Application of Conventional Solid-Phase Extraction for Multimycotoxin Analysis in Beers by Ultrahigh-Performance Liquid Chromatography–Tandem Mass Spectrometry

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A new analytical method has been developed and validated for the simultaneous analysis of mycotoxins (aflatoxins B1, B2, G1, G2, and M1, fumonisins B1 and B2, deoxynivalenol, ochratoxin A, HT-2 and T-2 toxins, and zearalenone) in beers. Mycotoxins were extracted by solid-phase extraction (SPE) using C18 as the cartridge. Several parameters such as type of sorbent, elution solvent, and dilution of the sample were evaluated. The separation and determination were carried out by ultrahigh performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). The method was validated, and mean recoveries ranging from 70 to 106% were obtained. Repeatability and intermediate precision, expressed as relative standard deviations, were lower than 21% for all mycotoxins and levels assayed. The limits of quantification were lower than 0.5 μg/L. The developed method has been applied for the analysis of several types of beers with different alcoholic content (nonalcoholic, normal, and special), and T2, HT-2 toxins, aflatoxin B1, and fumonisin B2 were detected. This methodology combines the simplicity of SPE using conventional cartridges and UHPLC-MS/MS, producing a rapid, sensitive, and reliable procedure.

KEYWORDS: Mycotoxins; beer; solid phase extraction; UHPLC; tandem mass spectrometry

INTRODUCTION

Mycotoxins are toxic natural secondary metabolites produced under particular environmental conditions by several molds on agricultural commodities in the field or during storage (*1*). They have been considered as the most important chronic dietary risk factor, and many of them are classified as cytotoxic, carcinogenic, mutagenic, or immunosuppressive compounds (*2*). They may occur in various products such as cereals (*3*), coffee beans (*4*), and beverages such as beer (*5*) and wine (*6*), and their presence can cause food and feed born intoxication. Because of their high toxicity, several national and international organizations have set up regulations for their control in foods in order to ensure food safety (*7*, *8*).

The presence of mycotoxins in beverages, such as beer, is mainly due to the transmission of these compounds from contaminated grains (barley and maize) during the brewing process (*9*). The presence of mycotoxins in beer is mainly due to the use of contaminated maize and barley or maize product used as a brewing adjunct. Most of the mycotoxins that can be found in cereals such as ochratoxin A, aflatoxins, fumonisins, and trichothecenes (deoxynivalenol, T-2, and HT-2 toxins) can also be detected in beers because they can survive the brewing process (*5*, *9*, *10*). For instance, between 13 and 32% of the ochratoxin A present in the original malt can be found in the beer (*11*). The

problem of mycotoxin contamination can be further complicated by the conjugation of the respective mycotoxin to certain function groups or molecules such as glycosyl or sulfate (*12*), and concentrations of these masked mycotoxins in beer can be higher than free mycotoxins (*13*, *14*). The harmful potential of these masked mycotoxins is that when contaminated food is ingested, attached functional groups might be cleaved by enzymatic action during the digestion process, releasing the unconjugated toxin.

Taking into account that the incidence of mycotoxins in beer has been described in several works (*15*, *16*), the development of reliable analytical methods has gained increasing interest. Although immunological techniques based on specific monoclonal and polyclonal antibodies are commercially available, they are used for rapid screening (*17*, *18*) and chromatographic methods based on gas chromatography (GC) (*19*), thin layer chromatography (TLC) (*20*), and liquid chromatography (LC) (*16*, *21*) for the simultaneous determination of mycotoxins. LC methods coupled to UV (*20*) or fluorescence detection (*22*) have been applied. Nowadays, LC coupled to mass spectrometry (MS) or tandem mass spectrometry (MS/MS) is mainly used for the simultaneous determination of several classes of mycotoxins (*16*, *19*) because it provides more accurate identification, better selectivity, and higher sensitivity than other detection techniques. However, one of the main problems during the development of multimycotoxin methods is that these compounds present different chemical and physicochemical properties, and generic extraction methods must be developed. Solid-phase extraction (SPE)

