Evaluation of QuEChERS for mycotoxin screening

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Author: Luis Ruano Miguel

Contact point: Chris Hopley

Christopher.hopley@lgcgroup.com

+44 (0) 2089437678

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Approved by:

Dr Julian Braybrook

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Summary

Various appropriate extraction and clean-up approaches have been applied to cereal matrices (maize flour, wheat flour, pasta, bread, breakfast cereals and beer), black peppercorn and groundnuts (peanuts).

The extracts were analysed for different mycotoxins (nivalenol, deoxynivalenol-3-glucoside, deoxynivalenol, Fusarenon-X, 3 & 15-acetyldeoxynivalenol, fumonensins B1 & B2, aflatoxins B1, B2, G1 & G2, diacetoxyscirpenol, T-2, HT-2, Zearalenone and ochratoxin A) using liquid chromatography with high resolution mass spectrometry with promising results.

A general strategy with described workflows based on QuEChERS is therefore proposed for the screening of mycotoxins in a variety of commodities susceptible to mycotoxin contamination. The proposed strategy represents an alternative to existing lengthy clean-up methods for specific mycotoxins.

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1 Background

Mycotoxins are toxic secondary metabolites produced by certain fungal species such as *Aspergillus* and *Fusarium* and can appear in a great variety of foods resulting from mould contamination in the field or during storage. Due to the complexity of food matrices and structural diversity of mycotoxins their simultaneous and sensitive analysis is challenging.

Extraction methods based on QuEChERS (quick, easy, cheap, effective, rugged, and safe[*]) have become more popular in the research field of mycotoxins due to its simplicity and effectiveness for isolating mycotoxins from complex matrices. First ideated for the analysis of pesticides, the successful application of this method for the analysis of mycotoxins has been reported recently [1]. Typically, samples are extracted with acetonitrile with partition of the water and acetonitrile phases being achieved using a combination of salts. The organic phase is then cleaned-up using dispersive Solid Phase Extraction (dSPE). A variety of sorbents are available; octadecyl silica (C18), primary secondary amine (PSA), and graphitized carbon black (GCB) are the most frequent.

The maximum concentrations of mycotoxins allowed in food for human consumption are set in Europe by Commission Regulation 1881/2006 [2] and Commission Recommendation 2013/165/EU [3] (see Table 1). (A maximum is also set in Regulation 1881/2006 for ergot alkaloids).

Different application notes and articles explore the possibilities of applying QuEChERS to the analysis of mycotoxins. However, they often apply it to one single food commodity. In order to support potential Government Chemist referee cases relating to these analytes, a screening approach for different matrices would be highly beneficial.

[*] Anastassiades M, Lehotay SJ, Stajnbaher D, Schenck FJ (2003) Residues and trace elements fast and easy multi-residue method employing acetonitrile extraction partitioning and ‘dispersive solid-phase extraction’. *JAOAC Int* 86:412‑431

Table 1. Summary of limits set by European legislation of mycotoxins in food for human consumption

Legislation	Mycotoxin	Food commodity	Limits (range for human consumption) [µg kg^{-1}]
Commission Recommendation 2013/165/EU.	T-2, HT-2 (sum of)	Cereals, cereal products	15 (infants and young children), 25–200.
Commission Regulation 1881/2006	Aflatoxins	Nuts, peanuts, almonds, pistachios, dried fruits, cereals, spices, milk	0.1 (B_1 in food for (infants and young children), 2–8 (B_1), 4–10 (sum of B_1, B_2, G_1, G_2) 0.025 - 0.050 (M1).
	Ochratoxin A	Cereals, dried fruit, coffee, wine, grape juice, spices, liquorice	0.50 (food for infants and young children), 2–80.
	Patulin	Apple, apple juice, cider	10 (baby food), 10–50.
	Deoxynivalenol	Cereals, cereal products	200 (baby food for infants and young children), 500–750.
	Zearalenone	Cereals, cereal products	20 (baby food for infants and young children), 50–100.
	Fumonisins	Maize	200 (baby food for infants and young children), 800–1000.
	Citrinin	Rice	2000

Routine analysis of mycotoxins carried out using LC-MS quantitation often uses triple quadrupole detectors (QqQ), which provide low resolution and are typically only capable of nominal mass determination. However QqQ detectors offer specific fragmentation and achieve high specificity when running in selected reaction monitoring (SRM) mode. Most recent models of high resolution mass spectrometers (HRMS) have overcome the drawbacks of initial HRMS models based on time of flight (ToF): mass resolution and linear range. Previous work carried out at the UK's National Measurement Laboratory (NML) at LGC has shown the feasibility of the use of a QuEChERS extraction with HRMS and accurate mass for the analysis of T-2 and HT-2 in cereals [1].

Even in the absence of known mycotoxins, they might still occur in conjugated form and be undetected by targeted detection techniques. Soluble conjugated mycotoxins have been nominated as “masked” or “modified” mycotoxins. These mycotoxins can emerge after metabolism by living plants, fungi and mammals or after food processing. In general, metabolised masked mycotoxins present less toxicity than their respective parent intact forms. However it is believed that many modified mycotoxins are hydrolysed into the parent compounds or released from the matrix during digestion [4,5]. Awareness of such altered forms of mycotoxins is increasing, but reliable analytical methods, measurement standards, and their occurrence and associated toxicity data are still lacking. A 2015 report from the European Food Safety Authority

(EFSA) [4] concluded that there is a need for more information on the chemical structures of modified mycotoxins and to identify modified mycotoxins not yet characterised. Likewise, it is also pointed out that properly validated and sensitive routine analytical methods for modified mycotoxins are needed. A more recent study [5] also recognised this, even if no hydrolysis occurred when using artificial digestive juices in the upper gut; the topic is still very complex and further studies are required using animal models.

The main problems encountered in the analysis of masked mycotoxins are the large number that may potentially exist and the lack of analytical standards. The use of HRMS as detection method could overcome these issues, with the help of specific software for data mining the full scan information in search of unknown compounds [6].

In this work, extraction and clean-up approaches based on QuEChERS were investigated for screening analysis in different matrices of a total of seventeen different mycotoxins (nivalenol, deoxynivalenol-3-glucoside, deoxynivalenol, Fusarenon-X, 3 & 15-acetyldeoxynivalenol, fumonensins B_1 & B_2, aflatoxins B_1, B_2, G_1 & G_2, diacetoxyscirpenol, T-2, HT-2, Zearalenone and ochratoxin A). The extracts have been analysed using LC with HRMS with promising results. A general strategy with different workflows has been proposed for the screening of mycotoxins in a variety of food commodities susceptible to mycotoxin contamination. This represents an alternative to already existing lengthy clean-up methods for specific mycotoxins. Satisfactory results have been obtained for cereal products and nuts.

2 Experimental details

2.1 Equipment

- Horizontal shaker.
- Head-over-heels mixer for 50 mL tubes.
- Centrifuge

(Note: specific equipment makes are not specified)

2.2 Chemicals

- Methanol and acetonitrile were obtained from LGC Standards (Teddington, UK).
- Formic acid was obtained from Thermo Fisher Scientific (Loughborough, UK).
- Acetic acid was obtained from Sigma-Aldrich (Dorset, UK).
- Ultrapure water (18 MΩ·cm) was obtained in-house using an Elga water purification unit (Veolia Water Technologies UK, High Wycombe, UK).
- All analytical standards were obtained from LGC Standards) as acetonitrile solutions. See Table 2 for a detailed list. Mixed dilutions of the stock solutions were made in acetonitrile at appropriate concentrations before fortification of the samples.

