

Application of a multi-toxin detect method to analyze mycotoxins occurrence in plant-based beverages

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ABSTRACT

In recent years, plant-based beverages have gained popularity on the market due to environmental and ethical concerns, as well as milk intolerances and allergies. However, raw materials employed in the manufacture of these products are susceptible to mycotoxin contamination. For this reason, a new method based on a QuEChERS extraction procedure followed by ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) detection was developed for the analysis of 29 mycotoxins in oat, rice, soy, and almond beverages. The method was validated in terms of linearity, detection and quantification limits, matrix effect, recoveries, accuracy and precision. Satisfactory performance characteristics were achieved, with recoveries above 70% for most mycotoxins. Several commercial samples were analyzed, aflatoxins were frequently detected in rice and almond beverages, while T-2 and HT-2 toxins were identified in oat-based products. In addition, emerging mycotoxins such as enniatins and beauvericin were detected in the four types of beverages.

1. Introduction

Plant-based beverages are diluted emulsions obtained from nuts, cereals, legumes, or seeds mixed with water, although they can include other ingredients (salt, sugar, natural additives...) to improve their nutritional value or flavour. In recent years, due to health problems such as intolerances or allergies, there has been an increase in the number of consumers of these types of products that do not include lactose in their composition (Reyes-Jurado, Soto-Reyes, Dávila-Rodríguez, Lorenzo-Leal, Jiménez-Munguía, Mani-López, et al., 2021).

The highest occurrence of mycotoxins tends to arise in agricultural commodities. Fungi can infect crops in the field or during the harvest and/or storage and, if the temperature and moisture conditions are appropriate, the agricultural products will be contaminated by mycotoxins (Lee & Ryu, 2017). Monitoring all stages of the agri-food chain is necessary to preserve food safety. Regulated mycotoxins are analyzed in certain foodstuffs; however, EU regulation does not contemplate plant-based beverages nor legumes or derived products (EC_2023/915, 2023). In addition, climate change may increase the risk related to mycotoxins since these metabolites can appear with more frequency in the matrices employed in the manufacture of plant-based beverages

(Medina, Akbar, Baazeem, Rodriguez, & Magan, 2017).

Emerging mycotoxins are compounds for which there is not enough data about their toxicity, although recent studies show a high incidence in different commodities (González-Jartín, Alfonso, Rodríguez, Sainz, Vieytes, & Botana, 2019). These toxins, which are not monitored in routine analysis, include, among others, beauvericin (BEA), enniatins (ENNs), fusaric acid (FA), sterigmatocystin (STC), mycophenolic acid (MPA), and cyclopiazonic acid (CPA) (Gruber-Dorninger, Novak, Nagl, & Berthiller, 2017). However, some emerging toxins namely alternariol (AOH) and alternariol methyl ether (AME) have recently begun to be monitored (EC_2022/553, 2022). There are reports on the incidence of these mycotoxins in raw materials employed in the manufacture of plant-based beverages; for example, BEA and ENNs have been detected in oat, rice, or soybean samples (García & Jarque, 2014; Pérez-Fuentes, Alvariño, Alfonso, González-Jartín, Gegunde, Vieytes, et al., 2021). A cytotoxic effect of emerging mycotoxins in human cells was reported, which suggests that more data about their occurrence and toxicity is needed to carry out a risk characterization (Cimbalo, Alonso-Garrido, Font, & Manyes, 2020). In addition, regulated and emerging mycotoxins can suffer changes in their chemical structure caused by fungi, plant, or animal metabolism, leading to another group of unregulated

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mycotoxins, known as modified mycotoxins, such as 3-acetyl-deoxynivalenol (3-AcDON) or 15-acetyl-deoxynivalenol (15-AcDON) which are modified forms of DON. Data on these mycotoxins are still scarce, and therefore it is necessary to develop methods for the detection of these compounds and study their occurrence in food (Pérez-Fuentes et al., 2021).

In recent decades, ultra-high liquid chromatography coupled mass spectrometry (UHPLC-MS/MS) has become the most employed technique for the analysis of mycotoxins in complex matrices (Medina, Akbar, Baazeem, Rodríguez, & Magan, 2017). This methodology presents some advantages including high selectivity and sensitivity and it also allows the detection of a wide range of toxins (EN17641, 2022; Wang, Zhang, Ma, & Li, 2022). There is not much data available on the presence of mycotoxins in plant-based beverages, Table 1 shows a compilation of the analytical techniques used for the determination of mycotoxins in these matrices (Arroyo-Manzanares, Hamed, García-Campaña, & Gámiz-Gracia, 2019; Hamed, Abdel-Hamid, Gámiz-Gracia, García-Campaña, & Arroyo-Manzanares, 2019; Hamed, Arroyo-Manzanares, García-Campaña, & Gámiz-Gracia, 2017; Miró-Abella, Herrero, Canela, Arola, Borrull, Ras, et al., 2017). The previously available methods are intended for the analysis of specific groups of toxins or allow the detection of a small number of compounds. Therefore, this study aimed to optimize and validate a new method to analyze mycotoxins in soy, oat, rice, and almond beverages, and to evaluate the presence of mycotoxins in these products.

