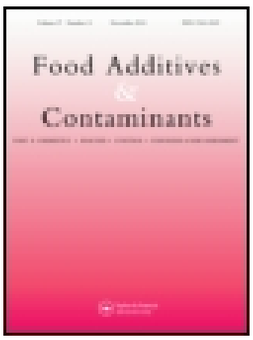

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A simple, rapid and novel method based on salting-out assisted liquid–liquid extraction for ochratoxin A determination in beer samples prior to ultra-high-performance liquid chromatography coupled to tandem mass spectrometry

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ABSTRACT

A novel, simple, easy and cheap sample treatment strategy based on salting-out assisted liquid-liquid extraction for ochratoxin A (OTA) ultra-trace analysis in beer samples using ultra-high-performance liquid chromatography-tandem mass spectrometry determination was developed. The factors involved in the efficiency of pre-treatment were studied employing factorial design in the screening phase and the optimal conditions of the significant variables on the analytical response were evaluated using a central composite face-centred design. Consequently, the amount of salt ($(NH_4)_2SO_4$), together with the volumes of sample, hydrophilic (acetone) and nonpolar (toluene) solvents, and times of vortexing and centrifugation were optimised. Under optimised conditions, the limits of detection and quantification were 0.02 µg l^{-1} and 0.08 µg l^{-1} respectively. OTA extraction recovery by SALLE was approximately 90% (0.2 µg l^{-1}). Furthermore, the methodology was in agreement with EU Directive requirements and was successfully applied for analysis of beer samples.

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Ochratoxin A; beer; salting-out assisted liquid-liquid extraction; ultra-high-performance liquid chromatography

Introduction

Some feeds, foods, agricultural products and alcoholic beverages such as wheat, maize, barley, coffee, cocoa, wine and beer might be contaminated with mycotoxins (EC 2002; Scudamore 2009; Duarte et al. 2010; Kedjebo et al. 2015), which are secondary metabolites produced by ubiquitous fungi *Aspergillus* and *Penicillium*. These mycotoxins are toxic compounds, ochratoxin A (OTA) being one of the most toxic of the group. The International Agency for Research on Cancer (IARC) has included this toxin in the 2B category as 'potential carcinogenic agent in humans' (IARC 1993), not only as initiator, but also as promoter of cancers and tumours (Akman et al. 2012; Pfohl-Leszkowicz and Manderville 2012). Also, OTA might be involved in the pathogenesis of various kidney diseases and be the cause of rare tumours in the kidney in certain endemic regions of the Balkan Peninsula. Furthermore, according to European Food Safe Authority (EFSA), it has also been found that OTA is a potent renal toxin in all animal species studied, where it was observed that it induced a typical karyomegaly and progressive nephropathy (EFSA 2006).

In this context, OTA has been detected in beers and its incidence in these samples from different worldwide regions has been reported (Medina et al. 2005; Rubert et al. 2011; Tamura et al. 2011; Deetae et al. 2013; Matumba et al. 2014; Soto et al. 2014). Furthermore, in a recent report about OTA dietary exposure in different populations, in men aged between 18 and 59 years old from Czech Republic, beer was the main dietary contributor with an intake of 2.60 ng kg^{-1} bw day^{-1} (Ostry et al. 2015).

OTA occurrence in beer may be explained since this is an alcoholic beverage from fermentation of barley and including wheat malt, cereals in which this mycotoxin has been widely detected (WHO/FAO 2001). Brewing processes vary from

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one industry to another, but the way the toxin is carried over into the beer is basically the same. Further, OTA is stable to boiling, hence its removal is difficult (Trivedi et al. 1992); thus after mashing, some OTA is recovered in the spent grains, but wort does contain OTA (Inoue et al. 2013). The concentration level of OTA along the brewing process decreases by <20% and although after fermentation yeasts retain part of the original OTA, the remaining content of this mycotoxin remains in the beer (BêLakova et al. 2011; Inoue et al. 2013).

In consequence, some countries and international organisations, as Commission Regulation of the European Community (EC) (European Commission 2006a), have established maximum allowable limits on the most widespread foods in order to protect consumers from risks related to mycotoxins. As mentioned above, while Commission Regulation (EC) 1881/2006 and its subsequent amendments (105/2010 and 594/2012) prescribes maximum levels of OTA in several foods, cereals, spices, fruits and wine (EC 2006a, 2010, 2012), such limit has not been established in beer; the OTA content in this drink must be controlled in the malt raw material (EC 2010). Nevertheless, several countries and traders have set up maximum allowable limits (MAL) for OTA content in beer, as is the case of Italy (0.2 μg l^{-1}), The Netherlands (0.3 μg l^{-1}), and Finland (0.5 μg l^{-1}); also, in several reports authors have taken a recommended limit of 0.2 μg l^{-1} (Medina et al. 2006; Soto et al. 2014). This maximum allowable limit of OTA in beer was considered in this work. The OTA incidence reported in beer from European countries has shown varied values from 14% to 100% (Soto et al. 2014), which indicates that this alcoholic beverage is prone to be contaminated with OTA. Also, although some authors found values near and above the assumed MAL (Medina et al. 2005; Soto et al. 2014), the OTA mean concentration levels in beer samples analysed from European countries were <0.2 μg l^{-1} (Soto et al. 2014).

