery [%]	atability [RSDr, %]
D3G	40	100	24	50	11,7
DON	40	100	24	102	10,7
AcDON	10	25	24	103	6,9
Z4S	0,4	1	24	68	17,7
bZG	4	10	24	91	5,2
aZG	4	10	24	91	9,0
Z4G	4	10	24	94	7
bZOL	4	10	24	90	7,9
aZOL	4	10	24	75	13,4
ZON	4	10	24	64	13,9

MAIZE GRAIN					
analyte	LOD [µg/kg]	LOQ [µg/kg]	# measurm.	recovery [%]	atability [RSDr, %]
D3G	40	100	24	100	5
DON	40	100	24	102	8,5
AcDON	10	25	24	88	10,4
Z4S	0,4	1	24	62	10,7
bZG	4	10	24	87	7,4
aZG	4	10	24	92	9,6
Z4G	4	10	24	79	8,1
bZOL	4	10	24	74	7,6
aZOL	4	10	24	54	8,3
ZON	4	10	24	65	9

PORRIDGE OATS					
analyte	LOD [µg/kg]	LOQ [µg/kg]	# measurm.	recovery [%]	atability [RSDr, %]
D3G	40	100	24	89	9,5
DON	40	100	24	103	8,5
AcDON	10	25	24	97	9,1
Z4S	0,4	1	24	75	11,9
bZG	4	10	24	82	9,2
aZG	4	10	24	93	8,2
Z4G	4	10	24	100	9,5
bZOL	4	10	24	88	7,4
aZOL	4	10	24	80	10,8
ZON	4	10	24	84	11,1

RYE GRAIN					
analyte	LOD [µg/kg]	LOQ [µg/kg]	# measurm.	recovery [%]	atability [RSDr, %]
D3G	40	100	24	92	10,6
DON	40	100	24	108	8,4
AcDON	10	25	24	89	7,2
Z4S	0,4	1	24	73	10
bZG	4	10	24	82	10,1
aZG	4	10	24	81	13,6
Z4G	4	10	24	85	9
bZOL	4	10	24	65	6,6
aZOL	4	10	24	58	12,2
ZON	4	10	24	50	10

GRISSINI					
analyte	LOD [µg/kg]	LOQ [µg/kg]	# measurm.	recovery [%]	atability [RSDr, %]
D3G	100	250	18	46	11,6
DON	40	100	24	96	11,3
AcDON	10	25	24	105	9,1
Z4S	0,4	1	24	71	11
bZG	4	10	24	94	9,8
aZG	4	10	24	89	9,1
Z4G	4	10	24	100	9,8
bZOL	4	10	24	94	11,8
aZOL	4	10	24	75	14
ZON	4	10	24	69	9,6

WHEAT GRAIN					
analyte	LOD [µg/kg]	LOQ [µg/kg]	# measurm.	recovery [%]	atability [RSDr, %]
D3G	40	100	24	98	11,3
DON	40	100	24	105	13
AcDON	10	25	24	93	9,3
Z4S	0,4	1	24	64	18,8
bZG	4	10	24	85	8,9
aZG	4	10	24	84	8,2
Z4G	4	10	24	88	9,5
bZOL	4	10	24	79	9,8
aZOL	4	10	24	55	10,6
ZON	4	10	24	84	11,7

Excel Table D: [FSA_Composites_screening.xls]

Results of the screening of 31 composites. Concentrations given in µg/kg (or µg/L for beer). In case of contamination, individual samples were re-analyzed, results for positive samples only are shown at the lower part of the table.

