Research Article

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Assessment of ochratoxin A occurrence in Argentine red wines using a novel sensitive quechers-solid phase extraction approach prior to ultra high performance liquid chromatography-tandem mass spectrometry methodology

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Abstract

BACKGROUND: The assessment of ochratoxin A (OTA) in wine is relevant for food safety and its continuous control allows to reduce the risk of intake. Thus, a novel sensitive QuEChERS-SPE (Quick, Easy, Cheap, Effective, Rugged and Safe – Solid Phase Extraction) pretreatment prior to liquid chromatography coupled to tandem mass spectrometry was developed for the determination of OTA in red wine samples from different grape-growing regions in Argentine.

RESULTS: A sensitive methodology was achieved and thus the limits of detection and quantification were 0.02 and 0.05 μg L^{-1}, respectively. Recoveries ranged from 89.0% to 105.3%. The method was applied to 136 red wine samples (Argentina's flagship varieties: Malbec and Cabernet Sauvignon) from ten grape-growing regions, during vintages 2013–2015. Although all of the samples investigated were contaminated with OTA (concentrations ranged from 0.02 to 0.98 μg L^{-1}), the levels detected were lower than the maximum allowable concentration limit of 2.0 μg L^{-1} established by international regulations.

CONCLUSION: The methodology proposed is suitable for reliable OTA analysis in red wines. Similarly, the values obtained from the samples analyzed were in accordance with the current regulations and, as a consequence, preventive actions to reduce this mycotoxin incidence can be undertaken.

Keywords: ochratoxin A; Argentinean red wines; QuEChERS; UHPLC-MS/MS

INTRODUCTION

Grapes and their derived products, such as grape juice, dry vine fruits and wine,[1,2] as well as other foodstuffs,[3] are frequently contaminated with ochratoxin A (OTA). This mycotoxin is a secondary metabolite produced by species of fungi belonging to the *Aspergillus* and *Penicillium* genera, and there is strong evidence that *Aspergillus carbonarius* is one of the main sources of OTA contamination in wine grapes.[4] Also, *A. carbonarius* grows at quite high temperatures and is resistant to ultraviolet light and sunlight. These characteristics provide a competitive advantage in vineyards and grape drying yards.[5] Otherwise, *Aspergillus* species belonging to section *Nigri*, commonly known as black Aspergilli, in particular, *A. carbonarius* and species belonging to the *A. niger* aggregate, have been isolated in Argentinian vineyards showing different potential of OTA production in wine, with *A. carbonarius* being the most ochratoxigenic strain as well.[6] Moreover, OTA is one of the most important mycotoxins of concern for human health. The major toxic effect of OTA is nephrotoxity, in addition to immunosuppressive, teratogenic, neurotoxic and carcinogenic properties.[7] The International Agency for Research on Cancer (IARC) classified OTA as a possible human carcinogen (group 2 B).[8] It now appears that OTA could be 'a complete carcinogen' (not only an initiator, but also a promoter) and that its mutagenicity has been revised, obliging reinforcement of its monitoring in food.[9–11] Several nephropathies affecting animals and humans

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have been attributed to OTA and this toxin has been reported as the possible causative agent of endemic kidney disease observed in the Balkans and related tumors in the urinary tract.[7] Moreover, this mycotoxin was shown to be stable in blood, with a half-life of approximately 35 days in serum.[12] On the other hand, after cereals, wine is the second most important source of contribution of OTA to the mean European total dietary intake. Thus, the consumption of wine can represent up to 10% of the contribution of OTA to the total European dietary intake.[13]

The most relevant factors that influence fungal growth on grapes and its OTA contamination of grapes and wine include: temperature and relative humidity in the month before harvesting the berries, the type of wine maceration (grape skin contact) and the percentage of damaged berries before vinification. In this sense, red wines are more susceptible to OTA contamination than rosé and white wines.[14] Also, OTA shows a high stability against degradation, possessing a high resistance to acidic conditions and high temperatures.[3] In this context, the biodiversity, toxigenic ability and potential contamination/production of OTA by *Aspergillus* section *Nigri* in Argentinean vineyards through different vintages and the effect of the agro-meteorological conditions have been evaluated.[15]

Furthermore, OTA content during wine production (i.e. from must to wine) in Bonarda and tempranillo from Argentine has been evaluated in a pilot scale vinification.[16] OTA levels during the vinification trials were observed to drop to an average of approximately 86.5% in both varieties of wine. The significant reduction of OTA during the vinification process might be a result of the partition of the OTA between the liquid and the solid phase because of extensive adsorption of OTA onto the solid parts of the grapes.