Table 2. List of stock solutions in acetonitrile, analytes, abbreviations and respective concentrations

Reference	Contents	Abbreviation	Concentration [mg/L]
Mycotoxins mix 2 B-MYC0700-1	3-Acetyldeoxynivalenol	3-AcDON	100
	15-Acetyldeoxynivalenol	15-AcDON	100
	Nivalenol	NIV	100
	Deoxynivalenol	DON	100
Mycotoxins mix 1 B-MYC0440-1	Fumonisin B1	FB1	50
	Fumonisin B2	FB2	50
Mycotoxins mix 4 B-MYC0750-1	3-Acetyldeoxynivalenol	3-AcDON	10
	Nivalenol	NIV	10
	Deoxynivalenol	DON	10
	Fusarenon-X	FusX	10
	Diacetoxyscirpenol	DAS	10
	HT-2	HT-2	10
	T-2	T-2	10
	Zearalenone	ZEN	10
Mycotoxins mix 9 B-MYC0310-5	Aflatoxin B1	AB1	1
	Aflatoxin B2	AB2	1
	Aflatoxin G1	AG1	1
	Aflatoxin G2	AG2	1
B-MYC0600-5	Zearalenone	ZEN	100
B-MYC0490-5	Ochratoxin A	OTA	10
B-MYC0360-1	15-Acetyldeoxynivalenol	15-AcDON	100
B-MYC0335-1	deoxynivalenol-3-glucoside	DON-3glc	50
B-MYC0540-1	T-2 toxin	T-2	100
B-MYC0560-1	HT-2 toxin	HT-2	100
B-MYC0550-1.2	13C24 T-2 toxin	13C24 T-2	25
B-MYC0565-1.2	13C22 HT-2 toxin	13C22 HT-2	25

2.3 Matrix materials

- Maize flour (TRS Fine Cornmeal, ASDA)
- Beer (Brewdog pale ale)
- Whole breakfast cereals (essential Waitrose bran flakes)
- Whole wheat semolina penne pasta (Tesco)
- Brown bread
- Cornflakes (Tesco)
- Brown wheat flour (Tesco)
- Oats, everyday value (Tesco)
- Whole Black Peppercorns (Tesco)
- Peanuts blank (Foods Team)
- Incurred materials:
    - Black pepper: FAPAS T04309QC from Fera Science (York, UK).
    - Maize: 2017 Multi-Mycotoxin PT from CODA-CERVA (NRL Belgium)
    - Maize: FAPAS T04312QC from Fera Science (York, UK)
    - Groundnuts (Foods Team).

2.4 Consumables

- 50-mL polypropylene tubes
- Unbuffered QuEChERS sachets: 1 g NaCl + 4 g $MgSO_4$ (Agilent ref. 5982-5550)

- EN buffered (citrate) QuEChERS sachets: DisQuE QuEChERS, 1 g trisodium citrate dihydrate, 0.5 g disodium hydrogencitrate sesquilhydrate, 1 g NaCl and 4 g $MgSO_4$ (Waters ref. 186006813).
- DisQuE QuEChERS, 900 mg $MgSO_4$ & 150 mg PSA, C18, 15 mL Tube, 50/pkg (Waters ref. 186004834).
- Bond Elut EMR-Lipid dispersive SPE (Agilent ref. 5982-1010).
- Bond Elut EMR-Lipid Polish Tube, NaCl/anhydrous $MgSO_4$. (Agilent ref. 5982-0101)

2.5 Sample preparation and extraction

All those samples that did not come as a homogeneous blend were comminuted to a fine powder and homogenised using a coffee grinder.

Details for sample extraction are specified in the Results and Discussion sections of the different experiments.

2.6 Liquid Chromatography method

- Instrument: Waters Acquity H-Class UPLC
- Column: Waters Symmetry C18, 3.5 µm, 4.6 × 75 mm PN WAT066224
- Column temperature: 40 ℃
- Sample tray temperature: 10 ℃
- Injection volume: 10 µL
- Solvent A: water/acetic acid: 100/0.5 (v/v)
- Solvent B: acetonitrile/methanol/acetic acid: 50/50/0.5 (v/v/v)
- Solvent gradient:

Time [min]	Flow rate [mL/min]	% B
0.0	0.50	5
1.0	0.50	5
9.0	0.50	100
9.6	0.50	100
9.7	0.65	100
12.0	0.65	100
12.5	0.65	5
14.9	0.65	5
15.0	0.50	5

2.7 Mass Spectrometry method

- Instrument: Waters Xevo G2-XS QToF
- Ion source: ESI
- Ionisation mode: positive or negative
- Collision energy: 6
- Acquisition mode: sensitivity, continuum
- Capillary voltage: 2 kV in negative, 3 kV in positive
- Cone voltage: 40 kV
- Source temperature 100 ℃
- Drying gas temperature: 500 ℃
- Cone gas: 100 L/h
- Drying gas: 600 L/h
- Mass range: 100–800 m/z

- Scan rate: 0.5 s/scan
- Lock mass: LeuEnk (m/z 556.2771, 3 kV; 10 µL/min, 0.5 s/scan, 1 scan every 30 s
- Divert valve timetable: to MS from 4 to 10. To waste all other times.

2.8 Data analysis

Data analysis was carried out using Targetlynx (Waters, Wilmslow, UK), the quantitation software provided with the instrument. The following parameters were used: mass windows: 15 ppm, smoothing: Savitzky–Golay, 2 iterations, width 1. The software generated extracted-ion chromatograms for each species along with peak areas automatically. Table 3 shows the list of detected compounds and their respective retention times, species detected and ionisation modes.

Table 3. List of all detected mycotoxins with their respective m/z, ionisation mode and retention times. Ticks indicate the preferred ionisation mode in matrix samples

		+ESI			−ESI			
Analyte	Molecular formula		Species detected	m/z		Species detected	m/z	Retention time [min]
NIV	$C_{15}H_{20}O_7$				➨	$+AcO^-$	371.134	4.5
DON-3glc	$C_{21}H_{30}O_{11}$		$+Na^+$	481.169	➨	$+AcO^-$	517.192	5.0
DON	$C_{15}H_{20}O_6$		$+H^+$	297.134	➨	$+AcO^-$	355.139	5.2
FusX	$C_{17}H_{22}O_8$		$+Na^+$	377.121	➨	$+AcO^-$	413.145	5.9
15-AcDON	$C_{17}H_{22}O_7$	➨	$+Na^+$	361.126				6.8
3-AcDON	$C_{17}H_{22}O_7$		+H+	339.144	➨	$+AcO^-$	397.150	6.8
FB-1	$C_{34}H_{59}NO_{15}$	➨	$+H^+$	722.396				6.9
AG-2	$C_{17}H_{14}O_7$	➨	$+Na^+$	353.064				7.2
AG-1	$C_{17}H_{12}O_7$	➨	$+Na^+$	351.048				7.5
AB-2	$C_{17}H_{14}O_6$	➨	$+Na^+$	337.069				7.6
FB-2	$C_{34}H_{59}NO_{14}$	➨	$+H^+$	706.401				7.8
AB-1	$C_{17}H_{12}O_6$	➨	$+Na^+$	335.053				7.8
DAS	$C_{19}H_{26}O_7$	➨	$+NH_4^+$	384.202				8.1
HT-2	$C_{22}H_{32}O_8$		$+Na^+$	447.200	➨	$+AcO^-$	483.223	8.7
T-2	$C_{24}H_{34}O_9$	➨	$+Na^+$	489.210				9.3
ZEN	$C_{18}H_{22}O_5$		+H+	319.155	➨	$-H^+$	317.139	9.5
OTA	$C_{20}H_{18}ClNO_6$	➨	$+H^+$	404.090		$-H^+$	402.074	9.7

3 Results and discussion

3.1 LC separation and MS detection

Several columns were tested for the separation of the different mycotoxins using solvent standards. Good separation among all the analytes with good peak shape was obtained with a Waters Symmetry C18 column (75×4.6 mm, 3.5 um, PN WAT066224). Separation conditions are indicated in 2.6. Example chromatograms are shown in Figures 1 and 2 for both positive and negative modes, respectively.