#	Composite	D3G	DON	AcDON	Z4S	b-ZG	a-ZG	Z4G	b-ZOL	a-ZOL	ZON
1	Wheat grain	**< LOQ**	**603**	< LOD	**6.4**	< LOD	< LOD	< LOD	< LOD	< LOD	**27**
2	Barley grain	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
3	Rye grain	< LOD	< LOD	< LOD	**2.1**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
4	Maize grain	< LOD	**< LOQ**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
5	Spelt grain	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
6	Oat grain	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
7	Wheat flour	< LOD	**237**	< LOD	**2.0**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
8	Wheat bread	**< LOQ**	**< LOQ**	< LOD	**< LOQ**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
9	Rye bread	< LOD	< LOD	< LOD	**< LOQ**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
10	Wheat bread wholemeal	**< LOQ**	**< LOQ**	< LOD	**2.2**	< LOD	< LOD	< LOD	< LOD	< LOD	**< LOQ**
11	Wheat semolina	< LOD	**< LOQ**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
12	Maize meal and flour	< LOD	< LOD	< LOD	**1.3**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
13	Extruded maize snacks	< LOD	< LOD	< LOD	**< LOQ**	< LOD	< LOD	< LOD	< LOD	< LOD	**< LOQ**
14	Biscuits	< LOD	**< LOQ**	< LOD	**1.5**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
15	Pasta	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
16	Beer (including wheat beer)	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
17	Canned maize	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
18	Breakfast cereals - corn flakes	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	**< LOQ**
19	Breakfast cereals - wheat	< LOD	**< LOQ**	< LOD	**1.8**	< LOD	< LOD	< LOD	< LOD	< LOD	**< LOQ**
20	Breakfast cereals - bran flakes	< LOD	**254**	< LOD	**6.1**	< LOD	< LOD	< LOD	< LOD	< LOD	**44**
21	Breakfast cereals - muesli	< LOD	< LOD	< LOD	**1.6**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
22	Breakfast cereals - oats	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
23	Crackers	< LOD	**248**	< LOD	**2.3**	< LOD	< LOD	< LOD	< LOD	< LOD	**< LOQ**
24	Baby food - wheat based	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
25	Baby food - oat based	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
26	Popcorn	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
27	Cereal snack bars	< LOD	< LOD	< LOD	**1.8**	< LOD	< LOD	< LOD	< LOD	< LOD	**< LOQ**
28	Buckwheat grain or flour	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
29	Quinoa grain or flour	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
30	Extruded oat snacks	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
31	Polenta	< LOD	< LOD	< LOD	**2.1**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD

#	Cereal food	D3G	DON	AcDON	Z4S	b-ZG	a-ZG	Z4G	b-ZOL	a-ZOL	ZON
1D	Wheat grain	**210**	**1760**	< LOD	**7.0**	< LOD	< LOD	< LOD	< LOD	< LOD	**51**
1E	Wheat grain	**109**	**936**	< LOD	**25**	< LOD	< LOD	< LOD	< LOD	< LOD	**76**
4B	Maize grain	< LOD	**129**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
4C	Maize grain	< LOD	**250**	< LOD	**1.7**	< LOD	< LOD	< LOD	< LOD	< LOD	**33**
7A	Wheat flour	< LOD	**307**	< LOD	**1.8**	< LOD	< LOD	< LOD	< LOD	< LOD	**18**
7B	Wheat flour	< LOD	**249**	< LOD	**3.5**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
7C	Wheat flour	< LOD	**286**	< LOD	**1.0**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
8A	Wheat bread	**42**	**160**	< LOD	**0.3**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
8B	Wheat bread	**40**	**149**	< LOD	**0.9**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
8C	Wheat bread	< LOD	**142**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
8D	Wheat bread	< LOD	**229**	< LOD	**0.2**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
10A	Wheat bread wholemeal	< LOD	**206**	< LOD	**2.8**	< LOD	< LOD	< LOD	< LOD	< LOD	**41**
10B	Wheat bread wholemeal	< LOD	**275**	< LOD	**1.2**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
11C	Wheat semolina	< LOD	**297**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
13A	Extruded maize snacks	< LOD	< LOD	< LOD	**1.2**	< LOD	< LOD	< LOD	< LOD	< LOD	**33**
13B	Extruded maize snacks	< LOD	< LOD	< LOD	**0.5**	< LOD	< LOD	< LOD	< LOD	< LOD	**19**
20A	Breakfast cereals - bran flakes	< LOD	**243**	< LOD	**4.2**	< LOD	< LOD	< LOD	< LOD	< LOD	**55**
20B	Breakfast cereals - bran flakes	< LOD	**241**	< LOD	**4.5**	< LOD	< LOD	< LOD	< LOD	< LOD	**40**
20C	Breakfast cereals - bran flakes	< LOD	**262**	< LOD	**5.8**	< LOD	< LOD	< LOD	< LOD	< LOD	**31**
23A	Crackers	< LOD	**324**	< LOD	**2.2**	< LOD	< LOD	< LOD	< LOD	< LOD	**19**
23B	Crackers	< LOD	**330**	< LOD	**2.9**	< LOD	< LOD	< LOD	< LOD	< LOD	**16**
23C	Crackers	< LOD	**132**	< LOD	**0.7**	< LOD	< LOD	< LOD	< LOD	< LOD	**15**