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Use of the modified quick easy cheap effective rugged and safe sample preparation approach for the simultaneous analysis of type A- and B-trichothecenes in wheat flour

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ABSTRACT

A suitable extraction and purification method for the simultaneous liquid chromatography–mass spectrometry (LC–MS) determination of five mycotoxins, three type A, diacetoxyscirpenol (DAS), T-2 toxin (T-2) and HT-2 toxin (HT-2), and two type B-trichothecenes, deoxynivalenol (DON) and nivalenol (NIV), has been optimised using a modified "Quick Easy Cheap Effective Rugged and Safe" (QuEChERS) method. Different solvents were studied in the extraction procedure to obtain better recoveries, which ranged from 86 to 108%, using a 85/15 (v/v) mixture of methanol/acetonitrile. The values obtained for recovery, repeatability and reproducibility of the optimized method are in agreement with Commission Directive 2005/26/EC for methods of analysis of *Fusarium* toxins. Finally, this optimized procedure was applied in wheat flour samples commercialized in Spain.

1. Introduction

Molds, as toxic secondary metabolites, can produce mycotoxins, including trichothecenes which are toxic sesquiterpenoid compounds composed of a central core of fused cyclohexene/tetrahydropyran rings. The major type A trichothecenes in *Fusarium* species include diacetoxyscirpenol (DAS), T-2 toxin (T-2) and HT-2 toxin (HT-2), both of which posses an isovalerate function at C-8 [1]. *F. sporotrichiodies* and *F. poae* are some of the major type A trichothecene producers whereas. *F. culmorum* and *F. graminearum* produce mainly type B-trichothecenes [2], including deoxynivalenol (DON) and nivalenol (NIV), which feature a ketone at C-8 [1]. These types of trichothecenes are responsible for a wide range of disorders in animals, including feed refusal, weight loss and vomiting, and they have been found to inhibit proteins, DNA and RNA synthesis, and to have immunosuppressive and cytotoxic effects [3–5]. Health risks associated with human exposure to *Fusarium* toxins are recognized worldwide and depend on their levels in a diversified diet. The major sources of dietary intake of trichothecenes are cereal products, in particular wheat and corn. Processing of cereal crops into foods and commercial or home preparation of cereal-based foods may decrease the level of mycotoxins but does not completely eliminate them [6].

The European Scientific Committee on Food (SCF) evaluated the *Fusarium* toxins [7], including DON, NIV, T-2 and HT-2 and established a fully tolerable daily intake (TDI) for DON at 1 mg kg^{-1} body weight day^{-1}, a temporary tolerable dietary intake (t-TDI) for NIV at 0.7 mg kg^{-1} body weight day^{-1}, and a combined t-TDI for T-2 and HT-2 at 0.06 mg kg^{-1} body weight day^{-1}.

These mycotoxins are soluble in organic solvents, such as chloroform, methanol, acetonitrile in acid medium and also in diluted aqueous sodium bicarbonate; its analysis is a multiple step process usually based on extraction, clean-up and determination. Frequently used clean-up and preconcentration procedures are solid-phase extraction [8], inmmunoaffinity columns [9] and solid-phase microextraction [10] which have resulted in new possibilities in sample treatment and advantages such as a substantial reduction of the extraction time and incorporation into on-line flow-analysis systems [11,12].

The 'Quick Easy Cheap Effective Rugged and Safe' (QuEChERS) sample preparation method has been introduced by Anastassiades et al. [13]. This method has many advantages over traditional techniques, high simple throughput, the use of smaller amounts of organic solvent and the use of no chlorinated solvents. QuEChERS extraction is a rapid and economic method for food contaminants extraction. This procedure has been applied with success in several nonfatty (<2%) and low-fat (2–20%) food matrixes, such milk, egg and avocado to determine compounds in foods such as pesticides [14], antibiotics [15] and acrylamide [16] among others. In this technique primary secondary amine (PSA) is used as sorbent to retain co-extractive compounds, such as sugar and fatty

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acids [17,18]. On the other hand, the extraction step products use anhydrous magnesium sulphate to reduce water in the sample, along with either sodium chloride. QuEChERS procedure can be performed with mixture of solvents.

The aim of this work is to apply QuEChERS method for the simultaneous determination of both type A- and B-trichothecenes by LC–MS in food and feed.