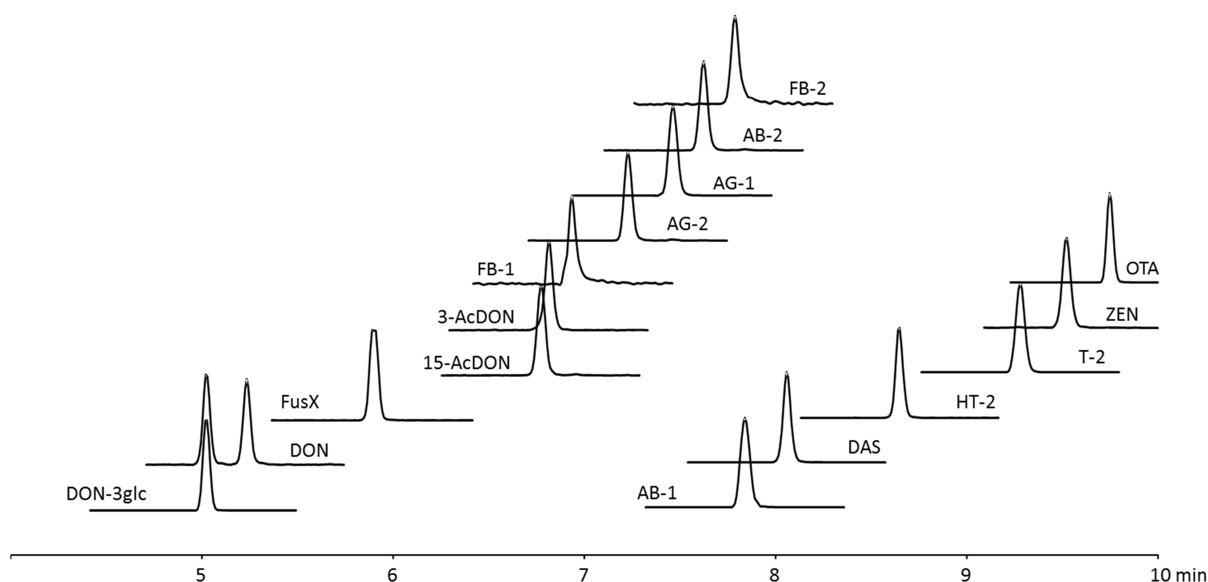


Figure 1. Chromatogram in positive ionisation mode of a standard solution. Chromatographic conditions in 2.6

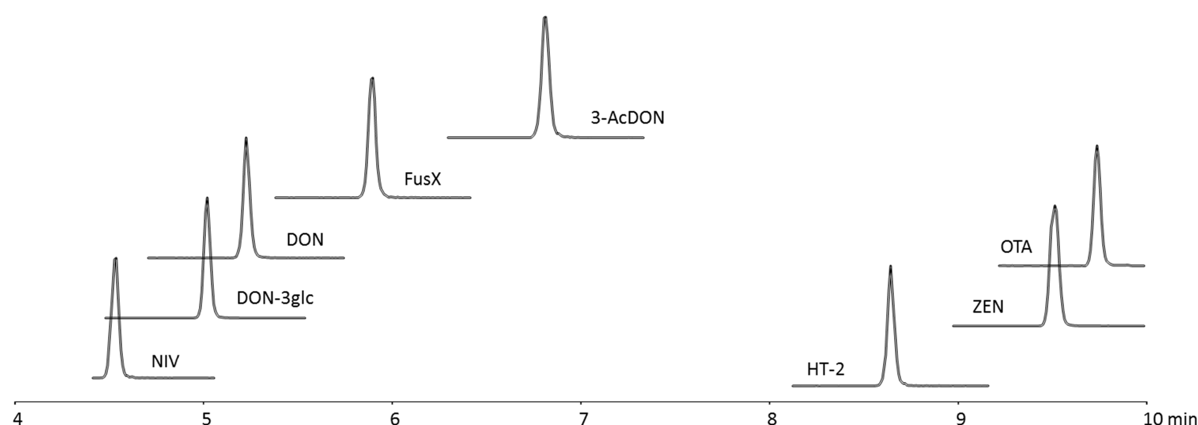


Figure 2. Chromatogram in negative ionisation mode of a standard solution. Chromatographic conditions in 2.6

Mobile phases were based on previous work [1]. Acetic acid at 0.5 % was needed for both the ionisation of analytes in positive ionisation mode and the formation of acetate adducts detected in negative ionisation mode. Results obtained using formic acid instead of acetic acid resulted in slightly lower sensitivities in negative mode.

A generic gradient from 5 % to 100 % organic phase was used for the separation with good results. Initial higher ratios of aqueous phase resulted in bad peak shape of the more polar compounds (NIV, DON-3glc and DON). Use of 100 % methanol in the organic mobile phase caused a significant increase in sensitivity for 3-AcDON, 15-AcDON and HT-2 in positive mode at the expense of longer retention times, worse peak shapes for all analytes and no separation between the isomers (3 and 15-AcDON) compared to separations obtained using 100% acetonitrile. Separation of these two isomers is convenient due to isobaric interferences. Final organic mobile phase was methanol/acetonitrile (1/1, v/v). Using these conditions a slight separation is obtained between 3 and 15-AcDON while still obtaining good results for HT-2 in negative mode.

3.2 First extractions

In order to check the general behaviour of the analytes during the extraction using QuEChERS variations and investigate possible matrix effects and interferences, an example matrix (maize flour, TRS cornmeal) was extracted and cleaned up following different methodologies.

The extraction was based on previous work [1] and the method described by Oplatowska-Stachowiak *et al.* for the analysis of mycotoxins in distiller's dried grain with solubles DDGS [7]. Briefly: 2 g of sample were weighed in 50-mL polypropylene tubes and vortexed for 30 seconds with 10 mL of 1 % formic acid in water. After 30 min, 10 mL of acetonitrile was added and the tubes were shaken for 30 min on a horizontal shaker.

Phase separation was induced by different methods:

A) salting-out by the addition of unbuffered QuEChERS salts
B) citrate buffered QuEChERS salts
(samples were vortexed immediately for 30 s to avoid salt agglomerations in both cases before shaking for 1 min)
C) by freezing the tubes at –20 °C for 48 h. Extrac ts were centrifuged for 5 min at 4000 rpm to help the solvent partition.

