



Identification of mycotoxins in yogurt samples using an optimized QuEChERS extraction and UHPLC-MS/MS detection

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Abstract

Yogurt, a milk-derived product, is susceptible to mycotoxin contamination. While various methods have been developed for the analysis of dairy products, only a few have been specifically validated for yogurt. In addition, these methods are primarily focus on detecting aflatoxins and zearalenone. This study aimed to conduct a preliminary investigation into the presence of regulated, emerging, and modified mycotoxins in natural and oat yogurts available in the Spanish market. For this, a QuEChERS-based extraction method was optimized and then validated to detect and quantify 32 mycotoxins using ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). The method was in-house validated for the analysis of natural and oat yogurt in terms of linearity, matrix effect, sensitivity, accuracy, and precision. Satisfactory performance characteristics were achieved; for most of the analytes, LOQs were lower than 2 ng/g, and recoveries ranged from 60 to 110% with a precision, expressed as the relative standard deviation of the recovery, lower than 15%. Subsequently, the validated method was applied to analyze commercial yogurt samples, revealing a notable incidence of beauvericin and enniatins, with some analogues found in up to 100% of the samples. Alternariol methyl ether was also frequently found, appearing in 50% of the samples. Additionally, the study identified regulated toxins such as fumonisins, ochratoxin A, and HT-2 toxin. These results provide new incidence data in yogurt, raising concerns about potential health risks for consumers.

Keywords Matrix effect · Mycotoxin · UHPLC-MS/MS · Yogurt · Validation

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Introduction

Yogurt, also spelled yoghurt, is a popular food around the world since it provides many essential nutrients. Crafted through a fermentation process facilitated by lactic acid bacteria, it has been related to the prevention of gut diseases and the reduction in cholesterol levels. In addition, it is possible to fortify it with bioactive functional ingredients such as fruits, cereals, aloe vera extracts, or essential oils (Ahmad et al. 2022).

Milk is the main ingredient in yogurt; to avoid microbiological risks, raw milk undergoes a heat treatment which destroys all pathogenic microorganisms and deactivates fungal spores. However, there are contaminants such as mycotoxins that are not altered at pasteurization temperatures and therefore resist this process (Ulusoy et al. 2022; Weerathilake et al. 2014). In this sense, it has been observed that the raw milk presents some emerging mycotoxins such as beauvericin (BEA) or enniatins (ENNs) (Gonzalez-Jartin

et al. 2021). Other toxins detected in milk include aflatoxins (AFs), zearalenone (ZEN), ochratoxin A (OTA), fumonisin B₁ (FB₁), and fumonisin B₂ (FB₂) (Flores-Flores et al. 2015; Leite et al. 2023). Furthermore, dairy products like cheese may present additional toxins coming from contamination during the manufacturing process such as roquefortine C (ROQC), citrinin (CTN), sterigmatocystin (STC), or mycophenolic acid (MPA) (Benkerroum 2016).

In the near future, an upswing in mycotoxin incidence is predictable, driven by the temperature rise associated with climate change (Chhaya et al. 2022). This shift may lead to the emergence of mycotoxins in new regions, exemplified by the expected appearance of aflatoxin B₁ (AFB₁) in Northern Europe (Battilani et al. 2016). This is especially concerning as AFs have been identified by the International Agency for Research on Cancer as human carcinogens (IARC 2002). The AFB₁ and other mycotoxins can cross the intestinal barrier and migrate into tissues and/or milk. After ingestion, the AFB₁ is hydroxylated by the animal metabolism, leading to aflatoxin M₁ (AFM₁) that is excreted in milk (Becker-Algeri et al. 2016).

To avoid toxic effects in humans, dairy products must be analyzed, and several analytical techniques can be employed. The AFM₁ can be detected by immunoassay such as enzyme-linked immunosorbent assay (ELISA) kits. A recent study used this technique to analyze 72 yogurts; the AFM₁ was detected in two. However, ELISA present some disadvantages such as low sensitivity and low specificity which can lead to inaccurate results (Cano-Sancho et al. 2010; Iqbal 2021; Matabaro et al. 2017).

The application of chromatographic techniques, combined either with fluorescence detection (FLD) or tandem mass spectrometry (MS/MS), offers remarkable selectivity and sensitivity. These methods are able to detect mycotoxins at very low levels and have become the most common way for confirmatory analysis (Shanakhat et al. 2018). For instance, a method based on high-pressure liquid chromatography (HPLC) coupled to photo-induced fluorescence detection (FLD) was developed to determine AFs in yogurt (Hamed et al. 2017). Similarly, a gas chromatography-MS/MS method was validated to detect ZEN and their modified forms in dairy products such as yogurt (D'Orazio et al. 2016). Nevertheless, the most widely employed technique is LC-MS/MS, as it enables the simultaneous determination of multiple mycotoxins in complex matrices. This makes it a highly suitable methodology, especially considering the potential coexistence of more than one mycotoxin in the same sample (González-Jartín et al. 2022).

The current study used ultra-high-performance liquid chromatography (UHPLC) coupled with an MS/MS method to explore the presence of mycotoxins in natural and oat yogurt. To detect the most relevant mycotoxins at trace levels, a swift extraction process was optimized aimed at

diminishing the matrix effect and enabling the simultaneous recovery of mycotoxins from samples in a single step.

