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Title: DETERMINATION OF MYCOTOXINS IN PLANT-BASED BEVERAGES USING QUECHERS AND LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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***Cover Letter**

Editorial Office

Food Chemistry

Tarragona, 9th September 2016

Dear Dr. Paul M. Finglas,

Please find attached the revised manuscript entitled “Determination of mycotoxins in plant-based beverages using QuEChERS and liquid chromatography-tandem mass spectrometry” by E. Miró-Abella, P. Herrero, N. Canela, Ll. Arola, F. Borrull, N. Fontanals and myself, to be considered for publication in Food Chemistry. This manuscript is new and original, and has been revised taking into account reviewers suggestions.

Looking forward to hearing from you.

Yours sincerely,

Dr. R. Ras

Reviewers' comments:

Reviewer #1:

Authors proposed a new method for the determination of mycotoxins in plant-based beverages. The topic fits the scope of "Food Chemistry". There are some issues that authors should address before the manuscript can be published in this journal.

Comments:
- Highlights: "QuEChERS" instead of "Quechers".

Sorry for the mistake. That term has been corrected.

- Title: Maybe "mycotoxins in plant-based" instead of "mycotoxins from plant-based".

We agree with the suggestion and the title has been modified.

- Abstract: Provide more information as recovery ranges obtained and LOQ values.

More information was added in the abstract according to the comment.

- Line 115: Include the three beverages covered by this study.

It was an error since the name of the studied beverages had not been included in the previous version. This has been included in the present version (line 118).

- Line 130: Indicate the stability of the mixed solutions.

The stability has been detailed in the manuscript (line 135).

- Line 167: Update SANCO reference. Currently is SANTE 11945/2015.

Accordingly with the suggestion, mentioned reference has been updated here and also in the same way the other times that appeared in the text (lines 176, 225, 322).

- Line 215: When instrumental linearity was studied, in addition to determination coefficient, provide linear range. Moreover, LODs and LOQs calculated in this Section (3.1) are instrumental lower limits. In this sense, instrumental LOQs are not provided.

In agreement with the comment, obtained LODs and LOQs values and the linear range were added to the commented section (line 236).

- Line 237-238: No formic acid was added in the original QuEChERS, so this sentence should be revised.

This has been corrected in the manuscript (line 262).

- Line 261: Set the ME range where matrix effect is acceptable.

This information has been inserted in the manuscript in consonance with that comment (line 294).

- Line 286: Specify the criteria used to estimate MQLs. Current Guidelines (as SANTE) indicate that these lower limits should provide suitable precision and recovery.

The criteria used was the same as for determine the instrumental limits. Nevertheless, to clarify it, and as suggested, we have included in the text (line 320).

- Line 300: Indicate the concentration levels used to estimate recovery. Currently more than one level is commonly used to ensure the trueness of the method in the whole linear range.

In order to study the recovery values we used two different concentrations for each analyte and we obtained similar results. For that reason, we detailed in Table 2 the concentration that was near to the values that can be found in real samples, and this concentration is which appear in the manuscript. To clarify it, this has been explained in the text (line 267).

- Line 308-315: This study is very interesting but I consider that it should be placed in Section 3.3.

Thank you for the suggestion. The paragraph with such information has been moved to the suggested section (line 345-352).

- Line 314: Indicate which representative matrix was used for quantification purposes.

Matrix information was added to the mentioned section in agreement with the suggestion (line 352).

- Line 351: Specify the meaning of "ER".

In agreement with the suggestion, the mistake has been modified (line 396).

- Figure Caption (Figure 1): Indicate the concentration of the mycotoxins detected.

Mycotoxin concentrations were added in Figure 1 in accordance with the comment.

- Figure 1: Some peaks (AFG2 and ZEA) are not well-resolved. This should be discussed in the text.

The reviewer is right. This has been discussed and introduced in the manuscript (line 372).

Reviewer #2:

The present manuscript describes the application of an already available method (by Anastassiades et al.) to soy, rice and oats beverages. Since the method has been applied to beverages without any further optimization, the only degree of novelty of the present work would be represented by the application to real samples. However method application to only beverage samples is reported.

To justify manuscript publication the number of beverage samples should be therefore increased either to add some novelty to the work and to demonstrate the usefulness of the presented method, providing data on mycotoxin co-occurrence in the matrices of interest.

Thank you for your suggestions, we are in agreement that the number of beverage samples is little but we wanted to focus our study only on beverages derived from cereals and there are only these three types commercialised, that have been never studied previously, as far as we know. There are also some derivatives with alcohol, such as beer on rice liquor but extraction process of alcoholic drinks would be different. Taking this into account, we made the study using three different commercial brands in order to increase the diversity and the number of studied samples, resulting in a total of 9 studied samples. This also allowed us to examine if there were differences between brands. In fact, no significant differences were found between them, as it can be seen in the study.

