



HAL
open science

Quantitative analysis of mycotoxins in cereal foods by Collision Cell Fragmentation - High Resolution Mass Spectrometry: performances and comparison with triple stage quadrupole detection.

Veronica Maria Teresa Lattanzio, Stefania Della Gatta, Michal Godula,
Angelo Visconti

► To cite this version:

Veronica Maria Teresa Lattanzio, Stefania Della Gatta, Michal Godula, Angelo Visconti. Quantitative analysis of mycotoxins in cereal foods by Collision Cell Fragmentation - High Resolution Mass Spectrometry: performances and comparison with triple stage quadrupole detection.. Food Additives and Contaminants, 2011, pp.1. 10.1080/19440049.2011.593192 . hal-00717081

HAL Id: hal-00717081

<https://hal.science/hal-00717081>

Submitted on 12 Jul 2012

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Quantitative analysis of mycotoxins in cereal foods by Collision Cell Fragmentation - High Resolution Mass Spectrometry: performances and comparison with triple stage quadrupole detection.

Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2011-153.R1
Manuscript Type:	Special Issue
Methods/Techniques:	Chromatography - LC/MS, In-house validation
Additives/Contaminants:	Mycotoxins - fusarium, Mycotoxins – aflatoxins, Mycotoxins – ochratoxin A, Mycotoxins – zearalenone
Food Types:	Bakery products, Cereals
Abstract:	<p>A liquid chromatography - high resolution mass spectrometry (LC-HRMS) method for the simultaneous determination of aflatoxins (B1, B2, G1, G2), ochratoxin A, deoxynivalenol, zearalenone, T-2 and HT-2 toxins in wheat flour, barley flour and crisp bread has been developed. Data presented show the process of obtaining. Mycotoxin fragmentation patterns obtained by High Energy Collision Dissociation (HCD) have been investigated to obtain quantitative and confirmatory information (two characteristic masses per mycotoxin) using Orbitrap™ based high resolution mass spectrometry. LC-HRMS (full scan) detection carried out by HCD allowed to monitor the pseudo-molecular ion and an additional characteristic fragment (for each mycotoxin) with mass accuracy in the range 0.1 – 3.9 ppm, meeting the current European regulatory requirements for LC-MS confirmatory analysis. A sample preparation procedure based on polymeric solid phase extraction cartridges was applied, allowing recoveries higher than 74% for all tested toxins, with relative standard deviation lower than 13%. Detection limits in the range 0.5 – 3.4 µg/kg were obtained for all tested matrices.</p> <p>A critical comparison between the proposed method and a validated method based on triple quadrupole mass spectrometry showed similar performances in terms of detection limits, recoveries and</p>

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

	repeatability, and matrix effects. Based on an efficient sample extraction and clean up, the LC-HCD-HRMS method developed herein represents a reliable and robust alternative tool for mycotoxin analysis in food matrices as compared to well established triple quadrupole based approaches.

SCHOLARONE™
Manuscripts

For Peer Review Only

1
2
3 **Quantitative analysis of mycotoxins in cereal foods by Collision Cell**
4 **Fragmentation - High Resolution Mass Spectrometry: performance and**
5 **comparison with triple stage quadrupole detection.**
6
7
8
9

10
11 **Veronica M. T. Lattanzio^{1,*}, Stefania Della Gatta¹, Michal Godula², Angelo Visconti¹**
12
13

14
15 ¹ *National Research Council of Italy, Institute of Sciences of Food Production, Via Amendola,*
16 *122/O, 70126 Bari, Italy*
17

18 ² *Thermo Fisher Scientific Praha, Slunečná 27 Praha 10, 100 00, Czech Republic*
19
20
21
22
23
24
25

26 * Corresponding Author

27 e-mail: veronica.lattanzio@ispa.cnr.it

28 Institute of Sciences of Food Production (ISPA)

29 National Research Council of Italy (CNR)

30 Via Amendola 122/O

31 70126 Bari - Italy

32 Tel: +39 080 5929364

33 Fax: +39 080 5929374
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60**ABSTRACT**

A liquid chromatography - high resolution mass spectrometry (LC-HRMS) method for the simultaneous determination of aflatoxins (B₁, B₂, G₁, G₂), ochratoxin A, deoxynivalenol, zearalenone, T-2 and HT-2 toxins in wheat flour, barley flour and crisp bread has been developed. Mycotoxin fragmentation patterns obtained by High Energy Collision Dissociation (HCD) have been investigated to obtain quantitative and confirmatory information (two characteristic masses per mycotoxin) using OrbitrapTM based high resolution mass spectrometry. LC-HRMS (full scan) detection carried out by HCD allows the monitoring of the pseudo-molecular ion and an additional characteristic fragment (for each mycotoxin) with mass accuracy in the range 0.1 – 3.9 ppm, meeting the current European regulatory requirements for LC-MS confirmatory analysis. A sample preparation procedure based on polymeric solid phase extraction cartridges was applied, allowing recoveries higher than 74% for nine mycotoxins, with relative standard deviation lower than 13%. Detection limits in the range 0.5 – 3.4 µg/kg were obtained for three cereal matrices. A critical comparison between the proposed method and a validated method based on triple quadrupole mass spectrometry showed similar performance in terms of detection limits, recoveries and repeatability, and matrix effects. Based on an efficient sample extraction and clean-up, the LC-HCD-HRMS method reported here represents a reliable and robust alternative tool for mycotoxin analysis in food matrices as compared to well established triple quadrupole based approaches.

Keywords: LC-HRMS, aflatoxins, ochratoxin A, trichothecenes, zearalenone, cereal foods

INTRODUCTION

Mycotoxins are naturally occurring toxic metabolites which can be produced by fungi infecting agricultural crops during the growth, drying, and subsequent storage. Especially environmental and biological factors such as water activity, temperature, humidity, and insect damage can have a great influence on growth of certain fungi and, therefore, on the spectrum of produced secondary metabolites (Leslie et al. eds 2008). Mycotoxins are small molecules with various chemical structures and, therefore, various biological effects. Generally mycotoxins are stable chemicals and cannot be destroyed during processing or by heat treatment. When present in food in sufficiently high amounts, these fungal metabolites can have toxic effects that range from acute to chronic symptoms. Some mycotoxins were shown to be mutagenic, teratogenic, or/and carcinogenic. Symptoms of intoxications range from skin irritation to immunosuppression, hepatotoxicity, and nephrotoxicity (WHO, 2001). Among chemical hazards, the contamination of foods and feeds by mycotoxins has been identified by the World Health Organization (WHO) as a significant source of food-borne illnesses (WHO, 2001).

Monitoring, control, risk assessment, and prevention of mycotoxins in foods and feeds are important issues worldwide associated with public health, agricultural production, food processing, and trade. Different countries have set regulatory maximum limits and guidelines for relevant mycotoxins such as aflatoxins (AB₁, AFB₂, AFG₁, AFG₂), ochratoxin A (OTA), fumonisins (FB₁, FB₂), deoxynivalenol (DON), and zearalenone (ZEN) (FAO 2004). In Europe, and in particular in the EU, regulatory and scientific interest in mycotoxins has undergone a significant development in the last decade, resulting in the harmonization of stringent legal limits for mycotoxins for approximately 50 toxin–food commodity combinations (European Commission 2006a, 2007). Maximum permitted levels for the sum of T-2 (T-2) and HT-2 (HT-2) toxins in cereals and derived products are currently under discussion.

Analytical methods for the identification and determination of major mycotoxins in food matrices considered by worldwide regulations need to be sensitive, selective and robust to provide accurate data for monitoring, risk assessment, quality control and research. The application of advanced methodologies based on high performance liquid chromatography hyphenated with mass spectrometry (LC-MS) offers a powerful tool to address these needs.

