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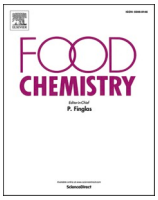

Detection of mycotoxins in cheese using an optimized analytical method based on a QuEChERS extraction and UHPLC-MS/MS quantification

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ABSTRACT

Mycotoxins can produce toxic effects on humans; hence, it is of high importance to determine their presence in food products. This work presents a reliable method for the quantification of 32 mycotoxins in cheese. The analysis procedure was optimized based on a QuEChERS extraction process and the ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) detection. The analysis method was validated for four cheese varieties (emmental, blue, brie and camembert) in terms of linearity, sensitivity, matrix effect, accuracy and precision. Satisfactory precision and accuracy values were achieved, with recoveries above 70% for most mycotoxins. The developed method was applied to the analysis of 38 commercial cheese samples. A high occurrence of beauvericin and enniatins were found, ranging from 31% for enniatin A to 100% for enniatin B. The ochratoxin A was detected in three samples at concentrations that may pose a risk to human health.

1. Introduction

Cheese is one of the most widely consumed dairy products worldwide, especially in Europe (Fox, Guinee, Cogan, & McSweeney, 2017). Different types of milk and a wide range of technologies can be employed to manufacture of a great variety of cheeses, which are diverse in morphological features, texture, and flavor. These dairy products are exposed to the growth of pathogenic and spoilage microorganisms such as bacteria, yeasts and filamentous fungi (Kure & Skaar, 2019).

Certain filamentous fungi belonging to *Aspergillus, Penicillum, Alternaria*, and *Fusarium* genera are known to produce toxic metabolic substances known as mycotoxins (Sainz, González-Jartín, Aguín, Mansilla, & Botana, 2018). Although more than 400 mycotoxins are known, only a few are of major concern because of their high toxicity. In this sense, maximum limits in food and feed have been established for some mycotoxins that are a public health problem at levels at which they can appear in food. This group includes aflatoxins (AFs), ochratoxin A (OTA), patulin (PAT), fumonisins (FBs), citrinin (CTN), zearalenone (ZEN), deoxynivalenol, T-2 toxin and HT-2 toxin (EC_1881, 2006).

The group of emerging mycotoxins comprises compounds for which there are little data about their toxicity and presence in food. This group includes, among others, enniatins (ENNs), beauvericin (BEA), sterigmatocystin (STC), mycophenolic acid (MPA) and *Alternaria* toxins (Pérez-Fuentes et al., 2021). Exposure to fungal toxins is not restricted to regulated and emerging mycotoxins since these compounds can be modified by fungi, plant or animal metabolism which leads to products with potential toxicity but not considered in the legislation. They are called modified mycotoxins (González-Jartín, Alfonso, Sainz, Vieytes, & Botana, 2018).

In some cases, the fungal contamination of cheese occurs intentionally since mould species are used for manufacturing and ripening to provide specific characteristics or prolong shelf-life (Soda, Madkor, & Tong, 2000). Fungi species employed with this purpose usually belong to the genus *Penicillium*, such as *P. roqueforti* and *P. camemberti*, which are used in the manufacture of blue and white cheeses, respectively (Hymery et al., 2014). The presence of mycotoxins in cheese depends on various factors such as water activity, temperature or the substrate (Becker-Algeri et al., 2016). For instance, *P. roqueforti* strains produce high amounts of roquefortine C (ROQC) when the ripening temperature is high. In addition, this fungus can produce PAT, penicillic acid and PR

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toxin. Similarly, *P. camemberti* produces cyclopiazonic acid (CPA) (Dobson, 2017).

Mycotoxins can also appear in cheese due to the use of contaminated milk. For example, if animals are exposed to feed contaminated with aflatoxin B_1 (AFB_1), this compound will be metabolized leading to aflatoxin M_1 (AFM_1), which will be excreted in milk (Fink-Gremmels, 2008). To avoid intoxications, the European legislation establishes a maximum level of 0.05 μg/kg for AFM_1 in dairy milk and milk for the manufacture of dairy products. However, other mycotoxins such as FBs, OTA, ZEN, cyclopiazonic acid (CPA), T-2 toxin, ROQC, ENNs and BEA can appear in this matrix (González-Jartín, Rodríguez-Cañás, et al., 2021). In cheese, certain mycotoxins have been detected including OTA, CTN, STC, CPA, ROQC, MPA and AFM_1 (Dobson, 2017). However, European legislation does not establish maximum limits for mycotoxins in this matrix. Nevertheless, the European Food Safety Authority (EFSA) has demanded more occurrence data on OTA in cheese to evaluate if its presence in this product may pose a risk to humans (EFSA, 2020).

Different methodologies have been proposed for the analysis of mycotoxins including screening techniques such as enzyme-linked immunosorbent assay (ELISA) and confirmation techniques like liquid chromatography (LC) coupled to mass spectrometry (MS), fluorescence (FD) or ultraviolet (UV) detection. Recently, Ultra-Performance Liquid Chromatography (UHPLC) coupled to tandem mass spectrometry (MS/MS) has become the technique of choice for analysis of wide range of contaminants in food, including mycotoxins, since it allows the simultaneous determination and accurate quantification of several analytes at low levels in a short run time (Rodríguez et al., 2017).

The high content of cheese in fat and protein and the chemical diversity of mycotoxins that can appear in this product make developing analytical procedures challenging. In fact, few methods currently available to extract mycotoxins from cheese. Some examples include a solid-phase microextraction procedure for the determination of CPA and MPA in white and fermented blue cheeses, respectively, or a solid–liquid extraction for the determination of nine mycotoxins in mould cheeses (Kokkonen, Jestoi, & Rizzo, 2005b).

In recent years, mycotoxin extraction procedures based on QuEChERS (Quick, Easy, Cheap, Effective, Rugged & Safe) methods have been developed for the analysis of complex matrices such as fruits, juices, milk, cereal products, eggs or beer (González-Jartín et al., 2019). In this context, the aim of this work was to develop a new method for the simultaneous analysis of the main types of regulated, emerging and modified mycotoxins that may be present in cheese by the combined use of a QuEChERS-based extraction and UHPLC-MS/MS detection.

2. Material and methods

2.1. Chemicals and reagents

Pure water was obtained from a Millipore Milli-Q Plus system (Millipore, USA). Methanol, acetonitrile, acetic acid glacial 100 %, anhydrous magnesium sulphate ($MgSO_4$) and sodium chloride (NaCl) were purchased from Panreac Química S.A. (Barcelona, Spain). Formic acid was from Merck (Madrid, Spain), ammonium formate and ammonium acetate from Fluka (Buchs, Switzerland), the Polygoprep^{TM} 60–50 C18 was supplied by Macherey-Nagel (Düren, Germany) and ultrafree-MC durapore membrane centrifugal filters (0.22 μm pore size) provided by Millipore (Billerica, USA).

