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Multi-mycotoxin analysis in eggs using a QuEChERS-based extraction procedure and ultra-high-pressure liquid chromatography coupled to triple quadrupole mass spectrometry

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ABSTRACT

A reliable and rapid method has been developed for the determination of 10 mycotoxins (beauvericin, enniatin A, A1, B1, citrinin, aflatoxin B1, B2, G1, G2 and ochratoxin A) in eggs at trace levels. Ultra-high-pressure liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS) has been used for the analysis of these compounds in less than 7 min. Mycotoxins have been extracted from egg samples using a QuEChERS-based extraction procedure (Quick, Easy, Cheap, Effective, Rugged and Safe) without applying any further clean-up step. Extraction, chromatographic and detection conditions were optimised in order to increase sample throughput and sensitivity. Matrix-matched calibration was used for quantification. Blank samples were fortified at 10, 25, 50 and 100 $\mu g\,kg^{-1}$, and recoveries ranged from 70% to 110%, except for ochratoxin A and aflatoxin G1 at 10 $\mu g\,kg^{-1}$, and aflatoxin G2 at 50 $\mu g\,kg^{-1}$. Relative standard deviations were lower than 25% in all the cases. Limits of detection ranged from 0.5 $\mu g\,kg^{-1}$ (for aflatoxins B1, B2 and G1) to 5 $\mu g\,kg^{-1}$ (for enniatin A, citrinin and ochratoxin A) and limits of quantification ranged from 1 $\mu g\,kg^{-1}$ (for aflatoxins B1, B2 and G1) to 10 $\mu g\,kg^{-1}$ (for enniatin A, citrinin and ochratoxin A). Seven samples were analyzed and aflatoxins B1, B2, G1, G2, and beauvericin were detected at trace levels.

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1. Introduction

Mycotoxins are secondary metabolites produced by many species of filamentous fungi. Currently, more than 400 mycotoxins have been identified in the world [1] and most of them can be categorized into *Aspergillus* mycotoxins (e.g. aflatoxins, ochratoxins), *Fusarium* mycotoxins (e.g. enniatins, beauvericin) and *Penicillium* mycotoxins (e.g. citrinin) [2,3]. The occurrence of these compounds depends on factors like strain of fungus, species, plant species, and environmental and ecological conditions such as humidity, temperature and presence of pests [4].

These compounds are toxic and pose a health hazard to humans and animals. This toxicity can range from the production of several hormonal disorders or immunosuppression to the induction of carcinogenic, teratogenic or mutagenic activities [5].

The presence of these contaminants and their metabolites in food of animal origin, such as meat, milk, eggs and cheese could be consequence of a carry over of these compounds into animal tissues after feeding of contaminated hay or corn [6]. Bearing in mind that egg is essential in diet and because the consumption is increasing worldwide [7], it is important to assure the safety of this product in terms of mycotoxins occurrence.

Among the different mycotoxins that can be found in eggs, aflatoxins are considered the most serious threat to public health due to the effects they can provoke. There are several compounds belonging to aflatoxins, such as B1, B2, G1 and G2. They have been detected at concentrations higher than 6 $\mu g\,kg^{-1}$ [8], and they are considered as Class 1 carcinogens by the International Agency for Research on Cancer (IARC). Aflatoxin B1 is considered the most toxic of them [2,9] and it can be metabolized by livestock into aflatoxin M1, which has also been detected at trace levels in eggs [8]. Ochratoxin A and citrinin can coexist in stored products and in cereal and cereal byproducts under optimal conditions, which may be part of feed and they can be found in animal origin products like eggs [10,11]. Despite their toxicity, ochratoxin A and citrinin are responsible of decreasing egg production and hatchability [12]. Other mycotoxins such as beauvericin and enniatins, including A, A1 and B1, are commonly present in harvested grains and because they are lipophilic contaminants, they can be bioaccumulated into egg yolks [13,14] at concentrations up to 7.5 $\mu g\,kg^{-1}$ [14].

Due to the high occurrence of these compounds in food and feed and their implication in pathologies, mycotoxins are a global concern and they are included in monitoring food program to minimise the levels in these products. Despite European legislation sets

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maximum levels of mycotoxin in foodstuffs [15], there is not specific legislation in eggs, and it should be established to assure food safety [5]. For this reason the development of analytical methods that allow unambiguous identification, quantification and detection at very low concentration levels is necessary. In this sense, it is difficult to develop an analytical method for the simultaneous determination of several mycotoxins, since they have different physicochemical properties. Thus, some of the developed analytical methods determine a single class of compounds [8,14], and very few multiclass methods for the determination of mycotoxins in egg have been proposed, analyzing simultaneously less than 7 compounds [16,17].

