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Analytical Methods

Determination of trichothecenes and zearalenones in grain cereal, flour and bread by liquid chromatography tandem mass spectrometry

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ABSTRACT

Although analytical methods have been already reported for legislated mycotoxins as trichothecenes and zearalenone (ZON) separately, we describe the optimization of a simple and rapid multimycotoxin method for the determination of a total of 12 mycotoxins simultaneously, nine trichothecenes (NIV, DON, FUS-X, DAS, 15-AcDON, 3-AcDON, NEO, HT-2, T-2 T2), and zearalenone and its metabolites (ZON, α-ZOL, β-ZOL), of different origin (wheat, oat, barley and spelt) and in three different products where these substance can be present (grain, flour and bread) reach the food chain and cause toxic effect either in humans or animals. The extraction procedure was based on a mixture of acetonitrile/water (84/16, v/v), which provided the highest recoveries and the lowest matrix effect. DON-d1 was used as internal standard (I.S.) which helped to compensate the significant matrix effect observed for some matrices, and to obtain high success in the method validation and to reach the parameters compiled in Commission Decision, 2002/657/EC. Analytes were determinate by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). Relative recoveries obtained were higher than 70% for the studied mycotoxins the four cereal. Good linearity ($r^2 > 0.992$) was obtained and quantification limits (2.5–25 ng/g) were below European regulatory levels. Repeatability, expressed as relative standard deviation, was always lower than 11%, whereas interday precision was lower than 11% for the developed method.

1. Introduction

Mycotoxins are secondary metabolites produced by many species of filamentous fungi, which grow on various agricultural commodities in the field site, and during postharvest (transport, processing and storage) (Sforza, Dall'Asta, & Marchelli, 2006). Cereals are plant products especially susceptible to be infested by fungi and in consequence, several classes of mycotoxins can simultaneously contaminate cereals. Food contamination due to mycotoxins, can compromise the safety of food and feed supplies and adversely affect health in humans and animals (JECFA, 2001).

The toxicity of the mycotoxins has lead to many countries to set up regulations for their control in food of plant origin that are intended for human or animal consumption (EU Commission 1881/2006; EU Commission 401/2006; FAO, 2003), and maximum levels in different foodstuffs, including cereals, have been fixed. These compounds are not only pathogenic to humans and animals but their phytotoxic nature is also of great importance in the global trade of cereal crops, as low yields may have devastating economical implications. The effects exhibited in plants, include growth retardation, wilting, chlorosis, necrosis and inhibition of germination (Rocha, Ansari, & Doohan, 2005). Moreover the European Union has also promoted several good agricultural practices from the cultivation to the distribution of cereals, such as crop rotation or dry storage, in order to minimize the levels of mycotoxins in cereals.

Most recently, the European Commission is interested in *Fusarium* toxin. At the present time two regulations are in forced, the Commission Regulation (EC) 1881/2006 and Commission Regulation (EC) 1126/2007, which lay down maximum levels for certain contaminants in foodstuffs including maximum levels for mycotoxins derived from *Fusarium* species, deoxynivalenol (DON) and zearalenone (ZON) ranged to 200–1750 and 20–400 ng/g, respectively, whereas for toxins HT-2 and T-2 (HT-2 and T-2) have not been fixed yet since aspect regarding its health damage and occurrence are still being considered, despite to be included in the Regulation (EC) 1881/2006. Recently the European Commission has request to European Food Safety Authority (EFSA) several scientific opinions on different mycotoxins produced by *Fusarium* species in food and/or feed, specially to ZON and three trichothecenes: HT-2, T-2 and nivalenol (NIV). In fact, safety and risk of them are being reviewed and possible increase of the maximum level (ML), for some of them in food, might be pruposed.

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Trichothecene mycotoxins, known as major secondary metabolites produced by *Fusarium* species, such as *Fusarium graminearum*, which can produce also ZON, are globally distributed, even in the more extreme environments (Nelson, Dignani, & Anaissie, 1994). Trichothecenes have contaminated field crops of wheat, barley, oat, spelt, maize and cause mycotoxicosis, a serious health hazard to humans and domestic animals, as reported by several authors (Bennett & Klich, 2003; CAST, 2003; Desjardins, Hohn, & McCormick, 1993; Rocha et al., 2005; Ueno, Nakajima, Sakai, Ishii, & Sato, 1973). In addition, trichothecenes can be acutely phytotoxic and act as virulence factors on sensitive cereal hosts (Jansen, von Wettstein, Schäfer, Kogel, Felk, & Maier, 2005).

Trichothecenes are divided into four groups (types A–D) according to their characteristic functional groups, being the types A and B, the most common. Type A is represented by HT-2 toxin (HT-2) and T-2 toxin (T2), but also include neosolaniol (NEO) and diacetoxyscirpenol (DAS). While type B include nivalenol (NIV), deoxynivalenol (DON), fusarenon-X (FUS-X), 15-acetyldeoxynivalenol (15-AcDON), 3-acetyldeoxynivalenol (3-AcDON), but is most frequently represented by DON.

On the other hand, zearalenones (ZONs), which include ZON and its metabolites: α-zearalenol (α-ZOL) and β-zearalenol (β-ZOL), are oestrogenic mycotoxins. ZONs also are commonly found in maize but barley, oats, wheat, rice, sorghum and soy beans they are also susceptible of contamination with *Fusarium* (*F. graminearum*). For ZON, in 2011, the EFSA has recently published the scientific opinion on the risks for public health (EFSA, 2011).