During last years, these beverages have been taking huge relevance according to actual lifestyle as well as with the increasing percentage of people suffering from allergies. The cereal based beverages, analysed in the present study, are allowed for allergic population, and also for children. We focused on getting very low limits of detection in order to obtain a method useful for the analysis of these fashioned drinks, including consumption for children where limits are below. These low limits were achieved for all samples.

As the suggestion, we agree that we have made only few changes in Anastassiades QuEChERS method (we introduced formic acid in the extraction buffer), but it is the one that performed best and a prove of this is its great applicability.

Other suggestion to improve the manuscript text are below:

- References mentioned in the Introduction are not always relevant. The whole reference list should be double checked. As an example, when discussing about mycotoxin toxicity (page 3 - lines 54-58) it would be more pertinent and exhaustive to mention evaluations by EFSA, JECFA, and/or IARC. By the way, the paper by Campone et al 2015 describes a method for aflatoxins

determination and not a toxicity study. More or less the same consideration applies to the last part of the introduction (pag 5 - lines 94-110) mentioning analytical methods for mycotoxin analysis. I would suggest to add in the reference list some review discussing the state-of-art of (multi) mycotoxin methods.

Thank you for the suggestion. Changes have been done in the introduction along with its references in order to improve it (lines 60 and 65). It was a mistake to use articles instead of evaluations of agencies when the text was related to toxicity. Also some reviews (Capriotti, Caruso, Cavaliere, Fogli, Samperi & Laganà, 2012; Köppen, Koch, Siegel, Merkel, Maul & Nehls, 2010) have been added in consonance with the recommendation (line 101).

- Results and discussion: Instrumental optimization. The whole paragraph describing optimization of conditions for mass spectrometry and liquid chromatography describes the setting of instrumental parameters that is routinely carried out in laboratories using LC-MS for determination of regulated mycotoxins. The relevant text should be deleted (or significantly shortened).

In agreement with the suggestion, we shortened the instrumental optimization section as far as possible.

- Results and discussion: Method validation. The authors should specify how they determined the limits of detection and the limits of quantification. For instance, the Quantification limit should be estimated according to official guidelines, as "the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy" (see CEN TR 16059 and ICH Q2A: Validation of Analytical Methods: Definitions and Terminology/III/5626/94).

Taking into account this comment and also suggested by Reviewer 1, the determination of limits of detection and quantification has been detailed in the manuscript (line 236).

Reviewer #3:

1 (Page 2, line 25): The abbreviation UHLPC-(ESI)MS/MS should be corrected to UHPLC-(ESI)MS/MS.

Sorry for the writing mistake, it has been corrected.

2 (Page 5, line 118): In this section, the authors should tell us the detail information of the beverages used in this work.

Some sample information was added to the mentioned section in agreement with the suggestion (line 143).

3 (Page 9, line 215): The authors should specify how to calculate LOD and LOQ.

This has been added in the text.

4 (Page 10, line 218): The LODs of other six mycotoxins in this work should be added.

We agree with the suggestion and LODs of all mycotoxins were added in this work. We did not detail this before, since each mycotoxin has different LODs according to its response in UHPLC-MS/MS. However, as the review suggests us to include it, we have done so (line 236).

5 (Page 11, line 243): A single concentration of spiked samples is not enough to study the recovery for every mycotoxin.

This has been also answered to reviewer 1 and in the text (line 267).

6 (Page 22): In the table 1, the rules of writing subscripts should be noticed and the abbreviation should keep the same, such as NH4 (NH4), AFG2 (AFG2).

Sorry for the mistakes. These abbreviations were corrected.

HIGHLIGHTS

- Mycotoxins are determined using ~~Quechers~~QuEChERS followed by LC-MS/MS
- The method is applied for the first time to analyse different plant-based beverages
- Recovery and matrix effect are similar among the different type of samples analysed
- Some mycotoxins are found in oat, soy and rice beverages

DETERMINATION OF MYCOTOXINS ~~FROM~~IN PLANT-BASED BEVERAGES USING QUECHERS AND LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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Abstract

A method was developed for the simultaneous determination of 11 mycotoxins in plant-based beverage matrices, using a QuEChERS extraction followed by ultra-high performance liquid chromatography coupled to tandem mass spectrometry detection (~~UHLPC~~UHPLC-(ESI)MS/MS). This multi-mycotoxin method was applied to analyse plant-based beverages such as soy, oat and rice.

QuEChERS extraction was applied obtaining suitable extraction recoveries between 80 and 91%, and good repeatability and reproducibility values. Method Quantification Limits were between 0.05 µg L^{-1} (for aflatoxin G_1 and aflatoxin B_1) and 15 µg L^{-1} (for deoxynivalenol and fumonisin B_2). This is the first time that plant-based beverages have been analysed, and certain mycotoxins, such as deoxynivalenol, aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, ochratoxin A, T-2 toxin and zearalenone, were found in the analysed samples, and some of them quantified between 0.1 µg L^{-1} and 19 µg L^{-1}.