While the official methodology for OTA determination in beer is based on High Performance Liquid Chromatography (HPLC) associated to Fluorescence Detection (FD) employing a previous step using an Immunoaffinity Colum (IAC) for sample pre-treatment (CEN 2003; Sáez et al. 2004; Lhotská et al. 2016), in recent years, other methods have been developed for the detection of this mycotoxin in beer including HPLC and ultra-high performance liquid chromatography (UHPLC) coupled to mass spectrometry (MS) and tandem mass spectrometry (MS/MS), and different sample treatments based on solid phase extraction (SPE) (Prelle et al. 2013; Rubert et al. 2013; Lhotská et al. 2016) as well as molecularly imprinted polymers (MIPs) (Yu and Lai 2006; Cao et al. 2013) and QuEChERS (Tamura et al. 2011).

An approach named salting-out assisted liquid–liquid extraction (SALLE), introduced by Matkovich (1973) for metal chelates extraction, has been used as isolation approach of several target compounds (e.g. drugs, metabolites, biogenic amines and polycyclic aromatic hydrocarbons) on different samples (e.g. rat serum, wine, food samples and soils) (Tang and Weng 2013; Ramos et al. 2014; Magiera and Kwietniowska 2016). This sample treatment follows the principles of liquid–liquid extraction, with the important advantage that the salting-out effect allows an efficient analyte extraction from aqueous samples. Furthermore, this approach has been applied for mycotoxin determination in pig urine prior to detection by HPLC-MS/MS (Song et al. 2013). However, to our knowledge, there are no previously reported strategies based on SALLE and UHPLC-MS/MS for OTA determination in beer samples.

As consequence, the aim of this work was to develop a convenient, robust, simple, rapid and selective sample preparation method based on SALLE for the extraction of an important mycotoxin, OTA, in beer samples prior to injection into a UHPLC-MS/MS system. The critical parameters that affect the extraction efficiency such as types and volumes/amounts of extraction solvent and salting-out reagents, and extraction time involved in the procedure were studied. In order to optimise these parameters, a factorial design and response surface design according to the response surface methodology (RSM) approach were employed. Furthermore, the methodology was validated taking into account international legislation and normatives (guidelines mainly from European Commission). The SALLE approach coupled with UHPLC-MS/MS was applied for the determination of ochratoxin A in beer samples, mainly from Argentina.

Materials and methods

Reagents

Ochratoxin A, analytical standard, was obtained from Fluka (Steinheim, Germany). Acetonitrile (ACN), methanol (MeOH), and water Optima® LC-MS grade were purchased from Fisher Scientific (Fair Lawn, New Jersey, USA). Formic acid was obtained from Fisher Scientific (Loughborough, UK). During the optimisation of SALLE, the following reagents were used: sodium chloride (NaCl) (Suprapur®), ammonium sulphate ($(NH_4)_2SO_4$) (ACROS Organics™ Thermo Fisher Scientific), sodium sulphate (Na_2SO_4) (Baker Analyzed™ – J.T. Baker™) and anhydrous magnesium sulphate ($MgSO_4$) from Sigma-Aldrich (Steinheim, Germany). Also, the following solvents were used: ethyl acetate ($C_4H_8O_2$) Optima®, acetone (C_3H_6O), dichloromethane (CH_2Cl_2), hexane (C_6H_{14}), toluene (C_7H_8) HPLC grade, cyclohexane (C_6H_{12}) and trichloromethane ($CHCl_3$) HPLC grade J.T. Baker™. All were obtained from Fisher Scientific (Fair Lawn, New Jersey, USA). Working standard solutions in ACN were prepared immediately before use by stepwise dilution from a 10 mg l^{-1} OTA stock standard solution.