On the other hand, the Scientific Committee for Food of the European Commission considered that it would be prudent to reduce the tolerable daily intake (less than 5 ng kg^{-1} body mass),[17] which indicates that OTA accumulation constitutes a considerable risk situation for consumers.[18,19] Recent trends in regulatory programs for food safety have focused on the emerging threat of mycotoxins in foods. The International Organization of Vine and Wine (OIV) has recommended a maximum allowable concentration limit of 2 μg L^{-1} for wines, which is the same as the maximum permitted level established and currently regulated by Commission Regulation (EC) No 1881/2006 and its subsequent amends in the European Community.[20] Also, some countries and marketers (e.g. Finland and some British supermarkets) have national laws and regulations for OTA and apply their own limits (sometimes as low as 0.5 μg L^{-1}).[21] The increased awareness of the potential risk for consumer health as a result of exposure to OTA through wine consumption requires each country to carry out systematic measurements of OTA levels of the wines offered in the domestic market. Also, OIV recommends applying preventive measures (e.g. identifying the species and strains of toxicogenic fungus present in their region; combining this information with regional risk factors, meteorological data and viticultural techniques; and proposing adapted management, communicate this information to growers, etc.) in viticulture regions in which the climatic conditions are favorable to the formation of OTA in vine products aiming to reduce epidemic risk which favors the onset of very damaging vine disease.[22]

As a consequence, it is imperative to obtain knowledge about the OTA concentration profile in wine production considering that, according to the OIV, Argentina is one of the largest wine producer worldwide (the fifth largest producer).[23] It is this essential to use the information obtained to reduce the presence of OTA in wine not only to improve wine quality, but also to maintain its safety. Therefore, continuous monitoring of OTA is needed to ensure wine quality. Thus, OTA levels have been detected in grape and wine samples from different vintage and regions in Argentine, ranging from 0.1 to 5.4 ng g^{-1} and from 0.01 to 4.82 ng mL^{-1}, respectively.[15] From the grape varieties assayed, Cabernet Sauvignon and Syrah have shown the highest levels of OTA contamination whereas, in wine samples, the mycotoxin has only been detected in the Syrah-based variety. Correlation analysis has shown that regions such as La Rioja and San Juan, with higher average temperatures, influenced the high *A. carbonarius* isolation percentages and the highest number of samples contaminated with OTA was observed. Higher temperatures during the day could favor fungal growth, whereas lower temperatures during night could favor OTA production.[15] The assessment of the OTA profile concentrations in wine from Argentina has been monitored and the results obtained are shown in Table 1.

Determination of OTA as a result of its particular chemical properties and the fact that it can be present in wine at low concentrations constitutes an analytical challenge; thus, reliable sample preparation protocols and subsequent sensible testing methods are needed. A reference method for the determination of OTA in wine has been established by the European Standard EN 14133[27] and the OIV (Method OIV-MA-AS315-10).[28] Both methods are based on the previous use of immunoaffinity columns (IACs) on the sample followed by high-performance liquid chromatography with fluorescence detection (HPLC). Other alternatives, such as liquid chromatography coupled to mass spectrometry or tandem mass spectrometry (LC-MS or LC-MS/MS) have also been reported for detection of OTA in wines.[29,30] Hence, sample preparation is a critical step in the analysis of OTA in food matrices, such as wine, because of the amount of endogenous compounds present that might interfere with an accurate analysis of the mycotoxin. To minimize matrix effects, different sample preparation procedures have been reported for the extraction of OTA from food matrices. Most of them have been based on the use of IACs.[28,31] However, the use of IACs is costly and sample preparation procedure is tedious and time consuming. Solid phase extraction (SPE)-based protocols have been reported for the removal of OTA from food matrices.[32–36] Also, recent studies have shown that the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction/purification procedure, initially proposed for pesticide analysis,[37] has been modified and successfully applied in food analysis. The characteristic features of the QuEChERS method are: (1) extraction with acetonitrile in a partitioning salting-out using anhydrous magnesium sulfate ($MgSO_4$) and sodium chloride (NaCl) and (2) purificatión using a dispersive solid phase extraction (dSPE) step that involves further clean-up using different sorbents, such as alumina, C_{18}, diatomaceous earths, graphitized black carbon, primary-secondary amine (PSA) and silica to remove interfering substances. QuEChERS-based approaches for the extraction of OTA in wine have also been reported. The determination of OTA using QuEChERS associated with LC-MS/MS employing electrospray ionization (ESI) and its respective evaluation of matrix effect has been carried out. A method using ACN as extraction solvent, $MgSO_4$ and sodium acetate (as buffer) in a single step associated with ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) system was proposed and signal suppression as a matrix effect was observed.[38] Additionally, another reported method for the analysis of OTA in wine was based on QuEChERS

Table 1. Summary of the published reports on ochratoxin A occurrence in Argentine wines

Vintage	Variety	Region	Number of samples	LoD[a]	Mean	Range	Reference
NM	NM	Argentina	12	0.021	0.035	0.028–0.042	Rosa *et al.*[24]
1996 1998 1999 2000 2001 2002	Borgoña Barbera, Cabernet Sauvignon, Malbec, Merlot, Pinot Noir, Syrah	Mendoza San Juan Neuquén-Rio Negro	54	0.008	NM	<2.0	Pacin *et al.*[25]
2008	NM[b]	North Mendoza North-West Mendoza High zone of Mendoza river South Mendoza San Juan La Rioja Neuquén-Rio Negro	47	0.01	NM[b]	0.02–4.82	Ponsone *et al.*[26]
2007 2008 2009 2010	Bonarda, Cabernet Sauvignon, Pinot Noir, Syrah	North Mendoza North-West Mendoza High zone of Mendoza river South Mendoza San Juan La Rioja Neuquén-Rio Negro	75	0.01	NM[b]	0.01–4.8	Chiotta *et al.*[15]

[a] LOD, limit of detection (μg L^{-1}).
[b] NM, not mentioned.

combined with liquid chromatography coupled to electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS) using a mixture of extraction solvents composed of acetonitrile and acetic acid (99:1), followed by extract clean-up by means of dSPE.[39] In this methodology, signal enhancement as a matrix effect was observed.