Keywords *Mycotoxin; Plant-based beverages; QuEChERS; UHPLC-(ESI)MS/MS*

1. Introduction

Mycotoxins are natural secondary metabolites produced by some species of filamentous fungi of the *Aspergillus*, *Penicillium* and *Fusarium* genera ~~(Richard, 2007)~~(Richard *et al.*, 2007). Over 400 types of mycotoxins are reported, classified by their structure, their biological source or the moment of production from preharvest on the plant culture to storage, transport or processing stages (Bhat, Rai, & Karim, 2010). Modern techniques and good practices of handling and preserving food and feed reduce the presence of mycotoxins. Nevertheless, these species also grow in cereals, fruit and milk (Bhat *et al*., 2010). Of all mycotoxins, aflatoxin B_1 (AFB_1) is the most potent carcinogen, but all mycotoxins are harmful in different ways, displaying acute and chronic toxicity, such as genotoxicity, carcinogenic toxicity, immunotoxicity (immunostimulatory or immunosuppressive), mutagenicity, nephrotoxicity and teratogenicity attributes ~~(Campone *et al.*, 2015; Sobrova *et al.*, 2010).~~(EFSA, 2007; EFSA 2014).

The main foods affected are cereals, nuts, dried fruit, coffee, cocoa, spices, oil seeds, dried peas, beans and several types of fruit, particularly apples, or sub-products produced from contaminated raw materials, such as wine and beer ~~(Piacentini, Savi, Olivo, & Scussel, 2015; Turner, Subrahmanyam, & Piletsky, 2009).~~(EFSA, 2013). Mycotoxins are a serious health risk present throughout the entire food chain as they display stability at high temperatures and withstand cooking processes (Bullerman & Bianchini, 2007). People can be intoxicated if they eat either contaminated food or products, such as eggs, meat and milk from animals that previously consumed these toxins. In order to reduce the effects of mycotoxin ingestion,

the European Union Commission Regulation establishes the maximum levels allowed in certain kinds of food for the major mycotoxins, such as aflatoxins (AFG_1, AFG_2, AFB_1, AFB_2), fumonisins (FB_1, FB_2), ochratoxin A (OTA), deoxynivalenol (DON) and zearalenone (ZEA) (EC, 2007), and recommends the maximum levels for the sum of T-2 toxin (T-2) and HT-2 toxin (HT-2) (EC, 2013). For example, the maximum level allowed in the case of AFB_1 in all cereals and all derivatives is 2.0 μg kg^{-1}. Consequently, this might be the maximum level permitted for oat- and rice-based products. However, this regulation does not consider the mycotoxin levels that may exist in legumes, such as soybeans. Soybeans are not a product that favours the production of certain mycotoxins, but there is still a risk as the presence of the main fungi contributor to aflatoxin production has been reported in this type of legume (Nesheim & Wood, 1995).

Over the last few years, the consumption of beverages of plant origin has increased for medical reasons (e.g. due to intolerances and allergies), or as part of an alternative lifestyle (Lawrence, Lopetcharat, & Drake, 2016; Mårtensson, Öste, & Holst, 2000). If the raw material contains mycotoxins, the resulting beverage will also probably contain these toxins. To analyse these mycotoxins during beverage production, it is important to note that, depending on the raw plant material composition, the beverage might be very different (Mäkinen, Uniacke-Lowe, O'Mahony, & Arendt, 2015), which results in different interferences between matrices when determining the analytes of interest. Considering these differences, finding a common method to determine different mycotoxins for all of the different types of beverages is challenging.

There are different extraction techniques suitable for mycotoxin isolation, such as liquid-liquid extraction (LLE) ~~(Aguilera-Luiz, Plaza-Bolaños, Romero-González, Vidal, & Frenich, 2011)~~ and solid-phase extraction (SPE) ~~(Aguilera-Luiz *et al.*, 2011)~~ for liquid samples, and pressurized liquid extraction (PLE) ~~(Campone *et al.*, 2015)~~ and solid-liquid extraction (SLE) ~~(Beltrán *et al.*, 2013)~~for solid samples, among others (Capriotti, Caruso, Cavaliere, Fogli, Samperi & Laganà, 2012; Köppen, Koch, Siegel, Merkel, Maul & Nehls, 2010). The method selection depends on the nature of the matrix, its characteristics and complexity. However, some of these methods are expensive, complex, and/or involve considerable consumption in terms of time and solvent. In order to minimize the sample treatment but prevent exposure to matrix effects, a Quick, Easy, Cheap, Effective, Rugged and Safe method (QuEChERS) is a suitable alternative. The QuEChERS method has been used for mycotoxin extraction from food, both in solid samples, such as dried fruit (Azaiez, Giusti, Sagratini, Mañes, & Fernández-Franzón, 2014), pseudocereals, spelt and rice (Arroyo-Manzanares, Huertas-Pérez, García-Campaña, & Gámiz-Gracia, 2014), and in liquid samples, such as wine (Pizzutti et al., 2014) and beer (Rodríguez-Carrasco, Fattore, Albrizio, Berrada, & Mañes, 2015). However, plant-based beverages have not previously been analysed and QuEChERS extraction could be a proper choice.

