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LC-MS/MS multimethod for mycotoxins after single extraction and validation data for peanut, pistachio, wheat, maize, cornflake, raisin and fig

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LC-MS/MS multi-method for mycotoxins after single extraction and validation data for peanut, pistachio, wheat, maize, cornflakes, raisins and figs

Abstract

Mycotoxin analysis is mainly carried out by high performance liquid chromatography after immunoaffinity column cleanup or in enzyme-linked immunosorbent assay tests. These methods basically imply determination of single compounds only. In the EU, legislation exists for the aflatoxins, ochratoxin A, and patulin in food. For deoxynivalenol, zearalenone and the fumonisins, legislation will get into force in 2007. To maintain several legal limits it would be preferable to determine all these mycotoxins by routine analysis in different types of matrices in one single extract. This would also be an advantage for HACCP control purposes. For this reason a multi-method was developed with which 33 mycotoxins in various types of products can be analysed simultaneously. The mycotoxins are extracted from the samples with an acetonitrile-water mixture, which is diluted with water and then directly injected in a LC-MS/MS system. The mycotoxins are separated by reversed phase HPLC and detected using an electrospray ionisation interface (ESI) and tandem MS, using MRM in the positive ion mode, to increase specificity for necessary quality control. With this method the following mycotoxins can be analysed in a single run within 30 min: aflatoxins B₁, B₂, G₁ and G₂, ochratoxin A, deoxynivalenol, zearalenone, T-2 toxin, HT-2 toxin, α -zearalenol, α -zearalanol, β -zearalanol, sterigmatocystin, cyclopiazonic acid, penicillic acid, fumonisins B₁, B₂ and B₃, diacetoxyscirpenol, 3- and 15 acetyl-deoxynivalenol, zearalanone, ergotamin, ergocornin, ergocristin, α -ergocryptin, citrinin, roquefortin C, fusarenone X, nivalenol, mycophenolic acid, alternariol and alternariol monomethyl

1
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3 ether. The limit of quantification for the aflatoxins and ochratoxin A is 1.0 µg/kg and for
4
5 deoxynivalenol 50 µg/kg. The quantification limits for the other mycotoxins are in the range of 10 –
6
7 200 µg/kg. The matrix effect and validation data are presented for 13-24 mycotoxins in peanuts,
8
9 pistachios, wheat, maize, cornflakes, raisins and figs. The method has been compared with the official
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11 EU method for the determination of aflatoxins in food and relevant FAPAS rounds. The advantage of
12
13 the multi-mycotoxin method has been proven by findings of more than one mycotoxin in for example
14
15 maize, buckwheat, figs and nuts. Examples are given of the application of the LC-MS/MS technique
16
17 for baby food, which is subject to lower limits for aflatoxin B₁ and ochratoxin A, for ergot alkaloids in
18
19 naturally contaminated rye and for freeze-dried silage samples.
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27 **Keywords:** multi-mycotoxin, matrix effect, ion suppression, LC-MS/MS, validation, baby food, ergot
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29 alkaloids, silage.
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34 **Introduction**

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39 Until now mycotoxins are mainly determined by single compound analytical methods based on
40
41 immunoaffinity column cleanup. These methods were studied for aflatoxins (Stroka et al., 2000),
42
43 ochratoxin A (Entwisle et al., 2000), fumonisins B₁ and B₂ (Visconti et al., 2001), deoxynivalenol
44
45 (Cahill et al., 1999) and zearalenone (Schuhmacher et al., 1998). Since some years immunoaffinity
46
47 columns are available for the simultaneous determination of aflatoxin & ochratoxin A (Chan et al.,
48
49 2004) and aflatoxin, ochratoxin A & zearalenone (Göbel et al., 2004). Despite minor possibilities for
50
51 cross-reactions, assays are generally considered to be so specific that confirmation is hardly necessary.
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3 Increasing quality demands changed these views in the nineties. Methods were developed in which
4
5 mass spectrometry was applied for confirmation purposes, still based on individual mycotoxins. Hurst
6
7 et al. (1991) applied thermospray mass spectrometry for the confirmation of aflatoxins in peanuts,
8
9 Xiao et al. (1995) used electron impact mass spectrometry to elucidate ochratoxin structures, and
10
11 Holcomb et al. (1993) choose fast atom bombardment mass spectrometry to confirm the presence of
12
13 fumonisin B₁ in food. In our laboratory the need for confirmation has emerged when application of
14
15 DON assays revealed peaks in the chromatogram, which could interfere with the DON peak. We
16
17 (Spanjer et al., 2002) started to use mass spectrometry (MS) by applying the Thermo Finnigan LCQ,
18
19 an ion trap detector, for confirmation of DON as described by Berger et al. (1999).
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27 Aflatoxins and ochratoxin A are already subject to European Union (EU) legislation for some years
28
29 (European Commission (EC), 2002 and 2004). EU legislation on DON and zearalenone came into
30
31 force (EC, 2005). The fumonisins B₁+B₂ will follow as per October 1, 2007 (EC, 2006). The European
32
33 Commission is considering legislation for *Fusarium* toxins like T-2, HT-2 and nivalenol. Facing all
34
35 this upcoming legislation there grew a need to analyse as many mycotoxins as possible in one liquid
36
37 chromatography-mass spectrometry (LC-MS) run. This principle originates from the mycological field,
38
39 where measurements are made preferably in crude fungal extracts to identify mould species
40
41 (Smedsgaard and Frisvad, 1996). These authors applied single quadrupole equipment to *Penicillium*
42
43 isolates, in which they identified 13 mycotoxins, of which ochratoxin A, citrinin, penicillic acid and
44
45 roquefortine C are the most important for food control. To perform quantification and identification at
46
47 the same time, a triple quadrupole is needed. In that case one transition reaction is used for
48
49 quantification and identification is obtained from a second transition. Kussak et al. (1995) applied this
50
51 technique for the determination of aflatoxins in dust, Lukacs et al. (1996) identified fumonisins in corn,
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3 and Razzazi-Fazeli et al. (1999) determined nivalenol and DON in wheat. However, these authors still
4
5 applied a cleanup step in sample preparation.
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10 Janssens and Franken (Technical Laboratory Rotterdam, the Netherlands) analysed trichothecenes,
11
12 fumonisins, aflatoxins and ochratoxin in feed after only one single extraction with acetonitrile-water.
13
14 We optimised, extended and validated their method for food, as mycotoxin limits for human
15
16 consumption are lower. Special attention was paid to the matrix effect. The result on 13 mycotoxins
17
18 was presented at the second World Mycotoxin Forum (Spanjer et al., 2003). Since then the number of
19
20 mycotoxins in this method has been more than doubled. Latest additions were the *Alternaria* toxins
21
22 (Scussel et al. 2006) that even recently were analysed with thin layer chromatography by Centeno and
23
24 Calvo (2002). The method has also been successfully applied to silage samples, as delivered by
25
26 Driehuis and Te Giffel (2003), who presented their results elsewhere. It can also be adapted to the
27
28 analysis of aflatoxin B₁ and ochratoxin A in baby food, for which lower legislative levels exist. This
29
30 made it necessary to apply immunoassay cleanup again as to reach the necessary LODs. With the
31
32 combined aflatoxin/ochratoxin column this could be done in one assay, as described for fluorescence
33
34 detection by Chan et al. (2004).
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43 **Materials and methods**

44 *Chemicals*

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46 Water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). Methanol (Merck KGaA,
47
48 Darmstadt, Germany) and acetonitrile (Labscan Limited, Dublin, Ireland) were HPLC-grade. Formic
49
50 acid (Merck) was analytical-reagent grade. All mycotoxins were purchased from Sigma-Aldrich
51
52 (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands).
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57 *Preparation of standard solutions*

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3 From all solid standards, individual stock standard solutions were prepared and stored in the freezer at
4
5 -18°C . Stock standard solutions of all mycotoxins were made in methanol, except for OTA (in
6
7 toluene/acetic acid), deoxynivalenol (DON, in acetonitrile), fumonisins (FB_1 , FB_2 , in 50%
8
9 acetonitrile/50% water, exceptionally stored in refrigerator, not freezer), ZEN (acetonitrile) and 3Ac-
10
11 DON (acetonitrile), respectively. For aflatoxin B_1 , ochratoxin A, DON and zearalenone, purity checks
12
13 are made by UV measurement since for these compounds molar absorptivities are known. As an
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15 independent check comparisons are made with measurements of standard solutions for aflatoxin B_1 ,
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17 ochratoxin A, DON, zearalenone and fumonisins, as delivered by R-Biopharm Rhone Ltd. (West of
18
19 Scotland Science Park, Unit 3.06 Kelvin Campus, Glasgow G20 0SP, Scotland) and Biopure
20
21 Referenzsubstanzen GmbH. (Technopark 1, 3430 Tulln, Austria), since these are often used in routine
22
23 analysis procedures and therefore available as well.
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32 From these individual stock standard solutions a stock-standard mixture was prepared in methanol, as
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34 to be used to prepare calibration curves in solvent and in matrix, as to determine ion suppression,
35
36 recovery and limit of detection (LOD). Stock standard mixture solution: an appropriate volume (μl) of
37
38 stock standard solution of each toxin was taken and transferred to a calibrated 200 ml flask and
39
40 methanol was added to make to volume in order to obtain the following concentrations: aflatoxins
41
42 (0.125 $\mu\text{g}/\text{ml}$ each); ochratoxin A (0.250 $\mu\text{g}/\text{ml}$), fumonisins, zearalenone (ZEN), alfa-zearalanol (α -
43
44 ZAL), beta-zearalanol (β -ZAL), zearalanone (ZAN), alfa-zearalenol (α -ZEL); roquefortine,
45
46 sterigmatocystin, cyclopiazonic acid, penicillic acid and citrinin (2.50 $\mu\text{g}/\text{ml}$ each), ergotamin (5.00
47
48 $\mu\text{g}/\text{ml}$) and trichothecenes (DON, DAS, T2, HT2, 3 and 15 Ac-DON) all 6.25 $\mu\text{g}/\text{ml}$ each. This stock
49
50 standard mixture is also kept in the freezer at -18°C . Both stock standard solutions and stock standard
51
52 mixture are stable for at least 2 years, when kept in the freezer at -18°C . This stability check was made
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58 by comparing the freshly made stock standard mixture with the former one.
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6 From the stock standard mixture, calibration solutions were prepared in 20% acetonitrile, by diluting
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8 25, 50, 100, 200, 400 μ l in volumes of 50 ml, resulting in concentration levels of 0.0625, 0.125, 0.25,
9
10 0.5 and 1.0 ng/ml for the aflatoxins, 0.125, 0.25, 0.5, 1.0 and 2.0 ng/ml for ochratoxin-A, 1.25, 2.50,
11
12 5.00, 10.0 and 20.0 ng/ml for fumonisins, zearalenone (ZEN), alfa-zearalanol (α -ZAL), beta-
13
14 zearalanol (β -ZAL), zearalanone (ZAN), alfa-zearalenol (α -ZEL); roquefortine, sterigmatocystin,
15
16 cyclopiazonic acid, penicillic acid and citrinin, 2.50, 5.00, 10.0, 20.0 and 40.0 ng/ml for ergotamin and
17
18 3.125, 6.25, 12.5, 25.0, 50.0 ng/ml for trichothecenes (DON, DAS, T2, HT2, 3 and 15 Ac-DON)
19
20 respectively.
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23 24 *Laboratory equipment*

