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Simultaneous determination of aflatoxins B1, B2, G1 and G2 in commercial rices using immunoaffinity column clean-up and HPLC-MS/MS

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ABSTRACT

Rice is frequently contaminated with aflatoxins, that are highly toxic fungal substances and strongly involved on hepatic cancer. In this work, different extraction and clean-up methods were evaluated for the simultaneous extraction and clean-up of aflatoxins B1, B2, G1 and G2 from rice. Favourable results were obtained by using methanol – water (80:20, v/v) extraction followed by immunoaffinity columns for clean-up, with recoveries of 86–92%, standard deviations between 5 and 11%, LOD ranged between 0.09 and 0.32 μg/kg, and LOQ between 0.31 and 1.06 μg/kg. Method validation and sample analysis were performed by using HPLC-MS/MS. Nine rice samples from different origin, varieties and specific characteristics, acquired in Spanish supermarkets were analysed. In two basmati samples from the same batch aflatoxin B1 was detected at (1.62 ± 0.08) μg/kg and (0.77 ± 0.03) μg/kg, both lower than the levels established by European Regulation for aflatoxin B1 in cereals.

1. Introduction

Mycotoxins are secondary metabolites produced by several phytopathogenic fungi. This family of compounds, which are produced by different fungi, includes a wide variety of low molecular weight organic molecules, with a great variety of chemical structures, toxicokinetic properties and biosynthetic origins (Kolawole, Meneely, Petchkongkaew, & Elliott, 2021). Among the main fungal producers of mycotoxins, *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* genus are the most common ones. These fungi are ubiquitous in the environment, and they usually colonize most crops during their growth and postharvest storage (Carvajal, 2013). It is possible that the same fungal specie could produce different types of mycotoxins, or that different fungal species could produce the same mycotoxin (Arce-López, Lizarraga, Vettorazzi, & González-Peñas, 2020). Nevertheless, the fungal presence and mycotoxin synthesis depends on different environmental factors such as temperature and relative humidity, land and crop properties, and the presence of other microorganisms and insects, because fungus colonization of crops does not imply the synthesis of mycotoxins, except in case that the previous factors reach specific balances (Pulina et al., 2014).

Diet is the main route of human exposition to mycotoxins and less frequent, inhalation and cutaneous contact with contaminated crops or surfaces with fungal mycotoxin producers (Waseem, Shah, Sajjad, Siddiqi, & Nafees, 2015). At present, up to four hundred different mycotoxins are known (Zhao, Chen, Shen, & Qu, 2017). Among them, attention focuses on those that produce relevant toxicities for animal and human health, as aflatoxins, fumonisins 1 and 2, zearalenone, deoxynivalenol, nivalenol, T-2 and HT-2 toxins, ocratoxin, patulin, citrinine and ergotic alcaloids (Spanish Agency for Consumption, n.d.).

Aflatoxins B1, B2, G1 and G2 are among the most toxic mycotoxins (Benkerroum, 2020). They are produced by *Aspergillus flavus*, *parasiticus* and *nomius* species under tropical and semi-arid tropical conditions (Kumar, Mahato, Kamle, Mohanta, & Kang, 2017). They contaminate a wide variety of foodstuffs such as cereals, nuts, vegetables, and spices, among others. In addition, aflatoxins M1 and M2 are aflatoxins from animal origin which are produced by animal metabolism from B and G original aflatoxins, respectively and are common in animal products (dairy products, meat, and eggs), according to Rushing & Selim, 2019. Moreover, mycotoxin cross-linked contamination using tools, surfaces or places that have been previously in contact with contaminated crops or foodstuffs is also frequent. Aflatoxins thermal stability avoids their destruction during typical food processing and cooking thermal treatments (Carvajal, 2013).

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Aflatoxins have acquired a great relevance because of their high toxicity (toxicity order is Aflatoxin B1 > Aflatoxin G1 > Aflatoxin B2 > Aflatoxin G2), that includes a broad range of acute and chronical effects over animal and human health including carcinogenicity, teratogenicity, hepatotoxicity, nephrotoxicity, endocrine disorders, immunosuppression, metabolic disorders, allergic reactions, reproductive deficiencies, fetal alterations; and, finally, death (Benkerroum, 2020). Carcinogenic effect of aflatoxins (especially for aflatoxin B1) is so powerful that they were classified as Group I carcinogenic agents by International Agency for Research on Cancer (IARC, 1993). Implication of aflatoxins in cancer development is especially frequent at hepatic level because this organ is responsible for the metabolization and detoxification of mycotoxins, among other toxins (Rushing & Selim, 2019). This process is carried out by the hepatic enzymes of the Cytochrome P450 Complex, that metabolize the original aflatoxins B1, B2, G1 and G2 into less toxic substances that are more easily excreted from the organism. However, some generated intermediates are reactive oxygen species (ROS) with high oxidant capacity. These reactive species, apart from alter cellular redox balance, can linked to proteins and deoxy guanosines from cellular DNA. This binding DNA-Adduct generates mutations in proliferative genes that alter cellular cycle, and, consequently, health hepatic cells turn into cancer cells (Marchese et al., 2018).

Cereals are among the most susceptible crops to aflatoxigenic fungi contamination. It is estimated that 25% of world cereal crops are contaminated by mycotoxins, and this fact is of special relevance for rice, as it is the second cereal more consumed worldwide (FAO, 2004). Rice demands large amounts of water for growing up, and this factor, in addition to warm temperatures and poor processing and storage conditions, makes this cereal especially susceptible to be colonized by aflatoxigenic fungi (Kumar et al., 2017). Although rice is processed by washing, peeling, drying and other specific physiochemical treatments before being commercialized, these treatments could not be effective enough to completely remove aflatoxins. Moreover, there are additional risks by under-processing in brown rice (this variety of rice is not peeled to keep the nutritional and digestive benefits of germ and bran that integrate external layers, according to Shen & Singh, 2021). Other risk factor could be the new agroecological techniques which avoid the use of pesticides. These agronomic trends could promote aflatoxigenic fungi colonization with a major risk of aflatoxin contamination over rice, in absence of methods for fungal control (Habschied et al., 2021).