The analysis of mycotoxins in egg is a difficult task because this is a complex matrix, and the chromatographic analysis requires the application of previous extraction and/or clean-up steps in order to remove proteins and lipids [18]. Generally, the extraction of mycotoxins from egg is based on a simple extraction using acetonitrile or a mixture of methanol and water [17], which allows the precipitation of proteins. Because the amount of coextractive compounds typically present in eggs, a clean-up procedure is usually applied using immunoaffinity columns [16], or conventional sorbents such as OASIS [17] or silica [14], increasing the analysis time due to sample treatment. However, it is necessary to develop generic extraction procedures that reduce sample handling and increase sample throughput. In this sense, in the last few years QuEChERS procedure (acronym name for Quick, Easy, Cheap, Effective, Rugged and Safe) has been developed [19]. This method is based on an extraction with acidified acetonitrile followed by an induced liquid–liquid partition after the addition of salts. It has been used for the extraction of a wide variety of compounds, such as pesticides [20], veterinary drug residues [21], and mycotoxins [22], from variety of matrices such as fruits and vegetables [23], and cereal products [24]. However, it has not been checked for the extraction of mycotoxins from eggs.

For the detection and quantification of mycotoxins, chromatographic techniques like gas chromatography (GC) and liquid chromatography (LC) coupled to mass spectrometry (MS) can be used. LC–MS is a suitable technique for the analysis of polar substances like mycotoxins, because no derivatization step is required as in GC–MS [25–27]. For instance, LC using several analysers such a single quadrupole [28], time of flight (TOF) [29] or triple quadrupole enabling tandem mass spectrometry (MS/MS) [30] have been the most applied methods [25]. In this sense, LC–MS/MS provides the highest sensitivity and specificity, detecting low levels of mycotoxins in complex matrices, reducing sample preparation and analysis time [31,32]. Furthermore, the application of ultra high pressure liquid chromatography (UHPLC) has decreased the analysis time by means of the reduction of particle size of stationary phase (<2 μm). Therefore, it provides significant advantages in relation to conventional LC, such as higher speed of analysis, resolution, sensitivity and peak capacity. UHPLC has been used for the detection of several types of mycotoxins in different matrices [32–34].

The purpose of this study has been the development of a simple and efficient UHPLC–MS/MS multi-mycotoxin analytical method for the simultaneous determination of enniatins A, A1, B1, aflatoxins B1, B2, G1, G2, citrinin, ochratoxin A and beauvericin in eggs at trace levels using a simple extraction procedure avoiding further clean-up steps.

2. Materials and methods

2.1. Reagents and chemicals

Beauvericin, citrinin and aflatoxins B1, B2, G1 and G2 were purchased from Sigma–Aldrich (Madrid, Spain). Enniatin A, A1 and B1 were obtained from Santa Cruz (Santa Cruz, CA, USA). Stock solution of ochratoxin A (in acetonitrile) was purchased from Riedel de Haën (Seelze, Germany).

First, stock standard solutions were prepared by exact weighing of those mycotoxins obtained in powder and dissolved in 10 mL of HPLC-grade acetonitrile (Sigma). Then, a multicompound working solution (2 mg L^{-1}) was prepared by combining suitable aliquots of each individual standard stock solution and diluting them with appropriate amounts of acetonitrile. These solutions were kept at 4 °C and renewed weekly.

Acetic acid (purity > 97%), formic acid (purity > 98%), ammonium formate and sodium sulphate anhydrous were obtained from Panreac (Barcelona, Spain). Sodium acetate anhydrous was purchased from J.T. Baker (Deventer, Holland). HPLC-grade methanol was supplied by Sigma. Ultrapure water was obtained from a Milli-Q Gradient water system (Millipore, Bedford, MA, USA). C18 Sep-Pak 200 mg/3 cm^3 and Oasis HLB 200 mg/3 cm^3 cartridges (Waters, Milford, MA, USA) were used to evaluate a clean-up step during the development of the extraction procedure.

2.2. Apparatus and software

Chromatographic analyses were performed in an ACQUITY UPLC™ system (Waters, Milford, MA, USA), using an Acquity UPLC BEH C_{18} column (100 mm × 2.1 mm), with 1.7 μm particle size, from Waters. MS/MS detection was performed using an Acquity TQD tandem quadrupole mass spectrometer (Waters, Manchester, UK). The instrument was operated using an electrospray (ESI) source in positive and negative ion mode. Data acquisition was performed using MassLynx 4.0 software with QuanLynx software (Waters). Centrifugations were performed in a high-volume centrifuge from Centronic (Barcelona, Spain). A Vortex mixer Heidolph, model Reax 2000 and an analytical AB204-S balance (Mettler Toledo, Greinfesee, Switzerland) were also used. A Reax-2 rotary agitator from Heidolph (Schwabach, Germany) was used for sample extraction.

2.3. UHPLC–MS/MS analysis

Chromatographic analyses were carried out using a gradient elution with eluent A being methanol and eluent B consisting on an aqueous solution of ammonium formate (5 mM). The analysis started with 25% of eluent A, which was increased linearly up to 100% in 3.75 min. This composition was held for further 1.25 min before being returned to 25% of eluent A in 0.5 min, followed by a re-equilibration time of 1 min, to give a total run time of 6.5 min. The flow rate was set at 0.30 mL min^{-1} and column temperature was maintained at 30 °C. Aliquots of 5 μL of sample extract were injected into the chromatographic system.

