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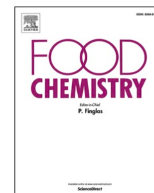

Analysis and evaluation of *in vitro* bioaccessibility of aflatoxins B1, B2, G1 and G2 in plant-based milks

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ABSTRACT

Plant-based milks emerge as a healthy and vegan alternative for human diet, but these foodstuffs are susceptible to be contaminated by aflatoxins. A new method based on SPE and HPLC-MS/MS analysis was optimized and validated to test the presence of aflatoxins B1, B2, G1 and G2 analysis in almond, oat, rice and soy commercial milks. Moreover, aflatoxin bioaccessibility was evaluated through an *in vitro* digestion assay applied to each type of spiked milk. Aflatoxins B1, B2 and G1 were detected in one soy milk sample below the LOQ, fulfilling the limits stablished by the European Legislation. The final bioaccessibility percentages were highly dependent on the type of mycotoxin and sample matrix, the highest and the lowest values were obtained for AFB2 (82%–92%) and AFG1 (15%–30%), whereas AFB1 (28%–50%) and AFG2 (32%–76%) values resulted more influenced by the plant-based milk matrix.

Chemical compounds analysed in this article

Aflatoxin B1 (Pubchem CID: 186907)
Aflatoxin B2 (Pubchem CID: 2724360)
Aflatoxin G1 (Pubchem CID: 14421)
Aflatoxin G2 (Pubchem CID: 2724362)

1. Introduction

The demand for non-animal origin alternatives to cow's milk has witnessed a significant rise in recent years, driven by a variety of factors. Many consumers opt for these alternatives due to clinical conditions, such as lactose intolerance, milk allergies or hypercholesterolemia (Hamed et al., 2019). Lifestyle choices, including veganism and vegetarianism, also contribute to the increasing preference for plant-based options (Gil-Serna et al., 2020; Mäkinen et al., 2016). Additionally, plant-based milks are regarded as functional food, further amplifying their popularity due to the growing demand for healthier products (Hamed et al., 2019).

It is important to note that the raw materials used in these plant-based milks, including cereals, nuts, and seeds, may pose a risk of mycotoxin contamination (EFSA, 2013). Consequently, the occurrence of mycotoxins should be taken into consideration as they can persist throughout food processing, maintaining their chemical and thermal stability, and therefore may be present in the final product (Bullerman & Bianchini, 2007; Gil-Serna et al., 2020).

Being recognized as carcinogenic agents to humans by the International Agency for Research on Cancer (IARC), aflatoxins are the most hazardous mycotoxins for human and animal health (Benkerroum, 2020). They are highly toxic secondary metabolites produced by fungi belonging to the *Aspergillus* genus, and these aflatoxin-producing fungi usually contaminate crops either during harvesting or storage and transport (Bennett & Klich, 2003).

The implication of aflatoxins in cancer development occurs mainly at hepatic level as this organ is responsible for the metabolization and detoxification of mycotoxins, among other toxins (Rushing & Selim, 2019) (Marchese et al., 2018). Besides being recognized as carcinogenic, aflatoxins can cause a broad range of acute and chronical effects on animal and human health, according to Benkerroum, 2020 and Romero-Sánchez et al., 2022.

Today, despite the efforts spent on decreasing the fungal infections of crops and food stocks, aflatoxin contamination is still a major risk for agricultural industry and consumers. Considering the above, the European Commission has regulated the maximum levels of aflatoxins allowed in food marketed in the EU- whether it is produced in the EU or imported.

Aflatoxin B1 is the only aflatoxin with maximum level regulated by the European Commission. Additionally, the total concentration of

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aflatoxins, which corresponds to the sum of aflatoxins B1, B2, G1 and G2, has also been regulated in multiple foodstuffs, so aflatoxin B1 and total aflatoxins concentrations in food for adult consumption has been set between 2 to 12 μg/kg and 4 to 15 μg/kg, respectively, depending on the commodity and its manufacture, according with the Commission Regulation (EU), 2023/915. However, specific aflatoxin limits for plant-based milks are not stablished according with current legislation. Although cereals, oilseeds and nuts are the main raw materials used for plant-based milks elaboration, the European Regulation stablishes that their aflatoxin limits should not be directly applied for derived products that contain <80% content of the raw material in the final product (case of plant-based milks). In these foodstuffs, Commission Regulation (EU), 2023/915 maintain the regulatory limits of the original raw materials, but stablishes that the final allowed aflatoxin content must be determined by considering the final percentage and the dilution or concentration factors of the raw material in the plant-based milk, but no more specifications are detailed in this regard.

A factor not considered when regulatory limits for aflatoxin levels in food matrices are stablished is that the amount of mycotoxin consumed does not always reflect the amount available to exert toxic action in a target organ of the body, as only a part of the ingested compound will be bioavailable (Versantvoort et al., 2005) (González-Arias et al., 2013). Therefore, this fraction is relevant for accurate risk assessment and should be considered to stablish legal limits of mycotoxin content in foodstuffs.

In order to obtain the oral bioavailability, it is essential to previously assess the bioaccessibility (Fernández-García et al., 2009). Food composition has a significant effect on mycotoxin bioaccessibility, as complex and diverse interactions can occur between the mycotoxin and the food matrix, according to González-Arias et al., 2013 and Romero-Sánchez et al., 2023.

The bioaccessibility of mycotoxins is greatly dependent on mycotoxin chemical structure and food matrix composition, highlighting the need for further studies on bioaccessibility of these fungal metabolites (Romero-Sánchez et al., 2023).

On the other hand, bioaccessibility studies for highly demanded food as plant-based drinks are scarce but needed. Moreover, bioaccessibility assays applied to plant-based milks is of increasing interest considering that these beverages can be made from different types of cereals (rice, oat, spelt wheat, corn); pseudocereals (quinoa); dried fruits (almond, hazelnut, walnut); or oilseeds (soy, peanut), among others, in addition to other components to improve the consistency and shelf-life of the product, as well as flavours are added during the manufacturing process. (González-Arias et al., 2013).

In accordance with the significant importance of aflatoxins, analytical techniques for their proper separation, detection and quantification in food, feed and beverages have been extensively developed. These toxins occur naturally at trace levels and are toxic even at low concentrations. Therefore, quantification methods of aflatoxins must be both reliable and sensitive in order to measure aflatoxin levels lower than those required by national or international regulations to monitor levels in the food chain (Tahir et al., 2018). Among the analytical techniques, high performance liquid chromatography (HPLC) coupled to mass spectrometer detectors are the method of choice for the separation and quantification of aflatoxins (FAO & WHO, 2018).

