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Analysis of mycotoxins in barley using ultra high liquid chromatography high resolution mass spectrometry: Comparison of efficiency and efficacy of different extraction procedures

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ABSTRACT

The effectiveness of four extraction methods (modified QuEChERS, matrix solid-phase dispersion (MSPD), solid–liquid extraction (SLE) and solid-phase extraction (SPE) clean-up) were evaluated for simultaneous determination of 32 mycotoxins produced by the genus *Fusarium*, *Claviceps*, *Aspergillus*, *Penicillium* and *Alternaria* in barley by ultra high pressure liquid chromatography coupled to ultra-high resolution mass spectrometry (UHPLC-Orbitrap® MS). The efficiency and efficacy of extraction methods were evaluated and compared in number of extracted mycotoxins and obtained recoveries. From the one point of view, QuEChERS procedure was fast and easy, as well as it was able to successfully extract all selected mycotoxins. On the other hand, SLE method, MSPD and SPE clean-up method did not extract adequately all selected mycotoxins and recoveries were not suitable enough. Thereby, method employing QuEChERS extraction connected with UHPLC-Orbitrap® MS was developed to quantify 32 mycotoxins in barley within this study. Analytical method was validated and recoveries ranged from 72% to 101% for selected mycotoxins with only one exception nivalenol (NIV) and deoxynivalenol-3-glucoside (D3G), which were lower than 67%. Relative standard deviations (RSD) were lower than 17.4% for all target mycotoxins. The lowest calibration levels (LCLs) ranged from 1 to 100 μg/ kg. Validated method was finally used for monitoring mycotoxins in a total of 15 Czech barley samples, when only *Fusarium* toxins representatives were detected in 53% of samples and the mycotoxins with the highest incidence were enniatins.

1. Introduction

Cultivated for over 10,000 years, barley is one of the oldest domesticated grain crops. There are different varieties of barley, which have been developed during a long time. Actually barley is the world's fourth most important crop and an important staple in many countries. The largest commercial producers of barley are Canada, United States, Russia, Germany, France and Spain [1,2]. Moreover, countries like Czech Republic, barley is a crop with a great economical importance, with a cultivation area of about 400,000 ha [3]. The use of barley is predominantly focused on the production of malt by malting process intended for beer production. Even though, the malt is also used for the manufacture of distilled spirits, such as whisky, as well as sirups, coffee substitutes, and some other cereal-based foods. Moreover, malt or barley derivatives are used for feed production. Thereby, this product is commonly consuming by humans and animals [4].

Normally, the plant of barley can easily grow in different climatic regions. Unfortunately, this particular capacity does vulnerable to be colonised by various toxinogenic fungi, some of them can be able to produce mycotoxins [5,6].

These toxins can cause both acute and chronic effects for humans and animals [7,8]. For this reason, well-known mycotoxins, such as aflatoxins (AFs), ochratoxin A (OTA) and some *Fusarium* toxins have been classified by International Agency for Research on Cancer (IARC) and regulated by European Union [9–11]. On the other hand, there are other mycotoxins, such as enniatins, beauvaricin or ergot alkaloids, which have not been classified nor legislated up to now. The starting point of the monitoring of mycotoxins began to be focused on legislated mycotoxins [12,13], but step-by-step the range was also extended to emerging mycotoxins. In fact, several recent works have been focused only on these new and emerging mycotoxins [14–16].

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In all the contexts, liquid chromatography tandem mass spectrometry is commonly used for mycotoxins analysis [17]. Most often, triple quadrupole (QqQ) has been widely accepted as the main tool in the identification and quantification of mycotoxins owing to its superior sensitivity, specificity and efficiency [12,13,15–17]. However, liquid chromatography coupled to ultra-high resolution mass spectrometry (HPLC-Orbitrap®) has been also included recently for routine mycotoxin analysis showing acceptable sensitivity and unambiguous identification [18–20].

The applicability of liquid chromatography triple quadrupole linear ion trap (HPLC-MS/MS) and HPLC-Orbitrap® has been recently evaluated for the analysis of mycotoxins in baby food. The comparison has highlighted that both instruments were complementary for determination of mycotoxins [21]. Orbitrap® technology has been therefore applied for routine analysis demonstrating some advantages: accurate mass, robust, sensitivity and unambiguous identification. In this research was a step further, different extraction procedures have been studied in deep using Orbitrap® technology. This issue has been normally carried out using QqQ analyzers in the mycotoxin field [22,23]. However, in this work the use of Orbitrap® MS technology demonstrated to be effective and a powerful tool for routine validation.

Overcome the drawbacks of detection, one of the main problems in a multi-mycotoxins analysis is to develop a method with rapid and simple extraction and purification step of these analytes from various food matrices, caused predominantly by great differences in physicochemical properties of these compounds. In fact, the extraction and the clean-up are the critical steps since they both determine the recoveries for all mycotoxins under investigation [24]. The varied structures of these mycotoxins make the extraction difficulties in using one standard extraction technique in order to detect different genera of toxins. Many extraction procedures have been already described in the literature, such as solid–liquid extraction (SLE) and liquid–liquid extraction (LLE), commonly linked with mass spectroscopy [17]. For example, the classic solid–liquid extraction (SLE) with or without clean-up methods have been mainly applied for cereals and derivates [24–27].

The last trends have been attractive alternatives, such as modified QuEChERS or matrix solid-phase dispersion (MSPD), which have been used for cereals and derivates [13,22,28–30]. These extractions have been demonstrated as reliable methods and they have been successfully applied to different matrices.

Thereby, the main aim of this work was to develop a robust analytical method for the simultaneous extraction and determination of 32 mycotoxins in barley. In this way, different extraction methods (SLE, solid-phase extraction (SPE) clean-up method, QuEChERS and MSPD) were compared and evaluated, as well as the selected procedure was applied to common agricultural samples. Analysis was carried out using ultra performance liquid chromatography coupled with Exactive Orbitrap® MS (UPLC–Orbitrap® MS). Finally, by comparison of existing methods results, it was able to optimize an analytical method according to the EU Commission Decision 2002/657/EC guidelines [31].