2. Experimental

2.1. Reagents and materials

Standards solutions of 100 μg/ml of five trichothecenes (DON, DAS, NIV, T-2 and HT-2) were purchased from Sigma–Aldrich (St. Louis, USA). Solvents (acetonitrile, methanol, formic acid, dichloromethane and ethyl acetate) were purchased from Merck. Primary secondary amine (PSA) was from Varian (USA). Anhydrous magnesium sulphate and sodium chloride were obtained from Sigma–Aldrich. Twenty white wheat flour samples were taken in Supermarkets from Valencia (Spain). All samples were stored at −18 °C prior to mycotoxin analysis.

2.2. Standard preparation

The stock solutions were diluted with acetonitrile in order to obtain multi-compounds working standard solutions (0.05–1.5 μg/ml concentration of each compound). The new working solutions were stored in a refrigerator at 4 °C.

2.3. Sample preparation

Some modifications from the original QuEChERS method were introduced to ensure efficient extraction of trichothecenes. All the samples were homogenized using a laboratory mill. 5 g of each homogenized samples were weighed into a 50 ml centrifuge tube, 10 ml of mixture of methanol:acetonitrile (85:15, v/v) were added and the tube was closed and shaken vigorously using a vortex for 1 min. After shaking, a mixture of 2 g of magnesium sulphate and 1 g of sodium chloride were added. The tube was closed and immediately vortex for 1 min and centrifuge for 10 min at 4000 r.p.m. An aliquot of 1 ml of the extract was transferred into a 2 ml centrifuge tube and 50 mg of PSA and 150 mg of magnesium sulphate were added. The tube was shaken for 1 min and centrifuged for 10 min at 4000 r.p.m. After centrifugation the cleaned extract was filtered through 0.45 μm nylon filter and transferred into a vial.

2.4. LC–MS analysis

LC–MS analysis was carried out on a Hewlett Packard HP-100 Series Model equipped with a binary solvent pump, an autosampler, a mass spectrometric detector (MSD) and an analytical work station. The MSD consisted of a standard atmospheric pressure ionisation (API) source configured as electrospray (ES). The interface was operated in positive ionization mode (API-ES+). A drying gas flow of 13 l/min and a drying gas (nitrogen) temperature of 350 °C were used for API-ES+ working. Dwell times (ms) for the mycotoxins analyzed were 700 for NIV, DON, and DAS and 349 for T-2 and HT-2. The most adequate capillary voltage was 4000 and the fragmentor was set at 100 V. Using this conditions, and with the mobile phase used, the ions obtained for NIV, DON, DAS, HT2 and T2 were the sodium adduct $[M+Na]^+$ at m/z 355, 319, 389, 447 and 489, respectively.

The analytical column used was a Luna C18 (250 mm × 4.6 mm ID 5 μm). As a mobile phase we used a mobile phase "A" (consisting of water:1% of formic acid) and mobile phase "B" (including methanol:1% of formic acid). The conditions for the mobile phase were set up at a constant flow of 0.5 ml/min with the following gradient: 0 min 40% B and 10 min 90% B until 25 min.

3. Results and discussion

Achieving maximum efficiency is probably the greatest concern in QuEChERS method development. Solvent choice is presented and some validation parameters were determined for analytical performance used. Finally, the selected procedure was used in real wheat flour samples commercialized in Spain.

3.1. Solvent election

The selection of a suitable extraction solvent is the first challenge in this method development. Mixture of several solvents, such as dichloromethane, ethyl acetate, acetonitrile, methanol, methanol/acetonitrile, methanol/acetonitrile/water and methanol/water, was tried out for the extraction of studied mycotoxins from wheat flour (Table 1). Methanol is the only solvent capable to extract all five mycotoxins with recoveries upper 50%. Acetonitrile present good recoveries for trichothecenes type A, but no with more polar NIV and DON. Only mixtures of methanol/acetonitrile between 75/25 and 95/5 (v:v) offers recoveries in agreement with Commission Directive 2005/26/EC [19] for methods of analysis of *Fusarium* toxins. Methanol/acetonitrile proportion finally used is selected for minor RSD values and better extract with the more polar DON and NIV. As reported in Fig. 1, the better recoveries, ranged from 86 to 108%, for the simultaneuous analysis of the studied mycotoxins is obtained with the mixture methanol:acetonitrile (85:15, v/v).

3.2. Analytical performance

For calibration and linearity, six levels of concentration (1500, 1125, 750, 375, 100 μg/kg and limit of quantification for each myco-

Table 1
Assessment of different extraction solvent (10 ml) in wheat flour (5 g) samples spiked at 500 μg/kg level.