The analytical standards AFB_1, aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), enniatin A (ENNA), enniatin A_1 ($ENNA_1$), enniatin B (ENNB), enniatin B_1 ($ENNB_1$), gliotoxin (GLIO), ROQC, and ZEN were from Sigma (Madrid, Spain). Analytics standards of 3-acetyldeoxynivalenol (3 Ac-DON), 15-acetyldeoxynivalenol (15-AcDON), AFM_1, alternariol (AOH), alternariol methyl ether (AME), CPA, CTN, diacetoxyscirpenol (DAS), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), hydrolyzed fumonisin B_1 (h-FB_1), HT-2 toxin, MPA, neosolaniol (NEO), STC, T-2 toxin, T-2 triol and zearalanone (ZOL) were provided by Romer Labs (Tulln, Austria). Circumdatin A (CTA) was from Santa Cruz Biotechnolgy (Santa Cruz, CA), OTA was obtained from Laboratorios CIFGA S.A. (Lugo, Spain) and BEA was supplied by Enzo (Barcelona, Spain). Cheese samples were purchased from local supermarkets.

2.2. Instrumentation

The UHPLC-MS/MS analysis was conducted on an Agilent 1290 Infinity UHPLC system coupled to a triple quadrupole mass spectrometer (Agilent 6460) equipped with an electrospray ionization (ESI) source (Agilent Technologies, Waldbronn, Germany). System control and data acquisition were carried out with Agilent MassHunter LCMS Acquisition Console software and data were also processed using QQQ Quantitative Analysis software.

2.3. Optimization of the extraction conditions

In order to optimize the extraction process, a first test was carried out in emmental cheese. In this sense, samples were artificially contaminated at 3 μg/kg of AFM_1, AFB_1, AFB_2, AFG_1, AFG_2, and at 80 μg/kg of AME, BEA, CTA, CTN, DAS, ENNA, $ENNA_1$, ENNB, $ENNB_1$, FB_1, FB_2, MPA, OTA, STC, T-2 toxin and ZEN. The matrix effect and recoveries were evaluated, as described below, after the extraction of a 0.5 g sample with 2 mL of a 2 % formic acid solution and 2 mL of acetonitrile, a d-SPE clean-up was applied by using 1.4 mL of organic phase, 0.4 g of $MgSO_4$ and 0.1 g of C18. In addition, a further clean-up step with hexane prior to d-SPE was studied. For this purpose, 2 mL of hexane were mixed with 1.4 mL of the organic extract and shaken in a vortex for 0.5 min. Then, hexane was discarded, and the obtained extract was submitted to the same d-SPE clean-up step. Finally, 1 mL of the resultant extracts was evaporated to dryness and reconstituted with 200 μL of sample solvent.

Next, in order to optimize the percentage of acid used for the extraction, samples were artificially contaminated with 3 μg/kg of AFB_1, AFB_2, AFG_1 and AFG_2 and 80 μg/kg of ZEN and OTA. In this case, 0.5 g of cheese were extracted with 2 mL of aqueous solution acidified with 0.5, 1, 2 and 4 % of formic acid or acetic acid, and 2 mL of acetonitrile. After mixing for 5 min, 0.8 g of $MgSO_4$ and 0.2 g of NaCl were added to induce the separation of two phases (organic and aqueous). After centrifugation, 1 mL of the resultant organic extract was evaporated to dryness and reconstituted with 200 μL acetonitrile/water/acetic acid 49/50/1 (v/v/v). Finally, samples were filtered and analysed by UHPLC-MS/MS in order to calculate the apparent recoveries (R_A).

Finally, the matrix effect was evaluated at different concentration levels of the matrix extract. In this sense, emmental cheese samples were contaminated with 3 μg/kg AFB_1, AFB_2, AFG_1, AFG_2 and AFM_1 and with 80 μg/kg of AME, CTA, CTN, DAS, FB_1, FB_2, MPA, OTA, STC, T-2 toxin and ZEN. Samples were extracted using the method previously described. However, after the extraction process, samples were concentrated 0, 2, 5, 10 and 20 times and R_A were studied.

2.4. Chromatography and mass spectrometric conditions

In order to separate the analytes, a Waters Acquity UPLC HSS T3 (1.8 μm, 100 mm × 2.1 mm) column was used at a temperature of 40 °C. Mobile phase A was composed of water with 1 % formic acid and 5 mM ammonium formate, and mobile phase B was composed of methanol/water/formic acid 97/2/1 (v/v/v) with 5 mM ammonium formate. The flow rate of the mobile phases was 0.3 mL/min, and a gradient elution of 13 min of duration was applied: after an initial hold time of 0.5 min at 0 %B, the gradient was increased to 14 %B within 0.5 min, and kept for 1.5 min. Later, the percentage of the eluent B was raised to 60 %B within 1 min and then maintained for 0.5 min. Next, the gradient reached the 100 %B within 4.5 min, and it was kept for 2 min. Finally, the gradient was changed to 0 % in 0.5 min and this percentage was hold for 2.5 min. The injection volume was set at 5 μL. The mass spectrometer (Agilent 6460) was operated in ESI modes using the following ionization source

parameters: nebulization gas (nitrogen) temperature 400 °C; nebulization gas flow, 12 L/min; drying gas (nitrogen) temperature, 350 °C; drying gas flow, 8 L/min; nebulizer, 45 psi; capillary voltage, 4000 V; nozzle voltage, 0 V. Analysis was carried out in negative and positive mode using a dynamic multiple reaction monitoring (dMRM) method monitoring two mass transitions for each mycotoxin. The fragmentor voltage (FV), collision energy (CE), cell accelerator voltage (CAV) and mass transitions had previously been optimized for most of the analysed compounds and are shown in the Table S1 (González-Jartín, Rodríguez-Cañás, et al., 2021). Fig. S1 shows the chromatogram of the 32 mycotoxins included in the present work.

2.5. Sample preparation

Samples consisting of the whole cheese we were milled in a food processor and then a QuEChERS extraction procedure was applied as follows: a 0.5 g portion of the homogenized sample was weighed in a Falcon tube and mixed with 2 mL of a 2 % of acetic acid solution using a multi-tube vortex (Nahita Blue, Auxilab, Spain) at 2500 rpm for 5 min. Then, 2 mL of acetonitrile were added, again samples were mixed using the multi-tube vortex at 2500 rpm (Nahita Blue, Auxilab, Spain), for 5 min. Next, in order to induce phase-phase partitioning 0.8 g of $MgSO_4$ and 0.2 g of NaCl were added, and the shaking step was repeated for 30 s. Next, samples were centrifuged for 10 min (3134 × *g*) using Gyrozen 1236R (Spain). Then, an aliquot of 500 μL of supernatant was diluted with 500 μL of aqueous acetic acid solution (2 %), the resultant extract was filtered through a 0.22 μm using centrifugal filters (5 min, 16000 × *g*) and analysed. In addition, some further steps may be included in the protocol in order to obtain a concentrated extract (see below).

2.6. Method validation

2.6.1. Calibration curves and performance characteristics

The performance characteristics of the analytical method were first evaluated in solvent. The linearity of the method was evaluated using the correlation coefficient (R) obtained with a linear regression model. In this sense, calibration curves were constructed in solvent at nine levels in a concentration range from 0.01 to 3 ng/mL for AFs, 0.19 to 50 ng/ml for BEA and ENNs, 4.69–600 ng/mL for ZOL and from 0.78 to 200 ng/mL for the rest of analysed mycotoxins. The within-batch repeatability of the analytical method was evaluated by calculating the relative standard deviation (RSD) of the slope of calibration curves for three consecutive batches, while the between-batch repeatability was measured through calibration curves constructed in three consecutive days. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated in solvent and in matrix following the guidance of the European Union Reference Laboratory (EU-RL) for each mycotoxin through the measurements of ten solvents namely an acetonitrile/water/acetic acid 49/50/1 (v/v/v) solution or ten extracts of blank samples (Wenzl, Haedrich, Schaechtele, Robouch, & J., 2016). The LOD was calculated with the following equation, where *Sb* is the standard deviation of the measurements of the blank, and *m* is the slope of the calibration curve. The LOQ was calculated as 3.3 times the LOD. Blank extracts of emmental, brie, camembert, and blue cheese were employed to calculate the LOD and LOQ in matrix.