2. Materials and methods

2.1. Chemicals and reagents

Standards of 3-acetyldeoxynivalenol (3-ADON), 15-acetydeoxynivalenol (15-ADON), deoxynivalenol (DON), deoxynivalenol-3-glucoside (D3G), fusarenon-X (FUSX), nivalenol (NIV), HT-2 toxin (HT-2), T-2 toxin (T-2), diacetoxyscirpenol (DAS), neosolaniol (NEO), aflatoxin B1 (AFB_1), aflatoxin B2 (AFB_2), aflatoxin G1 (AFG_1), aflatoxin G2 (AFG_2), ochratoxin A (OTA), fumonisin B1 (FB_1), fumonisin B2 (FB_2), fumonisin B3 (FB_3), sterigmatocystin (STER), zearalenone (ZEA), Penitrem A were supplied by Biopure (Tulln, Austria). Standards of beauvericin (BEA), altenuene, alternariol, ergocornine, ergocryptine, ergocrystine and ergosine were obtained from Sigma-Aldrich (Steinheim, Germany). On the other hand, enniatins A1 (ENA_1), A (ENA), B (ENB) and B1 (ENB_1) were purchased by Enzo Life Science (Lausen, Switzerland). The purity of standards was declared in the range 95%–98.9%, with the exception of ENB, which was $>90\%$.

Acetonitrile and methanol, both HPLC-grade, were supplied by Merck (Darmstadt, Germany). Deionized water was prepared from a Milli-Q system (Millipore, Bedford, MA, USA). Anhydrous magnesium sulphate, sodium chloride and ammonium formate and ammonium acetate ($\geq 99\%$ purity), were from Sigma-Aldrich (Steinheim, Germany).

Solid-phase used for MSPD was octadecy-silica (C18-E) (50 μm) bonded silica from Phenomenex (Torrance, USA). Oasis HLB 150 mg sorbent cartridges were from Waters Corp. (Milford, MA, USA).

2.2. Barley samples

A total of 15 spring barley samples were examined for selected mycotoxins. Samples were purchased from Czech farmers as a part of national projects. Barley samples were kept under the dark and dry conditions.

2.3. Extraction procedures

2.3.1. Matrix solid phase dispersion (MSPD)

Sample preparation was partially performed according to a previous research [32]. Barley samples were homogenized by mixing them thoroughly. Homogenized and representative 1 g portions were weighed and placed into a glass mortar (50 ml) and gently blended with 1 g of C_{18} for 5 min using a pestle, to obtain a homogeneous mixture. This mixture was introduced into a 100 mm × 9 mm i.d. glass column, and eluted dropwise with 1 mM ammonium formate in 10 ml of acetonitrile/methanol (50/50, *v*/*v*) by applying a slight vacuum. Then, an aliquot (1 mL) of extract was filtered through a 22 μm nylon filter prior to injection into the UPLC–Orbitrap MS.

2.3.2. QuEChERS

Modified QuEChERS procedure was employed to extract mycotoxins from the examined matrix [33,34]. Homogenized and representative portions of 2 g were weighed into a 50 mL PTFE centrifuge tube, and then 10 mL of 0.1% formic acid in deionised water were added. The mixture was mixed during 3 min and waited for the next step during 10 min. Afterwards, 10 ml acetonitrile were added, and consecutively the mixture was vigorously shaken (3 min). The following step, 4 g $MgSO_4$ and 1 g of NaCl were added and then the mixture was shaken 3 min again. Once the extraction was completed, the sample was centrifuged (5 min, 11.000 rpm, 20 °C). Then, an aliquot (1 mL) filtered through a 22 μm nylon filter before their injection into the UPLC–Orbitrap MS.

2.3.3. Solid–liquid extraction (SLE)

The classical SLE method was partially performed according to a previous work [35]. Representative portions of 2 g samples were accurately weighed and transferred to PTFE centrifuge tube (50 mL). Samples were extracted by shaking with 10mL acetonitrile/water/acetic acid (79:20:1, *v*/*v*/*v*) on an automatic shaker (IKA Laboratortechnik, Germany) for 90 min, and then centrifuged (5 min, 11,000 rpm, 20 °C). Afterwards, the supernatant extract

was two-fold diluted with HPLC-grade water, taking an aliquot of 0.5 mL and diluting to 1 mL. After that the sample was filtered through a 0.22 mm filter, consecutively the sample was injected.

2.3.4. Solid-phase extraction (SPE) clean-up method

The previous SLE extract was used for clean-up method. The extraction procedure was used according to Vendl et al. [36]. C_{18}-SPE clean-up procedure was performed with Oasis HLB cartridges (150 mg) from Waters (Milford, MA, USA). 2 mL of SLE extract were diluted with 30 mL of water in order to obtain a required maximum concentration of 5% organic solvent. The columns were pre-washed with 10 mL of acetonitrile, and further conditioned with 10 mL of 5% acetonitrile in deionized water. Consequently, diluted sample was loaded onto C_{18} cartridge. After that, SPE columns were washed with 10 ml of 5% acetonitrile in water. The cartridges were then dried for 30 min. In the last step, the mycotoxins were eluted by adding of 5 ml acetonitrile. Then, the extract was transferred into a 15 ml conical tube and evaporated to dryness at 35 °C with Buchi Rotavapor (Flawil, Switzerland). The residue was reconstituted to a final volume of 1 ml with methanol/water (50:50, *v*/*v*) and filtered through a 0.22 μm Millex-GN nylon filter, before the injection.