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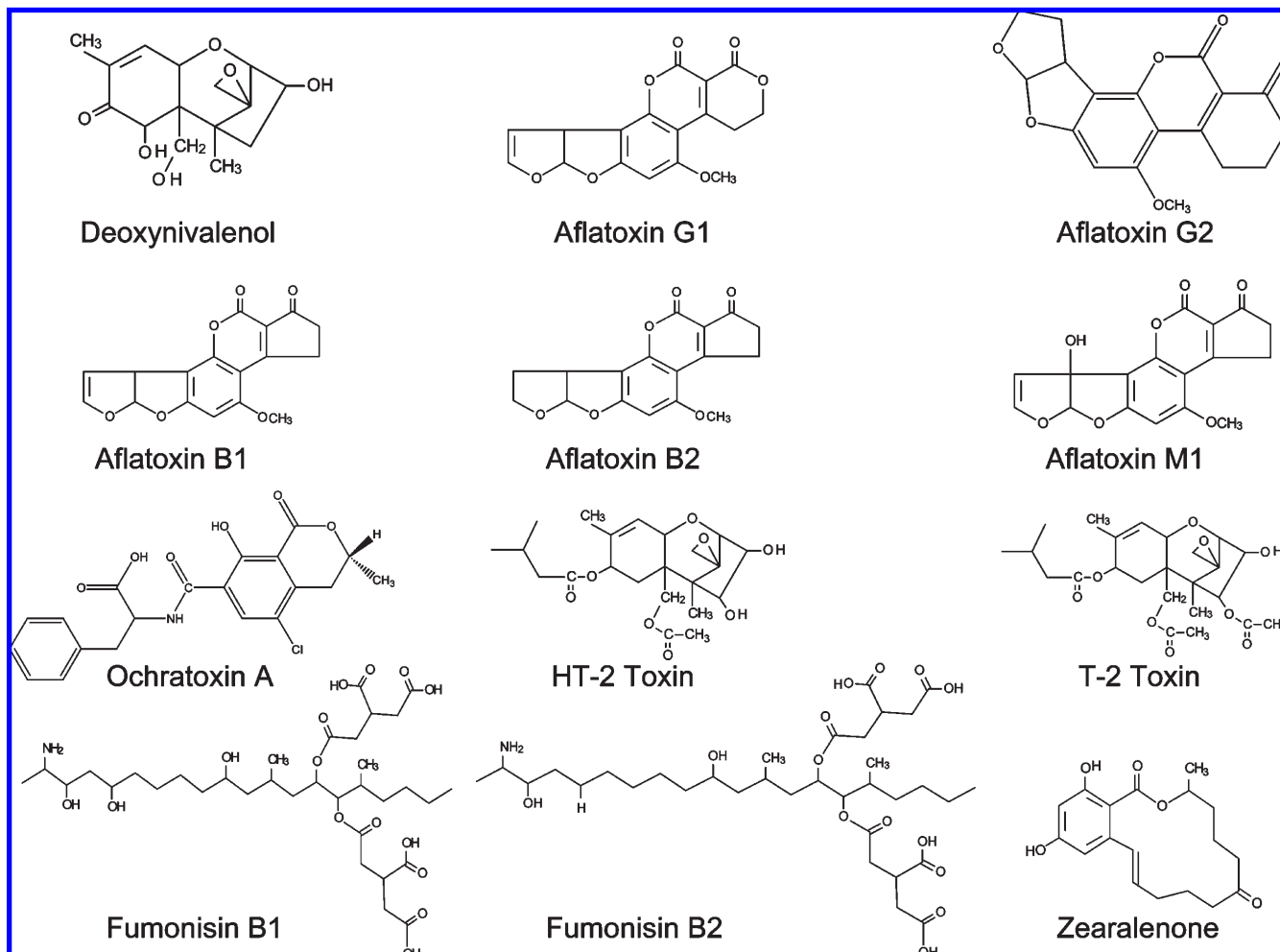
Figure 1. Chemical structures of the selected mycotoxins.

with immunoaffinity material is very popular in mycotoxin analysis because it is a very selective and time saving sample cleanup tool (*23*) for removing matrix compounds. This approach has been mainly used for the analysis of one mycotoxin or family of mycotoxins, although some works compared different immunoaffinity columns for multimycotoxin extraction (*24*). However, one of the problems of the immunoaffinity materials is the high cost, and other alternatives have been checked with conventional sorbents such as C18 (*5*, *25*), hydrophilic—lipophilic balanced copolymers (*10*), or ion exchangers (*21*). The cleanup of this extraction procedure prevents the LC-MS/MS system from being contaminated, and the cartridges are less expensive than immunoaffinity columns. Furthermore, other alternative procedures such as solid-phase microextracion (SPME) (*26*) and liquid-phase microextraction (LPME) (*27*) have been proposed for the determination of mycotoxins in beverages.

In relation to the analytical methods developed for the determination of mycotoxins in beer, up to now, most have been mainly focused on the determination of ochratoxin A (*5*, *26*). Only few methods have been developed for the simultaneous determination of mycotoxins in beer, even though a single fungal species can produce different toxins, or a single agricultural commodity can be contaminated with different fungal species, resulting in the co-occurrence of a number of different toxins (*28*). For instance, Ventura et al. (*10*) analyzed aflatoxins (B1, B2, G1, and G2) and ochratoxin A in beer, whereas trichothecenes such as HT-2 toxin, T-2 toxin, deoxynivalenol, and nivalenol were analyzed in malt and beer samples (*29*).

The aim of this work is to establish a reliable SPE method for the simultaneous extraction of several mycotoxins (ochratoxin A, aflatoxin B1, B2, G1, G2, M1, fumonisins B1 and B2, zearalenone, deoxynivalenol, and T-2 and HT-2 toxins) from several types of beers (nonalcoholic, normal, and special). After the extraction procedure, mycotoxins were determined by ultrahigh performance liquid chromatography (UHPLC) coupled to MS/MS. The use of UHPLC allows the reduction of analysis time and increases sensitivity because narrower peaks were obtained, and it can be applied in routine laboratory analysis because of its sample throughput.

MATERIALS AND METHODS

Chemicals and Reagents. The molecular structures of the target mycotoxins are shown in **Figure 1**. Aflatoxins B1, B2, G1, G2, zearalenone, and stock standard solution of fumonisin B1 and HT-2 toxin (in acetonitrile) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solution of Fumonisin B2 (in acetonitrile) was supplied by Fluka (Steinheim, Germany). Ochratoxin A, deoxynivalenol, T-2 toxin, and stock solution of aflatoxin M1 (in acetonitrile) were obtained from Biopure (Tulln, Austria).

Individual standard stock solutions (200 mg/L) were prepared by exact weighing of those mycotoxins obtained in powder and dissolved in 50 mL of HPLC-grade acetonitrile (J.T. Baker, Deventer, Holland). A working mixed standard solution at a concentration of 2 mg/L of each compound (except for aflatoxin M1 which was 0.2 mg/L) was prepared by combining suitable aliquots of each individual standard stock solution and diluting them with appropriate amounts of acetonitrile. These solutions were kept at 4 °C and renewed weekly.