The aim of this study is to develop a method for the simultaneous determination of 11 mycotoxins ~~in three different types of~~from soy, oat and rice plant-based beverages, using QuEChERS extraction followed by UHPLC-(ESI)MS/MS.

2. Materials and methods

2.1 Reagents and chemicals

The target mycotoxins, which are restricted or subject to recommendations by the European legislation (EC, 2007; EC, 2013), were four aflatoxins (AFB_1, AFB_2, AFG_1, and AFG_2), OTA and six Fusarium toxins (DON, ZEA, T-2, HT-2, FB_1 and FB_2). They were purchased (>99% purity) from Trilogy Analytical Laboratory (Washington, WA, USA). AFB_1, AFB_2, AFG_1 and AFG_2 were in acetonitrile (ACN) at 25·mg L^{-1}; ZEA, DON and OTA were in methanol (MeOH) at 25·mg L^{-1}, 100 mg L^{-1} and 10 mg L^{-1}, respectively; T-2 and HT-2 were in ACN at 100 mg L^{-1}; and a mixture of FB_1 and FB_2 was in ACN/water (50:50, v/v) at 100 mg L^{-1} and 30 mg L^{-1}, respectively. A mixed solution of all of the analytes was prepared at 1 mg L^{-1} for all of the analytes, except in the case of FB_2 at 0.3 mg L^{-1}, in MeOH/H_2O (1:1, v/v). Mixed solutions were stored at 4ºC~~.~~ for six months.

MeOH and ACN, both for LC-MS, were purchased from Panreac (Barcelona, Spain). Ultrapure-grade water was obtained from a MilliQ water purification system (Millipore, Darmstadt, Germany). Formic acid (HCOOH) ~98% and 10 M ammonium formate (NH_4HCOO) aqueous solution were purchased from Fluka (St. Louis, MO, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. QuEChERS extraction packets (4 g $MgSO_4$, 1 g NaCl) were obtained from Agilent Technologies (Waldbronn, Germany).

Real samples were soy, oat and rice plant-based beverages obtained from local supermarkets. Three different commercial brands were selected for each cereal.

146 It is important to take certain security measures when handling
147 mycotoxins, such as wearing double gloves (latex underneath and nitrile on
148 top) and cleaning all laboratory materials that have been in contact with
149 mycotoxins, including old solutions, with 20% commercial sodium hypochlorite
150 (NaClO).
151
152 **2.2 Liquid chromatography-mass spectrometry**
153 Chromatographic analyses were performed in an Agilent 1290 Infinity LC
154 Series coupled to a 6495 iFunnel Triple Quadrupole MS/MS with an
155 electrospray ionisation (ESI) interface, all from Agilent Technologies,
156 operating in positive ion mode. Chromatographic separation was performed
157 using a Cortecs UHPLC C_{18} column (100 mm x 2.1 mm, 1.6 µm) from Waters
158 (Wexford, Ireland).
159 The chromatographic separation was performed by gradient elution
160 using a binary mobile phase constituted of water (solvent A) and MeOH
161 (solvent B), both with 5 mM NH_4HCOO and 0.1% HCOOH. The elution started
162 at 10% of B and increased up to 50% in 4.5 min, then to 95% in 7.5 min,
163 remaining in isocratic mode for 2.5 min. The injection volume was 10 µL, the
164 flow rate was fixed at 0.45 mL min^{-1} and the column temperature was held at
165 40ºC. Samples were kept in the autosampler at 4ºC until analysis.
166 The source parameters were a capillary voltage of 4,000 V for
167 aflatoxins and 3,500 V for the rest of compounds, desolvation gas flow and
168 temperature of 18 L min^{-1} and 160ºC, nebulizer pressure of 35 psi, nozzle
169 voltage of 500 V, fragmentor voltage of 380 V, cell acceleration voltage of 5 V,
170 and sheath gas flow and temperature of 11 L min^{-1} and 350ºC. The high and

low pressure funnel parameters were, respectively, 180 and 150 V for aflatoxins and 150 and 90 V for the rest of compounds. The acquisition was performed in Multiple Reaction Monitoring (MRM) mode in positive polarity. For each analyte, three characteristic MRM transitions were monitored, in accordance with the European Commission guidelines ~~(SANCO Document, 2011).~~(SANTE Document, 2015). Four different time segments were also established in order to improve sensitivity. All of these parameters are specified in Table 1.