2.4. Ultra high pressure liquid chromatography Orbitrap® MS

The detection method has been optimized in a previous research [19]. An Accela U-HPLC system (Thermo Fisher Scientific, San Jose, CA, USA) was used for the separation of target analytes. It was equipped with an Acquity UPLC HSS T3 analytical column (100 mm × 2.1 mm i.d., 1.8 μm; Waters, Milford, MA, USA) held at 40 °C for the separation of sample components. As the mobile phase, 5 mM ammonium formate and 0.1% acid formic in water (A) and methanol (B) was used. The gradient was as follows: start with 5% B, linear increase to 50% B in 6 min, for next 4 min another linear increase to 95% B, keep up to 15 min, switching to 5% B in 15.1 min, and column equilibration for 3 min before the next injection start. The flow rate was 300 μL min^{-1}. The injection volume was 5 μL and the partial loop was used as an injection technique.

The operation parameters of the single-stage Orbitrap® mass spectrometer (Exactive; Thermo Fisher Scientific, Bremen, Germany) optimized for the heated electrospray interface (HESI-II; Thermo Fisher Scientific, Bremen, Germany) were as follows: sheath gas/aux gas: 35/10 arbitrary units, capillary temperature: 250 °C, heater temperature: 250 °C, capillary voltage: +60/−50 V, and spray voltage +4/−3.1 kV.

The system was operated in the full spectral acquisition mode in the mass range of m/z 100–1000 at resolving power settings of 50,000 FWHM at fixed acquisition rate of 2 spectrum s^{-1}. The method was developed in positive and negative ionization mode. The external mass axis calibration without the use of the specific lock mass was employed. For the mass accuracy estimation, mass at the apex of the chromatographic peak obtained as the extracted ion chromatogram was used. The calculated (exact) masses of analytes ions have been summarized in a previous work [19].

3. Results and discussion

3.1. Selection of Orbitrap® MS ionization mode.

Most of the published studies concerned with determination of multiple mycotoxins have used an electrospray ionization (ESI) source for ionization, however, a recent work has compared between ESI and atmospheric pressure chemical ionization (APCI) for multiple mycotoxins detection using Orbitrap® MS [19]. The authors concluded that using APCI enhancement in detectability of *Fusarium* toxins was archived, with the exception of OTA, which showed better ionization efficiency under ESI conditions. However, several limitations of APCI source were noted; on the one hand fumonisins did not show ionization efficiency under APCI conditions at all. On the other hand, these compounds require acidic conditions, which limit the ionization of other mycotoxins, mainly type B trichothecenes.

To keep in mind these premises, a compromise between sensitivity and identification was evaluated. At the end, 32 target mycotoxins were simultaneously detected by ESI. For this reason, this ionization mode was selected and LCLs were accepted knowing that they were higher than APCI except for OTA and fumonisins.

3.2. Optimization of proposed extraction methods

The proposed extraction methods have been partially performed. During this work, some parameters were evaluated again, as well as they were improved in order to extract selected mycotoxins. The efficiency and efficacy were evaluated and compared in number of compounds extracted and recoveries obtained.

For example, efficiency of MSPD extractions depends on type and amount of dispersing phase, the amount of sample and nature and volume of the eluting solvents [32]. In our study, the solid support was studied comparing between octy-silica (C_8) and octadecy-silica (C_{18}). At the end, C_{18} demonstrated to be the ideal support for multi-mycotoxins analysis when MSPD is used since the obtained recoveries were highest. However, in this study the eluting solvent could be reduced to 10 ml MeOH/ACN (50/50, *v*/*v*) demonstrating similar effectiveness to 20 ml (data not shown).

By contrast, SLE was partially used according to previous work [35]. This extraction was used in different ways. Firstly, it was used as clean-up using SPE cartridges, secondly without clean-up using diluted-and-shoot method. The SLE procedure without clean-up step used as diluted-and-shoot method demonstrated to be effective, crude extract and different diluted extracts (1+1, 1+2, 1+4) were evaluated (data not shown). At the end, the 1+1 diluted extracted showed acceptable recoveries for selected mycotoxins. Focusing on clean-up method, C_{18} cartridge was studied according to a previous work [36], in order to extract all selected mycotoxins.

Modified QuEChERS was evaluated according to previous works [33,34]. This extraction offers different alternatives, for example QuEChERS could be modified and it is an important advantage [37]. In our research the selected extraction did not require a clean-up step using (PSA) due to the low lipid content of the matrix, as well as by the presence of fumonisins which have an acidic nature, increasing the risk of their binding on the sorbent.

3.3. Comparison of proposed extraction procedures

MSPD, QuEChERS, SLE and SPE clean-up methods are commonly used for mycotoxins analysis. Even so, these methods have advantages and disadvantages. Among the four evaluated methods, QuEChERS is the fastest and cheapest procedure; because of pre-concentration and clean-up steps were not necessaries, as well as the glass decontamination. QuEChERS procedure was able to extract 10–15 samples in 1 h and 30 min, whereas MSPD and SLE methods took twice as long and clean-up method three times as long. For example, the time consuming could depend on the glass decontamination in MSPD and SPE steps or waiting time in SLE. Moreover, the cost of SPE columns, solid-phases, salts, solvents or the working time is important in order to decide the best option.

The use of external matrix-matched calibration or internal standard (IS) calibration can minimize the variations between samples. The best option should be the use of appropriate IS, which can overcome ion suppression/enhancement. However, this ideal analysis is difficult or expensive to do, because it is necessary two homologous analytes and IS (isotopically labeled, deutereted or analog) are not available for all target mycotoxins [38]. For this reason, matrix-matched calibration curves were applied for effective quantification for each extraction procedure.

Therefore, matrix-matched solutions were prepared by spiking barley in triplicate at eight concentrations levels into the analytical range: from 10 μg kg^{-1} to 1000 μg kg^{-1}. Calibration solutions for external matrix-assisted curve (eight-point calibration) were prepared in blank barley extracts (it was corroborated before the analysis that no mycotoxins were present) obtained following MSPD, modified QuEChERS, SLE, SPE-Clean up method (Section 2.3 *Extraction procedure*).

To determine the recoveries (%) obtained by each studied extraction, barley samples were spiked at adequate concentration before the extraction. The samples were left to stand 3 h at room temperature before the extraction to allow the evaporation of the solvent and to establish equilibration between the mycotoxins and barley. Consecutively, spiked samples were extracted and treated by the previously described protocols.