2.6.2. Matrix effect

Blank extracts of cheese were employed to construct calibration curves in matrix at the same concentration levels employed in solvent. The slopes of solvent and matrix extract curves were used to calculate the matrix effect defined as the signal suppression/enhancement (SSE) caused by matrix according to the following equation:. A value under 100 % means signal suppression and above 100 % signal enhancement.

2.6.3. Accuracy and precision

The accuracy and precision of the method were evaluated using the recovery from samples spiked before extraction. Blank samples were spiked in triplicate at 0.25 μg/kg of AFB_1, AFB_2, AFG_1, AFG_2, AFM_1; 8.5 μg/kg of BEA, ENNA, $ENNA_1$, ENNB, $ENNB_1$; 16.5 μg/kg of 15-AcDON, 3-AcDON, AOH, AME, CTA, CTN, CPA, DAS, FB_1, FB_2, GLIO, HT-2, h-FB_1, MPA, NEO, OTA, ROQC, STC, T2 toxin, T2 triol, ZEN; and 50 μg/kg of ZOL. Calibration curves constructed in solvent were employed to measure the concentration of each analyte in the extracts. In this way, the apparent recovery (R_A) was calculated. Next, the accuracy was calculated by applying the SSE factor to the R_A in order to obtain the recovery of the extraction (R_E), which was obtained by applying the following equation. The intra-day precision was studied through the relative standard deviation (RSD) of the R_A (%) by analysing samples in triplicate on the same day. In this sense, the intra-day RSD (RSD_r) was calculated according to the following equation:, where *SD* is the standard deviation of the measurements and is the average. On the other hand, inter-day precision (RSD_R) was evaluated by analysing samples in triplicate for three consecutive days.

3. Results and discussion

Cheese is a product susceptible to being contaminated by mycotoxins, but currently, available methods to detect toxins in this matrix are scarce and are focus on detecting a few compounds (Table S2). Therefore, the aim of this study was to develop a new method for the simultaneous analysis of regulated, emerging and modified mycotoxins in cheese. In this regard, a new UHPLC-MS/MS method and a new extraction procedure were developed and validated for the analysis of different varieties of cheese, namely: emmental, blue, brie and camembert.

3.1. Optimization of UHPLC-MS/MS conditions

The UHPLC-MS/MS method was based on a recently developed procedure for milk analysis (González-Jartín, Rodríguez-Cañás, et al., 2021). However, the presence of mycotoxins not included in the original method, such as CPA and GLIO, has been reported in cheeses (Table S2). Consequently, the detection method was modified to include these compounds. Mass transitions were optimized for each metabolite using MassHunter Optimizer software. In the case of CPA, the best performance was obtained in the negative ion mode. The predominant signal (precursor ion) was detected at *m/z* 335 and the product ions at *m/z* 154 and *m/z* 140. In the case of GLIO, the precursor ion was detected at *m/z* 327 and the product ions at *m/z* 263 and *m/z* 245. Mobile phases used for milk analysis were water containing 0.1 % of formic acid and 5 mM ammonium formate (A) and methanol (B). However, these chromatographic conditions were not suitable for CPA analysis since a well-defined peak was not obtained (Fig. 1A). The inclusion of CPA in multi-mycotoxin methods have previously been discarded by some authors since they were unable to obtained linear calibration curves. The instability of CPA may be caused by its low pKa (2.94). This toxin can be produced by fungi employed for cheese ripening, and therefore the chromatographic method was optimized for its detection (Peromingo, Rodriguez, Nunez, Silva, & Rodríguez, 2018). In this sense, ten mobile phases were evaluated (Table S3). The mobile phase A consisted in water and mobile phase B methanol/water mixtures, in both cases containing ammonium formate or ammonium acetate and formic acid or acetic acid. As shown in Fig. 1, no signal corresponding to the CPA appears when the percentage of formic or acetic acid was of 0.1 % (Fig. 1 A-E). Methanol is used more frequently than acetonitrile because it gives better results in terms of mycotoxin sensitivity. Similarly, ammonium formate and formic acid usually lead to an increase in mycotoxins signals (Desmarchelier et al., 2010). For this reason, acetonitrile, ammonium acetate and acetic acid were discarded for the following steps, which first consisted in an increase of the percentage of formic acid to