Materials and methods

Chemicals and reagents

Yogurt samples were purchased from local supermarkets. The natural yogurt samples have a fat content ranging between 2.5 and 3%, whereas the oat yogurt samples, comprising whole oats (1.5%) and walnuts (1%), exhibited a fat content between 3.9 and 4%. Pure water was obtained from a Millipore Milli-Q Plus system (Millipore, USA). Methanol, acetonitrile, acetic acid glacial 100%, anhydrous magnesium sulfate (MgSO₄), and sodium chloride (NaCl) were purchased from Panreac Química S.A. (Barcelona, Spain). Formic acid was from Merck (Madrid, Spain), while ammonium formate was from Fluka (Buchs, Switzerland). Additionally, ultrafree-MC Durapore membrane centrifugal filters with a pore size of 0.22 µm were supplied by Millipore (Billerica, USA).

The analytical standards, including AFB₁, aflatoxin B₂ (AFB₂), aflatoxin G₁ (AFG₁), aflatoxin G₂ (AFG₂), AFM₁, gliotoxin (GLIO), ROQC, enniatin A (ENNA), enniatin A₁ (ENNA₁), enniatin B (ENNB), enniatin B₁ (ENNB₁), and ZEN were acquired from Sigma (Madrid, Spain). OTA was from Laboratorios CIFGA S.A. (Lugo, Spain), circumdatin A (CTA) from Santa Cruz Biotechnology (Santa Cruz, CA), and beauvericin (BEA) from Enzo (Barcelona, Spain). The rest of the analytical standards used in this study were provided by Romer Labs (Tulln, Austria), including deoxynivalenol (DON), 3-acetyldeoxynivalenol (3 Ac-DON), 15-acetyldeoxynivalenol (15-AcDON), fusarenon X (FX), diacetoxyscirpenol (DAS), HT-2 toxin (HT-2), T-2, alternariol (AOH), alternariol methyl ether (AME), CPA, CTN, FB₁, FB₂, hydrolyzed fumonisin B₁ (h-FB₁), MPA, neosolaniol (NEO), STC, and zearalenone (ZAN).

Validation process

Limits of detection (LODs) and limits of quantification (LOQs) were calculated following the guidance of the European Union Reference Laboratory (EU-RL) (Wenzl 2016). The LOD for each compound was calculated by applying the following equation: $LOD = \frac{SD}{m} \times 3.9$ where *SD* was the standard deviation of 10 measurements of blank samples (samples with toxin levels lower than the LOD) and *m* was the slope of the curve of calibration. The LOQ was calculated with the equation $LOQ = LOD \times 3.3$

The effect of the matrix on the analytical signal was assessed by using the signal suppression/enhancement (SSE) factor according to the following equation:

$SSE(\%) = \frac{\text{slope calibration curve in matrix}}{\text{slope calibration curve in solvent}} \times 100$. In this sense, calibration curves were constructed in solvent and in blank matrix extracts, and their slopes were compared. The calibration levels ranged from 0.01 to 3 ng/mL in the case of AFs, from 0.78 to 200 ng/mL for FB₁, FB₂, HT-2, T-2, DON, OTA, ZEN, GLIO, MPA, AOH, AME, NEO, and STC; from 0.78 to 100 ng/mL for 15-AcDON, 3-AcDON, BEA, ENNA, ENNA₁, ENNB, ENNB₁, and h-FB₁; from 1.56 to 200 ng/mL for CTN; and from 1.95 to 500 for ZAN. To evaluate the linearity, the correlation coefficient (r^2) was determined using a linear regression model.

Accuracy and precision were assessed by calculating the average and relative standard deviation (RSD) of the recovery. To evaluate these parameters, blank samples were spiked with two different amounts of mycotoxins. The concentrations were selected based on the LOQs of each compound. For BEA and ENNs, samples were spiked at concentrations of 5 ng/g and 10 ng/g. For AFs, concentrations of 0.15 ng/g and 0.3 ng/g were utilized. ZAN was spiked at concentrations of 20 ng/g and 40 ng/g, while, for other mycotoxins, samples were contaminated at 10 ng/g and 20 ng/g.

First, the apparent recovery (R_A) (%) was calculated, and analytes were quantified by using solvent-based calibration curves. In this sense, the observed value of each toxin was divided by the spiked level and multiplied by 100. The RSD of the R_A values obtained for each compound were determined. In this way, intra-day precision was calculated using the RSD values obtained when the recovery was calculated in triplicate ($n = 3$) for samples analyzed on the same day, while inter-day precision was determined using samples analyzed on 3 consecutive days. Finally, the SSE factor was applied to the R_A obtaining the effective recovery ($R_E\%$) by using the following equation: $R_E\% = \frac{R_A\%}{SSE} \times 100$.

Sample analysis

Yogurt samples underwent homogenization, followed by the weighing of a 5 g portion into a 50-mL Falcon tube. Next, 10 mL of a 2% acetic acid solution was introduced and subjected to a 5-min mixing at 2500 rpm using a multi-vortex. Subsequently, 10 mL of acetonitrile were added, and the same mixing process was employed. A liquid-liquid partition was achieved by adding 4 g of MgSO₄ and 2 g of NaCl and a 30-s mixing in a vortex. Finally, samples were centrifuged for 10 min at 3134 × g, and a 1000 μL aliquot of the supernatant was evaporated to dryness and then reconstituted with 500 μL of acetonitrile/water/acetic acid (49/50/1 v/v/v). This reconstitution involved vortex mixing for 30 s followed by sonication for 1 min. Finally, the extract was filtered through a 0.22-μm centrifugal filter for 5 min at 10,000 rpm in preparation for analysis.

An Agilent 1290 Infinity UHPLC system coupled to a triple quadrupole mass spectrometer (Agilent 6460) equipped with an Agilent Jet Stream ESI source (Agilent Technologies, Waldbronn, Germany) was employed for UHPLC-MS/MS detection. The analysis was carried out in positive and negative modes and dynamic multiple reaction monitoring (dMRM) acquisition. Chromatographic and mass spectrometric conditions (Tables S1 and S2) have been previously optimized (Gonzalez-Jartin, et al. 2021; Rodríguez-Cañás et al. 2024).