In the present study, the development of a methodology based on the optimization of an original and robust combined QuEChERS-SPE strategy for sampling clean-up and the extraction of OTA coupled to UHPLC-MS/MS analysis of red wine samples was developed. Although our group recently reported a methodology based on SPE extraction, this procedure reached a minimization of the matrix effect of approximately 80%. In the present study, a sample clean-up greater than 90% was achieved, which is more compatible with the ultra-trace OTA concentration levels allowed in red wine samples by legislation. In this context, the sensitivity was highly improved and the observed initial matrix effect on the signal of the analyte was substantially reduced. Thus, reliable measurements from Argentinian wines from different regions were evaluated. Therefore, the present study aimed: (i) to assess a novel sample treatment procedure based on a QuEChERS-SPE extraction/clean-up step to improve the removal conditions of OTA from the red wine samples and, consequently, reduce the matrix effect and (ii) to screen the ocurrence and concentrations of OTA in red wine samples from different Argentine grape-growing regions during three vintages from 2013 to 2015. The information obtained could be used as valuable tool to support and optimize the knowledge about grape and wine management, during culture time (pre- and post-harvest), as well as OTA content in grapes from different grape-growing regions. In addition, because it is important to decrease the risk of OTA contamination, the proposed analytical methodology can be used for the rigorous monitoring of this mycotoxin and assist in the application of agroalimentary strategies that decrease its occurrence in wine.

MATERIALS AND METHODS

Reagents and standard solutions

Ochratoxin A, analytical standard ≥98% for HPLC, was obtained from Sigma (St Louis, MO, USA). The working solutions were prepared and preserved according to the AOAC method.[40] Acetonitrile (ACN), methanol (MeOH), ammonium acetate, ammonium formate, acetic acid, formic acid, and water Optima® LC-MS grade were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Magnesium sulfate anhydrous ($MgSO_4$) was obtained from Sigma-Aldrich (Steinheim, Germany). Suprapur® Sodium chloride (NaCl), disodium hydrogen phosphate, potassium dihydrogen phosphate and EMSURE® trisodium citrate dehydrate were purchased from Merck (Darmstadt, Germany). AOAC formulation ISOLUTE® QuEChERS (AOAC Method 2007.01; QuEChERS extraction tube: 6 g of $MgSO_4$ and 1.5 g of sodium acetate) extraction tubes were supplied by Biotage (Charlotte, NC, USA). Bulk sorbents as alumina and PSA (Agilent Technologies, New Castle, DE, USA), C_{18} endcapped and GPC (Agilent Technologies, Mulgrave, Australia) and silica (Agilent Technologies, Waldbronn, Germany) were employed for dSPE. For the solid phase extraction step, 3-mL and 60-mg ISOLUTE® Myco cartridges (Biotage, USA) were used.

Working standard solutions were prepared in ACN by stepwise dilution of a 10 mg L^{-1} OTA stock standard solution immediately before use. Quantification was achieved by making spiked red wine samples with proper amounts of the analyte. The OTA solutions in ACN were maintained at −4 °C, protected from light, and kept in amber flasks. Intermediate spiked samples of red wine without previously detected OTA were prepared before analysis.

Sample origin

Ten grape-growing regions located in Argentina were surveyed in three consecutive periods (2012/13, 2013/14 and 2014/15). The major wine regions of Argentina are located in the western part of the country. Thus, the geographical areas selected for the sampling were located in Salta-Catamarca-Calchaquies Valley