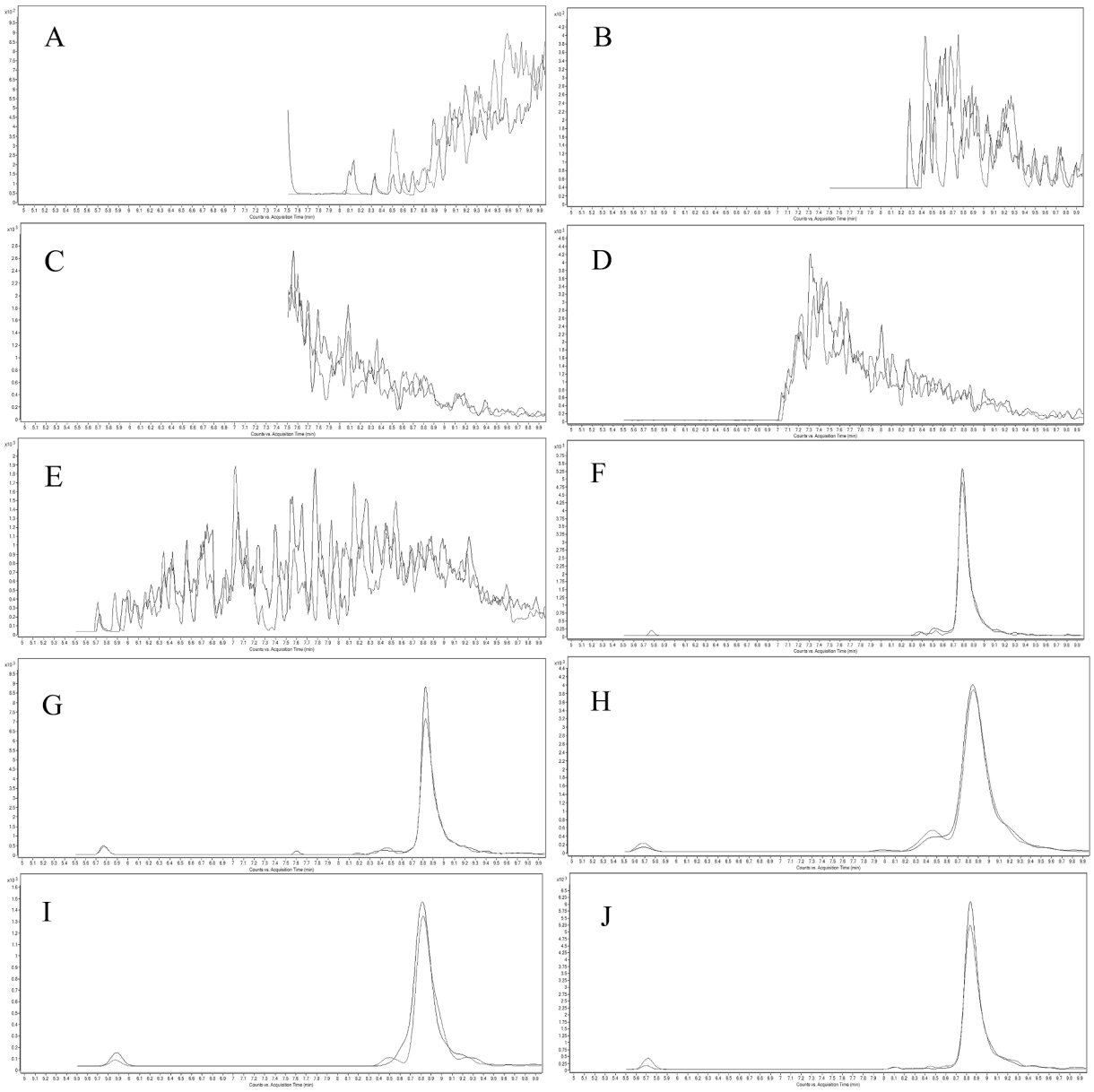

Fig. 1. Optimization of UHPLC conditions. Chromatograms of cyclopiazonic acid at 400 ng/mL obtained with mobile phase 1 (A), 2 (B), 3 (C), 4 (D), 5 (E), 6 (F), 7 (G), 8 (H), 9 (I), 10 (J).

0.5 %, in this way, a narrow peak is achieved (Fig. 1 F). Then, the percentage of formic acid was increased to 1 % leading to an increase in the intensity of the signal (Fig. 1 G). However, the inclusion of 2 % formic acid led to a broader peak (Fig. 1 H). Finally, the amount of ammonium formate was doubled (10 mM) (Fig. 1 I) and halved (2.5 mM) (Fig. 1 J). The higher intensity was observed when using the 5 mM of ammonium formate (Fig. 1 G). Therefore, a mobile phase A composed of water with 1 % formic acid and 5 mM ammonium formate, pH of 2.5, and a mobile phase B composed of methanol/water/formic acid 97/2/1 (v/v/v) with 5 mM ammonium formate, pH of 4, were chosen for analysis. With the new conditions, a well-defined CPA peak was obtained while the sensitivity for most of the analytes included in the method such as OTA or AFM_1 was maintained. However, the sensitivity to some compounds such as deoxynivalenol and its analogues deepoxy-deoxynivalenol and deoxynivalenol 3-glucoside was diminished, the probability of finding these toxins in cheese is very low so they were eliminated from the method (Koesukwiwat, Lehotay, Mastovska, Dorweiler, & Leepipatpiboon, 2010).

3.2. Optimization of extraction procedure

The original QuEChERS method was designed for pesticide analysis in fruits and vegetables. Later, it was extended to extract different compounds from several matrices (Panda, Dash, Manickam, & Boczkaj, 2021). The first step of the QuEChERS procedure is a solid–liquid or liquid–liquid extraction. The solvent most commonly employed is acetonitrile, which enables the extraction of analytes showing different polarities. This solvent is usually combined with water and different acids. In this sense, the addition of water helps the organic solvent to enter the food matrix, and the addition of acidic is useful to break the bonds between analytes and food components (Yan et al., 2020). The second step, sometimes omitted, consists of a further clean-up to remove interfering compounds, for which different sorbents such as C18, primary secondary amine (PSA) and graphitized carbon black (GCB) can be employed. In addition, other solvents, such as hexane, have been used in some protocols (Dong et al., 2019).

The different compounds present in the matrix may interference in

the analysis, reducing or enhancing intensity of analytical signal of the target mycotoxin. This effect is of especial concern in complicate matrices such as cheese. Therefore, different clean-up steps were evaluated, all of them based on the use of C18 as a dispersive solid phase extraction (d-SPE). The procedure was initially optimized in emmental cheese with the aim of reducing the matrix effect and maximize toxins extraction. For the initial evaluation the employed method was based in a previous one developed for the analysis of milk (González-Jartín, Rodríguez-Cañás, et al., 2021). A portion of 0.5 g of sample and 2 mL of acetonitrile were chosen because the volume of the final extract was enough to perform the clean-up test. The salts used were $MgSO_4$ and NaCl in a proportion of 4:1 since is the most employed one in QuEChERS extractions (González-Curbelo et al., 2015). As shown in Fig. S3, there was not much difference in the matrix effect between samples treated with or without the application of d-SPE step with C18. However, a reduction in the R_E of BEA, ENNs and CTN was observed when C18 was included for sample treatment (Fig. S3). In this sense, low recoveries of BEA and ENNs had previously been reported when using d-SPE (González-Jartín, Rodríguez-Cañás, et al., 2021). BEA and ENNs are lipophilic compounds therefore it is possible that the removal of lipids by C18 also remove some mycotoxins attached to them. The combination of C18 and hexane was proposed for the analysis of mycotoxins in egg since the hexane is highly effective in the elimination of lipids (Capriotti, Cavaliere, Piovesana, Samperi, & Laganà, 2012). However, their use was discarded since, as shown in Fig. S3, yielded low recoveries for compounds like CTN. It has been previously observed that low recoveries for CTN can be a consequence of incomplete solvent extraction, the adsorbents used in the clean-up step, as well as the evaporating process of the extract (Koesukwiwat, Sanguankaew, & Leepipatpiboon, 2014). Consequently, an extraction procedure without a clean-up step was chosen for sample analysis.

On the other hand, as the recovery for some compounds was low, the percentage of acid was optimized. As shown in Fig. 2, the increase of the percentage of formic acid causes a decrease in the R_A. For instance, in the case of OTA, the R_A was reduced from 24 % to 9 % when the percentage of this addictive was increased from 0.5 to 4 %. In general, aqueous solution containing acetic acid resulted in higher R_A. Although there are low differences in the recovery obtained with the employed percentages of acetic acid, the 2 % acetic acid solution was chosen for extraction because it gave higher R_A values especially for AFG_1 and, this percentage of addictive has previously been successfully employed for the analysis of mycotoxin in several complex matrices (González-Jartín et al., 2019; González-Jartín, Alfonso, Sainz, Vieytes, & Botana, 2021).

Finally, it was studied how the concentration of the sample modify the matrix effect. Samples were extracted using the method previously described. However, after the extraction process, samples were concentrated 0, 2, 5, 10 and 20 times and R_A were studied. As shown in Fig. S4, the concentration of the sample leads to a decrease in the R_A due to a progressive increase in the matrix effect. Even when the sample was not concentrated, there was a low R_A for many toxins, which suggests a high matrix effect. Finally, the matrix effect (SSE factor) was calculated using calibration curves constructed at nine levels in solvent and in matrix extract diluted (1/1 v/v) with sample solvent for the four varieties of cheese studied. The SSE factor in the diluted extract was compared with a that observed when the extract was five times concentrated. For most toxins the SSE factor is highly reduced when the extract was diluted (Fig. S5). In the case of AFs, the concentration of blue and brie extracts causes an important increase in the suppression of the signal, while in emmental and camembert low differences are observed. Therefore, the diluted extract was chosen for analysis.