For enforcement purposes, it is essential to have available precise and reliable analytical methods applicable at the regulatory levels for the relevant mycotoxins and commodities and although analytical procedures employed may be different, laboratories should be obtaining the same result. The development of multimycotoxin methods with one common sample preparation and a single final determination is highly desirable. In this regard, the most critical step is the extraction that should allow good recoveries for all mycotoxins under investigation in a specific food matrix.

Several authors have reported that high proportions of methanol or acetonitrile (75%) in the extraction solvent are suitable for the extraction of most mycotoxins, such as trichothecenes, ZON, ochratoxin-A, and aflatoxins in any matrix (Eriksen & Pettersson, 2004; Juan, González, Soriano, Moltó, & Mañes, 2005; Juan, Moltó, Lino, & Mañes, 2008; Juan, Zinedine, Moltó, Idrissi, & Mañes, 2008; Rocha et al., 2005).

Most analytical techniques used for the determination of trichothecenes proposed their extraction from the food matrix into a solvent followed by a clean up to remove any impurities/interfering materials plus to concentrate the extract and its quantification. In general, organic-solvent extraction is used to allow further concentration of the analyte for accurate measurement at low levels. The choice of solvent or solvent/water mixture used is driven by the chemical characteristics of the mycotoxin being analyzed, the sample type, health and safety issues and also the analytical technique being used.

Another important consideration is the extraction efficiency. It cannot be assumed that the mycotoxins will be totally dispersed into the liquid phase, however the key issue is that the recovery values determined are consistent. As a result, during method development, scientists investigate various solvents and solvent/water ratios to ascertain the most reproducible, efficient combinations for particular analytical methodologies (Diaz, 2005; Gilbert, 2000; Langseth & Rundberget, 1998; Lattanzio, Pascale, & Visconti, 2009; Mateo, Llorens, Mateo, & Jiménez, 2001; Meneely, Ricci, van Egmond, & Elliott, 2011; Schneider, Curtui, Seidler, Dietrich, Usleber, & Martlbauer, 2004).

Various physical techniques have been reported by mixing sample and extraction solvent (e.g., shakers or blenders). Blending tends to offer a more rapid approach. In fact Ueno et al. (1997) described a 5-min procedure for types A and B trichothecenes extraction. Although sample shaking invariably involves lengthier time periods of 60–90 min (Krska, Baumgartner, & Josephs, 2000; Sinha, Savard, & Lau, 1995; Ueno et al., 1997), Kolosova et al. (2008) recently reported accurate, reliable results after shaking for only 10 min.

Following the extraction, the extracted solution is often removed of any impurities/interfering materials in addition to concentrating the extract prior to analysis and quantification.

A number of clean-up strategies have been employed to facilitate the accurate measurement of trichothecenes in cereal products including solid-phase extraction (SPE) columns, immunoaffinity columns (IACs), Quick Easy Cheap Effective Rugged and Safe (QuEChERS), ion-exchange columns and the more traditional liquid–liquid partitioning (Gilbert, 2000; Langseth & Rundberget, 1998; Lattanzio et al., 2009; Mateo et al., 2001; Meneely et al., 2011; Schneider et al., 2004; Sospedra, Blesa, Soriano, & Mañes, 2010).

The disadvantage of using columns for the multianalysis trichothecenes is that these toxins often differ greatly with respect to polarity and solubility, so recovery for some may be compromised as well as the cost of analysis in addition to longer works out process and methodology (Klotzel, Lauber, & Humpf, 2006; Ren et al., 2007; Santini, Ferracane, Somma, Aragón, & Ritieni, 2009; Sulyok, Berthiller, Krska, & Schuhmacher, 2006). There are many ways to analyze substances, and to our knowledge, few reports where combining an extraction method and liquid chromatography coupled to mass spectrometry (LC–MS/MS), without column clean-up, for multianalysis trichothecenes in food are published. In all of them a good efficiency, selectivity and rapid analysis is shown.

In this study, we present the validation of a new multimycotoxin developed method for the analysis of 12 mycotoxins:nine trichothecenes (NIV, DON, FUS-X, DAS, 15-AcDON, 3-AcDON, NEO, HT-2 toxin (HT-2), T-2 toxin (T2)), and three zearalenones (ZON, α-ZOL and β-ZOL), in a wide variety of cereal matrices (wheat, oat, barley and spelt), two market format modalities (flour and grain) and one final product (wheat bread). Previously it was necessary to study different extraction solvents used by other authors for this kind of analysis, and to compare them. An internal standard was used in order to help the simultaneous determination of all mycotoxins.

2. Materials and methods

2.1. Chemical and reagents

Trichothecene standards, NIV, DON, FUS-X, DAS, 15-AcDON, 3-AcDON, NEO, HT-2, T2, ZON, α-ZOL, β-ZOL, deoxynivalenol-d1 (DON-d1) were purchased from Sigma–Aldrich (Milan, Italy).

Acetonitrile, methanol and water for LC mobile phase and organic solvents were HPLC grade from Merck (Darmstadt, Germany), while acetic acid was obtained from Fluka (Milan, Italy).

The individual stock solutions of trichothecenes were prepared in acetonitrile: NEO and HT-2 at concentration of 0.5 mg/mL, DON, 3-AcDON, 15-AcDON, NIV and ZON at concentration of 5 mg/mL, DAS, FUS-X, T-2, α-ZOL and β-ZOL at concentration of 2.5 mg/mL. Internal standard DON-d1 at 2 μg/mL was prepared by dilution of individual stock solutions of 100 μg/mL in acetonitrile.