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Table 1. Retention Time Windows (RTWs) and UHPLC-MS/MS Parameters

compound	RTW (min)	acquisition function	dwell time (s)	cone voltage (V)	quantitation transition (m/z)[a]	confirmation transition (m/z)[a]
deoxynivalenol	1.35−1.48	1	0.200	25	297.4 > 249.4 (10)	297.4 > 231.3 (15)
aflatoxin G2	2.42−2.51	2	0.015	60	331.4 > 313.5 (25)	331.4 > 245.3 (30)
aflatoxin M1	2.57−2.70	2	0.015	50	329.4 > 273.4 (20)	329.4 > 259.3 (25)
aflatoxin G1	2.58−2.67	2	0.015	45	329.2 > 243.1 (25)	329.2 > 311.4 (25)
aflatoxin B2	2.73−2.83	2	0.015	50	315.2 > 259.2 (30)	315.2 > 243.3 (35)
aflatoxin B1	2.88−2.97	2	0.015	30	313.3 > 285.5 (25)	313.3 > 241.3 (30)
fumonisin B1	3.31−3.48	3	0.025	45	723.1 > 334.7 (40)	723.1 > 352.8 (35)
ochratoxin A	3.34−3.46	3	0.025	25	404.2 > 239.2 (20)	404.2 > 358.2 (15)
HT-2 toxin	3.44−3.54	3	0.025	25	442.6 > 263.4 (15)	442.6 > 215.3 (15)
T-2 toxin	3.78−3.90	4	0.025	25	484.7 > 215.3 (20)	484.7 > 245.4 (15)
fumonisin B2	3.90−4.09	4	0.025	55	707.1 > 336.7 (30)	707.1 > 354.7 (30)
zearalenone	4.09−4.21	4	0.025	30	319.5 > 301.6 (10)	319.5 > 283.6 (12)

[a] Collision energies (eV) are given in parentheses.

Ammonium formate was obtained from Panreac (Barcelona, Spain). HPLC-grade methanol was supplied by Sigma. Ultrapure water was obtained from a Milli-Q Gradient water system (Millipore, Bedford, MA, USA). Oasis HLB SPE (200 mg) and C18 (200 mg) cartridges were purchased from Waters (Milford, MA, USA) for the optimization of the SPE procedure.

Apparatus and Software. Chromatographic analyses were performed using an ACQUITY UPLC system (Waters). The column used was a 100 mm × 2.1 mm i.d., 1.7 μm particle size Acquity UPLC BEH C18 column (Waters). Mass spectrometry analysis was performed using an Acquity TQD tandem quadrupole mass spectrometer (Waters, Manchester, UK). The instrument was operated using an electrospray (ESI) source in positive mode. Data acquisition was performed using MassLynx 4.0 software with QuanLynx software (Waters).

A Vortex mixer Heidolph, model Reax 2000, and an analytical AB204-S balance (Mettler Toledo, Greinfesee, Switzerland) were also used. An extraction manifold from Waters connected to a Büchi Vac V-500 (Flawil, Switzerland) vacuum system was used for SPE experiments.

UHPLC-MS/MS Analysis. Chromatographic separation was carried out with a mobile phase consisting of methanol, eluent A, and an aqueous solution of 5 mM ammonium formate, eluent B, at a flow rate of 0.35 mL/min. The analysis started with 25% of eluent A, which was increased linearly up to 100% in 3.75 min. This composition was held for 1.25 min before being returned to 25% of eluent A in 0.5 min, followed by a re-equilibration time of 1 min to give a total run time of 6.5 min. The analytical column was maintained at 30 °C, and the injection volume was 5 μL.

All mycotoxins were detected using ESI in positive mode. The capillary voltage was 3.5 kV, and the extractor voltage was set at 3 V. The source and desolvation temperatures were 120 and 350 °C, respectively. The desolvation gas (nitrogen) and cone gas (also nitrogen) were set at flow rates of 600 L/h and 80 L/h, respectively. Collision-induced dissociation was performed using argon as the collision gas at a pressure of 4 × 10^{-3} mbar in the collision cell. The specific MS/MS parameters for each mycotoxin are shown in **Table 1**.

Sample Preparation. Beer samples were processed using the following procedure. First, samples were degassed by sonication for 20 min. Then, 10 mL of beer was loaded onto a C18 cartridge (200 mg) previously conditioned with 5 mL of a mixture of acetonitrile/methanol (60:40 v/v) and 5 mL of water. After the samples were passed through the cartridges, they were washed with 5 mL of water, and the cartridges were vacuum-dried for 30 min. Analytes were eluted by adding 2 mL of the same mixture of acetonitrile/methanol. The extracts were filtered through a 0.20 μm Millex-GN nylon filter (Millipore, Carrightwohill, Ireland) and collected into a vial. Finally, 5 μL was injected onto the UHPLC system.

Method Validation. For the preparation of standards and recovery studies, blank beer samples (those samples showing the absence of the target compounds) were used. For the evaluation of matrix effect, blank samples from each type of beer (nonalcoholic, normal, and special) were extracted, and the sample extracts were spiked with concentrations ranging from 1 to 100 μg/L (10 times lower for aflatoxin M1).

Linearity was evaluated using matrix-matched calibration by analyzing blank samples of beer at five concentration levels between 0.5 and 100 μg/L (except for aflatoxin M1, which was 10 times lower). Precision and trueness were evaluated by spiking blank samples. Repeatability was performed spiking blank beer at two concentration levels (0.5 and 5 μg/L, 10 times lower for aflatoxin M1), using five replicates for each concentration level in one day. Intermediate precision was evaluated at 1 μg/L (0.1 μg/L for aflatoxin M1), and spiked samples were analyzed at six different days. Trueness (estimated in terms of recovery) was determined for five replicates at 0.5 and 5 μg/L concentrations. Limits of detection (LODs) and quantification (LOQs) were calculated as the concentrations for which signal-to-noise ratios were 3 and 10, respectively.