3.3. Method validation

The method was validated in terms of linearity, LODs, LOQs, matrix effect, accuracy, and precision. First, calibration curves were constructed in an acetonitrile/water/acetic acid mixture 49/50/1 (v/v/v). This solution had previously been optimized for the simultaneous analysis of several mycotoxins with different polarities (González-Jartín et al., 2019). In this solvent, the linearity of calibration curves constructed at nine levels was acceptable with a regression coefficient (R) higher than 0.995 (Table 1). In addition, repeatability of the analytical method was evaluated within-batch and between-batch. In this sense, a low deviation of the slope of calibration curves was found within- and between-batch with variations, in general, lower than 10 % and 15 %, respectively. Once the performance of the analytical method was verified, the LODs and LOQs were studied. As shown in Table 1, LODs in

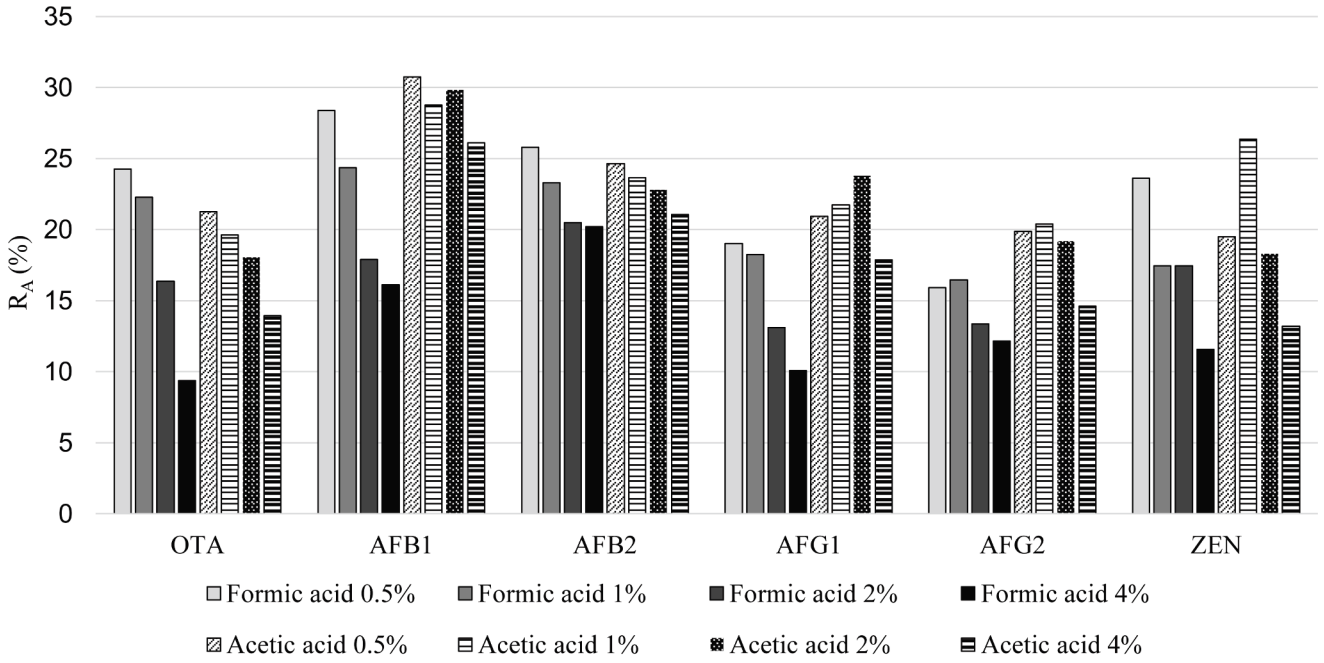

Fig. 2. Optimization of extraction process. R_A of OTA, AFB_1, AFB_2, AFG_1, AFG_2 and ZEN using formic acid or acetic acid at 0.5 %, 1 %, 2 % and 4 % for the extraction procedure.

Table 1
Performance characteristics of the analysis method in solvent. [1]Linear range (ng/mL) are usually of nine levels of concentration for each mycotoxin.

Mycotoxin	R^2	LODs (ng/ml)	LOQs (ng/ml)	Linear Range[1] ng/mL	Linear equation y = ax + b	Within batch (RSD_r %)	Between-batch (RSD_R %)
15-AcDON	0.997	0.490	1.616	0.78–200	y = 3606.67x + 1293.01	2.71	11.20
3-AcDON	0.999	0.480	1.585	0.78–200	y = 2059.78x-1391.03	5.05	12.79
AFB_1	0.998	0.004	0.012	0.01–3	y = 162230.21x-6188.52	4.32	16.32
AFB_2	0.995	0.002	0.006	0.01–3	y = 119786.81x-4154.80	2.06	14.35
AFG_1	0.995	0.006	0.020	0.01–3	y = 150853.39x-3987	2.63	15.27
AFG_2	0.995	0.008	0.027	0.01–3	y = 38236.76x-1217	1.65	15.51
AFM_1	0.999	0.007	0.023	0.01–3	y = 16674.07x-288.05	6.49	13.90
AOH	0.996	0.848	2.798	1.56–200	y = 832.30x-1204.97	10.85	18.30
AME	0.999	0.025	0.084	0.78–200	y = 5853.46x-3535.69	3.81	18.54
BEA	1.000	0.190	0.628	0.19–50	y = 1382.08x + 673.28	9.90	14.49
CTA	0.996	0.005	0.017	0.78–200	y = 8345.99x-15044.75	2.38	16.05
CTN	0.999	0.428	1.414	0.78–200	y = 1022221.84x + 81636.69	2.10	11.69
CPA	0.996	0.359	1.183	0.78–200	y = 377.45x-1790.46	3.83	12.99
DAS	0.997	0.069	0.227	0.78–200	y = 14229.20x-33995.12	3.95	12.77
ENNA	0.998	0.074	0.243	0.19–50	y = 5664.37x-2508.23	7.22	9.50
$ENNA_1$	0.999	0.006	0.021	0.19–50	y = 12708.56x-4734.26	7.07	8.95
ENNB	1.000	0.277	0.914	0.39–50	y = 2017.02x-825.68	7.88	13.00
$ENNB_1$	0.998	0.033	0.110	0.19–50	y = 5062.96x-4242.51	9.45	13.22
FB_1	0.999	0.793	2.618	1.56–200	y = 445.08x-1152	5.17	12.83
FB_2	0.996	0.028	0.094	0.78–200	y = 789.36x-1865.87	5.04	14.75
GLIO	1.000	2.177	7.185	3.13–200	y = 663.67x + 304.44	10.83	13.00
HT-2 TOXIN	0.996	0.237	0.781	0.78–200	y = 2857.95x-5137.20	4.51	14.65
h-FB_1	0.998	1.108	3.655	1.56–200	y = 1735.99–5115.82	6.51	11.27
MPA	0.995	0.193	0.638	0.78–200	y = 10739.48x-24549.54	3.73	12.34
NEO	0.997	0.088	0.291	0.78–200	y = 7172.70x-22088.37	2.49	13.72
OTA	1.000	0.139	0.460	0.78–200	y = 3944.63x-22369.58	3.11	12.62
ROQC	0.998	0.024	0.079	0.78–200	y = 131795.16x + 165756.34	1.69	13.49
STC	0.998	0.038	0.127	0.78–200	y = 22338.87x + 18351.06	2.95	11.35
T-2 TOXIN	0.999	0.163	0.539	0.78–200	y = 7991.51x-16428.10	4.79	14.48
T2 TRIOL	0.996	0.721	2.378	0.78–200	y = 1551.23x-3858.17	5.27	19.55
ZEN	0.997	0.753	2.486	0.78–200	y = 470.18x-942.92	8.71	16.81
ZOL	0.997	2.923	9.646	4.69–600	y = 200.29x-520.49	4.65	18.75