A) and B) and acetonitrile upper layers were cleaned up by:

X) dispersive SPE: 6 mL added to PSA/C18/$MgSO_4$ 15 mL tubes
Y) dispersive SPE: 1 mL added to PSA/$MgSO_4$ 2 mL tubes, dSPE tubes were centrifuged before taking aliquots for evaporation
Z) freezing 1 mL of extract at –20 °C for 3 h.

Aliquots (0.75 mL) of acetonitrile extracts of different combinations (phase separation/clean up) were evaporated and reconstituted in 0.25 mL of MeOH/water/formic acid (50/50/0.1, v/v/v). Extracts were fortified before reconstitution to yield a concentration equivalent to 100 µg/kg in sample. 20 µL of the final extracts were run on the LC-MS.

After the qualitative analysis of the results the following conclusions could be drawn:

- Comparing the TIC and UV (210 and 254 nm) chromatograms with that of a solvent standard at the same level, the extracts after a dSPE step looked cleaner (less interferences) compared to those without
- Looking at the extracted exact masses of all the analytes the d-SPE'ed extracts shown less suppression but still notable, especially in positive ionisation mode. Hence it could be concluded that:
    - The accurate determination of the concentration of mycotoxins after a QuEChERS extraction must be accompanied by the use of isotopically labelled analogues as internal standards. Screening would be still possible without, though
    - Any differences between the effects of two different dSPE phases (with and without C18) were unremarkable
    - The suppression in negative ionisation mode is less intense, with signals comparable to those of the solvent standard, except for 3 and 15-AcDON and FusX
    - The matrix employed contains large amounts of fumonisins. They contain carboxylic acids in their structure and are captured almost completely by the PSA sorbent of the dispersive SPE phase. These are only seen in the non-dSPEed extracts, but they tend to saturate the detector. As it will be explained later (3.7) the legal limits for this family of compounds allow injecting diluted extracts without the need of further clean-up
    - Weak signals were observed for 3 and 15-AcDON, their ionisation seems to be suppressed to a great extent both in positive and negative ionisation modes.
    - No signal was observed for FusX in any of the extracts.

The following experiments were carried out using the A-X phase separation combinations. Acidic mycotoxins (ochratoxins and fumonisins) have to be extracted at low pH values and buffered salts could reduce the recovery for these compounds. No differences were observed between the extracts treated with or without C18 phase, but samples susceptible to containing mycotoxins often exhibit fatty extractable material and a dSPE phase containing C18 was chosen for all subsequent work. Final injection volume was lowered to 10 µL due to better S/N ratio and less suppression found when less volume is injected.

The maize matrix already tested was analysed again with the chosen extraction method together with a bran breakfast cereals sample. Different sample sizes were tested (2 and 5 g). In this case the matrices were fortified before the extraction (pre-extraction spiked matrix extracts, PrEMS), at 10 and 50 µg kg^{-1} (except aflatoxins: 1 and 5 µg kg^{-1}) and their absolute signals compared against post-extraction spiked matrix extracts (PoEMS). The matrices exhibited different behaviours, with the cereals showing an increased background noise and severe suppression of the ionisation. An indication of the total amount of background matrix eluted throughout the analysis can be deduced from the UV absorption chromatograms (Figure 3).

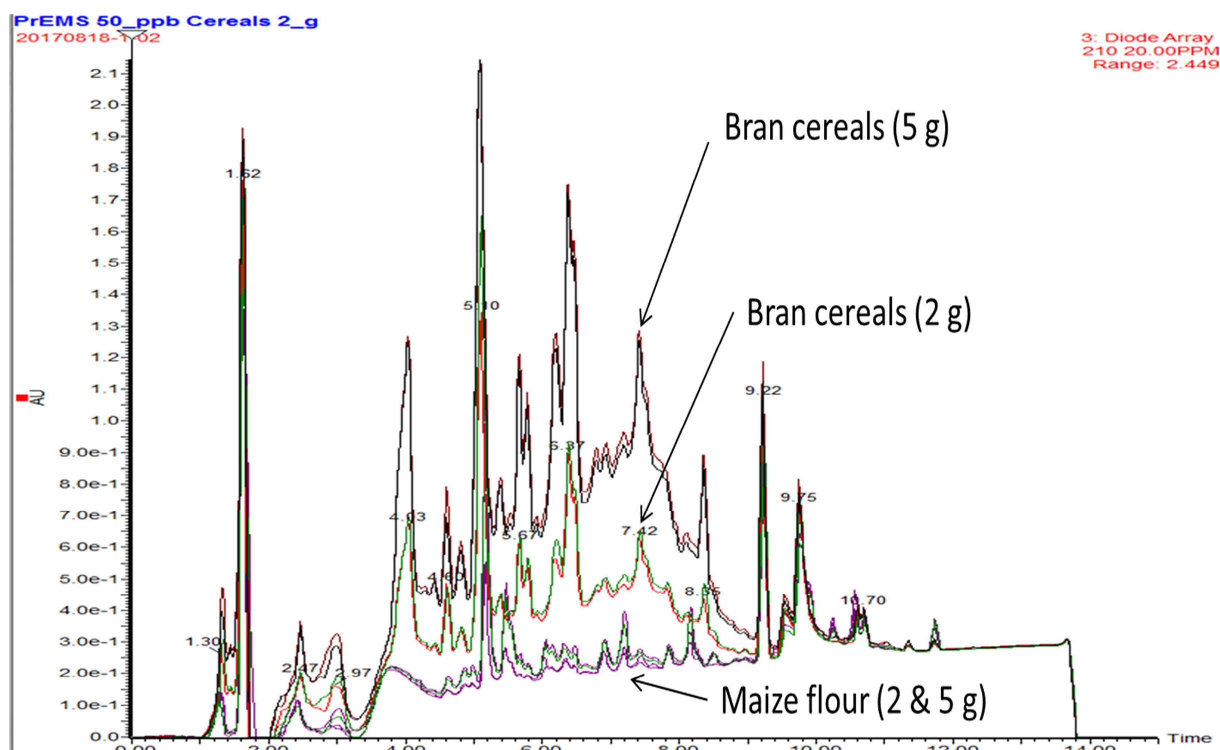


Figure 3. UV chromatograms (absorbance at 210 nm) of maize flour and bran cereals QuEChERS extracts (2 & 5 g sample size)

Bran cereal samples yielded very dirty extracts with complete MS suppression of the analytes, with the exception of ZEN in negative mode. This behaviour indicates great matrix-to-matrix variation even for samples within the same commodity (dry cereal products with moderate content of salt and fat). Further experiments studied the performance of the method with a larger variety of cereal matrices (see Section 3.3).

Better results were obtained from the maize samples. Less matrix effects can be obtained using 2 g of sample. Acceptable signal-to-noise and recoveries are found for the following compounds at the lower level: 3-AcDON, AB1, AG1, T-2 and OTA (positive ESI) and ZEN, DON, 3-DONglc, NIV and OTA (negative ESI).

It should be taken into account that the concentrations used in this experiment are lower than the limits set in the legislation, with few exceptions. This fact shows the potential of a QuEChERS screening method at higher levels.