1
2
3 Control laboratories are not forced to use official methods as published by AOAC International or
4 the European Standardization Organization (CEN). However, for each mycotoxin, the values of
5 recovery, repeatability and reproducibility of the analytical method selected by each laboratory
6 must fall within the range of acceptability as prescribed by the Commission Regulation 401/2006
7 (European Commission 2006b). Different types and modes of mass spectrometric detectors provide
8 different degrees of selectivity, which relates to the confidence in identification. The requirements
9 for identification are specified in official documents such as the Commission Decision 2002/657/EC
10 (European Commission 2002) and the Document SANCO/10684/2009 (DG SANCO 2009), the
11 latter specifically dedicated to pesticide residues.
12
13
14
15
16
17
18
19

20
21 Even though some efforts have been made to develop multi-detection methods, combining UV and
22 fluorescence detection in series (Ofitserova et al. 2009, Soleimany et al. 2011) until now the
23 preferred option in multi-mycotoxin analysis is the use of tandem mass spectrometry (mostly based
24 on triple quadrupole mass analyzer) for selective detection and confirmation of target mycotoxins
25 (Songsermsakul and Razzazi-Fazeli 2008, Lattanzio et al. 2011). Therefore, while the potential of
26 tandem mass spectrometry to enable low detection limits and good repeatability has been
27 documented even for complex matrices (Songsermsakul and Razzazi-Fazeli 2008, Lattanzio et al.
28 2007, Lattanzio et al. 2011), there is still limited information on the performance of other types of
29 mass analyzers in multiple mycotoxins analysis in relation to sample preparation strategy. An
30 increasing interest in the application of LC-MS instrumentation delivering high mass accuracy and
31 resolution to mycotoxin analysis is demonstrated by very recent publications as reported in Table 1.
32 One of the key advantages of approaches based on high resolution mass spectrometry (HRMS) is
33 the possibility to obtain full spectral information on target analytes but also to detect and
34 characterize unknown compounds (mycotoxin metabolites and/or conjugation products) in sample
35 extracts on the basis of accurate mass measurements. The few reports on the application of HRMS
36 hyphenated with (ultra)-high performance liquid chromatography for mycotoxin determination in
37 foods show satisfactory performance in terms of recoveries and detection limits (Tanaka 2006 et al.,
38 Vaclavik et al. 2010), but do not fulfill current EU legislation requirements for mass spectrometric
39 detection. In particular, when HRMS is applied for confirmatory analysis it is necessary to provide
40 at least 2 characteristic masses for a target analyte to obtain a sufficient number of identification
41 points (European Commission 2002).
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59

60 A very attractive option for full scan analysis in the field of food safety is now represented by
OrbitrapTM technology (Kellmann et al. 2009), as shown in Table 1, where 4 out of 6 applications

1
2
3 use this technology. Recently the OrbitrapTM mass analyzer has been introduced in a benchtop
4 format without the linear ion trap that normally performs ion accumulation, fragmentation, and
5 analysis of the fragments. This instrument, termed ExactiveTM, is capable of generating
6 fragmentation information in a nonselective manner using a collision cell without precursor ion
7 selection. In this instrument ions are passed through the C-trap into a multipole collision cell where
8 they are fragmented using High Energy Collision Dissociation (HCD). Thereafter, the HCD cell
9 voltages are ramped and ions are transferred back into the C-trap from where they are injected into
10 the OrbitrapTM for detection. In this way structural information can be obtained on compounds of
11 interest and fragment ions can be used for confirmation in targeted analyses.
12
13
14
15
16
17
18
19
20

21 In this study we explored the possibility to employ OrbitrapTM based HRMS for the quantitative
22 determination of regulated mycotoxins in small cereal grains and related foods. An LC-HCD-
23 HRMS method for the simultaneous determination of aflatoxins (B₁, B₂, G₁, G₂), ochratoxin A,
24 deoxynivalenol, zearalenone, T-2 and HT-2 toxins based on OrbitrapTM mass spectrometry has been
25 developed. The use and optimization of HCD conditions to obtain quantitative and confirmatory
26 information, in compliance with EU legislation requirements, are reported together with the
27 performance of the whole analytical procedure in terms of recovery, repeatability and detection
28 limits. A critical comparison between the newly developed method and a recently validated LC-
29 MS/MS method based on triple quadrupole mass analyzer (Lattanzio et al. 2011) is also reported
30 confirming the reliability of the LC-HCD-HRMS method for quantitative and confirmatory analysis
31 of mycotoxins in foods at regulatory levels.
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

MATERIALS and METHODS

Chemicals and Reagents

Acetonitrile, methanol (both HPLC grade) and glacial acetic acid were purchased from Mallinckrodt Baker (Milan, Italy). Ultrapure water was produced by a Millipore Milli-Q system (Millipore, Bedford, MA, USA). Ammonium acetate (for mass spectrometry) was from Sigma-Aldrich (Milan, Italy). Standard mycotoxin solutions and ^{13}C labeled mycotoxin solutions were purchased from Biopure Referenzsubstanzen GmbH (Tulln, Austria). Oasis[®] HLB columns (3mL, 60mg) were purchased from Waters (Milan, Italy). Filter papers (Whatman no. 4) were obtained from Whatman International Ltd (Maidstone, UK). Micro Spin Filter Tubes, (0.20 μm , RC, GRACE) were from Alltech (Deerfield, IL, USA).

Barley flour, durum wheat flour, wheat based and rye based crisp bread were purchased from local retail market. FAPAS[®] proficiency testing materials, namely DON in wheat flour no. T2256, ZEN in breakfast cereals no. T2257, Aflatoxins in maize no. T04148 were obtained from Food and Environment Research Agency (FERA, York, UK).

LC-HRMS equipment and parameters

LC-HRMS analyses were performed on a benchtop single stage mass spectrometer Exactive[™] equipped with a heated electrospray ion source (HESI II) (Thermo Fisher Scientific, Bremen, Germany), coupled to a HPLC system Accela (Thermo Fisher Scientific, San Jose, USA). For the LC-HRMS analyses the HESI II interface was used in positive ion mode, with the following settings: sheath gas: 30 arbitrary units, auxiliary gas: 10 arbitrary units, sweep gas: 0 arbitrary units, capillary voltage: 4000 V. The mass spectrometer operated in a scan range from 50 to 1000 m/z with a resolving power of 100.000 FWHM (full width at half maximum). For the HCD experiments the mass spectrometer operated in positive ion mode MS scans using the HCD cell at different collision energies, namely 10, 20, 30, 35, and 40 eV. The Xcalibur software (version 2.1.0) was used for data acquisition and processing.

The analytical column was a Kinetex C18 (100 mm \times 2.10 mm, 2.6 μm particles) (Phenomenex, Torrance, CA, USA), preceded by KrudKatcher Ultra In-Line Filter (0.5 μm Porosity \times 0.004 in. ID). The flow rate of the mobile phase was 200 $\mu\text{L}/\text{min}$, while the injection volume was 20 μL . The column effluent was directly transferred into the HESI II interface, without splitting. The column oven was set at 40°C. Eluent A was water and eluent B was methanol, both containing 0.5% acetic

1
2
3 acid and 1 mM ammonium acetate. A gradient elution was performed by changing the mobile phase
4 composition as follows. The proportion of eluent B was linearly increased from 10% to 40% in 4
5 min, then increased to 60% in 27 min, and kept constant for 5 min. The column was re-equilibrated
6 with 10% eluent B for 7 min. Before LC-MS/MS analysis all samples were filtered through 0.20
7 μm regenerated cellulose filter.
8
9

10
11
12
13 For comparison purposes a QTrap[®] MS/MS system, from Applied Biosystems (Foster City, CA,
14 USA), equipped with an electrospray interface and a 1100 series micro-LC system comprising a
15 binary pump and a microautosampler from Agilent Technologies (Waldbronn, Germany) was used.
16 The mass spectrometer operated in multiple reaction monitoring (MRM) mode, monitoring 2
17 characteristic fragments (1 quantifier, 1 qualifier) according to parameters described by Lattanzio et
18 al. (Lattanzio et al. 2011). LC conditions were identical to those used above for LC-HRMS analysis.
19 The Analyst software (version 1.4.1) was used for data acquisition and processing.
20
21
22
23
24
25
26
27

28 Mycotoxin detection limits for LC-HRMS and LC-MS/MS methods were experimentally
29 determined by spiking blank samples at different levels down to the lowest detectable
30 concentration.
31
32
33