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27 The usual laboratory equipment and following mixers: a high shearing mixer, Silverson model EX
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29 (Silverson Machines Ltd, Waterside, Chesham, UK), a mini shaker model KMS2 and a horizontal
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31 shaker model HS501/digital from IKA (IKA Werke GmbH & Co. KG, Staufen, Germany).
32
33

34 *LC-MS system*

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36 The LC-MS/MS system consisted of a Waters Alliance 2695 separation module with a 100 μ l injection
37
38 loop (Waters, Milford, MA, USA) coupled to a Quattro Ultima triple quadrupole mass spectrometer
39
40 (Waters-Micromass, Manchester, UK) equipped with an electrospray interface. A 20 μ L aliquot is
41
42 injected on an Alltima C₁₈ (150x3.2 mm, 5 μ m) column (Alltech, Breda, the Netherlands) at 30°C
43
44 column temperature. The gradient was composed of solvents A (0.1% formic acid in water) and B
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46 (0.1% formic acid in acetonitrile) at a flow rate of 0.3 ml/min (Table 1).
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53 [Insert Table 1 about here]
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3 The MS was operated in the positive mode at a capillary voltage of 2.5 kV, a desolvation temperature
4 of 450°C, a desolvation gas flow of 600 L/h, a variable cone voltage, a cone gas flow of 100 L/h and
5 nebuliser gas fully open, all nitrogen. For MRM the collision gas was argon at 0.8 bar on pressure
6 regulator. Ionisation and MS-MS collision energy settings were optimised while continuously infusing
7 the separate mycotoxin solutions (1 - 5 µg/ml) at a flow rate of 5- 10 µl/min by means of a syringe
8 pump. For each mycotoxin the parent and two daughter ions were recorded, which are listed in Table 2.
9
10 The first is used for quantification and the second for confirmation. Quantification was done by
11 matrix-matched standard calibration. All results were corrected for matrix-effect. Dwell time was 0.02
12 s. Masslynx and Quanlynx software was used for data acquisition and processing.
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27 [Insert Table 2 about here]
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32 *Extraction*

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34 Dry milling: samples less than 1.5 kg are dry milled. Of the ground sample material 25 gram is mixed
35 with 100 mL 80/20 v/v acetonitrile-water and placed in the horizontal shaker during 2 hours. One
36 millilitre of the clear extract is diluted with 3 ml water and mixed. Cloudy solutions were filtrated
37 using a 0.45 µm membrane filter (Schleicher & Schull, Spartan 13). Samples larger than 1.5 kg are
38 slurry mixed with water by means of a high shearing mixer (Spanjer et al. 2006). Usually to one part
39 of sample, 1.5 parts of water is added. Other slurry-ratios of sample to water are possible. In such
40 cases the slurry-amount for the extraction is adjusted. For extraction 50 grams slurry (1: 1.5) is taken
41 to which 60 ml acetonitrile is added. This mixture is shaken for two hours in the horizontal shaker. 1
42 ml of the clear extract is diluted with 2.55 ml water and handled as described at the dry sample
43 handling procedure. To investigate the matrix-effect, 7 standards were prepared in 20% acetonitrile as
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3 well as added to non-contaminated, i.e. blank matrix after extraction and injected 6 times each. Figs
4
5 and raisins are extracted in 70% methanol-water instead of acetonitrile-water. This is due to the fact
6
7 that the high sugar content of these matrices causes layer separation of the acetonitrile and water, a
8
9 phenomenon that has been thoroughly investigated by Stroka et al. (1999). Silage samples were taken
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11 by Driehuis and Te Giffel (2003) and delivered as a milled powder, for which they applied a freeze-
12
13 drying procedure, 5 g of which were extracted.
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16 17 *Validation*

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19 Validation of the method is carried out for 13-24 mycotoxins in 7 different matrices. For the validation
20
21 of the method, two extra standard solutions were prepared, by diluting 800 μ l and 1600 μ l of the stock
22
23 standard mixture mixture in 50 ml 20% acetonitrile, resulting in two- and four times the highest
24
25 concentration of the individual standard concentrations as mentioned before (i.e. the concentrations for
26
27 the aflatoxins will be 2.0 and 4.0 ng/ml respectively). Three levels of contamination were prepared in
28
29 matrix. Aflatoxins (1, 2 and 16 μ g/kg each), OTA (2, 4 and 32 μ g/kg), fumonisins B₁ and B₂ and ZON
30
31 (20, 40 and 320 μ g/kg each), trichothecenes DON, DAS, T-2, HT-2 and 3 Acetyl-DON (40, 80 and
32
33 640 μ g/kg each). The three levels and a blank level were injected 6 times each in the LC-MS/MS
34
35 system, resulting in 18 datapoints.
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40 41 *Ergot alkaloids*

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43 From a naturally contaminated rye sample (0.4% m/m) the ergot kernels were sorted out and processed
44
45 according to the general sample preparation procedure. The extract was injected in the LC-MS system
46
47 at parent scan mode at m/z 268, which represents the protonated lysergamide part of the molecule, a
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49 characteristic fragment for ergot alkaloids, as to investigate the presence of all possible natural
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51 constituents. After this experiment a number of ergot alkaloid standards were obtained, to add to the
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3 standard mixture. This lead to validation of ergotamin, ergocornin, ergocristin and α -ergocryptin in
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6 whole wheat flour at levels of 100, 1000 and 2000 $\mu\text{g}/\text{kg}$ in the MRM mode of the multimethod.
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10 **Results**

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12 All chromatographic and mass spectrometric data are presented in table 2: parent and daughter ions,
13
14 cone (V) and collision (eV) voltages, ratios and retention times (min) and references for fragment
15
16 clarification. Validation data are given in tables 3-9 for peanut, pistachio, wheat, maize, cornflake,
17
18 raisin and fig. The calibration curves are of the type $Y = aX + b$, with a weight factor $1/X$ and
19
20 consisted of 7 data points.
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27 [Insert Tables 3-9 about here]
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32 The recoveries in several food types, which we gathered in 2005 and 2006 while applying the
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34 multimethod for analysis of routine samples, are listed in table 10.
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39 [Insert Table 10 about here]
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44 Figure 1 gives an impression of peak shape of 26 mycotoxines as they appear in the chromatogram.

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46 Figures 2 and 3 show some examples of multi-mycotoxin measurements. Figure 2 gives an example of
47
48 a maize sample as run with this multi-mycotoxin method. The following peaks could be identified: at
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50 11.26 min, fumonisin B₁ (4,200 $\mu\text{g}/\text{kg}$); at 12.04 min, fumonisin B₃ (only qualitative, because of lack
51
52 of standard); at 12.49 min, fumonisin B₂ (1,200 $\mu\text{g}/\text{kg}$); and at 13.76 min, aflatoxin B₁ (30 $\mu\text{g}/\text{kg}$). It is
53
54 common practice to detect fumonisins, zearalenone and DON in maize, since these mycotoxins are
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3 formed in the field by *Fusarium* species. The presence of aflatoxin B₁ in this maize sample is an
4
5 indication for the occurrence of heat stress during growth in the field (Abbas et al., 2002). In Figure 3
6
7 an example is given of a buckwheat sample with zearalenone, ochratoxin A and sterigmatocystin
8
9 levels around 35 µg/kg, and a trace of roquefortin C. Both examples show the advantage of the multi-
10
11 mycotoxin method above single methods. In figure 4 an example is given of the measurement on a
12
13 silage sample. Figure 5 shows the individual peaks of ergot alkaloids when running the LC-MS in the
14
15 parent mode on an extract of naturally contaminated rye kernels, sorted out of an inspected lot.
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22 [Insert Figures 1-5 about here]
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27 Discussion

28
29 To separate these different type of mycotoxins a gradient is needed, which starts with a high
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31 percentage of water. Figure 1 gives an impression of 26 mycotoxins. It shows that for nivalenol it is
32
33 not possible to obtain a chromatographic peak, even not at a level of 400 µg/kg and for citrinin and
34
35 cyclopiazonic acid a slight tailing could not be prevented. In the ESI⁺ mode the most prominent ion is
36
37 the protonated molecular [M+H]⁺ ion, the parent m/z. To validate the identity of the parent, these ions
38
39 are fragmented into daughter ions with argon gas in the collision cell of the triple quadrupole.
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42

43 Quantification is carried out on the primary transition. Each mycotoxin is confirmed by the second
44
45 transition and the ratio between primary and secondary daughter ion is calculated. Differences in
46
47 sensitivity between positive or negative electrospray were not investigated systematically in this study.
48
49