Recovery ± SD (%) (n = 5)	Mycotoxin				
	DON	NIV	DAS	T-2	HT-2
Dichloromethane	–*	–*	52 ± 8.4	40 ± 7.9	–*
Ethyl acetate	42 ± 7.7	–*	60 ± 7.7	–*	–*
Acetonitrile	–*	–*	80 ± 7.4	74 ± 7.3	92 ± 8.1
Methanol/acetonitrile (20/80, v/v)	–*	–*	104 ± 7.2	97 ± 7.2	119 ± 9.1
Methanol/acetonitrile (50/50, v/v)	–*	–*	95 ± 7.5	82 ± 7.9	113 ± 9.0
Methanol/acetonitrile (80/20, v/v)	78 ± 6.6	92 ± 7.9	95 ± 7.3	90 ± 7.4	91 ± 7.4
Methanol	58 ± 8.2	50 ± 8.1	80 ± 7.4	81 ± 7.3	86 ± 7.6
Methanol/acetonitrile (80/20, v/v) + 5 ml water	–*	42 ± 8.2	91 ± 6.4	79 ± 6.8	85 ± 6.7
Methanol + 5 ml water	–*	–*	68 ± 7.4	50 ± 8.0	–*

–*, mean recovery value is <20%.

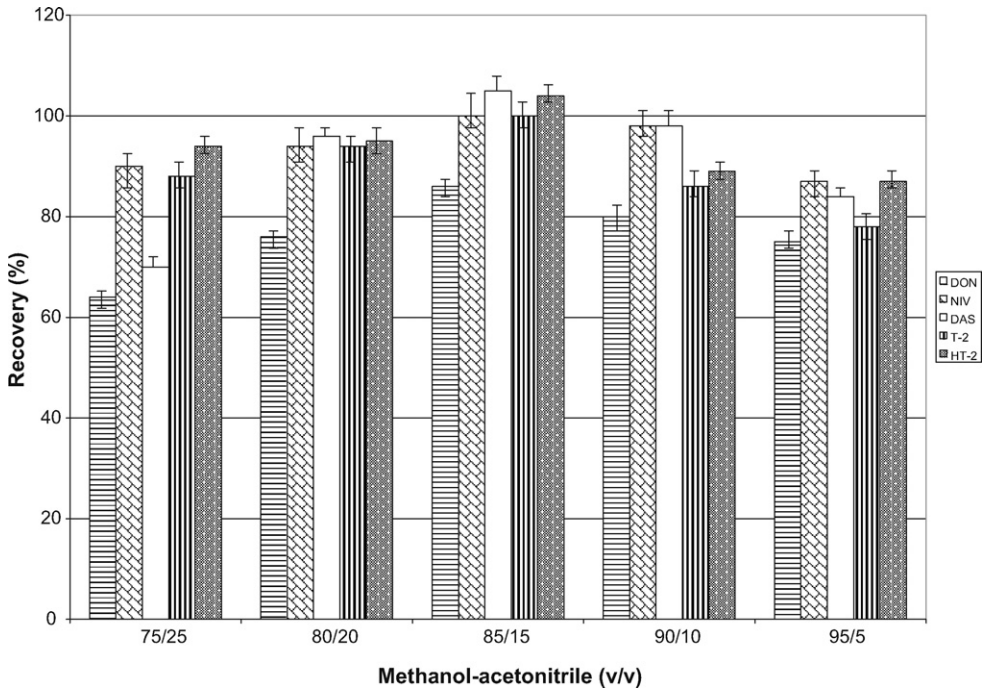

Fig. 1. Effect of different mixture of methanol-acetonitrile about extraction of fortified wheat flour samples (error bars are ±S.D.; $n = 5$) at a level of 500 μg kg^{-1} of studied mycotoxins.

toxin) were tested in triplicate. The calibration plots from 100 to 500 μg/kg were linear with $r^2 > 0.99$.

Table 2 reflected the values of performance characteristic including limits of detection (LOD) and quantification (LOQ), recoveries, repeatability and reproducibility obtained in wheat flour samples spiked at 500 μg/kg by quintuplicate using methanol–acetonitrile (85:15, v/v) as eluting solvent. Fig. 1 shows the LC–MS chromatogram obtained following the proposed extraction procedure for wheat flour fortified with studied mycotoxins at 500 μg/kg level.