In order to successfully detect and quantify aflatoxins in plant-based milks, it is essential to optimize the sample pretreatment and clean-up steps to extract the aflatoxins from the matrix; to preconcentrate the extracted analytes as much as possible; and to mitigate the matrix effects that might interfere with accurate quantification (Gil-Serna et al., 2020). The nutritional complexity of plant-based milks, characterized by high contents of proteins, carbohydrates, lipids and fiber, requires a careful optimization of sample treatment procedures. Moreover, the differences between the nutritional composition of the most consumed plant-based milks (soy, oat, rice and almond) and even between the same beverage obtained from different manufacturers, poses an additional challenge in order to develop new extraction and clean-up methods simultaneously valid for different types of plant-based drinks and from different commercial brands (Silva et al., 2020).

Several works have studied the occurrence of different mycotoxins in plant-based milks, especially oat, soy, rice- and almond-based milks, which are the most consumed worldwide. Despite previous analyses of certain plant-based beverages like tiger nut milk (Rubert et al., 2011; Sebastià et al., 2010), the comprehensive investigation of mycotoxin presence in commonly consumed plant-based milk alternatives was unexplored until 2017. Moreover, the high and rapid rise in consumption of plant-based drinks, the multiple and unknown origins of the raw materials that compose the basis of this foodstuffs, and the high risk that supposes oral exposure to aflatoxins, require the development of reliable methods for the analysis of aflatoxins from commercial plant-based milk samples to monitoring the current population exposure, and *in vitro* digestion studies to evaluate aflatoxin behaviour and bioaccessibility from different types of plant-based milks.

To address the imperative of ensuring food safety concerning these milk substitutes, we have optimized a sample extraction and clean-up procedure and validated an analytical method by using High Performance Liquid Chromatography coupled to Tandem Mass Spectrometry (HPLC-MS/MS) for the accurate quantification of aflatoxins B1, B2, G1 and G2 in rice, almond, oat and soy drinks. In addition, recognizing the importance of bioaccessibility evaluation in aflatoxin risk assessment in food and feed, this study includes an evaluation of the bioaccessibility of aflatoxins B1, B2, G1 and G2 in the afore mentioned food matrices.

2. Materials and methods

2.1. Chemicals and reagents

A Milli-Q Millipore system (Bedford, MA, USA) was used to produce the ultrapure water. Acetonitrile (ACN) LC-MS grade employed for chromatography was acquired from Fisher Scientific (Fair Lawn, NJ, USA); ACN and methanol (MeOH) supergradient grade for extractions and formic acid (HCOOH) for HPLC-MS/MS were purchased from Scharlab (Barcelona, Spain). Solid phase extraction (SPE) cartridges Oasis HLB containing 60 mg and 200 mg of sorbent were supplied by Waters Corporation (Milford, MA, USA).

The salts $CaCl_2·2H_2O$, $NaHCO_3$, $MgCl_2·6H_2O$ as well as mucin, bile salts, tripsin (10,000 U/mg), α-amylase (400 U/mg), pepsin (≥400 U/mg) and pancreatin (4 x USP) employed in the preparation of saliva, gastric and duodenal fluids were purchased from Merck (Darmstadt, Germany). NaCl and KCl were supplied by Panreac (Barcelona, España) and KH_2PO_4 by Riedel de Haën (Kufstein, Austria).

Aflatoxins analytical solid standards were purchased from Sigma Aldrich (St. Luis, Missouri, USA): (3*S*,7*R*)-11-methoxy-6,8,19-trioxapentacyclo[10.7.0.02,9.03,7.013,17]-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[10.7.0.02,9.03,7.013,17]nonadeca-1,9,11,13(17)-tetraene-16,18-dione (Aflatoxin B2, ≥98%), 11-methoxy-6,8,16,20-tetraoxapentacyclo-[10.8.0.02,9.03,7.013,18]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[10.8.0.02,9.03,7.013,18]icosa-1,9,11,13(18)-tetraene-17,19-dione (Aflatoxin G2, ≥98%). Individual stock solutions were prepared by conveniently diluting with ACN 1 mg of mycotoxin to get a final concentration of 100 mg/L. A work solution containing a mixture of the four aflatoxins at 20 mg/L in ACN was prepared from the individual stock solutions. All the standards were stored at −20 °C until their use to prevent their degradation.

2.2. Plant-based milks samples

Plant based milk samples from four different brands (A, B, C and D) were selected for each type of milk and acquired from several supermarkets of Madrid (Spain). In accordance with the current consumption

trends the following beverages were selected: soy milk (soy content 13–15%), almond milk (almond content 4–4.5%), oat milk (oat content 15–16%) and rice milk (rice content 15–17%). All the samples were stored in darkness at room temperature until the analysis. The specific nutritional composition from the manufacturer of the plant-based milks that were assessed through *in vitro* digestion is detailed on Table 1.

2.3. Instrumentation

Analysis were carried out through a high performance liquid chromatograph coupled to a triple quadrupole mass spectrometer detector (HPLC-MS/QqQ). Firstly, analyte separation was developed by a HPLC Model 1200 Series (Agilent Technologies, Madrid, Spain). The chromatographic system consists of a G1312A binary pump, a G1329A degasser, a G1329A automatic thermostatic injector and a G1316A thermostatic module column.

Reversed-phase separation of aflatoxins was achieved by a Synergy™ Fusion-RP C18 (150 mm × 3 mm, ID 4 μm, pore size 80 Å) analytical column (Phenomenex, Torrance, CA, USA). Volume sample of injection was 5 μL and the flow rate was set at 0.5 mL/min at 40 °*C. mobile* phase consisted of ultrapure water with HCOOH 0.1% (eluent A) and ACN with HCOOH 0.1% (eluent B). The percentage of B changed as follows along the chromatographic gradient: 0 min, 30%; 7 min, 60%; 8.0 min, 100%; 12 min, 100%; 13 min, 30%, and the last condition was kept during 4 min to re-equilibrate the column.

Aflatoxin detection and quantification was done with a G6410B triple quadrupole mass spectrometer (Agilent Technologies, Madrid, Spain). Analysis were performed in positive electrospray ionization mode using the Multiple Reaction Monitoring (MRM) mode with a dwell time of 100 ms for the transitions corresponding to aflatoxins B1, B2, G1 and 150 ms for aflatoxin G2 transitions. ESI capillary voltage was set at 4.0 kV, fragmentation voltage at 152 V, and current chamber was established at 0.16 μA. The nebulisation system was set at 55 psi and the applied drying gas flow rate was 12 L/min at 300 °C. Selected transitions and collision energies (CE) for aflatoxins are detailed on **Table S1**, whereas chromatograms and MS/MS spectrum of the aflatoxins are provided on **Fig. S1** in the Supplementary Material. For data acquisition and processing, Masshunter Data Acquisition B.04.01, Masshunter Qualitative Analysis B.07.00 and Masshunter Quantitative Analysis B.07.00. were employed (Agilent Technologies, Waldbroon, Germany).