Samples. Samples with different alcoholic content (including nonalcoholic beers) were purchased from local supermarkets in Almeria (Spain). The samples were stored at 4 °C in the dark prior to analysis. All samples were analyzed following the procedure described above.

RESULTS AND DISCUSSION

Despite of the wide use of immunoaffinity columns during the extraction of mycotoxins from beers, the use of SPE cartridges such as Oasis HLB and C18 has several advantages such as the relatively low-cost and the common use in laboratories. The aim of this work has been the development and validation of a new extraction procedure for the simultaneous determination of several classes of mycotoxins in beers, using a chromatographic method based on UHPLC-MS/MS, which has recently been developed (*30*).

Optimization of the Extraction Procedure. In order to optimize the extraction step by SPE, several variables were evaluated. For the optimization process, blank normal beer was spiked with 10 μg/L of mycotoxins. First the type of cartridge was studied, and Oasis HLB and C18 were evaluated, taking into account their previous used for the extraction of some of the selected mycotoxins (*5*, *10*). For this experiment, methanol was selected as the elution solvent. The sample was not diluted before the extraction, and the cartridge was washed with 5 mL of water before the elution step. **Figure 2A** shows the obtained results, and it can be observed that for aflatoxins higher peak areas were obtained when the C18 cartridge was selected, although no significant differences were observed between both types of cartridges. However, Oasis HLB cartridges provided higher peak areas for ochratoxin A, whereas other mycotoxins present similar responses when both cartridges were studied. C18 cartridges were selected for further experiments because they provide slightly better responses for aflatoxins.

Second, the organic solvent used for the elution of the mycotoxins from the cartridge was evaluated. Mycotoxins were eluted with either 2 mL of acetonitrile or 2 mL of methanol showing the obtained results in **Figure 2B**. It can be observed that methanol provides higher peak areas than acetonitrile for deoxynivalenol, aflatoxin M1, fumonisin B1 and B2, and ochratoxin A. However, aflatoxins (B1, B2, G1, and G2) and HT-2 and T-2
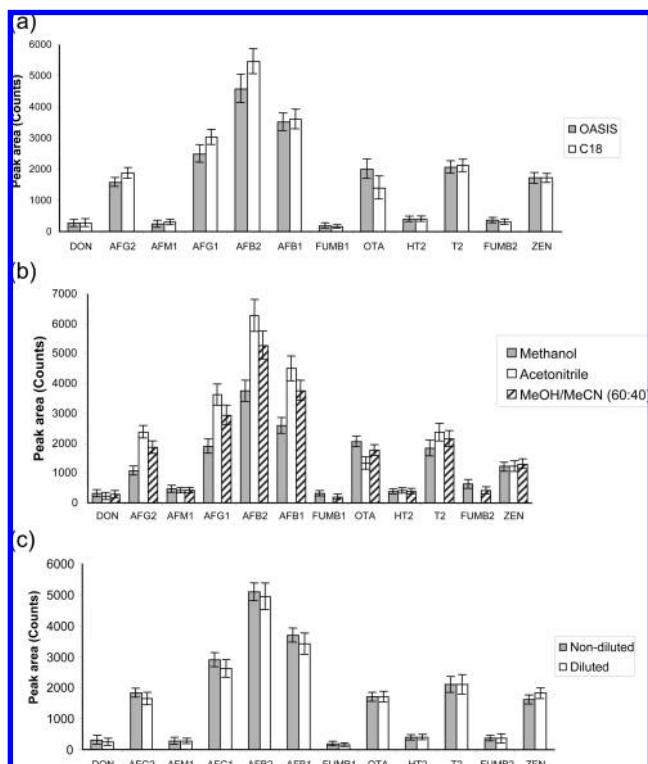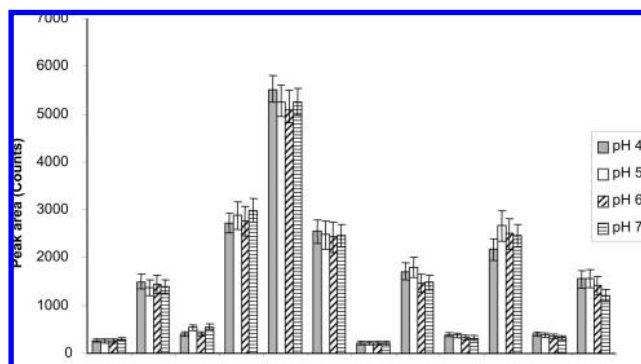
Figure 2. Effect of (**A**) type of cartridge, (**B**) type of solvent, and (**C**) dilution of sample on the extraction of the selected mycotoxins, when a blank normal beer sample was spiked at 10 μg/L. Peak areas are given in counts corresponding to the quantification ions indicated in **Table 1**. Error bars indicated standard deviation (*n* = 3). Mycotoxin abbreviations: DON, deoxynivalenol; AFG2, aflatoxin G2; AFM1, aflatoxin M1; AFG1, aflatoxin G1; AFB2, aflatoxin B2; AFB1, aflatoxin B1; FUMB1, fumonisin B1; OTA, ochratoxin A; HT2, HT-2 toxin; T2, T-2 toxin; FUMB2, fumonisin B2; ZEN, zearalenone. Other abbreviations: MeOH, methanol; MeCN, acetonitrile.