2.4 Sample preparation

For the extraction of soy, oat and rice plant-based beverages, the original QuEChERS extraction method (Anastassiades, Lehotay, Štajnbaher, & Schenck, 2003) was used just with the addition of formic acid in the extraction buffer. Briefly, 10 mL of sample was added to a 50 mL centrifuge tube with 10 mL ACN with 1% HCOOH and shaken for 3 min. Then, 4 g of $MgSO_4$ and 1 g of NaCl were added to the solution, and shaken vigorously for 3 min. Afterwards, the tubes were centrifuged at 10,000 rpm at 20ºC for 5 min. Finally, 1 mL aliquot of the supernatant phase (organic layer) was diluted 1:1 (v/v) with solvent A of the mobile phase, and filtered with a 0.2 µm nylon filter (GVS Filter Technology, Indianapolis, IN, USA). The extracts were stored at 4ºC until analysis in order to preserve their stability.

3. Results and discussion

3.1 Instrumental optimisation

With the aim of identifying the optimal conditions for the ESI of mycotoxins, different concentrations of HCOOH (0-0.3%) and NH_4HCOO (0-10 mM) on mobile phase were tested, since the addition of buffers to the mobile phase allows a reduction in sodium adducts, improving analyte ionisation (Campone *et al*., 2015). The addition of HCOOH is important, especially in the case of fumonisins (FB_1 and FB_2), because it increases their sensitivity and improves their peak shape (Zollner & Mayer-Helm, 2006). However, higher buffer concentrations cause ion suppression (Beltrán, Ibáñez, Sancho, & Hernández, 2009). After testing the different mobile phase compositions, the best one was 0.1% HCOOH and 5 mM NH_4HCOO (pH 3.1), which allows the highest level of ionisation for all of the analytes in a suitable chromatographic separation under the gradient applied. Under these conditions, all of the mycotoxins are better ionised in positive mode, presenting an abundance of $[M+H]^+$ ion, except for the T-2 and HT-2 toxins, which were ionised as ammonium adducts $[M+NH_4]^+$ in a more abundant form. ~~This is also in line with previous works, in which the presence of $[M+NH_4]^+$ adducts for T-2 and HT-2 were also reported (Arroyo-Manzanares et al., 2014; Azaiez et al., 2014; Beltrán et al., 2013, 2009; Jackson, Kudupoje, & Yiannikouris, 2012; Lattanzio, Ciasca, Powers, & Visconti, 2014). Moreover, in the present work, the cationic form of ZEA was the most abundant, in accordance with some authors (Arroyo-Manzanares *et al*., 2014), rather than the anionic form, which is selected in some of the studies (Beltrán *et al*., 2013, 2009; Jackson *et al*., 2012; Lattanzio *et al*., 2014).~~ All these adducts are well study and reported by previous works (Arroyo-Manzanares *et al*., 2014; Azaiez *et al*., 2014; Beltrán

et al., 2009; Jackson, Kudupoje, & Yiannikouris, 2012; Lattanzio, Ciasca, Powers, & Visconti, 2014).

Once the precursor ions were selected, different collision energies were applied to obtain three product ions for each mycotoxin and thus three MRM transitions, which are specified in Table 1. These three selected transitions enable the correct identification of every toxin as recommended by the EU directive ~~(SANCO Document, 2011)~~ (SANTE Document, 2015) and most of them have previously been reported in the literature ~~(Arroyo-Manzanares *et al.*, 2014; Azaiez *et al.*, 2014; Beltrán *et al.*, 2009; Jackson, Kudupoje, & Yiannikouris, 2012; Lattanzio, Ciasca, Powers, & Visconti, 2014).~~(Arroyo-Manzanares *et al.*, 2014; Beltrán *et al.*, 2009; Jackson *et al.*, 2012; Lattanzio *et al.*, 2014).

After studying the instrumental linearity (with $r^2 \geq 0.992$), the detection limits (LOD) and quantification limits (LOQ) were determined by adopting the criteria of a signal-to-noise ratio (S/N) equivalent to 3 and 10, respectively. ~~The LODs were between 0.25 ng L^{-1} (for AFG_2, AFG_1, AFB_2 and AFB_1) and 0.25 µg L^{-1} (for HT-2).~~

Obtained LODs were 0.001 µg L^{-1} (for AFG_2, AFG_1, AFB_2 and AFB_1), 0.04 µg L^{-1} (for FB_1, FB_2 and ZEA), 0.01 µg L^{-1} (for OTA and T-2), 0.1 µg L^{-1} (for DON) and finally 0.25 µg L^{-1} (for HT-2). Regarding to obtained LOQs they were 0.003 µg L^{-1} (for AFG_2, AFG_1, AFB_2 and AFB_1), 0.2 µg L^{-1} (for FB_1, FB_2 and ZEA), 0.03 µg L^{-1} (for OTA and T-2), 0.3 µg L^{-1} (for DON) and finally 0.9 µg L^{-1} (for HT-2). Linear range was from LOQ to 100 µg L^{-1} (for AFG_2, AFG_1, AFB_2, AFB_1 and OTA), to 500 µg L^{-1} (for DON, FB_2 and T-2) and to 1000 µg L^{-1} (for FB_1, HT-2 and ZEA).