Mass spectrometry

Mass spectrometry analyses were performed on a Xevo™ TQ triple quadrupole mass spectrometer with a *Z*-Spray™ electrospray ionisation source – multi-mode source ESI/APCI/ESCi® (Waters, Milford, USA). The source was operated in a positive (ES+) mode at a 350°C desolvation temperature with N_2 as the nebuliser and the source temperature was kept at 120°C. The capillary voltage was maintained at 3.5 kV and the extractor voltage was set at 1.0 kV. Ultrapure nitrogen was used as desolvation gas with a flow of 800 l h^{-1}. Argon was used as collision gas at a flow of 0.18 ml min^{-1}. Detection was performed in multiple reaction monitoring (MRM) mode of selected ions at the first (Q_1) and third quadrupole (Q_3). To choose the fragmentation patterns of *m/z* (Q_1) → *m/z* (Q_3) for the analyte in MRM mode, direct infusion (via syringe pump) into the MS of OTA (1 mg l^{-1}) standard solution in ACN was performed and the product ion scan mass spectra were recorded. The OTA quantification transition in MRM mode was (m/z) 404.1 → 239.2 produced at collision energy of 25 eV. The transitions used for confirmation were (m/z) 404.1 → 341.1 and 404.1 → 358.2 produced at collision energies of 25 and 20 eV; respectively. The values optimised for the dwell time and cone voltage parameters were of 0.25 s and 20 V; respectively. The data were acquired using MassLynx version 4.1 Mass Spectrometry Software (Waters, Milford, USA).

Chromatography

An Acquity™ Ultra High Performance LC system (Waters, Milford) equipped with autosampler injection and pump systems (Waters, Milford) was used. The autosampler vial tray was maintained at 15°C. The needle was washed with appropriate mixtures of ACN and water. The separation was performed by injecting 25 µl sample onto an ACQUITY UPLC® BEH C_{18} (Waters, Milford, USA) analytical column with 2.1 mm internal diameter × 50 mm length, and 1.7 µm particle size. The binary mobile phases consisted of water with 0.1% (v/v) of formic acid (A) and ACN with 0.1% (v/v) of formic acid (B) delivered at 0.35 ml min^{-1}. The composition of the isocratic elution programme was 30% A and 70% B. Under the mentioned conditions, OTA retention time was 0.45 ± 0.03 min within a total chromatographic run time of 2.0 min. The column was held at a temperature of 30 °C. Under aforementioned conditions, no sample contamination or sample to sample carryover was observed.

Samples and conditioning

Quantification was achieved by preparing spiked beer samples with appropriate amounts of OTA. The solutions were maintained at −4°C, protected from light, and kept in amber flasks. Intermediate spiked samples of beer without previously detected OTA were prepared. Beer samples (Lager, Pale, Pale Ale, Wizen, Strong Pale Lager/Bock, Dunkelweizen, Pilsener, Ale Kölsch, Porter) were purchased from local liquor stores mainly from Argentine, but also from Germany, Czech Republic, Holland and Russia. These beer samples were classified according to the malt type as barley, wheat or a blend of both. A total of 21 beer samples

from different types and styles as mentioned above were analysed. Before sample treatment, a volume of 250 ml of beer was degassed by sonication during 1 h until no foam and gas were observed, to avoid their interference in the OTA analysis.

Sample preparation

For sample treatment based on SALLE prior to OTA determination by UHPLC-MS/MS, an aliquot of 6.0 ml of beer sample was placed into a 15 ml PTFE tube with screw cap and conical base. Then, for the salting-out step, aliquots of 1.5 ml of toluene and 3.5 ml of acetone, and a mass of 1.0 g of $(NH_4)_2SO_4$ were added to the sample. This obtained mixture was vortexed at 1100 rpm during 40 s (0.65 min). In order to achieve phase separation, centrifugation for 9.0 min at 2880 × *g* at room temperature was performed. The supernatant layer was removed and placed in glass tube to evaporate until dryness under a nitrogen stream at 35°C. Finally, the evaporated sample was reconstituted in 250 µl of MeOH and filtered through a 0.2 µm nylon syringe filter to be transferred into a sample vial with insert for analyses. Operative conditions mentioned above are summarised in the Table 1 (Supplementary Data 1 depicts a process diagram of the methodology).

Optimization: experimental designs

In order to find out the optimal values for each variable (factor) involved in SALLE, an experimental design approach was employed. Hence, a RSM was used to optimise the main factors involved. For the screening phase, a fractional design was utilised to select the significant factors. Also, to assess the suitability of the model, curvature of response surface and the optimal operational conditions, a central composite face-cantered design (CCF) was applied to assess the factors selected previously. Moreover, the desirability function was used to select the optimal experimental conditions evaluated in CCF according to the RSM (Vera Candioti et al. 2014). Experimental design, and the obtained models were evaluated using Design Expert 8.0.0 (Stat-Ease, Inc., Minneapolis, USA).

Table 1. Optimum values for the factors studied in the central face-cantered composite design model.