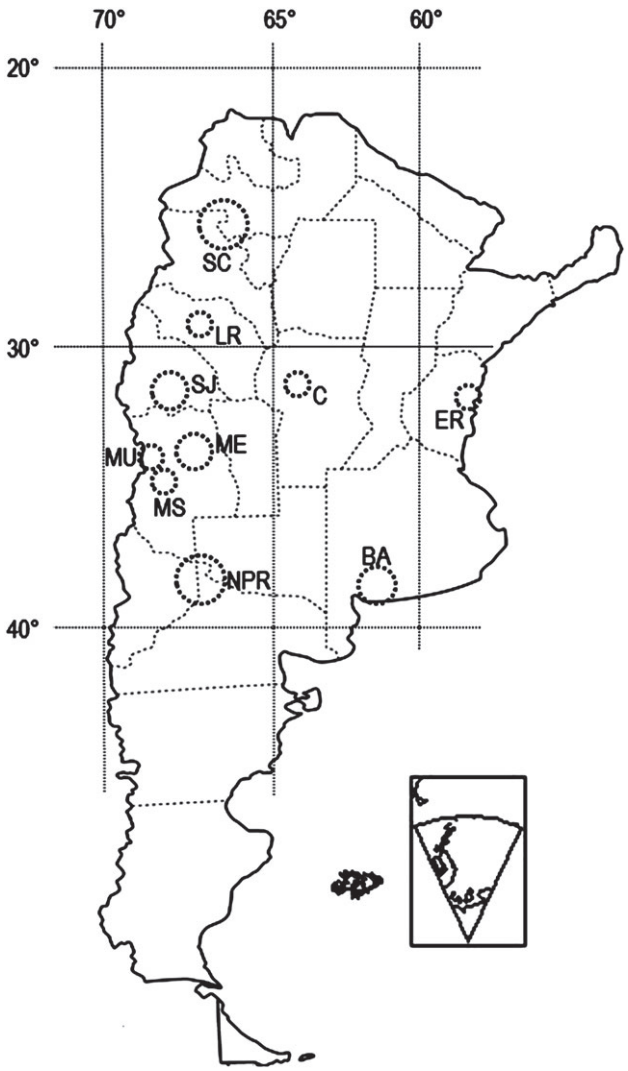

Figure 1. Locations of the grape-growing regions monitored: Salta-Catamarca-Calchaquies Valley (SC), La Rioja (LR), San Juan-Tulum Valley (SJ), Mendoza East (ME), Mendoza-Uco Valley (MU), Mendoza South (MS), La Pampa-Neuquen-Rio Negro (NPR), Buenos Aires South (BA), Cordoba (C) and Entre Rios (ER).

(25–26°S, 65–66°W), La Rioja (29°S, 67°W), San Juan-Tulum Valley (31–32°S, 68°W), Mendoza East (33°S, 67–68°W), Mendoza-Uco Valley (33°S, 69–70°W), Mendoza South (34–35°S, 68–69°W), La Pampa-Neuquen-Rio Negro (37–39°S, 67–69°W). In addition, other eastern regions considered as a 'new grape-growing region', located in Buenos Aires South (37–39°S, 61–62°W), Cordoba-Colonia Caroya (34–35°S, 68–69°W) and Entre Rios-Concordia (31–32°S, 58°W), were also evaluated (Fig. 1). According to the recognized Köppen Climate Classification, climate in these regions has been defined as cold desert (BWk) for Salta-Catamarca, La Rioja, San Juan, Mendoza East and La Pampa-Neuquen-Rio Negro; cold steppe (BSk) for Mendoza-Uco Valley and Mendoza South; warm temperate with fully humid and warm summer (Cfb) for Cordoba and Buenos Aires South; and warm temperate with fully humid and hot summer (Cfa) for Entre Rios.

Phenological growth stages of the grapevine cover the period between dormancy and leaf fall. In Argentina, this period starts in October/November with flowering, followed by veraison in December depending on the variety and the location. All fields were sampled in harvest, between March and May. Also, soil textural classes in the vineyards sampled were mainly sandy loam, silt loam and clay loam.

Malbec and Cabernet Sauvignon red wine varieties were sampled in each region as the principal varieties of production of each grape-growing region and vintage noted above. In the sampling plan, all samples were collected with a ripeness measured between 20 and 27.5 °Brix; most of the samples were collected with optimal hydration and health status. Bunches employed for vinification were collected in paper bags. Next, berries were mechanical crushed and the fermentation was carried out in stainless steel tanks for must production and vinification. Potassium metabisulfite ($K_2S_2O_5$) was employed as only additive during vinification. This process was performed at the Instituto Nacional de Vitivinicultura (Argentina). Wine samples (total volume of 1 L aliquoted into 50-mL subsamples) were obtained from the cellar during bottling and stored at 10 °C until analysis.

Meteorological data

Air temperature and rainfall meteorological data (from September to May) for each sample and respective vintage were supplied by the Servicio Meteorologico Nacional (Argentina) from weather stations located as close as possible to each vineyard. It is important to note that maximum temperatures monitored during each year (2013–2015) were above the values registered in the last 55 years, being extremely hot in the case of summer of 2013/14. The mean temperature anomalies for 2013, 2014 and 2015 were +0.58 °C, +0.60 °C and +0.71 °C, respectively. These anomalies indicate that the years monitored were warmer than the reference value. Also, the rainfall rate for 2013 was below average (−10 % rainfall anomaly), being a dry year, whereas rainfall rates for 2014 and 2015 were above average (+21 % and + 9% rainfall anomalies), indicating wetter years.

Mass spectrometry

Mass spectrometry analyses were performed on a Quattro Premier™ XE Micromass MS Technologies triple quadrupole mass spectrometer with a *Z*-Spray™ electrospray ionization source (Waters, Milford, USA). The source was operated in a positive (ES+) mode at a desolvation temperature of 350 °C with N_2 as the nebulizer 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 mode of selected ions. The OTA quantification transition was (*m/z*) 404.1 → 239.2, which was produced at a collision energy of 25 eV. The transitions used for confirmation purposes were (*m/z*) 404.1 → 341.1 and 404.1 → 358.2, produced at an collision energy of 25 and 20 eV, respectively. The values optimized for the dwell time and cone voltage parameters were 0.08 s and 20 V, respectively. The data were acquired using mass spectrometry software (MassLynx, version 4.1; Waters).