3.3 Study of 7 cereal matrices

A similar study was conducted on seven different cereal-based products following a similar extraction protocol: 2 g of sample were hydrated with 10 mL water (1% formic acid) in 50 mL polypropylene tubes (except beer: 8 mL + 100 µL formic acid). After 30 min 10 mL of acetonitrile and couple of ceramic homogenisers were added and the tubes were shaken for 30 min on a rotary mixer for 20 min. A sachet of 5 g of unbuffered QuEChERS salts was added and the tubes were vortexed for 30 s and then centrifuged at 4000 rpm for 5 min. 6 mL of the supernatant were added to 15-mL tubes containing PSA+C18+$MgSO_4$ and vortexed for 30 s. The tubes were centrifuged for 5 min at 3500 rpm. 1 mL of the extract was evaporated and reconstituted in 330 µL of MeOH/water (1/1). The final extracts were filtered through 0.22-µm nylon spin filters.

The matrices were:

- Brown bread
- Maize flour, TRS cornmeal
- Corn flakes, cheap value Tesco
- Brown wheat flour, Tesco
- Whole pasta, Tesco
- Oats, everyday value Tesco
- Beer, Brewdog pale ale

Samples were fortified before the extraction (PrEMS) at 20 and 100 μg kg^{-1} except DON-3glc (10 and 50 μg kg^{-1}) and aflatoxins (2 and 10 μg kg^{-1}). Blank extracts were fortified after the extraction (PoEMS) at the higher level to estimate the extraction efficiency.

An overview of the results is shown in Table 4. Despite the initial data obtained from the QuEChERS extraction applied to the bran cereal sample (3.2), general good analyte behaviour was observed from the different cereal matrices with the exception of 15-AcDON and fumonisins. As mentioned above, fumonisins are sequestrated by the PSA contained in the dSPE phase and an alternative approach will be proposed.

Extraction efficiency and sensitivity was moderated for the two more polar compounds: NIV and DON-3glc. It should be noted however that the fortification levels are still low for these compounds (compared to the limits for the only legislated type B trichothecene: DON, > 500 μg kg^{-1}) and better results are expected at higher concentrations. Recoveries should not represent a problem for screening methods as long as the sensitivity is sufficient and pre-extracted matrix matched standards are used for quantitation, especially if an isotopically labelled internal standard is used.

The analysis of the blanks of some of the matrices studied presented detectable and sometimes significant amounts of some mycotoxins: 3-DONglc (oats), DON (maize flour, bread and wheat flower), T-2 (oats), ZEN (maize flour, corn flakes and bread), HT-2 (oats and pasta), NIV (maize flour). For fumonisins in maize flour, a very intense signal was observed even after the clean-up with dispersive PSA indicating very high levels of these mycotoxins in this sample.

All mycotoxins were fortified at levels at least equal to those listed in the current legislation, with the exception of OTA (limit in cereal products: 3 μg kg^{-1}). As will be explained later, further clean-up or different strategy is needed in order to achieve this level.

Table 4. Overview of results obtained after fortification of 7 cereal-based products with mycotoxins at two different levels: signal to noise and recovery (rec)

Aflatoxin	Beer	Bread	Corn flakes	Wheat flour	Maize flour	Oats	Whole pasta
NIV	5 ☹ 25 😐 Rec 😐	20 ☹ 100 😐 Rec ☺	20 ☹ 100 ☹ Rec ☹	20 ☹ 100 😐 Rec 😐	20 ☹ 100 😐 Rec 😐	20 ☹ 100 😐 Rec 😐	20 ☹ 100 😐 Rec 😐
DON-3glc	2.5 ☹ 12.5 ☹ Rec ☹	10 😐 50 😐 Rec 😐	10 ☹ 50 😐 Rec ☹	10 😐 50 ☺ Rec 😐	10 😐 50 ☺ Rec 😐	10 ☹ 50 ☺ Rec 😐	10 ☹ 50 ☺ Rec 😐
DON	5 😐 25 ☺ Rec ☺	20 😐 100 ☺ Rec ☺	20 😐 100 ☺ Rec 😐	20 ☺ 100 ☺ Rec ☺	20 😐 100 ☺ Rec ☺	20 😐 100 😐 Rec ☺	20 ☺ 100 ☺ Rec ☺
FusX	5 ☹ 25 ☺ Rec ☺	20 ☹ 100 ☺ Rec ☺	20 ☹ 100 😐 Rec 😐	20 😐 100 ☺ Rec ☺	20 ☹ 100 ☺ Rec ☺	20 😐 100 ☺ Rec ☺	20 ☺ 100 ☺ Rec ☺
15-AcDON	➔	➔	➔	➔	20 ☹ 100 ☺ Rec ☺	➔	➔
3-AcDON	5 ☹ 25 ☺ Rec ☺	20 ☹ 100 ☺ Rec ☺	20 😐 100 ☺ Rec 😐	20 ☺ 100 ☺ Rec ☺	20 😐 100 ☺ Rec ☺	20 😐 100 ☺ Rec ☺	20 😐 100 ☺ Rec ☺
Fumonisins	➔	➔	➔	➔	➔	➔	➔
Aflatoxins	0.5 ☺ 2.5 ☺ Rec ☺	2 ☺ 10 ☺ Rec ☺	2 ☺ 10 ☺ Rec 😐	2 ☺ 10 ☺ Rec ☺	2 ☺ 10 ☺ Rec ☺	2 ☺ 10 ☺ Rec ☺	2 ☺ 10 ☺ Rec ☺
DAS	5 ☺ 25 ☺ Rec ☺	20 ☹ 100 ☺ Rec ☺	20 😐 100 ☺ Rec 😐	20 ☹ 100 ☺ Rec ☺	20 ☺ 100 ☺ Rec ☺	20 ☺ 100 ☺ Rec ☺	20 ☺ 100 ☺ Rec ☺
HT-2	5 ☺ 25 ☺ Rec ☺	20 😐 100 ☺ Rec ☺	20 😐 100 ☺ Rec 😐	20 ☺ 100 ☺ Rec ☺	20 ☺ 100 ☺ Rec ☺	20 ☺ 100 ☺ Rec ☺	20 ☺ 100 ☺ Rec ☺
T-2	5 ☺ 25 ☺ Rec ☺	20 ☺ 100 ☺ Rec ☺	20 ☺ 100 ☺ Rec ☺	20 ☺ 100 ☺ Rec ☺	20 ☺ 100 ☺ Rec ☺	20 ☺ 100 ☺ Rec ☺	20 ☺ 100 ☺ Rec ☺
ZEN	5 😐 25 ☺ Rec ☺	20 ☺ 100 ☺ Rec ☺	20 ☺ 100 ☺ Rec 😐	20 ☺ 100 ☺ Rec ☺	20 ☺ 100 ☺ Rec ☺	20 ☺ 100 ☺ Rec ☺	20 ☺ 100 ☺ Rec ☺
OTA	5 ☹ 25 ☺ Rec ☹	20 ☺ 100 ☺ Rec ☺	20 ☺ 100 ☺ Rec 😐	20 ☺ 100 ☺ Rec ☺	20 ☺ 100 ☺ Rec ☺	20 ☺ 100 ☺ Rec ☺	20 ☺ 100 ☺ Rec ☺

Legend:

➔: not detected (15-AcDON), or meaningless results (fumonisins)
☹: S/N < 3, Rec < 30
😐: S/N 3–10 , 30 < Rec < 70
☺: S/N > 10, Rec > 70

3.4 Incurred cereal samples

The method already described in Section 3.3 for cereal products was applied to two different incurred samples:

- Maize: 2017 Multi-Mycotoxin PT from CODA-CERVA (NRL Belgium)
- Maize: T04312QC from FAPAS.