For MS/MS detection, the ionisation source parameters in positive mode were: capillary voltage 3.5 kV, extractor voltage 4 V, source temperature 120 °C, desolvation temperature 350 °C, cone gas flow 80 L h^{-1} and desolvation gas flow 550 L h^{-1} (both gases were nitrogen). The ionisation source parameters in negative mode were the same except the capillary voltage, which was set at 2.5 kV. Collision-induced dissociation was performed using argon as collision gas at a pressure of 4×10^{-3} mbar in the collision cell. The optimum MS/MS conditions of the mycotoxins were performed by column injection of individual standards at 500 μg L^{-1}. Full-scan mass spectra and product ion scan were acquired in order to obtain at least one precursor and two product ions for each compound for both identification and quantification purposes, selecting the most abundant product ion for quantification and the second one for confirmation. The multiple reaction monitoring (MRM) transitions and

Table 1
Retention time windows (RTW) and MS/MS parameters of the selected mycotoxins.

Compound	Abbreviation	ESI	Function	RTW (min)	Cone voltage (V)	Quantification transition (*m/z*)[a]	Confirmation transition (*m/z*)[a]
Aflatoxin G2	AFG2	+	1	2.45–2.63	60	331.4 > 313.5 (25)	331.4 > 245.3 (30)
Aflatoxin G1	AFG1	+	1	2.62–2.67	50	329.2 > 243.1 (25)	329.2 > 311.4 (25)
Aflatoxin B2	AFB2	+	1	2.70–2.85	50	315.2 > 259.2 (30)	315.2 > 243.3 (35)
Citrinin	CIT	–	2	2.80–2.96	60	249.1 > 205.4 (20)	249.1 > 177.3 (25)
Aflatoxin B1	AFB1	+	3	2.85–3.02	30	313.3 > 285.5 (25)	313.3 > 241.3 (30)
Ochratoxin A	OTA	+	3	3.04–3.19	25	404.2 > 239.2 (20)	404.2 > 358.2 (15)
Beauvericin	BEA	+	4	4.57–4.72	45	784.4 > 244.1 (20)	784.4 > 262.6 (30)
Enniatin B1	ENNB1	+	4	4.62–4.76	40	655.0 > 196.1 (20)	655.0 > 210.2 (25)
Enniatin A1	ENNA1	+	4	4.70–4.89	30	668.9 > 210.4 (35)	668.9 > 228.3 (30)
Enniatin A	ENNA	+	4	4.76–4.84	20	705.1 > 232.4 (55)	705.1 > 350.6 (60)

[a] Collision energies (eV) are given in brackets.

the applied cone voltages and collision energies are summarised in Table 1.

2.4. Sample preparation

For the extraction of mycotoxins, ten fresh eggs (albumen and yolk combined) were homogenized at room temperature under continuous agitation for 5 min and they were stored in polypropylene tubes under refrigeration (<5 °C). After that, a simple extraction procedure was applied: 2 g of homogenized fresh egg were weighed in a polypropylene centrifuge tube (40 mL). 10 mL of a methanol/water solution (80/20, v/v) with 1% acetic acid, 4 g of sodium sulphate anhydrous and 1 g of sodium acetate anhydrous were added and the mixture was vortexed for 2 min. After that, the tube was put into a rack in the rotary agitator for 30 min at 60 rpm. After centrifugation at 5000 × *g* for 5 min, 1 mL of the supernatant layer was taken and filtered through a Millex-GN nylon filter (0.20 μm, Millipore, Carrightwohill, Ireland), before the sample extract was injected into the UHPLC–MS/MS system.

During the optimization of the extraction procedure, a clean-up step was evaluated. Thus, the supernatant layer, obtained after the centrifugation step described previously, was transferred onto an SPE cartridge (C_{18} or Oasis HLB) and it was let flow through the cartridge. The cleaned extract was collected in a vial for injection into the UHPLC–MS/MS system.

2.5. Validation study

Performance characteristics of the optimized method were established by a validation procedure with spiked egg samples, studying matrix effect, linearity, trueness, repeatability, inter-day precision, limits of detection (LOD) and quantification (LOQ) and selectivity.

Linearity was tested by spiking blank extract eggs at five concentration levels within the range of 1–200 μg kg^{-1} for aflatoxins G1, B2 and B1, from 2 to 200 μg kg^{-1} for aflatoxin G2 and beuvericin, from 5 to 200 μg kg^{-1} for enniatin B1 and A1 and from 10 to 200 μg kg^{-1} for citrinin, ochratoxin A and enniatin A.

Recovery was studied by spiking blank samples at four fortification levels, 10, 25, 50 and 100 μg kg^{-1}, processing five samples in each experiment. Precision was evaluated through repeatability and inter-day precision. Repeatability was evaluated at the four concentration levels of the recovery studies, performing five replicates for each level. For inter-day precision, one spiked sample at 25 μg kg^{-1} was analyzed daily for a period of five days.

LODs and LOQs were calculated analyzing blank samples spiked at 0.1, 0.5, 1, 2, 5 and 10 μg kg^{-1}, and they were determined as the lowest concentration of the selected compounds that produce chromatographic peak at signal to noise ratio (S/N) of 3 and 10 respectively.