Chromatography

An Acquity™ Ultra High Performance LC system (Waters) equipped with autosampler injection and pump systems (Waters) was used. The autosampler vial tray was maintained at 15 °C. The needle was washed with appropriate mixtures of acetonitrile and water. The separation was performed by injecting 25 μL of sample

Table 2. Summary of the optimized conditions for the QuEChERS-SPE procedure

	Operating conditions
QuEChERS Step	
Mixing	Sample (5 mL); NH_4CH_3COO (75 mg) ; ACN (5 mL)
Salting-out	$MgSO_4$:NaCl (4:1) 2.5 g
Centrifuged	3100 × *g* for 3 min
Load/dilution	Water: Supernatant (70:30) (17 mL)
SPE Step	
Conditioning	ACN (2 mL); 1 mL min^{-1}
Equilibrate	H_2O (2 mL); 1 mL min^{-1}
Wash interference 1	H_2O (5 mL); 1 mL min^{-1}
Wash interference 2	H_2O/ACN (60:40) (5 mL); 1 mL min^{-1}
Elute	2.65 mmol L^{-1} of formic acid in MeOH (2 mL)
Evaporate	Drying under nitrogen stream (30 °C)
Reconstitute	MeOH (0.25 mL); transfer to UHPLC vials

onto an ACQUITY UPLC® BEH C_{18} (Waters) analytical column (internal diameter 2.1 mm, length 50 mm, particle size 1.7 µm). The binary mobile phases consisted of water with 2.65 mmol L^{-1} formic acid (A) and acetonitrile with 2.65 mmol L^{-1} formic acid (B) delivered at 0.4 mL min^{-1}. The composition of the isocratic elution program was 30% A and 70% B. Under the described conditions, the OTA retention time was 0.5 min within a total chromatographic run time of 2.0 min. The column was held at a temperature of 30 °C. Under the above conditions, no sample contamination or sample-to-sample carry-over was observed.

Sample treatment

A volume of 5 mL of red wine sample was transferred to a 15-mL polytetrafluoroethylene tube with screw caps and then 75 mg of ammonium acetate (additive buffer system) was added, and the tube was shaken for 10 s in a vortex. Subsequently, 5 mL of ACN was added to the sample and vortexed for 1 min. Later, 2.5 g of the salting-out mixture composed of anhydrous magnesium sulfate and sodium chloride (4:1) was added. Each tube was vortexed for 30 s and centrifuged at 3100 × *g* for 3 min. Afterward, an aliquot (5 mL) from the supernatant (upper phase of the extract-acetonitrile phase) was diluted in 12 mL of water (water supernatant dilution 70:30) to allow clean-up using a SPE Myco® cartridge. This procedure was shown to be of crucial importance for maximizing the sensitivity of OTA and minimizing the presence of interfering compounds in the extract. The conditions of QuEChERS and SPE procedure applied were developed previously by our group.[41] The optimized conditions are provided in Table 2.

Statistical analysis

Data on OTA contamination in wine sampled different vintage, grape-growing regions and variety were analyzed using different statistical tools: one-way and two-way analysis of variance (ANOVA) and its non-parametric counterpart, the Kruskal–Wallis test, as well as the Welsh Test when different variances were assumed for the analysis. Prior to ANOVA analysis, Bartlett's method (normal data were evaluated and assumed) was used for homogeneity of variance. Additionally, after ANOVA, Tukey's test and the Games-Howell post-hoc tests were employed. Moreover, the Mann–Whitney (Wilcoxon) *W*-test was used to evaluate whether groups of samples differ from each other. Pearson correlation was applied to evaluate the relationship between OTA concentration and soil texture. All statistical analyses were performed using Minitab, version 17.1.0 (Minitab Inc., State College, PA, USA) and OriginPro, version 9.1.0 (OriginLab Corp., Northampton, MA, USA).

RESULTS

Optimization QuEChERS-SPE

To reduce the matrix effect on the OTA signal, a pretreatment was implemented consisting of an extraction by salting-out, which used an excess of ACN, $MgSO_4$ and NaCl to separate the aqueous and organic phases, in accordance with the QuEChERS approach to red wine samples. In this sense, the extraction performance of QuEChERS was evaluated with respect to the salting-out step using several additives. Thus, acetic acid, ammonium acetate, ammonium bicarbonate, ammonium formate, AOAC formulation (extraction tube: 6 g of $MgSO_4$ and 1.5 g of sodium acetate), formic acid, disodium-hydrogen phosphate/potassium-dihydrogen phosphate and trisodium citrate dihydrate as buffers systems or additives in the salting-out step were assayed.

The additive-buffer was selected with the aim of obtaining a signal improvement as a result of the reduction of the signal suppression effect. In this sense, initial sample signal suppression was approximately 95%; subsequently, after applying the salting-out step without additive, the effect was reduced to 45% and, considering the use of an optimal additive, the observed detrimental outcome on the OTA signal was approximately 30%. The results obtained demonstrated that the extraction was most effective when ammonium acetate was employed as an additive (Fig. 2). As a consequence, ammonium acetate was used in further assays. In addition, the concentration of ammonium acetate was evaluated to increase the OTA signal in the salting-out extract. As shown in Fig. 2(b), the optimal concentration value for the additive buffer was 15 mg mL^{-1}.