2.4. Determination of aflatoxins in commercial plant-based milks

20 mL of each plant-based milk were firstly stirred at 99 rpm for 30 min using an Intelli-Mixer RM-2 M (Elmi, Riga, Latvia). Subsequently, 10 mL of MeOH were added, followed by another 30-min stirring incubation. The resulting samples were centrifuged at 16,400 xg using an Eppendorf Centrifuge 5810 R (Eppendorf, Hamburg, Germany) for 10 min.

Next, 25 mL of the supernatant were transferred to a pure polypropylene tube (Eppendorf, Hamburg, Germany), where 12.5 mL of n-hexane were then added and the mixture was shaken manually. The addition of the organic solvent was followed by another 10-min centrifugation at 16,400*g*, leading to the separation of two phases – an aqueous phase and an organic phase - with a noticeable interphase.

Table 1
Nutritional composition in terms of fats, carbohydrates and proteins (g/L) provided by the manufacturer of each type of plant-based milk (almond, oat, rice, and soy) employed for the *in vitro* digestion model.

Component	*In vitro* digested plant-based milk			
	Almond milk	Oat milk	Rice milk	Soy milk
Fats (g/L)	22	8	9	20
Carbohydrates (g/L)	3	93	110	12
Proteins (g/L)	8	13	1	35

Both the above organic phase and the interphase were removed. Then, a 15 mL aliquot of the aqueous phase was taken and diluted with Milli-Q water to reach a final volume of 50 mL. Half of this resulting dilution (25 mL) was passed drop by drop, with the assistance of a vacuum pump, through a previously conditioned HLB cartridge containing 60 mg of sorbent. The cartridges were previously conditioned with 6 mL of MeOH followed by 6 mL of Milli-Q water. After the 25 mL solution had been passed through the cartridges, the columns were washed with 20 mL of Milli-Q water and completely dried under vacuum for 5 min. Aflatoxins were then eluted from the cartridges by adding 5 mL of MeOH, which was subsequently evaporated to dryness by nitrogen stream. Finally, the extracts were reconstituted in 0.1 mL of Milli-Q water for the analysis by HPLC-MS/MS.

2.5. Validation study of the analysis methodology for aflatoxins on commercial plant-based milks

For the aflatoxin extraction and clean-up methodology from milk commercial samples (Section 2.4), precision and accuracy were evaluated for each plant-based milk type by spiking of 20 mL of plant –based milks at 2.5 μg/kg with each aflatoxin (equivalent to an initial concentration of 2.5 μg/L and a final concentration of 125 μg/L) before the application of the extraction method. Analysis were performed in triplicate to evaluate precision by standard deviations and RSD calculations. For each aflatoxin, the area of the spiked sample was compared to the area of a blank extract that was spiked before the injection sample at the same final concentration as follows (1):

$$\%Recovery = \frac{Peak\ area\ of\ spiked\ sample}{Peak\ area\ of\ purified\ spiked\ extract} \times 100 \quad (1)$$

The recovery percentages and RSD values were evaluated at intraday and inter-day levels, with two different determinations per day along two different days.

In addition, the aflatoxin recoveries considering only the losses caused by the matrix factor (MF) was calculated by comparing the area of blank purified extracts that were spiked with each aflatoxin before the injection at 125 μg/L with the area of a stock solution at the same concentration, as follows (2):

$$\%MF = \frac{Peak\ area\ of\ purified\ spiked\ extract}{Area\ Stock\ Solution} \times 100 \quad (2)$$

This method was also 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 extracts from each plant-based milk that were spiked before the injection at four known final concentrations (25, 50, 125 and 250 μg/L), and results were evaluated in terms of slope and squared correlation coefficient (R^2). Peak areas of real samples were interpolated on matrix calibration line to get their real concentration. The LOD and LOQ were estimated by the method based on signal-to-noise (giving a signal-to-noise (S/N) ratio of 3 for LOD and 10 for LOQ), applied for the determination method and for the analytical instrument. In the first case, the aflatoxin concentration of the spiked milk samples was considered as the most reliable detectable and quantifiable for the determination method; in the second case, aflatoxin pure stock solutions were considered for the instrument.

2.6. In vitro digestion model

In vitro digestion model is an assay that simulates the physiological conditions that take place during human gastrointestinal digestion. For this purpose, solutions with different chemical and enzymatical composition were prepared to simulate the compositional properties of the salivary, gastric, and duodenal fluids, and were added sequentially on the foodstuff. In each step, the mixture was homogenized and

incubated at different times. In this work, the digestive model used by (Romero-Sánchez et al., 2023) was taken as a reference.

The *in vitro* digestion model was applied to 10 mL of each plant-based milk sample with a sequential addition of each artificial digestive fluid previously prepared (the composition of the digestive fluids is detailed in Table 2).

The digestive incubations were performed under oscillating agitation in rotate mode using an Incubator Genie (Scientific Industries, Inc., USA) at 35 rpm and 37 °C in darkness for all the digestive steps. Firstly, 5 mL of the salivary fluid was added to the beverage sample. The resulting mixture was vortexed, its pH was adjusted at 6.8 and was then incubated for 15 min. Next, 12 mL of the gastric fluid was applied to each sample, the resulting mixture was vortexed, and its pH was adjusted at 1.8, followed by a 2-h incubation. Finally, 17 mL of the duodenal fluid was introduced into the mixture, vortexed, the pH was adjusted at 8.2 and the final extract was incubated for 2 h. Once the incubations were finished, the digestive extracts were subjected to a thermal shock at −80 °C to denature the digestive enzymes and thereby to stop the digestive process.

2.7. Digestive extracts clean-up

In order to determine the bioaccessibility of aflatoxins B1, B2, G1 and G2, the digestive extracts were thawed at room temperature and centrifuged at 16,400*g* for 10 min. Subsequently, a 20 mL aliquot was transferred from each supernatant to a pure polypropylene tube (Eppendorf SE, Hamburg, Germany), where 10 mL of n-hexane were then added and the mixture was shaken manually. The addition of the organic solvent was followed by centrifugation at 16,400*g* for 10 min, leading to the separation of two phases – an aqueous phase and an organic phase - with a remarkable interphase. Then, the above organic phase and the interphase are removed, and a 10 mL aliquot of the aqueous phase was taken and diluted with Milli-Q water to reach a final volume of 50 mL. Half of this resulting dilution (25 mL) was passed drop by drop through a HLB cartridge containing 200 mg of sorbent, with the assistance of a vacuum pump. The cartridges were previously conditioned with 6 mL of MeOH followed by 6 mL of Milli-Q water. After the 25 mL solution had been passed through the cartridges, the columns were washed with 20 mL of Milli-Q water and completely dried under vacuum for 5 min. Aflatoxins were then eluted from the cartridges by adding 10 mL of ACN, which was subsequently evaporated to dryness by nitrogen stream. Finally, the extracts were reconstituted in 0.2 mL of Milli-Q water for the analysis by HPLC-MS/MS.