3.2 QuEChERS extraction optimisation

Initially, a simple solid-liquid extraction method successfully applied by Beltr~~a~~án *et al*. (Beltrán *et al.*, 2013) for solid matrices was adapted for these liquid matrices. To specify, the method involved mixing 250 µL of plant-based beverage with 1 mL of ACN 0.1% HCOOH, which was then shaken for 20 min, and centrifuged at 4,000 rpm for 10 min, before adding a supernatant aliquot diluted with aqueous solvent of the mobile phase (1:4, v/v). However, the high sugar content of the extracts caused a loss in the reproducibility of the results obtained. Thus, to solve this problem a pretreatment with QuEChERS was applied.

With respect to the different QuEChERS methods (the European Committee for Standardization (CEN) Method 15662, the AOAC Official Method 2007.01 and the original QuEChERS method (Anastassiades *et al*., 2003)), different studies (Koesukwiwat, Sanguankaew, & Leepipatpiboon, 2014; Martínez-Domínguez, Romero-González, & Garrido Frenich, 2016; Rubert *et al.,* 2014) have shown that there are no significant differences between them. Thus, considering the simplicity of the original QuEChERS method, it was selected for the present study~~.~~ with the extraction buffer with formic acid.

Prior to recovery studies, the samples (oat, soy and rice plant-based beverages) were analysed in order to subtract the possible signal of analytes present. ~~Then, the samples were spiked to different mycotoxin concentrations~~ Then, analytes were added to fortify samples at two different group concentrations to calculate extraction recoveries. One concentration group

was near to the highest concentration range and was at 50 µg L^{-1} (for AFG_2, AFG_1, AFB_2, AFB_1 and OTA), at 250 µg L^{-1} (for DON, FB_2 and T-2) and at 500 µg L^{-1} (for FB_1, HT-2 and ZEA). The other concentration group was lower than the previous but analytes concentrations were according to their sensitivity in UHPLC-(ESI)MS/MS, with the aim of obtaining similar analyte response values. To do so, samples were spiked to concentrations of 10 µg L^{-1} of AFB_1, AFB_2, AFG_1, AFG_2, ZEA, OTA, FB_1 and T-2, 3 µg L^{-1} of FB_2, 50 µg L^{-1} of DON and 100 µg L^{-1} of HT-2. Obtained results were similar at both concentrations and finally only lower concentrations were used to calculate extraction recoveries because there were near to the real concentrations that usually appear in real samples.

Extraction recoveries (ER) were calculated by comparing the analyte concentration when the sample was spiked before and after extraction. Matrix effects (ME) were calculated by comparing the concentration when the sample was spiked after extraction with the calibration standard response, as well as taking into account the analyte concentration in non-spiked samples. ER and ME percentages were calculated according to following equations:

$$\%ER = \frac{C_{before} - C_{non\text{-}spiked}}{C_{after} - C_{non\text{-}spiked}} \times 100 \qquad \%ME = \left(\frac{C_{after} - C_{non\text{-}spiked}}{C_{calibration\ curve}} \times 100 \right) - 100$$

The results, which are detailed in Table 2, show excellent extraction recoveries in all matrices, with values between 80% and 91%. The recoveries obtained were in accordance with previously reported recoveries in liquid matrices, such as wine (Pizzutti *et al*., 2014) and beer (Rodríguez-Carrasco *et al*., 2015). With respect to the ME, values among the three types of matrices

were similar but differed depending on the mycotoxin, as can be observed in Table 2. As can be seen, all of the ME values were acceptable with values up to 45%, with the exception of DON, FB_1 and FB_2. DON underwent ion suppression in all of the matrices, and the high values obtained might be attributed to the polar nature of the analyte ~~(Sobrova et al., 2010~~ (Sobrova, Adam, Vasatkova, Beklova, Zeman & Kizek, 2010, Wang & Li, 2015). In contrast, FB_1 and FB_2 displayed significant ion enhancement, especially in the case of FB_2. This fumonisin enhancement was also previously observed in cereal grains (Jackson *et al*., 2012) and in liquid and powder milk ~~(Wang & Li, 2015)~~(Wang *et al.*, 2015), where these mycotoxins showed strong ion enhancement. In view of these ME values, different attempts to reduce them were tested. However, none of these attempts were successful for the other mycotoxins studied. Thus, this ME was assumed in the rest of the study.

3.3 Method validation

The method validation was performed before its application to sample analysis, for the 11 selected mycotoxins in three different liquid matrices: oat, soy and rice beverages.