On the other hand, as noted above, these studies were performed because conventional QuEChERS procedures normally involve the use of dSPE sorbents for clean-up step after salting-out extraction. Next, several dSPE sorbents, such as alumina, C_{18}, GPC, PSA and silica, were employed for this purpose. The whole supernatant salting–out extract (5 mL) was subjected to a clean-up process using different mixtures of each of the mentioned sorbents plus $MgSO_4$. Extract from salting-out and reagents was placed into a screw cap test tube and vortexed for 1 min and then centrifuged at 3000 × *g* for 5 min. The supernatant was dried down under a nitrogen stream and reconstituted with 0.5 mL of MeOH. The recoveries for OTA were calculated and ranged between 30 and 40% for alumina, C_{18} and silica, and were below 15% for GPC and PSA. Unfortunately, signal suppression continued to be the main drawback when dSPE sorbents were employed in the clean-up step and this strategy was not used for further studies.

As a consequence, a second clean-up approach for removing interferents based on using SPE was evaluated to improve the OTA response. As noted, the SPE procedure was developed previously by our group.[41] However, some modifications were required to be able to hyphenate QuEChERS to SPE and, consequently, to favor the retention/elution of OTA into the packed sorbent of the cartridge. Thus, the extract or supernatant from the salting-out step, mainly composed of ACN, was diluted into water previously used for loading the SPE Myco® cartridges to favor retention of the analyte. The water/supernatant (ACN) ratio was evaluated in the range from 40:60 to 90:10 (v/v). As shown in Fig. 3, the

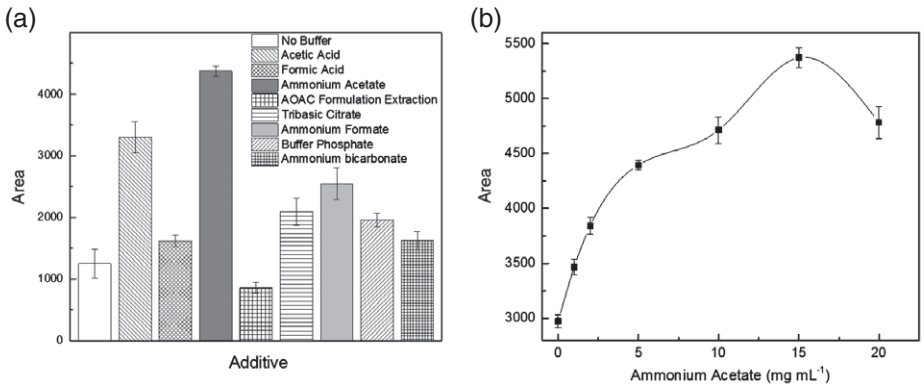

Figure 2. (a) Evaluation of the effect of the nature of the additive on the OTA signal used for the salting-out step. (b) Optimization of the concentration of the selected additive on the OTA signal used for the salting-out step.

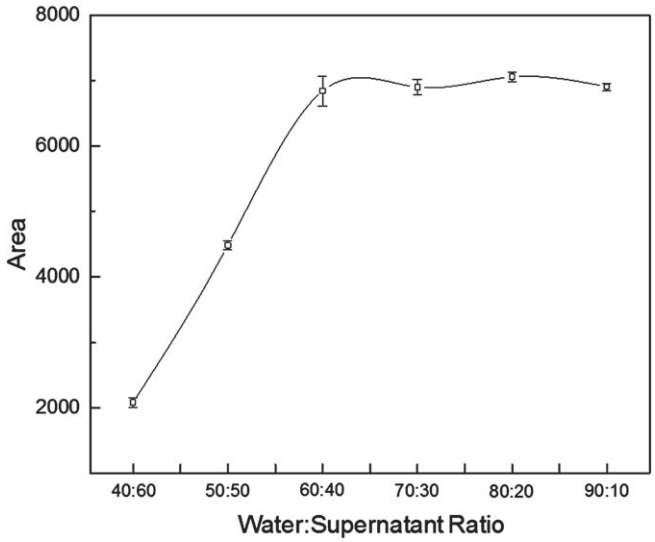

Figure 3. Evaluation of water content in the supernatant dilution before cartridge loading.

optimum OTA signal was obtained within the range 40:60 to 90:10 (v/v) for water:supernatant. Thus, a 70:30 [water/supernatant (v/v)] content for the supernatant dilution was selected for subsequent assays.

Evaluation of QuEChERS-SPE performance on the matrix effect

One of the main drawbacks of a method using LC-atmospheric pressure ionization-MS for complex samples is related to ionization effects as a result of co-eluting matrix components.[42] Matrix effects can cause suppression or enhancement of the target analytes and might hamper accurate mass spectrometric quantitation, leading to false results. In this sense, the matrix effect (produced, for example, by sugars, polyphenolic, carotenoids and anthocyanins compounds) was evaluated by comparing slopes from both the standard calibration curve (OTA in MeOH) and spiked wine samples after they had been treated using QuEChERS-SPE and then calculating the signal suppression/enhancement (SSE%) using an equation that compares the slopes obtained:

$$SSE\% = \frac{Slope_{(\text{spiked extract})}}{Slope_{(\text{spiked solvent})}} \times 100 \quad (1)$$