Table 2
Chemical and enzymatical composition of each digestive fluid (g/L) that were sequentially applied on the *in vitro* digestion model to each plant-based milk.

Component (g/L)	Digestive Fluid		
	Salivary	Gastric	Duodenal
NaCl	1.82	4.26	–
$CaCl_2 \cdot 2H_2O$	0.47	–	0.55
Na_2SO_4	4.57	–	–
$NaHCO_3$	0.64	–	0.96
KCl	1.62	1.16	0.34
KH_2PO_4	2.00	0.41	–
$MgCl_2 \cdot 6H_2O$	–	–	0.24
Mucin	2.53	4.38	–
Urea	0.29	–	0.32
Uric acid	0.03	–	–
Bile salts	–	–	0.93
a-amylase	1.10	–	–
Pepsin	–	1.60	–
Pancreatin	–	–	0.90
Tripsin	–	–	0.24
pH	6.8 ± 0.2	1.8 ± 0.2	8.2 ± 0.2

2.8. Analytical performance of the in vitro digestion procedure

Bioaccessibility calculation was based on the formula below (3). On the basis of this formula, 10 mL of each plant-based milk sample were spiked simultaneously with all the aflatoxins at initial concentration of 100 μg/L by triplicate (equivalent to 100 μg/kg) to evaluate the precision of the assay, and submitted to the *in vitro* digestion procedure along with blank plant-based milk samples without aflatoxins (named as blank sample). After the *in vitro* digestion, 20 mL of final digested extracts from blank samples were spiked at 23 μg/L for each type of aflatoxin before the clean-up procedure. 23 μg/L concentration was selected as represents the expected concentration after *in vitro* digestion if aflatoxins exhibited a 100% of bioaccessibility (considering the dilution factors applied during *in vitro* digestion). Finally, both types of samples were preconcentrated at a final concentration of 568 μg/L after the clean-up of digestive extracts.

Finally, the area of each analyte obtained from the spiked sample was compared to the area of the blank extract spiked after the *in vitro* digestion and before the clean-up procedure:

$$\%B = \frac{Peak\ area\ of\ spiked\ sample}{Peak\ area\ of\ spiked\ digestive\ extract} \times 100 \quad (3)$$

In this way, the losses derived exclusively from the clean-up procedure and the matrix effect were corrected with this formula (because both types of samples were spiked previous to the clean-up procedure, so both suffered the same losses due to the matrix effect and the clean-up method), so the only losses taken into account in the calculation are those derived exclusively from the *in vitro* digestion procedure (because the sample was spiked before digestion and the extract was spiked after digestion).

The clean-up method applied to the digestive extracts after the *in vitro* digestion model was evaluated in order to test the recovery of the analytes (AFB1, AFB2, AFG1 and AFG2) from each type of milk. For this purpose, the 20 mL aliquots taken from the blank digestive extracts were spiked at 23 μg/L with the four aflatoxins, and its area was compared to the area of a blank digestive extract that was spiked after the clean-up method at a 568 μg/L, exactly the final expected concentration after the clean-up procedure if there were not aflatoxin losses during the clean-up method, as follows (4):

$$\%Recovery = \frac{Peak\ area\ of\ spiked\ digestive\ extract}{Peak\ area\ of\ purified\ spiked\ digestive\ extract} \times 100 \quad (4)$$

Finally, LOD and LOQ for each type of plant-based milk sample spiked after the *in vitro* digestion were calculated based on S/N ratio of 3 and of 10, respectively, for the *in vitro* digestion process.

3. Results and discussion

3.1. Determination of aflatoxins in commercial plant-based milks

The presence of aflatoxins B1, B2, G1 and G2 has been evaluated in twenty commercial samples of almond, oat, rice and soy milks, and from four commercial brands (A, B, C and D) for each type of milk. All the samples were acquired on different supermarkets from Madrid (Spain) and selected according to a representative trend of current consumption. For the analysis of these samples, a method based on simultaneous extraction of aflatoxins with MeOH combined with SPE clean-up by using HLB cartridges to purify and concentrate the analytes was previously optimized in the same study.

The results obtained evidenced the presence of low intense peak signals in all the samples tested. To confirm that these signals corresponded to mycotoxins, signal ratios between quantification and confirmative transitions (Q/q) were calculated for each positive sample and compared with the Q/q average ratio of the matrix-matched calibration points. Q/q ratio values obtained were within ±30% of the allowed

tolerance range for AFB1, AFB2 and AFG1 on soy milk (sample A), compared with Q/q average ratio of matrix-matched calibration, according to the European Commission guidance for mycotoxin identification on foodstuffs (European Commission, 2017). Therefore, these samples were contaminated with the aflatoxins mentioned at levels below the quantification limits of the applied method (<LOQ), or not measurable, whereas the rest of the samples were not contaminated with aflatoxins, or aflatoxins were below the detection limits of the method (<LOD). Fig. 1 shows the chromatograms of the aflatoxins detected below the LOQ whose Q/q ratios were in accordance with the Q/q average ratio from the matrix-matched calibration.

Considering that LOD and LOQ of this method are much lower than the maximum limits for aflatoxins in cereals, dry fruits, legumes and derived products stablished by the Commission Regulation (EU), 2023/915, we can conclude that the analysed samples accomplished with the current legislation. However, it should be taken into account the ambiguity of this legislation regarding to derived products which contain <80% of the regulated raw materials. In these cases, the maximum final aflatoxin level depends on the initial raw material content and the final dilution level applied to the raw material for the elaboration of the derived product (Commission Regulation (EU), 2023/915). In the current study, the absence of measurable aflatoxin levels in these commercial samples is in accordance with the results obtained by Hamed et al., 2019, that analysed aflatoxin presence in eleven samples of rice, oat and almond milk, and six additional products enriched with oat and almond marketed in Granada (Spain), and no quantifiable contamination was found. In a similar approach, Rodríguez-Cañás et al., 2024 analysed simultaneously aflatoxins along with other 25 emerging