Factor	Optimum value
Acetone volume	3.5 ml
Toluene volume	1.5 ml
Sample volume	6.0 ml
Vortexing time	0.6 min – 40 seg
Centrifugation time	9 min (2880 × *g*)

Limit of detection and limit of quantification

According to the calibration approach proposed by EU Reference Laboratories for mycotoxins (Wenzl et al. 2016), the estimation of the limits of detection (LOD) and the limits of quantification (LOQ) was carried out employing the Equations (1) and (2); respectively.

$$\mathrm{LOD} = 3.8\frac{S_{y,x}}{b}\sqrt{1.1 + \frac{\bar{x}^2}{\sum_{i=1}^{n}(x_i - \bar{x})^2}} \quad (1)$$

$$\mathrm{LOQ} = 3.3^{*}\mathrm{LOD} \quad (2)$$

Where $\bar{x}$ represents the mean concentration level, $S_{y,x}$ the standard error of the residuals (regression error), b slope of the calibration curve and $\bar{x}_i$ content value of OTA at calibration level i.

Precision, recovery, and trueness

With the aim to evaluate the methodology performance, precision, recovery and trueness (accuracy) were calculated. Repeatability (intraday precision) and intermediate precision (inter-day precision) as terms of precision were evaluated. Accuracy of the methodology, trueness – expressed as bias (%) – of the measurements through recovery of additions of known amounts of OTA to blank beer samples, were studied. As consequence, beer samples were spiked at different OTA concentration levels, that is five replicates of blanks and at 0.1 µg l^{-1}, 0.2 µg l^{-1}, 0.4 µg l^{-1} and 1.0 µg l^{-1}; and were analysed under the optimal conditions described later. Also, intermediate precision was evaluated by means of a similar procedure during four consecutive weeks. The values obtained in these assays were compared to the requirements established by current norm for OTA (EC 2006b).

Results and discussion

Solvents and salts for SALLE of OTA in beer

As mentioned above, the aim of this work was to develop an efficient, simple, and fast treatment to determine OTA in beer samples. During the screening step, the OTA extraction efficiency in spiked beer samples employing different salts and hydrophilic solvents were studied. As consequence, hydrophilic solvents such as acetone, acetonitrile, ethyl acetate, and methanol containing salts, such as magnesium sulphate ($MgSO_4$), sodium chloride (NaCl), ammonium sulphate ($(NH_4)_2SO_4$) and sodium sulphate (Na_2SO_4), following the Hofmeister series for anions and cations to enhance the salting-out effect, were tested. As can be seen in Figure 1, the extractions carried out with ammonium sulphate or magnesium sulphate with either acetone or ACN showed the highest recoveries (%) for OTA. During these assays, spiked beer samples with an OTA concentration level of 0.6 μg l^{-1} were employed.

On the other hand, the use of an organic nonpolar solvent was studied with the aim to enhance the OTA extraction and reduce the matrix components that affect the analytical response signal. As consequence, different nonpolar solvents as chloroform, cyclohexane, dichloromethane, hexane and toluene were evaluated. Thus as shown in Figure 1, toluene improved the extraction efficiency of OTA and was selected as nonpolar solvent. The use of toluene has been reported for previous liquid-liquid extraction methodologies for OTA in wine, must, and grape juices (Bellí et al. 2002).

While ACN and $MgSO_4$ or $(NH_4)_2SO_4$ as extraction components demonstrated satisfactory recoveries, asymmetrical chromatography peaks of OTA were observed when ACN was used. On the other hand, when $MgSO_4$ (Figure 1(a)) or $(NH_4)_2SO_4$ (Figure 1(b)) and acetone were employed as system extraction, the highest recovery values for OTA were obtained (Figure 1(a,c)) as well as good chromatographic peaks. As a result, ammonium sulphate, acetone and toluene (Figure 1(b)) were selected for further SALLE experiments. Also, for preliminary and further experiments, mixing was performed using a vortex shaker at 1100 rpm and phase separation through centrifugation during 5 min at 2880 × *g* were used.

Experimental designs: significant variables and optimal conditions for SALLE procedure

In order to optimise the experimental SALLE conditions, an initial screening to identify the variables and factors with significant effects followed by an optimisation step of the crucial factors to determine the best analytical conditions was performed. Therefore, the main factors suspected to affect the extraction efficiency in the sample treatment based on SALLE were evaluated. The analysed variables were: amount of salt (0.5 – 2.0 g of (NH4)2SO4), acetone volume (3.0 – 5.0 ml), toluene volume (1.5 – 2.0 ml), volume of sample (6.0 – 8.0 ml), centrifugation time (9 – 11 min) and vortexing time (0.6 – 1.8 min). The experimental ranges