The high risks of aflatoxins for animal and human health added to their prevalence on cereals have promoted the establishment of different legislation to limit aflatoxins content in cereals, among other susceptible foodstuff, for animal and human consumption. One of the most restrictive legislations is Commission Regulation (EC) No 1881/2006, that establishes a maximum limit of aflatoxin B1 at 2 μg/kg in processed cereals for human consumption, and a maximum limit of the sum of aflatoxins B1, B2, G1 and G2 at 4 μg/kg. This Regulation affects to all cereals that are consumed in the European Union countries, regardless of the cultivation origin. However, European Community legislation has not established any preferent method for aflatoxins determination in foodstuffs yet. Traditional methods for aflatoxins extraction include simple extraction with different organic and polar solvent combinations, and extraction combined with Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) salts method. QuEChERS method is particularly effective for mycotoxins extraction (Perestrelo et al., 2019). Nevertheless, this method usually sweeps along much matrix interferences, so a clean-up method to minimize matrix residues present after extraction is usually needed. Among the most typical techniques for cleaning up, are QuEChERS salts with solid phase extraction (SPE) cartridges (Krska, Welzig, Berthiller, Molinelli, & Mizaikoff, 2005). However, typical SPE cartridges are not capable enough to retain only aflatoxins, because they have an insufficiently selective composition. This limitation, added with a poor clean-up capacity of QuEChERS sorbents depending on the food matrix, has increased interest in the use of immunoaffinity columns (IAC) (Scott, Banting, & Trucksess, 1997). These columns contain specific antibodies that retain only aflatoxins B1, B2, G1 and G2 simultaneously, and allow a more exhaustive cleaning of the extracts, providing high aflatoxin recoveries, and these advantages make IAC method a promising alternative for aflatoxins clean-up (Şenyuva & Gilbert, 2010) (Krska et al., 2005).

The objective of this study is to analyse aflatoxins B1, B2, G1 and G2 in rice samples from different varieties, origins, processing by different treatments and growing with organic and no organic techniques. For getting this purpose, a method for extraction, clean-up and simultaneously analysis of these aflatoxins in rice, by employing IACs and HPLC-MS/MS has been optimized. This work contributes to increase the global knowledge about mycotoxin contamination on basic foodstuffs from the common diet and to confirm if a representative selection of cereals commercialized in Spanish supermarkets complies with the restrictive limits established by European Regulation (i.e. 2 μg/kg for each aflatoxin). Studies about the presence of aflatoxins in cereals obtained using new organic culture techniques are scarce, so this study provides new data that might help to confirm if new crop techniques without pesticides could involve additional risks of aflatoxin presence in rice.

2. Materials and methods

2.1. Chemicals and materials

Ultrapure water was obtained by using a Milli-Q Millipore system (Bedford, MA, USA). Acetonitrile (ACN) LC-MS grade used for chromatography was purchased from Fisher Scientific (Fair Lawn, NJ, USA); ACN and methanol (MeOH) supergradient grade for extractions and formic acid (HCOOH) for HPLC-MS/MS were acquired from Scharlab (Barcelona, Spain). QuEChERS salt combinations selected for initial extraction assays (4 g magnesium sulphate (Mg_2SO_4), 1 g sodium cloride (NaCl), 1 g sodium citrate ($Na_3C_6H_5O_7$) and 0.5 g sodium hydrogen citrate hexahydrate ($Na_2HC_6H_5O_7{\cdot}6H_2O$), were purchased from Scharlab (QUEXTENAK1 extraction kit reference). Solid phase extraction (SPE) cartridges Extrabond EBH (60 mg, 3 mL), Extrabond EB2 (200 mg, 6 mL) and Extrabond EAX (200 mg, 6 mL) were obtained from Scharlab; Oasis MCX (60 mg, 3 mL) and Oasis MAX (150 mg, 6 mL) cartridges were from Waters (Massachusetts, USA) and Afla-Clean IAC were supplied from LCtech GmbH (Obertaufkirchen, Germany). Filters of polyvinylidene fluorure (PVDF), and glass microfiber filters (GMF/A) with a 1.6 μm pore size, were provided by Scharlab. Buffer salts disodium hidrogenphosphate dodecahydrate ($Na_2HPO_4{\cdot}12H_2O$), sodium dihydrogenphosphate monohydrate (NaH_2PO_4) and sodium cloride (NaCl) for PBS preparation were acquired from Panreac (Barcelona, Spain). Aflatoxins analytical solid standards were purchased from Sigma Aldrich (St. Luis, Missouri, USA): (3*S*,7*R*)-11-methoxy-6,8,19-trioxapentacyclo[10.7.0.0^{2,9}.0^{3,7}.0^{13,17}]-nonadeca-1,4,9,11,13(17)-pentaene-16,18-dione (Aflatoxin B1, ≥97.0%), (3*S*,7*R*)-11-methoxy-6,8,19-trioxapentacyclo[10.7.0.0^{2,9}.0^{3,7}.0^{13,17}]nonadeca-1,9,11,13(17)-tetraene-16,18-dione (Aflatoxin B2, ≥98%), 11-methoxy-6,8,16,20-tetraoxapentacyclo-[10.8.0.0^{2,9}.0^{3,7}.0^{13,18}]icosa-1,4,9,11,13(18)-pentaene-17,19-dione (Aflatoxin G1, ≥98%) and (3*S*,7*R*)-11-methoxy-6,8,16,20-tetraoxapentacyclo[10.8.0.0^{2,9}.0^{3,7}.0^{13,18}]icosa-1,9,11,13(18)-tetraene-17,19-dione (Aflatoxin G2, ≥98%).