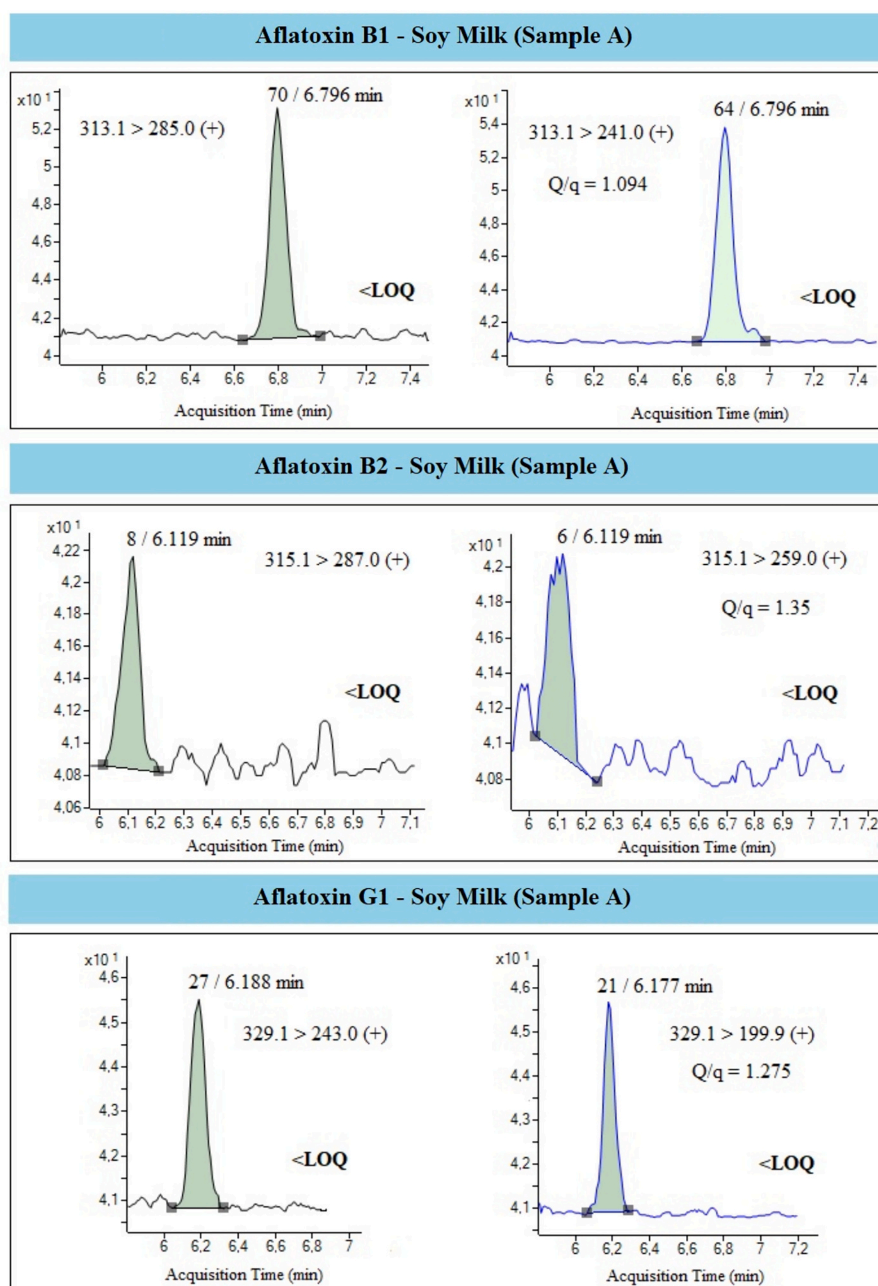

Fig. 1. Chromatograms for the quantification and confirmation transitions of each aflatoxin detected below the LOQ in commercial plant-based milks samples: AFB1 in soy milk (Sample A); AFB2 in soy milk (Sample A); and AFG1 in soy milk (Sample A). The area and the retention time for each transition is detailed and the Q/q ratios has been calculated (coincident with Q/q ratios from the matrix-matched calibration points).

mycotoxins in almond, oat, rice and soy sample milks from Galicia (Spain). In this case, AFB1 and AFB2 were detected in almond and rice samples (28%–33% of prevalence), with values below the LOQ, and only AFB1 was detected and quantified at 0.5 μg/L in soy milk (11% of prevalence). However, the low values of this study contrasted with the high presence of other mycotoxins as beauvericin, enniatins or fumonisins from the same study.

On the other hand, our results disagree with other studies of aflatoxin analysis in plant-based milks commercialized in other parts of Spain. For instance, Miró-Abella et al., 2017 monitored the presence of aflatoxins, among other mycotoxins, in oat, rice and soy milks acquired in Catalonia (Spain). In this study, oat milk samples from different brands were found to be contaminated with AFB1 at 0.2–0.3 μg/L, AFB2 at 0.4 μg/L and AFG1 at 0.1 μg/L, whereas AFB1, AFG1 and AFG2 were detected in other samples of oat, soy and rice at levels below the LOQ. In the same line, Juan et al., 2022 developed a multi-mycotoxin analysis in almond, oat, rice, and soy milks acquired from Valencia (Spain), and they found AFB2 at 0.7 μg/L in almond milk samples and AFG1 at 0.1 μg/L in oat milk samples. In a more international context, Mohebbi et al., 2022 found AFB1 contaminations in a range between 0.054 and 0.234 μg/L in five soy milk samples purchased from Iran, similar to Rezaeefar et al., 2022, who also found lower AFB1 levels between 1.6 and 3.9 ng/L in eight soy milk samples acquired in the same country.

Other studies have analysed aflatoxins in horchata, a vegetable drink elaborated with tiger-nut. In this drink, Arranz et al., 2006 found AFB1 at 0.06 μg/L in 1 sample out of 22 analysed, whereas Sebastià et al., 2010 quantified AFB1 at 1.2–3.1 μg/L, AFB2 at 1.3–2.2 μg/L and AFG2 at 1.3–1.4 μg/L present in 3 samples of 25, and Rubert et al., 2011 also quantified AFB1 at 0.8–1.7 μg/L, AFB2 at 1.1–1.6 μg/L and AFG2 at 1.2–2.3 μg/L in 15 samples out of a total of 190. Tiger-nut drinks were purchased in Valencia (Spain), in all the studies. To the best of our knowledge, there are no more monitoring studies on aflatoxins in vegetable drinks apart from those monitored above.