0.25 mL aliquots of the extracts were taken before the dispersive SPE step in order to be able to detect and quantify fumonisins (which are retained in the dSPE phase) and diluted with 0.75 µL water before the injection on the LC-MS. This parallel line of work represents a simplification of

the general QuEChERS workflow and it should also be valid for the screening of mycotoxins for which high levels are expected.

The fortification levels were chosen to be closer to the ones set by the legislation than in previous analyses. In addition labelled T-2 and HT-2 were added to all samples at 50 µg kg^{-1}. A blank sample more resembling the incurred matrices was included (TRS Fine Cornmeal). Levels are shown in Table 5.

Table 5. Fortification levels (in µg kg^{-1}) of blank and incurred maize samples

		Incurred		Blanks	
Analyte	**(limit in maize)**	**2017 CODA-CERVA PT**	**FAPAS T04312QC**	**TRS fine Cornmeal**	**Tesco cornflakes**
OTA	(3)	0, 6		0, 3, 6	
Aflatoxins	(2)	0, 2		0, 1, 2	
ZEN	(75)	0, 60		0, 30, 60	
T-2 & HT-2	(100)	0, 100		0, 50, 100	
DON NIV 3,15-AcDON fumonisins	(750) (1000)	0, 400		0, 200, 400	

Blank samples were fortified after the extraction (PoEMS) to have an estimate of the extraction efficiency (recovery).

Positive results were obtained for several compounds both from blank and incurred samples.. Concentrations were estimated by the standard addition method using the fortified samples and results are shown in Table 6. Absolute responses are exhibited for all compounds except T-2 and HT-2, for which area ratios with their labelled analogue were used.

Although some compounds (DON, ZEN and HT-2) are better detected in negative mode, good results are also obtained using positive ionisation. The good response of aflatoxins allows their detection even in the diluted, non-cleaned-up sample, although more reliable results are obtained after a clean-up and pre-concentration step.

Sensitivity of OTA is very weak, with S/N ratio of about 3 for the low-level PrEMS. Estimated concentration of the FAPAS sample is about 3.2 µg kg^{-1} (reported value of 2.2 µg kg^{-1}). Further work is needed to improve the detection and quantitation of OTA at such low levels. It was however detected in the CODA-CERVA sample at high levels.

In general, good agreement was found between the estimated concentrations and the levels given by the FAPAS T04312QC certificate, indicating the potential of a QuEChERS strategy for the screening of different types of mycotoxins. The use of internal standards and more calibration levels would be needed for a more accurate quantitation. The results of the CODA-CERVA PT have not been made public yet, but results will be compared if they become available.

Table 6. Estimated concentrations (µg kg-1) in maize matrices by standard addition
ND: not detected, D: detected but not quantified

		Dilute-shoot			Dispersive SPE			Certificate[(a)]
Mode	**Analyte**	**TRS fine Cornmeal**	**2017 CODA-CERVA PT**	**FAPAS T04312QC**	**TRS fine Cornmeal**	**2017 CODA-CERVA PT**	**FAPAS T04312QC**	**FAPAS T04312QC**
–	NIV	ND	151	ND	ND	75	ND	–
– +	DON	89 (216)	669 (1313)	551 (818)	29 (115)	867 (919)	646 (580)	731 (486–976)
+	15-AcDON	ND	1118	ND	ND	1504	ND	–
-	3-AcDON	ND	ND	ND	ND	ND	ND	–
+	FB1	768	D [(b)]	336	–	–	–	463 (297–629)
+	FB2	258	D [(b)]	78	–	–	–	108 (61–156)
+	AB1	ND	ND	5	ND	13.1	3	3.1 (1.7–4.4)
+	AB2	ND	ND	ND	ND	ND	ND	–
+	AG1	ND	2.6	D	ND	1.9	ND	–
+	AG2	ND	ND	ND	ND	ND	ND	–
– +	HT-2	ND	94	75	ND	62 (354)	66 (130)	120 (67–173)
+	T-2	ND	949	80	ND	3053	73	109 (61–157)
– +	ZEN	ND	454 (D)	122 (102)	ND	544 (857)	125 (201)	141 (80–201)
+	OTA	–	–	–	ND	D	3.2	2.2 (1.2–3.1)

(a) Between brackets range for |z|≤2
(b) Estimated concentration of fumonisins in the CODA-CERVA PT sample is very high in comparison to the fortification level. It is not possible to predict the concentration, but it is of the order of several thousand µg kg^{-1}.

3.5 Incurred black pepper

A spice sample (black pepper) incurred with aflatoxins and OTA (FAPAS T04309QC) was extracted using the same method applied in Section 3.3 for cereal products and run on the LC-HRMS. A sample of black peppercorns from the supermarket was also extracted and analysed after grinding and fortification at 2 levels with aflatoxins (1.25 and 2.5 µg kg^{-1}) and OTA (15 and 30 µg kg^{-1}).

Extracts looked very dark throughout the extraction process. Final dried extracts were very oily (Figure 4), (oleoresins [8]). These were readily soluble in methanol, but the solution became a pale suspension fllowing addition of small amounts of water, with the appearance of dark precipitate after ultracentrifugation. Only addition of significant amounts of acetonitrile gave rise to clear solutions, indicating the elevated lipophilic character of these extracts.

Figure 4. Black pepper extracts after a QuEChERS extraction and final solvent evaporation

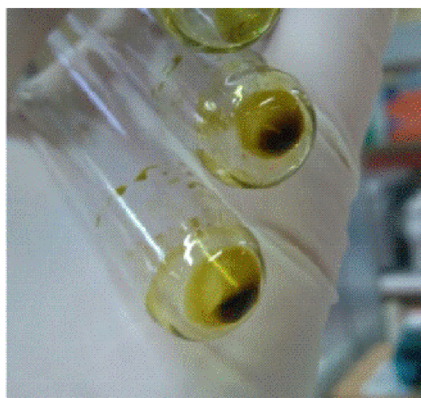


Figure 5. Reconstituted black pepper QuEChERS extracts after spin filter (0.2 µm) centrifugation. From left to right, water/methanol/acetonitrile (v/v): 1/3/0, 1/1/0, 3/1/0, 2/1/1, 1/1/2

It was not possible to detect any of the sought mycotoxins in any of the extracts due to the increased noise and suppression of the signal. Due to the hydrophobic characteristics of the extracts, application of the extraction method used for nuts (Section 3.6) may well have given better results, but further work is needed for this specific matrix.

The huge variety among the different potential spices prone to mycotoxin contamination should be stressed. "Spices" cannot be considered as a single commodity and extraction should be optimised on a case-by-case basis.

3.6 Incurred nuts

A Government Chemist referee sample [11] confirmed to be positive for aflatoxin B1 and blank peanut matrix was used to prepare PrEMS and PoEMS standards. Samples were prepared as a slurry made of ground sample/water (2/3, m/m).

Blank peanut matrix was fortified with aflatoxins at 1 and 2 µg kg^{-1}. The positive sample was fortified, pre-extraction, at 2 µg kg^{-1}.