On the other hand, working standard solutions with DON, 3-AcDON, 15-AcDON, NIV, FUS-X, DAS, NEO, T-2, HT-2, ZON, α-ZOL and β-ZOL at concentrations of 100 and 2 μg/mL were prepared. All these solutions were kept in safety conditions at −20 °C. All working standard solutions were prepared immediately before use by diluting the stock solution with methanol.

Whatman No. 4 type filter papers (Maidstone, England).

Syringe filters PTFE (politetrafluoroethylene membrane, 15 mm, diameter 0.2 μm) were provided from Phenomenex® (Castel Maggiore, Italy).

2.2. Instrumentation

2.2.1. Liquid chromatography parameters

LC analysis was performed using a system consisting of two micro pumps (HPLC Series 200, PerkinElmer®, Waltham, MA, USA). Separation was attained on a Phenomenex® (Castel Maggiore, Italy) Gemini C18 (150 mm × 2.0 mm, I.D. 5 μm particle size, 110 A) preceded by a SecurityGuard™ cartridge C18 (4.0 × 3.0 mm I.D. 5 μm). Mobile phase A consisted of an $H_2O/CH_3OH/CH_3COOH$ mixture (89:10:1, v/v/v) containing 5 mM ammonium acetate, while mobile phase B: $H_2O/CH_3OH/CH_3COOH$ mixture (2:97:1, v/v/v) containing 5 mM ammonium acetate.

The following gradient was applied: initial condition 55% B; 0–3 min, 70% B; 3–8 min, 100% B; 8–11 min constant at 100% B; 11–13 min returning to the initial conditions and maintain during 2 min 55% B. Flow rate was set to 0.2 mL/min, while the injection volume was 20 μL.

2.2.2. MS/MS parameters

The triple quadrupole mass spectrometry detector (MS/MS) was an API 3000 (Applied Biosystems, Ontario, Canada). All analyses were performed with an TurboIonSpray™ mode (TIS) interface with the following settings: turbo probe temperature 450 °C, heather gas flow 8 μL/min, nebulizer setting 10, position lateral 8 and position horizontal 10 (depending on use in positive or negative mode).

The declustering potential (DP) and collision energy (CE) were optimized for each compound by direct infusion of standard solutions (10 μg/mL) into the mass spectrometer at a flow rate of 8 μL/min, using a Model 11 syringe pump (Harvard Apparatus, Holliston, MA, USA) and a solution of $H_2O/CH_3OH/CH_3COOH$ 5 mM ammonium acetate (29:70:1, v/v/v) as liquid carrier at a flow rate of 0.2 mL/min.

The acquisition was carried out by multiple reaction monitoring (MRM) both in the negative and positive ion mode for each compound. Data acquisition and processing were performed using Analyst software v. 1.4.

2.3. Sample extracts preparation

Due to the diversity of the matrices and compounds evaluated, the matrix effect was studied. So that one common procedure was used while different mixture of solvent was proved. Previously the grain and bread samples (100 g) were ground in a laboratory mill for 3 min. The procedure used was: 5 g of samples was transferred into 50 mL PTFE centrifugal tube, 25 mL of each mixture of solvents studied (acetonitrile:methanol (60:40, v/v), acetonitrile:methanol (40:60, v/v), acetonitrile:water (84:16, v/v) and acetonitrile:water (16:84, v/v)) was added and stirred for 1 h. The mixed was centrifugated at 4500 rpm for 5 min at 5 °C and supernatant phase was paper filtered. Five millilitres of the filtrate were evaporated using a centrifugal evaporator (Savant).

Dry extracts produced by each method were then dissolved in 1 mL of CH_3OH/H_2O mixture (70:30, v/v) by vortexing vigorously and finally filtered through a 0.22 μm cellulose filter (Chemtek Analytica, Bologna, Italy) before LC–MS/MS analysis.

2.4. Matrix effect measurements

Matrix-induced signal suppression must be taken into consideration in extraction procedures and specially for a multimycotoxin analysis. It can be omitted by using an appropriate calibration method such as matrix-matched calibration standards, standard addition or by the use of adequate internal standards as previously reported for other analyzed compounds (Krska et al., 2007).

The matrix effect was assessed by employing matrix-matched standards and calculating the area ratio, which is defined as the absolute matrix effect (ME%).

2.5. Method validation

The method was validated for linearity, accuracy, precision (repeatability and reproducibility) and sensitivity. For that, it was validated as a quantitative confirmatory method according to the EU Commission Decision, 2002/657/EC (Commission Decision, 2002).

Linearity was evaluated using the standard calibration curves that were constructed for each mycotoxins by plotting the signal intensity versus the analyte concentration and the internal standard (I.S.) and obtaining the areas ratios (area analyte/area internal standard). Calibration curves were constructed from the peak area ratio of each analyte to I.S. DON-d1.

The accuracy was evaluated by compound individually, calculating the recoveries. Recovery experiments were conducted at two different levels for each matrix, one at limits of quantification (LQs) and the other 10 times LQs, added before the corresponding extraction procedure.

Intraday precision was assessed by calculating the relative standard deviation (RSD_r), calculated from results generated under repeatability conditions of six determinations per concentration in a single day. Interday precision was calculated by the relative standard deviation (RSD_R) calculated from results generated under reproducibility conditions by one determination per concentration on 6 days.