The disparity of results related to the presence or absence of aflatoxins in this type of food products may be related to the presence of the toxins in the original raw materials employed for drink elaboration, added to the inadequate storage conditions, the mixing and the specific processing of the plant-based milks (Baker et al., 2014 and Juan et al., 2022). Raw materials such as cereals, nuts or legumes are highly susceptible to the colonization by ubiquitous aflatoxigenic fungus during their growing and storage, which produce aflatoxins at variable levels under a combination of factors that are not entirely clarified. Then, the aflatoxigenic contamination can be extended during the blending and storage processes of the raw materials (Gil-Serna et al., 2020; Lin et al., 2023). In addition, the specific processes for plant-based milks elaboration also promotes the aflatoxin extraction from the raw materials to the final liquid beverage through the soaking and the wet milling processing (McClements et al., 2019; Tahir et al., 2018). On the other hand, the addition to the original vegetable extracts of substances to enhance the homogenisation, flavours, preservation and nutritional enrichment of the final beverage (*i.e.* emulgents, fats, sugars, salts, vitamins, minerals and synthetic additives) suppose a factor that may promote the masking of aflatoxins in the final beverage, so masked aflatoxins may not be detected along the analysis if the employed methods are not able to disrupt these interactions (Romero-Sánchez et al., 2023). Finally, the high chemical and thermal stability of aflatoxins confer them resistance to the common processing methods applied to the vegetable drinks (Alshannaq & Yu, 2017; Kabak, 2009). Moreover, a concise European Legislation to establish clear and homogeneous limits for mycotoxins in plant-based derived milks is required in order to decrease the risks derived from the exposition to these mycotoxins for the consumers (Marchese et al., 2018).

3.2. *In vitro* bioaccessibility of plant-based milk samples

In this *in vitro* assay, almond, oat, rice and soy milk samples were spiked simultaneously with aflatoxins B1, B2, G1 and G2 at 100 μg/L (equivalent to 100 μg/kg approximately) and subjected to an *in vitro* digestion process step-by-step through the sequential addition of artificial salivary, gastric and duodenal fluids to establish the final bioaccessibility values for each aflatoxin and the influence of the milk composition over each analyte tested. Once finished the digestion, the final duodenal extracts were purified, preconcentrated and analysed by HPLC-MS/MS. The final bioaccessibility percentages for each aflatoxin from each type of milk are presented on Fig. 2.

Considering that plant-based milks are water-rich liquid foods with a complex composition and that aflatoxins may be therefore found as free form in the aqueous media or linked with the milk components (masked forms), milk samples were incubated overnight after spiking to favour the possible interactions established between the aflatoxins and the matrix components of the milks. Moreover, the clean-up method for the duodenal extracts deliberately did not include any aflatoxin extraction phase to avoid that masked aflatoxins remaining after the digestion could be released, so the only analysed aflatoxins were those released to the final digestive extract in their free original form, because these free forms are the immediately bioaccessible for intestinal absorption, whereas masked aflatoxins are not considered as potentially susceptible to be absorbed by the intestine. Consequently, aflatoxins masked by the milk components or even adsorbed to the digestive components that could not be released to the soluble fraction of the duodenal extract have not been considered in the current study (*i.e.* the interaction between the analytes with the duodenal matrix components was corrected by spiking the duodenal extracts with the aflatoxins). Regarding the digestion process, large intestinal digestion step has not been taken into account because aflatoxins are mainly absorbed in the small intestine (Kabak & Ozbey, 2012).

The values obtained were processed by a multifactorial ANOVA to establish the individual contribution of both factors involved in the assay (*i.e.* plant-based milk and analyte) to the final variability of the results, the statistical differences between both factors and among the groups belonging to each factor, that is, between the plant-based milk types and between the aflatoxin types (Fig. 3). Firstly, multifactorial ANOVA confirmed that both plant-based milk and analyte factors involved in the assay are statistically significant with p-values <0.05, and statistically significant differences were observed between both factors. This trend confirms that milk and analyte factors are determinant in the final bioaccessibility of aflatoxins.

Regarding the constituent groups of each factor, ANOVA established significant differences between the analytes and between the plant-based milks. Although the behaviour differences observed between the aflatoxin groups were higher than between the based-plant milks groups, as shown in the ANOVA diagram from Fig. 3. So, all the aflatoxins showed statistical differences with respect to each other (no homogeneous groups). However, in the case of the plant-based milks groups, significant differences were found between almond - soy, almond - rice and oat - rice, but homogeneous groups were established between almond - oat, oat - soy and soy - rice. A Fisher LSD test was performed to confirm the differences between the groups belonging to the analyte and milk factors (**Fig. S2** from Supplementary Material).

The differences obtained in the ANOVA test are in accordance with the results observed in Fig. 2, as we can appreciate that each type of aflatoxin was released from the foodstuff to the final digestive extract according to a single pattern of behaviour. Meanwhile AFB2 is bioaccessible at high levels (82%–92%) and AFG1 is poorly bioaccessible (15%–30%) regardless of the milk type, AFB1 (28%–50%) and AFG2 (32%–76%) present values broadly dependent on the milk matrix. These differences of bioaccessibility between the aflatoxin types contrast significantly with their highly structural similarity. Influence of milk composition on aflatoxins bioaccessibility is relevant, so while AFB2 and AFG1 bioaccessibility are independent of the compositional differences between the matrix milks (Table 1), AFB1 and AFG2 bioaccessibility are strongly dependent on specific composition of the milk. The major AFB1

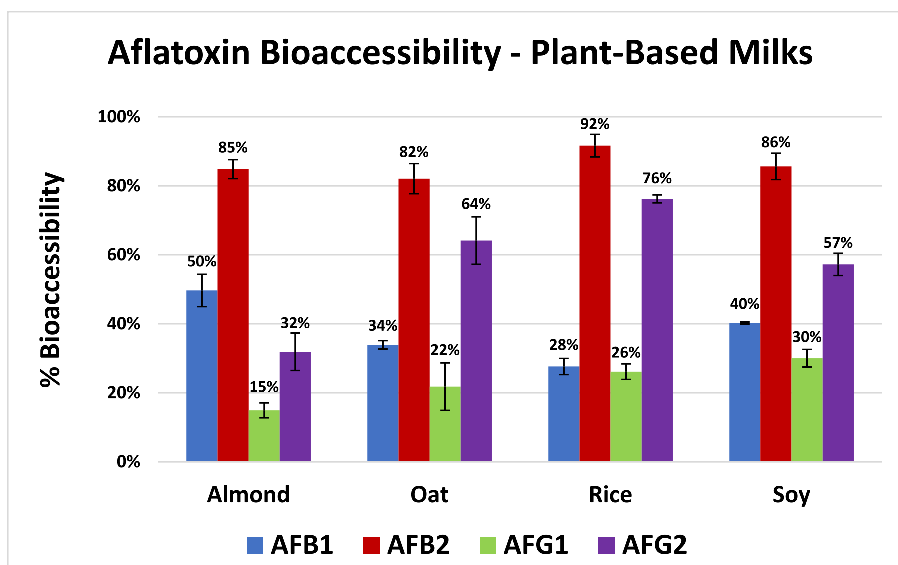

Fig. 2. Bioaccessibility percentages and standard deviations obtained for aflatoxin B1, B2, G1 and G2 from spiked almond, oat, rice and soy milks after the *in vitro* digestion assay.