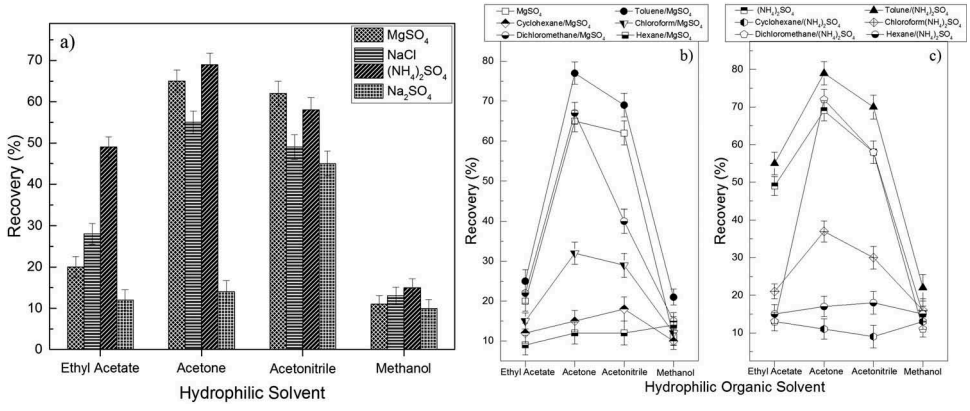

Figure 1. (a) Effect of the hydrophilic solvent and salt on the extraction efficiency (Recovery (%)) of OTA in beer. Hydrophilic organic solvents and nonpolar solvents for the salting-out assisted liquid–liquid extraction of OTA in beer samples when either (b) $MgSO_4$ or (c) $(NH_4)_2SO_4$. Spiked OTA concentration: of 0.6 μg l^{-1}.

for each variable were selected according to previous experiments. Thus a fractional factorial 26–1 design was chosen based on a total of 32 runs. The recovery (%) was selected as analytical response of the spiked samples at a constant OTA concentration level (0.6 μg l^{-1}). Then the proposed model was evaluated in order to fulfil the ANOVA assumptions.

The main factors that statistically affect the extraction efficiency in the sample treatment were chosen according to the Pareto chart. This graph shows the absolute effect of each variable as bar height and may be used for comparing its statistical significance. Also, there are two limits plotted as approximations at a significance level (p = 0.05): based on the Bonferroni corrected t and a standard t. As consequence, the parameters considered as significant effects were above the t-value limit. As can be seen in the Figure 2, the variables with significant effect were the volumes of sample, acetone, and toluene (although the latter was near to the threshold, that is $p \sim 0.05$). Moreover, a deviation from normality of the selected variables was observed ($p > 0.05$) when the Shapiro-Wilk test was applied, verifying this way an agreement with that observed in the Pareto chart as well. Moreover, as it can be seen in Figure 2 for the Pareto chart, non-significant interactions were found.

Considering its flexibility and the fact that it can be built from the previously constructed fractional experimental design by adding central and axial points, a central composite face-centred design was applied. Consequently, 50 runs (2^{k-1} + 2k+Cp = 32 + 10 + 8) based on combinations of the previously selected independent variables were performed and, as was mentioned above, 32 runs from the previous fractional factorial design were used in the central composite face-centred design construction. A quadratic model for the response (recovery (%)) was fitted. As a consequence, sample volume and acetone volume were found to be the significant factors or principal effects for the final second order fitted model. Interestingly, this time, the toluene volume was found non-significant. The response surface corresponding to the model fitted considering the most significant factors is shown in Figure 3(a)).

In order to obtain the maximum of OTA response (expressed as recovery (%)) employing a reduced time of vortexing and centrifugation as well as a lower volume of acetone and toluene in the method, the desirability function was studied from the fitted model. As can be seen in Figure 3(b)), the maximum recovery for OTA was obtained under the conditions described in Table 1.

Also, the coefficient of determination (R^2) and the adjusted coefficient of determination (R^2adj) were assessed. Thus, R^2adj of 0.7201 shows an optimal fit for the second-order model.