Sensitivity was evaluated by limit of detection (LD) and limit of quantification (LQ) values.

3. Results and discussion

3.1. Chromatography and mass spectrometry optimized conditions

The optimized MRM parameters (MRM transitions, interface parameters, and MS/MS parameters) were obtained by infusion experiments performed in both positive and negative ion mode for all the analytes (Table 1). The ion mode was chosen based on the more selective and sensitive signal intensities detection.

Most mycotoxins exhibited higher precursor ion signal intensities or better fragmentation patterns (i.e. a higher number of characteristic product ions suitable for MRM detection) in negative ion mode than in positive mode (DON, FUS-X, 3-AcDON, NIV, 15-AcDON, ZON, α-ZOL and β-ZOL); however, HT-2, T-2, DAS and NEO showed higher (about three times) signal intensities in positive mode.

In this work, a single run by switching the ion source polarity between positive and negative modes in 15 min the chromatographic run was performed. For each analyte two transitions were monitored. In spite of the high number of MRM transitions (26) to be monitored within one period, including positive and negative polarity, 100 ms as dwell time was used, leading to a fine sensitivity; this allowed us to obtain enough data points per chromatographic peak. The use of two transitions gave four identification points, corresponding to one precursor (1 point) and two product ions (3 points). This parameter fulfills the European Commission requirements (at least three identification points) for confirmation of compounds listed in group B where the mycotoxins studied are included (Directive 96/23/EC, 1996). The most sensitive transition was used for quantitation (quantifier), while the others were used to identify confirmation (qualifiers).

Table 1
MS/MS parameters for mycotoxin detection by the multiple reaction monitoring (MRM) method.

Analyte	Precursor ion Q_1 (m/z)	Product ion Q_3 (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)
NIV	371.0	281[A]	−60	−10	−30	−7
		311			−30	−7
DON	355.1	265	−15	−8	−21.7	−9.2
		295[A]			−10.0	−10.0
FUS-X	413.0	58.6[A]	−30	−4	−35	−10
		263.2			−17	−24
15-AcDON	397.1	336.9	−10	−10	−13	−16
		150.3			−35	−8
3-AcDON	397.3	336.0	−50	−10	−10	−12
		307.2[A]			−21	−10
DAS	384.3	105	35	7	45	4
		307.3			15	9
NEO	400.2	185.0	45	8	25	5
		305.1			17	9
HT-2	442.4	263[A]	14	7	18	7
		215			18	6
T-2	484.2	305.2	14	7	19.6	10
		185[A]			29	16
ZON	317.0	131[A]	−22	−12	−5	−35
		174			−13	−35
α-ZOL	319	160[A]	−75	−10	−28	−7
		275			−28	−7
β-ZOL	319	160	−75	−10	−28	−7
		275[A]			−28	−7
d1-DON[a]	356.3	217	−35	−12	−35	−12
		140			−35	−12

Q_1 First quadrupole; Q_3 third quadrupole; DP: declustering potential; EP: entrance potential; CE: collision energy; CXP: collision cell exit potential. Dewll (msec): 100.
[A] Transitions used for quantitation.
[a] Internal standard; (Analite abbreviation identification as in Fig. 1).

Excellent separation of the 12 mycotoxins was obtained under the optimized LC–MS/MS conditions mentioned, as shown in Fig. 1.

3.2. Extraction

Given the chemical diversity of mycotoxins investigated herein, the optimization of the extraction solvent was the most challenging step for the method development. Choosing the extraction solvent was based on its frequency of use by other authors for mycotoxins (Cavalieri, Foglia, Pastorini, Samperi, & Laganà, 2005; Commission Decision, 2002; Diaz, 2005; Klotzel et al., 2006; Krska et al., 2007; Lattanzio, Solfrizzo, Powers, & Visconti, 2007; Meneely et al., 2011; Ren et al., 2007; Santini et al., 2009; Sospedra et al., 2010; Sulyok, Krska, & Schuhmacher, 2010; Sulyok et al., 2006; Tanaka, Takino, Sugita-Konishi, & Tanaka, 2006). As mentioned in reports published by other authors, solvent combination can guarantee high recoveries of trichothecenes of different polarity. Currently, the acetonitrile/water (84/16, v/v) mixture is the most widely used solvent for multimycotoxins extraction. In fact, many reports show that the use of these solvents for the extraction yielded less interfering compounds of the matrix compared to other mixtures as methanol/water.

Among the various extraction mixtures used to extract trichothecenes and ZON from cereals, it has been acetonitrile/water (Ren et al., 2007; Santini et al., 2009; Vendl, Berthiller, Crews, & Krska, 2009) and methanol/water (Sospedra et al., 2010; Tanaka, Sugita-Konishi, Takino, Tanaka, Toriba, & Hayakawa, 2010) mixed at different ratios the most commonly solvents used. Sulyok et al., used acetonitrile/water over methanol/water since low recoveries for ZON were found with the latter (Sulyok et al., 2006).

Moreover, trichothecenes and ZONs are generally extracted either by conventional liquid shaking for a period varying from 30 min to 1 h (Fazekas & Tar, 2001; Tanaka, Yoneda, Inoue, Sugiura, & Ueno, 2000) or by blending for few minutes (Krska & Josephs, 2001). Taking as a starting point these scientific reports, an extraction solvent method performed by Santini et al. (2009) to analyze NIV, DON, FUS-X, 3-AcDON, HT-2, T-2, NEO, ZON and zearalanone (ZAN), in wheat and maize, was used as our reference.