34 ***Sample preparation (extraction and cleanup)***

35 Sample were extracted and purified according to Lattanzio et al. 2011 (Lattanzio et al. 2011).
36 Ground samples (10 g) were extracted with 50 mL acetonitrile/water 84/16 (v/v) for 60 min on an
37 orbital shaker. The extract was filtered through filter paper (Whatman no. 4), and 5 mL of filtrate
38 (equivalent to 1 g sample) were evaporated to dryness at 50°C under a stream of air. The residue
39 was reconstituted with 100 μL of methanol then 900 μL water were added (to obtain a
40 methanol/water ratio of 10/90 (v/v). The Oasis[®] HLB column was activated and conditioned prior
41 to use as follows. The column was attached to a vacuum manifold, conditioned with 2 mL
42 methanol, and equilibrated with 2 mL methanol/water 10/90 (v/v). Then, the reconstituted sample
43 extract was passed through the column at flow rate of about one drop per second. The column was
44 washed with 1 mL methanol/water 20/80 (v/v) and dried. Afterwards the toxins were eluted with 1
45 mL methanol. The methanol eluate was dried down under air stream, and the residue was
46 redissolved in an appropriate amount of LC mobile phase. In particular, for barley flour, wheat
47 flour, wheat based crisp bread and FAPAS[®] reference materials, the residue was redissolved with
48 200 μL of methanol/water 20/80 by vortexing for 1 min. For rye based crisp bread, the residue was
49 redissolved in 500 μL of methanol/water 20/80. Aliquots of 20 μL (equivalent to 100 mg sample for
50
51
52
53
54
55
56
57
58
59
60

1
2
3 barley flour, wheat flour, wheat based crisp bread or 40 mg sample for rye based crisp bread) were
4 analyzed by LC-HRMS and LC-MS/MS.
5
6
7

8 *Mycotoxin solutions*

9
10 As stock solutions, certified calibrant solutions of DON 100 µg/mL, AFB₁ 2 µg/mL, AFB₂ 0.5
11 µg/mL, AFG₁ 2 µg/mL, AFG₂ 0.5 µg/mL, T-2 100 µg/mL, HT-2 100 µg/mL, ZEN 100 µg/mL,
12 OTA 10 µg/mL, and ¹³C DON 25 µg/mL, ¹³C AFB₁ 0.5 µg/mL, ¹³C AFB₂ 0.5 µg/mL, ¹³C AFG₁ 0.5
13 µg/mL, ¹³C AFG₂ 0.5µg/mL, ¹³C T-2 25 µg/mL, ¹³C HT-2 25 µg/mL, ¹³C ZEN 25 µg/mL, ¹³C OTA
14 10 µg/mL were used.
15
16
17
18

19
20 For optimization of HCD conditions, solutions of individual mycotoxins and ¹³C labeled standards
21 were prepared at concentrations of 5 µg/mL for DON, AFB₁, AFG₁, T-2, HT-2, ZEN, 2 µg/mL for
22 AFB₂ AFG₂, and 1 µg/mL for OTA by diluting adequate amounts of mycotoxin standard solutions
23 with methanol/water 20/80 (v/v).
24
25
26
27

28
29 For spiking experiments a mixed standard solution of 75 µg/mL DON, 0.5 µg/mL AFB₁, 0.1 µg/mL
30 AFB₂, 0.3 µg/mL AFG₁, 0.1 µg/mL AFG₂, 5 µg/mL T-2, 5 µg/mL HT-2, 7.5 µg/mL ZEN and 0.3
31 µg/mL OTA was prepared by drying down appropriate amounts of commercial standard solutions
32 and redissolving them with methanol.
33
34
35
36

37
38 For calibration purposes a mixed standard solution of DON (5 µg/mL), ZEN, T-2, HT-2 (1 µg/mL)
39 AFB₁, AFG₁, OTA (0.1 µg/mL), AFB₂ and AFG₂ (0.025 µg/mL) was prepared by drying down
40 appropriate amounts of commercial standard solutions and re-dissolving them with methanol.
41
42

43 Calibrant solutions for standard calibration curves (6 points) were prepared in the LC mobile phase
44 (methanol/water 40/60 (v/v), containing 1mM ammonium acetate and 0.1% acetic acid) by
45 dissolving adequate amounts of mixed standard solution, previously evaporated to dryness under air
46 stream. Calibrant solutions for external matrix assisted calibration (6 points) were prepared in blank
47 sample extract solutions passed through Oasis[®] HLB columns, according to the above described
48 procedure. Appropriate volumes of mixed standard solution were added to the methanolic eluate
49 before drying it down. Then the residue was re-dissolved with 200 µL of methanol/water 20/80 by
50 vortexing for 1 min. For rye based crisp bread, the residue was re-dissolved in 500 µL of
51 methanol/water 20/80. Twenty microliters were analyzed by LC-MS/MS.
52
53
54
55
56
57
58
59
60

Standard and matrix-matched calibrations were performed in the range 2-100 ng injected for DON, 0.4-20 ng for T-2, HT-2 and ZEN, 0.04-2 ng for AFB₁, AFG₁ and OTA, 0.01-0.5 ng for AFB₂ and AFG₂. For matrix-matched calibration the absolute amount of toxins injected refer to 100 mg sample for barley flour, durum wheat flour, wheat based crisp bread or 40 mg sample for rye based crisp bread.

Recovery experiments

Recovery experiments for the whole analytical procedure were carried out in triplicate at contamination levels of: 300 µg/kg DON, 2 µg/kg AFB₁, 0.4 µg/kg AFB₂, 1.2 µg/kg AFG₁, 0.4 µg/kg AFG₂, 20 µg/kg HT-2, 20 µg/kg T-2, 30 µg/kg ZEN, 1.2 µg/kg OTA. Samples were spiked with 40 µL of mycotoxin mixed solution to obtain the desired level. Spiked samples were left overnight to allow solvent evaporation prior to extraction. In all experiments, the amounts of recovered toxins were calculated by external matrix-matched calibration.

Analysis of reference materials

DON, ZEN and aflatoxins reference materials were analyzed in triplicate. Samples (10 g) were extracted and purified by Oasis[®] HLB as described in "Sample preparation" section. The methanol eluate from the Oasis[®] HLB column was added with an appropriate amount of ¹³C-labeled internal standard stock solution before drying it down (4 µL ¹³C ZEN, 28 µL ¹³C DON, 10 µL ¹³C AFB₁ and ¹³C AFG₁, 4 µL ¹³C AFB₂ and ¹³C AFG₂).

Uncertainty of measurements (U_f) for each mycotoxin was calculated using the formula reported in the Commission Regulation (EC) No 401/2006:

$$U_f = \left[\left(\frac{LOD}{2} \right)^2 + (\alpha \times C)^2 \right]^{\frac{1}{2}}$$

where:

U_f is the maximum standard uncertainty (µg/kg)

LOD is the limit of detection of the method (µg/kg).

α is a constant, numeric factor to be used depending on the value of C. α values to be used are set out in EC regulation No 401/2006 and were 0.2 for aflatoxins, 0.18 for ZEN, and 0.15 for DON.

C is the concentration of interest (µg/kg).

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

RESULTS

LC-HCD-HRMS optimization

The European official guidelines for confirmatory analysis (European Commission 2002, DG SANCO 2009) recommend to use at least 2 diagnostic ions (preferably including the quasi molecular ion) when high resolution mass spectrometry is used for routine screening and quantitation of residues in food. At the same time, both ions should be measured with resolution > 10.000 FWHM and mass accuracy < 5 ppm. The use of HCD to comply with these requirements was evaluated.

In our study, after acquisition of LC-HRMS chromatograms of standard mycotoxins in positive and negative ion modes, signal intensities of protonated $[M+H]^+$ and deprotonated $[M-H]^-$ molecular ion, ammonium $[M+NH_4]^+$ and acetate $[M+CH_3COO]^-$ adducts were compared for each mycotoxin. For quantitative purposes ions with the highest intensity were chosen, namely $[M+H]^+$ for all toxins but T-2 and HT-2 toxins that were detected as $[M+NH_4]^+$ adducts (Table 2).

Using a HCD cell in the ExactiveTM instrument it was possible to obtain a second ion for all tested mycotoxins. For HCD experiments, standard solutions of individual toxins were analyzed. LC-HCD-HRMS chromatograms were acquired for all mycotoxins at different collision energy values to set, for each mycotoxin, a collision energy value giving comparable intensity for precursor ion and the selected fragment ion. Optimized collision energy and selected ions (molecular ion and characteristic fragment) for each mycotoxin are reported in Table 2. The acquisition method was divided into three scan events in order to use the optimal collision energy value for the detection of selected ions for each mycotoxin. Table 2 also reports selected ions for the detection of ^{13}C labeled mycotoxins to be used as internal standards.