50 In literature several studies can be found on this topic. Razzazi-Fazeli et al. (1999, 2002) considered
51
52 the positive ion mode to be preferred for type A (e.g. T-2 and HT-2) and the negative mode for type B
53
54 trichothecenes (e.g. nivalenol and DON). Lagana et al. (2003) proved this for trichothecenes in maize
55
56 by data for the water/acetonitril/formic acid eluent. Biselli et al. (2004), Sulyok et al. (2006) and Ren
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2
3 et al. (2007) dealt with this item by running a sample extract twice, both in positive and negative mode.
4
5 This doubles analysis time and gives raise to possible errors, i.e. combining the wrong runs for one
6
7 sample. The main aim of this study was to end up with a single run multimethod for mycotoxins that
8
9 are subject to present and upcoming EU food legislation. In the positive mode all EU limits for
10
11 aflatoxin B₁, ochratoxin A and DON were met. Therefore, it was preferred not to switch polarity, since
12
13 T-2 and HT-2, the next important mycotoxins of interest, have distinctly more response in the positive
14
15 mode (Razazzi-Fazeli et al., 2002). Another factor is that in an opposite mode mostly another buffer is
16
17 preferred, i.e. formiate buffer, instead of formic acid, which does not make it even practical to switch
18
19 polarity during a single LC run, even when it is technically possible in the MS. In a preliminary test
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21 we observed an increase in sensitivity for T2 and DAS, but a dramatically decrease for OTA when
22
23 changing from formic acid to ammonium formiate buffer. This again led to the conclusion to maintain
24
25 the positive mode in the multi-mycotoxin method for the entire run, since this mycotoxin is the next
26
27 best regulated after the aflatoxins. The recent work of Delmulle et al. (2006) confirmed this view.
28
29 They ran 16 mycotoxins on a Micromass Quattro Micro triple quadrupole mass spectrometer. After
30
31 investigations in both modes they found out that not all toxins were detectable in the negative mode. In
32
33 the positive mode they achieved LOD values of 1, 2.3 and 50 µg/kg for aflatoxin B₁, ochratoxin A and
34
35 DON, respectively. They extracted mycotoxins directly from cellulose filters that were applied as
36
37 indoor air collectors in buildings. Compared to food and feed this type of samples is apparently rather
38
39 “clean” by itself, because even when the sample is mainly composed of water, there exists a matrix
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41 effect, as is shown by Hartmann et al. (2007) who analysed zearalenone in river water, drainage water
42
43 and waste water effluent. Mohamed et al. (2006) concluded that positive ionisation mode also revealed
44
45 a higher total ion current for all ergot alkaloids, with sensitivity increased up to 50-fold for ergotamine
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47 compared to the negative one. All together this confirmed us to stick at the choice we made for
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49 positive mode to be applied in general for mycotoxins.
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6 In all references but the one of Delmulle et al. (2006) as mentioned in the above paragraph, some kind
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8 of cleanup by a cartridge or assay was applied. Using cleanup indirectly implies a restriction of the
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10 types of mycotoxins that can be analysed, as any cleanup will influence the presence of compounds in
11
12 the finally extracted aliquot to be injected in a chromatographic system. It led to methods, which
13
14 mostly were only suitable to handle *Fusarium* mycotoxins like trichothecenes, fumonisins and
15
16 zearalenone derivatives (Tuomi et al., 1998; Razzazi-Fazeli et al., 1999, 2002; Biselli et al., 2004;
17
18 Klötzel et al., 2005; Berthiller et al., 2005; Cavaliere et al., 2005 and Hartmann et al. 2007). At our
19
20 starting point with this multi-mycotoxin LC-MS method in 2001, we intended to analyze as many
21
22 mycotoxins as possible, so to apply crude extracts only. Therefore ion suppression by the matrix
23
24 components can be expected, as has been investigated basically by Nelson and Dolan (2002). In
25
26 practice it is more common to prepare calibration curves in solvent and matrix, and calculate the
27
28 matrix effect by comparison of the results. This was done first for quantification of diarrhetic shellfish
29
30 toxins in scallops (Ito and Tsukada, 2002), and the mycotoxin penitrem A in a food mixture
31
32 (Rundberget and Wilkins, 2002). We did this in our first presentation for aflatoxin B₁, ochratoxin A
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34 and DON in a peanut and a cornflake sample (Spanjer et al. 2003). These three mycotoxins were
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36 chosen because at that time they were already subject to EU legislation. The two matrices were chosen
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38 to investigate the difference in matrix effect for these mycotoxins, nevertheless the fact that it is rather
39
40 weird to expect DON in a peanut sample. The presented graphs clearly indicated that the matrix effect
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42 depends on the analyte as well as on the matrix, which makes it obligatory to determine ion
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44 suppression for every single separate matrix-mycotoxin combination. Regarding the number of
45
46 mycotoxins and matrices this would result in an enormous validation task. For practical reasons we
47
48 focussed in this study on the most relevant combinations, which limited our choice to 13-24
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50 mycotoxins in the 7 matrices peanut, pistachio, wheat, maize, cornflakes, raisins and figs. From the
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3 data in tables 3-9 it becomes clear that ion suppression in these matrices varies between a minor and a
4 huge effect. For aflatoxin B₁, all matrices showed a moderate matrix effect, except wheat, where this
5 method revealed a matrix effect of around 50%. For ochratoxin A the effect was less. The opposite
6 holds for DON. This mycotoxin has less matrix effect in wheat, but a huge matrix effect in peanuts,
7 raisins and figs. It can generally be assumed that DON is absent in peanut, raisin and fig samples,
8 however this does not hold for maize and cornflake samples. Sulyok et al. (2006) found no matrix
9 effect for aflatoxin B₁, ochratoxin A and DON in wheat, but 80% signal decrease for aflatoxin B₁ in
10 maize. They applied dry milling of samples and another type of MS/MS. Both factors will influence
11 the matrix effect as well. Biselli and Hummert (2005) measured signal suppression up to 40% for
12 DON and T-2 in ground soybeans. Hartmann et al. (2007) experienced 28-58% ion suppression for
13 zearalenone, while going from river to waste water samples. Both examples underline the necessity to
14 evaluate every matrix/mycotoxin combination again, even when it is mainly composed of water.
15 Compared to the application of HPLC after immunoaffinity cleanup this may look a more complicated
16 and more time-consuming sample handling, but recently Castegnaro et al. (2006) reported several
17 drawbacks in this field as well. They detected false-positive presence of aflatoxin G₁ in maize,
18 underestimation of ochratoxin A in some breakfast cereals and coffee and interfering compounds
19 while applying fumonisin antibodies. They emphasized the point of necessary validation of every
20 method using immunoaffinity for each type of matrix. From that perspective the application of matrix-
21 matched calibration for LC-MS analysis is not more laborious at all and delivers above all the
22 identification of the MS.
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53 The fact that mycotoxins can be found in almost every food or feed sample, also complicates analysis
54 in a sense of finding an appropriate internal standard. Tuomi et al. (1998) applied reserpine, Cavaliere
55 et al. (2005) nafcillin and Paepens et al. (2005) have chosen (2S, 3R)-2-aminododecane-1, 3-diol as
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3 internal standards, which compounds differ completely from mycotoxins, as to avoid intervention of
4 naturally present compounds in a sample. However, this has the disadvantage of different behaviour of
5 such compounds during extraction and the processes in the ion source of the mass spectrometer. On
6 the other hand, Berger et al. (1999), Plattner et al. (2003), and Biselli et al. (2004) applied verrucarol,
7 Berthiller et al. (2005) zearalanone, Ren et al. (2006) zearalenone and both Klötzel et al. (2005) and
8 Delmulle et al. (2006) applied de-epoxy-deoxynivalenol as internal standards. The chemical structures
9 of these compounds are all more or less related to mycotoxins, which will reveal similar chemical
10 behaviour during extraction and analysis, but has the disadvantage that their complete absence in a
11 naturally contaminated sample cannot be fully guaranteed. This is a drawback to the expected
12 precision of the analysis, even when the methods are focused on *Fusarium* toxins only. It is known
13 that verrucarol is likely to be present in samples of mouldy interior finishes from buildings with
14 moisture problems (Tuomi et al., 1998). Its presence in food was questioned by Cavaliere et al. (2005),
15 who for that reason chose 17 α -estradiol and penicillin, which nevertheless also can be present in
16 nature. The same holds for the corticosteroid dexamethasone which was applied by Razzazi et al.
17 (2003) when determining the major B-trichothecenes and the de-epoxy-metabolite of deoxynivalenol
18 in pig urine and maize. This de-epoxy-DON is basically found in vivo in animals, but it can also be
19 present in milk, as has been shown by Sørensen and Elbæk (2005). The only way to guarantee
20 application of compounds that cannot be found in any food or feed sample is to apply isotope labelled
21 isomers. Hartl et al. (1999) applied deuterated fumonisin B₁ for analysis of this mycotoxin in corn.
22 Rundberget and Wilkins (2002) and Razzazi-Fazeli et al. (2002) applied deuterated T-2 toxin as
23 internal standard for determination of several *Fusarium* toxins. Lindenmeier et al. (2004) reported the
24 use of deuterated Ochratoxin A while analysing raisins. Asam and Rychlik (2006) synthesized four
25 carbon-13 labelled type A-trichothecenes (T2, HT2, MAS and DAS) and applied these as internal
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standards for quantification in foods by LC tandem MS after cleanup on multifunctional columns. A year later they did the same for type B-trichothecenes (DON, 3 and 15 Ac-DON and fusarenon X) in foods and feeds (Asam and Rychlik 2007). Basically this approach is the correct one, but when analysis has to be perfect, it implies the use of a labelled compound for every single mycotoxin of interest in the analytical method, as already noticed by Tuomi in 1998. But not all these compounds are commercially available yet. Since then carbon-13 labelled DON (Häubl et al. 2006) became commercially available at a company that also supplies ¹³C labelled fumonisin B₁ and T-2 Toxin (www.biopure.at). The deuterated zearalenone as applied by Hartmann et al. (2007) can be obtained from RIVM (Bilthoven, The Netherlands). But running labelled compounds for every mycotoxin, as it should be, would cause a significant raise in analysis costs. The alternative, standard addition, has the same drawback, as one has to add all mycotoxins of interest at different levels to the same sample and analyse all these combinations, which multiplies analysis time and costs by the same factor. Altogether this led to the common practice to apply matrix-matched calibration in those cases when after application of the multi-method, one or more mycotoxins exceed the legal limit. This is done in this study as well. The mycotoxin of interest is added to blank matrix, which is diluted to the same extent as the sample. The analytical result is corrected for matrix-effect. For exact identification of a compound, both the ion ratio and retention time have to fit within the maximum tolerances.

For the mycotoxins presented in table 10 can be concluded that recoveries fulfil the requirements as regulated by EC 401/2006. The fumonisins are missing, since these showed too low recoveries when applying the single extraction step as is done in this method. This is a common feature when apolar compounds like these are extracted with a large portion of acetonitrile, as reported likewise by Sulyok et al. (2006). Several experiments were done to improve the extraction efficiency for the fumonisins.