Results and discussion

Yogurt stands as a globally consumed dairy product, with approximately 6% of the populace including it in its diet daily (Fisberg and Machado 2015). A potential concern associated with this product is the presence of mycotoxins from milk. In addition, this product is added with ingredients that can also contain mycotoxins such as fruits or cereals (Rodríguez-Cañás et al. 2024). Nevertheless, the detection of these contaminants in yogurt remains a challenge, resulting in limited available data regarding the occurrence of mycotoxins in this dairy product. Therefore, a new method based on a QuEChERS extraction process was optimized for the recovery of mycotoxins in natural and oat yogurt. The method was validated for the detection of toxins reported in both milk, such as AFM₁, and oat, including trichothecenes, fumonisins, AFs, and OTA (Jia et al. 2014; Tarazona et al. 2021).

Optimization of extraction conditions

A successful analytical method hinges on an efficient extraction procedure. Hence, it is imperative to develop methods with optimal recoveries for all targeted mycotoxins. In this context, QuEChERS protocols have proven effective in analyzing complex matrices. Nevertheless, the extraction process involves several critical steps that significantly impact the attainment of successful recoveries.

The nature of the sample and the analytes play a fundamental role in the choice of solvents. The first step consists of moistening the sample, which is why extractions are initially carried out using water, and then an organic solvent must be added to recover the lipophilic compounds (Perestrelo et al. 2019). The water employed for moistening is usually acidified, which increases the recovery of some toxins. Therefore, the first step was to evaluate the acid conditions since it was previously observed that both the nature of the acid and its concentration can affect the extraction efficiency (Rodríguez-Cañás et al. 2023). In this sense, 5 g of yogurt were extracted with 10 mL of water containing 0.5%, 1%, 2%, and 3% acetic acid or formic acid by

mixing at 2500 rpm for 5 min (multi-tube vortex, Nahita Blue, Auxilab, Spain). The extraction protocol was followed by adding 10 mL of acetonitrile and, after vortex mixing, 4 g of MgSO_4 and 1 g of NaCl were added to induce phase separation. Finally, 1 mL of the supernatant was evaporated to dryness and reconstituted with 200 μL of acetonitrile/water/acetic acid (49/50/1 v/v/v). The extracts were filtered through 0.22 μm and analyzed by UHPLC-MS/MS. For each condition, a yogurt sample ($n = 1$) had been spiked before extraction with 5 ng/g of DON, OTA, T-2, ZEN; 2.5 ng/g of BEA and ENNs; and 0.075 ng/g of AFs. The comparison of the R_A shows that, although there are no important differences, acetic acid yields higher recoveries (Fig. S1). Within the conditions tested using this acid, it was decided to use a 2% concentration since it shows a slight increase in the signal for ENNs, which have previously given low recoveries in dairy products such as cheese (Rodríguez-Cañás et al. 2023).

The next step was to test different volumes of water and acetonitrile. The R_A (%) was evaluated ($n = 1$) using the ratios (v:v) 1:1, 2:1, 3:1, and 1:2. The utilization of higher acetonitrile-to-water ratios (10 mL of acetonitrile vs 5 mL of water) for AFs led to increased recoveries (Fig. S2). This trend was also observed for regulated toxins such as DON, OTA, T-2 toxin, and ZEN. On the contrary, for ENNs and BEA, the recovery was enhanced with higher water proportions. Due to the lipophilic nature and low solubility of these mycotoxins in water, it is plausible that the role of water was

moistening the matrix, facilitating the subsequent penetration of acetonitrile. Based on the findings, extraction using 10 mL water and 10 mL acetonitrile emerges as the compromise choice for the extraction of all toxins.

Next, a separation between aqueous and organic phases must be induced. For this procedure, it is commonly employed the addition of anhydrous MgSO_4 and NaCl. It is known that NaCl forces polar compounds to go into the organic phase, while MgSO_4 induces phase partitioning (Santana-Mayor et al. 2019). Therefore, the ratio of salts may have a remarkable influence on the recovery; three tests with different proportions of salts were carried out to evaluate it. In all cases, 4 g of MgSO_4 was added with 1, 1.5, or 2 g of NaCl; the R_A (%) was evaluated by duplicate ($n = 2$); and results are shown in Fig. 1. In the case of lipophilic compounds as BEA and ENNs, no differences were observed; however, this parameter shows an important effect in the R_A (%) of hydrophilic compounds as AFs. For instance, the R_A (%) for AFM_1 was increased from 23 to 34%, when the amount of NaCl was doubled. Given this, the extraction was carried out by using 4 g of MgSO_4 and 2 g of NaCl.

The final step in the optimization process involved the study of the most suitable concentration for the extract. Analyzing a concentrated extract allows the detection of low toxin amounts, but it also carries the risk of increasing the matrix effect due to an increase in the background noise, resulting in low improvements or even a decrease in overall