To assess the interference of matrix compounds in HCD spectra of target mycotoxins, LC-HCD-HRMS spectra were acquired for each mycotoxin in standard solutions and matrix extracts (wheat flour, barley flour, wheat and rye based crisp bread) passed throughout Oasis[®] HLB column. Figure 1 shows HCD spectra of AFB₁ (1 ng injected, corresponding to 25 μ g/kg in matrix extract) in standard solution (A), in barley flour extract (B) and wheat flour extract (C) after SPE column clean-up. Notwithstanding the presence of peaks attributable to matrix compounds, the main AFB₁

1
2
3 characteristic fragments obtained in HCD spectrum of standard solution were easily detected in
4 wheat and barley flour extracts too. Similar results were obtained for all mycotoxins.

5
6
7 Figure 2 shows a LC-HCD-HRMS chromatogram of a spiked barley flour extract acquired by using
8 a resolving power of 100,000 FWHM. Chromatographic profiles of the two selected diagnostic ions
9 for each mycotoxin, obtained by applying a narrow mass window of 5 ppm, allowed to perform
10 unbiased identification and reliable quantification of all target mycotoxins in matrix extracts after
11 SPE clean-up.
12
13
14
15

16
17 The influence of the matrix compounds (overlapping with nearby masses) on mass accuracy of the
18 ions selected for mycotoxin monitoring was therefore evaluated. Table 3 reports a comparison
19 between mass accuracy values for the two diagnostic ions of each mycotoxin, measured in standard
20 solution and wheat flour, barley flour and wheat- and rye-based crisp bread extracts after SPE
21 clean-up. Values in Table 3 are the average of 3 replicated injections, and refer to 1 ng mycotoxin
22 injected. Similar mass accuracy values were obtained for mycotoxin ions monitored in standard
23 solutions and matrix extracts indicating that under our experimental conditions mass accuracy was
24 not affected by the presence of matrix interfering components. The variations in mass accuracy
25 observed between standard solutions and matrix extracts, and among the different matrices were
26 within the range of instrumental variability. The high selectivity of the detection technique allowed
27 to obtain mass accuracy below 5 ppm in all cases (ranging from 0.1 to 3.9 ppm).
28
29
30
31
32
33
34
35
36
37

38 ***Method performance and comparison between MS/MS and HRMS approaches***

39
40 To evaluate the applicability of the developed LC-HCD-HRMS method for
41 quantitative/confirmatory analysis of mycotoxins in cereal foods at regulatory levels, performance
42 such as recoveries, repeatability, detection limits and matrix effects were studied.
43
44

45 A previously developed sample preparation procedure based on acetonitrile/water extraction
46 followed by SPE clean-up through polymeric cartridges was applied (Lattanzio et al. 2011), then
47 samples were analyzed by LC-HCD-HRMS using the selected conditions reported in Table 2. For
48 comparison purposes, samples were also analyzed by a previously validated LC-MS/MS method
49 using a triple quadrupole mass analyzer operating in MRM mode (Lattanzio et al. 2011).
50
51
52
53
54
55

56 ***Matrix effects: matrix assisted calibration and signal suppression/enhancement***

57 Matrix assisted calibration curves were linear over the working range with r values in the range
58 0.996-1.000 for the LC-HCD-HRMS method and 0.997-0.999 for the LC-MS/MS method. Matrix
59 effects were evaluated by comparing 6 points standard and matrix assisted calibration graphs and by
60

1
2
3 calculating the signal suppression/enhancement (SSE) for each mycotoxin. Table 4 reports the SSE
4 % values obtained by comparing standard and matrix assisted calibration in barley flour extract
5 using LC-HCD-HRMS and LC-MS/MS methodologies after SPE clean-up. A slight ion suppression
6 was observed in most of cases (a SSE of 100% indicated that matrix had no effect on the MS
7 signal). Similar SSE values were obtained for both methods suggesting that matrix effects are likely
8 to be independent from the design of the ion source and the monitoring mode, whereas major
9 factors determining SSE are molecular structure, sample preparation protocol, and column
10 chromatography.
11
12
13
14
15
16
17

18 *Recoveries, repeatability and detection limits*

19 Recovery and repeatability were evaluated for each toxin at contamination levels equal to or lower
20 than EU maximum permitted levels in durum wheat flour, wheat and rye based crisp bread.
21 Quantitative analysis in both LC-MS systems was performed by matrix-assisted calibration.
22 Recoveries of the developed LC-HRMS method ranged from 74% to 105 % with relative standard
23 deviations lower than 13%, in compliance with the EU criteria for acceptance of analytical methods
24 of mycotoxins (European Commission 2006b). Similar recoveries and repeatability values were
25 obtained by analyzing samples with the LC-MS/MS method. Detailed results are reported in Table
26 5.
27
28
29
30
31
32
33
34
35

36 Table 6 reports a comparison of detection limits in durum wheat flour, barley flour, wheat- and rye-
37 based crisp bread obtained by LC-HRMS, with and without the HCD cell, and LC-MS/MS
38 methodologies after SPE clean-up.
39
40
41
42

43 Similar LOD were obtained for all toxins in all tested matrices using LC-HCD-HRMS (range 0.1-
44 2.9 $\mu\text{g}/\text{kg}$) or LC-MS/MS (range 0.1-59.2 $\mu\text{g}/\text{kg}$). Higher LODs (from 2.8 to 59.2 $\mu\text{g}/\text{kg}$) were
45 obtained for DON and ZEN using MRM, since they were detected in negative ion mode suffering
46 of higher baseline noise.
47
48
49
50

51 *Analysis of certified reference materials*

52 To demonstrate the trueness of generated data, the LC-HCD-HRMS method was also applied to
53 analyze FAPAS[®] reference materials. Mycotoxins concentrations were obtained by means of ¹³C
54 isotopically labeled standard addition, and their accuracy was controlled considering the FAPAS
55 assigned value. The experimentally determined concentrations of target analytes were within the
56 satisfactory range for all tested toxins (Table 7). Figure 3 shows extracted ion chromatograms of
57
58
59
60

1
2
3 DON and ^{13}C DON in FAPAS[®] wheat flour and of ZEN and ^{13}C ZEN in FAPAS[®] breakfast cereals.
4
5 Quantifier and confirmatory ion are shown for each mycotoxin and its relevant internal standard.
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Peer Review Only

DISCUSSION

Recent publications reveal an increasing interest on the application of high resolution mass spectrometry to food safety issues. However there is still limited information on the performance of the full scan HRMS approach for reliable quantitative analysis of food contaminants as compared with well established triple quadrupole based methodologies. A basic difference exists between the two detection approaches. By using MRM, the instrument is “instructed” to specifically monitor selected ion masses and ignore other ions. This permits very selective and sensitive detection and analysis when monitoring the presence of one or several chemical compounds in complex food matrices. When full scan acquisition is performed there is no selection *a priori* of the target analytes. This approach however requires high resolution power to correctly detect target masses in a selected range of m/z values. Main advantage of full scan high resolution MS is the possibility to perform untargeted analysis, i.e. to apply post run data processing to search for further analytes that were not considered at the beginning of the analysis.

In the field of mycotoxin analysis this trend is very well reflected by recent studies (Table 1) exploring the potential of application of HRMS, mostly based on OrbitrapTM technology, as a tool for obtaining quantitative determination and full spectral information in an unique analysis. Important requirements to be fulfilled when using full scan MS detection are high selectivity due to the complexity of food matrices, adequate sensitivity to enable mycotoxin detection at regulatory levels (in the $\mu\text{g}/\text{kg}$ range), confirmation of compound identity and accurate quantification of target analytes to comply with acceptance criteria for analytical methods (European Commission 2002, 2006b). Available reports show OrbitrapTM based HRMS to be a very promising technology to address these issues, even though some problems remain to be undertaken and solved. Difficulties when using single stage MS are encountered to obtain confirmatory ions or to detect them with adequate mass accuracy at low concentrations (Zachariasova et al. 2010a). High detection limits, unsuitable to assess mycotoxin contamination at regulatory levels, are obtained when poor sample preparation is applied to recover a wide range of different analytes (Herebian et al. 2009).