toxins present higher responses when acetonitrile was used as the elution solvent. It must be pointed out that fumonisins (B1 and B2) were not extracted if acetonitrile was applied, as was observed previously for the extraction of these mycotoxins from other samples (*30*), where the use of 100% of acetonitrile as extractant solvent was not appropriate. Because it was difficult to find an organic solvent for the simultaneous extraction of all mycotoxins, different ratios of methanol/acetonitrile were studied, showing in **Figure 2B** the obtained results when a mixture of methanol/ acetonitrile (60:40 v/v) was used. This was selected as the more adequate elution solvent for the simultaneous extraction of the selected mycotoxins because it provides suitable elution for all selected mycotoxins. Furthermore, it was noted that the cartridge must be washed before the elution step. Elution was carried out after the cartridge was washed with 5 mL of water, and the results were compared with those obtained when the washing step was not applied. Mycotoxins were poorly recovered if no washing step was used; therefore, the addition of a washing step with water was mandatory for the recovery of the target mycotoxins.

Ethanol is one of the major constituents of beer, and it can influence the extraction of mycotoxins from alcoholic beverages. Therefore, it could be necessary to dilute the sample prior to the extraction in order to reduce the alcoholic content. In this work, 10 mL of beer was diluted with 10 mL of water before the extraction step, and the results were compared with those obtained when 10 mL of beer was directly extracted. The results are shown in **Figure 2C**, and it can be observed that dilution does not improve the extraction of the mycotoxins. Only the extraction

Figure 3. Effect of pH on the extraction of the selected mycotoxins, when a blank normal beer sample was spiked at 10 μg/L. Error bars indicated standard deviation (*n* = 3). Mycotoxin abbreviations are indicated in **Figure 2**.

of zearalanone improved when the dilution was applied, whereas the rest of the mycotoxins presented similar or better responses when no dilution was used. Consequently, no dilution of sample was carried out in order to minimize sample handling.

The effect of the extraction pH was also evaluated. Blank, normal beer samples spiked with 10 μg/L of the target mycotoxins were adjusted to different pH values (from 4 to 7). No significant differences were obtained for the selected mycotoxins (**Figure 3**), although slightly better responses were obtained for ochratoxin A and zearalenone at lower pH values. Taking into account the fact that beers have a pH close to 4.5, no pH adjustment was carried out in this work.

Finally, **Figure 4** shows a typical chromatogram of a blank beer sample spiked with 5 μg/L (0.5 μg/L for aflatoxin M1) of the selected mycotoxins, and no interferences were observed. It must be highlighted that although complete resolution for all of the mycotoxins was not obtained, they can be resolved because of the high specificity of MS/MS detection. Furthermore, no significant broad peaks were obtained despite the fact mycotoxins were injected in acetonitrile/methanol (60:40 v/v).

Evaluation of Matrix Effect: Influence of the Type of Beer. It is well known that when electrospray ionization is used the presence of matrix components can affect the ionization of the target compounds, reducing or enhancing the response compared with that of standards and solvents, despite the application of extraction and cleanup procedures. The best way to compensate the matrix effect is the use of isotope internal standards which are commercially available or can be synthesized in the laboratory (*31*). However, labeled internal standards are expensive, and they are not available for some of the selected mycotoxins, and other approaches such as matrix-matched calibration can be used. To evaluate the presence and extension of this effect, different types of beers were selected. For that purpose, nonalcoholic beer (< 1% v/v), normal beer, and special beers (original gravity, understood as a measure of the fermentable and unfermentable substances in the wort before fermentation, higher than 13% v/v, and alcoholic content > 5%) (*32*) were studied. Standards of different concentrations were analyzed in pure solvent and in the three matrixes. The slopes were compared, and analysis of covariance was carried out in order to compare the slopes obtained for the different mycotoxins in the solvent and matrixes evaluated (*33*). **Table 2** shows the obtained results, and for all of the mycotoxins, the slopes are statistically different (*P* was lower than 5% for all of the mycotoxins). It can be observed that when special beer was studied, a significant enhancement was observed for all of the mycotoxins, whereas
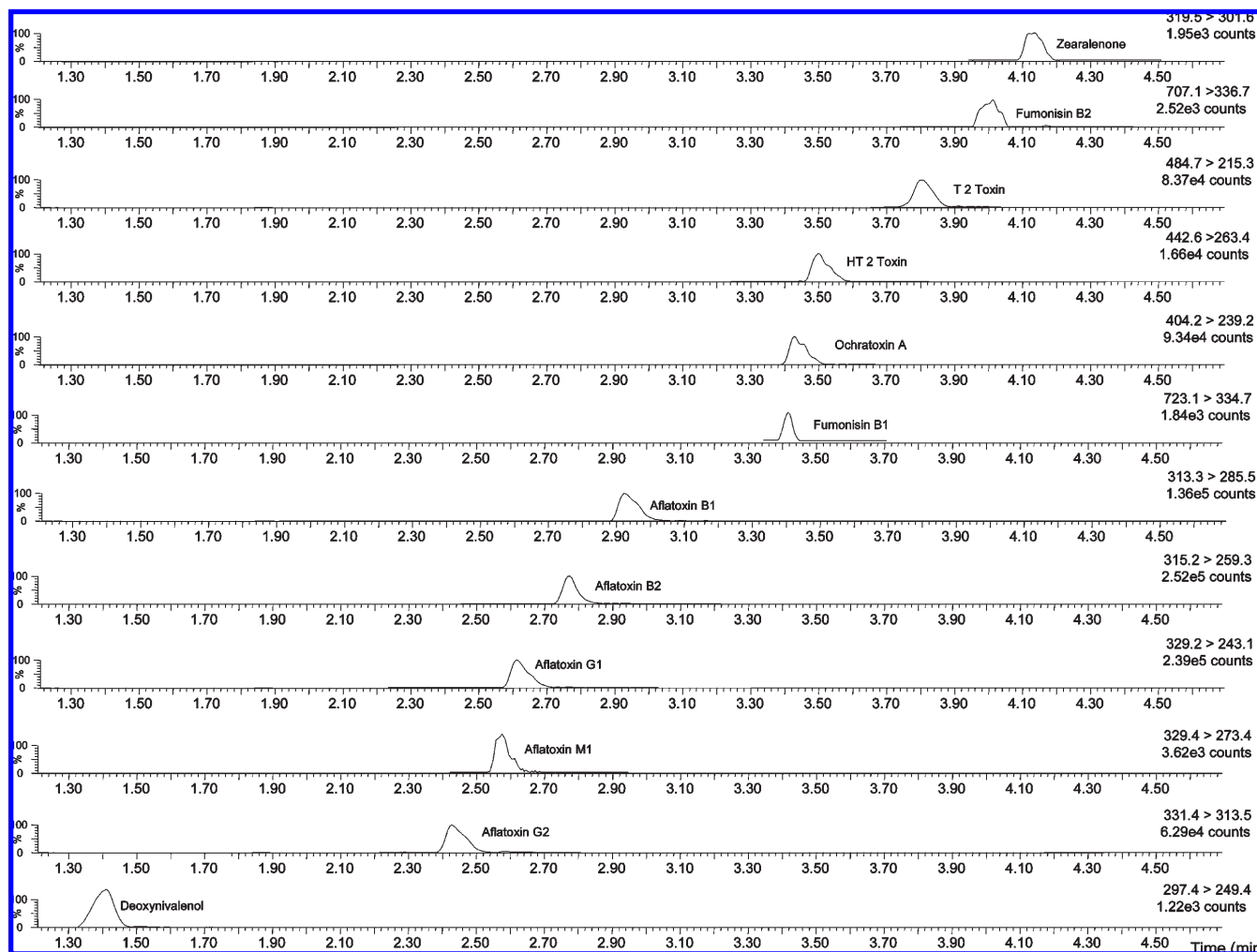
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Figure 4. UHPLC-MS/MS chromatograms obtained from a blank beer sample spiked at 5 μg/L (0.5 μg/L for aflatoxin M1).