2. Materials and methods

2.1. Chemicals and materials

The following analytical standards were used in the study: aflatoxin B₁ (AFB₁), aflatoxin B₂ (AFB₂), aflatoxin G₁ (AFG₁), aflatoxin G₂ (AFG₂), enniatin A (ENNA), enniatin A₁ (ENNA₁), enniatin B (ENNB), enniatin B₁ (ENNB₁), gliotoxin (GLIO), and zearalenone (ZEN), which were purchased from Sigma in Madrid, Spain. Ochratoxin A (OTA) standard solution was obtained from Laboratorios CIFGA S.A. in Lugo, Spain, while BEA was supplied by Enzo in Barcelona, Spain, and circumdatin A (CTA) was obtained from Santa Cruz Biotechnology in Santa Cruz, CA. Analytical standards of 3-acetyldeoxynivalenol (3-AcDON), 15-acetyldeoxynivalenol (15-AcDON), alternariol (AOH), alternariol methyl ether (AME), citrinin (CTN), diacetoxyscirpenol (DAS), fumonisin B₁ (FB₁), fumonisin B₂ (FB₂), hydrolyzed fumonisin B₁ (h-FB₁), HT-2 toxin (HT-2), mycophenolic acid (MPA), neosolaniol (NEO), STC, T-2 toxin (T-2), T-2 triol and zearalanone (ZOL) were provided by Romer Labs in Tulln, Austria.

The chemicals used in the study were purchased from the following suppliers: Methanol, acetonitrile, acetic acid glacial 100%, anhydrous magnesium sulphate (MgSO₄), and sodium chloride (NaCl) were purchased from Panreac Química S.A. in Barcelona, Spain. Ammonium formate was obtained from Fluka in Buchs, Switzerland, and formic acid

from Merck in Madrid, Spain. The Polygoprep™ 60-50 C18 was supplied by Macherey-Nagel in Düren, Germany, and ultrafree-MC Durapore membrane centrifugal filters (0.22 µm pore size) were provided by Millipore in Billerica, USA. A Millipore Milli-Q Plus system (Millipore, USA) was employed to obtain pure water.

2.2. Samples

A diverse selection of plant-based beverage samples from different brands was purchased from local supermarkets, namely: 16 oat beverage samples, with oat percentages ranging between 11% and 15%, 6 rice beverage samples (15%–17% of rice), 7 almond beverage samples (2%–14% almond), and finally, 9 soy beverage samples (8%–15% of soy). All samples contain other secondary ingredients such as stabilizers, thickeners, aromas, salt, sugar, and vitamins. All samples were storage at 4 °C until required for sample preparation and analysis.

2.3. UHPLC-MS/MS conditions

Sample analysis was conducted using an Agilent 1290 Infinity ultra-high-performance liquid chromatography (UHPLC) system coupled with an Agilent 6460 Triple Quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source, both of which were manufactured by Agilent Technologies in Waldbronn, Germany. Agilent MassHunter LCMS Acquisition Console software was used for system control and data acquisition, while QQQ Quantitative Analysis software was used for data processing.

For chromatographic analysis, an Acquity UPLC HSS T3 column (1.8 µm, 100 mm × 2.1 mm) from Waters was used at a temperature of 40 °C, with a flow rate of 0.3 mL/min. Mobile phase A was composed of water with 5 mM ammonium formate and 0.1% formic acid, while mobile phase B was methanol 100%. The injection volume was set at 5 µL and a gradient elution of 13 min was employed as follows: after 0.5 min at 0% B, the gradient was raised to 14% B within 0.5 min, and maintained for 1.5 min. Then, the percentage of the eluent B was increased to 60% B within 1 min and later held for 0.5 min. Next, it was increased to 100% B within 4.5 min, and maintained for 2 min. Finally, the gradient was back to 0% B in 0.5 min and this percentage was kept for 2.5 min.

Mass spectrometer parameters including the fragmentor voltage (FV), collision energy (CE), cell accelerator voltage (CAV) and mass transitions for each analyte had previously been described (González-Jartín, Rodríguez-Cañás, Alfonso, Sainz, Vieytes, Gomes, et al., 2021). Analysis was carried out in positive and negative modes and dynamic multiple reaction monitoring (dMRM) acquisition. In this sense, two *m/z* transitions were monitored for each mycotoxin (Table S1). In order to identify a compound, the maximum tolerances for the ion ratio measurements, with respect to their relative intensities, were set at 30% and the retention time retention time (RT) with a tolerance of ±0.1 min in relation to the RT of the analyte in the calibration curve. The ionization source parameters were the following: nebulizer, 45 psi; nebulization

Table 1

Available methods for determination of mycotoxins in plant-based beverages.

Food items	Analyte	Analytical technique	LOQs (µg/L)	Levels (µg/L)	Reference
Oat, soybean, and rice beverage	<i>Fusarium</i> toxins (FB ₁ , FB ₂ , T-2, HT-2, FX, DON, ZEN)	Salting-out-assisted liquid–liquid extraction (SALLE) followed by UHPLC-MS/MS	3.2–57.7	192–263 µg/L (only for DON)	(Hamed, Arroyo-Manzanares, García-Campaña, & Gámiz-Gracia, 2017)
Soy, oat and rice beverage	BEA and ENNA, ENNA ₁ , ENNB, ENNB ₁	SALLE followed by UHPLC-MS/MS	0.3–0.8	0.4–26	(Arroyo-Manzanares, Hamed, García-Campaña, & Gámiz-Gracia, 2019)
Oat, rice, coconut, almond, birdseed beverages	AFB ₁ , AFB ₂ , AFG ₁ and AFG ₂	Dispersive liquid–liquid microextraction (DMLLE) followed by HPLC-FLD QuEChERS by LC-FLD	0.5	–	(Hamed, Abdel-Hamid, Gámiz-Gracia, García-Campaña, & Arroyo-Manzanares, 2019)
Soy, oat and rice beverage	11 mycotoxins (DON, AFB ₁ , AFB ₂ , AFG ₁ , AFG ₂ , HT-2, FB ₁ , FB ₂ , T-2, OTA, ZEN)	QuEChERS followed by UHPLC-MS/MS	0.05–15	0.1–19	(Miró-Abella, Herrero, Canela, Arola, Borrull, Ras, et al., 2017)