For our multimycotoxin extraction procedure of trichothecenes, ZON and its metabolites (α-ZOL and β-ZOL), from different kinds of cereal and cereal products samples, it was necessary to study slight different solvent polarity for their extraction. The solvent mixtures used and the recovery obtained are shown in Fig. 2. Two different mixtures of acetonitrile/methanol (extraction 1: 60:40, v/v; extraction 2: 40:60, v/v) and two acetonitrile/water mixtures (extraction 3: 84:16, v/v; extraction 4: 16:84, v/v) were proved, while the mixture methanol/water was dismissed according to the disadvantages indicate previously by other authors.

Recovery experiments were performed in triplicate by spiking blank samples at 100 μg/kg. Therefore, recovery data reported are within the criteria approved by the European Committee for Standardization, for the acceptability of analytical methods for ZON, DON and HT-2 and T-2, at levels $\geqslant$100 μg/kg, recoveries ranged between 70–120%, 20–110% and 30–160%, respectively (Commission Directive, 2005).

The best recoveries were obtained when the solvents reported for extraction 1 and 3 were used, which mixture solvent had higher proportion of acetonitrile, as shown in Fig. 2. However in these two extractions better recoveries were observed for some mycotoxins than others. The "extraction 1" was more adequate for DON, 3-AcDON, 15-AcDON, while "extraction 3" was better for HT-2, T-2, ZON, α-ZOL and β-ZOL. These performances were observed in all the matrices studied. Fig. 2 shows the recoveries obtained for wheat matrices. Comparing each mycotoxin recoveries of both extractions, the "extraction 3" was chosen because of the high difference recoveries existing between extraction methods for HT-2, T-2, ZON, α-ZOL and β-ZOL. Observing the recoveries of "extraction 3" between the studied matrices (Fig. 2) the differences were not significant, but lower recoveries were obtained for bread than for grain and flour.

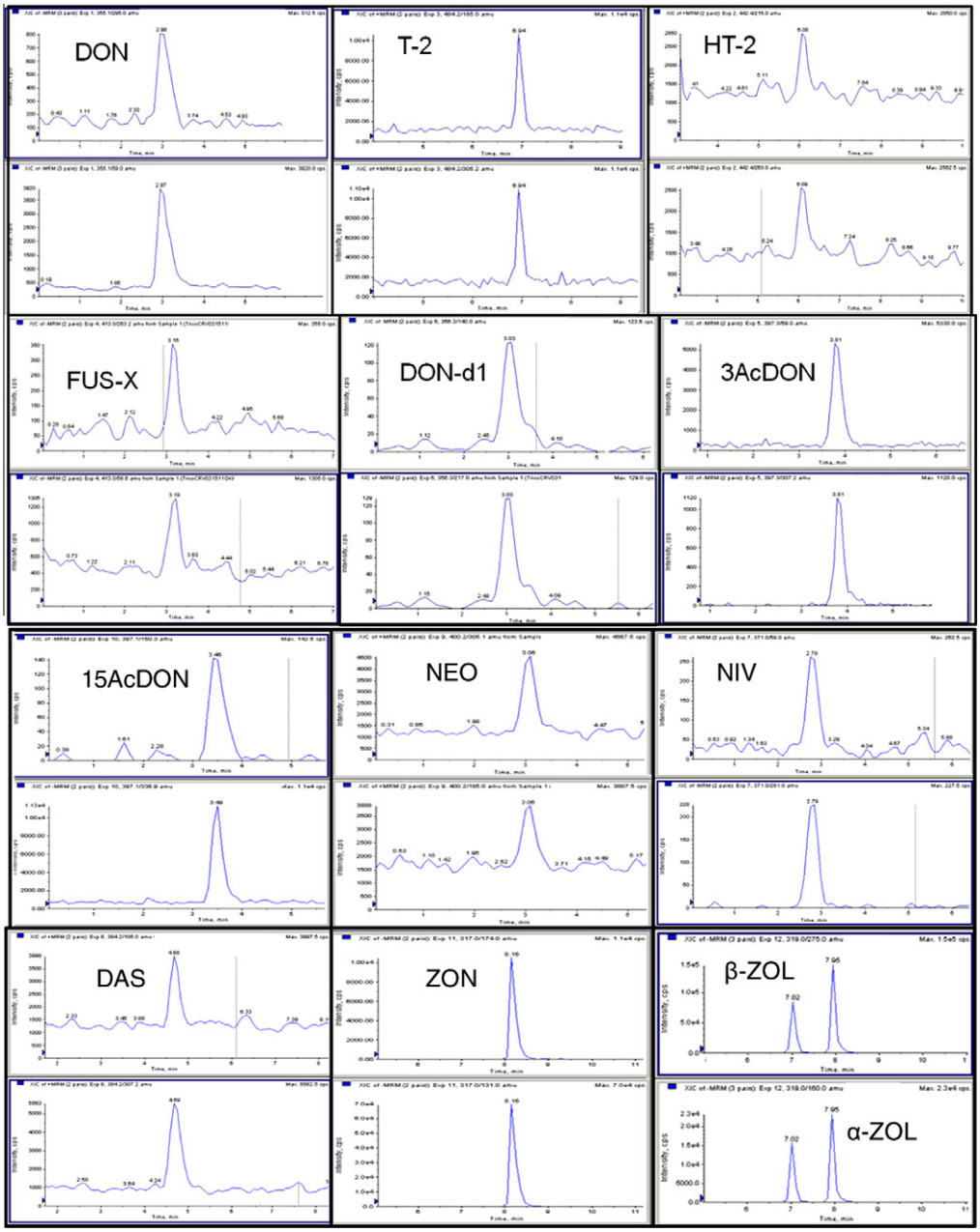

Fig. 1. Chromatograms of blank flour wheat sample spiked with studied mycotoxin at 50 μg/kg.