In this work a LC-HRMS method for routine analysis of major mycotoxins in cereal foods at regulatory levels has been developed. The use of an OrbitrapTM mass analyzer equipped with a HCD collision cell allowed to obtain two characteristic ions, namely the molecular ion and one fragment, for each mycotoxin, overcoming the problem of obtaining confirmatory ions in single stage full scan mass spectrometry.

1
2
3 Quite satisfactory mass accuracy values, in the range 0.1-3.9 ppm, were obtained for the two
4 monitored ions of each mycotoxin in each tested matrix (Table 3), making possible unbiased
5 analyte identification in complex food extracts. The influence of food matrix components on mass
6 accuracy has been deeply discussed in a recent report (Kellman et al. 2009) showing how the
7 presence of matrix compounds can affect the correct assignment of masses. At this regard it is
8 important to emphasize that, besides the ability of a mass spectrometer to resolve two close peaks
9 on the m/z scale, the accuracy of mass assignments is mainly affected by the ratio of the analyte
10 concentration relative to co-eluting matrix compounds. Data obtained in this study confirmed that,
11 given the complexity of real food matrices, key parameters to guarantee correct mass assignments
12 are the resolution power of the mass spectrometer combined with appropriate extract clean-up and
13 chromatographic separation of target mycotoxins from matrix interfering compounds. To date no
14 criteria for mass accuracy have been set in the 2002/657/EC, that requires only a minimum
15 resolving power of 10,000 FWHM. The need of higher resolving power (up to 100,000 FWHM) in
16 analysis of food contaminants has been recently highlighted by several authors (Kellman et al.
17 2009, Van der Heeft et al. 2009, Thurman et al. 2006) that agreed on the need to incorporate mass
18 accuracy into identification points system defined in the Commission Decision. At this regard it
19 should be noted that mass accuracy < 5 ppm is already required for the analysis of pesticide
20 residues in foods and feeds according to the recent Document No. SANCO/10684/2009.

21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37 Based on obtained detection limits and results of recovery experiments, the whole analytical
38 procedure proposed herein can be considered suitable for accurate quantification of aflatoxins,
39 ochratoxin A, deoxynivalenol, zearalenone, T-2 and HT-2 toxins at levels close to or lower than
40 maximum permitted levels in the tested cereal foods.

41
42
43
44
45
46 A critical comparison between the proposed LC-HCD-HRMS method and a validated LC-MS/MS
47 method (Lattanzio et al. 2011) revealed that by applying adequate sample preparation and
48 chromatographic conditions similar method performance by using either LC-MS/MS or LC-HCD-
49 HRMS could be obtained. The two methodologies gave similar matrix effects, recovery values and
50 detection limits. Furthermore 4 identification points were earned either monitoring two MRM
51 transition or two characteristic ions with high resolution (100,000 FWHM in the present work). The
52 general conclusion is therefore that by using either the LC-MS/MS or the LC-HCD-HRMS
53 approach the quality of generated data is the same and legislation requirements can be fulfilled with
54 respect to recoveries, detection limits and criteria for MS detection.

1
2
3 To date, the only example of a similar comparison reports confusing results (Herebian et al. 2009)
4 leading to the wrong perception that the whole method performance can be dramatically affected by
5 the use of different LC-MS instrumentation. When comparing LC-MRM and LC-HRMS (full scan)
6 detection modes in multi-mycotoxin analysis of crude extracts the authors observed different
7 performance between the two approaches in terms of matrix effects. As an example, for aflatoxin B₁
8 65% and 51% SSE were observed in maize and wheat respectively using the MRM detection,
9 whereas 130% SSE was observed in the same samples when using full scan HRMS detection.
10 Similar results were obtained for other mycotoxins. Furthermore an unexplainable increase of
11 matrix effects was observed for some toxins after dilution of the sample extract. Results of our
12 study clearly indicate that when adequate sample preparation and good chromatography are applied,
13 matrix effects are almost independent from the instrumentation design or the detection approach.
14 Methods based on direct injection of crude extract very often show poor detection limits, inadequate
15 to comply with current regulatory levels. For example, Herebian et al. (Herebian et al. 2009), even
16 using a very sophisticated instrumentation (LTQ-OrbitrapTM, Thermo Fisher), reported for
17 wheat/maize LOD values of 2000 µg/kg for DON, 20 µg/kg for aflatoxins, 20 µg/kg for OTA, 100
18 µg/kg for ZEN, 20 µg/kg for T-2 and 100 µg/kg for HT-2. In the present study LODs of one-two
19 orders on magnitude lower, in the range 0.5 – 3.4 µg/kg, were obtained for all mycotoxins in all
20 investigated matrices by using a more simple and less expensive instrumentation (ExactiveTM,
21 Thermo Fisher), coupled with a suitable sample preparation protocol.
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37

38 CONCLUSION

39
40 In the present study a LC-HCD-HRMS method for the simultaneous determination of aflatoxins
41 (B₁, B₂, G₁, G₂), ochratoxin A, deoxynivalenol, zearalenone, T-2 and HT-2 toxins in cereal-based
42 foods has been developed. The process of obtaining quantitative and confirmatory information
43 using OrbitrapTM based high resolution mass spectrometry has been shown. In three food matrices
44 (wheat flour, barley flour and crisp bread) two ions were detected for each toxin with suitable mass
45 accuracy meeting European regulatory requirements for confirmatory analysis. A sample
46 preparation protocol based on polymeric solid phase extraction cartridges was applied. The
47 resulting analytical procedure exhibited satisfactory recoveries and adequate detection limits to
48 assess mycotoxin contamination in cereal foods at regulatory levels. A critical comparison between
49 the proposed method and a validated method based on triple quadrupole mass spectrometry has
50 been carried out with respect to sensitivity, recoveries and repeatability, and matrix effects.
51 Comparable performance were obtained with the two procedures revealing that the LC-HCD-
52 HRMS is a reliable and robust alternative tool for routine analysis of major mycotoxins in foods,
53
54
55
56
57
58
59
60

1
2
3 with the additional advantage of possibility to perform retrospective analysis, i.e. to search for
4 mycotoxin metabolites/conjugates (degradation products or “masked” forms) at a second stage
5 without the need to re-analyze the sample. At this regards it should be pointed out that sample
6 preparation itself implies some selection of recovered analytes. The present method is based on a
7 general extraction solvent, and clean-up on SPE columns containing a balanced lipophilic
8 hydrophilic polymer able to retain compounds with a wide range of polarity. This sample
9 preparation procedure can be considered a sort of “generic” protocol enabling to recover
10 compounds in a wide range of polarities, keeping open the perspective of retrospective investigation
11 on mycotoxins metabolites and structurally related compounds.
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

REFERENCES

DG SANCO. 2009. Document No. SANCO/10684/2009. Method validation and quality control procedures for pesticide residues analysis in food and feed.

European Commission. 2002, Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Off J Eur Commun. L 221/8.

European Commission 2006a, Commission Regulation of 19 December 2006 setting maximum levels of certain contaminants in foodstuffs. Off. J. Eur Union, L364/5.

European Commission. 2006b, Commission Regulation of 23 February 2006: Laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Off J Eur Commun L 70/12.

European Commission. 2007. Commission Regulation of 28 September 2007 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards *Fusarium* toxins in maize and maize products. Off. J. Eur Union L255/14.

FAO. 2004. Food and Agriculture Organization, 2004. Worldwide regulations for mycotoxins in food and feed in 2003, Food and Nutrition Paper no. 81, Rome, Italy.

Herebian D, Zuhlke S, Lamshoft M, Spiteller M. 2009. Multi-mycotoxin analysis in complex biological matrices using LC-ESI/MS: Experimental study using triple stage quadrupole and LTQ-Orbitrap. J Sep Sci 32: 939-948.

Kellmann M, Muenster H, Zomer P, Mol H. 2009. Full Scan MS in comprehensive qualitative and quantitative residue analysis in food and feed matrices: how much resolving power is required? J Am Soc Mass Spectrom 20:1464-1476.