gas flow, 12 L/min; drying gas flow, 8 L/min; nebulization gas temperature 400 °C; drying gas temperature, 350 °C; capillary voltage, 4000 V; nozzle voltage, 0 V. In all cases, the gas used was nitrogen.

2.4. Optimization of the procedure

The extraction procedure was optimized following a protocol previously proposed for the analysis of cheese (Rodríguez-Cañás, González-Jartín, Alvarino, Alfonso, Vieytes, & Botana, 2023). In this sense, the feasibility of sample concentration and the use of a clean-up step were investigated, while the effectiveness of different solvents was not evaluated, 0.5% formic acid was employed due to its high efficacy in the extraction of mycotoxins from milk, as previously demonstrated (González-Jartín et al., 2021). Moreover, given the unique nature of the matrix, the contamination levels for optimization were reduced as follows: Five mL of sample were artificially contaminated with 0.19 µg/L of aflatoxins; 6.25 µg/L of BEA, ENNs and h-FB1; 12.5 µg/L of other regulated (DON, FB1, FB2, ZEN, HT-2, T-2, OTA, CTN), emerging and modified (AOH, AME, MPA, NEO, GLIO, STC, DAS, T-2 triol) toxins. Next, the samples were extracted using 5 mL of 0.5% formic acid in acetonitrile. Phase separation was induced by adding 4 g of MgSO₄ and 0.6 g of NaCl, followed by agitation and centrifugation (at 3134 × g for 10 min). To assess the feasibility of analysing a concentrated sample, the organic phase (1.5 mL) was evaporated to dryness and the resulting extract was redissolved in 1.5, 0.6, 0.3, 0.15, and 0.075 mL of the analysis solvent (a mixture of acetonitrile, water, and acetic acid at a ratio of 49:50:1 v/v/v). The effectiveness of a cleaning step was evaluated by adding 1.125 g of MgSO₄ and 0.375 mg of C18 to 2.5 mL of the organic phase. Next, 1 mL of the obtained extract was evaporated to dryness and then redissolved in 0.2 mL of the analysis solvent.

2.5. Sample extraction

Five mL of sample were mixed with 5 mL of 0.5% formic acid in acetonitrile in a polypropylene tube using a multi-tube vortex (Nahita Blue, Auxilab, Spain) at 2500 rpm for 5 min. Then, a mixture of 4 g of MgSO₄ and 0.6 g of NaCl was added for the partitioning step, which was achieved by shaking using a vortex mixer for 30 s and centrifugation at 3134g, 4 °C, for 10 min. An aliquot of 1 mL of the supernatant was evaporated to dryness in a centrifugal evaporator (Jouan RC 1022, Thermo Electron Corp.) and reconstituted with 200 µL of the analysis solvent. Finally, the extract was filtered through 0.22 µm by using centrifugal filters (5 min at 16,000g).

2.6. Method validation

The method was validated for the target mycotoxins in plant-based beverages in terms of linearity, limits of detection (LODs) quantification (LOQs), matrix effect, accuracy, and precision. Nine-point calibration curves were constructed in the analysis solvent (mixture of acetonitrile/water/acetic acid 49/50/1 v/v/v) and in the blank matrix. In this way, the linearity of the method was evaluated, and the correlation coefficients (R) were calculated. The range of concentrations were: 0.01–3 ng/mL for aflatoxins except for AFG₂ (0.05–3 ng/mL); 0.78–100 ng/mL for 15-AcDON, 3-AcDON, BEA, ENNs, and h-FB1; 1.56–200 ng/mL for CTN; 1.95–500 for ZOL, and 0.78–200 ng/mL for other toxins.

LODs and LOQs were calculated according to the EURL guide (Wenzl, Haedrich, Schaechtele, Piotr, Stroka, Eppe, et al., 2016). In this sense, LODs were assessed according to the equation $LOD = 3.9 \times \frac{S_b}{m}$, where S_b is the standard deviation of the measurement of ten blank samples (plant-based beverage extracts) prepared following the extraction procedure previously described and m is the slope of the calibration curve. Next, LOQs were calculated by multiplying the LODs of each mycotoxin by a factor of 3.3.

The matrix effect for each mycotoxin was evaluated in triplicate in each matrix (soy, oat, rice, and almond) by determining the signal suppression/enhancement (SSE) factor. The accuracy and precision of the method were evaluated through recovery experiments by spiking blank samples with mycotoxins at three levels of contamination (Table S2). Recovery experiments were performed in triplicate on the same day to assess repeatability and in three consecutive days, by different operators, to evaluate within-lab reproducibility. The apparent recovery (R_A) and the relative standard deviation (RSD) were calculated. Precision was evaluated on the same day using RSD_r (intra-day precision) and over three consecutive days using RSD_R (inter-day precision). Finally, the accuracy was assessed by determining the recovery of the extraction (R_E). The SSE, R_A , RSD, and R_E were calculated according to the previously described equations (Rodríguez-Cañás, González-Jartín, Alvarino, Alfonso, Vieytes, & Botana, 2023). In order to calculate the concentration of toxins in samples, the quantification was conducted using solvent based calibration curves and then the data was corrected with the SSE and R_E factors.