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3 The solvent/sample ratio was varied, the extraction solvent was acidified and the water content of the
4 extraction solvent was increased, as to decrease the percentage of acetonitrile. The conclusion of these
5 experiments was that increasing the water content of the extraction solvent was the most convenient
6 way to improve extraction efficiency and thus recoveries for fumonisins and leave the multimethod
7 unchanged for all other mycotoxins. A higher solvent/sample ratio increased the recovery, but has the
8 disadvantage to repeat the extraction once again from the beginning. The addition of formic acid to the
9 extract showed almost the same improvement, but this also means repetition of the extraction. When
10 the measurement has to be repeated in order to improve the determination of fumonisins, it is most
11 practical to use the first extract again for the quantification of the fumonisins. The first run in the
12 multimethod gives a good qualitative estimate as to see whether legislative excess can be expected. If
13 it turns out that fumonisins have to be quantified precisely, 100 ml of water is added to the original
14 sample extract, shaken again for another hour and re-run with the LC-MS/MS. Using extra water in the
15 extraction solvent yielded recoveries of 98% at RSD = 3.4 % for fumonisin B₁ and 90% at RSD =
16 2.8% for fumonisin B₂ (n=8), which fulfil the criteria of the EU regulation again. Since determination
17 of fumonisins implies to re-run the extract a second time, less data are available when compared with
18 the numbers in table 10. When quantifying fumonisins, a standard control sample is run in that series.
19 This allows to judge on the performance for fumonisin analysis by evaluating the data of the
20 measurements on the standard control sample instead. During last 1.5 year this revealed following
21 data: means and RSD values of 1136 µg/kg and 8.3% for fumonisin B₁ and 235 µg/kg and 10.5% for
22 fumonisin B₂ respectively, both at n=12.
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53 To evaluate the performance of this multimethod, at first a comparison was made between
54 measurement results obtained when aflatoxin B₁ was determined in peanut with LC-fluorescence
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3 (CEN method) and when analysed by the presented LC-MS method. Until now 128 data points
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5 resulted in mean and variance values of 2.2 $\mu\text{g}/\text{kg}$ and 22.6% for CEN and 2.1 $\mu\text{g}/\text{kg}$ and 22.0% for
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7 the LC-MS method respectively. From the t-test on these data ($t\text{-calculated} = 0.98$ and $t\text{-critical} = 1.98$)
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9 can be concluded that both methods do not differ significantly. In the mean time the method was
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11 applied in different laboratory proficiency tests (FAPAS 2005 and 2006). The results for several
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13 rounds are listed in table 11. It illustrates that performance is good for the tested mycotoxin/matrix
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15 combinations, including fumonisin results, where we applied the procedure as described above.
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22 [Insert Table 11 about here]
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27 Application as a single method is a good starting point to explore the prospects for the simultaneous
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29 determination of different mycotoxins in a sample. Finding more mycotoxins in one single sample has
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31 been done before, simply by applying more single methods to an extract, e.g. by Vargas et al. (2001)
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33 who determined aflatoxins B₁, B₂, G₁ and G₂, zearalenone and fumonisin B₁ in Brazilian corn, by Park
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35 et al. (2002) who investigated the natural co-occurrence of aflatoxin B₁, fumonisin B₁ and ochratoxin
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37 A in barley and corn foods from Korea, by Eskola et al. (2002) who analysed ochratoxin A and
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39 zearalenone in cereals in Finland, by Pierard et al. (2003) who applied OTA and MycoSep cleanup to
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41 analyse wheat and by Domijan et al. (2005) who determined fumonisins B₁ and B₂, zearalenone and
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43 ochratoxin A of maize samples in Croatia. These studies are examples of investigations where not only
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45 *Fusarium* toxins are determined, but also other types like aflatoxin B₁ and ochratoxin A. In these
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47 studies, two or three single methods were applied to determine the specific mycotoxins of interest,
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49 which implied three different cleanup procedures and detection methods. Different from this approach,
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51 Aresta et al. (2003) simultaneously determined ochratoxin A, cyclopiazonic, mycophenolic and
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53 tenuazonic acids in cornflakes by a single solid-phase microextraction. Unfortunately these
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3 mycotoxins are not the most relevant ones when considering the EU legislation. With the multi-
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5 mycotoxin method presented here, the results as mentioned above could have been obtained after one
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7 run, as can be seen in figures 2 and 3. This also turned out in everyday practice where we found
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9 fumonisin B₁ in figs and ochratoxin A in pistachio in routine samples. The Italian Institute of Sciences
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11 of Food Production in Bari was the first to report the presence of toxigenic fungi, able to produce other
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13 mycotoxins then aflatoxin, in figs (Logrieco et al., 2003). Two years later they reported the results of
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15 chemical analysis on rotten fig fruits, showing fumonisin contamination at a low level in some samples
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17 (Moretti et al., 2005). These finding are comparable with those of ochratoxin A in pistachio nuts.
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19 Bayman et al. (2002) reported the isolation of fungi with the ability to produce ochratoxin A and the
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21 British Food Standards Agency reported the actual analysis of it in a mycotoxin survey (Matthews,
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23 2002). With this multi-method we also determined ochratoxin A in several nut types in 2006, apart
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25 from aflatoxin as well. The sensitivity of the method is very good when EU legislation is taken into
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27 account. Comparison of the LODs in tables 3-9 with the directive EC 1881/2006 reveals that they all
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29 can cope with the limits. Suylok et al. (2006) presented the only other method that is applied directly
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31 on the crude extract. Comparison of LODs for wheat in their table 6 with our data in table 5 shows
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33 some discrepancy for aflatoxin B₁ and ochratoxin A data. Both mycotoxins for which most legislation
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35 exists. Regarding the EC limit in wheat of 2 µg/kg for aflatoxin B₁ and 3 µg/kg for ochratoxin A, they
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37 reported LODs of 8 and 3.5 µg/kg respectively, where this method achieved 0.5 and 1 µg/kg. For
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39 *Fusarium* mycotoxins both methods reach the EC limits. The differences between the methods are
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41 twofold. They dilute 1 + 1 with eluent, i.e. by a factor 2 and their MS equipment is different, which
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43 implies another ionization chamber design. Both factors influence ion suppression and thus sensitivity.
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55 Regarding other mycotoxins of interest it has already been proven by Biselli et al. (2004) and
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57 Kokkonen et al. (2005) that aflatoxin M₁ can be dealt with in the same way. For patulin this is
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3 impossible due to its small size and high polarity. As shown by Sulyok et al. (2006) patulin can be run
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5 under the same LC-MS conditions, however, the extraction procedure is completely different when
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7 compared with the one for other mycotoxins. If patulin cannot be extracted from a sample, it is not
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9 useful to implement it in a method of analysis for routine samples. This conclusion was confirmed by
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11 Garon et al. (2006). To determine patulin we apply the LC-MS method of Sewram et al. (2000) as a
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13 single method. Regarding the ergot alkaloids we first got an impression of the presence of ergot
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15 alkaloids in a naturally contaminated sample. Figure 5 shows the major peaks in the chromatogram of
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17 an extract of *Claviceps purpurea* kernels, which were sorted out of a naturally contaminated lot of rye,
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19 while running the mass spectrometer in the parent mode. Five of these alkaloids could be identified as
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21 indicated. Epimerisation was not noticed, due to the fact that extraction is performed with 80/20 v/v %
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23 acetonitril-water, which is a neutral mixture. The eluent contains 0.1% formic acid, but retention time
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25 appeared to be too short to cause significant formation of epimers. Since alkaloids are LSD-type
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27 compounds it is difficult to get standards. At first we were only able to obtain ergotamin, ergocornin,
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29 ergocristin and α -ergocryptin, which we incorporated in this multi-mycotoxin method. Compared to
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31 the findings of others we missed ergonovin as detected by Mohamed et al. (2006) and by Diers (2006),
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33 who applied the name ergometrin. The variation in ergot alkaloid pattern in nature is well known, but
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35 few data exist. Diers (2006) studied the 6 different ergot alkaloids as mentioned here, i.e. the 5 we
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37 found in the contaminated rye sample and ergometrin. She found variations for ergotamine content
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39 from 20-40% and 30-50% for ergocristin. These findings are slightly different from the data that are
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41 presented in the EFSA opinion on feed: ergocristine 31 % and ergotamine 17 % (EFSA, 2006).
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43 Agreement seems to exist on the fact that ergometrine, ergosine, ergotamine, ergocornine,
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45 ergocryptine and ergocristine are the 6 most important ergot alkaloids. For this reason we will extend
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47 the method up to this number of indicator ergot alkaloids.
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6 To lower the limit of determination to the level of the EU limits for baby food (0.1 µg/kg for aflatoxin
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8 B₁ and 0.5 µg/kg for ochratoxin A (EC, 2004)) it was necessary to return to immunoaffinity cleanup.
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10 This was made easier by the introduction of a new type of immunoaffinity column composed of
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12 antibodies specific both for aflatoxins and ochratoxin A. The application is similar when compared to
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14 single mycotoxin analysis and is described by Chan et al. (2004). Unfortunately, these authors did not
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16 get lower than a limit of quantification of 0.2 µg/kg for both mycotoxins. Therefore, we adapted
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18 sample preparation with extra concentration steps. A test was done with a crisp bread sample at a level
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20 of 0.011 µg aflatoxin B₁ and 0.173 µg ochratoxin A per kilo. The corresponding standard deviation
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22 data were 0.0011 and 0.0220, with relative standard deviation (RSD) values of 10 and 13% (n=5). Due
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24 to the difficulties to extract spices and herbs the application of this combined assay column was also
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26 ideal to analyse these matrices properly on aflatoxin B₁ and ochratoxin A. This has the drawback of
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28 missing information on other mycotoxins, but for spices there is no legislation on other mycotoxins yet.
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30 Unfortunately for babyfood all limits are set lower one by another, so these samples may need several
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32 assays again in the future. For silage this method yielded recoveries between 86 and 111 % for DON,
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34 aflatoxin B₁ and zearalenone at levels as those mentioned in tables 3-9. Garon et al. (2006) reported
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36 65% recovery for DON at a level of 100-213 µg/kg, aflatoxin B₁ at a level of 4-34 µg/kg and
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38 zearalenone at 23-41 µg/kg. For citrinin, which we investigated partly in our validation study, they
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40 yielded 80% at a level of 4-25 µg/kg.
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50 51 **Conclusions**

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53 The multi-mycotoxin method as presented here has proven to be a good starting point for further
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55 development of sample analysis in one single run, without any cleanup. The EU limits are met for
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57 those mycotoxin-matrix combinations that are most commonly controlled in practice. The method
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3 gives relevant information about the presence of 33 mycotoxins in a run time of 30 min. Further
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5 research is planned regarding extraction of specific sample types, matrix effects, baby food limits and
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7 update of validation data. Changing the liquid chromatography system into an ultra performance liquid
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9 chromatography system is considered as well, as to cut analysis time further and to improve peak
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11 shape, especially for nivalenol. This might also lead to gain of sensitivity, as to reach lower, i.e. baby
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13 food, limits.
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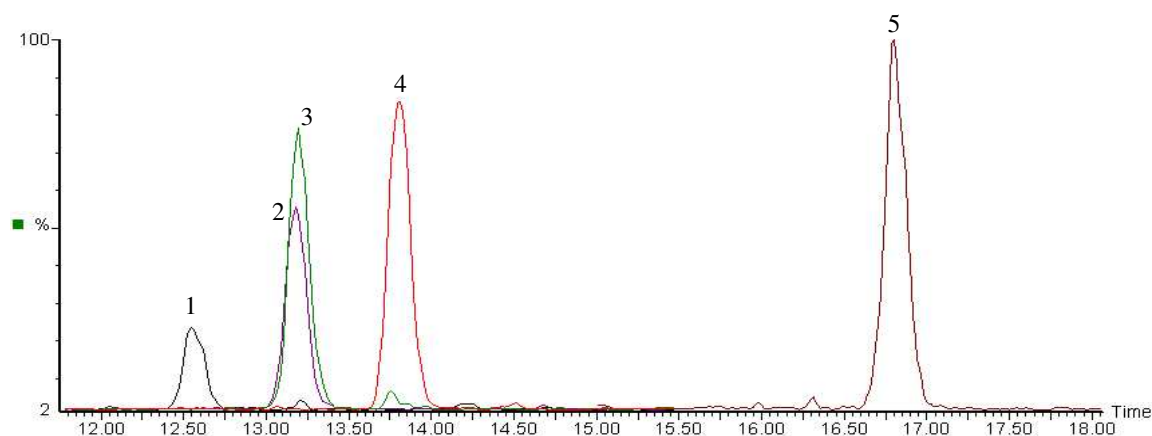
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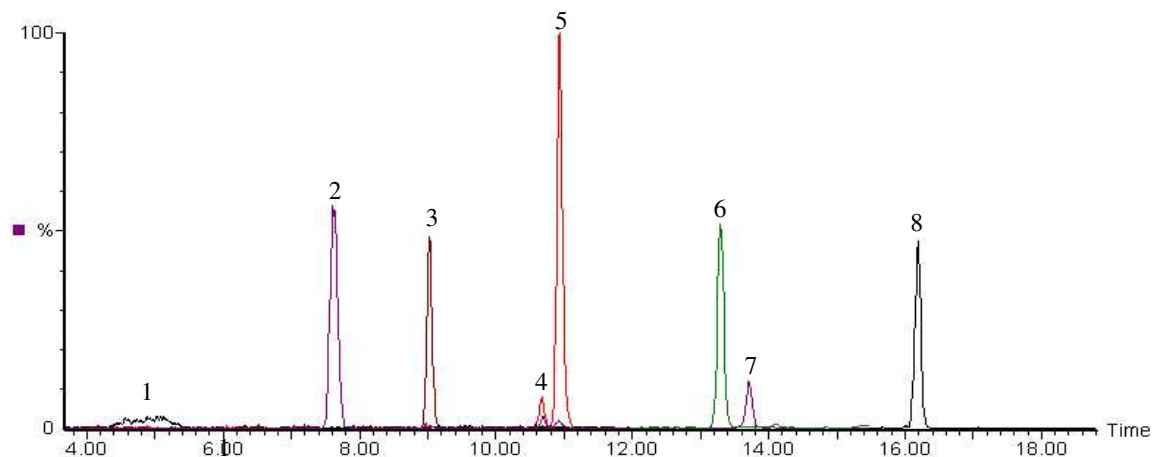
Figure 1. Chromatographic peak shapes as they appear after injecting a spiked pistachio extract.

(a) Aflatoxins and Ochratoxin A.