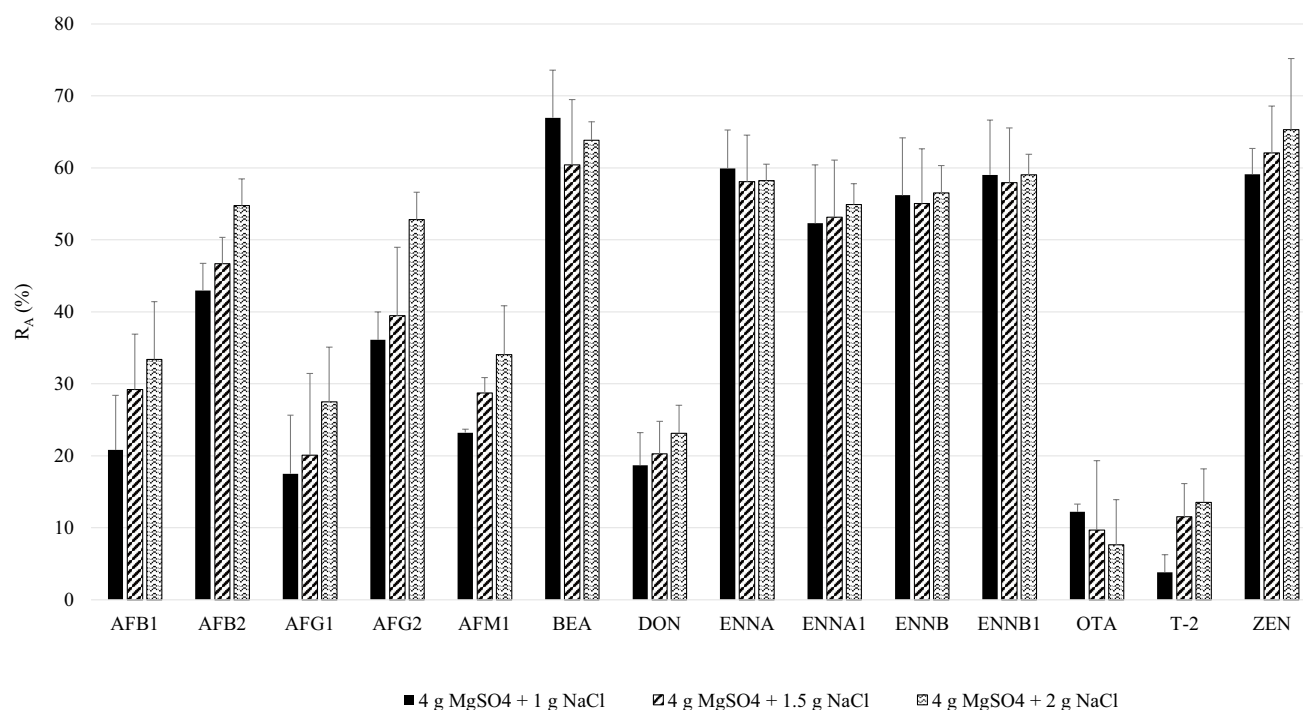


Fig. 1 Optimization of extraction process. R_A (%) obtained by applying a QuEChERS-based extraction using 4 g of MgSO_4 combined with 1, 1.5, and 2 g of NaCl

sensitivity (Keevil 2013). One approach to maximize the sensitivity of the method is to evaluate the effect of pre-concentration or dilution of the extract. The results usually vary greatly according to the mycotoxin and the matrix (Castilla-Fernández et al. 2022). Therefore, to assess this parameter, samples were spiked and extracted as it was previously explained. Finally, 1 mL of the extract was evaporated to dryness, and the residue was reconstituted with 0.5, 0.2, 0.1, or 0.05 mL of acetonitrile/water/acetic acid (49/50/1 v/v/v). Therefore, the sample was analyzed at 0, 2, 5, 10, 15, and 20 times concentrated ($n=1$), and the $R_A\%$ was checked. When the extract is concentrated beyond 10 times, notably low $R_A\%$ was observed, particularly for AFs (Fig. S3). This signal reduction may be attributed to a substantial increase in the matrix effect. Accordingly, when the sample was not concentrated, a signal enhancement of AFs was observed,

with recoveries exceeding 100%. Finally, a five-time concentrated sample was chosen for analysis as it strikes a balance between heightened sensitivity and the adverse effects of the matrix effect.

Method validation

There are no available certified reference materials to validate the analysis of mycotoxins in yogurt. Therefore, the method was in-house validated by using artificially contaminated samples. This approach allowed us to study the performance characteristics of the method.

The LOQs ranged between 0.002 ng/g and 13.78 ng/g (Table 1). The AFM₁ is regulated by European Union in milk, with a maximum permitted level of 0.05 ng/g (EC_2023/915). However, there is data about its presence

Table 1 Limits of quantification of the analysis method in natural and oat yogurt

Analyte	Natural yogurt			Oat yogurt		
	r^2	LOD (ng/g)	LOQ (ng/g)	r^2	LOD (ng/g)	LOQ (ng/g)
15-AcDON	0.999	0.24	0.78	0.999	0.42	1.39
3-AcDON	0.996	0.35	1.16	0.999	0.52	1.73
AFB ₁	0.998	0.003	0.01	0.999	0.003	0.01
AFB ₂	0.997	0.009	0.03	0.999	0.024	0.08
AFG ₁	0.997	0.009	0.03	0.999	0.024	0.08
AFG ₂	0.998	0.024	0.08	0.998	0.009	0.03
AFM ₁	0.999	0.002	0.01	0.999	0.002	0.01
AOH	0.999	2.39	7.89	0.998	0.74	2.43
AME	0.999	0.20	0.67	0.999	0.29	0.96
BEA	0.999	0.02	0.07	0.999	0.07	0.22
CTN	0.997	1.04	3.42	0.997	0.62	2.04
DAS	0.997	0.05	0.18	0.999	0.04	0.13
DON	0.998	0.66	2.18	0.999	0.32	1.07
DOM-1	0.999	2.68	8.86	0.999	1.65	5.44
ENNA	0.998	0.02	0.05	0.999	0.16	0.52
ENNA ₁	0.999	0.01	0.02	0.999	0.06	0.21
ENNB	0.999	0.01	0.04	0.996	0.16	0.54
ENNB ₁	0.999	0.01	0.04	0.997	0.09	0.31
FB ₁	0.999	0.08	0.28	0.998	0.18	0.61
FB ₂	0.995	0.36	1.19	0.997	0.48	1.58
FX	0.999	1.80	5.93	0.999	4.18	13.78
GLIO	0.999	0.42	1.39	0.998	0.11	0.36
HT-2	0.999	0.43	1.42	0.999	0.46	1.52
h-FB1	0.999	0.40	1.33	0.999	0.08	0.26
MPA	0.999	0.03	0.10	0.999	0.02	0.07
NEO	0.999	0.27	0.90	0.999	0.78	2.57
OTA	0.999	0.07	0.22	0.999	0.24	0.80
ROQC	0.999	0.04	0.12	-	-	-
STC	0.999	0.13	0.43	0.999	0.15	0.50
T-2	0.999	0.08	0.28	0.999	0.04	0.13
ZEN	0.999	0.61	2.02	0.999	0.96	3.16
ZAN	0.999	0.27	0.88	0.999	0.33	1.08