3. Results and discussion

Raw materials employed in the manufacture of plant-based beverages include nuts, cereals, legumes, or seeds, all of them can be contaminated in the field or during storage with a wide range of mycotoxins and consequently, a carry-over of toxins to plant-based beverages may occur. The European Regulation establishes maximum levels of mycotoxins in certain raw materials. For example, it set maximum residue levels of aflatoxins in almonds, cereals, and rice, as well as levels for OTA in soybeans and DON in oat grains. Nevertheless, the presence of mycotoxins in plant-based beverages has not been regulated and there are not many studies dealing with the presence of mycotoxins in the final product, whose consumption has increased significantly in recent years (García & Jarque, 2014; Reyes-Jurado et al., 2021). Consequently, the aim of this study was to develop a method applicable to the analysis of several mycotoxins in the most consumed plant-based beverages and, on the other hand, to carry out a preliminary study on the incidence of toxins in these products.

3.1. Optimization of extraction procedure

In the present study, a QuEChERS based extraction was applied. This technique has ever increased relevance for the analysis of different contaminants, including mycotoxins since it was first proposed in 2003 for the analysis of pesticides (Xu, Xu, Han, Qiu, & Hou, 2019). This methodology, which allows the simultaneous extraction of analytes from complex matrices, consists of a solid-liquid or liquid-liquid extraction, followed by a phase partition which is achieved by the addition of a mixture of salts, commonly MgSO₄ and NaCl since they have shown an improved extraction efficiency. A clean-up step such as a dispersive solid phase extraction (d-SPE) is sometimes applied, although many authors have ruled out this step since it reduces the recovery of some mycotoxins or does not produce significant improvements in the matrices effect (Koesukwiwat, Sanguankaew, & Leepipatpiboon, 2014). The effectivity of the QuEChERS extraction is affected by several factors, such as the amount of acid that is added or the relation between the amount of solvent and matrix. A buffered QuEChERS extraction employing potassium dihydrogen phosphate and acetonitrile with 5% formic acid was previously proposed to determine aflatoxins in plant-based beverages although low recoveries were achieved (Hamed, Abdel-Hamid, Gámiz-Gracia, García-Campana, & Arroyo-Manzanares, 2019). Another method using acetonitrile with 1% formic acid was applied to the analysis of 11 regulated mycotoxins in oat, soy and rice based products (Miró-Abella et al., 2017).

In order to develop a new extraction procedure applicable to regulated and emerging toxins, conditions previously used for cow milk analysis were chosen since the method achieved good results (González-

Jartín et al., 2021). In this sense, acetonitrile acidified with 0.5% formic acid was employed to extract 5 mL of plant-based beverages as described in Material and Methods. The extract was evaporated to dryness and redissolved in analysis solvent to reach a final extract 0, 2.5, 5, 10 and 20 times concentrated. In this way, the R_A was evaluated in five different conditions, with a different concentration of the final extract. As shown in Fig. S1, in general, when the concentration of the extract increases, the R_A % decreases, and therefore there is a suppression of the analytical signal caused by some compounds present in the matrix. However, rice and almond beverages show higher R_A % values when the sample was analysed 2.5 times concentrated. Therefore, there is an enhancement of the analytical signal in comparison with the not concentrate extract. In soybean and oat beverages, although there is an enhancement of the signal for some toxins such as AME or DAS, most of the analysed compounds do not show this effect. In the case of lipophilic toxins, namely BEA and ENNs the highest R_A % values were achieved when the extract was not concentrated especially in oat, rice, and almond beverages. For certain mycotoxins, such as T-2 triol the suppression is very pronounced in all matrices. For analysis, a five times concentrated sample was chosen as the compromise level which allows the detection of low mycotoxin levels, and, at the same time, the matrix effect is not too pronounced.

Next, it was studied whether the application of an additional clean-up step was effective in reducing the matrix effect. The adsorbent employed was C18, because is commonly employed in QuEChERS procedures (Zhao, Liu, Zhang, Zhou, & Yang, 2021). As shown in Fig. S2, in general, there is a suppression of the signal although for certain mycotoxins such as h-FB1 or ZEN an enhancement was observed. When the d-SPE was applied, in the case of almond beverages the differences between the extraction without and with clean-up steps were low. A reduction in the matrix effect was observed when the C18 was employed, especially for BEA and ENNs. For example, in the case of rice beverage, ENNB₁ gave an SSE value of 63% in comparison with a value of 8% without the clean-up step. On the contrary, the cleaned extract

yielded higher suppression rates for AFs, especially in the case of oat beverage. Therefore, due to the great relevance of aflatoxins and the need to have methods especially applicable to their detection the extraction without a clean-up step was chosen.

3.2. Method validation

The linearity was studied by using calibration curves constructed at nine levels of concentration. Linearities of the method were acceptable since most of the R values were higher than 0.997 in the studied concentration range (Table 2). In addition, it was checked that the back-calculated concentration was in the range of 80–120%.