Individual stock standard solutions were prepared by dissolving 1 mg in ACN, up to a final concentration of 100 mg/L. The individual stock solutions were used for preparing a working solution containing a mixture of the four aflatoxins at 25 mg/L in ACN. All the standards were stored at −20 °C until their use to prevent their degradation.

Phosphate buffer saline work solution (1X PBS) was prepared by fivefold dilution with water of a solution containing $Na_2HPO_4{\cdot}12H_2O$ (140.06 mmol/L), $NaH_2PO_4{\cdot}1H_2O$ (70.01 mmol/L) and NaCl (727.24 mmol/L), with a final pH value of 7.2.

Table 1
Retention times, precursor and product ions, and collision energies selected for HPLC-MS/MS analysis.

Compound	Retention Time, min	Precursor Ion, m/z $[M + H]^+$	Quantification Transition	Confirmation Transitions	
			Product Ion I, m/z (CE, V)	Product Ion II, m/z (CE, V)	Product Ion III, m/z (CE, V)
Aflatoxin B1	5.49	313.1	285.1 (−25)	241.1 (−38)	213.1 (−46)
Aflatoxin B2	5.31	315.2	287.1 (−26)	259.0 (−30)	243.0 (−40)
Aflatoxin G1	5.26	329.0	243.1 (−29)	200.0 (−41)	311.1 (−23)
Aflatoxin G2	5.06	331.0	313.1 (−26)	245.0 (−30)	189.0 (−45)

2.2. Rice samples

Rice samples were obtained from several supermarkets of Madrid (Spain), in accordance with a previous selection that considers the current consumption trends of this cereal (variety, origin, culture technique and industrial processing). The following types of rice were selected: round rice (from Spain), brown round rice (Spain), organic round rice (Spain), organic long rice (EU), organic brown long rice (EU), basmati rice (Pakistan), Thai rice (Thailand) and organic brown basmati rice (India). All the samples were completely grinded and homogenized with a blender jar and were kept in darkness at room temperature until the analysis.

2.3. Instrumentation

Development and method optimization was carried out using a capillary Liquid Chromatograph coupled to diode array and mass spectrometer detectors (cHPLC-DAD/MS) Model 1100 Series, (Agilent Technologies, Madrid, Spain). This chromatographical system consists of a G1376A binary capillary pump, a G1379A degasser and an external stainless-steel loop with a volume injection of 10 μL placed into a Rheodyne injection valve with six channels. Reversed-phase separation of aflatoxins was carried out on a Synergy^{TM} Fusion C18 (150 mm × 0.3 mm, ID 4 μm, pore size 80 Å) analytical column (Phenomenex, Torrance, CA, USA). Volume sample for injection was 10 μL and flow rate was set at 10 μL/min. Mobile phase consisted of ultrapure water with HCOOH 0.05% (eluent A) and ACN (eluent B). The percentage of B changed as follows: 0 min, 30%; 3 min, 60%; 7.5 min, 60%; 11 min, 30%, and the last condition was kept during 4 min to rebalance the column.

A G1315B diode array detector (Agilent Technologies, Madrid, Spain) with 500 nL and 10 mm pathlength coupled to a single quadrupole mass spectrometer with an electrospray ionization source (Model 6120 Series, Agilent Technologies) were used. Positive ESI mode was chosen for instrument performance, capillary voltage was set at 3000 V and analyte ionization voltage, 150 eV. Nitrogen was employed as nebulizing gas at 35 psig with a drying flow of 12 L/min at 350 °C. For data acquisition and processing, Agilent ChemStation software for Windows (Agilent Technologies) was employed.

The wavelengths measured (360 nm for quantification and 375 nm for confirmation) were previously selected using a diode array G6860A UV/Vis spectrophotometer (Agilent Technologies), equipped with the HP Chemstation software.

Confirmatory analyses by HPLC-MS/MS were performed in a triple quadrupole mass spectrometer system (LC-MS-8030, Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with a Nexera LC-30AD solvent delivery unit, a Prominence DGU-20A5 degasser, a Nexera SIL-30AC autosampler with temperature-controlled tray and a Prominence CTO-20AC column oven. Chromatographic separation was carried out using a Synergy^{TM} Fusion C18 column (150 mm × 3 mm, ID 4 μm, pore size 80 Å) from Phenomenex, Torrance, CA, USA. The injection volume onto the column was 20 μL, the flow rate was 0.3 mL/min and the elution gradient was the same than the used in in cHPLC-DAD/MS system.

Analysis were done in positive electrospray ionization mode using the Multiple Reaction Monitoring (MRM) mode with a dwell time of 100 msec for all the transitions. ESI ionization voltage was set at 4.5 kV, detector voltage at 2.18 kV, and current interface was established at 2.1 μA. Nitrogen was applied as nebulizing gas at 1.5 L/min and as drying gas at 15 L/min. Desolvation line temperature was established at 250 °C and heat block temperature at 400 °C. Argon was used for collision-induced dissociation (CID) at 230 kPa in the collision cell. LabSolutions LCMS software (Shimadzu) was employed for data acquisition and processing. Selected transitions and collision energies (CE) for aflatoxins are detailed on Table 1.

2.4. Optimization parameters for aflatoxins extraction and clean-up using QuEChERS and SPE cartridges

The optimization of extraction and clean-up was carried out only for aflatoxin B1, as the four aflatoxins have a very similar structure (Fig. 1) and therefore, they show a very similar physiochemical behaviour. Therefore, it is expected that the four aflatoxins will show a similar behaviour during the extraction and clean-up steps (Carvajal, 2013).