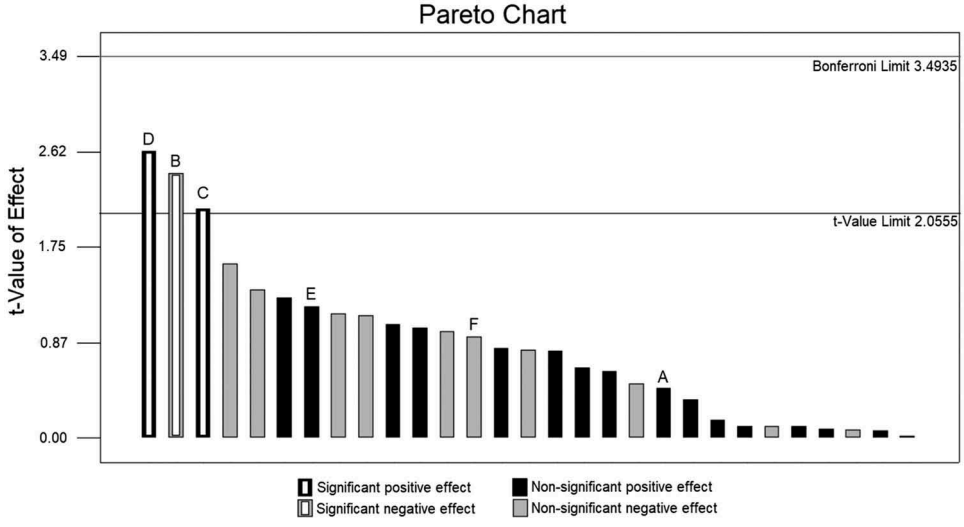

Figure 2. Pareto chart employed to select significant effects in the full factorial design. Acetone volume (B), toluene volume (C), sample volume (D) were observed as significant effects; while amount of $(NH_4)_2SO_4$ (A), centrifugation time (E) and vortexing time (F) were statistically non-significant.

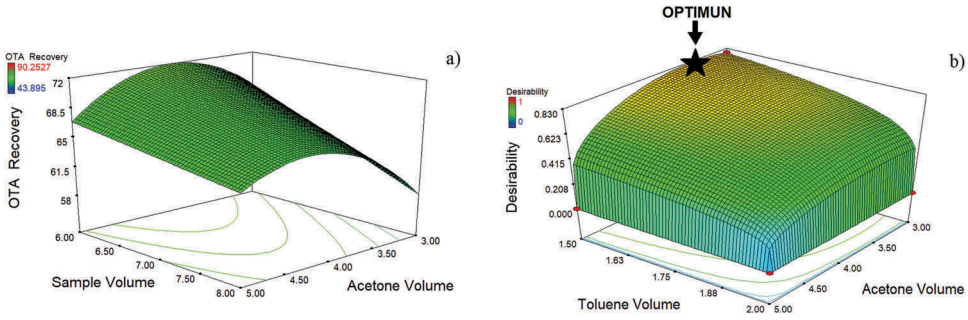

Figure 3. (a) Response surface plot for the response (OTA Recovery) for optimisation of the factors: sample and acetone volume. (b) Response surface plot for the desirability function surface and optimal toluene and acetone volume.

The values for each variable presented in Table 1 were verified and the theoretical recovery was contrasted with the obtained experimental recovery. Consequently, no significant differences between the expected (88.2%) and the experimental values (90.1%, n = 5, RSD% = 5.6%) were observed when the t-test for means differences was applied.

Matrix effect evaluation

Components in complex matrices (e.g. beer) might affect the analyte's signal and could induce enhancement or suppression on the analytical signal and untrusted results. Consequently, the extent of this effect (signal suppression SS%) was evaluated and calculated by means of the quotient of both the slope of the calibration curve from spiked samples treated with the SALLE methodology and the slope of the calibration curve obtained from OTA standards in pure methanol solvent, using Equation (3). As a result, signal suppression was reduced from almost 95% (in this case spiked samples without a sample treatment were employed) to slightly less than approximately 16% when the SALLE pre-treatment was applied to OTA analysis in beer samples prior to UHPLC-MS/MS system. Thus, a significant improvement of the signal, and consequently of the sensitivity is attained with the optimised pre-treatment. The latest demonstrated the importance of applying the SALLE strategy for OTA determination at trace levels in beer samples.

$$SS\% = 100 - \left[\left(\frac{b_{\text{sample}}}{b_{\text{standards}}}\right)100\right] \quad (3)$$

Method validation

Normality and homoscedasticity were assumed for the calculation of the LOD and LOQ. These values were calculated as mentioned in the preceding text and the obtained values were 0.02 μg l^{-1} and 0.08 μg l^{-1}; respectively. These values were compatible and lower than the maximum concentration levels established by countries including Italy (0.2 μg l^{-1}) (Soto et al. 2014). Also, a symmetrical shape for OTA chromatography peak in the spiked beer samples was observed when the transition (m/z) 404.1 → 239.2 was employed for quantification (Supplementary Data 2).

Furthermore, the results for precision and accuracy of the method are shown in Table 2. According to these results, low variability for the methodology was observed and the intra-day precision was in agreement with the current

Table 2. Precision, recovery (%) and bias (%) for OTA determination in beer samples by SALLE sample treatment prior to UHPLC-MS/MS.