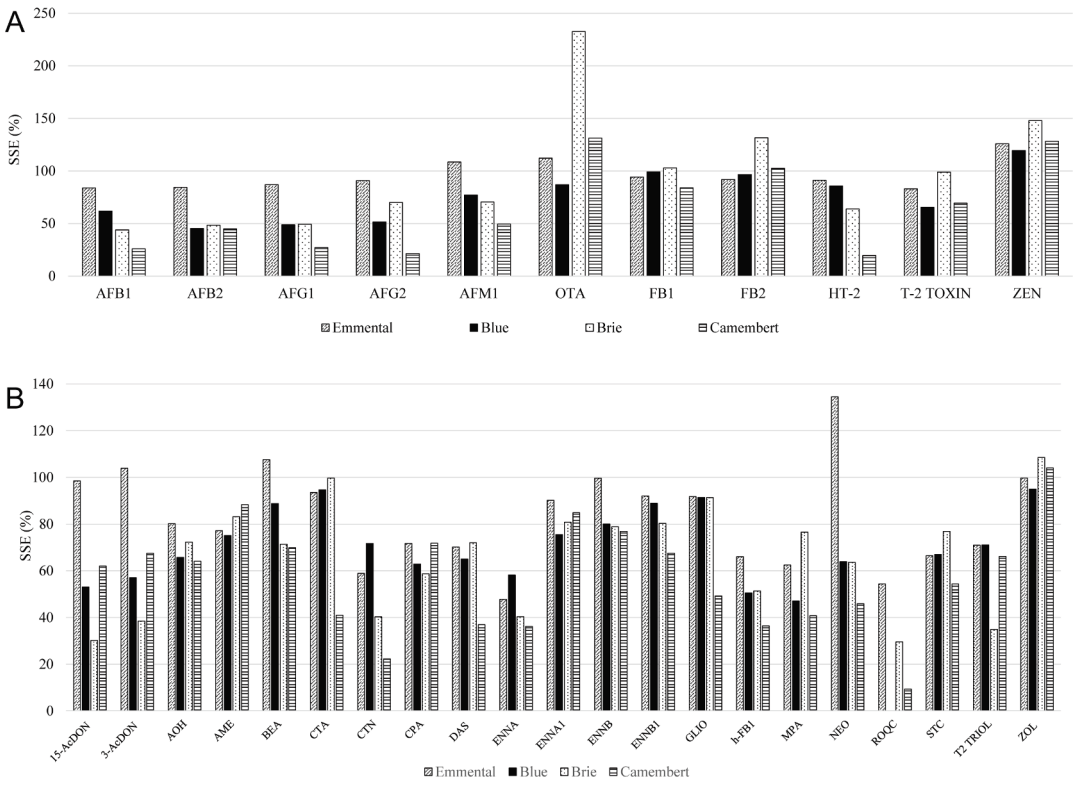

Fig. 3. Matrix effect of the analysis method. SSE value of emmental (diagonal stripes columns), blue (dark columns), brie (dotted columns), and camembert (horizontal stripes columns) obtained for regulated mycotoxins (A) and emerging/modified mycotoxins (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

solvent were between 0.002 and 2.923 ng/mL and LOQs in solvent were between 0.006 and 9.646 ng/mL, and the method is linear over a wide range of concentrations. After verifying that the performance method in solvent was adequate, they were calculated in matrix for each cheese variety, namely emmental (Table S4), blue (Table S5), brie (Table S6) and camembert (Table S7). The LODs obtained were between 0.020 μg/kg and 5.873 μg/kg. The LOQ, for instance, for AFM_1 in emmental cheese was 0.282 μg/kg, 0.244 μg/kg in blue, 0.232 μg/kg in brie and 0.224 μg/kg in camembert. Other authors had reported LOQs values of 0.6 μg/kg for AFM1 in mould cheese, 0.125 μg/kg in white cheese and 0.02 μg/kg in hard cheese (Kokkonen et al., 2005b; Škrbić, Antić, & Živančev, 2015). In the case of OTA, LOQs ranging from 0.723 to 2.272 μg/kg were obtained. Similar LOQs of OTA had been reported in grated cheese, 1 μg/kg (Biancardi, Piro, Galaverna, & Dall'Asta, 2013). However, unlike previous methods, the one proposed in this work allows the simultaneous detection of multiple toxins.

Next, the matrix effect was assessed; this phenomenon, caused by compounds that coelute with the target analyte, may lead to a suppression or enhancement of the mycotoxin signal. To correct the matrix effect, different approaches can be followed including matrix-matched calibration, standard addition or stable isotopically labelled standards (Varga et al., 2012). In this case, the SSE factor was calculated by comparing the slope of the calibration curves constructed in solvent and in matrix. Due to the variations that may exist between different batches of cheese, this factor was determined in triplicate for each cheese (Tables S4-S7). In general, there is an important matrix effect translated into a suppression of the signal for the majority of mycotoxins. As shown in Fig. 3 A, SSE values are around 60–80 % for regulated mycotoxins in emmental and blue cheeses. However, in camembert and brie cheeses there is a higher signal suppression. The mycotoxins less affected by matrix were the FBs, with SSE values from 83.96 % to 131.59 %, while the most affected compound was the HT-2 toxin, whose SSE value varied from 63.83 % in brie to 19.58 % camembert. On the other hand, the matrix barely affects the signal of OTA, although in brie there is an important enhancement of the signal (Fig. 3 A, Table S6). In the case of emerging mycotoxins, it must be highlighted and important signal suppression of ENNs in all cheeses. Similarly, the signal of ROQC is highly suppressed in camembert cheese, in blue cheese it was not possible to calculate this value because samples with low levels of ROQC were not found. In general, the emmental variety was the less affected by matrix. On the other hand, the matrix also causes a broadening of the peak shape of CPA (Fig. S6).

Finally, the precision and accuracy of the analysis method were evaluated. As shown in Fig. 4 A and Tables S4-S7, good R_E values were obtained for regulated mycotoxins, all of them higher than 60 % with RSD values lower than the 15 %. For instance, the R_E of OTA varied from 103 % in blue cheese to 111 % in emmental with RSD values were lower than 20 %. In the case of AFM_1, the obtained R_E was between 79 % and 101 %. In Europe, there are no specific methods for the determination of mycotoxins in food. However, the proposed procedure must be in compliance with the legislation. In this sense, the developed method shows recoveries and RSD values which conform to European regulations. In the case of emerging toxins, accuracy values were, in general, above 60 % (Fig. 4 B). In the case of BEA, the R_E was satisfactory only for emmental cheese; similarly, low R_E values were obtained for ENNs in brie and camembert cheese (Fig. 4 B). On the other hand, in the case of blue cheese, it was not possible calculate the accuracy of ROQC because no blank samples were found since this toxin is produced in high concentrations by the fungi used during cheese ripening (Maragos, 2022). However, the R_E of this mycotoxin in the other cheese varieties was adequate ranging from 83 % to 108 % (Fig. 4 B).