2.4.1. Optimization of extraction conditions

The effect of the composition of the extraction solvent, the presence of QuEChERS salts after the extraction, the extraction times and the extraction technique were tested. In all the experiments performed, 5 g of ground rice (Spain) in triplicate were taken and then, the samples were spiked with aflatoxin B1 at 100 μg/L and incubated in darkness during 1 h. In the first assay, samples were extracted with different composition solvents: pure ACN, pure MeOH, ultrapure H_2O, ACN-ultrapure water (80:20, v/v), and MeOH-ultrapure water (80:20, v/v). The mixture ACN-ultrapure water (80:20, v/v) was the most effective for extraction, so it was selected to test the other extraction factors. In the second experiment, extractive QuEChERS addition was tested. In the third assay, samples were extracted by using different extraction techniques: magnetic stirrer (950 rpm) during 30 min and 1 h, and two different shaking techniques, magnetic stirrer at 950 rpm and orbital shaker at 950 U/min (both during 1 h). Finally, all the extracts were centrifugated at 5000 rpm during 15 min, 1 mL supernatant aliquot was evaporated to dryness by vacuum pressure, reconstituted in 1 mL of ultrapure water and transferred into glass vials for cHPLC-DAD/MS analysis.

2.4.2. Optimization of clean-up conditions

After the optimization of the extraction method, several clean-up steps were evaluated. For this purpose, firstly two filters were tested (PVDF and GMF/A) to evaluate if aflatoxin B1 was retained on them. For this assay an aqueous solution of aflatoxin B1 at 100 μg/L was employed. Moreover, both filters were tested again using solution of MeOH at the same concentration to compare the loss with regard the solvent nature.

Five SPE cartridges were also tested: two of them were polymeric (Extrabond EBH and Extrabond EB2); another one was a mixed mode cationic exchange (MCX); and other two, mixed mode anionic exchange (MAX and EAX). 3 mL of a 100 μg/L aflatoxin B1 aqueous solution were passed through the cartridges (evenly conditioned) and, after drying under vacuum, aflatoxin B1 was eluted with ACN. Finally, aflatoxin loss after reconstitution of the final evaporated extracts was also studied. For this assay, stock solutions of aflatoxin B1 at 100 μg/L were evaporated at dryness under a gentle nitrogen stream and reconstituted with ultrapure water and ultrapure water with different ACN or MeOH organic

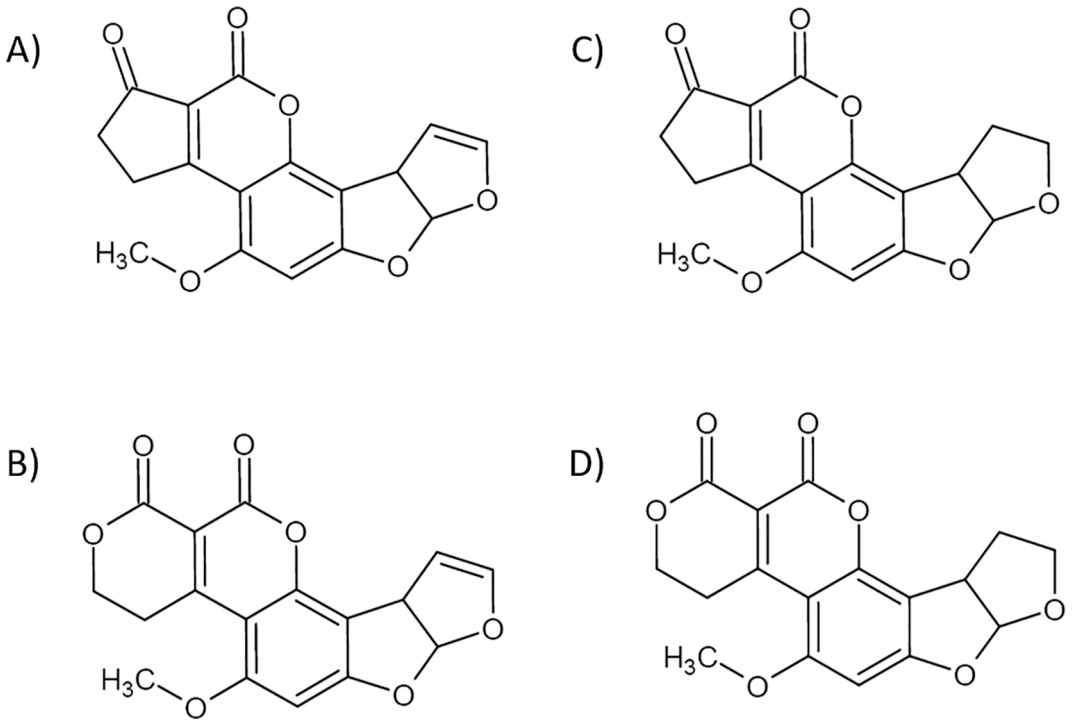

Fig. 1. Molecular structures of aflatoxin B1 (A), aflatoxin G1 (B), aflatoxin B2 (C) and aflatoxin G2 (D).

percentages: 10%, 20% and 30% of each type of organic solvent. Then, signals of reconstituted samples were compared with an aqueous stock solution, not evaporated, at the same concentration.

2.5. Optimization and determination of aflatoxins in rice by using immunoaffinity column (IAC) clean-up and HPLC-MS/MS

Different organic solvents for aflatoxins extraction prior to the IAC clean-up were tested: MeOH-ultrapure water (80:20, v/v), which is the recommended extraction mixture by the IAC manufacturer (LCtech IAC Data Sheets and Extraction Protocols, n.d.), and ACN-ultrapure water (80:20, v/v), which was the most effective mixture according to the extraction tests previously mentioned. Once determined the best extractant, the following protocol was applied to determine aflatoxins in rice. 20 g of grinded rice samples were spiked at 1.43 μg/kg and were kept during 1 h in darkness before the extraction. Then, 100 mL of MeOH-ultrapure water (80:20, v/v) were added and the extract was stirred at 950 rpm by magnetic stirring during 1 h in darkness. Then the solution was kept at rest during 5 min to induce rice sedimentation. Liquid supernatant was taken and centrifugated at 5000 rpm during 45 min. 7 mL aliquot from the supernatant was diluted in 43 mL PBS 1X and filtered through a PVDF syringe filter. IACs were conditioned with 3 mL PBS and then a 5 mL aliquot of the diluted filtered extract was charged onto the column. The solution was passed drop by drop by gravity. The columns were washed with 20 mL of ultrapure water by applying a slight vacuum pressure and completely dried under vacuum. Then, 2 mL of MeOH were charged onto the column and kept in contact with the matrix column during 10 min to induce antibodies denaturation. Finally, aflatoxins were eluted drop by drop from the cartridge, organic solvent was evaporated to dryness by nitrogen stream and extracts were reconstituted in 0.1 mL of ultrapure water for the analysis with HPLC-MS/MS.