Spiked concentration level (μg l^{-1}) (n = 5)	Intra-day RSD (%)[a]	Inter-day RSD (%)[a]	Recovery (%)[b]	Bias (%)[c]
0.1	6.7	13.5	90.7 ± 4.1	9.3
0.2	5.9	11.2	90.4 ± 2.4	9.6
0.4	5.2	14.2	92.6 ± 4.6	7.4
1.0	7.5	13.0	91.5 ± 2.8	8.5

[a]RSD (%) = Relative standard deviation; [b]Recovery (%) = [(Measured content/Spiked level)×100] and ± standard deviation associated; [c]Bias (%) = [((Measured content − Spiked level)/Spiked level) × 100].

legislation (European Commission 2006b). Otherwise, at a concentration level of 0.1 μg l^{-1}, inter-day precision (relative standard deviation (%)) was 16.3%, lower than 23% which corresponds to the stipulated value in the norm for concentrations levels below 100.0 μg l^{-1} (European Commission, 2006b). Additionally, accuracy in terms of recovery and trueness (bias (%)) was in accordance to the same regulation, which establishes as an acceptable bias values between −50% and +20% for concentration levels ≤ 1 μg l^{-1}, −30% and +10% for concentration range > 1 μg l^{-1} – 10 μg l^{-1}.

Linearity

Linearity of the methodology from the calibration curves for spiked beer samples at OTA concentration levels from the LOQ up to approximately 1.0 μg l^{-1} (considering the concentrations mentioned in Table 2), including blank samples, was evaluated. As a result, linearity was adequate with a determination coefficient (R^2) of 0.9982. Also, linear regression was statistically acceptable at the studied concentration levels and this model showed a satisfactory fit when the F-test for lack of fit ($p = 0.05$) was evaluated according to Olivieri (2015) (as can be seen for the calibration curve in the Supplementary Data 3).

Determination of OTA in beer samples and methodological comparison

As mentioned in previous sections, cereals employed in brewing as malt, either wheat or barley, might be contaminated with OTA; then, beer might be contaminated as well. As a consequence, the optimised and herein proposed methodology was applied to beer samples in order to evaluate the mycotoxin content in different types of samples. A total of 21 beer samples from different provenance and types were analysed using the SALLE – UHPLC-MS/MS methodology (Table 3). As can be observed, all the analysed samples were contaminated with OTA at concentration levels ranging from <LOQ (OTA was detected but non-quantifiable <0.08 μg l^{-1}) to 0.26 μg l^{-1}. Both mean and statistical mode of the OTA concentration were 0.12 μg l^{-1}, while the statistical median was 0.105 μg l^{-1}. Moreover, only one beer sample with a concentration level of 0.26 μg l^{-1} exceeded the maximum limit allowable (0.2 μg l^{-1}) by Italy (Soto et al. 2014).

As an analytical comparison, Table 4 summarises some selected works reported in the literature focused on the use of either HPLC or UHPLC coupled to FD or MS/MS for OTA determination in beer samples. As mentioned earlier, methodologies based on HPLC-FD (i.e. official methodology (Visconti et al. 2001)) have shown high sensitivity. However, some of these methods require the use of

Table 3. OTA analysis in beer samples by mean of SALLE-UHPLC-MS/MS.

Origin	Type	Cereal Type	Alcoholic content (% v/v)	OTA concentration level (μg l^{-1})
Argentina	Ale Kölsch	Barley	5.0	<0.08
Argentina	Lager	Barley	4.6	<0.08
Argentina	Lager	Barley	4.7	0.08
Argentina	Lager	Barley	4.8	<0.08
Argentina	Lager	Barley	5.0	<0.08
Argentina	Pale Ale	Barley	5.5	0.26
Argentina	Pilsener	Barley	4.8	<0.08
Argentina	Porter	Barley	5.5	0.11
Argentina	Scotch Ale	Barley	6.0	0.08
Argentina	Strong Ale/Barley Wine	Barley	10.0	0.12
Argentina	Strong Lager/Bock	Barley	6.3	0.09
Argentina	Strong Lager/Double Bock	Barley	4.8	0.12
Argentina	Weizen	Wheat	3.0	<0.08
Czech Republic	Lager	Barley	5.0	<0.08
Germany	Dunkelweizen	Wheat/Barley	5.3	<0.08
Germany	Lager	Barley	4.8	<0.08
Germany	Lager	Barley	7.9	<0.08
Germany	Pale	Barley	0.5	<0.08
Germany	Weizen	Wheat/Barley	4.0	<0.08
Netherlands	Strong Pale Lager/Bock	Barley/Wheat	7.9	<0.08
Russia	Lager	Barley	4.7	0.10

Table 4. Comparison of analytical performance of different methodologies for OTA determination in beer samples.