in dairy products such as yogurt at lower concentrations, namely 0.02 and 0.005 ng/g (Jia et al. 2014). The proposed method allows the detection of these levels since the LOD was 0.002 ng/g, while the LOQs were 0.007 and 0.008 ng/g for natural and oat yogurt, respectively; therefore, these limits are adequate to quantify the mycotoxin at the maximum permitted level (EC_2023/915). In the case of the ROQC, it was not possible to calculate the quantification limit in oat yogurt because no blank samples were available.

Several approaches can be used to correct matrix effect including matrix-matched calibration, standard addition, or stable isotopically labeled standards. For instance, isotope-labeled mycotoxins were employed to analyze dairy products (Yang et al. 2021; Zhang et al. 2013). However, the number of toxins for which this kind of standards are available is low, mainly regulated mycotoxins. On the other hand, since BEA and ENNs seem to be ubiquitous contaminants, it is difficult to obtain blank matrices to apply matrix-matched calibration (Akinyemi et al. 2022; Pietruszka et al. 2023). Therefore, in this work, the impact of the matrix on the analytical signal was assessed by using the SSE factor. Figure 2 displays the SSE values for each mycotoxin in both natural and oat yogurt; these values were comparable in both matrices. For example, in the case of AFM₁, the signal was diminished by 40% in both matrices, aligning with findings reported in dairy products (Xie et al. 2015). However, notable differences were observed for other compounds such as CTN, HT-2, and OTA. Specifically, OTA exhibited a 23% signal reduction in oat yogurt, while in natural yogurt, the analytical signal was suppressed by almost 50%. The less affected compounds were ENNs and BEA, with SSE values varying from 83 to 96%. These emerging mycotoxins had shown a similar behavior in milk (Gonzalez-Jartin, et al. 2021).

Finally, accuracy and precision were evaluated. The accuracy was adequate since, in general, recoveries higher than 70% were obtained for most of target compounds (Fig. 3). In natural yogurt, recoveries ranged from 61 (DON) to 112% (AOH) and from 60 (AFG₁) to 120% (ZEN) in oat yogurt. For instance, the AFM₁ shows satisfactory recovery in both matrices (81–110%). Error bars in Fig. 3 show the RSD values (intra-day precision) obtained for the different mycotoxins, which are lower than 15%, while the inter-day precision shows RSD values lower than 20% (Table S3). Therefore, this method conforms to the minimum performance characteristic that methods should meet for the analysis of regulated mycotoxins according to European legislation (EC_2023/2782). Emerging mycotoxins such as BEA and ENNs also shows good recoveries, with values ranging from 62 to 97%. The lower fat content in yogurt may account for the reduced matrix effect seen in lipophilic compounds such as ENNs compared to cheese, leading to more accurate results (Rodríguez-Cañás et al. 2023). The yogurts used in

this study presented a fat content ranging between 2.5 and 4%. Considering that yogurts with higher fat contents are available, it is necessary to evaluate the method's suitability through SSE and recovery studies before its application to that kind of matrices.

Several methods have been developed for the analysis of dairy products; however, most of them are only validated for milk. For example, a method using QuEChERS extraction followed by UHPLC—Orbitrap—detection was previously proposed for analyzing 58 toxins in dairy products, although the validation was conducted in milk (Jia et al. 2014). Similarly, good performance was achieved by using an extraction method involving a stable isotope dilution assay followed by LC—MS/MS (Yang et al. 2021). Regarding methods specifically developed for yogurt analysis, ELISA technique has been utilized for the detection of AFM₁ in yogurt. However, complete validations are not available. The LOD reported for this technique is approximately 0.02 µg/kg (LOQ) (Cano-Sancho et al. 2010; Mason et al. 2015). Therefore, their sensitivity is improved by the proposed method which is 0.008 µg/kg. The detection limits for other AFs (ranging from 0.01 to 0.08 µg/kg) are also lower compared to those achieved by a method based on dispersive liquid-liquid microextraction (DLLME) coupled to HPLC-FLD (ranging from 0.05 to 0.18 µg/kg). The other two methods developed for yogurt analysis are focused on detecting ZEN and its modified forms, with LOQs ranging from 0.9 to 15 µg/kg (D'Orazio et al. 2016; González-Sálamo et al. 2017). The proposed method was validated for the analysis of ZEN and ZAN; however, it did not include the main metabolites from ZEN and alpha- and beta-zearalenol (ZOL). This omission could pose a limitation for the method, as these compounds have been sporadically reported in milk (Prelusky et al. 1990). Therefore, while various methods have been developed for analyzing dairy products, only a few have undergone specific validation for yogurt. Moreover, those focused on this matrix were validated for AFs and ZEN (Table S4). In contrast, the proposed method enables the detection of a broader range of mycotoxins across different classes while maintaining or enhancing sensitivity and accuracy. Additionally, the validation process has been successfully conducted for both natural and oat yogurt samples, yielding comparable results, thus indicating its adaptability to other types of fortified yogurts.