The percentage of the quotient of the slopes in the spiked and pure solvent samples was used as an indicator of the extent of ion suppression or signal enhancement. No signal enhancement was observed, although there was a response reduction of approximately 95% as a result of matrix interference for red wine without applying any sample treatment procedure. As already noted, for the already reported SPE-based methodology developed by our group, 20% of the matrix effect was still observed.[41] Thus, to reduce the effect of the components in the matrix of the wine samples and to improve the OTA signal, the QuEChERS-SPE approach proposed in the present study was developed. For this methodology, only < 8% OTA signal reduction was observed. The results obtained are shown in Fig. 4. The QuEChERS-SPE strategy was also demonstrated to be robust in terms of the wine varieties evaluated, which included, for optimization purposes, not only the two varieties under study (i.e. Malbec and Cabernet-Sauvignon), but also Bonarda, Tempranillo, Syrah and Merlot. After applying the QuEChERS-SPE sample treatment, a comparable matrix effect reduction was observed in all of the wine varieties.

Method validation

Limit of detection (LoD) and limit of quantification (LoQ)

Taking into account the behavior of the compound during ionization (i.e. ion suppression as a result of matrix components) and its influence on the variability and calibration results, an approach with spiked samples was preferred instead of using blank samples (from which the signal-to-noise ratio is commonly obtained and used for the calculation of the LoD and LoQ). Thus, a calibration based on spiked samples was performed. This approach considered at least a five-point calibration curve, linearity, normality and constant variance (homoscedasticity).[43] As result, the values of merit were calculated using the equations (2) and (3), respectively:

$$LoD = \frac{3.3S_{y/x}}{b}\sqrt{\frac{1}{m}+\frac{1}{n}+\frac{\bar{x}^2}{\sum_{i=1}^{n}(x_i-\bar{x})^2}} \quad (2)$$

$$LoQ = \frac{10S_{y/x}}{b}\sqrt{\frac{1}{m}+\frac{1}{n}+\frac{\bar{x}^2}{\sum_{i=1}^{n}(x_i-\bar{x})^2}} \quad (3)$$

where $\bar{x}$ corresponds to the mean concentration, $S_{y/x}$ is the residual standard deviation, b is the slope of the calibration curve, m is the

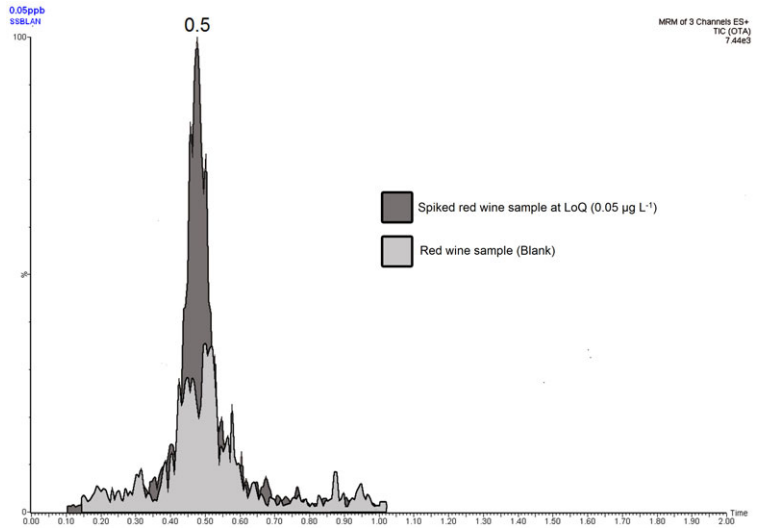

Figure 4. Chromatograms of red wine samples. Spiked sample at a 0.05 µg L^{-1} (LoQ) concentration level (dark gray-filled peak); blank red wine sample (light gray-filled peak).

number of replicates per concentration level of the spiked samples and n is the number of concentration levels for spiked samples: $i = 1, 2 \ldots I$. Thus, the obtained LoD and LoQ values were 0.02 and 0.05 µg L^{-1}, respectively.

Precision, recovery and trueness

To evaluate the methodology, precision recovery and trueness were calculated. The precision of the whole method was evaluated in terms of repeatability (intraday precision) and reproducibility (interday precision). Also, it is acceptable to study the trueness [expressed as bias (%)] of the measurements through recovery of additions of known amounts of the analyte to a blank matrix. Red wine spiked samples composed of five blanks; ten replicates at the maximum allowable concentration limit (2.0 µg L^{-1}); and five replicates at 0.05, 0.1, 0.5 and 1.0 µg L^{-1} were analyzed under the conditions described above. Reproducibility was evaluated with a similar procedure during five different weeks. The results are shown in Table 3.

Linearity

Linearity was evaluated from values closer to the LoQ up to approximately 20 µg L^{-1}. The linearity of the calibration curves for spiked red wine samples was satisfactory with determination coefficients (r^2) of 0.9991. The F-test ($P = 0.05$) demonstrated that linear regression was statistically acceptable in the working range and this model showed goodness of fit.

Levels of OTA in Argentinean red wine

Wine samples ($n = 136$: $n = 70$ for Malbec and $n = 66$ for Cabernet Sauvignon) were obtained from the grape-growing regions mentioned above. Table 4 shows the concentration levels of OTA in the Argentinean wines sampled to conduct this survey.