Sample treatment	Separation/Detection	LOD – LOQ (μg l^{-1})	Beer style/Type	Number of samples (*n*)	Reference
IAC (OchraTest™)	HPLC-FD	0.01–0.2	NM[a]	12	(Visconti et al. 2001)
Addition of alkali solution/Zn salt, LLE, SPE; and IAC (OchraTest™)	HPLC-FD/HPLC-Ion Trap-MS	0.0008–0.0025	NM[a]	69	(Medina et al. 2006)
Protein Precipitation with acetone and SPE (Anion exchange/reversed phase)	UHPLC–Ion Trap-MS/MS	LoD = 0.4 μg kg^{-1}	NM[a]	NM[a]	(Reinsch et al. 2007)
IAC	UHPLC-FD	0.0003–0.001	Blended, Dark, Lager, Nonalcoholic, Pale, Special beers	115	(BêLakova et al. 2011)
SPE (Oasis HLB®)	UHPLC-Hybrid Linear Ion Trap-HRMS	LoQ = 0.03	Ale, Pale, Red Ale, and Stout	25	(Rubert et al. 2011)
Modified QuEChERS	UHPLC-QqQ-MS/MS	LoQ = 0.25	NM[a]	24	(Tamura et al. 2011)
'Dilute and Shot' and stable isotope dilution	UHPLC – QqQ-MS/MS	LoD = 0.1	NM[a]	76	(Al-Taher et al. 2013)
SPE based on MIP	HPLC-FD/HPLC-QqQ-MS/MS	0.025–0.08	NM[a]	10	(Cao et al. 2013)
SPE	HPLC-QqQ-MS/MS	0.75–2.5	Ale, Pale Lager, Red Ale, Stout	49	(Rubert et al. 2013)
On-line SPE-HPLC	HPLC-FD	0.003–0.01	Dark Lager, Light Lager,Wheat	49	(Lhotská et al. 2016)
SALLE	UHPLC-MS/MS	0.02–0.08	Ale Kölsch, Dunkelweizen, Lager, Pale, Pale Ale, Pilsener, Porter, Strong Pale Lager/Bock, Wizen	21	This Work

Fluorescence detection (FD); high-performance liquid chromatography (HPLC); immunoaffinity column (IAC); liquid–liquid extraction (LLE); mass spectrometry (MS); molecularly imprinted polymer (MIP); quick, easy, cheap, efficient, roughness and safe (QuEChERS); salting-out assisted liquid–liquid extraction (SALLE); solid phase extraction (SPE); tandem mass spectrometry (MS/MS); triple quadrupole (QqQ); ultra-high-performance liquid chromatography.
[a]NM = Not mentioned.

IAC, as sample preparation strategy previous to HPLC-FD, for a non-equivocal OTA detection. In this sense, there have been reported other alternative approaches (addition of buffers or salt-SPE, liquid-liquid extraction or SPE) to reduce the cost of analysis. Thus, methodologies based on either HPLC or UHPLC coupled MS/MS have allowed unambiguous OTA determination without the use of IAC, though also require a sample treatment in order to extract OTA from samples and reduce matrix effects. As a consequence, different clean-up procedures such as 'Dilute and Shot' – stable isotope dilution, modified QuEChERS, and SPE (commercial and MIP) have been employed (Table 4). However these approaches sometimes are expensive, time consuming, and require a special effort/know-how. Therefore, in contrast to others, the SALLE-approach herein described is an excellent alternative for sample treatment that allows performing a simple and easy analysis for relatively non-expensive OTA determination in beer samples.

A comparison of the LOD and LOQ values reported in Table 4 with the ones obtained for the proposed methodology was not feasible since most of such values were calculated using the ratio-S/N approach and differ this way from the herein obtained based on the EU Reference Laboratories for mycotoxins approach (Wenzl et al. 2016). However, the LOD and LOQ calculated for the described methodology were suitable for the OTA determination in beer samples at low concentrations levels (<0.2 μg l^{-1}). Furthermore, as summarised in the Table 4, several styles/types of beer and beers with different alcohol contents were studied.

Conclusion

A novel sample treatment based on SALLE applied prior to the sample injection to the UHPLC-MS/MS system was developed. Thus a simple, cheap, fast, and effective to reduce matrix effect methodology for the analysis of ochratoxin A in beer samples was achieved. Also, this methodology was in agreement with the rigorous sensitivity requirements of current normatives for the determination of mycotoxins in food. In this context, both LOD

and LOQ were lower than the maximum allowable limit of OTA in beer samples proposed by Italy. We used an experimental design approach to find out the optimal conditions of SALLE. This optimisation approach resulted in saving time and materials, while arriving at conclusions with statistical meaning. Finally, it was observed that SALLE is suitable and robust for OTA determination in different types and origins of beers.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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