Finally selectivity was evaluated extracting and analyzing blank egg samples. Identification of the target mycotoxins was carried out by searching the characteristic transitions of the compounds in the appropriate retention time windows (RTWs), which were obtained by mean retention time ± three standard deviation of the retention time of ten blank samples spiked at 25 μg kg^{-1} for each compound (see Table 1).

2.6. Samples

Seven egg samples were purchased from local supermarkets of Almeria (Spain) and were analyzed before the expiration date. All samples were analyzed following the procedure described above and those samples showing the absence of the target compounds were used as blank samples in the preparation of standards and recovery studies. In order to ensure the reliability of the results, an internal quality control was used when the proposed method was applied. This quality control consisted of a matrix-matched calibration, a matrix blank, in order to eliminate false positives by contamination in the extraction process, and a spiked blank sample at 25 μg kg^{-1}, in order to evaluate the recovery of the proposed method.

3. Results and discussion

3.1. Optimisation of the UHPLC–MS/MS determination

The chromatographic and MS/MS method was based on a previously reported UHPLC–MS/MS methodology [32,35]. New mycotoxins (citrinin, beauvericin, enniatin B1, enniatin A1, enniatin A) have been included in this study, considering that they may be found in eggs [14]. ESI positive and negative ion modes were evaluated, observing that all mycotoxins were detectable in ESI positive, except citrinin. Unexpectedly, this mycotoxin was more sensitive in negative ion mode, despite it has been usually detected in positive mode in bibliography [36]. Table 1 shows the MS/MS transitions, as well as the cone voltages and collision energies optimized for each compound. Under the experimental conditions, protonated molecules, $[M+H]^+$, (deprotonated molecule for citrinin, $[M-H]^-$) were observed for all the compounds, except for enniatin A, which mainly formed the sodium adduct $[M+Na]^+$, and they were used as precursor ions. In relation to product ions, the most intense ions for beauvericin, enniatin A1 and enniatin A were 244.1, 210.4 and 232.4 respectively, which corresponded to [monomer with phenylmethyl residue + H − H_2O]$^+$ for beauvericin, [monomer with *sec*-butyl residue + H − H_2O]$^+$ for enniatin A1 [37] and [monomer with *sec*-butyl residue + Na − H_2O]$^+$ for enniatin A. For enniatin B1, the most intense ion was 196.1, corresponding to [monomer with *iso*-propyl + H − H_2O]$^+$ as it was observed previously [38]. In relation to citrinin, which is a carboxylic acid, the most intense product ion was 205.4, corresponding to the loss of

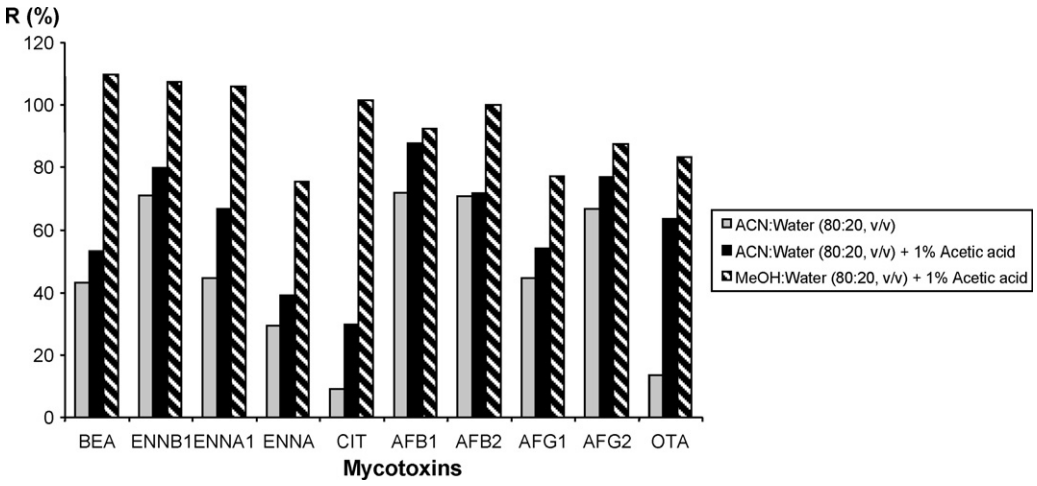

Fig. 1. Effect of type of solvent on the extraction recovery of mycotoxins in egg. *Abbreviations*: ACN, acetonitrile; MEOH, methanol. Compound abbreviations are indicated in Table 1.