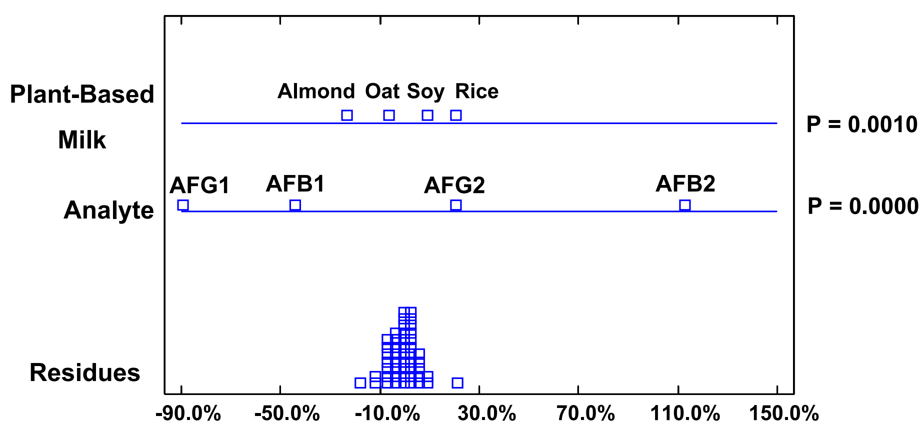

Fig. 3. Multifactorial ANOVA diagram for analyte (AFB1, AFB2, AFG1 and AFG2) and based-plant milk (almond, oat, rice, soy) factors evaluated and p-values obtained.

bioaccessibility value (50%) obtained from almond milk seems to be related with the high fats content of this type of milk, but the high carbohydrates content in oat and rice milks reduce the AFB1 bioaccessibility until 34% and 28%, while the 40% AFB1 bioaccessibility from soy milk represents an intermediate value between the almond and the oat and rice milks. This trend could be related to the intermediate composition of soy milk (similar fats content to the almond milk but more carbohydrates, according to Table 1) and its higher protein content regarding to the rest of milks. Likewise, the opposite trend is observed for AFG2, as its bioaccessibility is higher from the oat and rice milks (64% and 76%) in contrast to the almond milk (32%), whereas intermediate values are obtained for AFG2 from the soy milk (57%).

Thus, fat, carbohydrate and protein content play an important role in the final bioaccessibility of AFB1 and AFG2 (Tahir et al., 2018). Considering that AFB1 is the most lipophilic aflatoxin (United States Environmental Protection Agency (EPA), 2012) due to the presence of an additional double bond (absent on type 2 aflatoxins) and the absence of the additional lactone group (present on type G aflatoxins), as well as the major presence of emulgents as much fats contain the plant-based milks (Gil-Serna et al., 2020), maybe the emulgent action from the fatty milks could increase the final solubility of the fats and the AFB1 bioaccessibility in the duodenal extracts (Fernández-García et al., 2009). Regarding to the higher bioaccessibility values for AFG2 from rice and oat milks, this phenomenon could be related to the higher polarity of this aflatoxin according to the United States Environmental Protection Agency (EPA), 2012, whose diffusion to the final aqueous digestive extract could be easier from milks with a high polar composition as rice or oat milks. The relatively low AFB1 bioaccessibility values $<50\%$ obtained from all the plant-based milks contrast significantly with the high values close to 100% usually obtained by other authors for AFB1 from solid foods with variable composition, as the developed by Versantvoort et al., 2005 on peanut slurry or by Kabak et al., 2009 on pistachio nuts and infant processed pasta, among others; even for aflatoxin M1 (the main mammal hydroxylated metabolite derived from AFB1) from cow milk, bioaccessibility ratios between 81%–86% were found by Kabak & Ozbey, 2012, which implicates a higher risk of these foodstuffs related to AFB1exposure compared to plant-based milks, considering that AFB1 is the most toxic and cancerous aflatoxin (Marchese et al., 2018).

Although this is the first study to test the aflatoxin bioaccessibility

from plant-based milks, there are behaviour patterns common to other *in vitro* digestion studies with aflatoxins from other foodstuffs. This is the case of the assay developed by Romero-Sánchez et al., 2023 to evaluate the bioaccessibility of aflatoxins B1, B2, G1 and G2 from white and brown rice artificially spiked, where the authors also obtained important bioaccessibility differences between the food matrices, but the digestive trends among the aflatoxins were more homogeneous. However, a similar assay developed by Da Silva et al., 2019 with the same rice types established important differences in behaviour between the aflatoxins as well as between the matrices, in line with the trend observed in the present study. So, even from the same matrix, the bioaccessibility patterns can differ between the aflatoxin types, which can be due to the structural differences between aflatoxins and, to a lesser extent, to the experimental digestion conditions applied, the aflatoxin level or even the contamination type (natural or artificial) applied over the foodstuff (Hur et al., 2011; Kabak et al., 2009).

Considering that the final bioaccessible aflatoxin is the free toxin present in the soluble fraction of the duodenal extract, it is possible that the aflatoxins were initially linked to the milk matrix components and the digestive physio-chemical conditions provoked the leakage of these interactions and their release; or even a part from the aflatoxins remained at the original free form in the initial milk, and then were transferred to the final duodenal extract by mixing with the initial milk matrix without extractive action from the digestive fluids, according with Fernández-García et al., 2009. Aflatoxins which were not detected along the *in vitro* digestion could be formed by masked aflatoxins linked with matrix components (González-Arias et al., 2013), or aflatoxins partially degraded by some digestion conditions as the strongly acidic gastric pH at 1.8. This is in agreement with the ability of acidic substances as hydrochloric acid (used to adjust the gastric pH) of disturbing the aflatoxin structure, decreasing the concentration of the original aflatoxin, as Kabak et al., 2006 reviewed. Romero-Sánchez et al., 2023 also observed a reduction on the aflatoxin bioaccessibility from spiked rice during the gastric digestion step. Based on these hypothesis, new studies would be necessary to determine how the different physio-chemical digestive parameters affect aflatoxins behaviour.