Table 2. Evaluation of Matrix Effect by Comparing the Slopes (L/μg) of the Calibration Curves Using Matrix-Matched Calibration and Solvent-Based Standards

mycotoxins	solvent	nonalcoholic beer	normal beer	extra beer	P (%)[a]
deoxynivalenol	1.20	0.59	0.55	1.62	4.7
aflatoxin G2	3.30	2.67	2.61	16.22	0.9
aflatoxin M1	3.60	6.05	5.76	15.96	1.2
aflatoxin G1	10.62	14.13	14.20	35.87	1.8
aflatoxin B2	14.27	9.74	9.46	28.17	2.7
aflatoxin B1	14.17	12.09	12.04	31.55	3.0
fumonisin B1	2.22	1.23	1.19	19.2	0.8
ochratoxin A	1.37	7.76	7.59	33.85	0.5
HT-2 toxin	3.41	4.47	4.47	7.68	4.2
T-2 toxin	15.64	25.26	25.50	46.28	2.2
fumonisin B2	3.48	7.45	7.64	10.7	4.0
zearalenone	1.14	1.35	1.47	4.89	3.2

[a] The *P*-values (in %) were calculated using the procedure indicated in ref *30*.

for the other two types of beer, a matrix enhancement or suppression was observed, depending on the mycotoxin. For instance, matrix suppression was observed for deoxynivalenol, aflatoxins G2 and B2, and fumonisin B1, whereas for the other mycotoxins, a matrix enhancement was noted. It can be indicated that no significant difference between nonalcoholic and normal beer was observed, whereas special beer always provides different slopes. These results can be attributed to the different characteristics of the evaluated beers, such as original gravity. In order to

minimize this matrix enhancement, special beer was diluted from 1:1 to 1:4, but no significant differences were observed. Taking into account the obtained results, a common representative matrix can be used for the analysis of nonalcoholic and normal beers, whereas for the analysis of special beer or high alcoholic content beers, a specific matrix (blank special beer) must be used to quantify this type of sample.

In order to check it, blank beer samples (nonalcoholic, normal, and special) were spiked at 2 μg/L (0.2 μg/L for aflatoxin M1), and five replicates were quantified using a matrix-matched calibration prepared in normal beer for non-alcoholic and normal beer, and special beer to quantify this type of beer. The data are shown in **Table 3** where the *P* values were obtained when an independent sample *t*-test was used to compare the results obtained in nonalcoholic and normal beer (the same calibration curve was used for both types of beer). No significant differences were observed except for aflatoxin B1. However, for both beers, recoveries were within 70 and 110%, indicating that normal beer can be used as a representative matrix for the determination of mycotoxins in nonalcoholic and normal beer. In addition, special beer recoveries ranged from 70.5 to 98.5%, indicating the suitability of the proposed method, and no losses of mycotoxins were observed when high alcoholic beers were checked.

Finally, it must be indicated that when performing matrix-matched calibration, representative matrixes must be selected in order to correct the matrix effect. However, some differences in

the matrix effect within a given matrix can be detected (*34*), and other approaches such as standard addition methodology should be used.

Validation of the Optimized Method. Performance characteristics of the optimized method were established by a validation procedure, studying linearity, trueness, repeatability and intermediate precision, sensitivity, and selectivity. In this case, the validation procedure has been carried out in normal beers.