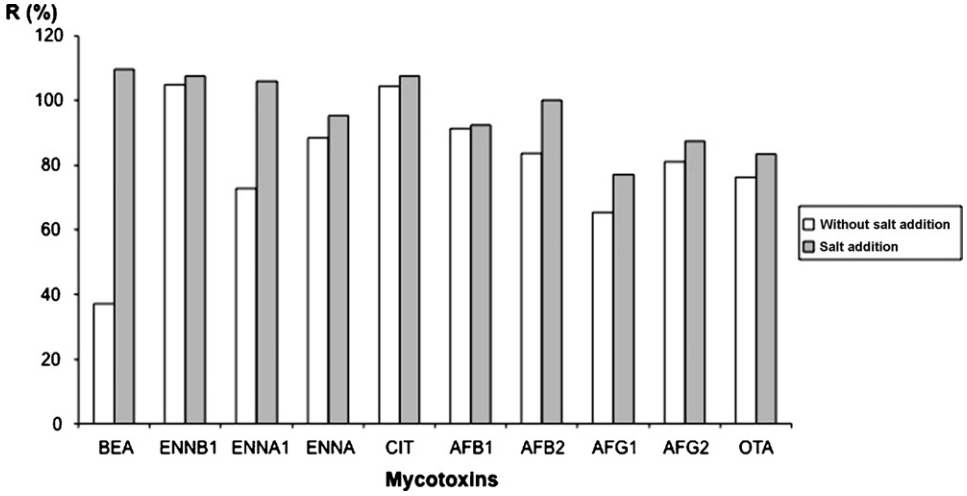

Fig. 2. Effect of salt addition (sodium sulphate anhydrous and sodium acetate anhydrous) on the extraction recovery of mycotoxins in egg. Compound abbreviations are indicated in Table 1.

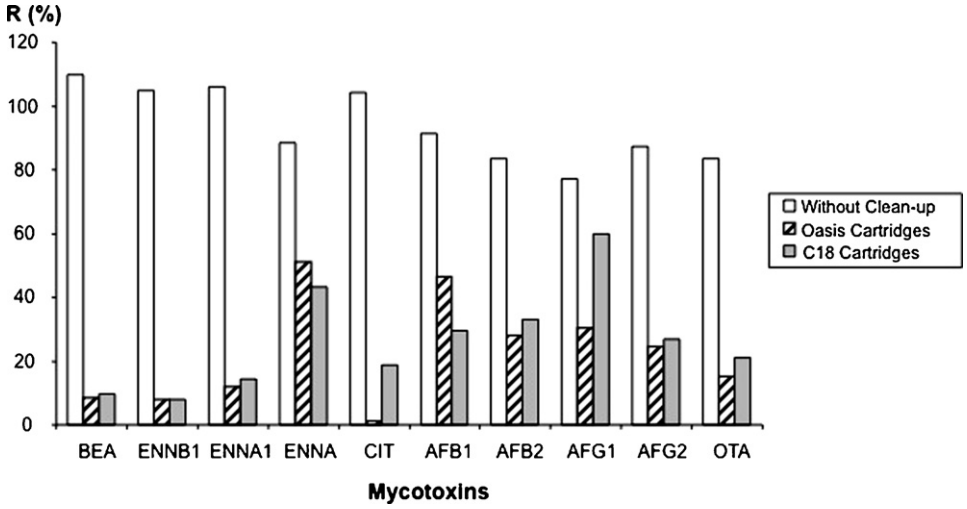

Fig. 3. Effect of the clean-up step on the recovery of mycotoxins in egg. Compound abbreviations are indicated in Table 1.