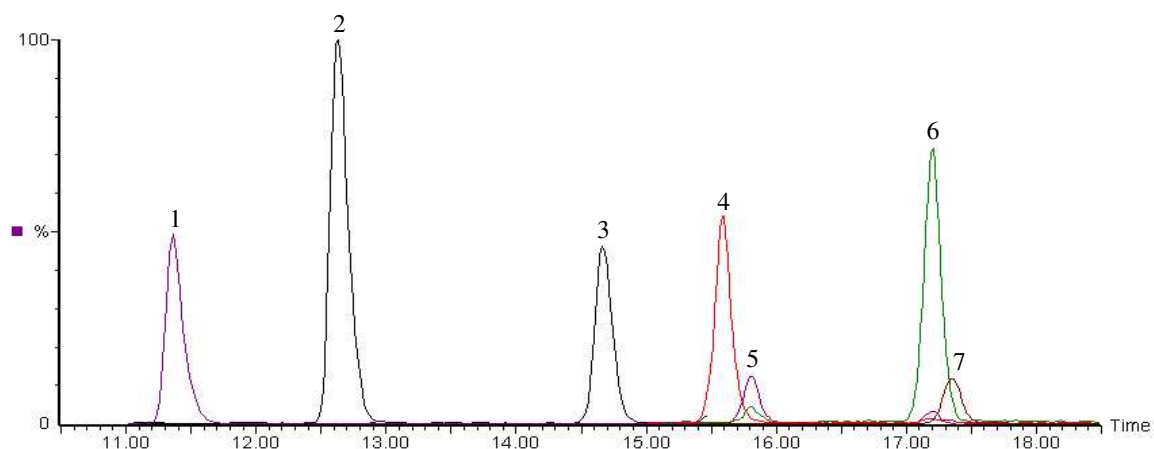
1: Aflatoxin G₂, 2: Aflatoxin G₁, 3: Aflatoxin B₂, 4: Aflatoxin B₁ and 5: Ochratoxin A.
Aflatoxins at a level of 0.125 ng/ml, corresponding with 2 µg/kg. Ochratoxin A at a level of 0.25 ng/ml, corresponding with 4 µg/kg.

(b) Trichothecenes.



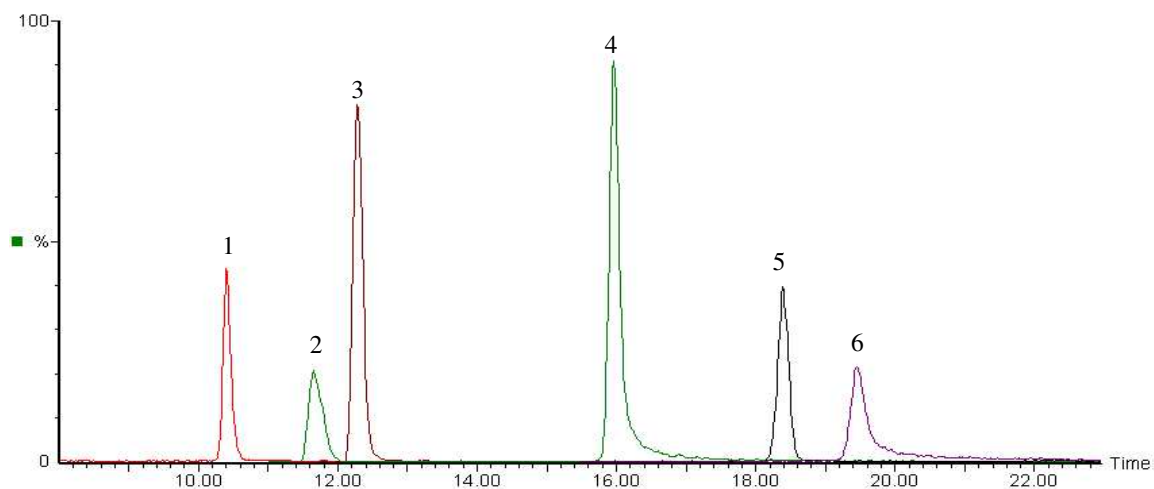
1: NIV, 2: DON, 3: Fusarenon, 4: 15-Ac-DON, 5: 3-Ac-DON, 6: DAS, 7: HT2-toxin and 8: T2-toxin.
All trichothecenes at a level of 25 ng/ml, corresponding with 400 µg/kg.

(c) Fumonisin and the Zeara-group.



1: Fumonisin B₁, 2: Fumonisin B₂, 3: β -ZAL, 4: α -ZAL, 5: α -ZEL, 6: ZAN and 7: ZEN, all at a level of 10 ng/ml, corresponding with 160 μ g/kg.

(d) Other mycotoxins.



1: Penicillic acid, 2: Ergotamin, 3: Roquefortin C, 4: Citrinin, 5: Sterigmatocystin and 6: Cyclopiazonic acid. Roquefortin C and sterigmatocystin at a level of 2.5 ng/ml, corresponding with 40 μ g/kg. The other mycotoxins at a level of 10 ng/ml, corresponding with 160 μ g/kg.

Figure 2. LC-MS data of a maize sample containing aflatoxin B₁ (30 µg/kg) and fumonisins B₁ (4200 µg/kg), B₂ (1200 µg/kg) and B₃. Top left, the chromatogram with 4 peaks; top right, and bottom left and right, the separate peaks characterised by the ion transitions of the MS as to prove their structure. The peak eluting before the fumonisin B₂ peak belongs to fumonisin B₃. Since both fumonisins are isomers, they have the same transitions in the MS/MS spectra. Fumonisin B₃ could not be quantified, since there is no standard available.

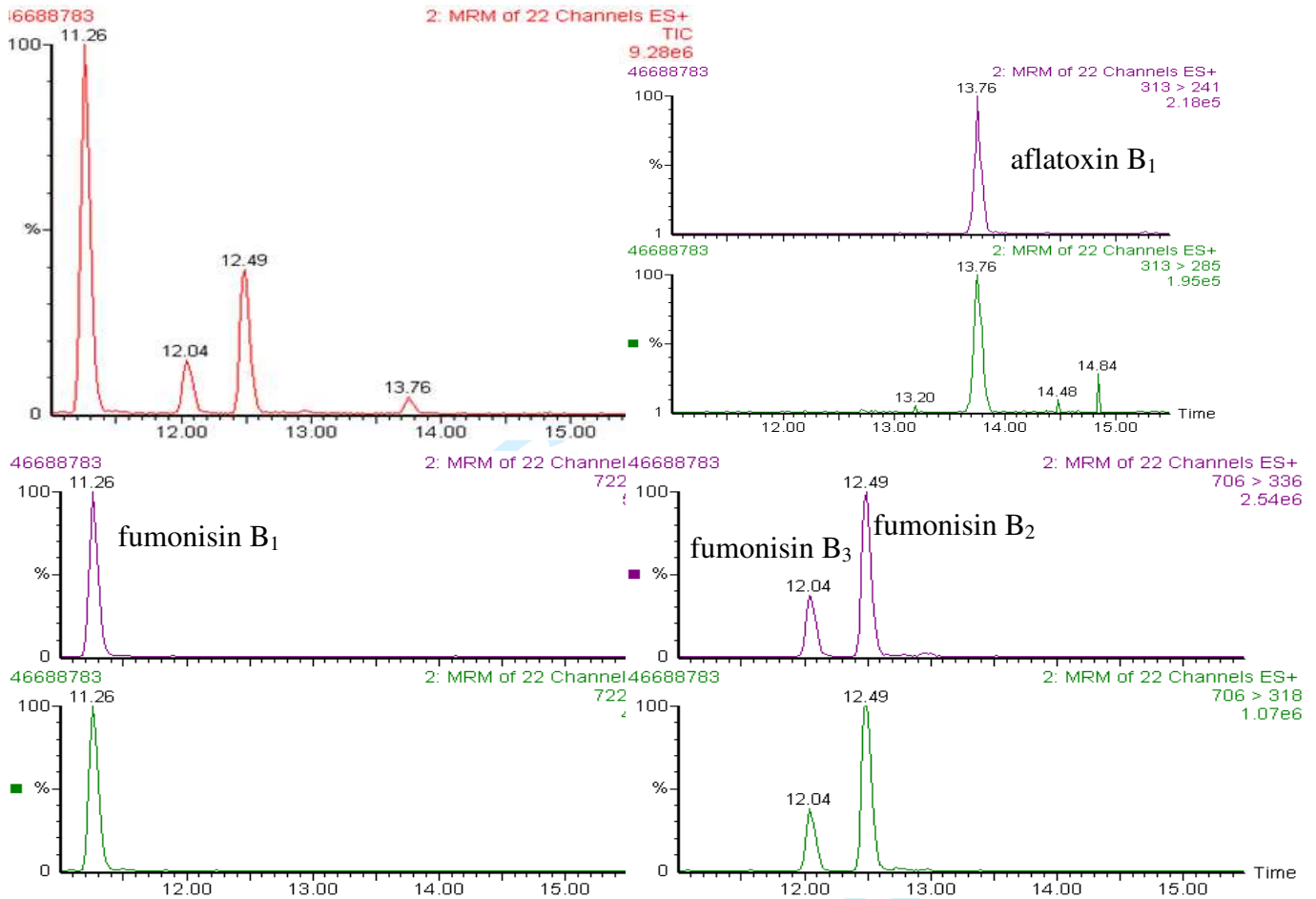


Figure 3. LC-MS data of a buckwheat sample containing several mycotoxins, i.e. ochratoxin A (40 $\mu\text{g}/\text{kg}$), zearalenone (34 $\mu\text{g}/\text{kg}$), sterigmatocystin (36 $\mu\text{g}/\text{kg}$) and roquefortin C (1 $\mu\text{g}/\text{kg}$).

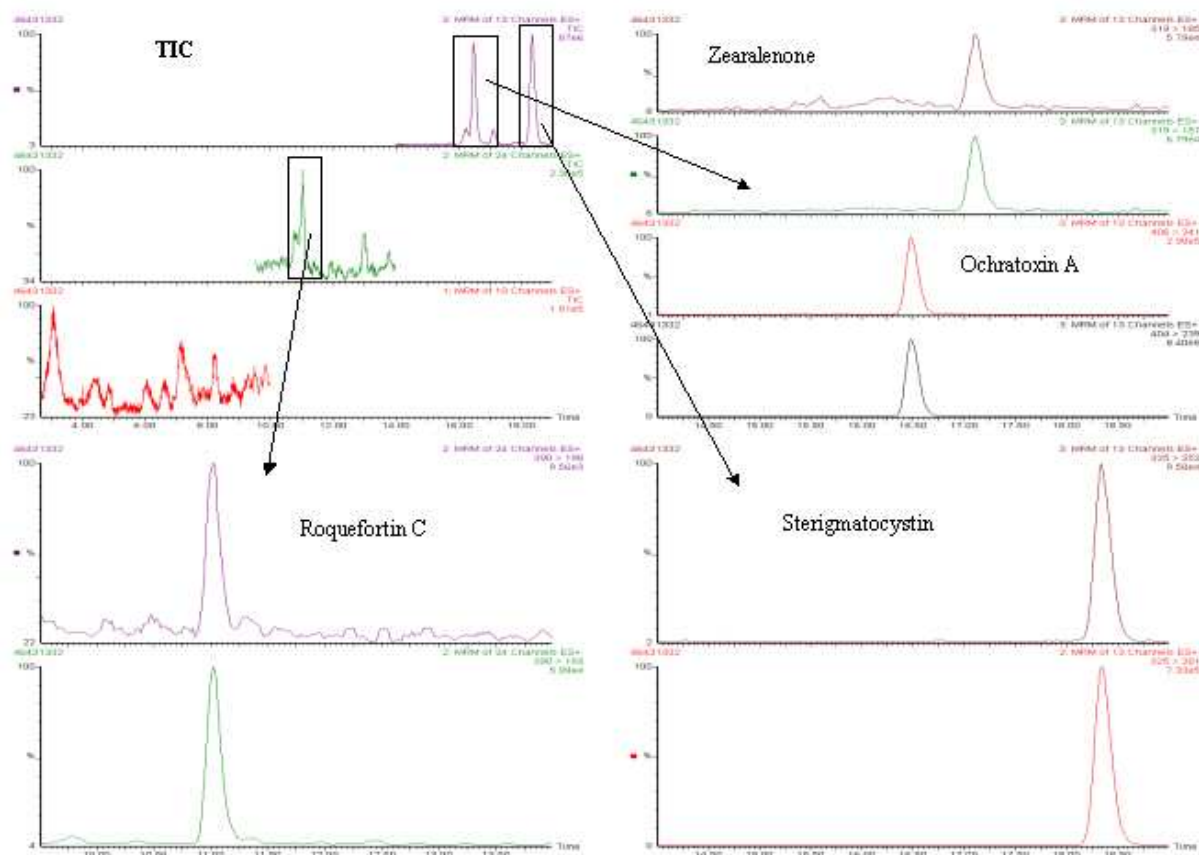


Figure 4. LC-MS data of a freeze-dried mais silage sample containing several mycotoxins, i.e. DON (2500 µg/kg), zearalenone (240 µg/kg) and 15-Acetyl-DON (960 µg/kg).