3.3. Analytical method validation

The developed method was validated as a quantitative confirmatory method according to the EU Commission Decision, 2002/657/EC (Commission Decision, 2002) and the parameters taking into account for this purpose were: instrumental linearity, accuracy, precision (repeatability and reproducibility) and sensitivity.

Calibration curves used for linearity evaluation were constructed from the peak area ratio of each analyte to the I.S. In the same manner, matrix-matched standards of the studied mycotoxins were prepared using sample treatment, by adding known amount of working solution to the obtained extracts in order to reach the desired concentration range. The signal intensities obtained were plotted.

The linearity in the response in LC–MS/MS was obtained in triplicate by spiking six concentrations, 10, 20, 50, 100, 250 and 500 ng/g. The linear regression coefficient of calibration curves are presented in Table 2, showing that good results were achieved, with corresponding correlation coefficients (r^2) higher than 0.992.

The accuracy was evaluated for each compound individually by calculating the recoveries. Recovery experiments were conducted at two different levels for each matrix, one between 5 and 50 μg/kg (limits of quantification, LQs) and the other between 50 and 500 μg/kg (10 times LQs). In both cases, mycotoxins were added before the corresponding extraction procedure.

The precision values are calculated from the Horwitz equation for the relative standard deviation (RSD_R) calculated from results generated under reproducibility and repeatability conditions.

Intraday precision was assessed by calculating the RSD of six determinations per concentration in a single day and interday precision by one determination per concentration on 6 days.

The values of intraday precision ($n = 6$) and interday precision ($n = 6$) for wheat, oat, barley and spelt were inferior to 10% and 14%, respectively. In Table 3 is shown the results of precision for grain samples at fortified level of two times the LQ. The recoveries obtained ranged for wheat: 73–98%; oat: 75–96%; barley: 73–99%; and spelt: 78–99%. It collects also the precision (RSDs) of theses