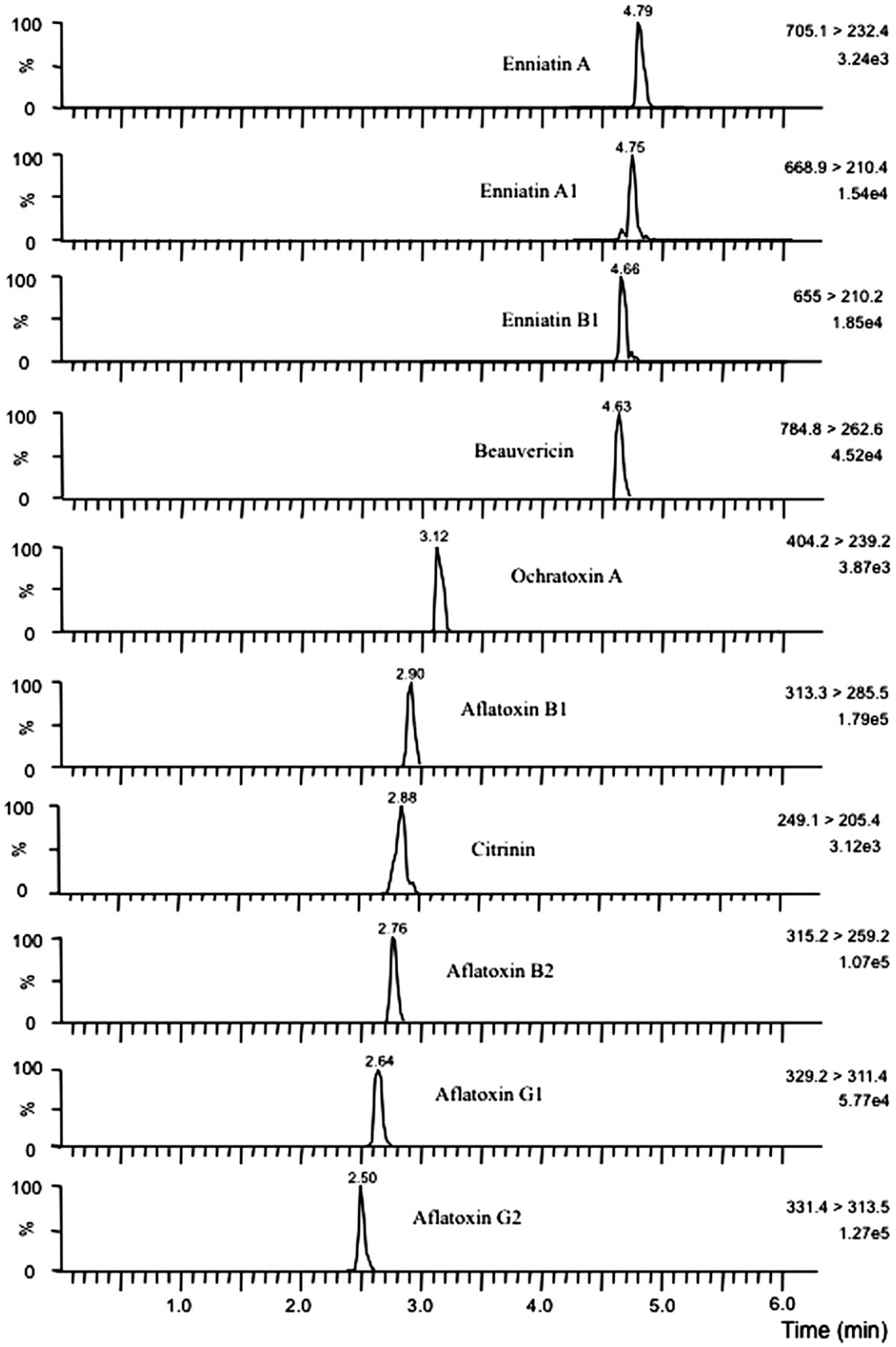

Fig. 4. UHPLC–MS/MS chromatogram obtained from a blank egg sample spiked at 25 μg kg^{-1}.

Table 2
Validation parameters of the developed method.

Compound	Recovery[a]				Interday precision[b]	LOD ($\mu g\,kg^{-1}$)	LOQ ($\mu g\,kg^{-1}$)
	10 $\mu g\,kg^{-1}$	25 $\mu g\,kg^{-1}$	50 $\mu g\,kg^{-1}$	100 $\mu g\,kg^{-1}$			
AFG2	80.5 (14.7)	89.3 (11.4)	114.2 (9.3)	84.6 (6.2)	24.7	1.0	2.0
AFG1	65.1 (18.4)	77.2 (15.0)	77.0 (10.9)	72.9 (8.5)	20.2	0.5	1.0
AFB2	79.2 (15.1)	70.3 (10.6)	74.7 (9.4)	73.1 (7.8)	12.3	0.5	1.0
CIT	85.6 (24.5)	94.0 (16.3)	70.0 (14.9)	83.2 (12.5)	22.5	5.0	10.0
AFB1	76.5 (18.6)	78.9 (16.8)	78.2 (11.1)	99.7 (9.3)	15.8	0.5	1.0
OTA	63.3 (20.2)	102.8 (11.6)	109.8 (10.4)	93.4 (5.3)	15.6	5.0	10.0
BEA	79.6 (18.6)	90.9 (9.6)	109.7 (7.5)	101.7 (7.1)	17.2	1.0	2.0
ENNB1	83.0 (18.7)	73.2 (15.4)	72.5 (11.1)	78.3 (10.3)	12.9	2.0	5.0
ENNA1	103.4 (21.4)	87.9 (20.1)	75.1 (15.1)	74.4 (13.1)	22.4	2.0	5.0
ENNA	101.2 (17.1)	93.4 (14.9)	75.4 (9.0)	101.6 (11.3)	20.9	5.0	10.0

[a] Repeatability values, expressed as RSD, are given in brackets ($n = 5$).
[b] RSD values obtained at 25 $\mu g\,kg^{-1}$. Samples were analyzed on 5 consecutive days.

CO_2 [citrinin–H–CO_2]$^-$. Other parameters such as desolvation and cone gas flow, source and desolvation temperature and capillary voltages were studied, selecting the optimum conditions indicated in Section 2.3.

In relation to the chromatographic conditions described in a previous work [32], the gradient profile was slightly modified to include the new compounds and to achieve an adequate retention of the target compounds. All the mycotoxins were eluted with high selectivity in less than 6.5 min, including cleaning and re-equilibration steps, and good MS sensitivity were achieved. Using the conditions described in Section 2.3, retention time ranges from 2.50 (aflatoxin G2) to 4.79 min (enniatin A), and the analytes were distributed in four overlapping acquisition functions, using a maximum of four mycotoxins (8 transitions) per function (see Table 1). One problem was the co-elution of citrinin (ionized in negative mode) between aflatoxins B1 and B2, which are ionized in positive mode. For that case, the multiple reaction monitoring (MRM) cycle time, understood as the sum of the dwell times of all the MRM channels, inter-channel delay times between successive MRM and inter-scan delay times during polarity switching is critical. In order to obtain at least 10 points per peak, an inter-scan delay time of 20 ms was used and dwell time of 15 ms. For the rest of mycotoxins a dwell time was slightly higher (25 ms) in order to increase the reproducibility of the peak shape. Other co-eluted compounds were beauvericin and enniatin B1, and enniatin A1 and A, but the use of MS/MS enabled an accurate analysis of these compounds, considering that different precursor ions were selected.