For LOD and LOQ of the mycotoxin were experimentally calculated by considering a signal value 3 and 10 times, respectively, that of the background noise given by the software, on the basis of five independent determinations. LOD values ranged from 1 to 30 μg/kg and LOQ values ranged from 4 to 100 μg/kg. The technique of standard additions in the flour samples was used to calculate the recovery of this method adding to five levels and in triplicate of the standard solution of studied mycotoxins. The slopes of the lines thus obtained for each of the mycotoxins were compared with the corresponding slopes obtained in the calibration with standards, according to the Student's *t*-criterion [20]. In Table 2, data for the recovery of each mycotoxin, with values ranging from 86.2 to 108.5%, is demonstrated.

For repeatability and reproducibility, five series of five extractions of a flour samples spiked, at 500 μg/kg, with the studied mycotoxins. The corresponding relative standard deviations (RSD) were calculated and shown in Table 2. The RSD obtained for intra-day variation ($n = 5$) ranged from 3.4 to 6.7%. The inter-day variation showed RSD values between 5.6 and 9.0%. These values lower than 10% confirming the good reproducibility and repeatability of this technique. Furthermore, the values obtained for recovery, repeatability and reproducibility of the optimized method are in agreement with Commission Directive 2005/26/EC [19] for methods of analysis of *Fusarium* toxins. A graph of the experimental results, corresponding to the optimized QuEChERS of mycotoxin in a spiked wheat flour sample, is shown in Fig. 1.

3.3. Application of the optimized method to real flour samples

The improved QuEChERS method was applied for the determination of five trichothecenes in twenty white wheat flour samples commercialized in Spain. The studied mycotoxins were not detected in any of the studied samples. A chromatogram of a real sample is shown in Fig. 2B. Neither T-2 nor HT-2, DON and other mycotoxins were detected in flour, as well as in a variety of other wheat-based products and in whole wheat in the study of Valente-Soares and Furlani [21]. Betchel et al. [22] suggested that DON and NIV were localized at the site of their production rather than being transported from the kernel surface to the interior. Schollenberger et al. [23] reflected that DON, NIV, T-2 and HT-2 were restricted mainly to the outer parts of the original wheat kernels.

In conclusion, this paper describes a simple, rapid, economic and effective procedure for sample extraction using QuEChERS method and liquid chromatography with mass spectrometric detection by

Table 2
Performance characteristic obtained in wheat flour samples spiked with five trichothecenes at 500 μg/kg.

Mycotoxin	Limit of detection (LOD, μg kg^{-1})	Limit of quantification (LOQ, μg kg^{-1})	Recovery (%)	Repeatability (RSD, %) ($n = 5$)	Reproducibility (RSD, %) (5 different days)
DON	3	10	86	6.7	8.2
NIV	30	100	100	6.5	8.1
DAS	1.5	5	108	3.4	8.1
T-2	1	4	93	4.0	5.6
HT-2	5	18	104	4.7	9.0

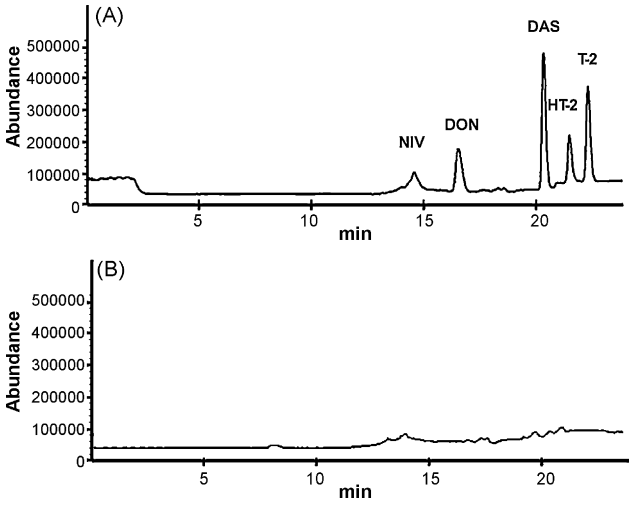

Fig. 2. LC–MS chromatograms obtained after QuEChERS extraction; (A) extract of wheat flour fortified with 500 μg kg^{-1} of NIV, DON, DAS, HT-2 and T-2 toxins respectively and, (B) extract of sample non-fortified.

selected ion monitoring (LC/MS-SIM) for the simultaneous quantification in wheat flours of five A- and B-trichothecenes (T-2, HT-2, DAS, DON and NIV). This method has many advantages over traditional techniques such as having no derivatisation step, high simple throughput and using smaller amounts of organic solvent.

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