Specific QuEChERS kits are available in the market for the analysis of foods with high content of fat (e.g. avocado, meat, formula milk, salmon or nuts). these were used for the clean-up of the nut extracts. For this analysis the following procedure was applied:

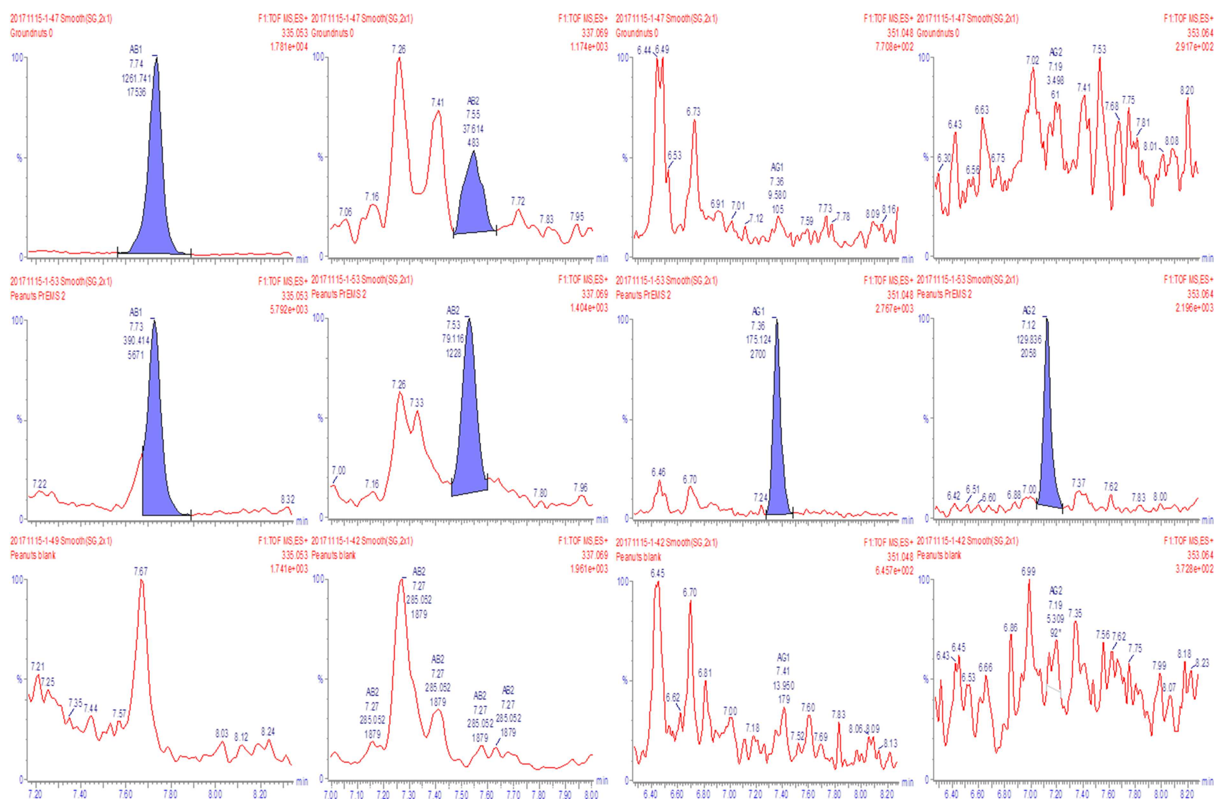
- 5 g of nut aqueous slurry weighed (composition: 2 g sample + 3 g water) in 50 mL tubes
- 5 mL water (1% formic acid) added and vortexed well to homogenise
- 10 mL ACN and 2 ceramic homogenisers added

- Tubes were shaken horizontally for 30 min
- 5 g of unbuffered QuEChERS salts added and tubes were immediately vortexed and shaken for 1 min
- Centrifugation 5 min at 4000 rpm
  (To this point the extraction corresponds to that for cereal products, see above)
- 5 mL water added to 15 mL **BondElut EMR-lipid dSPE** tubes
- 5 mL of the ACN supernatant added to the wetted EMR-lipid dSPE tubes
- Tubes vortexed and shaken for 2 min
- Centrifugation 5 min at 4000 rpm
- Supernatant added to tubes with **BondElut EMR-Lipid Polish** Tube and vortexed and shaken for 1 min
- Centrifugation 5 min at 4000 rpm
- 1 mL evaporated and reconstituted in 150 µL methanol (first) and then 450 µL water added
- Filtered through 0.22 µm nylon spin filters.

All analysed mycotoxins gave a S/N ratio greater than 3 at the lower level (1 µg kg^{-1}) in the PrEMS. Comparison of slopes between the PoEMS and PrEMS calibration solutions allowed estimation of extraction efficiencies (recoveries) of around 50 %. The use of adequate internal standards and matrix matched calibration solutions should compensate for losses during the extraction to yield a more accurate quantitation of these compounds. The incurred sample gave an intense signal for AB1, while detection of AB2 was more doubtful (S/N < 3). AG1 and AG2 were not detected.

Figure 6 shows the extracted chromatograms of the 4 aflatoxins (B1, B2, G1 & G2) in the incurred sample, the blank matrix, and the blank fortified at the legal limit (2 µg kg^{-1}).

Figure 6. Extracted chromatograms (10 ppm) of aflatoxins (left to right: A1, A2, B1, B2). Top to bottom: incurred sample, peanut blank fortified at the legal limit (2 μg kg^{-1}) and peanut blank



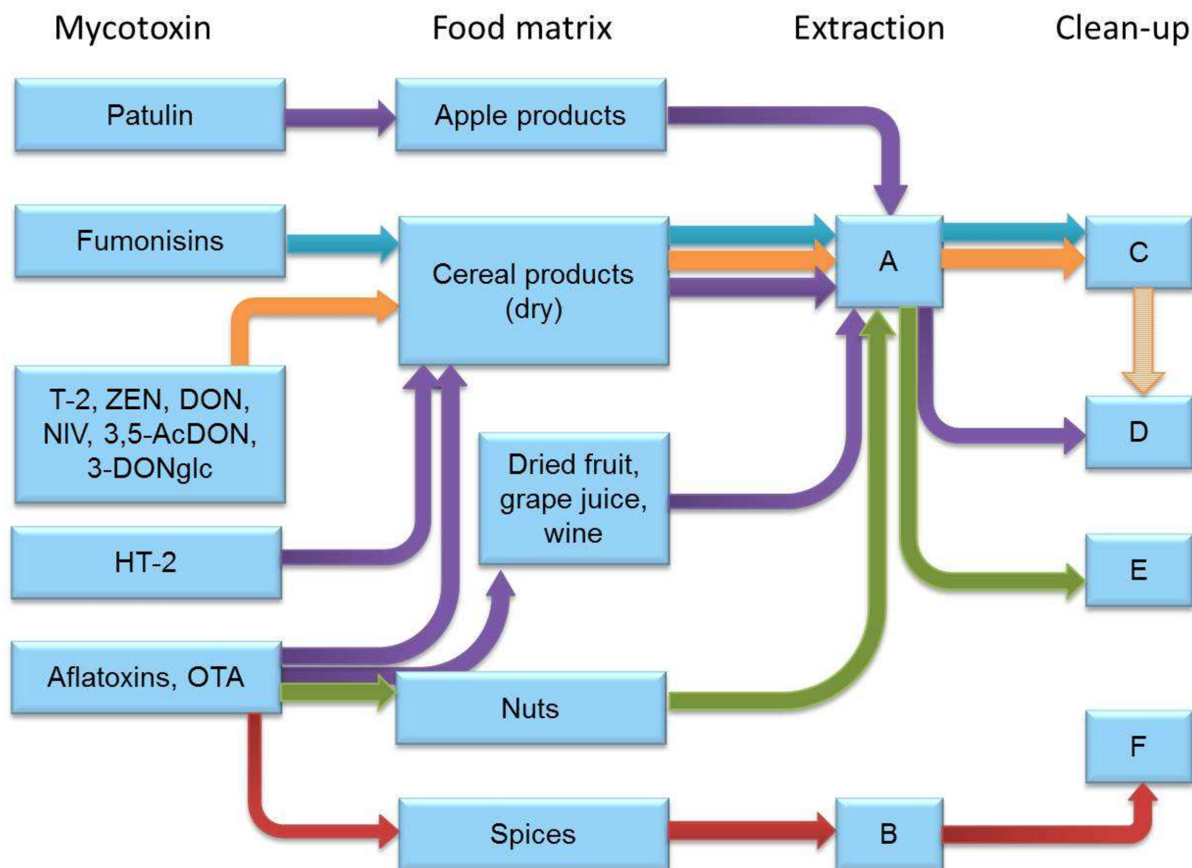
The peanut blank used for the preparation of PrEMS and PoEMS presented a small interference for Aflatoxin B1 at a retention time close to that of the analyte. Although retention times are not exactly the same, a better separation between this analyte and the interference is desirable to avoid false positive results. The use of a detector with different selectivity (e.g. MS/MS) could help to address this.