3.2. Optimisation of the extraction procedure

In multiclass mycotoxin methods, the critical step is the extraction and clean-up procedure, especially when complex matrix such as eggs, which consist of albumen and ovum with very different composition, are analyzed. To reduce sample handling and increase throughput, a simple extraction QuEChERS-based procedure was used before chromatographic determination. Several parameters that influence the extraction of mycotoxins from egg were optimized spiking blank egg samples at 50 $\mu g\,kg^{-1}$.

Despite QuEChERS uses acetonitrile acidified with acetic acid, and bearing in mind that conventional extraction procedures of mycotoxins from different samples use a mixture of acetonitrile/water or methanol/water [17], the extraction solvent was evaluated first, using the extraction procedure described in Section 2.4. Thus, different solvents such a mixture of acetonitrile/water (80:20, v/v), a mixture of acetonitrile/water (80:20, v/v) with 1% acetic acid, and a mixture of methanol/water (80:20, v/v) with 1% acetic acid were checked. The obtained results are shown in Fig. 1. It can be observed that the worst results were obtained with the mixture acetonitrile/water without acid, but when this mixture was acidified, better results were obtained. However, the best results were obtained when a mixture of methanol/water (80:20, v/v) acidified with 1% acetic acid was used, increasing the recovery of some compounds such as enniatins, citrinin and ochratoxin A. Therefore, it was used for further experiments.

An important characteristic of the QuEChERS procedure is the addition of salts to separate water from the extraction solvent [39].

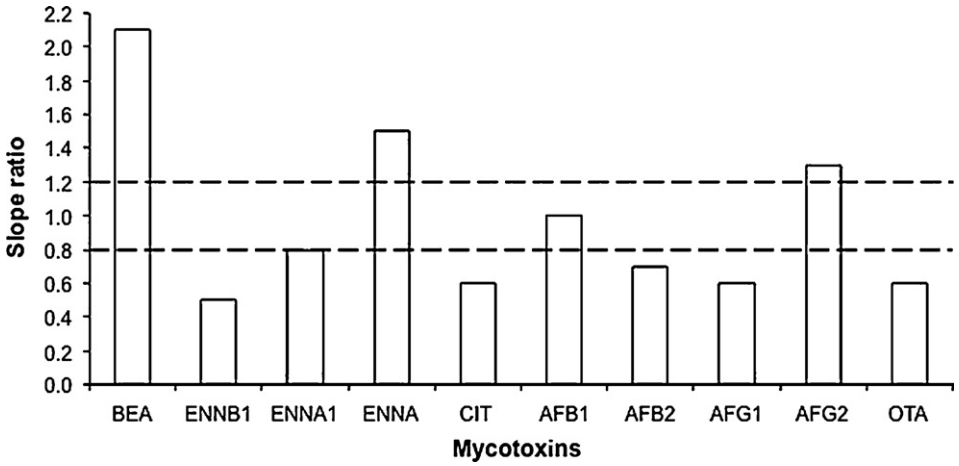

Fig. 5. Slope ratios between matrix-matched and solvent calibration of the target mycotoxins. Compliance interval covering the range between 0.8 and 1.2 for tolerable matrix effect has been plotted. Compound abbreviations are indicated in Table 1.

Table 3
Mycotoxins detected in analyzed eggs.

Mycotoxin	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
AFG2					<2 μg/kg
AFG1	<1 μg/kg	<1 μg/kg	<1 μg/kg		
AFB2			<1 μg/kg	<1 μg/kg	
AFB1				<1 μg/kg	
BEA	<2 μg/kg				

Bearing in mind that egg has water content higher than 70% and water is also added during the extraction, it is necessary the addition of salts to induce phase separation (organic and aqueous phase) and avoid the need of post-extraction solvent evaporation steps. Therefore, the influence of salts (in this case, sodium sulphate anhydrous and sodium acetate anhydrous) was evaluated. Fig. 2 shows the obtained results when the extraction was carried out with and without salts. It can be observed that the addition of salts improves the recovery of the selected compounds, and facilitates the partition of the water and extraction solvent, and salts were used for further experiments.

Then, the extraction time was evaluated, studying 5, 30 and 60 min. It can be highlighted that the recovery of the compounds increased from 5 to 30 min and then, it decreased or kept constant. Therefore, 30 min was selected as extraction time for further experiments. It must be indicated that although extraction time could be considered too long, a large number of samples can be extracted simultaneously, increasing sample throughput.

A clean-up procedure after the extraction was evaluated considering that some interfering compounds could be co-extracted, interfering with mycotoxin detection and reducing the lifetime of the column. Conventional SPE cartridges were used, testing OASIS HLB and C_{18} as sorbents, and using the procedure described in Section 2.4. The obtained results are shown in Fig. 3 and it can be observed that poorer results were obtained when clean-up procedure was applied. Therefore no clean-up was used for further experiments, observing that the lifetime of the column was not affected by the direct injection of the extract.

Finally, Fig. 4 shows a typical chromatogram of a blank egg sample spiked with 25 $\mu g\,kg^{-1}$ of the target mycotoxins. It can be observed that the optimized extraction procedure coupled to UHPLC–MS/MS provides a clean chromatogram without interferences. Furthermore, it can be observed that complete resolution was not obtained but MS/MS detection allows the selective analysis of all the compounds.