Analysis of commercial samples

Dairy products are prone to mycotoxin contamination, primarily through the carryover of contaminants from animal feed to milk. Additionally, there is a risk of direct contamination by fungi (Becker-Algeri et al. 2016). On the other hand, it is known that oats are susceptible to contamination with several mycotoxins; therefore, their inclusion in

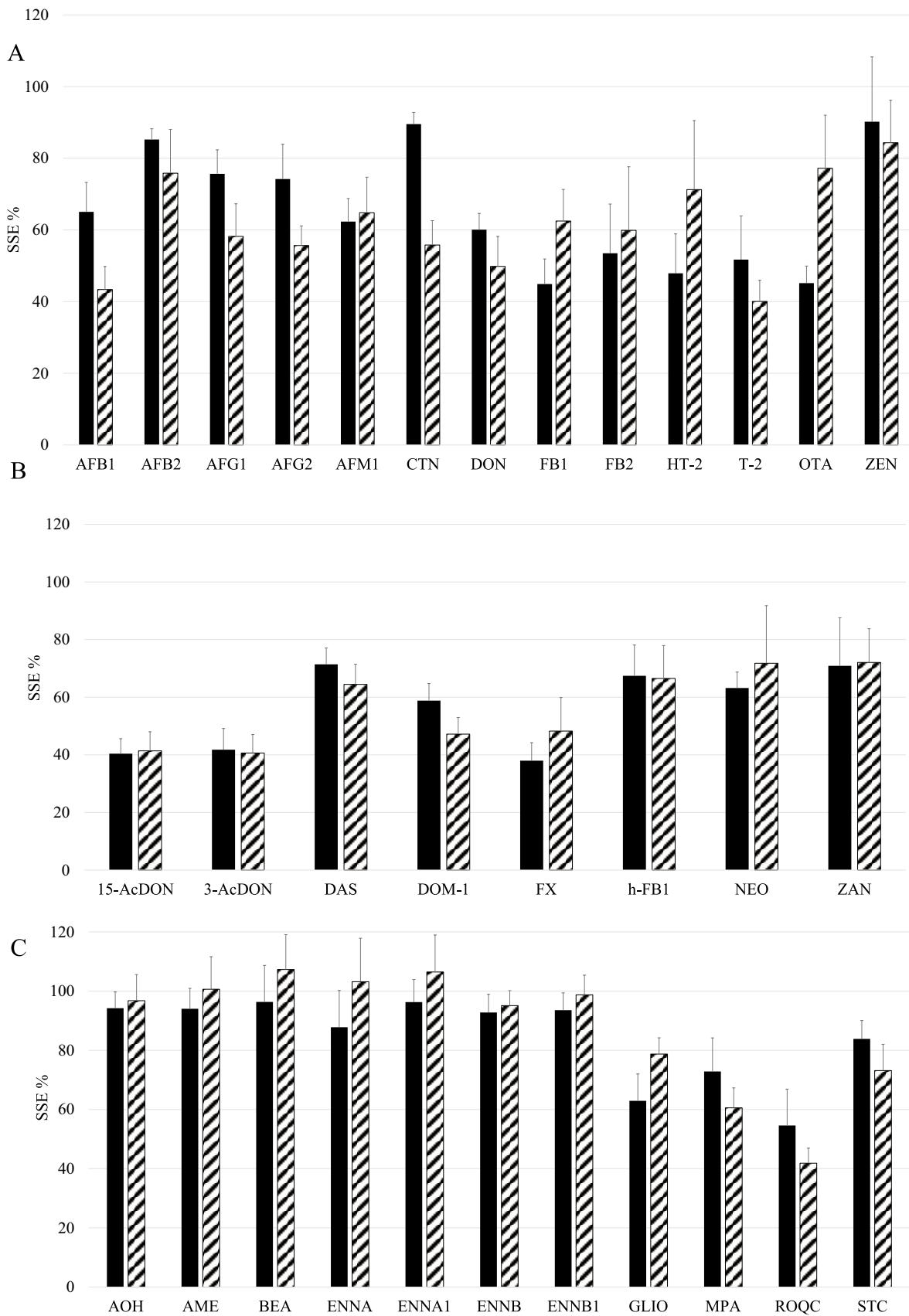


Fig. 2 Matrix effect of mycotoxins in yogurt. SSE values in natural yogurt (dark columns) and in oat yogurt (diagonal stripes columns). Results for regulated **A**, modified **B**, and emerging **C** toxins