3.7 General strategy

Based on the findings from this study it is possible to sketch out a general strategy with different workflows depending on the combination analyte/matrix (Figure 7).

Figure 7. General plan for the extraction and clean-up by QuEChERS of diferent mycotoxins in different commodities

- **A: Acidified acetonitrile extraction followed by salting-out partition using unbuffered salts**
- **B: To be investigated. See main text**
- **C: Dilute and shoot: dilution of the acetonitrile phase 1-to-4 with water**
- **D: dispersive SPE. 1 mL of the acetonitrile phase cleaned up with PSA/C18/$MgSO_4$. Extract evaporated and reconstituted in smaller volume of methanol/water mixture**
- **E: dSPE specific for samples with high fat content, see Section 3.6**
- **F: To be investigated. See main text.**



Several of the matrices shown in Figure 7 have not been tested in this work, e.g. apple and dried fruits, and the suggested approach will have to be confirmed. The proposed QuEChERS extraction/clean-up is based in this case on the comprehensive literature already published for analysis of pesticides [12]. For example, it is necessary to keep the acetonitrile/water ratio constant for the extraction of the analytes from the matrix by, for example, adding the required amount of water to the sample (e.g. flour) dependent on the water content of the original sample.

Several of the mycotoxins (T-2, ZEN, DON, NIV, 3,5-AcDON and 3-DONglc) provide good sensitivity at the limits set in the legislation and a simplified approach diluting the extract after the salting-out step should be sufficient to detect them at these levels. If a specific sample provides an increased noise or signal suppression, the saved extracts can then be further cleaned-up using the dSPE step. Fumonisins represent a different case. They are retained in the dispersive SPE phase and the dilute-and-shoot workflow must be applied.

Legal limits for OTA are very low (for example, 2 μg kg^{-1} for grape juice and various wine matrices and 3 μg kg^{-1} for cereals) for the sensitivity obtained in this work. Limits of detection (LOD) are too close to the legal limits to extend the use of the proposed approach to all samples. More development work is required to enhance the method sensitivity for this compound (for example better, or different, clean-up, separation or detection).

Samples with high concentration of fats (e.g. nuts, oilseeds and some spices) need to be extracted and cleaned up using a QuEChERS approach specific for high-fat content samples (see Section 3.6). With slight modification, corn oil (legislated for fumonisins) could also be cleaned-up using this approach.

Extraction and clean-up methods for spices should be decided and tested on a case-by-case basis due to the great variety of different matrices (dried herbs, berries, grains, nuts, powdered dried fruits etc.) that can produce interferences for specific compounds or dirty extracts with increased background noise.

3.8 Masked mycotoxins

The use of HRMS allows untargeted analysis and interrogation of the chromatograms registered in full-scan to search for analytes for which analytical standards are not available, such as masked or modified mycotoxins.

However, the enormous number of the possible different metabolites and large data sets, specific data-mining software is needed for a proper and exhaustive search of masked mycotoxins. Some of the targeted masked mycotoxins for which analytical standards were available (DON-3glc, 3 & 15-AcDON) have been analysed routinely during this work but their presence has not been found (with the exception of 15-AcDON, where high levels for 15-AcDON in the CODA-CERVA PT sample indicate probably a spiked sample).

4 Conclusions

Different extraction and clean-up approaches based on QuEChERS have been applied for the screening analysis of mycotoxins in various matrices. The extracts have been analysed using LC with a high resolution MS with promising results. A general strategy with different workflows has been proposed for the screening of mycotoxins in a variety of commodities susceptible of mycotoxins contamination. This represents an alternative to already existing lengthy clean-up methods specific for individual mycotoxins. Satisfactory results have been obtained for cereal products and nuts. Good sensitivities have been obtained for all legislated mycotoxins, with the exception of OTA for which additional development is required to guarantee unequivocal detection at the legal limits. Further work is also needed to identify extraction and clean-up methods for spices, on a case-by-case basis due to the great variety of different matrices that can produce interferences for specific compounds or dirty extracts with increased background.

The results obtained in this work are linked to the characteristics of the detection method employed: high resolution mass spectrometry. One of the potential advantages of this approach is to be able to perform untargeted full scan experiments of exact masses with high specificity and good sensitivity. An unambiguous identification of the analytes will need at least two exact m/z using HRMS; this may be challenging for all the scoped mycotoxins.

5 References

1. McCullough, Bryan J., and Christopher J. Hopley. “A Validated LC-HRMS Method for the Detection of T-2 and HT-2 Toxins in Cereals” Journal of the Association of Public Analysts (Online) 45 (2017): 041-073.

2. Commission regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs (Text with EEA relevance) (OJ L 364, 20.12.2006, p. 5)

3. Commission recommendation of 27 March 2013 on the presence of T-2 and HT-2 toxin in cereals and cereal products (Text with EEA relevance) (2013/165/EU).

4 Berthiller, Franz, et al. "Formation, determination and significance of masked and other conjugated mycotoxins." Analytical and Bioanalytical Chemistry 395.5 (2009): 1243–1252.

5 Berthiller, Franz, et al. “Introduction to Masked Mycotoxins.” Masked Mycotoxins in Food: Formation, Occurrence and Toxicological Relevance (book) Chapter 1 (2015) 1–13.?

6 “Scientific Opinion on the risks for human and animal health related to the presence of modified forms of certain mycotoxins in food and feed.” EFSA Journal 2014; 12(12):3916.

7 Crews, Colin et al. “Evaluation of Masked Mycotoxins in Food and Their Release and Uptake in the Gut.” FS 102101. March 2016. Fera Science Ltd., York (UK).

8 Righetti, Laura, et al. "Recent advances and future challenges in modified mycotoxin analysis: why HRMS has become a key instrument in food contaminant research." Toxins 8.12 (2016) 361.

9 Oplatowska-Stachowiak, Michalina, et al. "Determination of the mycotoxin content in distiller’s dried grain with solubles using a multianalyte UHPLC–MS/MS method." Journal of agricultural and food chemistry 63.43 (2015): 9441-9451.

10 Meghwal, M., and T. K. Goswami. "Chemical composition, nutritional, medicinal and functional properties of black pepper: A review." Open Access Sci Rep 1.2 (2012): 1–5.