Lattanzio VMT, Solfrizzo M, Powers S, Visconti A. 2007. Simultaneous determination of aflatoxins, ochratoxin A and *Fusarium* toxins in maize by liquid chromatography/tandem mass spectrometry after multitoxin immunoaffinity cleanup. Rapid Commun Mass Spectrom 21:3253-3261.

Lattanzio VMT, Della Gatta S, Suman M, Visconti A. 2011. Development and in house validation of a robust and sensitive solid phase extraction - LC-MS/MS method for the quantitative determination of aflatoxins B₁, B₂, G₁, G₂, ochratoxin A, deoxynivalenol, zearalenone, T-2 and HT-2 toxins in cereal based foods. Rapid Commun Mass Spectrom 25:1-12.

1
2
3 Leslie JF, Bandyopadhyay R, Visconti A eds. 2008. *Mycotoxins. Detection Methods,*
4 *Management, Public Health and Agricultural Trade.* CAB International Press, Wallingford, UK.
5
6

7 Ofitserova M, Nerkar S, Pickering M, Torma L, Thiex N. 2009. Multiresidue mycotoxin analysis
8 in corn grain by column high-performance liquid chromatography with postcolumn photochemical
9 and chemical derivatization: single-laboratory validation. *J AOAC Int.* 92:15-25.
10
11

12 Soleimany F, Jinap S, Rahmani A, Khatib A. 2011. Simultaneous detection of 12 mycotoxins in
13 cereals using RP-HPLC-PDA-FLD with PHRED and a post-column derivatization system. *Food*
14 *Addit Contam* 28:494-501.
15
16
17

18 Songsermsakul P, Razzazi-Fazeli E. 2008. A review of recent trends in applications of liquid
19 chromatography-mass spectrometry for determination of mycotoxins. *J Liquid Chromatogr Rel*
20 *Technol* 31:1641-1686.
21
22
23

24 Tanaka H, Takino M, Sugita-Konishi Y, Tanaka T. 2006. Development of a liquid
25 chromatography/time-of-flight mass spectrometric method for the simultaneous determination of
26 trichothecenes, zearalenone and aflatoxins in foodstuffs. *Rapid Commun Mass Spectrom* 20:1422-
27 1428.
28
29
30

31 Thurman EM, Ferrer I, Zweigenbaum JA. 2006. High resolution and accurate mass analysis of
32 xenobiotics in food. *Anal. Chem.* 78:6702-6708.
33
34
35

36 Vaclavik L, Zachariasova M, Hrbek V, Hajslova J. 2010. Analysis of multiple mycotoxins in
37 cereals under ambient conditions using direct analysis in real time (DART) ionization coupled to
38 high resolution mass spectrometry. *Talanta* 82:1950-1957.
39
40
41

42 Van der Heeft E, Bolcj YJC, Beumer B, Nijrolder AWJM, Stolket AAM, Nielen MWF. 2009.
43 Full-scan accurate mass selectivity of ultra-performance liquid-chromatography combined with
44 time-of-flight and Orbitrap mass spectrometry in hormone and veterinary drug residue analysis.
45 *JAm. Soc Mass Spectrom* 20:451-463.
46
47
48

49 World Health Organization (WHO) 2001, *Evaluation of certain mycotoxins in food.* Fifty-sixth
50 report of the Joint FAO/WHO Expert Committee on Food Additives, WHO Technical Report Series
51 906, WHO, Geneva, Switzerland.
52
53
54

55 Zachariasova M, Cajka T, Godula M, Malachova A, Veprikova Z, Hajslova J. 2010. Analysis of
56 multiple mycotoxins in beer employing (ultra)-high-resolution mass spectrometry. *Rapid Commun*
57 *Mass Spectrom* 24:3335-3367.
58
59
60

1
2
3 Zachariasova M, Lacina O, Malachova A, Kostelanska M, Poutska J, Godula M, Hajslova J.
4
5 2010. Novel approaches in analysis of *Fusarium* mycotoxins in cereals employing ultra
6
7 performance liquid chromatography coupled with high resolution mass spectrometry. Anal Chim
8
9 Acta 662:51-61.
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Peer Review Only

Table 1. Overview of LC-HRMS methods for multi-mycotoxin analysis in foods and related performance.

Reference	Target mycotoxins-matrix	Extraction solvent – clean-up	LOD range (µg/kg)	Recovery range (%)	Type of LC-MS detection	Mass accuracy*
Tanaka et al. 2006	13 mycotoxins (including DON, ZEN, HT-2, T-2, AFs) in corn, wheat, cornflakes, biscuits.	Acetonitrile/water (85:15) Multisep #226	Wheat: 0.1-3.8 Corn: 0.1-4.9	Wheat: 71-132 Corn: 81-133	LC-APCI-TOFMS	≤ 2.5 ppm
Zachariasova et al. 2010a	11 mycotoxins (including DON, HT-2, T-2, ZEN, FBs) in maize, wheat, barley	Water 0.1% formic acid/acetonitrile (QuEChERS)	Maize:5-50	Maize: 43-120	UPLC-ESI-TOF-MS	≤ 9.6 ppm
		Water 0.1% formic acid/acetonitrile (1:1) Direct injection	Maize:10-30	Not tested	UPLC-APCI-Orbitrap™ MS	≤ 5 ppm
Vaclavik et al. 2010	12 mycotoxins (including DON, de-epoxyDON, acetyl-DON, nivalenol, ZEN) in wheat and maize	Water/acetonitrile (3:4) QuEChERS	Maize/Wheat: 80-100	Maize/Wheat: 95-118	DART- Orbitrap™ MS	≤ 3.8 ppm
Zachariasova et al. 2010b	32 mycotoxins (including DON, HT-2, T-2, ZEN, AFs, OTA) in beer	Acetonitrile	0.5 – 60 µg/L	86-124	UPLC-APCI-Orbitrap™ MS	≤ 5 ppm (confirmatory ion, in opposite polarity)
Herebian et al. 2009	32 mycotoxins (including DON, HT-2, T-2, ZEN, FBs, AFs, OTA) in wheat and maize	Acetonitrile/water/acetic acid, (79:20:1) Direct injection	Maize:0.4-2000 Wheat:0.4-200	Maize:68-152 Wheat:87-131	LC-ESI-LTQ-Orbitrap™	≤ 1 ppm

*absolute value (1 ion detected)

Table 2. Selected ions, adduct specification, theoretical mass, collision energy value for mycotoxin detection and quantification.

Mycotoxin	Molecular formula	Calculated mass	Mycotoxin	Molecular formula	Calculated mass	HCD (eV)
DON [M+H] ⁺	C ₁₅ H ₂₁ O ₆	297.13381	¹³C DON	¹³ C ₁₅ H ₂₁ O ₆	312.18406	15
	C ₁₄ H ₁₅ O ₃	231.10157*		¹³ C ₁₄ H ₁₅ O ₃	245.14847*	
AFB₁ [M+H] ⁺	C ₁₇ H ₁₃ O ₆	313.07066	¹³C AFB₁	¹³ C ₁₇ H ₁₃ O ₆	330.12816	35
	C ₁₄ H ₉ O ₄	241.04953*		¹³ C ₁₄ H ₉ O ₄	255.09643*	
AFB₂ [M+H] ⁺	C ₁₇ H ₁₅ O ₆	315.08631	¹³C AFB₂	¹³ C ₁₇ H ₁₅ O ₆	332.14326	35
	C ₁₆ H ₁₅ O ₅	287.09140*		¹³ C ₁₆ H ₁₅ O ₅	303.14500*	
AFG₁ [M+H] ⁺	C ₁₇ H ₁₃ O ₇	329.06558	¹³C AFG₁	¹³ C ₁₇ H ₁₃ O ₇	346.12308	35
	C ₁₄ H ₁₁ O ₄	243.06518*		¹³ C ₁₄ H ₁₁ O ₄	257.11208*	
AFG₂ [M+H] ⁺	C ₁₇ H ₁₅ O ₇	331.08123	¹³C AFG₂	¹³ C ₁₇ H ₁₅ O ₇	348.13818	35
	C ₁₄ H ₁₃ O ₄	245.30808*		¹³ C ₁₄ H ₁₃ O ₄	259.35498*	
HT-2 [M+NH ₄] ⁺	C ₂₂ H ₃₆ NO ₈	442.24409	¹³C HT-2	¹³ C ₂₂ H ₃₆ NO ₈	447.29124	10
	C ₁₅ H ₁₇ O ₃	245.11722*		¹³ C ₁₅ H ₁₇ O ₃	260.16754*	
T-2 [M+NH ₄] ⁺	C ₂₄ H ₃₈ NO ₉	484.25460*	¹³C T-2	¹³ C ₂₄ H ₃₈ NO ₉	491.30851*	10
	C ₁₄ H ₁₅ O ₂	215.10660		¹³ C ₁₄ H ₁₅ O ₂	229.15350	
ZEN [M+H] ⁺	C ₁₈ H ₂₃ O ₅	319.15450*	¹³C ZEN	¹³ C ₁₈ H ₂₃ O ₅	337.21485*	10
	C ₁₈ H ₁₉ O ₃	283.13287		¹³ C ₁₈ H ₁₉ O ₃	301.19317	
OTA [M+H] ⁺	C ₂₀ H ₁₉ NO ₆ Cl	404.08950*	¹³C OTA	¹³ C ₂₀ H ₁₉ ClNO ₆	424.15709*	10
	C ₁₉ H ₁₇ NO ₄ Cl	358.08406		¹³ C ₁₉ H ₁₇ O ₄ NCl	377.14771	