2.6. Validation of IAC clean-up and HPLC-MS/MS methodology

Precision and accuracy were evaluated from recovery experiments of 20 g of blank rice samples spiked at 1.43 μg/kg before loading the sample in the IAC. For each aflatoxin, its area was compared to area of a blank rice extract that was spiked after passing through the IAC at the same concentration as follows:

$$\%Recovery = \frac{Peak\ area\ of\ Spiked\ Sample}{Peak\ area\ of\ Purified\ Spiked\ Extract} \times 100 \quad (1)$$

The HPLC-MS/MS method was evaluated in terms of linearity, detection limit (LOD) and quantification limit (LOQ). Linear range was established in a matrix-matched calibration curve for each aflatoxin by least squares linear regression representation, that was made by using peak areas of samples with respect to the known analyte concentrations, and results were evaluated in terms of slope and squared correlation coefficient (R2). Peak areas of real spiked samples were interpolated on matrix calibration line to get their real concentration. The LOD and LOQ were estimated considering the concentration of the lowest point of the matrix matched calibration curve of each aflatoxin giving a signal to noise (S/N) ratio of 3 and of 10, respectively.

3. Results and discussion

3.1. Optimization of extractive and clean-up conditions

With the purpose of selecting the optimal extraction and clean-up conditions for aflatoxins from rice samples, the following factors were compared: 1) extractive efficiency of different solvents and mixtures, QuEChERS presence during the extraction and other extraction parameters; and 2) aflatoxin losses caused by different factors involved in the cleaning up of the extracts.

3.1.1. Effect of extractive solvents, QuEChERS and other extraction conditions on aflatoxin recovery

Firstly, several factors involved in aflatoxin extraction were evaluated to establish the best extraction conditions.

3.1.1.1. Extraction solvent

Extractive efficiency using the following miscible solvent combinations were evaluated: (a) ACN; (b) MeOH; (c) ultrapure water; (d) ACN-ultrapure water (80:20, v/v); (e) MeOH-ultrapure water (80:20, v/v).

Each solvent combination was added over the rice samples previously spiked with aflatoxin B1. Recoveries were calculated using equation (1) and they were as follows: 76% (d) > 69% (b) > 66% (a) > 48% (e) > 20% (c). Recoveries are low in general, with important differences between solvents, because the lowest recovery for ultrapure water is below 20% and the highest recovery for ACN-ultrapure water (80:20, v/v) is about 76%.

3.1.1.2. Presence of QuEChERS salts after aflatoxin extraction

Then, we tested if the addition of QuEChERS salts could improve aflatoxin B1 extraction in combination with the mixture ACN-ultrapure water (80:20, v/v). QuEChERS with magnesium sulphate, sodium cloride, sodium citrate and sodium hydrogen citrate sesquihydrate were selected because of the predominant use of magnesium sulphate and sodium cloride for aflatoxin extraction from cereals, according with studies cited in Perestrelo et al., 2019. However, in our study, the extractive efficiency of aflatoxin B1 from rice using QuEChERS was about 56%, around 20% lower compared with their absence using the same combination solvent, ACN-ultrapure water (80:20, v/v).

3.1.1.3. Other extraction parameters

Finally, extraction times (30 min versus 1 h) and extraction techniques (magnetic stirrer at 950 rpm versus orbital shaker at 950 U/min) were compared. In all conditions tested, recoveries over 100% were obtained. Therefore, we concluded that aflatoxins do not suffer losses under these extraction conditions.

3.1.2. Effect of different clean-up protocols on aflatoxin recovery.

Secondly, different factors involved in aflatoxin clean-up at different steps were evaluated.

3.1.2.1. Filters

A comparison between PVDF (0.22 μm pore size) and GMF/A (1.6 μm pore size) filters, that were selected because they were recommended by LCtech IAC manufacturer for aflatoxins clean-up (LCtech IAC Data Sheets and Extraction Protocols, n.d.) gave the following recoveries: 92% (PVDF) and 98% (GMF/A) using an aqueous aflatoxin solution and 100% recovery for both filters using an organic aflatoxin solution. Considering these results, both filters allow aflatoxin B1 recoveries above 90%. Although the best recovery using aqueous solutions were obtained for GMF/A filters, PVDF filter was selected because of its best suitability for handling small sample volumes; this filter is designed to filter small sample volumes by coupling to syringe, while GMF/A is specifically designed to filter large sample volumes by a vacuum system.

3.1.2.2. Solid phase extraction cartridges

Then, we tested the extraction efficiency of different SPE cartridges: Two polymeric cartridges which can be used for a wide range of target compounds with quite distinct polarities; two anion exchange cartridges, suitable for both anionic and nonpolar compounds; and a cation exchange cartridge, suitable for compounds with cationic groups and nonpolar compounds. For this purpose, 3 mL of a 100 μg/L aflatoxin B1 aqueous solution was charged onto each SPE cartridge and eluted with pure ACN, giving the following recoveries: EBH (79%) and EB2 (72%); MAX (99%) and EAX (69%); and MCX (84%). Generic nature of traditional tested SPE cartridges prevents an exclusive retention of aflatoxins, and this fact causes undesirable retention of matrix residues from the extract according to Wells, 2013. To improve selectivity of the clean-up procedure, immunoaffinity columns (IAC) were evaluated. IAC retain only aflatoxins B1, B2, G1 and G2 and this exclusivity prevents undesirable interferents retention. IAC yielded recoveries of 86% for aflatoxin B1; 90% for aflatoxin B2; 87% for aflatoxin G1; and 92% for aflatoxin G2 in spiked rice samples (see section 2.6). These satisfactory results are in accordance with Sugita-Konishi et al., 2006 and Bradburn, Coker, & Blunden, 1995. So, IAC cartridges were selected for the clean-up of rice samples.