Overview of recoveries data for MSPD, QuEChERS, SLE and SPE clean-up methods is summarized in Table 1; recovery study was carried out by spiking selected mycotoxins in blank barley at 250 μg kg^{-1}. In Table 1 could be observed that MSPD, QuEChERS and SLE showed an acceptable range of recoveries which were higher than 60% for the most of selected compounds. However, SPE clean-up method presented low recoveries, which ranged from < 50% to 80%. Recoveries were higher than 65.4% for type A and B trichothecenes, aflatoxins and fumonisins. Moreover, they were not upper than 58.5% for Penitrem A and some mycotoxins, such as ergot alkaloids. In our research HLB cartridges showed an unsuccessful efficiency for target mycotoxins. In other studies, C_{18} cartridges were compared with other specific columns and their recoveries were improved successfully for some compounds [26,36]. However, in our research C_{18} was used looking for a wide number of mycotoxins, but it was rejected due to low recoveries. Obviously, owing to the poor recoveries obtained, SPE clean-up method was rejected for the study.

The following step was to evaluate deeply the recoveries obtained by other techniques, as it shows in Table 2. Delving into every detail, it was observed that MSPD was able to extract all

Table 1
Preliminary recovery studies for selected mycotoxins. Blank barley was spiked at 250 μg/ kg for targeted mycotoxins.

Recovery, (average, $n=5$)		Extraction methods			
		MSPD	QuEChERS	SLE	Clean-up
Percent of 32 mycotoxins	< 50	2	0	1	6
	50–60	1	0	0	10
	60–70	11	2	5	14
	70–80	13	17	2	2
	80–90	5	12	17	0
	90–100	0	1	7	0
	100–110	0	0	0	0

Table 2
Recovery data for MSPD, QuEChERS and SLE method in blank barley at 100 μg kg^{-1} except type B trichothecenes and fumonisins which were spiked at 250 μg kg^{-1}. In brackets are given % RSD ($n=5$).

Toxin classification	Mycotoxin	Extraction method		
		MSPD	QuEChERS	SLE
Fusarium toxins	NIV	68.2 (14)	65.2 (12)	69.1 (16)
	D3G	60.1 (22)	64.1 (16)	67.2 (18)
	DON	77.9 (9)	87.9 (9)	83.1 (6)
	3-ADON	72.1 (18)	85.1 (12)	83.2 (14)
	15-ADON	70.9 (21)	83.9 (11)	88.2 (19)
	FUSX	67.9 (15)	81.1 (14)	83.1 (7)
	NEO	71.1 (12)	76.7 (5)	87.2 (5)
	DAS	76.5 (8)	86.1 (6)	92.8 (20)
	HT-2	71.5 (12)	88.2 (10)	93.3 (15)
	T-2	75.3 (20)	93.4 (12)	92.3 (18)
	ZEA	66.7 (16)	71.8 (13)	91 (10)
	FB_1	87.1 (15)	83.3 (8)	61.1 (19)
	FB_2	86.1 (13)	88.1 (7)	64.2 (12)
	FB_3	81.3 (17)	82.8 (9)	60.7 (11)
	ENA	68.6 (11)	77.3 (12)	81.1 (15)
	ENA_1	69.1 (8)	74.4 (11)	80.2 (13)
	ENB	74.1 (11)	76.1 (12)	85.1 (16)
	ENB_1	67.1 (6)	70.5 (12)	90.1 (10)
	BEA	69.3 (19)	72.8 (15)	80.1 (20)
Ergot alkaloids toxins	Ergosine	60.1 (21)	74.1 (9)	88.1 (4)
	Ergocornine	47.3 (18)	76.4 (11)	91.1 (13)
	Ergocryptine	56.7 (23)	71.9 (16)	90.6 (18)
	Ergochristine	63.4 (27)	76.6 (10)	79.6 (11)
Aspergillus toxins	AFB_1	73.1 (14)	81.9 (9)	82.1 (12)
	AFB_2	76.6 (17)	81.2 (10)	85.1 (13)
	AFG_1	81.1 (18)	78.2 (12)	83.2 (14)
	AFG_2	71.7 (16)	75.3 (11)	80.1 (17)
	STER	73.5 (20)	85.3 (7)	81.5 (24)
Penicillium, *Claviceps Aspergillus* and *Alternaria* toxins	OTA	68.9 (12)	86.9 (7)	79.2 (10)
	Penitrem A	42.5 (13)	73.4 (10)	46.1 (16)
	Altenuen	76.5 (15)	85.7 (14)	83.1 (8)
	Alternariol	81.1 (18)	89.4 (9)	83.3 (26)

selected mycotoxins; recoveries ranged from 66.7% to 87.1%, but Penitrem A, D3G and some ergot alkaloids showed lower recoveries and high RSDs (%). SLE showed the highest recoveries, as well as, all selected mycotoxins were completely extracted. The problem was that Penitrem A and fumonisins showed lower recoveries. QuEChERS did not present the highest recoveries, but this method was able to extract adequately all selected mycotoxins. The recoveries ranged from 64.1% to 93.4% without exception. Thereby, the recoveries obtained were into acceptable range and RSDs were lower than 20% (Table 2).

To sum up, modified QuEChERS was selected for further studies in order to take advantage its potential for simultaneous extraction of selected compounds. The data comparison showed that QuEChERS offered acceptable range of recoveries and low RSDs. Furthermore, QuEChERS gave low time consuming during the extraction procedure, as well as, it was easier and cheaper than MSPD, SLE and SPE clean-up. For these reasons, QuEChERS was the most efficient and effective extraction procedure evaluated.

3.4. Validation of the QuEChERS procedure

Validation of the method was performed according to following directive and guide on that subject [39,40]. The following parameters were studied: confirmation of identity, specificity/selectivity, linearity, lowest calibration level (LCL), precision as repeatability and within-lab reproducibility, process efficiency and recovery.