3.3. Validation of analysis method for aflatoxins in commercial plant-based milks and in vitro digestion assay

The methodology for the analysis of aflatoxins B1, B2, G1 and G2 in commercial samples of plant-based milks was evaluated in terms of accuracy and precision. Firstly, LOD and LOQ were calculated for each aflatoxin in almond, oat, rice and soy milk matrices for the determination method. LOD values ranged between 4 ng/kg and 39 ng/kg, and LOQ values between 14 ng/kg and 131 ng/kg in the initial samples. LOD and LOQ values for the determination method are detailed on **Table S2** in the Supplementary Material. These values are far below the limits established by the current legislation for aflatoxin B1 and the total content of aflatoxins on cereals, legumes (2–4 μg/kg) and nuts (8–10 μg/kg) and their derived products, even with high dilution levels. On the other hand, LOD and LOQ range values for the analytical instrument were between 0.21 and 1.53 μg/L and between 0.69 and 5.09 μg/L. LOD and LOQ values for the analytical instrument are detailed on **Table S3** in the Supplementary Material. Linearity of the matrix-matched calibration curve was satisfactory within the range from 25 μg/L to 250 μg/L (equivalent to a range from 0.5 μg/kg to 5 μg/kg in the initial drink) for each type of aflatoxin present in the four plant-based milks, with R^2 values >0.99 in all cases. Regarding the recoveries obtained in this method, blank samples of each matrix were spiked by triplicate with each aflatoxin at 2.5 μg/kg previous to the application of the extraction and clean-up methodology. Recovery assays were repeated to test the intra-day (two assays per day) and inter-day (two different days) reproducibility, and the final range of media recoveries ranged from 73% to 98% in all matrices, with the only exception of AFG2 in oat milk with a recovery of 46%, and standard deviations resulted between 1% and 6%, and RSD range was between 1% and 8% in all cases. No differences were found between the recovery values obtained between different days nor into the same day in terms of accuracy or precision. The average from all the recovery results is represented in Fig. 4. Considering that the Commission Regulation (EC) No. 401/2006, 2006 establishes minimal recovery values between 70 and 110% in samples spiked at 1–10 μg/kg for aflatoxin extraction methodologies from foodstuffs, all the recoveries are in accordance with the legislation, except the recovery of AFG2 from oat milk. This low recovery value could be attributed to the presence of specific components in oat milk able to interact only with AFG2, thus preventing their extraction and retention on the cartridge. Regarding the matrix factor (MF), the final range of recoveries considering only the losses caused by the matrix effect ranged from 127% to 137% in rice milk, from 42% to 66% in oat milk, from 72% to 94% in almond milk, and from 46% to 73% in soy milk.

For the bioaccessibility test, the methodology applied to purify the aflatoxins from the digested extracts of spiked plant-based milks was evaluated to establish the aflatoxin losses exclusively due to the clean-up method. In this case, the range of recoveries for all the aflatoxins achieved by the clean-up methodology applied over the final spiked digestive extracts obtained from plant-based milks was 84%–100% for almond milk extracts, 96%–104% for oat milk extracts, 89%–99% for rice milk extracts, and 106%–116% for soy milk extracts. On the other hand, LOD and LOQ of all aflatoxins in the plant-based milk digestive extracts for the *in vitro* digestion methodology resulted in the following ranges: LOD at 0.42–13.31 μg/L and LOQ at 1.41–44.38 μg/L for almond milk; LOD at 0.56–1.80 μg/L and LOQ at 1.86–6.00 μg/L for oat milk; LOD at 0.15–2.29 μg/L and LOQ at 0.50–7.64 μg/L for rice milk; and LOD at 0.16–2.21 μg/L and LOQ at 0.53–7.38 μg/L for soy milk.

4. Conclusions

Considering the growing implementation of the plant-based milks as healthy and vegan dietary alternatives in addition to their contamination susceptibility by aflatoxigenic fungus, the current optimized method to analyse the aflatoxins B1, B2, G1 and G2 from almond, oat, rice and soy milks represents a useful tool to test the presence of these highly toxic substances in commercial samples.

This method of analysis has detected AFB1, AFB2 and AFG1 in at least one sample of soy milk and, although the contaminations could not be quantified, the presence of these aflatoxins on plant-based milks suppose a health risk for the customer, especially AFB1.

On the other hand, the *in vitro* digestion assay performed over almond, oat, rice and soy milks has demonstrated the high influence of the aflatoxin structure and the plant-based nutritional composition on the final bioaccessibility of aflatoxins. However, more *in vitro* digestion studies are needed to elucidate the specific interaction between aflatoxins and the matrix components of each type of milk, to detect sources of aflatoxin losses during the digestion, and to extend aflatoxin bioaccessibility studies to other plant-based milks, as new types of plant-based beverages are continuously entering the market.

Finally, the moderate bioaccessibility observed for AFB1 from the plant-based milks tested supposes a human exposure barrier against this hazardous aflatoxin, that suppose an additional health advantage for plant-based milks compared to other contaminated foodstuffs.

CRediT authorship contribution statement

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

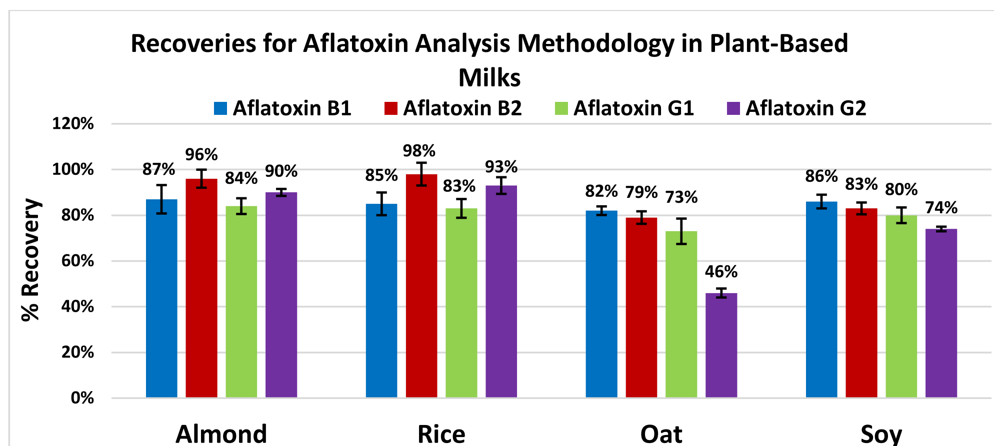

Fig. 4. Mean recovery percentages and standard deviations obtained for aflatoxin B1, B2, G1 and G2 from spiked almond, rice, oat and soy milks after the application of the plant-based milks analysis method.

Funding acquisition, Conceptualization.

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.2024.140538.

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