3.1.2.3. Evaporation and reconstitution of purified extracts

Evaluation of aflatoxin recoveries after the evaporation to dryness of the ACN extract and reconstitution yielded 100% recovery using pure water. However, recoveries got worse when the percentage of organic solvent used for reconstitution increased: 64%, 57%, 61% (using water with 10%, 20% and 30% of ACN, respectively); and 80%, 73%, 61% (using water with 10%, 20%, and 30% of MeOH, respectively). On the other hand, we compared the area of a 100% aqueous standard solution versus an aqueous solution with 5% of ACN at the same concentration. In this case, the signal of a standard with 5% ACN was 18% less intense. Then, we decided to reconstitute the extracts in 100% water.

3.1.3. Effect of extractant composition over retention capacity of IAC

Once confirmed the highest extractive efficiency of ACN-ultrapure water (80:20, v/v) and the highest efficiency of IAC for the clean-up of rice extracts, the effect of different organic solvent present in extraction mixture over IAC retention was evaluated. For this purpose, two different extractive mixtures were compared: ACN-ultrapure water (80:20, v/v), the one with greatest extractive power in the extraction test (section 3.1.1.1), and MeOH-ultrapure water (80:20, v/v) (recommended by the manufacturer). Results are expressed as recovery percentages of spiked rice samples (by triplicate per condition) regarding blank rice extracts spiked at the same concentration after applying extraction and IAC method (equation (1)). Aflatoxin B1 recovery using ACN-ultrapure water was 65% and 86% recovery was obtained using MeOH-ultrapure water. Moreover, using ACN the standard deviation was 33.7%, in contrast to the deviation obtained when using MeOH (1.1%). Considering that both extraction mixtures were diluted at the same proportion with PBS buffer prior to loading in IAC, and that ACN is in principle tolerated by IAC antibodies according to the study of Ibáñez-Vea, Martínez, González-Peñas, Lizarraga, & López de Cerain, 2011 (where IAC antibodies resisted extracts with until 39% of ACN content), these results might be due to two possibilities. First possibility could be attributed to a greater affinity of ACN than MeOH for aflatoxins, and this fact might complicate aflatoxin interaction with IAC antibody. This hypothesis concurs with the greatest extractive and elution capacity of ACN in preliminary assays and with ACN predominant use for aflatoxins extraction in other papers according to Perestrelo et al., 2019, Qian et al., 2018, and Zhao et al., 2017, among others. The second possibility consists of IAC antibody design from this manufacturer makes it more susceptible to be denatured in contact with ACN. This hypothesis is in accordance with Scott et al., 1997, where a greater denaturing power of ACN over IAC antibodies was observed. In the same line, Stroka, Petz, Joerissen, & Anklam, 1999 experimentally demonstrated the excessive denaturing power of ACN over IAC antibodies. They recommended ACN dilutions of at least 7% before charging the extract on IAC. By contrast, MeOH can be tolerated by antibodies even at dilutions of 30%, according with Şenyuva & Gilbert, 2010, Holcomb' & Thompson, 1991, Barmark et al., 1994, Suárez-Bonnet et al., 2013, among others. Consequently, MeOH-ultrapure water (80:20, v/v) was employed as extractant before performed IAC clean-up.

3.2. Validation of IAC clean-up and HPLC-MS/MS methodology

LODs and LOQs were lower than the maximum limits established in cereals (i.e. 2 μg/kg and 4 μg/kg for individual and total aflatoxins), according to Commission Regulation (EC) No 1881/2006. The lowest LOD and LOQ were found for aflatoxin G1 at 0.09 μg/kg and 0.31 μg/kg, and highest limits for aflatoxin G2 at 0.32 μg/kg and 1.06 μg/kg (Table 2). All aflatoxins showed satisfactory linearity in the range from 1 to 25 ug/L, that is equivalent to the range 0.71 μg/kg to 17.83 μg/kg in rice. For all the calibration curves, quadratic correlation coefficients above 0.999 were obtained (Table 2).

Recoveries were satisfactory for all the compounds (86% for Aflatoxin B1, 90% for Aflatoxin B2, 87% for Aflatoxin G1 and 92% for Aflatoxin G2), with standard deviations between 5 and 11% in all cases. These recoveries were obtained from spiked rice samples at 1.43 μg/kg, that was equal to 20 μg/L. These values are in accordance with Commission Regulation (EC) No. 401/2006, that establishes minimal recoveries between 80–110% for > 10 μg/kg spiked samples; 70–110%

Table 2
Matrix-matched calibration parameters, LOD and LOQ for aflatoxins B1, B2, G1 and G2.

Compound	Molecular Ion (*m/z*) $[M + H]^+$	Transition Ion I (*m/z*)	Linear Range* (μg/L)	LOD (μg/kg)	LOQ (μg/kg)	Calibration equation (Matrix Calibration)		
						$y = ax + b$		
						a	*b*	*R2*
Aflatoxin B1	313.3	285.1	1–25	0.16	0.54	4128.7	15.139	0.9993
Aflatoxin B2	315.3	287.1	1–25	0.26	0.87	5081.9	−3495.4	0.9993
Aflatoxin G1	329.3	243.1	1–25	0.09	0.31	10.542	−5417.1	0.9995
Aflatoxin G2	331.3	313.1	1–25	0.32	1.06	3803.4	−3180.4	0.9994

*Linear range was tested by using n = 4 levels of concentration (1, 2, 10 and 25 μg/L) in matrix-matched calibration curves.

recoveries for spiked samples between 1–10 μg/kg; and 50–120% recoveries for < 1 ug/kg spiked samples, in application to any method for aflatoxin extraction and purification from cereals. The validation parameters of this method (i.e. recoveries, LOD and LOQ) were compared with other methods for aflatoxins in cereals and other foodstuffs reported in literature (Supplementary Material Table A1).