Confirmation of identity was based on the following criteria: (i) the measured accurate mass of $[M+H]^+$, $[M+NH4]^+$ or $[M-H]^-$ and $[M+HCOOH]^-$ must fit the theoretical accurate mass with a mass tolerance set at ± 5 ppm and (ii) the retention time window was set to $\pm 2\%$ from that of a calibration standard.

The LCLs were determined as previous works [19,41]. Table 3 gives LCLs for target mycotoxins in barley. The LCLs ranged between 1 to 100 $\mu g\ kg^{-1}$ for ENB and NIV, respectability. Based on LCLs obtained values the method proved to be sensitive and it allows us to assess the compliance of all the mycotoxins and matrix with the Commission Regulation no. 1881/2006 [10].

It is well known that the presence of matrix components in the extract (co-eluting compounds), which can affect the ionization of the compounds when ESI is used producing the so-called matrix effects (ME). There are different ways, which could be applied to compensate matrix effects. Although the best way to compensate the matrix effect is the use of isotope internal standards, these compounds are not available for some of the studied mycotoxins, as well as they are expensive for routine analysis. Another form to avoid matrix effects is the use matrix-matched calibration curves for effective quantitative determinations of mycotoxins in barley. The ME was calculated for each mycotoxin in barley, as the percentage of the matrix-matched calibration slope (B) divided by the slope of the standard calibration in solvent (A); the ratio $(B/A \times 100)$ is defined as the matrix effect. A value of 100% indicates that there is no absolute matrix effect. There is signal enhancement if the value is $>100\%$ and signal suppression if the value is $<100\%$. In this way, the linearity in the response was

Table 3
Validation modified QuEChERS method. Lowest calibration Levels (LCLs), matrix effects (ME), percentage recovery and repeatability (% RSD) at three levels used for validation, and Inter-day precision (% RSD) at medium level.

Mycotoxin	LCL ($\mu g\ kg^{-1}$)	ME[a]	Intra-day[c]			Inter-day[d]
			Low level 25 $\mu g\ kg^{-1}$	Medium level 50 $\mu g\ kg^{-1}$	High level 100 $\mu g\ kg^{-1}$	Medium level 50 $\mu g\ kg^{-1}$
NIV	100	86.1	62.8 (6.8)[b]	66.3 (5.5)[b]	67.1 (5.2)[b]	9.1[b]
D3G	25	68.9	61.8 (8.8)[b]	63.9 (7.5)[b]	65.1 (7.2)[b]	6.1[b]
DON	5	81.2	86.9 (6.7)[b]	87.1 (5.9)[b]	90.2 (8.2)[b]	8.2[b]
3-ADON	50	88.1	95.9 (16.1)[b]	83.9 (8.5)[b]	81.1 (9.1)[b]	12.5
15-ADON	50	85.1	92.8 (13.4)[b]	80.1 (7.9)[b]	79.3 (10.2)[b]	14.4[b]
FUSX	100	77.8	89.1 (7.8)[b]	91.5 (6.9)[b]	90.1 (9.9)[b]	7.7[b]
NEO	1	109.2	83.9 (11.7)	77.5 (9.1)	77.7 (8.9)	11.1
DAS	1	111.9	93.9 (6.1)	88.3 (7.2)	86.7 (9.3)	9.6
HT-2	1	99.4	101.1 (10.1)	95.5 (9.7)	93.2 (9.9)	12.9
T-2	1	123.1	95.1 (10.1)	95.5 (9.1)	93.4 (9.5)	10.9
ZEA	1	91.2	81.8 (7.8)	77.8 (6.9)	79.8 (10.1)	10.3
FB_1	50	112.1	83.9 (6.4)[b]	89.6 (7.9)[b]	84.9 (6.9)[b]	8.6[b]
FB_2	10	103.4	81.7 (5.8)[b]	87.2 (8.5)[b]	82.3 (7.9)[b]	9.1[b]
FB_3	10	107.1	85.9 (4.7)[b]	89.1 (9.4)[b]	87.1 (8.3)[b]	12.6[b]
ENA	5	77.9	84.1 (6.2)	81.8 (6.3)	80.1 (6.3)	7.1
ENA_1	1	82.9	80.1 (5.2)	79.8 (7.2)	80.4 (5.7)	6.9
ENB	1	88.1	84.1 (7.1)	86.5 (5.6)	83.4 (6.1)	7.5
ENB_1	1	78.1	79.1 (11.9)	78.9 (8.1)	77.6 (7.9)	11.8
BEA	1	110.1	78.2 (14.6)	74.1 (10.1)	73.1 (10.2)	17.4
Ergosine	1	110.1	78.8 (10.8)	76.3 (7.1)	74.8 (6.9)	9.3
Ergocornine	2.5	66.1	76.3 (11.1)	74.1 (9.1)	70.1 (12.2)	9.9
Ergocryptine	2.5	86.3	74.8 (8.8)	76.6 (4.4)	71.9 (5.9)	7.9
Ergochristine	2.5	69.8	76.8 (10.1)	82.2 (9.6)	78.4 (11.2)	11.3
AFB_1	1	82.3	74.7 (5.2)	75.7 (8.6)	86.1 (9.1)	10.8
AFB_2	1	71.2	73.7 (9.2)	77.1 (12.1)	81.2 (3.1)	11.9
AFG_1	1	68.9	71.4 (14.1)	79.5 (4.5)	78.9 (6.4)	8.1
AFG_2	1	98.1	72.3 (11.8)	75.7 (7.4)	76.3 (7.1)	10.2
STER	2.5	128.1	86.5 (5.3)	81.8 (3.8)	85.2 (5.9)	5.6
OTA	10	103.9	96.3 (4.2)	87.9 (1.5)	88.8 (4.1)	4.3
Penitrem A	50	114.1	88.8 (10.4)[b]	84.8 (7.4)[b]	79.8 (5.8)[b]	7.9[b]
Altenuene	2.5	109.8	92.7 (7.2)	80.9 (7.9)	79.8 (9.3)	8.3
Alternariol	2.5	111.1	94.1 (7.7)	92.4 (7.1)	89.8 (8.4)	8.1

[a] ME%: (slope matrix matched calibration/slope standard in solvent) $\times$ 100
[b] The spiking levels of type B trichothecenes, fumonisins and Penitrem A were 150, 300, 600 $\mu g\ kg^{-1}$.
[c] Number of replicates: 10.
[d] Different days: 5.

calculated using standard solutions and matrix-matched solutions were prepared by spiking barley in triplicate at six concentrations levels into the analytical range: from LCL to 100 times this LCL. Type A trichothecenes, altenuene, STER and BEA showed great signal enhancement. However, matrix suppresses the response for AFG_1, D3G, ergocristine and ergocornine. Thereby, matrix-matched calibration was used.