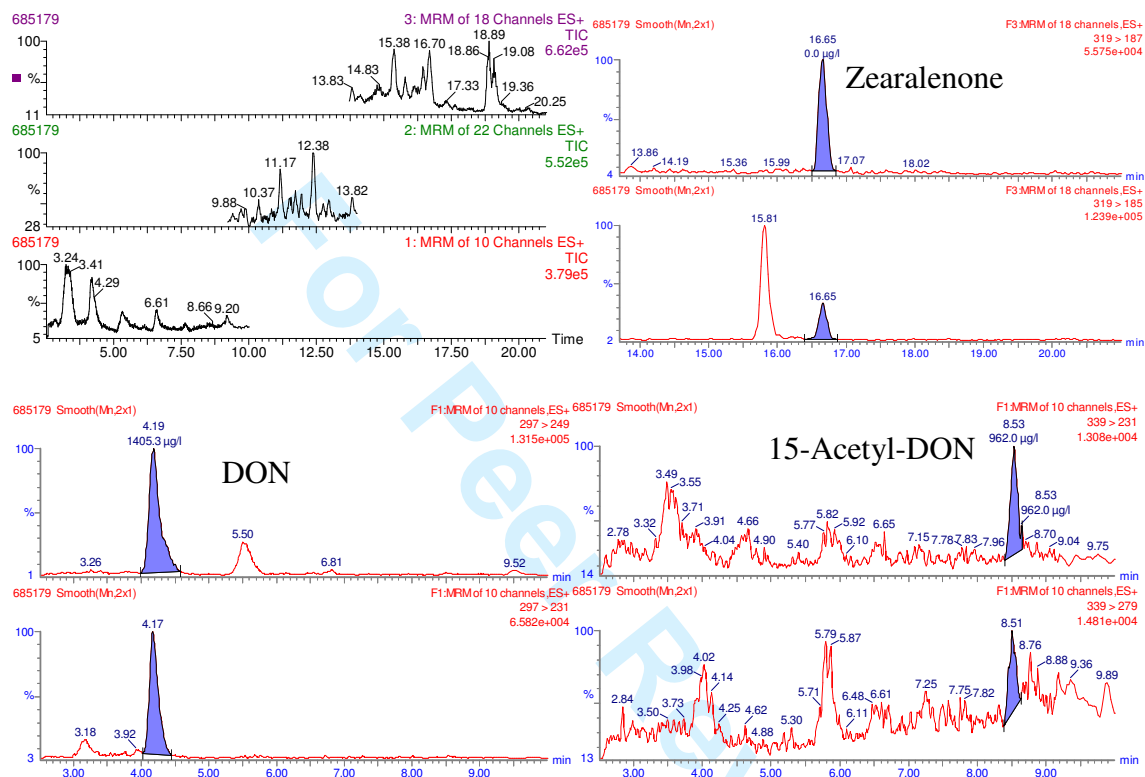


Figure 5. Chromatogram of ergot alkaloids extracted from a naturally contaminated rye sample with LC-MS in parent scan mode at m/z 268.

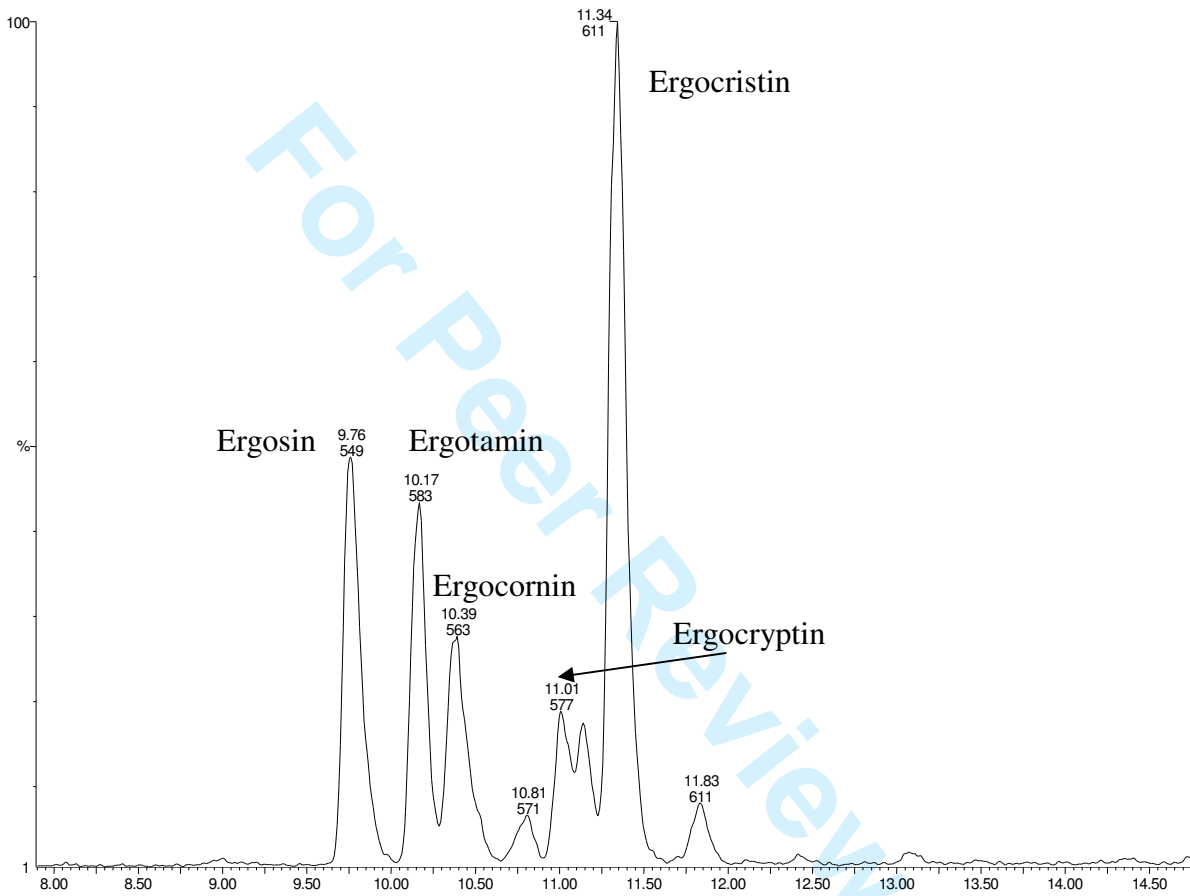


Table 1. Gradient programme of the LC system.

Step	Time (min)	Solvent A (%)	Solvent B (%)	Flow (mL/min)	Curve
1	0.00	90	10	0.3	1
2	12.00	30	70	0.3	6
3	16.00	30	70	0.3	6
4	17.50	10	90	0.3	6
5	20.00	10	90	0.3	1
6	21.00	90	10	0.3	6
5	25.00	90	10	0.3	1

Table 2. Overview of all relevant information regarding the investigated mycotoxins: parent and daughter ions, cone (V) and collision (eV) voltages, ratios and retention times (min) and references for fragment clarification.

Mycotoxin	Parent	Primary	Secondary	Ratio	Cone	Coll.	eV	RT	Reference
Aflatoxin B ₁	313	241	285	1.2	90	37/23	13.8	Kussak <i>et al.</i> (1995)	
Aflatoxin B ₂	315	259	287	1.0	100	30/24	13.2	Kussak <i>et al.</i> (1995)	
Aflatoxin G ₁	329	243	283	1.8	90	25/24	13.2	Kussak <i>et al.</i> (1995)	
Aflatoxin G ₂	331	245	275	2.4	100	29/29	12.6	Kussak <i>et al.</i> (1995)	
Alternariol	259	185	213	6.4	35	30/26	14.4	Lau <i>et al.</i> (2003)	
Alternariol-methylether	273	184	199	0.9	35	35/27	17.8	Lau <i>et al.</i> (2003)	
Citrinin	251	205	191	1.1	80	25/25	16.2	Shu & Lin (2002)	
Cyclopiazonic acid	337	196	182	0.6	45	26/20	20.0	Losito <i>et al.</i> (2005)	
Deoxynivalenol	297	249	231	2.5	35	10/10	7.8	Berger <i>et al.</i> (1999)	
3-Acetyl-DON	339	231	279	2.8	35	15/12	11.0	Berger <i>et al.</i> (1999)	
15-Acetyl-DON	339	231	279	1.3	35	15/12	10.8	Berger <i>et al.</i> (1999)	
Diacetoxyscirpenol	367	307	289	2.5	35	13/10	13.4	Razzazi <i>et al.</i> (2002)	
Ergotamin	582	268	208	0.4	58	30/42	12.0	Shelby <i>et al.</i> (1997)	
Ergocornin	562	268	544	4.0	58	25/17	11.3	Lehner <i>et al.</i> (2004)	
Ergocristin	610	268	592	4.0	58	27/17	12.6	Lehner <i>et al.</i> (2004)	
α -Ergocryptin	576	268	558	4.0	58	27/18	12.1	Lehner <i>et al.</i> (2004)	
Fumonisin B ₁	722	334	352	1.1	75	38/34	11.5	Lukacs <i>et al.</i> (1996)	
Fumonisin B ₂	706	336	318	2.2	75	36/36	12.9	Lukacs <i>et al.</i> (1996)	
Fumonisin B ₃	706	336	318	1.7	75	36/36	12.6	Lukacs <i>et al.</i> (1996)	
Fusarenon X	355	247	229	1.2	40	13/17	9.1	Klötzel <i>et al.</i> (2005)	
HT-2 toxin	425	263	105	2.0	50	10/36	13.8	Razzazi <i>et al.</i> (2002)	
Mycophenolic acid	321	207	159	2.7	70	23/35	14.2	Rundberget <i>et al.</i> (2002)	
Nivalenol	313	175	295	0.9	35	19/15	5.3	Razzazi <i>et al.</i> (1999)	
Ochratoxin A	404	239	241	3.0	50	22/22	17.0	Xiao <i>et al.</i> (1995)	
Penicillic acid	171	125	153	0.9	35	12/7	10.5	Kokkonen <i>et al.</i> (2005)	
Roquefortin C	390	193	322	1.7	90	26/18	12.7	Rundberget <i>et al.</i> (2002)	
Sterigmatocystin	325	281	253	7.7	85	32/39	18.7	Nielsen <i>et al.</i> (2003)	
T-2 toxin	467	305	245	1.2	40	8/8	16.3	Razzazi <i>et al.</i> (2002)	
Zearalanone	321	303	285	26	45	14/16	17.4	Bennekom <i>et al.</i> (2002)	
Zearalenone	319	187	185	1.6	35	17/23	17.6	Bennekom <i>et al.</i> (2002)	
α -Zearalanol	323	305	277	2.7	35	8/15	15.6	Bennekom <i>et al.</i> (2002)	
α -Zearalenol	321	303	285	0.4	40	7/11	15.6	Bennekom <i>et al.</i> (2002)	
β -Zearalanol	323	305	277	11.2	35	8/15	14.7	Bennekom <i>et al.</i> (2002)	