Method linearity was assayed by performing calibration curves using beer samples spiked with the selected mycotoxins (matrix-based external calibration). Calibration curves were obtained by least-squares linear regression analysis of the peak area versus concentration, and the response was linear in the assayed range with determination coefficients higher than 0.98 in all cases.

The trueness of the method was evaluated through recovery studies, spiking blank samples at two fortification levels (0.5 and 5 μg/L, 10 times lower for aflatoxin M1), processing five samples in each experiment. The obtained results are shown in **Table 4**. It can be observed that for 0.5 μg/L, recoveries ranged from 76.7% (aflatoxin B1) to 102.0% (aflatoxin B2 and fumonisin B2), and from 70.2% (fumonisin B2) to 106.0% (HT-2 toxin) when the samples were spiked with 5.0 μg/L.

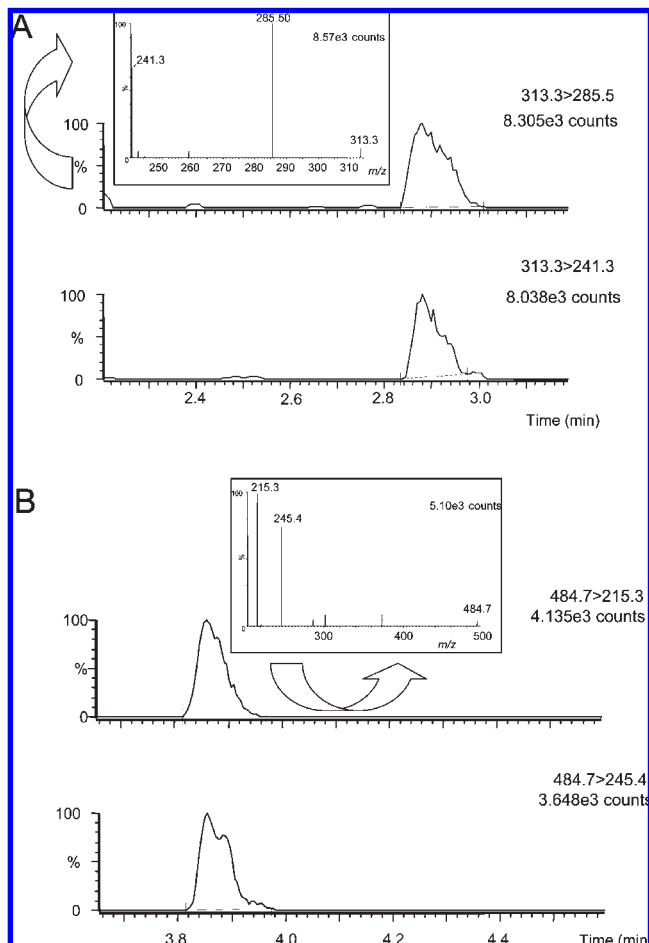Repeatability was evaluated at the two concentration levels assayed for the recovery studies, performing five replicates for

each level (**Table 4**), whereas intermediate precision was studied analyzing one spiked sample at 1 μg/L (0.1 μg/L for aflatoxin M1) during six consecutive days (**Table 4**). It can be observed that repeatability and intermediate precision, expressed as RSD, were lower than 20% for all target mycotoxins, except for fuminisin B1 and B2, which present values slightly higher than 20% (intermediate precision).

LODs and LOQs were calculated, analyzing blank samples spiked at 0.05, 0.1, 0.2, 0.5, and 1 μg/L, and they were determined as the lowest concentrations of analyte for which signal-to-noise ratios were 3 and 10, respectively (**Table 4**). LOQs ranged from 0.05 μg/L (aflatoxin M1) to 0.45 μg/L (deoxynivalenol), which allows the determination of these type of compounds at trace levels. These values are similar to or lower than others reported previously (*10*). In addition, the obtained value for ochratoxin A (0.07 μg/L) was below the guidance levels established by several European countries (*26*).

Finally, the selectivity of the method was evaluated by the analysis of blank samples. The absence of any chromatographic signal at the same retention time as the target mycotoxins indicated that no matrix or chemical compounds are extracted and give a false positive signal. Identification of the target mycotoxins was carried out by searching in the appropriate retention time windows (RTWs), which were given by the mean retention time ± three standard deviations of the retention time of 10 blank samples spiked at 5 μg/L for each compound (**Table 1**). After identification by RTW, each compound was confirmed by comparison of the signal intensity ratios of the two transitions (quantification and confirmation) with those obtained from the calibration standards. Confirmation was reliable if the experimental ion ratio for each compound fell within the tolerance laid down in European Commission Decision 2002/657 (*35*), which provides maximum permitted tolerances depending on the estimated ion ratio. For LC-MS/MS, the ion ratio must be within ±20% (relative, not absolute value) of the ion intensity of the reference spectrum for ions > 50% relative abundance, ±25% for ion ratios <20−50%, ±30% for ion ratios >10−20%, and ±50% for ion ratios ≤10%.