Linearity was then evaluated. Peak area was selected as response and good linearity within LCL and 100 times LCL (six-point calibration) was found with determination coefficients higher than 0.9922 in all the cases.

Trueness was evaluated through recovery studies. Recoveries ($n=10$), they were carried out spiking barley at three levels (Table 3). The precision of the method, expressed as relative standard deviation (%RSD), was estimated by the repeated analysis ($n=10$) of a spiked barley at these levels during the same day (intra-day) and on different five days (inter-day).

Recoveries ranged from 71.4% to 101.1% for all mycotoxins assayed at concentration levels evaluated (Table 3), except for NIV and D3G, which were lower than 67.1%. Good recoveries were therefore obtained throughout the developed QuEChERS method. Precision of the overall method was studied by performing intra-day and inter-day precision experiments, showing the results in Table 3. It can be observed that repeatability, expressed as RSD was lower than 16.1% for intra-day experiments and for inter-day precision, RSDs were always lower than 17.4% for three spiked levels.

Thus, the method was successfully validated according to the criteria specified in Commission Decision 2002/657/EC for quantitative confirmation method [16]. Furthermore, the specificity of the methods was demonstrated by the analysis of blank barley and spiked samples.

Table 4
Occurrence of target mycotoxins in barley, expressed as μg kg^{-1}.

	DON	ENB	ENB_1	ENA	ENA_1	HT-2	T-2
Sample 1	33.1	87.7	139	100.5	108.9	78.5	30.5
Sample 2	43.3	2029	1821	340	698	26.2	8.8
Sample 3	38.1	95.6	101.1	75.6	93.5	30.5	< LCL
Sample 4	49.1	< LCL	< LCL	< LCL	< LCL	< LCL	< LCL
Sample 5	25.1	< LCL	< LCL	< LCL	< LCL	< LCL	< LCL
Sample 6	< LCL	< LCL	< LCL	< LCL	< LCL	< LCL	< LCL
Sample 7	31.2	< LCL	< LCL	< LCL	< LCL	< LCL	< LCL
Sample 8	< LCL	< LCL	< LCL	< LCL	< LCL	< LCL	< LCL
Sample 9	< LCL	< LCL	< LCL	< LCL	< LCL	< LCL	< LCL
Sample 10	< LCL	< LCL	< LCL	< LCL	< LCL	< LCL	< LCL
Sample 11	< LCL	< LCL	< LCL	< LCL	< LCL	< LCL	< LCL
Sample 12	36.5	< LCL	< LCL	< LCL	< LCL	< LCL	< LCL
Sample 13	< LCL	< LCL	< LCL	< LCL	< LCL	< LCL	< LCL
Sample 14	< LCL	19.4	28.5	21.9	25.3	< LCL	< LCL
Sample 15	< LCL	< LCL	< LCL	< LCL	< LCL	< LCL	< LCL

< LCLs: Lower than LCL level.