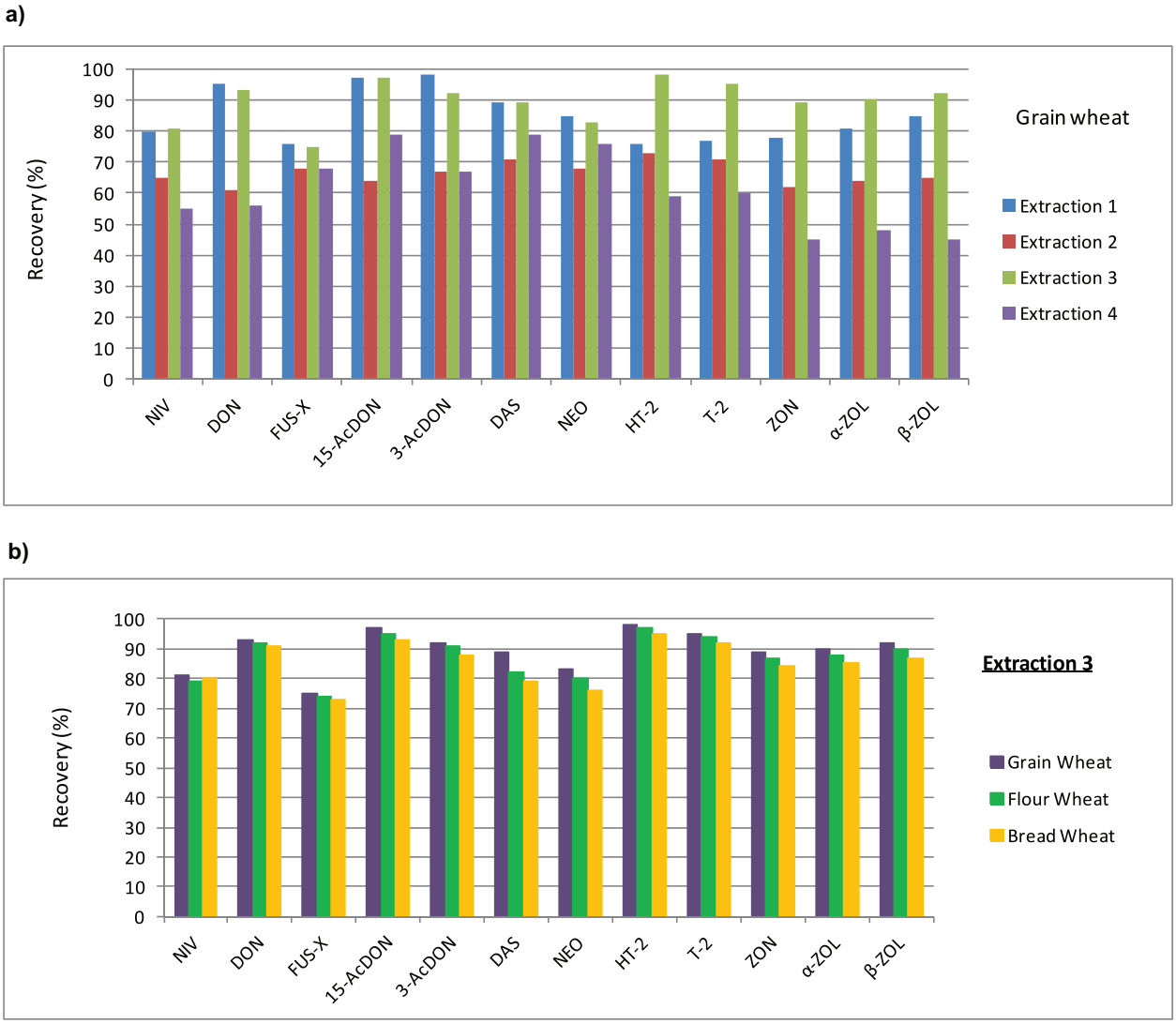

Fig. 2. Recoveries experiments performed in triplicate by spiking blank samples at 100 μg/kg. (a) Recoveries obtained with studied extraction methods ("extraction 1" acetonitrile:methanol (60:40, v/v), "extraction 2" acetonitrile:methanol (40:60, v/v), "extraction 3" acetonitrile:water (84:16, v/v) and "extraction 4" acetonitrile:water (16:84, v/v)) in grain wheat; and (b) recoveries obtained with extraction 3 in the studied products of wheat.

Table 2
Linearity equation's parameters of mycotoxin in matrix-matched samples using as internal standard DON-d1.

Compound	Wheat			Oat			Barley			Spelt		
	Range (0–500 ng g^{-1})			Range (0–500 ng g^{-1})			Range (0–500 ng g^{-1})			Range (0–500 ng g^{-1})		
	Slope (a)	Coefficient correlation (r^2)	ME%	Slope (a)	Coefficient correlation (r^2)	ME%	Slope (a)	Coefficient correlation (r^2)	ME%	Slope (a)	Coefficient correlation (r^2)	ME%
NIV	69.0	0.9966	72	107.7	0.9960	70	101.4	0.9983	70	100.9	0.9980	73
DON	482.2	0.9994	89	574.6	0.9920	77	752.9	0.9989	75	852.9	0.9930	88
FUS-X	640.4	0.9930	68	559.8	0.9960	67	561.9	0.9985	65	374.2	0.9958	69
15-AcDON	990.5	0.9990	90	857.2	0.9960	84	738.8	0.9952	83	856.2	0.9950	90
3-AcDON	183.5	0.9987	85	346.5	0.9920	78	298.5	0.9970	75	299.0	0.9950	84
DAS	3651.4	0.9989	83	4709.3	0.9970	75	5601.7	0.9930	74	6147.8	0.9920	83
NEO	4553.1	0.9996	89	3637.8	0.9992	83	5927	0.9951	81	5537.6	0.9973	88
HT-2	1930	0.9991	102	3637.0	0.9992	100	4355	0.9980	99	3651	0.9990	102
T-2	4355	0.9990	110	6342.4	0.9950	100	4763	0.9940	102	6444.1	0.9963	110
ZON	2000	0.9950	93	1440	0.9921	91	2140	0.9999	90	2200	0.9999	93
α-ZOL	3060	0.9980	95	1700	0.9950	92	3370	0.9994	90	2760	0.9980	93
β-ZOL	1450	0.9959	96	2180	0.9999	92	2130	0.9998	91	2140	0.9970	94

Compound abbreviation identification as in Fig. 1; ME% is area ratio of standard solution and matrix-matched standard multiplied per 100.

samples, which ranged for wheat: 2.4–11; oat: 2.8–13; barley: 2.8–15; and spelt: 2.4–12. It demonstrates to be satisfactory for the 12 mycotoxins in all the matrices studied.

Sensitivity was evaluated by limit of detection (LD) and limit of quantification (LQ) values. The LD was estimated from blank extract, spiked with decreasing concentrations of the analytes, where the re-

Table 3
LD and LC for wheat, oat, barley and spelt grain samples (n = 20) spiked at level two times the LQ.