* quantifier ion

Table 3. Mass accuracy of quantifier and qualifier ions for each mycotoxin, measured in LC-HCD-HRMS chromatograms of standard solutions and cereal food extracts after SPE clean-up.

Mycotoxin	Calculated mass	Mass accuracy (ppm)**				
		Standard solution	Wheat flour	Barley flour	Crisp bread (wheat based)	Crisp bread (rye based)
DON	297.13381	2.2	0.2	0.6	2.1	3.9
	231.10157*	1.0	0.4	0.3	1.5	1.9
AFG ₂	331.08123	0.9	0.3	1.7	0.3	0.8
	245.30808*	0.5	0.6	1.1	0.6	1.5
AFG ₁	329.06558	1.2	0.2	1.5	1.5	1.8
	243.06518*	1.2	0.5	1.3	0.8	1.3
AFB ₂	315.08631	1.2	0.2	1.6	2.2	0.9
	287.09140*	1.1	0.4	1.7	3.3	1.5
AFB ₁	313.07066	1.1	0.1	1.3	0.4	2.6
	241.04953*	1.0	0.3	1.2	0.4	1.2
HT-2	442.24409	1.2	0.2	0.3	1.7	1.6
	245.11722*	1.2	0.6	1.2	1.5	1.6
T-2	484.25460*	0.7	0.4	1.7	1.6	1.9
	215.10660	0.4	0.1	0.5	0.1	1.6
ZEN	319.15450*	1.0	0.1	0.6	1.5	2.2
	283.13287	0.9	0.4	2.1	1.9	1.5
OTA	404.08950*	0.8	0.1	0.5	2.9	1.6
	358.08406	1.0	0.3	0.8	2.5	1.5

* quantifier ion

** absolute value, average of triplicate injections of 1 ng toxin (relevant to 40 mg matrix for wheat and barley flour and wheat based crisp bread, and to 100 mg matrix for rye based crisp bread).

Table 4. SSE % values obtained by comparing standard and matrix assisted calibration in barley flour extract using LC-HCD-HRMS and LC-MRM methodologies after SPE clean-up.

Mycotoxin	Calibration range ($\mu\text{g}/\text{kg}$)	SSE, %	
		HCD-HRMS	MRM
DON	50-2500	76	95
AFG₂	0.25-12.5	100	95
AFG₁	1-50	100	89
AFB₂	0.25-12.5	67	88
AFB₁	1-50	100	88
HT-2	10-500	90	95
T-2	10-500	94	103
ZEN	10-500	65	70
OTA	1-50	87	106

Table 5 Comparison of recovery and repeatability values obtained in durum wheat flour, wheat-based and rye-based crisp bread by using LC-HRMS and LC-MRM methodologies after SPE clean up.

		Recoveries, % (RSDr, %)								
		DON	AFG ₂	AFG ₁	AFB ₂	AFB ₁	HT-2	T-2	ZEN	OTA
Spiking level (µg/kg)		300	0.4	1.2	0.4	2	20	20	30	1.2
Wheat flour	MRM	95 (2)	n.d.	82 (4)	84 (6)	89 (4)	95 (4)	92 (4)	95 (9)	74 (7)
	HCD-HRMS	102 (5)	90 (8)	89 (0)	95 (2)	81 (6)	104 (4)	98 (6)	76 (6)	97 (9)
Wheat crisp bread	MRM	100 (0)	n.d.	106 (5)	85 (10)	102 (6)	107 (2)	108(6)	84 (5)	101 (3)
	HCD-HRMS	104 (0)	102 (5)	104 (4)	80 (2)	102 (2)	105 (1)	103(1)	85 (1)	93 (2)
Rye crisp bread	MRM	95 (3)	91 (7)	79 (2)	85 (7)	77 (3)	97 (2)	91 (3)	96 (7)	82 (2)
	HCD-HRMS	105 (1)	93 (2)	95 (6)	93 (8)	87 (4)	100 (3)	95 (3)	101 (9)	74 (13)

Table 6 Comparison of detection limits in durum wheat flour, barley flour and wheat- and rye-based crisp bread by using LC-HRMS, with and without HCD, and LC-MRM methodologies after SPE clean-up.

	Detection limits ($\mu\text{g}/\text{kg}$)											
	Wheat flour			Barley flour			Crisp bread (wheat based)			Crisp bread (rye based)		
	HRMS	HCD-HRMS	MRM	HRMS	HCD-HRMS	MRM	HRMS	HCD-HRMS	MRM	HRMS	HCD-HRMS	MRM
DON	0.2	1.6	3.9	0.2	1.8	10.3	0.3	3.4	29.0	0.5	2.3	59.2
AFG₂	0.1	1.5	0.1	0.1	0.5	0.2	0.1	0.2	0.4	0.1	0.5	1.9
AFG₁	0.1	0.6	0.2	0.1	1.1	0.7	0.2	0.1	0.7	0.3	1.2	2.6
AFB₂	0.1	0.7	0.3	0.1	0.5	0.3	0.1	0.2	0.4	0.1	0.5	1.1
AFB₁	0.1	1.0	0.3	0.1	1.0	0.5	0.1	0.4	0.5	0.1	1.6	1.1
HT-2	0.3	1.7	0.3	0.2	2.5	1.1	0.3	1.0	0.5	0.4	1.7	1.7
T-2	0.2	1.0	0.2	0.2	0.5	0.5	0.3	2.9	0.5	0.5	1.6	0.9
ZEN	0.8	1.0	2.8	0.3	1.4	4.0	0.4	1.0	2.2	1.6	2.3	5.8
OTA	0.2	1.4	0.1	0.6	1.9	0.3	0.5	0.4	0.1	0.5	2.9	0.4

Table 7. Results of analysis of reference materials, by LC-HCD-HRMS after SPE clean-up

Material description	Target analyte	Assigned value, $\mu\text{g}/\text{kg}$	Satisfactory range, $\mu\text{g}/\text{kg}$	Level of ^{13}C-IS addition, $\mu\text{g}/\text{kg}$	Results obtained by HRMS analysis, $\mu\text{g}/\text{kg}$
wheat flour FAPAS T2256	DON	774	517 - 1032	700	756 ± 113.4
breakfast cereals FAPAS T2257	ZEN	69.5	38.9 - 100.1	100	48.6 ± 9.8
maize, FAPAS T04148	AFs	AFB ₁ 5.07	AFB ₁ 2.84 - 7.30	AFB ₁ 5.0	AFB₁ 5.3 ± 1.3
		AFB ₂ 1.06	AFB ₂ 0.60 - 1.53	AFB ₂ 2.0	AFB₂ 1.0 ± 0.3
		AFG ₁ 2.97	AFG ₁ 1.66 - 4.27	AFG ₁ 5.0	AFG₁ 2.2 ± 0.8
		AFG ₂ 1.25	AFG ₂ 0.70 - 1.80	AFG ₂ 2.0	AFG₂ 1.2 ± 0.4

Figure 1. HCD spectra of aflatoxin B₁ in standard solution (A) and in barley flour (B) and wheat flour (C) extracts after clean-up by Oasis[®] HLB column. Amount of injected toxin: 1 ng, corresponding to 25 µg/kg in matrix extracts.