LODs and LOQs were calculated following the guidance of the EU-RL by the measurement of ten solvents or blank samples (Wenzl et al., 2016). In the case of oat beverage, LODs and LOQs are indicated in Table 2, while data for rice, soy and almond beverages are indicated in Tables S3, S4 and S5, respectively. The LOQs in oat beverage were from 0.007 µg/L (AFG₂) to 10.7 µg/L (DON), in soy beverage were from 0.03 µg/L (AFB₂) to 29 µg/L (CTN), in almond beverage were from 0.003 µg/L (BEA and ENNB₁) to 1.1 µg/L (CTN) and in rice beverage were from 0.001 µg/L (AFG₁ and ENNB₁) to 2 µg/L (CTN). The obtained LOQs improve those previously reported in the literature for some mycotoxins. For instance, LOQs of 10 µg/L and 0.5 µg/L were reported for HT-2 and T-2 in oat beverage, while the LOQs achieved by the present method are 0.4 µg/L and 0.1 µg/L, respectively (Miró-Abella et al., 2017). Although the way that has been employed to calculate LOQ has been widely used in the bibliography, currently, the latest SANTE document establishes this level as the lowest one that has been validated by applying the complete analytical method (SANTE, 2021).

The matrix effect was calculated as the suppression or enhancement of the signal caused by the presence of coeluting compounds. This phenomenon can affect the accuracy of the method and therefore it is necessary to correct the data; for instance, using the SSE factor, internal standards, or matrix-matched calibration (Fabregat-Cabello, Zomer,

Table 2
Performance characteristics of the method in oat beverage. ^{a, b} Expressed as µg/L. ¹Relative Standard Deviation evaluated in terms of reproducibility (inter-day precision) calculated from the results of the extraction recovery obtained with the 3rd level of contamination.

Analyte	LOD ^a	LOQ ^b	r^2	SSE	Recovery %						
					Level 1	RSD _r %	Level 2	RSD _r %	Level 3	RSD _r %	RSD _R (%) ¹
15-AcDON	2.44	8.06	0.997	46	84	4	77	5	94	6	15
3-AcDON	0.11	0.37	0.995	46	70	19	84	12	79	6	10
AFB ₁	0.013	0.042	0.999	51	81	2	95	14	87	5	12
AFB ₂	0.008	0.028	0.998	54	85	4	79	8	92	2	13
AFG ₁	0.008	0.025	0.997	37	92	13	103	2	98	8	11
AFG ₂	0.002	0.007	0.995	35	84	10	85	4	84	6	13
FB ₁	0.59	1.94	0.999	143	80	9	83	9	86	10	15
FB ₂	0.06	0.21	0.997	121	93	3	98	8	88	14	20
HT-2	0.14	0.47	0.999	44	101	5	105	4	103	13	17
T-2	0.03	0.12	0.999	73	104	7	95	10	99	2	7
CTN	0.21	0.70	0.998	47	114	12	93	11	87	13	15
DON	3.24	10.68	0.998	21	127	20	136	6	100	17	19
OTA	0.02	0.06	0.999	98	106	4	98	3	101	4	6
ZEN	0.01	0.04	0.999	83	112	5	101	8	101	2	15
GLIO	0.40	1.32	0.999	112	122	7	118	9	98	8	18
MPA	0.03	0.11	0.999	106	101	5	103	6	94	2	17
AOH	0.10	0.32	0.999	68	110	2	99	6	102	7	11
AME	0.005	0.02	0.999	99	109	2	96	2	93	3	14
NEO	0.25	0.86	0.999	38	108	4	99	5	99	3	19
DAS	0.01	0.04	0.999	89	92	1	95	2	91	10	16
STC	0.002	0.009	0.999	80	93	0	100	1	110	1	22
T-2 TRIOL	0.028	0.09	0.999	27	108	4	83	5	74	8	13
ZOL	0.03	0.08	0.997	92	74	6	80	1	90	4	10
BEA	0.006	0.019	0.999	49	71	4	86	2	84	11	13
ENNA	0.004	0.015	0.999	103	88	4	89	3	87	6	22
ENNA ₁	0.007	0.023	0.999	57	80	3	75	6	92	15	19
ENNB	0.004	0.013	0.998	25	87	13	65	2	66	7	18
ENNB ₁	0.005	0.018	0.998	43	86	10	73	5	100	3	20
h-FB ₁	0.03	0.10	0.999	209	82	2	79	2	72	9	9

Sancho, Roig-Navaroo, & Mol, 2016). In this work, the SSE factor was calculated for all mycotoxins (Fig. 1). In general, signal suppression was observed, in the rice beverage the SSE factor vary from 11% for ENNB₁ to 125% for ZOL, in almond beverage from 33% for DON to 153% for h-FB₁, in oat beverage from 20% for DON to 209% for h-FB₁ and in soy beverage from 19% for T2 triol to 218% for h-FB₁. Therefore, a signal enhancement is usually observed for FB₁, FB₂, and h-FB₁. In the case of DON, signal suppression occurred in all matrices. These results were in agreement with other data previously reported in rice and soy beverages (Miró-Abella et al., 2017). The matrix effect for HT-2 is more pronounced in oat, rice, and soy beverages (SSE values from 44% to 58%) than in almond (SSE of 85%). Previously, a high matrix effect was also observed for HT-2 and DON, with a signal suppression of 39% was observed in oat, soy, rice, and bird seed beverage (Hamed, Arroyo-Manzanares, García-Campaña, & Gámiz-Gracia, 2017).