Mycotoxin	Wheat				Oat				Barley				Spelt			
	LD (μg/kg)	LQ (μg/kg)	Intra-day R ± RSDr (%)	Inter-day R ± RSDr (%)	LD (μg/kg)	LQ (μg/kg)	Intra-day R ± RSDr (%)	Inter-day R ± RSDr (%)	LD (μg/kg)	LQ (μg/kg)	Intra-day R ± RSDr (%)	Inter-day R ± RSDr (%)	LD (μg/kg)	LQ (μg/kg)	Intra-day R ± RSDr (%)	Inter-day R ± RSDr (%)
NIV	5.5	15	81 ± 5.6	80 ± 7.1	8	20	82 ± 7.0	79 ± 7.0	6	15	80 ± 6.3	75 ± 11	6	15	85 ± 7.3	83 ± 7.3
DON	1	10	93 ± 3.0	90 ± 4.0	2	12	95 ± 2.8	90 ± 6.1	1	10	91 ± 3.1	95 ± 5.4	1	10	93 ± 2.9	95 ± 4.2
FUS-X	5.5	20	75 ± 9.0	73 ± 11	8	22	76 ± 9.3	77 ± 9.0	5	20	73 ± 10	76 ± 10	5	25	78 ± 9.8	81 ± 11
15-AcDON	2	10	97 ± 4.5	95 ± 6.0	5	12	95 ± 5.1	96 ± 8.0	2	10	93 ± 5.2	90 ± 9	2	10	97 ± 4.7	99 ± 7.3
3-AcDON	2	10	92 ± 3.6	95 ± 5.3	5	12	92 ± 3.7	93 ± 5.2	2	10	93 ± 3.3	93 ± 8.1	2	10	94 ± 3.5	98 ± 10
DAS	2	10	89 ± 6.5	80 ± 8.0	8	12	90 ± 8.0	84 ± 11	2	10	93 ± 7.6	93 ± 15	2	10	94 ± 7.7	93 ± 11
NEO	5.5	20	83 ± 5.5	82 ± 7.0	8	22	85 ± 4.7	75 ± 13	5	20	76 ± 5.7	76 ± 14	5	20	85 ± 5.9	91 ± 9.0
HT-2	2	6	98 ± 4.3	95 ± 5.1	2	9	96 ± 4.2	86 ± 6.0	4	8	95 ± 4.6	95 ± 9.4	2	6	95 ± 2.4	95 ± 6.3
T-2	3	8	95 ± 11	90 ± 11	3	11	90 ± 10	89 ± 10	5	10	92 ± 8.0	92 ± 10	3	8	92 ± 12	95 ± 12
ZON	1.5	5	89 ± 2.4	88 ± 5.0	2	5	86 ± 2.5	83 ± 5.0	2	5	95 ± 3.0	99 ± 8	1.5	5	94 ± 2.9	94 ± 5.0
α-ZOL	1.5	5	90 ± 3.1	90 ± 5.0	2	5	88 ± 3.2	90 ± 8.0	2	5	90 ± 2.8	93 ± 7.3	1.5	5	89 ± 3.2	87 ± 5.7
β-ZOL	2	2.5	92 ± 2.8	90 ± 6.2	2	2.5	95 ± 2.7	93 ± 10.1	2	5	87 ± 2.9	90 ± 9	2	2.5	87 ± 3.0	90 ± 6.2

LD = 3 *S/N* (20 representative blank samples); LQ = 10 *S/N* (20 representative samples spiked at CCα level) (compound abbreviation identification as in Fig. 1).

sponse of the qualifier ion was equal to three times the response of the blank extract ($n = 20$). Once evaluated, three samples were spiked at the estimated levels and extracted according to the proposed procedure. The LQ (RSD ⩽15% and an accuracy ⩾73 ± 15%) was preliminarily estimated, in the same way as the LD but using the criterion of $S/N \geqslant 10$ for the qualifier ion.

The limits of detection and quantification of studied mycotoxin by using LC ESI (+) are shown in Table 3.

3.4. Matrix effect

In this work due to the diversity of the matrices and compounds evaluated, matrix effect is different, and it made necessary the evaluation of this effect.

According to other authors, recovery are highly dependent on the analyte-matrix combination and the efficiency of extraction prior to MS analysis (Juan, Igualada, Moragues, León, & Mañes, 2010; Meneely et al., 2011; Santini et al., 2009). To study this effect, matrix-matched calibration was performed.

The matrix interference is defined as the area ratio between the area of matrix-matched standard (blank cereal extract resolved in a standard solution with the same analyte concentration of the standard solution to compare) and the standard solution. The internal standard studied and used for this method was DON-d1.

The area ratio (B/A × 100) is defined as the absolute matrix effect (ME%). A correspond to: MS/MS area of standard solution and B: MS/MS area of matrix-matched standard. 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%. Tests carried out in this work were conducted in triplicate on blank flour and milled grain samples, purchased from a local supermarket, and spiked to obtain the experimental concentration of each mycotoxin for the LC–MS/MS analysis. All samples used were first examined for the presence of possible contaminants.

In order to evaluate the possible differences in matrix-induced signal, between bread, flour and grain of wheat and between flour and grain of the other three studied cereals varieties (oat, barley and spelt) matrices were spiked at three levels and extracted with the optimized method. Results obtained show similar matrix effect in all of the three levels studied. A signal enhancement for HT-2 and T-2 and a signal suppression for the other mycotoxins, with higher suppression for FUS-X and NIV, were observed. The ME obtained in the four flour matrices spiked at two times the LQ, is shown in Table 3.

4. Conclusions

In this study is presented and developed a method simple, rapid, quantitative, performed with a simple liquid mixture extraction without clean-up step and by using a fast determination technique, triple quadrupole mass spectrometer (LC–MS/MS).

Mass spectrometer detection allowed obtaining unambiguous identification of all mycotoxins mentioned throughout the text below μg/kg levels, fulfilling the requirements established by the European Union. Sensitivity was high due to the low LDs and LQs obtained which were better than those reported by other authors for these natural toxic compounds, although the analyzed number of them has been lower than the presented in this study. Furthermore, the use of internal standard helped to compensate the significant matrix effect observed for some matrices and to obtain high success in the method validation and to reach the parameters compiled in Commission Decision 2002/657/EC (Commission Decision, 2002).

Chromatographic analysis time was performed in 15 min, which would be able to meet the requirement for a high throughput determination.

Recoveries and precision (RSDs) collected in Table 3, demonstrated to be satisfactory for the 12 mycotoxins in all the matrices studied.

The optimization of this method presented for 12 mycotoxins, in four cereal matrices, two format market modalities and one final cereal product, which means a wide variety of possible sample combinations to analyze in laboratories, displays the high possibility of being chosen as a method used for routine analysis for any laboratory, but especially for those of reference where the number of received samples is high.

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