For the method validation, linear range, limits of detection (MDL) and limits of quantification (MQL), accuracy, repeatability and reproducibility were studied. All of the above parameters were calculated when 10 mL of sample were analysed following the procedure described above. In order to compensate for the ME, the matrix-matched calibration approach was studied for each matrix. The linear range was between the MQLs and 200 µg L^{-1} for AFB_1, AFB_2, AFG_1, AFG_2, OTA and ZEA, at 600 µg L^{-1} for FB_2, and at 2,000

319 µg L^{-1} for DON, HT-2, FB_1 and T-2. The linearity of the method was good with
320 $r^2 \geq 0.993$ in all matrices.

321 MDL and MQL were estimated in the same way than instrumental limits
322 detailed previously. Taking into account current guidelines (SANTE
323 Document, 2015) obtained limits afford suitable precision, accuracy and
324 recovery results making them acceptable. The MQLs are all shown in Table 3,
325 which are in line with the response provided in the instrumental UHPLC-
326 MS/MS. The MDLs in the present study were between 0.02 µg L^{-1} and 0.4 µg
327 L^{-1} for AFG_2, AFG_1, AFB_2, AFB_1, FB_1, T-2, OTA and ZEA, and, for the rest of
328 compounds, they were between 2 µg L^{-1} and 5 µg L^{-1}. The maximum
329 mycotoxin limits established for certain food commodities by the European
330 Union Commission Regulation (EC, 2006) were used as reference values for
331 the studied samples, because of the lack of regulation. If these regulated
332 levels are taken as a reference, the MQLs obtained are between 10 and 100
333 times lower.

334 The method repeatability (intra-day, n=5) and reproducibility (inter-day,
335 n=5), expressed as relative standard deviation (%RSD), were tested at
336 concentration levels that correspond to ten times the MQLs of each
337 compound. Good repeatability and reproducibility results were obtained, all
338 below 9% and 19%, respectively, in accordance with the guidelines.

339 With respect to accuracy, the obtained results were excellent for all
340 three matrices and all of the analytes. The accuracy values for the oat-based
341 beverage were between 82% and 110%, while the values for soy were
342 between 91% and 112%, and, in the case of rice, the values were between

91% and 110%. As can be observed, there were no significant differences between the matrices.

~~**3.4 Application to beverage samples**~~

Prior to analysis of different samples, a comparison was performed between matrix-matched calibration curves obtained for three matrices in order to identify whether there were significant differences between them. Firstly, slope standard deviations (S_b) of each matrix were compared using the F-Fisher test, and then the T-student test for the slope (b) comparison was applied, with $\alpha=0.05$. The results showed that all of the slopes were comparable. In consequence, a single matrix-matched curve could be used for studying all of the different plant beverage matrices. The matrix-matched curve selected in the present study was the obtained from rice.

3.4 Application to beverage samples

The developed methodology was applied for the analysis in triplicate of three types of plant-based beverages (soy, oat and rice) from three different commercial brands obtained from local supermarkets. Some of the studied mycotoxins were detected and/or quantified (at very low concentrations) in the samples, and the results are shown in Table 3.

In the case of oat beverages, DON, AFG_2, AFG_1, AFB_2, AFB_1, HT-2, T-2, OTA and ZEA were found in some of the analysed samples. One interesting feature is that the concentrations found for the analytes in samples B and C were very similar. This is explained by the fact that, although they are from different brands, they were found to come from the same source. With respect to DON, it is also widely detected in solid oat samples (Jestoi, *et al.,*

2004; Juan, Ritieni, & Mañes, 2013). HT-2 was found in one sample and T-2 was quantified in all of them, in line with the literature, which confirms that HT-2 and T-2 are predominantly detected in oats and oat-based products ~~(Köppen *et al.*, 2015).~~ (Köppen, Bremser, Stephan, Klein-Hartwig, Rasenko & Koch, 2015). As an example, Figure 1 shows the MRM chromatograms for one of the oat samples analysed. In this figure, AFG_2 and ZEA showed poor resolution, although peak separations were tried to improve without success. Nevertheless, it should be taken into consideration that the concentration of these compounds is below MQL. In any case, analytes identifications were always performed with all the obtained product ions.

With respect to soy beverages, AFG_2, AFG_1 and AFB_1 were detected in one or two of the soy samples studied, in agreement with the previous literature, which found these aflatoxins in soybean samples and soy derivatives ~~(Xie et al., 2014).~~(Xie, Lai, Saini, Shan, Cui & Liu, 2014). Furthermore, OTA and ZEA were found in some of the analysed samples.

Finally, with regard to rice beverages, DON, AFB_1, OTA and ZEA were found in some of the studied samples. These results agree with those obtained by some authors, who found these toxins in different types of grain rice samples (Arroyo-Manzanares ~~et al.,~~ *et al.*, 2014; Serrano, Font, Ruiz, & Ferrer, 2012). With respect to AFG_2, AFG_1 and AFB_2, they were not detected in rice beverage samples, which is also in agreement with other studies that analyse rice samples (Serrano *et al.*, 2012).