Therefore, the developed method allows the detection of 32 mycotoxins in cheese, fulfilling the minimum performance characteristic set in the legislation for regulated mycotoxins (EC_401, 2006). On the other

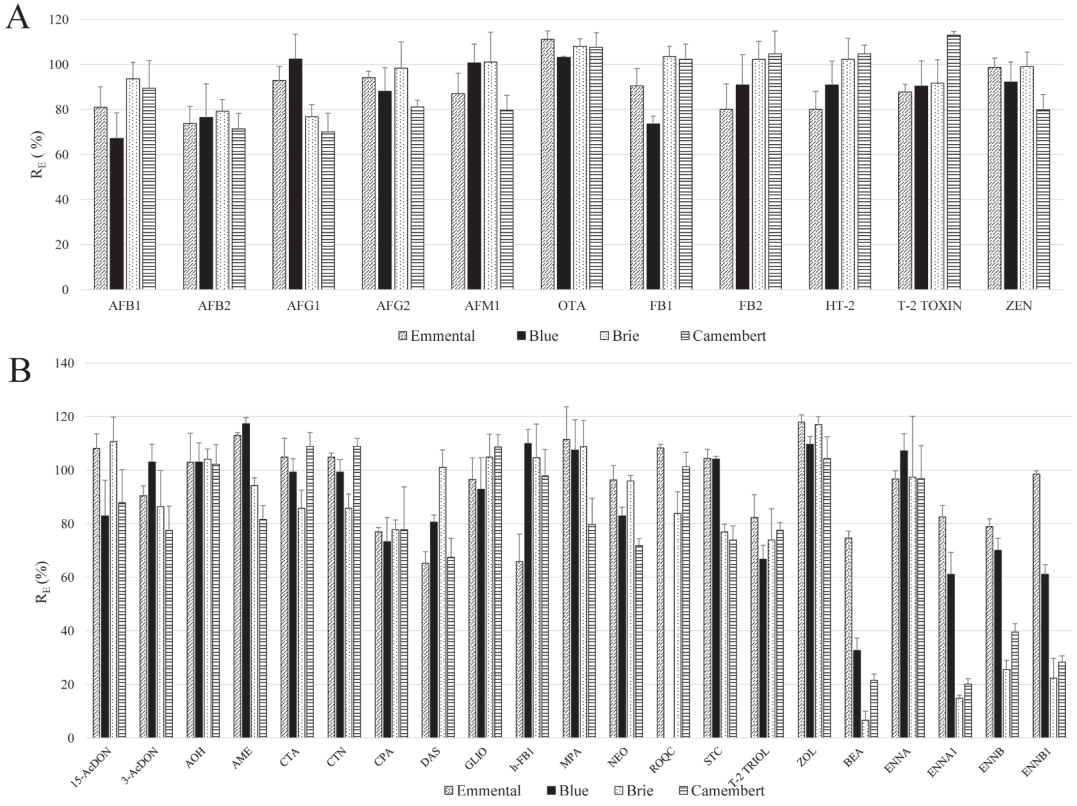

Fig. 4. Accuracy of the analysis method. R_E value of emmental (diagonal stripes columns), blue (dark columns), brie (dotted columns), and camembert (horizontal stripes columns) obtained for regulated mycotoxins (A) and emerging/modified mycotoxins (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hand, it represents a clear improvement over currently available methods that are summarized in table S2. An important part of these methods only allows the analysis of one or two mycotoxins at the same time, mainly AFM_1, MPA, CPA or CTN, using different techniques such as TLC, HPLC-UV or HPLC-FL. On the other hand, some works using mass spectrometry have been found, most of which are only optimized for the detection of OTA, STC, or CPA. Regarding the methods available for the simultaneous detection of multiple toxins there is a method that allows the quantification of AFs, OTA, MPA, penicillic acid and ROQC in blue and white cheese (Kokkonen, Jestoi, & Rizzo, 2005a). In addition, other method was identified for the analysis of 18 toxins including: MPA, ROQC, chanoclavine, ergometrinine, ergometrine, festuclavine, agroclavine, OTA, STC in cheese (Sulyok, Krska, & Schuhmacher, 2010). Therefore, among the validated methods, the proposed one detects the highest number of toxins, 32 compounds, and it was validated for some of the most important varieties of commercial cheeses. Another advantage of the optimized method is the low LOQ achieved, for instance the LOQ of AFM_1 varied from 0.086 μg/kg in emmental to 0.068 μg/kg in camembert, so they are about 10 times lower than the previously proposed method (Kokkonen et al., 2005b).

According to the EU Regulation, milk destined for the manufacture of dairy products, such as cheese, should not contain AFM_1 in a concentration higher than 0.05 μg/kg (EC_1881, 2006). However, there is no specific regulation for AFM_1 in cheese, although milk is concentrated during cheese manufacturing which can lead to an increase of toxin in the final product. On the other hand, Austria and Turkey stablish the limit of 0.25 μg/kg of AFM_1 in cheese (Becker-Algeri et al., 2016). Another mycotoxin whose presence in cheese is causing concern is the OTA (Younis, Ibrahim, Awad, & El Bardisy, 2016). At the moment, in the EU, only Slovakia has set a limit for OTA in cheese, 5 μg/kg (Duarte, Lino, & Pena, 2010). The proposed method allows the quantification of OTA at levels lower than 5 μg/kg, and AFM_1 can be quantified at level of 0.25 μg/kg in blue, brie and camembert cheese. However, in order to develop a method capable of detecting mycotoxins at lower levels, a further concentration step was included in the extraction protocol. In order to reduce the LOQs, mycotoxins were extracted with the developed method and analyzed in the extract 5 times concentrated. In this sense, 1 mL of the organic extract was dried using a centrifugal evaporator, then it was reconstituted with 200 μL of the acetonitrile/water/acetic acid mixture 49/50/1 (v/v/v) and filtered by using centrifugal filters of 0.22 μm. Consequently, the whole procedure was evaluated for the analysis of mycotoxins whose probability of being detected in milk is high. In this sense, AFM_1 is the most common aflatoxin detected in animal milk (Womack, Sparks, & Brown, 2016). Other mycotoxins have already been detected in this dairy product such as FBs, OTA, ZEN and trichothecenes like DAS, HT-2 toxin and T-2 toxin (Becker-Algeri et al., 2016). Also, AME had recently been reported for first time in animal milk (Akinyemi, Braun, Windisch, Warth, & Ezekiel, 2022). On the other hand, CTN, ROQC and MPA are frequently found in cheeses (Dobson, 2017). Consequently, the performance of the method was evaluated in the extract 5 times concentrated for the analysis of AFM_1, AFB_1, AFB_2, AFG_1, AFG_2, AME, CTA, CTN, DAS, FB_1, FB_2, HT-2 toxin, h-FB_1, MPA, OTA, ROQC, STC, T-2 toxin, T-2 triol, ZEN, ZOL, 15-AcDON and 3-AcDON. Using these conditions, LODs, LOQs, SSE values were calculated as detailed before. As shown in Table S8, smaller LODs and LOQs values were achieved for certain mycotoxins in concentrated extract, as in the case of AFs. For instance, the LOQ for AFM_1 was reduced to 0.020 μg/kg in emmental, 0.031 μg/kg in blue cheese, 0.036 μg/kg in brie and 0.028 μg/kg in camembert. For some toxins, such as OTA in camembert cheese there are low differences in the LOQ in the concentrated (0.985 μg/kg) and diluted matrix (0.750 μg/kg), while for some compounds the sample concentration increases the LOQ as in the case of MPA in camembert cheese, which was increased from 1.183 to 5.731 μg/kg. The main effect caused by sample concentration was an important increase in the signal suppression. For instance, in the case of AFM_1, as shown in Table S8, when the matrix was concentrated, the SSE value decreases from 77.08 % to 43.19 % in blue cheese, this reduction in the analytical signal was more pronounced for compounds like CTN with a decrease from 71.67 % to 16.03 %. The use of a method with a higher matrix effect can lead to a greater error in the accuracy of the method. Therefore, the accuracy of contaminated cheese was studied at 3 different levels (Table S9). As shown in Fig. 5 and Table S10, the R_E in the 3 levels was, in general, between 60 % and 100 %. Although, there are some exceptions, like CTN in the case of brie. On the other hand, RSD values were lower than 20 % (Table S10). Therefore, if necessary, the developed method could be used with a concentration step prior to analysis for the detection of toxins at very low levels. For example, the AFM_1 can be detected at 0.05 μg/kg in all cheeses, and therefore it reaches the sensitivity needed for the analysis of this compound at levels allowed in milk.