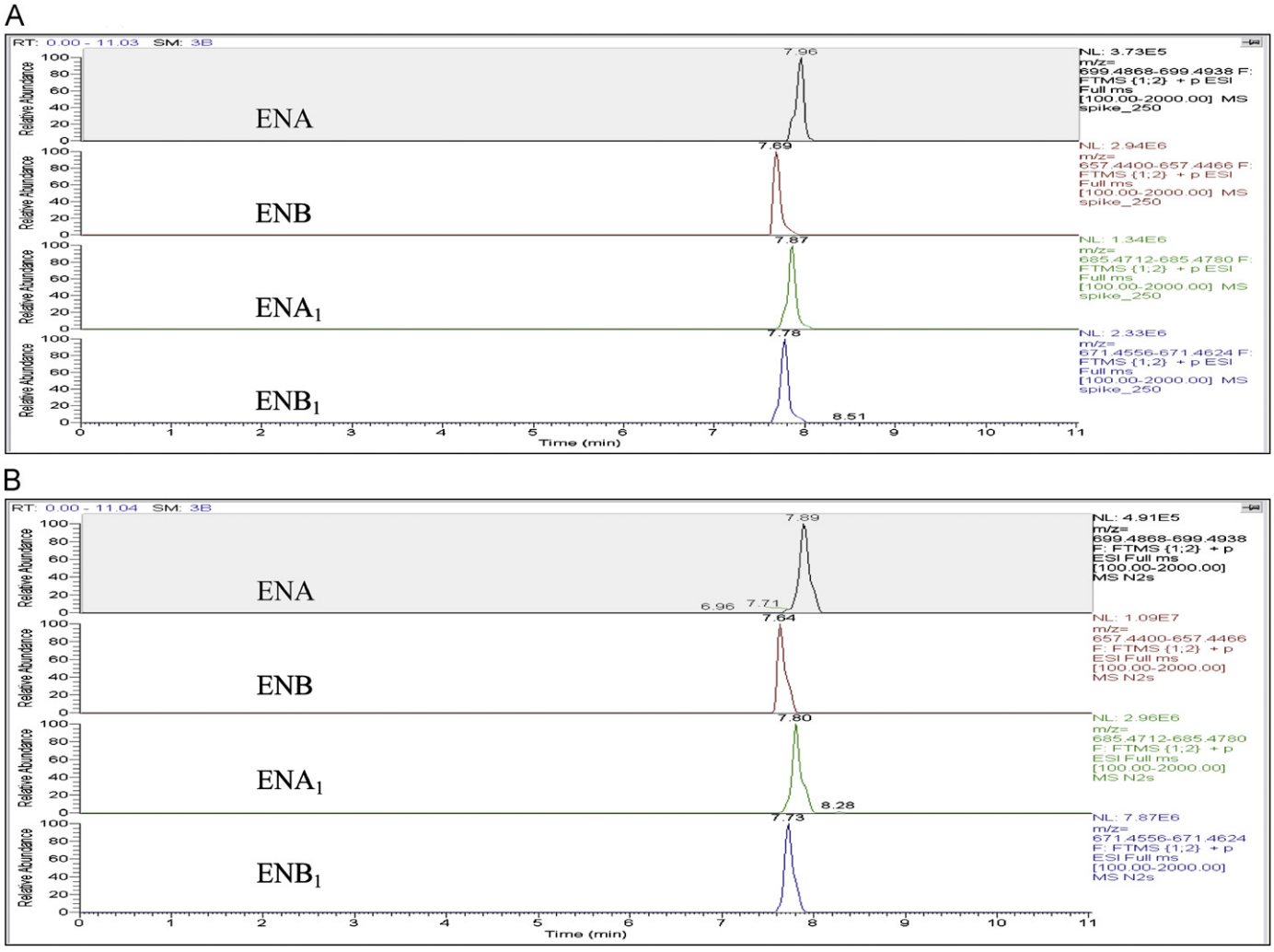

Fig. 1. Chromatograms for enniatin A, A1, B, B1 spiked at 250 μg kg^{-1} (A). Positive barley, sample 2, extracted ion chromatograms for enniatins (B).

3.5. Analysis of barley samples

The developed analytical method was applied for testing of 15 barley samples from Czech Republic. Within this monitoring, several *Fusarium* toxins were identified (Table 4). In this research *Alternaria*, *Aspergillus*, *Claviceps* and *Penicillium* toxins were not detected, although several works have demonstrated the presence of these toxins in barley [42,43]. In this research, enniatins were commonly detected in barley samples, as well as type A and B trichothecenes. By contrast, the presence of trichothecenes has been commonly related in barley [44,45], but the presence of enniatins have not been commonly cited up to now [46]. At the end, in total 7 samples out of 15 tested samples the co-occurrence of *Fusarium* mycotoxins was presented, but in all cases the calculated concentrations were lower than those established by European directives [10]. However, the calculated concentrations for enniatins were considerable.

Fig. 1 shows a chromatogram of a spiked barley at 250 $\mu g\ kg^{-1}$ and a positive sample in which were identified enniatins: ENA, ENA_1, ENB and ENB_1. Thereby, *Fusarium* mycotoxins were identified and quantified in barely samples. Moreover, it is important to keep in mind that it was difficult to find samples without enniatins for validation study.

The confirmation of positive samples was carried out, according to previous criteria cited above. Furthermore, an internal quality control was carried out for every batch of samples to check if the system was under control, and it implied a matrix-matched calibration, a matrix blank and a spiked barley sample at low concentration level. This quality control was very important to guarantee accuracy of the analysis. Mycotoxin analysis in the raw material could assure the quality of the raw material, as well as its derivate. In this form, it could minimize the public health risk.

4. Conclusion

The simultaneous extraction of 32 mycotoxins from barley was difficult, because of the great structural variability of these mycotoxins, as well as, it was a compromise between sensitivity and detection. The selection of the extraction procedure depends on the group of mycotoxins to be extracted. Moreover, if the number of mycotoxins is extended different extraction procedures should be studied and compared in deep. In fact, the extraction is the critical step because of it has to extract selected compounds and to reach acceptable recoveries.

At the end, the efficiency and efficacy of modified QuEChERS demonstrated to be superior to SLE, MSPD, and SPE clean-up method. The developed analytical method could extract selected compounds from barley at low cost, reducing time consuming and increasing throughput.

The validated UHPLC-Orbitrap® MS was confirmed to be an accurate, precise, and sensitivity methodology for the detection of 32 mycotoxins in barley samples. This instrument allowed target mycotoxins to be analyzed, but ultra-high resolution mass spectrometry could have been used to identify non-target mycotoxins.

Finally, the validated method was used to analyze commercialize barley samples, detecting *Fusarium* toxins at low concentrations. To sum up, in our research it has been demonstrated the applicability of QuEChERS for this type of organic contaminants as well as the excellent sensitivity obtained using liquid chromatography ultra-high resolution mass spectrometry.