3.3. Validation of the proposed method

Method validation was performed in terms of matrix-effect, linearity, trueness, precision (repeatability and inter-day precision), LODs, LOQs and selectivity.

It is well known that the presence of matrix components can affect the ionization of the analytes when ESI is used. 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 selected analytes or they are expensive, and other approaches such as matrix-matched calibration can be used. To evaluate the matrix effect, several concentrations (from 5 to 200 $\mu g\,kg^{-1}$) were analyzed in pure solvent and in blank egg samples. Fig. 5 shows slope ratios matrix/solvent for each compound. Signal suppression or enhancement effect was considered tolerable if the value was between 0.8 and 1.2. The values outside this range indicate a strong matrix effect. It can be observed that there was a strong matrix effect for most of the mycotoxins evaluated, except for aflatoxin B1 and enniatin A1. Furthermore, matrix suppresses the response for the rest of the compounds, except for aflatoxin G2, beauvericin and enniatin A, for which the signal increases. In order to avoid the matrix effect, matrix-matched calibration standard curves were used to quantify mycotoxins in egg samples.

Linearity was then evaluated. Peak area was selected as response and good linearity within the tested interval was found with determination coefficients higher than 0.98 in all the cases. Furthermore, a test for linearity based on the analysis of the residual variance from a regression into parts due to "lack of fit" and "pure error" was carried out, obtaining that the calibration model was significantly linear for all the evaluated compounds.

Trueness was evaluated through recovery studies. Recoveries ranged from 70 to 110% for all the mycotoxins assayed and levels evaluated (Table 2), except for ochratoxin A at 10 $\mu g\,kg^{-1}$, with recovery values of 63.3%, aflatoxin G1 at 10 $\mu g\,kg^{-1}$ (65.1%) and aflatoxin G2 at 50 $\mu g\,kg^{-1}$ (114.2%). Therefore, good recoveries from egg samples were obtained throughout the developed method, indicating the suitability of the proposed extraction procedure for the simultaneous extraction of several types of mycotoxins from eggs.

Precision of the overall method was studied by performing repeatability and inter-day precision experiments, showing the obtained results in Table 2. It can be observed that repeatability, expressed as RSD (relative standard deviation) was equal or lower than 20%, except for ochratoxin A at 10 $\mu g\,kg^{-1}$ (20.2%), enniatin A1 at 10 $\mu g\,kg^{-1}$ and 50 $\mu g\,kg^{-1}$ (21.4 and 20.1% respectively) and citrinin at 10 $\mu g\,kg^{-1}$ (24.5%). For inter-day precision, RSDs were always lower than 25%, indicating the stability of the proposed method.

LODs and LOQs were evaluated, showing in Table 2 the obtained values. In general good values were obtained and LODs ranged from 0.5 $\mu g\,kg^{-1}$ (aflatoxin G1, B1 and B2) to 5 $\mu g\,kg^{-1}$ (citrinin, ochratoxin A and enniatin A). LOQs ranged from 1 $\mu g\,kg^{-1}$ (aflatoxin G1, B1 and B2) to 10 $\mu g\,kg^{-1}$ (citrinin, ochratoxin A and enniatin A).

Finally, the selectivity of the method was studied. The absence of any signal at the same elution time as the target compounds indicated that there were no matrix interferences that could have given a positive signal.

In order to show the feasibility of the proposed method, when the validation parameters were compared with previous methods [14,17], slightly higher LOQs were obtained although similar or better recoveries were achieved. However, it must be highlighted that the other methods were only focused on a single class of mycotoxins, such as beauvericin and enniatins [14] or longer extraction procedures, including clean-up steps were required [17].

3.4. Sample analysis

The develop method was applied to egg samples. Seven samples from different lots collected from stores located in Almeria were analyzed. Mycotoxins were not detected in two samples and trace levels (<LOQ) of some mycotoxins were detected in five samples (Table 3). Thus, traces of aflatoxin G1 were detected in three samples, aflatoxin B2 was detected in two samples and beauvericin, aflatoxin B1 and G2 were detected in one sample (Table 3). It must be highlighted that beauvaricin and aflatoxin G1 were detected simultaneously in one sample, indicating the suitability of the proposed method for the simultaneous determination of compounds belonging to different types of mycotoxins.

4. Conclusions

A new method based on QuEChERS extraction procedure and UHPLC–MS/MS was developed for the simultaneous determination of different classes of mycotoxins in egg. The extraction procedure, using as extractant solvent a mixture of methanol/water

acidified with acetic acid, is simple and no further clean-up step was necessary, increasing sample throughput. Furthermore, the use of UHPLC coupled to MS/MS allows a fast determination of the selected compounds. The method gives quantitative results for the assayed mycotoxins, providing good validation parameters in terms of linearity, trueness, precision and LOQ. Although strong matrix effect was observed, it was successfully compensated using matrix-matched calibration. Finally, this method was applied to seven real samples and trace levels were detected in five of them. Considering the advantages of the proposed method, this could be applied for regular monitoring of mycotoxins in eggs by routine laboratories.

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