Table 3. Validation results in peanut slurry (n=18)

Compound	Calibration curve			Cal. range (µg/kg)	Validation levels (µg/kg)	Recovery (%)	RSDr (%)	Max RSDr (%)	Reporting limit (µg/kg)	LOD (µg/kg)	Matrix- effect (%)
	a	b	r ²								
Aflatoxin B ₁	48284	943	0.9932	1-64	1, 2, 16	118	5.2	20-30	1.0	0.5	-19
Aflatoxin B ₂	33041	1528	0.9930	1-64	1, 2, 16	119	10.9	20-30	1.0	0.5	-5
Aflatoxin G ₁	34812	1475	0.9930	1-64	1, 2, 16	117	9.1	20-30	1.0	0.5	4
Aflatoxin G ₂	20363	272	0.9944	1-64	1, 2, 16	114	13.6	20-30	1.0	0.5	21
Ochratoxin A	63526	726	0.9955	2-128	2, 4, 30	91	12.7	20-30	2.0	0.5	-13
DON	994	8113	0.9886	50-3200	50, 100, 780	96	11.4	12-18	150	75	-67
Fumonisin B ₁	33610	-3890	0.9914	10-640	10, 20, 160	26	61.7	15-23	20	5	18
Fumonisin B ₂	61988	4068	0.9970	10-640	10, 20, 160	41	46.6	15-23	20	5	-2
Zearalenone	6086	2234	0.9903	20-1280	20, 40, 320	100	19.8	13-20	40	5	-7
DAS	4456	617	0.9990	50-3200	50, 100, 780	118	8	12-18	100	10	-17
T2-Toxin	3482	1998	0.9976	50-3200	50, 100, 780	117	8.1	12-18	100	10	-3
HT2-Toxin	2165	798	0.9995	50-3200	50, 100, 780	126	8.3	12-18	100	10	-26
3-Acetyl-DON	3277	100	0.9970	50-3200	50, 100, 780	119	8.5	12-18	100	10	-40

Table 4. Validation results in pistachio slurry (n=18)

Compound	Calibration curve			Cal. Range ($\mu\text{g}/\text{kg}$)	Validation levels ($\mu\text{g}/\text{kg}$)	Recovery (%)	RSDr (%)	Max RSDr (%)	Reporting limit ($\mu\text{g}/\text{kg}$)	LOD ($\mu\text{g}/\text{kg}$)	Matrix- effect (%)
	a	b	r^2								
Aflatoxin B ₁	60609	-392	0.9979	1 - 64	1, 2, 10	106	10.8	20 - 30	1.0	0.5	32
Aflatoxin B ₂	33572	702	0.9967	1 - 64	1, 2, 10	99	11.6	20 - 30	1.0	0.5	29
Aflatoxin G ₁	26183	-947	0.9943	1 - 64	1, 2, 10	93	9.4	20 - 30	1.0	0.5	-4
Aflatoxin G ₂	15712	322	0.9940	1 - 64	1, 2, 10	85	42	20 - 30	1.0	10	31
Ochratoxin A	16551	-414	0.9886	2 - 128	2, 4, 20	102	28	20 - 30	2.0	1.0	-7
DON	212	90	0.9906	50 - 3200	50, 100, 500	91	26	12 - 18	100	50	15
Fumonisin B ₁	5869	-2017	0.9918	20 - 1280	20, 40, 200	79	9.3	15 - 23	20	10	-10
Fumonisin B ₂	11953	-3020	0.9885	20 - 1280	20, 40, 200	86	10.3	15 - 23	20	10	-1
DAS	330	-776	0.9693	50 - 3200	50, 100, 500	97	20.9	12 - 18	50	25	-11
T2-Toxin	102	5	0.9538	50 - 3200	50, 100, 500	88	20.7	12 - 18	100	50	-12
HT2-Toxin	103	59	0.9646	50 - 3200	50, 100, 500	83	27.0	12 - 18	100	50	-25
3-Ac-DON	286	274	0.9944	50 - 3200	50, 100, 500	100	18.9	12 - 18	100	50	11
ZEN	903	21	0.9853	20 - 1280	20, 40, 200	101	16.6	15 - 23	20	10	7
Penicillic acid	2834	-427	0.9959	20 - 1280	20, 40, 200	107	7.5	15 - 23	20	10	13
Fusarenon-X	129	-57	0.9799	50 - 3200	50, 100, 500	85	23.2	12 - 18	100	50	24
Ergotamin				40 - 2560	40, 80, 400	98	9.5	15 - 23	40	20	26
Roquefortin	33076	47626	0.9925	20 - 1280	20, 40, 200	101	11.0	15 - 23	20	10	43
β -ZAL	2111	-830	0.9659	20 - 1280	20, 40, 200	92	14.7	15 - 23	40	10	-7
α -ZAL	2332	-618	0.9628	20 - 1280	20, 40, 200	96	19.7	15 - 23	40	10	-3
Citrinin	22476	1578	0.9993	20 - 1280	20, 40, 200	53	17.7	15 - 23	20	100	5
ZAN	5129	-58	0.9739	20 - 1280	20, 40, 200	94	17.2	15 - 23	20	10	7
Cyclopiazonic acid	6093	-7949	0.9872	20 - 1280	20, 40, 200	59	25	15 - 23	200	200	-32
Sterigmatocystein	60056	59007	0.9974	20 - 1280	20, 40, 200	82	14.1	15 - 23	20	10	8
α -ZEL	926	-282	0.9750	20 - 1280	20, 40, 200	88	20.7	15 - 23	40	40	-3

Table 5. Validation results in wheat slurry (n=18)

Compound	Calibration curve			Cal. range (µg/kg)	Validation levels (µg/kg)	Recovery (%)	RSDr (%)	Max RSDr (%)	Reporting limit (µg/kg)	LOD (µg/kg)	Matrix- effect (%)
	a	b	r ²								
Aflatoxin B ₁	64120	314	0.9728	1-64	1, 2, 10	87	14.9	20-30	1.0	0.5	-53
Aflatoxin B ₂	65165	153	0.9887	1-64	1, 2, 10	92	9.8	20-30	1.0	0.5	-32
Aflatoxin G ₁	71493	490	0.9877	1-64	1, 2, 10	92	9	20-30	1.0	0.5	-24
Aflatoxin G ₂	40235	376	0.9887	1-64	1, 2, 10	90	19	20-30	1.0	0.5	5
Ochratoxin A	49176	1666	0.9736	2-128	1.5, 3, 15	94	16.3	20-30	2	1	-32
DON	2811	1769	0.9977	50-3200	25, 50, 750	91	12.8	12-18	40	20	-23
Fumonisin B ₁	25656	1127	0.9957	10-640	10, 20, 160	59	24.9	15-23	20	10	16
Fumonisin B ₂	25581	5659	0.9912	10-640	5, 10, 150	51	57.4	15-23	20	10	-7
Zearalenone	7373	-6392	0.9778	20-1280	25, 50, 500	80	41.6	13-20	40	5	-29
DAS	2345	-1824	0.9884	50-3200	25, 50, 500	35	29.7	12-18	100	5	-10
T2-Toxin	733	554	0.8896	50-3200	25, 50, 500	38	57.5	12-18	100	20	-39
HT2-Toxin	353	159	0.9864	50-3200	25, 50, 500	84	41	12-18	100	50	-65
3-Acetyl-DON	2232	3485	0.993	50-3200	5, 10, 150	115	24.2	12-18	100	50	-14

Table 6. Validation results in maize slurry (n=18)

Compound	Calibration curve			Cal. Range ($\mu\text{g}/\text{kg}$)	Validation levels ($\mu\text{g}/\text{kg}$)	Recovery (%)	RSDr (%)	Max RSDr (%)	Reporting limit ($\mu\text{g}/\text{kg}$)	LOD ($\mu\text{g}/\text{kg}$)	Matrix- effect (%)
	a	b	r^2								
Aflatoxin B ₁	59401	-456	0.9958	1 - 64	1, 2, 10	93	13.4	20 - 30	1.0	0.5	17
Aflatoxin B ₂	40546	1506	0.9949	1 - 64	1, 2, 10	87	27	20 - 30	2.0	1.0	21
Aflatoxin G ₁	35053	385	0.9926	1 - 64	1, 2, 10	99	21	20 - 30	2.0	1.0	-1
Aflatoxin G ₂	20208	-302	0.9972	1 - 64	1, 2, 10	108	14.7	20 - 30	1.0	0.5	15
Ochratoxin A	31924	275	0.9822	2 - 128	2, 4, 20	81	11.9	20 - 30	2.0	1.0	11
DON	1238	722	0.9934	50 - 3200	50, 100, 500	104	12.2	12 - 18	50	50	15
Fumonisin B ₁	12726	-584	0.9970	20 - 1280	20, 40, 200	20	72	15 - 23	200	100	-17
Fumonisin B ₂	29590	-2798	0.9960	20 - 1280	20, 40, 200	46	7.7	15 - 23	200	100	-2
DAS	1707	34	0.9954	50 - 3200	50, 100, 500	104	7.4	12 - 18	50	25	8
T2-Toxin	1204	1253	0.9925	50 - 3200	50, 100, 500	102	10.5	12 - 18	50	25	13
HT2-Toxin	718	769	0.9966	50 - 3200	50, 100, 500	100	11.5	12 - 18	50	25	20
3-Ac-DON	1292	407	0.9939	50 - 3200	50, 100, 500	102	17.8	12 - 18	100	25	21
ZEN	3301	638	0.9836	20 - 1280	20, 40, 200	100	10.9	15 - 23	20	10	12
Penicillic acid	12869	-624	0.9958	20 - 1280	20, 40, 200	103	6.5	15 - 23	20	10	12
Fusarenon-X	592	87	0.9952	50 - 3200	50, 100, 500	97	17.2	12 - 18	100	50	11
Ergotamin	5560	8253	0.9982	40 - 2560	40, 80, 400	100	10.6	15 - 23	40	20	45
Roquefortin	71857	159492	0.9889	20 - 1280	20, 40, 200	99	7.9	15 - 23	20	10	46
β -ZAL	16972	4905	0.9905	20 - 1280	20, 40, 200	103	7.6	15 - 23	20	10	15
α -ZAL	17681	3076	0.9942	20 - 1280	20, 40, 200	94	26	15 - 23	20	10	11
Citrinin	27106	47666	0.9813	20 - 1280	20, 40, 200	13	105	15 - 23	200	100	6
ZAN	17496	6787	0.9867	20 - 1280	20, 40, 200	101	6.7	15 - 23	20	10	11
Cyclopiazonic acid	16140	-6998	0.9787	20 - 1280	20, 40, 200	19	105	15 - 23	200	200	-177
Sterigmatocystin	55332	48561	0.9868	20 - 1280	20, 40, 200	109	10.9	15 - 23	20	10	-18
α -ZEL	3117	1597	0.9899	20 - 1280	20, 40, 200	100	21.9	15 - 23	40	20	17