Sample Analysis. The optimized method was applied to analyze 15 samples of beer, including nonalcoholic (*n* = 4), normal beer (*n* = 10), and special (*n* = 1) beers. In order to ensure the reliability of the results when the proposed method is applied, an internal quality control was used. This quality control consisted of a matrix-based external calibration using representative blank beers, a reagent blank to detect possible chemical interferences, a matrix blank in order to eliminate

Table 3. Evaluation of Recovery (%) in Different Beer Samples Using the Suitable Matrix-Matched Calibration Curve for Each Type of Sample Spiked at 2 μg/L

	recovery (%)[a]			
mycotoxins	nonalcoholic beer	normal beer	special beer	*P* (%)[b]
deoxynivalenol	96.3 (12.4)	83.7 (9.9)	81.9 (18.9)	8.9
aflatoxin G2	90.4 (5.9)	87.0 (5.1)	89.1 (8.3)	30.5
aflatoxin M1[c]	79.0 (12.2)	75.9 (10.1)	72.9 (10.9)	58.9
aflatoxin G1	95.7 (6.5)	102.5 (7.2)	89.9 (8.8)	15.4
aflatoxin B2	103.9 (9.9)	97.1 (5.1)	98.5 (7.4)	22.0
aflatoxin B1	77.3 (5.1)	98.8 (3.4)	91.3 (6.1)	0.0
fumonisin B1	74.8 (9.1)	76.2 (7.5)	70.5 (11.2)	73.4
ochratoxin A	103.5 (8.3)	104.6 (4.9)	85.8 (12.8)	81.2
HT-2 toxin	97.9 (13.3)	99.8 (6.9)	95.2 (13.5)	78.0
T-2 toxin	93.3 (8.3)	96.2 (7.1)	90.5 (10.6)	54.8
fumonisin B2	74.2 (9.1)	79.2 (8.0)	75.3 (12.4)	26.2
zearalenone	91.7 (12.5)	82.9 (9.4)	71.8 (13.1)	19.3

[a] RSD values are given in parentheses (*n* = 5). [b] The *P* values (in %) were obtained when the *t*-test was used to compare the data obtained from nonalcoholic and normal beers. [c] 0.2 μg/L for aflatoxin M1.

Table 4. Validation Parameters of the Optimized Method

				recovery (%)[a]		
mycotoxin	R^2	LOD (μg/L)	LOQ (μg/L)	0.5 μg/L	5 μg/L	intermediate precision
deoxynivalenol	0.9855	0.14	0.45	92.4 (9.7)	77.4 (8.7)	80.6 (9.2)
aflatoxin G2	0.9904	0.08	0.27	88.0 (15.9)	93.0 (9.3)	90.6 (12.4)
aflatoxin M1	0.9943	0.02	0.07	89.6 (9.3)	81.2 (9.1)	70.4 (12.9)
aflatoxin G1	0.9972	0.03	0.10	96.0 (6.1)	89.4 (4.9)	95.4 (11.5)
aflatoxin B2	0.9868	0.05	0.17	102.0 (7.6)	80.7 (6.9)	86.6 (7.7)
aflatoxin B1	0.9872	0.04	0.13	76.7 (14.1)	104.9 (8.2)	99.2 (13.3)
fumonisin B1	0.9823	0.07	0.23	85.0 (10.1)	70.2 (18.3)	81.3 (20.2)
ochratoxin A	0.9912	0.02	0.07	78.2 (14.2)	104.7 (9.0)	83.6 (16.2)
HT-2 toxin	0.9993	0.06	0.20	93.4 (11.7)	106.0 (6.3)	105.7 (19.8)
T-2 toxin	0.9904	0.07	0.23	80.7 (14.7)	109.4 (8.9)	92.8 (12.8)
fumonisin B2	0.9832	0.09	0.30	102.0 (11.1)	76.1 (10.5)	86.6 (20.4)
zearalenone	0.9891	0.10	0.30	91.0 (11.9)	79.5 (9.3)	83.7 (17.8)

[a] Relative standard deviation is given in parentheses (*n* = 5). [b] 0.05 and 0.50 μg/L for aflatoxin M1. [c] Obtained at 1 μg/L (0.1 μg/L for aflatoxin) and 6 replicates.

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Figure 5. UHPLC–MS/MS chromatograms for (**A**) normal beer containing aflatoxin B1 at 0.6 μg/L and (**B**) normal beer containing T2 toxin at 1.0 μg/L. Quantification and confirmation transitions are shown for both compounds along with the corresponding spectra for the detected mycotoxins.

false positives by contamination in the extraction process, and a spiked blank sample at 1 μg/L in order to evaluate the recovery of the proposed method.

When the samples were analyzed by the proposed method, only four samples contained traces of the target mycotoxins. HT-2 toxin was detected in the special beer sample at 1.2 μg/L. Fumonisin B2, T-2, and HT-2 toxins, and aflatoxin B1 were detected in three normal beers at 2.8 μg/L, 1.0 μg/L, 0.9 μg/L, and 0.6 μg/L, respectively. No mycotoxins were detected in nonalcoholic beers.

Figure 5 shows two positive samples of aflatoxin B1 and T-2 toxin as examples of real samples analyzed, observing that no interfering peaks appear on the chromatogram, showing the high selectivity of the extraction procedure in combination with UHPLC-MS/MS, providing a suitable procedure for the determination of mycotoxins in beers.

In conclusion, this work presents a suitable method for the extraction of several classes of mycotoxins by SPE using conventional cartridges (C18 sorbent). The extraction procedure allows the preconcentration of the mycotoxins, obtaining LOQs lower than 0.5 μg/L. The problem of possible interfering compounds is overcome by the use of UHPLC-MS/MS. Nonalcoholic and normal beer can be quantified using a representative matrix, whereas beers with high alcoholic content ($>5\%$) require a similar blank matrix for quantification purposes.

SAFETY

Mycotoxins are dangerous compounds, and they should be handled with care and with the appropriate safety precautions. To minimize exposure, they should be handled only in a fume hood, and gloves and protective clothing should be worn. Contaminated glassware should be treated with 3% sodium hypochlorite. Then, they should be washed with detergent and rinsed with water and methanol before reuse.

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