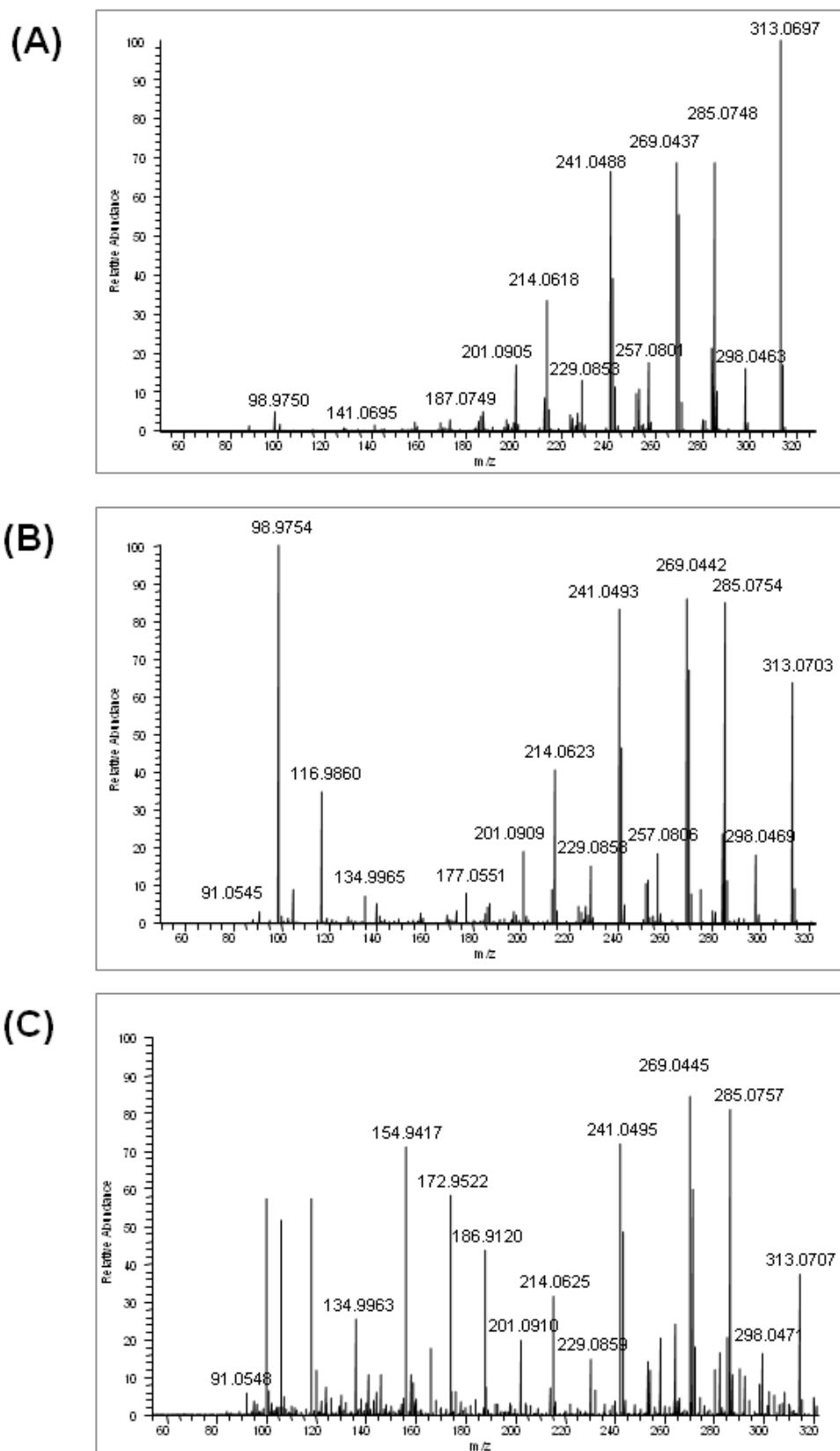


Figure 2. LC-HCD-HRMS chromatogram of a barley flour extract spiked with 12 µg/kg of DON, , T-2, HT-2, ZEN, OTA, 3 µg/kg of AFG₁, AFB₁ and 1 µg/kg of AFG₂, AFB₂.

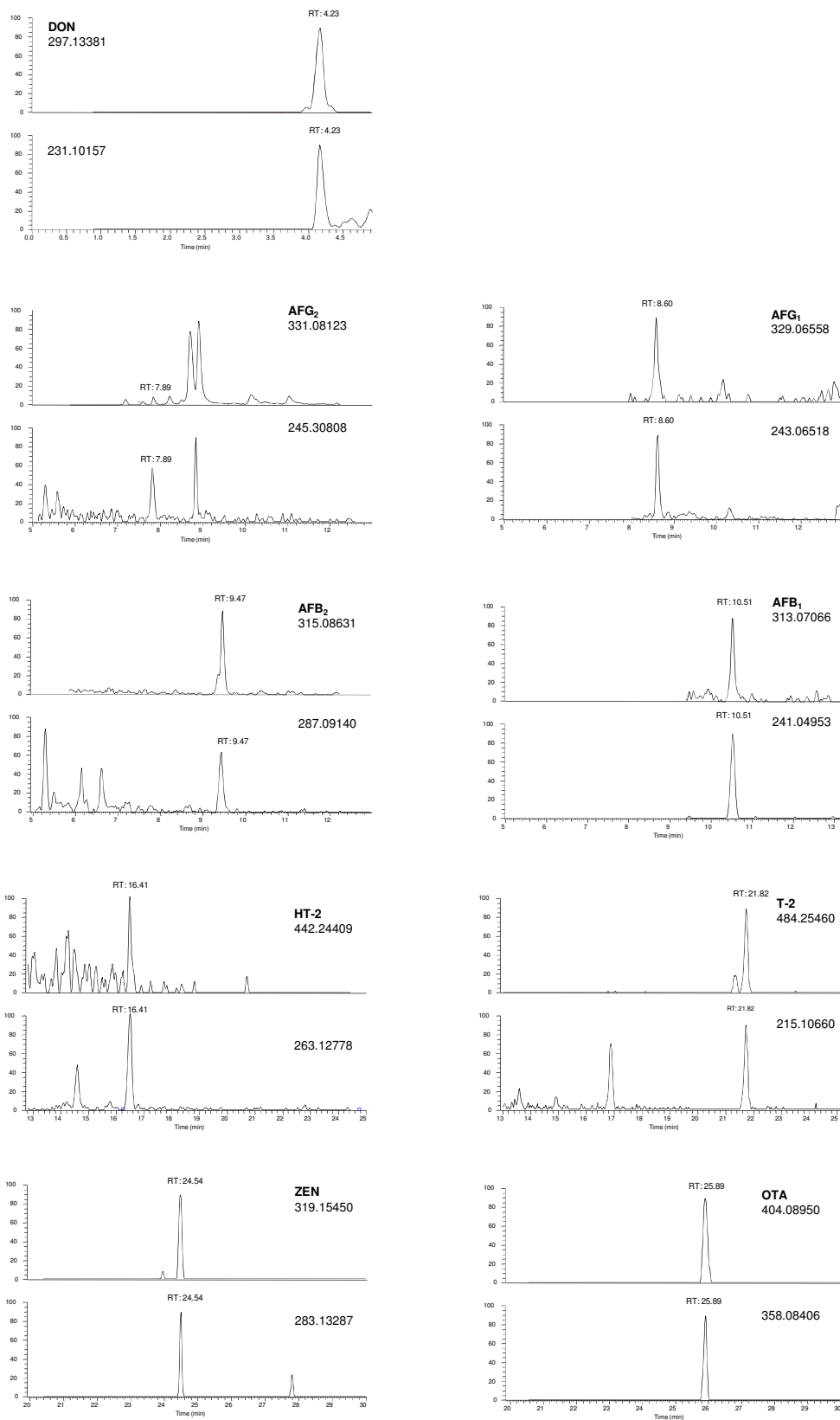


Figure 3. Extracted ion chromatograms of DON and ^{13}C DON in FAPAS[®] wheat flour (A) and of ZEN and ^{13}C ZEN in FAPAS[®] breakfast cereals (B).