4. Conclusions

This is the first study in which plant-based beverages have been analysed to determine the presence of several mycotoxins. A sensitive, reliable and multi-analyte method were developed for the quantification of eleven mycotoxins using QuEChERS extraction followed by UHPLC-(ESI)MS/MS.

The applied QuEChERS approach was suitable for the extraction of the target mycotoxins from this kind of matrices, as shown by the ~~ER~~extraction recovery values obtained above 80%, and with ME values comparable to other studies that determine mycotoxins in other matrices.

The method was applied to the analysis of different plant-based beverages and some of the mycotoxins were found at low µg L^{-1} levels.

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561 **Table 1**. LC-MS/MS parameters for mycotoxin determination.

Start time (min)	Mycotoxin	Retention Time (min)	Precursor ion (m/z)		Dwell time (ms)	Product ion (m/z)*	CE (eV)
0	DON	2.2	297.1	$[DON + H]^+$	170	249.1 (100) 231.1 (57) 203.1 (61)	10 10 12
3	AFG_2	4.9	331.1	$[AFG_2 + H]^+$	40	313.1 (100) 245.1 (40) 257.0 (65)	25 25 35
	AFG_1	5.2	329.1	$[AFG_1 + H]^+$		243.0 (100) 200.0 (60) 283.0 (35)	30 45 25
	AFB_2	5.5	315.1	$[AFB_2 + H]^+$		287.0 (100) 259.0 (93) 243.0 (43)	30 30 45
	AFB_1	5.8	313.1	$[AFB_1 + H]^+$		241.0 (100) 285.1 (99) 213.0 (60)	42 25 50
6.1	HT-2	6.3	442.2	$[HT\text{-}2 + NH_4]^+$	55	215.0 (100) 263.0 (89) 197.0 (48)	12 15 20
	FB_1	6.5	722.4	$[FB_1 + H]^+$		334.1 (100) 352.1 (77) 703.9 (78)	42 35 35
	T-2	6.8	484.2	$[T\text{-}2 + NH_4]^+$		215.1 (100) 185.1 (82) 305.2 (95)	15 15 10
7	FB_2	7.0	706.4	$[FB_2 + H]^+$	55	336.1 (100) 318.1 (65) 74.1 (29)	45 40 35
	OTA	7.1	404.1	$[OTA + H]^+$		239.0 (100) 220.8 (41) 193.0 (34)	30 45 45
	ZEA	7.2	319.2	$[ZEA + H]^+$		283.1 (100) 187.1 (59) 203.0 (39)	15 20 20

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567 **Table 2**. Extraction recoveries (%) and matrix effects (%) obtained for the three
568 samples studied spiked with the analyte mixture. See the text for information about
569 concentrations.

Mycotoxin	Oat		Soy		Rice	
	Extraction recoveries	Matrix effects	Extraction recoveries	Matrix effects	Extraction recoveries	Matrix effects
DON	87	-52	84	-56	87	-56
AFG_2	88	5	87	-10	86	-12
AFG_1	89	35	87	34	87	12
AFB_2	88	0	86	-7	85	-11
AFB_1	86	43	88	38	86	19
HT-2	90	-2	88	-6	88	-6
FB_1	80	76	82	63	85	75
T-2	86	13	89	-8	86	6
FB_2	89	>100	80	>100	83	>100
OTA	91	23	89	21	87	1
ZEA	88	16	90	3	87	-5

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575 **Table 3.** Mycotoxin concentration (µg L^{-1}) found in the analysed beverage samples.

Mycotoxin	MQL* (µg L^{-1})	Oat			Soy			Rice		
		A	B	C	A	B	C	A	B	C
DON	15	<MQL	<MQL	<MQL	-	-	-	-	19	15
AFG_2	0.5	-	<MQL	<MQL	-	<MQL	<MQL	-	-	-
AFG_1	0.05	-	0.1	-	-	<MQL	<MQL	-	-	-
AFB_2	0.1	-	0.4	0.4	-	-	-	-	-	-
AFB_1	0.05	-	0.3	0.2	-	<MQL	-	-	<MQL	-
HT-2	10	<MQL	-	-	-	-	-	-	-	-
FB_1	2	-	-	-	-	-	-	-	-	-
T-2	0.5	1.3	1.2	0.4	-	-	-	-	-	-
FB_2	15	-	-	-	-	-	-	-	-	-
OTA	0.1	-	0.2	0.2	-	<MQL	0.1	-	<MQL	-
ZEA	2	-	<MQL	<MQL	-	<MQL	-	-	<MQL	<MQL

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578 (-) Not detected

579 *MQL average between the studied beverages

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Figure caption

Figure 1. MRM chromatograms of quantitative transitions for detected mycotoxins in an oat sample. "*" denotes analyte's peak.

Figure(s)

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