Next, the accuracy of the method was studied through of recovery at three concentration levels (Table S2). Recoveries are indicated in Fig. 2 and Table 3, S3, S4, and S5. Recoveries vary according to the matrix and the level of contamination, but, in general, values higher than 80% were achieved (Fig. 2), although there are some exceptions as HT-2 and T-2 triol in soy beverage, with values of 45% and 48%, respectively; and CTN in rice beverage with a 21% of recovery. In the case of AFs, values higher than 65% were obtained for all matrices in the three levels of contamination (0.02, 0.09 and 0.19 µg/L). In literature, good recoveries have also been obtained using a method based on a dispersive liquid-liquid microextraction followed by high-performance liquid chromatography with fluorescence detection (DLLME-HPLC-FL) (Hamed, Abdel-Hamid, Gámiz-Gracia, García-Campaña, & Arroyo-Manzanares, 2019). In the case of lipophilic toxins, BEA and ENNs, the recoveries were satisfactory (64%–107%) when samples were spiked at concentrations between 0.6 µg/L and 2.5 µg/L. A salting-out assisted liquid-liquid extraction (SALLE) was applied to extract BEA and ENNs of oat, rice and soy beverages, the obtained recoveries varied from 84% to 97% when samples were spiked at 1, 10 and 50 µg/L (Arroyo-Manzanares, Hamed, García-Campaña, & Gámiz-Gracia, 2019). Similarly, a method

to determinate *Fusarium* toxins in plant-based beverages was previously proposed showing recoveries around 80% in two levels of contamination of up to 300 µg/L (Hamed, Arroyo-Manzanares, García-Campaña, & Gámiz-Gracia, 2017). The proposed method also achieves good recoveries for these toxins and their modified forms at much lower contamination levels, more similar to the levels at which the toxins are present in samples. As described above, precision was studied through of RSD of recoveries obtained by triplicate in the same day and in three days consecutives. Intra-day and inter-day precision was high, with RSD values lower than 20% in both cases.

Levels of contamination used for the study of the recovery and their RSD were lower than those used in previously published methods, but still, a high accuracy and precision were obtained. In this sense, the method meets the performance criteria of the Commission Regulation (EC) N° 401/2006. Therefore, although the proposed method greatly broadens the target toxins, it maintains adequate performance characteristic.

3.3. Application to real samples

As mentioned before, previously available methods were intended to the analysis of specific groups of toxins and allow the detection of a small number of compounds. Consequently, in order to know the impact of mycotoxins in plant-based beverages, first a new method applicable to the analysis of several mycotoxins was validated and next it was applied to the analysis of 38 plant-based beverages including 9 of soy, 6 of rice, 16 of oat and 7 of almond. Samples were collected in the local market and belong to different brands or batches. The results are indicated in the Table 3, a high incidence of emerging mycotoxins was found, especially for BEA and ENNs. These results are in concordance with previously reported data (Arroyo-Manzanares, Hamed, García-Campaña, & Gámiz-Gracia, 2019). Similarly, the AME was found in the four varieties of the plant-based beverages, the STC was detected in rice, soy and oat, while MPA was found in almond, soy and oat. Regarding regulated toxins, aflatoxins were mainly detected in almond and rice,