3.3. Analysis of aflatoxins B1, B2, G1 and G2 in commercial rice samples by using IAC clean-up and HPLC-MS/MS

The presence of aflatoxins B1, B2, G1 and G2 has been evaluated on a set of rice samples which include rice of different varieties (round, long, basmati or Thai), different culture origin (Spain, Italy, Pakistan, Thailand, or India), subjected to different processing treatments (husked rice and brown rice), and to different cultivation techniques (traditional and organic farming). Samples were acquired from different supermarkets located in Madrid (Spain) and sample selection was as representative as possible of current consumer trends. We analysed the presence of aflatoxins in these samples to confirm compliance with Commission Regulation (EC) No 1881/2006 applied to rice marketed within the EU. The samples were processed using IAC optimized method and analysed by HPLC-MS/MS technique. The results are present on Table 3.

Aflatoxins were only detected and quantified in samples 6 and 7. Sample 6 only contained aflatoxin B1 at (1.62 ± 0.08) μg/kg. In order to evaluate if this contamination was punctual or spread over the whole batch, sample 7 was analysed (same variety, origin, commercial brand and batch as sample 6). Aflatoxin B1 was detected and quantified in sample 7 at (0.77 ± 0.03) μg/kg. Signal peaks of aflatoxin B1 transitions on sample 6 are shown on Fig. 2.

Peak area ratios between the quantification transition (*Q*) and the two confirmation transitions (q_1 and q_2) from this sample were compared with the ion ratios from the same basmati rice previously spiked with aflatoxins at 17.86 μg/kg (Fig. A1 in Supplementary Material). Experimental deviation between ion-ratios from both samples were within the tolerance range (±30%), according to *Guidance Document on identification of mycotoxins in food and feed* (European Commission, 2017).

The fact that two different food samples from the same batch were contaminated in different proportion with aflatoxin B1 might be due to the heterogeneous nature of fungal contaminations over solid foodstuffs as cereals. Mycotoxigenic contaminations can occur at any step of rice cultivation, harvest, processed or storage until its commercialization, according to Leite, Freitas, Silva, Barbosa, & Ramos, 2021. Many factors can influence the proliferation of mycotoxins in food, such as the climatic conditions of the area of origin of the cereal crop (temperature, humidity, droughts and specific soil microbiome, among others); packing and storage conditions; cleaning methods and other processing treatments applied during the manufacturing processes (washing, peeling, pelleting and other heat treatments to change the properties of the rice, and presence or absence of physical treatments to remove mycotoxins, etc.) or cleaning conditions of warehouses (to avoid cross-linking contaminations). Moreover, some factors like ubiquitous fungal presence, random mixing of spiked and not spiked grains, in addition to poor control of temperature, humidity and cleaning conditions of grain storage places, contribute to spread of punctual mycotoxigenic fungal contaminations all over rice batch and between batches, according to Jolanta Wawrzyniak, 2021, and fungal colonization and proliferation over rice can produce (or not) fungal synthesis of aflatoxins, depending on ambient conditions. Nevertheless, none of the two Basmati rice samples exceeded the 2 μg/kg limit for aflatoxin B1 established by Commission Regulation (EC) No 1881/2006 that is currently in force for cereals marketed in EU countries. However, compliance with legislation is not enough to ensure contaminated samples safety, since a minimal aflatoxin B1 intake of 1 ng/kg/person/day may be enough to increase liver cancer risk, according to the European Commission (Suárez-Bonnet et al., 2013).

Absence of aflatoxins is especially relevant for samples 3, 5 and 9 (brown rice) or samples 2, 4, 5 and 9 (organic farming). Brown rice are characterized by maintaining the outer layer of bran and germ during its processing because of nutritional and digestive benefits of these grain components. The bran can be especially vulnerable to mycotoxigenic fungus, according to Salim, Sukor, Ismail, & Selamat, 2021. This fact might involve an additional risk from brown rice to aflatoxin contamination, as Jettanajit & Nhujak, 2016 demonstrated, who found different mycotoxins on brown rice (but not aflatoxins), and Ruadrew, Craft, & Aidoo, 2013, who detected aflatoxins in brown rice. Regarding rice from

Table 3
Range of aflatoxin contamination (μg/kg) in different types of rice samples.

N° Sample	Type of Rice	Origin	Concentration in μg/kg			
			Aflatoxin B1	Aflatoxin B2	Aflatoxin G1	Aflatoxin G2
1	Round rice	Spain (EU)	nd	nd	nd	nd
2	Organic round rice	Spain (EU)	nd	nd	nd	nd
3	Brown rice	Spain (EU)	nd	nd	nd	nd
4	Organic long rice	Italy (EU)	nd	nd	nd	nd
5	Organic long brown rice	Italy (EU)	nd	nd	nd	nd
6	Basmati rice (1)	Pakistan	1.62 ± 0.08	nd	nd	nd
7	Basmati rice (2)	Pakistan	0.77 ± 0.03	nd	nd	nd
8	Thai rice	Thailandia	nd	nd	nd	nd
9	Organic brown Basmati rice	India	nd	nd	nd	nd

nd: Aflatoxin below the LOD established limit.
(1)(2): Samples from the same batch.