3.4. Application to real samples

A total of 38 cheese samples were analyzed, including 12 samples of emmental cheese, 10 of blue, 9 of brie and 7 of camembert. The results obtained are present in Table S11. BEA was found in all samples, the highest levels were detected in brie cheese, in concentrations ranging from 0.7 μg/kg to 29 μg/kg. A high incidence of ENNs was also found, but these emerging compounds were detected in low amounts. These mycotoxins had already been found in milk, and therefore a carry-over to cheese may occur (González-Jartín, Rodríguez-Cañás, et al., 2021). All the blue samples contained ROQC in concentrations higher than 250 μg/kg, although these results were not corrected for the recovery nor the matrix effect since no blank samples were found to calculate these parameters. In camembert cheese, this mycotoxin was detected in a concentration ranging from 3 μg/kg to 189 μg/kg. Although there are no reports about human health problems related to this mycotoxin, their toxicity is still not well characterized (Maragos, 2022). CTN was found in four samples, the maximum amount was detected in a blue cheese sample, with a concentration of 71 μg/kg. This mycotoxin had already been reported in cheese (EFSA, 2012). OTA was detected in brie, blue, and camembert cheeses in concentration ranging for 4.8 μg/kg to 9.6 μg/kg, respectively. Maximum levels of OTA have not been stablished in cheese or other dairy products, but the EFSA has requested more data on the presence of OTA in cheese to carry out a risk assess since high amounts of this mycotoxin were detected in traditional semi-hard cheeses (EFSA, 2020). In this sense, the concentrations found in the samples are similar to the maximum amount of OTA allowed in coffee (5 μg/kg), and much higher than those allowed for processed cereal-based foods and baby foods for infants and young children (0.5 μg/kg) (EC_1881, 2006). CPA was founded in three varieties of cheeses. The highest level (810 μg/kg) was in a brie sample, this mycotoxin was reported with a similar contamination level in taleggio cheese (Finoli, Vecchio, Galli, & Franzetti, 1999).

4. Conclusion

A new method was developed to quantify regulated, emerging and modified mycotoxins in cheese. The method, based on a QuEChERS extraction and UHPLC-MS/MS detection, allows the simultaneous detection of 32 mycotoxins in four varieties of cheese (emmental, blue, brie and camembert). Although the method has a large matrix effect, good accuracy and precision results have been obtained for most of the toxins in four commercial varieties of cheese. In addition, it significantly increases the number of mycotoxins that can be detected in a single analysis and improves the quantification limits of previously published methods. It was found a high occurrence of the emerging mycotoxins in samples obtained from local supermarkets; in addition, our data suggest that the presence of OTA in cheese may pose a risk to human health.

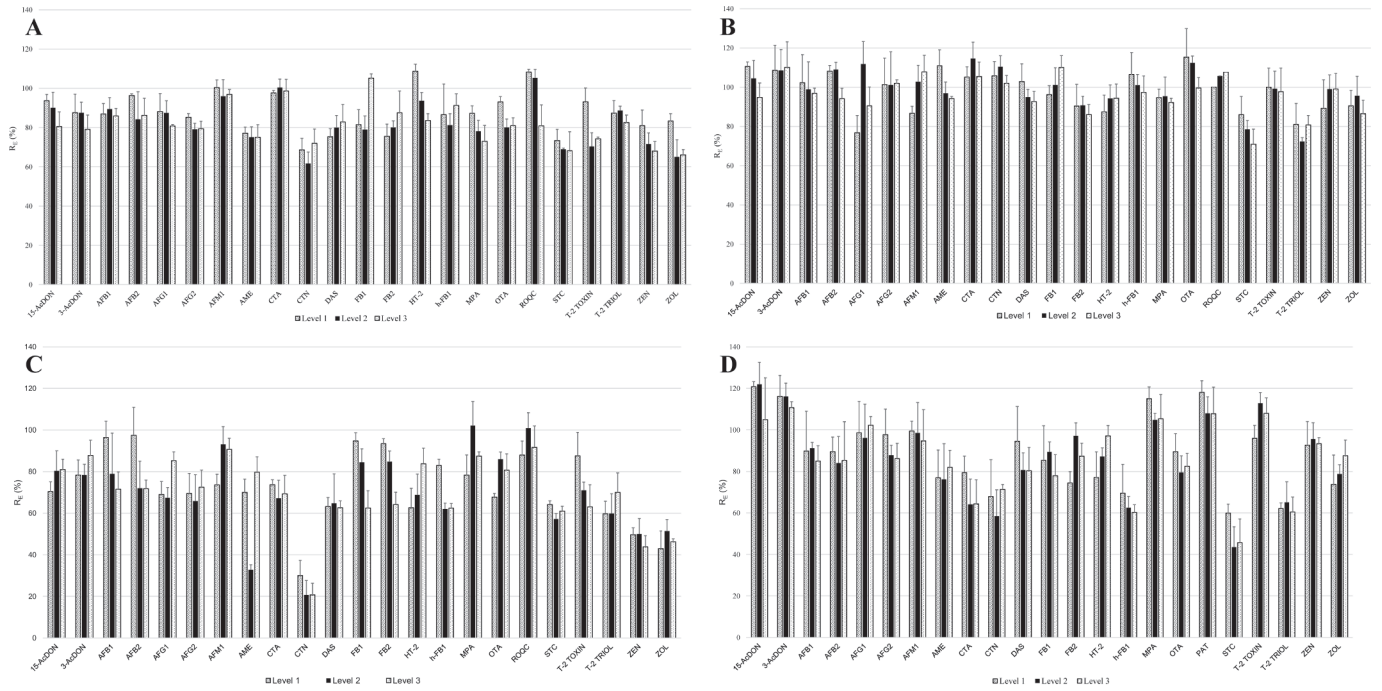

Fig. 5. Accuracy from the concentrated extract. R_E value calculated in the five times concentrated extract for three contamination levels in emmental (A), blue, (B) brie (C) and camembert (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

CRediT authorship contribution statement

Inés Rodríguez-Cañás: Investigation, Writing – original draft. **Jesús M. González-Jartín:** Investigation, Writing – review & editing. **Rebeca Alvariño:** Investigation. **Amparo Alfonso:** Methodology, Writing – review & editing. **Mercedes R. Vieytes:** Methodology. **Luis M. Botana:** Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.135182.

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