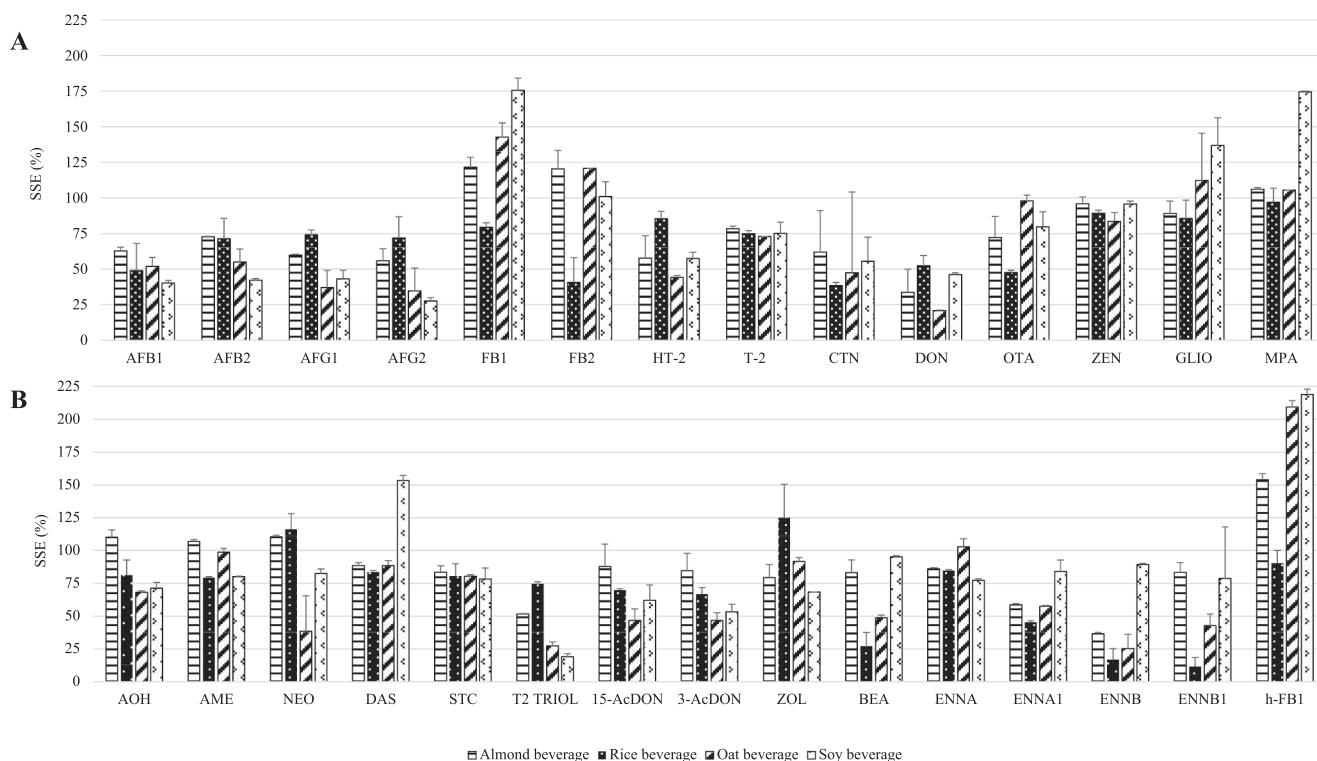


Fig. 1. Matrix effect of the analysis method. SSE value of oat, rice, soy, and almond beverages obtained for regulated mycotoxins (A) and emerging/modified mycotoxins (B).

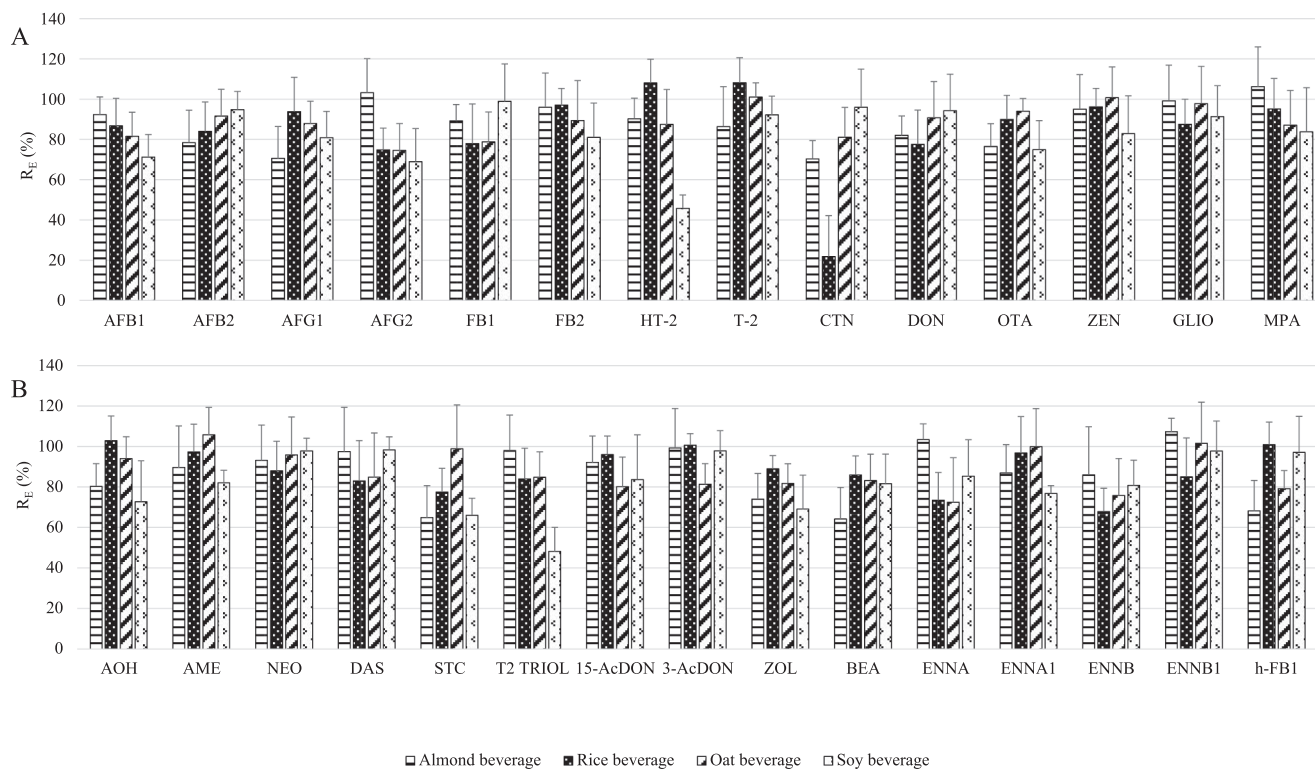


Fig. 2. Accuracy of the analysis method. R_E value of oat, rice, soy, and almond beverages obtained for regulated mycotoxins (A) and emerging/modified mycotoxins (B).

Table 3

Analysis of commercial samples. Percentage of positive samples (%), mean concentration and range of mycotoxins expressed as $\mu\text{g/L}$.