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References

[1] European Commission forecasts average crop production for 2010 in the EU despite extreme weather. MEMO/10/361.
[2] United States Department of Agriculture Circular Series WAP 10-10 October 2010.
[3] 〈http://www.cspas.cz/index.asp?lang=2〉.
[4] C. Griffey, W. Brooks, M. Kurantz, W. Thomason, F. Taylor, D. Obert, R. Moreau, R. Flores, M. Sohn, K. Hicks, J. Cereal Sci. 51 (2010) 41–49.
[5] C.M. Placinta, J.P.F. D'Mello, A.M.C Macdonald, Anim. Feed Sci. Technol. 78 (1999) 21–37.
[6] V. Kumar, M.S. Basu, T.P. Rajendran, Crop Prot. 27 (2008) 891–905.
[7] G.S. Shephard, Food Addit. Contam. 25 (2008) 146–151.
[8] J.L. Richard, Int. J. Food Microbiol. 119 (2007) 3–10.
[9] IARC (International Agency for Research on Cancer), in: IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Some Naturally Occurring Substances; Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins, IARC (Ed), Geneve, vol. 56, 1993. pp. 489–521.
[10] Commission Regulation (EC) 1881/2006 of Decembre 19th 2006 replacing Regulation (EC) 466/2001 setting maximum levels for certain contaminants in foodstuffs. Off. J. Eur. Commun. L364: pp. 5–24.
[11] Commission Regulation (EU) 165/2010 of 26 February 2010 amending Regulation (EC) 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins. Off. J. Eur. Commun. 2010, L50, pp. 8–12.
[12] A.L. Capriotti, P. Foglia, R. Gubbiotti, C. Roccia, R. Samperi, A. Laganà, J. Chromatogr. A 1217 (2010) 6044–6051.
[13] A.G. Frenich, J.L. Martínez Vidal, R. Romero-González, M.M. Aguilera-Luiz, Food Chem. 117 (2009) 705–712.
[14] M. Jestoi, M. Rokka, E. Järvenpää, K. Peltonen, Food Chem. 115 (2009) 1120–1127.
[15] A.G. Frenich, R. Romero-González, M.L. Gómez-Pérez, J.L. Martínez Vidal, J Chromatogr. A 1218 (2011) 4349–4356.
[16] N. Mahnine, G. Meca, A. Elabidi, M. Fekhaoui, A. Saoiabi, G. Font, J. Mañes, A. Zinedin, Food Chem. 124 (2011) 481–485.
[17] N.W. Turner, S. Subrahmanyam, S.A. Piletsky, Anal. Chim. Acta 632 (2009) 168–180.
[18] J. Rubert, J. Mañes, K.J. James, C. Soler, Food Addit. Contam. Part A Chem. Anal. Control Exposure Risk Assessment 28 (2011) 1438–1446.
[19] M. Zachariasova, T. Cajka, M. Godula, A. Malachova, Z. Veprikova, J. Hajslova., Rapid Commun. Mass Spectrom. 24 (2010) 3357–3367.
[20] A. Malachova, Z. Dzuman, Z. Veprikova, M. Vaclavikova, M. Zachariasova, J. Hajslova, J. Agric. Food Chem. 59 (2011) 12990–12997.
[21] J. Rubert, K.J. James, J. Mañes, C. Soler, J. Chromatogr. A 1223 (2012) 84–92.
[22] A. Desmarchelier, J.M. Oberson, P. Tella, E. Gremaud, W. Seefelder, P. Mottier, J. Agric. Food Chem. 58 (2010) 7510–7519.
[23] M.M. Aguilera-Luiz, P. Plaza-Bolaños, R. Romero-González, J.L.M Vidal, A.G. Frenich, Anal. Bioanal. Chem. 399 (2011) 2863–2875.
[24] V.M.T. Lanttanzio, M. Solfrizzo, S. Powers, A. Visconti, Rapid. Commun. Mass Spectrom. 21 (2007) 3253–3261.
[25] M. Sulyok, F. Berthiller, R. Krska, R. Schuhmacher, Rapid Commun. Mass Spectrom. 20 (2006) 2649–2659.
[26] E. Beltrán, M. Ibáñez, J.V. Sancho, F. Hernández, Rapid Commun. Mass Spectrom. 23 (2009) 1801–1809.
[27] Y. Ren, Y. Zhang, S. Shao, Z. Cai, L. Feng, H. Pan, Z. Wang, J. Chromatogr. A 1143 (2007) 48–64.
[28] J. Blesa, J.M. Soriano, J.C. Moltó, R. Marín, J. Mañes, J. Chromatogr. A 1011 (2003) 49–54.
[29] J. Rubert, C. Soler, J. Mañes, Talanta 82 (2010) 567–574.
[30] I. Sospedra, J. Blesa, J.M. Soriano, J. Mañes, J. Chromatogr. A 1217 (2010) 1437–1440.
[31] Commission Decision 2002/657/EC of 12 of August 2002, Concerning the performance of analytical methods and the interpretation of the results. Off. J. Eur. Commun. 2002, L221–L232.
[32] J. Rubert, C. Soler, J. Mañes, Talanta 85 (2011) 206–215.
[33] M. Zachariasova, O. Lacina, A. Malachova, M. Kostelanska, J. Poustka, M. Godula, J. Hajslova, Anal. Chim. Acta 662 (2010) 51–61.
[34] L. Vaclavik, M. Zachariasova, V. Hrbek, J. Hajslova, Talanta 82 (2010) 1950–1957.
[35] M. Sulyok, F. Berthiller, R. Krska, R. Schuhmacher, Rapid Commun. Mass Spectrom. 20 (2006) 2649–2659.
[36] O. Vendl, F. Berthiller, C. Crews, R. Krska, Anal. Bioanal. Chem. 395 (2009) 1347–1354.
[37] M. Annastassiades, K. Mastovska, S.J. Lehotay, J. Chromatogr. A 1015 (2003) 163–184.
[38] J. Rubert, K.J. James, J. Mañes, C. Soler, Food Chem. Toxicol. 50 (2012) 2034–2041.
[39] Commission Decision 2002/657/EC of 12 August 2002, Implementing Council Directive (EC) 96/23 concerning the performance of analytical methods and the interpretation of the results (text with EEA relevance), Off. J. Eur. Commun. 2002, L221.

[40] EURACHEM Working Group, The fitness for purpose of analytical methods a laboratory guide to method validation and related topics, LGC, Teddington, 1998.
[41] T. Cajka, J. Hajslova, J. Chromatogr. A 1058 (2004) 251–261.
[42] M. Ibáñez-Vea, L.A. Corcuera, R. Remiro, M.T. Murillo-Arbizu, E. González-Peñas, E. Lizarraga, Food Chem. 127 (2011) 351–358.
[43] A. Medina, F.M. Valle-Algarra, R. Mateo, J.V. Gimeno-Adelantado, F. Mateo, M. Jiménez, Int. J. Food Microbiol. 108 (2006) 196–203.
[44] M. Ibáñez-Vea, E. Lizarraga, E. González-Peñas, A. López de Cerain, Food Control 25 (2012) 81–88.
[45] D. de Smet, S. Monbaliu, P. Dubruel, C. van Peteghem, E. Schacht, S. de Saeger, J. Chromatogr. A 1217 (2010) 2879–2886.
[46] S. Uhlig, M. Torp, B.T. Heier, Food Chem. 94 (2006) 193–201.