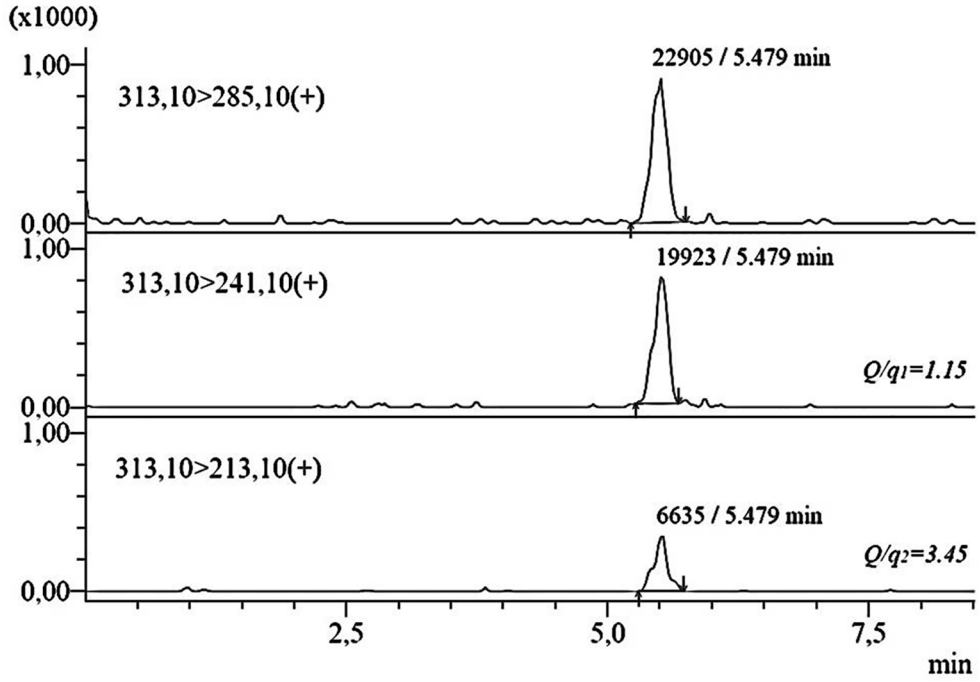

Fig. 2. Signal peaks detected and quantified for aflatoxin B1 transitions in the Basmati rice sample from Pakistan (sample N° 6). Quantification transition (Q), confirmation transitions (q1 and q2) and ratios between Q and q1 / q2 are shown.

organic farming, is important to keep in mind that pesticides and fungicides absence during culture growing improve mycotoxigenic fungal colonization, so more aflatoxin contamination risk is possible. However, emerging development of biological strategies as alternatives to chemical treatments for pest control in crops have counteracted these risks in organic crops, according to Pellan et al., 2021.

Results obtained from rice samples in the current work are not in agreement with previous works reported in the literature. Suárez-Bonnet et al., 2013 carried out the determination of aflatoxins in rice samples grown in Spain, Mexico, Pakistan, France, and USA and marketed in Mexico. Most of the samples analysed were seriously contaminated with aflatoxins (up to 138 μg/kg for total aflatoxins). In the same line, Iqbal, Mustafa, Asi, & Jinap, 2014 detected high levels of aflatoxin B1 (from 4.9 to 8.8 μg/kg) and total aflatoxins (from 8.9 to 12.5 μg/kg) in basmati rice from Pakistan. Fredlund et al., 2009 reported aflatoxins contaminations in rice samples from different origin that were marketed in Sweden, that resulted on 71% of basmati and 21% of Thai rice samples contaminated with aflatoxin B1, some of them above the European Regulation. Similarly, Ruadrew et al., 2013 detected contaminations up to 14.7 μg/kg for total aflatoxins on brown oriental rice marketed on Scotland. Differences in aflatoxin contamination between this work and the contamination levels found in other previous studies could be related to the fact that aflatoxin appearance can occur in cereals stored after the official control tests have been carried out (i.e. cereals can be contaminated by fungal spores that are not detected, and, after passing mycotoxin controls, bad storage conditions can cause fungus proliferation and aflatoxin synthesis). On the contrary, in a study of one hundred rice samples marketed in different English regions (Fredlund et al., 2009) and in other work based on the analysis of rice and other cereals marketed in the northern of Spain (Ibáñez-Vea et al., 2011), aflatoxin levels were below the limits established by the European Regulations. Finally, it is important to highlight that aflatoxin B1 is usually the most prevalent over aflatoxins B2, G1 and G2 in cereals, although the four aflatoxins are synthesized from the same metabolic precursors by mycotoxigenic fungus (Kolawole et al., 2021). This fact is of special interest for consumer safety due to the more toxic nature of aflatoxin B1.

4. Conclusions

Optimization of extraction and clean-up methods for aflatoxins from foodstuffs depends on physiochemical properties of the analytes and food matrix. In this sense, food matrix composition is a limitation factor that requires the best method adaptation to get suitable recoveries of analytes in accordance with established limits by legislation. The method optimized in this work based on the use of IAC is a feasible and valid alternative for the simultaneous determination of aflatoxins B1, B2, G1, G2 in different types of rice.

Rice samples with different properties and origins have been analysed. The most consumed types of rice by the population have been selected. Only two samples were contaminated with aflatoxin B1 but without exceeding the established limits by European Regulations.

The different results found between studies on aflatoxin contaminations in rice along the last decades could make convenient further focusing on the specific patterns of aflatoxin contamination in rice, i.e., on rice varieties, origins and specific characteristics. In this sense, specific processing methods and emerging organic farming techniques could make necessary more studies for evaluating additional risks of aflatoxins (and other mycotoxins) contamination for rice and other cereals subjected to these methods.

CRediT authorship contribution statement

Iván Romero-Sánchez: Conceptualization, Resources, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. **Lorena Ramírez-García:** Resources, Investigation, Methodology, Validation. **Emma Gracia-Lor:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. **Yolanda Madrid-Albarrán:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

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

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