Toxin	Almond		Oat		Rice		Soy	
	%	Mean (range) $\mu\text{g/L}$	%	Mean (range) $\mu\text{g/L}$	%	Mean (range) $\mu\text{g/L}$	%	Mean (range) $\mu\text{g/L}$
AFB ₁	28	0.03 (<LOQ – 0.04)	0		33	0.05 (<LOQ – 0.09)	11	0.05
AFB ₂	0		0		33	<LOQ	0	
AME	100	0.3 (<LOQ – 0.32)	87	0.22 (<LOQ – 0.31)	100	0.28 (0.17–0.33)	44	0.33 (0.29–0.37)
BEA	100	0.36 (0.05–2)	94	0.08 (0.03–0.5)	83	0.18 (0.08–0.33)	66	0.39 (<LOQ – 2.47)
ENNA	85	0.08 (<LOQ – 0.1)	100	0.07 (0.03–0.14)	66	0.05 (0.03–0.07)	11	0.19
ENNA ₁	85	0.14 (<LOQ – 0.2)	81	0.09 (<LOQ – 0.2)	66	0.07 (<LOQ – 0.1)	22	0.19 (0.12–0.23)
ENNB	100	0.16 (0.07–0.3)	100	0.07 (0.01–0.22)	66	0.08 (<LOQ – 0.2)	11	0.50
ENNB ₁	100	0.08 (0.04–0.12)	100	0.1 (<LOQ – 0.34)	83	0.22 (<LOQ – 0.3)	11	0.29
FB ₁	42	0.8 (0.72–0.84)	6	5.99	0		0	
FB ₂	42	1 (0.75–1.3)	6	0.72	0		0	
HT-2	0		37	0.65 (<LOQ – 0.9)	0		0	
T2	0		50	0.6 (<LOQ – 0.7)	0		0	
MPA	28	0.2 (<LOQ – 0.27)	25	0.2 (<LOQ – 0.22)	0		22	0.12 (0.11–0.13)
OTA	14	0.186 (<LOQ – 0.186)	6	0.74	0		0	
STC	0		43	0.11 (<LOQ – 0.125)	100	0.19 (<LOQ – 0.25)	66	0.16 (<LOQ – 0.19)

FBs were in almond while HT-2 and T-2 toxins were present in oat samples.

In the case of oat beverages, AME, BEA, ENNAs, FBs, HT-2, T-2, MPA, OTA and STC were found. With respect to T-2, it was identified in 7 samples with a concentration ranging from 0.55 to 0.74 $\mu\text{g/L}$, while the HT-2 was found in 9 samples (concentration ranging from 0.49 to 0.87 $\mu\text{g/L}$). These mycotoxins were only detected in this matrix. In addition, there are previous reports on the presence of these type-A trichothecenes in oat beverage (Miró-Abella et al., 2017). T-2 and HT-2 usually contaminate oat crops, and therefore an important carry-over is observed (Edwards, Barrier-Guillot, Clasen, Hietaniemi, & Petterson, 2009; Janavičienė, Mankevičienė, Kochiieru, & Venslovas, 2022). In this matrix, the OTA was detected in one sample at 0.741 $\mu\text{g/L}$, which is close to the maximum amount of this toxin allowed in some products like wine (EC_2023/915, 2023).

In the rice beverage, AFB₁ was detected in five samples in a concentration that ranged from the LOQ to 0.09 $\mu\text{g/L}$, STC, an aflatoxin biosynthetic precursor, was detected and in all samples (Anjorin, Ariyo, Peter, Sulyok, & Krska, 2021). In the case of soy beverage, the AFB₁ was detected in one sample at 0.06 $\mu\text{g/L}$, the emerging compounds NEO and MPA were also detected at trace levels. These results are in agreement with previous data that reported the contamination of soy beverage with AFB₁ in the range of 1.6 to 3.9 ng/L (Rezaeefar, Nemati, Farajzadeh, Afshar Mogaddam, & Lotfipour, 2022). Regarding the almond beverage, OTA was present in three samples, but in only one with concentration above the LOQ. AFB₁ was found in five samples, in a concentration lower than 0.04 $\mu\text{g/L}$ and only one sample was contaminated with AFB₂ with levels below the LOQ. In this case, there can also happened a carry-over from raw materials since almonds are usually contaminated with aflatoxins. The observed levels are close to the maximum amount of

aflatoxin M₁ allowed in milk (EC_2023/915, 2023). Therefore, the obtained data revealed that soy beverage is the matrix with the lowest levels of contamination, and, in order to avoid risks to consumers, AFB₁ should be monitored in rice and almond beverage, and T-2 and HT-2 toxin in oats.

4. Conclusions

A new method based on a QuEChERS extraction procedure followed by UHPLC-MS/MS detection was developed for the simultaneous analysis of 29 mycotoxins in plant-based beverages. The method was validated for the determination of mycotoxins in oat, rice, soy and almond beverages, and shows good performance characteristics including linearity, repeatability, accuracy, and precision. This study revealed that plant-based beverages are susceptible to mycotoxin contamination, especially beauvericin and enniatins, T-2 and HT-2 were found in oat beverage samples, and aflatoxins in rice and almond. Therefore, further investigation is needed to provide incidence data and evaluate the risk for consumers.

CRedit authorship contribution statement

Inés Rodríguez-Cañás: Investigation. **Jesús M. González-Jartín:** Investigation, Writing – original draft, Writing – review & editing. **Amparo Alfonso:** Methodology, Writing – review & editing. **Rebeca Alvaríño:** Investigation. **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.2023.137427>.

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