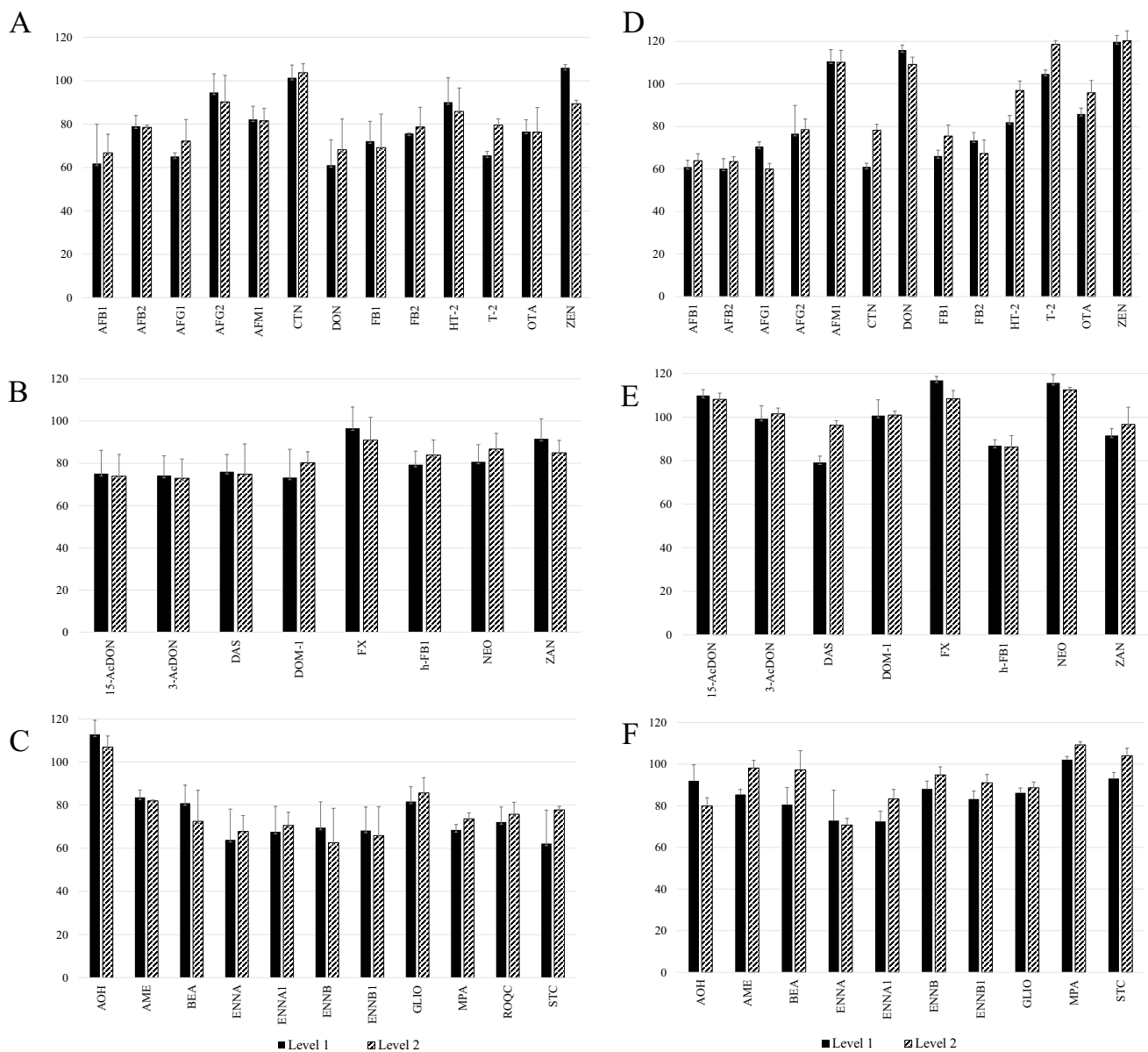


Fig. 3 Recovery of mycotoxins from yogurt. $R_E\%$ values for regulated **A**, modified **B**, and emerging **C** toxins in natural yogurt and for regulated **D**, modified **E**, and emerging **F** toxins in oat yogurt

yogurt could cause the direct contamination of these products (Becker-Algeri et al. 2016).

In order to carry out a first assessment on the presence of mycotoxins in the Spanish market, a total of 27 natural yogurt samples and 16 oat yogurt samples were obtained in local markets and analyzed with the described method. As shown in Fig. 4, HT-2 was found in an oat yogurt sample (6.25%); this mycotoxin is probable that proceed from the added oat, since it is commonly detected in this cereal (Pettersson et al. 2011). The presence of OTA in 10% of natural yogurt is not surprising, since in previous studies, OTA had been reported in milk and cheese (Keyvan et al. 2018; Rodríguez-Cañás et al. 2023; Sakin et al. 2018; Zhang

et al. 2022). Most of the samples tested positive for ENNs and BEA; these ubiquitous *Fusarium* mycotoxins have been found in dairy cow feed as well as milk on a regular basis. Consequently, their occurrence in yogurt seems possible (González-Jartín et al. 2022; Gonzalez-Jartin, et al. 2021; Pietruszka et al. 2023; Rodríguez-Blanco et al. 2020). Fumonisin were primarily detected in natural yogurt, with FB_1 found in 40% of samples and FB_2 in 26%, albeit in minimal amounts. In the case of oat yogurt, 1 out of the 16 samples showed these toxins at a level lower than the LOQ. The presence of fumonisins in milk has not been widely studied, but it was reported that 80% of commercial milk samples in Italy showed an average contamination level of 0.26 ppbs.