Table 7. Validation results in dry milled cornflakes (n=18)

Compound	Calibration curve			Cal. range ($\mu\text{g}/\text{kg}$)	Validation levels ($\mu\text{g}/\text{kg}$)	Recovery (%)	RSDr (%)	Max RSDr (%)	Reporting limit ($\mu\text{g}/\text{kg}$)	LOD ($\mu\text{g}/\text{kg}$)	Matrix- effect (%)
	a	b	r^2								
Aflatoxin B ₁	27735	214	0.9983	1-64	1, 2, 16	101	12.6	20-30	1.0	0.5	-19
Aflatoxin B ₂	24749	787	0.9973	1-64	1, 2, 16	106	8	20-30	1.0	0.5	-6
Aflatoxin G ₁	6928	218	0.9916	1-64	1, 2, 16	99	27.6	20-30	3.4	1.7	-1
Aflatoxin G ₂	12810	189	0.9976	1-64	1, 2, 16	107	16.4	20-30	1.0	0.5	-5
Ochratoxin A	37687	332	0.9909	2-128	2, 4, 30	108	9.4	20-30	2.0	1.0	-1
DON	1177	284	0.9971	50-3200	50, 100, 780	91	7.2	12-18	150	75	-34
Fumonisin B ₁	19434	2940	0.9988	10-640	10, 20, 160	58	13.8	15-23	20	5	10
Fumonisin B ₂	41920	4697	0.9989	10-640	10, 20, 160	88	6.5	15-23	20	5	10
Zearalenone	2954	826	0.9877	20-1280	20, 40, 320	108	6.3	13-20	40	15	-6
DAS	4690	-319	0.9993	50-3200	50, 100, 780	104	4.6	12-18	100	10	-18
T2-Toxin	3325	2325	0.9996	50-3200	50, 100, 780	111	3.7	12-18	100	10	-5
HT2-Toxin	1931	244	0.9993	50-3200	50, 100, 780	110	4.9	12-18	100	10	-14
3-Acetyl-DON	4171	-486	0.9994	50-3200	50, 100, 780	102	6.4	12-18	100	10	-23

Table 8. Validation results in raisin slurry (n=18)

Compound	Calibration curve			Cal. range ($\mu\text{g}/\text{kg}$)	Validation levels ($\mu\text{g}/\text{kg}$)	Recovery (%)	RSDr (%)	Max RSDr (%)	Reporting limit ($\mu\text{g}/\text{kg}$)	LOD ($\mu\text{g}/\text{kg}$)	Matrix- effect (%)
	a	b	r^2								
Aflatoxin B ₁	39637	-436	0.9857	1-64	1, 2, 16	95	11.3	20-30	1.0	0.5	6
Aflatoxin B ₂	23265	728	0.9951	1-64	1, 2, 16	110	12.9	20-30	1.0	0.5	14
Aflatoxin G ₁	33805	143	0.9970	1-64	1, 2, 16	98	8.4	20-30	1.0	0.5	44
Aflatoxin G ₂	12109	41	0.9956	1-64	1, 2, 16	103	13.5	20-30	2.0	1.0	19
Ochratoxin A	5244	-29	0.9919	2-128	2, 4, 30	103	5.4	20-30	16.0	8.0	7
DON	1306	-150	0.9985	50-3200	50, 100, 780	98	3.9	12-18	18	9	-40
Fumonisin B ₁	38186	-7381	0.9986	10-640	10, 20, 160	89	5.8	15-23	20	5	3
Fumonisin B ₂	47530	844	0.9987	10-640	10, 20, 160	87	4.8	15-23	20	1	2
Zearalenone	61790	-805	0.9946	20-1280	20, 40, 320	95	4.4	13-20	40	2	1
DAS	3693	-1256	0.9985	50-3200	50, 100, 780	92	4.9	12-18	100	20	-4
T2-Toxin	2049	953	0.9963	50-3200	50, 100, 780	89	6.3	12-18	100	15	-5
HT2-Toxin	1845	-310	0.9980	50-3200	50, 100, 780	94	7	12-18	100	15	-5
3-Acetyl-DON	2421	-1083	0.9992	50-3200	50, 100, 780	98	5	12-18	100	10	-8

Table 9. Validation results in fig slurry (n=18)

Compound	Calibration curve		Cal. range ($\mu\text{g}/\text{kg}$)	Validation levels ($\mu\text{g}/\text{kg}$)	Recovery (%)	RSDr (%)	Max RSDr (%)	Reporting limit ($\mu\text{g}/\text{kg}$)	LOD ($\mu\text{g}/\text{kg}$)	Matrix- effect (%)	
	a	b									r^2
Aflatoxin B ₁	2054	32	0.9879	1-64	1, 2, 16	84	11.7	20-30	1.0	0.5	-3
Aflatoxin B ₂	1503	-32	0.9820	1-64	1, 2, 16	77	12.9	20-30	8.0	4	-12
Aflatoxin G ₁	2658	2	0.9910	1-64	1, 2, 16	74	13.1	20-30	3.0	1.5	-4
Aflatoxin G ₂	978	-33	0.9893	1-64	1, 2, 16	91	14.8	20-30	10.0	5	-8
Ochratoxin A	1667	-904	0.9867	2-128	2, 4, 30	74	12.8	20-30	14.0	7.0	-5
DON	297	112	0.9961	50-3200	50, 100, 780	97	12.8	12-18	250	125	-47
Fumonisin B ₁	7313	15281	0.9954	10-640	10, 20, 160	64	36.4	15-23	30	15	1
Fumonisin B ₂	15924	-150	0.9958	10-640	10, 20, 160	76	17.3	15-23	20	5	-1
Zearalenone	19473	-648	0.9953	20-1280	20, 40, 320	76	9.1	13-20	5	1	-11
DAS	415	71	0.9958	50-3200	50, 100, 780	86	9.5	12-18	100	10	-2
T2-Toxin	254	164	0.9900	50-3200	50, 100, 780	82	11.2	12-18	100	10	-11
HT2-Toxin	362	-185	0.9938	50-3200	50, 100, 780	84	13.3	12-18	100	10	-14
3-Acetyl-DON	564	291	0.9960	50-3200	50, 100, 780	82	10.2	12-18	100	10	-21

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Table 10. Recovery data for different mycotoxins in several types of food.

Mycotoxin	Recovery (%)	RSD%	n
Aflatoxin B ₁	104	17	152
Aflatoxin B ₂	105	18	151
Aflatoxin G ₁	103	17	152
Aflatoxin G ₂	107	21	143
Ochratoxin A	99	15	163
Deoxynivalenol	101	18	150
Diacetoxyscirpenol	108	16	153
T2-Toxin	110	19	152
HT2-Toxin	105	20	149
3-Ac-Don	105	18	148
ZEN	103	17	150
Penicillic acid	101	17	153
Fusarenon X	102	20	148
Ergotamin	105	16	149
Roquefortin	104	13	150
β-ZAL	107	17	152
α-ZAL	104	14	147
Citrinin	80	20	151
ZAN	104	16	150
Cyclopiazonic acid	85	25	150
Sterigmatocystin	101	21	152
α-ZEL	107	17	150

Table 11. FAPAS test results as obtained in the indicated rounds in 2005 and 2006, n.d. = not detected.

Compound	Commodity	Reference ($\mu\text{g}/\text{kg}$)	Our result ($\mu\text{g}/\text{kg}$)	Accuracy (%)	z-score	Fapas report
Aflatoxin B ₁	fig	4.36	5.2	119	0.9	483
Aflatoxin B ₂	fig	1.62	2.1	130	1.4	483
Aflatoxin G ₁	fig	3.09	2.9	94	-0.3	483
Aflatoxin G ₂	fig	0.88	0.9	102	0.1	483
Aflatoxins total	fig	10.0	11.1	111	0.5	483
Aflatoxin B ₁	maize	4.69	6.2	132	1.5	489
Aflatoxin B ₂	maize	2.54	2.6	102	0.1	489
Aflatoxin G ₁	maize	3.27	2.5	76	-1.1	489
Aflatoxin G ₂	maize	1.67	< 1			489
Aflatoxins total	maize	12.1	11.3	93	-0.3	489
Aflatoxin B ₁	pistachio	3.53	3.7	105	0.2	490
Aflatoxin B ₂	pistachio	0.85	1.0	118	0.8	490
Aflatoxin G ₁	pistachio	2.73	2.9	106	0.3	490
Aflatoxin G ₂	pistachio	0.95	1.0	105	0.2	490
Aflatoxins total	pistachio	7.92	8.3	105	0.6	490
Aflatoxin B ₁	chili powder	6.89	6.1	89	-0.5	493
Aflatoxin B ₂	chili powder	0.49	< 1			493
Aflatoxin G ₁	chili powder	n.d.	n.d.			493
Aflatoxin G ₂	chili powder	n.d.	n.d.			493
Aflatoxins total	chili powder	7.53	6.1	81	-0.9	493
Aflatoxin B ₁	baby food	0.145	0.19	131	1.4	494
Aflatoxin B ₁	hazelnut	2.36	3.1	131	1.4	495
Aflatoxin B ₂	hazelnut	0.93	1.2	129	1.3	495
Aflatoxin G ₁	hazelnut	1.75	2.5	143	2.0	495
Aflatoxin G ₂	hazelnut	0.43	< 1			495
Aflatoxins total	hazelnut	5.39	6.8	126	1.2	495
Ochratoxin A	baby food	0.89	0.81	91	-0.4	1734
Ochratoxin A	paprika powder	56.1	60.8	108	0.4	1735
Ochratoxin A	wine	1.8	2.2	122	1.0	1736
Ochratoxin A	barley	8.44	7.1	84	-0.7	1739
Ochratoxin A	dried vine fruit (raisins)	8.3	9.4	113	0.6	1740
Ochratoxin A	baby food	0.60	0.66	110	0.4	1742
Ochratoxin A	animal feed	8.03	9.3	116	0.7	1748

Ochratoxin A	barley	6.16	5.4	88	-0.6	1749
Ochratoxin A	dried vine fruit	8.5	10.3	121	1.0	1750
Ochratoxin A	baby food	0.90	0.88	98	-0.1	1752
Ochratoxin A	paprika powder	18.3	20.8	114	0.6	1753
Fumonisin B ₁	maize	520.0	718	138	1.5	2215
Fumonisin B ₂	maize	230.1	318	138	1.5	2215
Zearalenone	maize	157.8	197	125	1.2	2219
Zearalenone	animal feed	274	233	85	-0.8	2225
DON	dried paste	640	583	91	-0.5	2226
Fumonisin B ₁	maize	2281	2900	127	1.1	2228
Fumonisin B ₂	maize	719	810	113	0.3	2228
DON	wheat flour	1184	1558	132	2.0	2229
Zearalenone	breakfast cereal	52.7	52	99	-0.1	2231