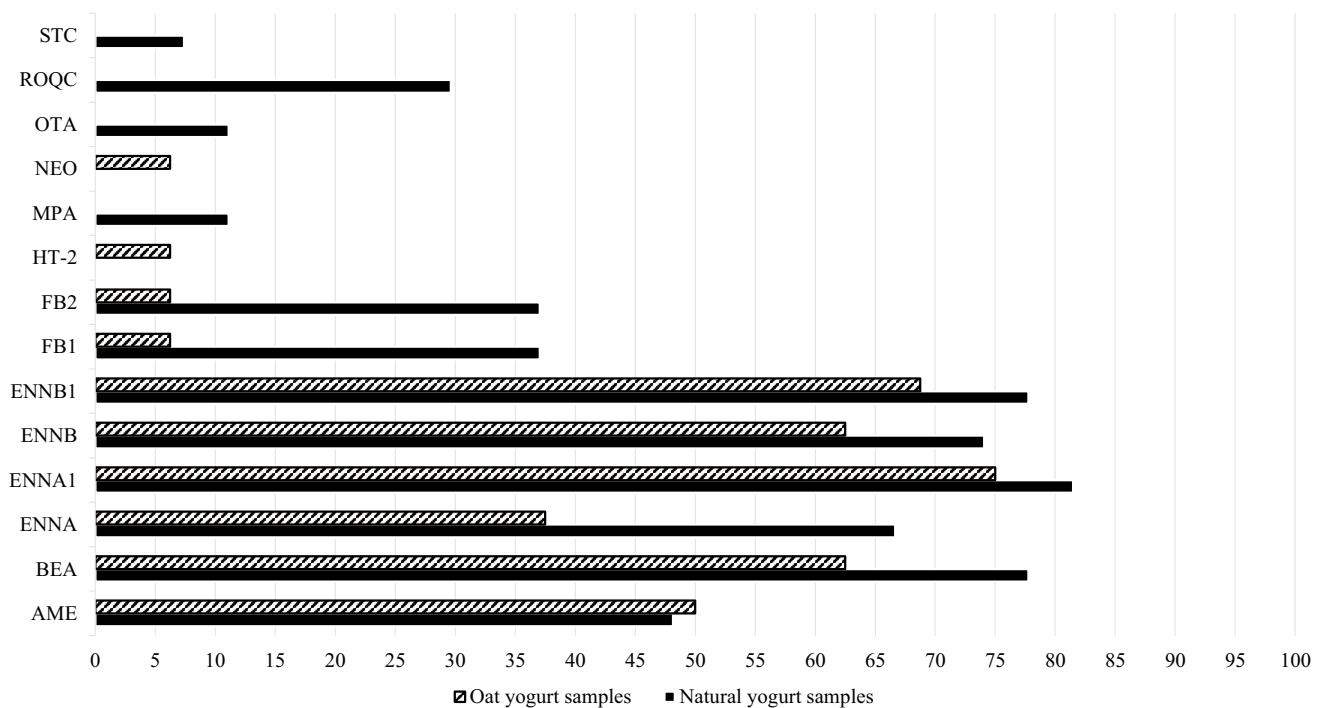


Fig. 4 Positive samples of natural yogurt and oat yogurt. Percentage of each mycotoxin in samples

Similarly, other studies have reported low levels of fumonisins in milk, while there are no reports of their presence in yogurt (Benkerroum 2016; Gazzotti et al. 2009). Another frequently found toxin was AME, detected in approximately half of the samples of both natural and oat yogurt. *Alternaria* toxins are commonly found in various grains, particularly when there is high moisture content in the field (De Colliet al. 2021). For instance, in Canada, up to 75% of cereal-based samples have tested positive for AME (Scott et al. 2012). The transfer of this toxin to milk has been recently reported, AME was found in 9% of cow milk samples from Nigeria,

and it was also detected in camel and goat milk (Akinyemi et al. 2022).

Table 2 shows the average concentrations in samples. For example, of the 81% of natural yogurt samples positive for ENNA₁, the average contamination level was 0.90 ng/g. Comparable levels of ENNS have been noted in milk by various researchers. It has been emphasized that these contaminants, albeit detected only in trace quantities, do not imply any immediate hazard to consumers (Pietruszka et al. 2023). In the case of the ROQC, all oat yogurt samples were positive, but showed a signal to noise ratio lower than 10,

Table 2 Statistic components of the analysis of yogurt samples^a

Analyte	Natural yogurt				Oat yogurt			
	25% percentile	Median	Maximum	Mean	25% percentile	Median	75% percentile	Mean
AME	0.75	0.77	1.48	0.88	2.67	2.67	2.67	2.67
BEA	0.09	0.11	3.11	0.43	0.09	0.11	0.20	0.13
ENNA	0.71	1.36	22.89	3.58	1.27	2.05	2.20	1.84
ENNA ₁	0.15	0.21	9.27	0.90	0.19	0.21	0.63	0.37
ENNB	0.09	0.29	8.36	0.80	0.28	0.43	0.62	0.58
ENNB ₁	0.25	0.47	8.43	1.15	0.44	0.69	1.19	0.79
FB ₁	0.76	1.99	7.39	2.69	-	-	-	-
FB ₂	3.22	5.05	12.28	5.65	-	-	-	-
OTA	1.00	1.54	4.31	2.28	-	-	-	-
ROQC	0.12	0.16	0.19	0.16	-	-	-	-

^aConcentration data (expressed in ng/g) for samples with levels above the LOQ

and therefore, they were not quantified. Eight natural yogurt samples were positive and presented an average concentration of 0.16 ng/g. In summary, emerging mycotoxins are present in yogurt at low concentrations but exhibit a high frequency of occurrence.

The present study revealed the susceptibility of yogurt, both natural and oat-based, to mycotoxin contamination. The optimized QuEChERS-type extraction method coupled with UHPLC-MS/MS provided a robust analytical approach capable of detecting and quantifying 32 mycotoxins, and the in-house validation demotes that the method is adequate. Emerging mycotoxins, specifically BEA and ENNs, has a high frequency of appearance in the samples analyzed. Furthermore, OTA has detected three samples of the natural yogurt. In addition, the detection of HT-2 in oat yogurt represents a novel observation. These results underscore the significance of monitoring mycotoxin levels in yogurt products for consumer safety.

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Data availability No datasets were generated or analyzed during the current study.

Declarations

Competing interest The authors declare no competing interests.

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