DISCUSSION

Methodology's performance

According to the results reported above, a low variability for the methodology was observed and the intraday precision was in agreement with the current legislation.[44] On the other hand, the interday precision was 21.3% (relative SD) at a concentration level of 0.05 µg L^{-1}, which was lower than the indicative value, ≤ 60%, for concentrations lower than 1 µg L^{-1} (1 µg kg^{-1}), reported by the Commission Regulation (EC) No. 401/2006.[44] Moreover, recovery of the developed methodology was in agreement with the same regulation, which establishes that, for a concentration range < 1 µg L^{-1}, an acceptable recovery is between 50% and 120% and, for a concentration range > 1–10 µg L^{-1}, an acceptable recovery is between 70% and 110%.

The values calculated for LoD and LoQ were compatible and much lower than (100- and 40-fold, respectively) the maximum allowable concentration limit of 2.0 µg L^{-1} established previously.[20] In addition, the analytical performance of the proposed methodology was in agreement with many methods reported in the current literature. A satisfactory shape and peak symmetry for typical chromatogram of a red wine spiked sample with OTA were obtained (Fig. 4).

Distribution of OTA in Argentinean red wine

Although OTA contamination was present in almost all of the wines samples analyzed, OTA levels detected were lower than the maximum allowable concentration limit of 2 µg L^{-1}, ranging from 0.02 to 0.98 µg L^{-1}. From the total of the wine sample analyzed, 135 (99%) the OTA levels determined were above the detection limit and therefore were considered as positive. Also, as noted above, only three samples (2% of the total of samples) were higher than the concentration limit of 0.5 µg L^{-1} established by some countries and marketers.[21] Furthermore, the concentration levels of OTA detected in wine samples (2013–2015) were less than or equal to those mentioned in other published reports on OTA concentration in Argentine wines (Table 1). The highest level of 0.98 µg L^{-1} was found in only one wine sample analyzed from the Buenos Aires region for the 2013/14 vintage.

Levels of OTA according to the vintages

First, to evaluate the normality of data from each region in the studied vintages (2013–2015), considering the OTA concentrations found, probability plots ($P > 0.05$) were employed. From the findings, normality was assumed for all grape-growing regions and

Table 3. Precision, Recovery (%) and Bias (%) for the QuEChERS-SPE-UHPLC-MS/MS

Concentration level (μg L^{-1})	*n*	Intra-day RSD (%)[a]	Inter-day RSD (%)[a]	Recovery (%)[b]	Bias (%)[c]
0.05	5	15.1	18.6	92.0 ± 2.6	−8.0
0.1	5	16.5	17.9	97.8 ± 4.1	−2.2
0.5	5	13.5	16.2	98.2 ± 3.2	−1.8
1.0	5	12.9	13.3	105.3 ± 1.7	5.3
2.0	10	8.5	10.1	102.6 ± 2.0	2.6

[a] RSD (%) = relative standard deviation;
[b] recovery (%) = [(measured content/spiked level) × 100] and ± SD;
[c] bias (%) = ([(measured content − spiked level)/spiked level] × 100).

Table 4. OTA occurrence in the Argentinean grape-wine regions monitored

		2012/13		2013/14		2014/15	
Grape-growing regions	*n*	Mean [a]	Range [a]	Mean [a]	Range [a]	Mean [a]	Range [a]
Salta-Catamarca-Calchaquies Valley	16	0.07	< LoQ – 0.11	0.09	< LoQ – 0.19	0.15	0.12 – 0.19
La Rioja	12	0.16	0.06 – 0.25	0.17	0.10 – 0.24	0.18	0.09 – 0.28
San Juan-Tulum Valley	17	0.14	< LoQ – 0.21	0.13	0.08 – 0.31	0.14	0.05 – 0.50
Mendoza East	17	0.10	< LoQ – 0.05	0.11	0.05 – 0.16	0.17	< LoQ – 0.62
Mendoza-Uco Valley	9	< LoQ	0.05 – 0.25	0.09	0.07 – 0.10	0.10	0.06 – 0.15
Mendoza South	17	< LoQ	< LoQ – 0.04	0.07	< LoQ – 0.11	0.05	< LoQ – 0.08
Neuquén – La Pampa – Rio Negro	15	< LoQ	< LoQ – 0.07	0.05	< LoQ – 0.11	0.06	0.05 – 0.08
Buenos Aires South	12	0.05	< LoQ – 0.07	0.26	< LoQ – 0.98	0.12	0.09 – 0.13
Cordoba-Colonia Caroya	13	0.09	< LoQ – 0.18	0.06	< LoQ – 0.09	0.09	0.05 – 0.12
Entre Rios-Concordia	8	0.06	0.05 – 0.07	< LoQ	< LoQ	0.08	0.06 – 0.11

[a] Mean and range concentrations (μg L^{-1}).
LoQ, limit of quantification: 0.05 μg L^{-1}.

vintages assessed. Then, the equal variance test (Bartlett's method; $P < 0.05$) was applied for each vintage as well. Although non-equal variances were assumed for the 2012/13 and the 2014/15 vintages, no significant differences were observed for the 2013/14 vintage variances. As a consequence, the ANOVA Welch's test was used to evaluate the statistically different variances; thus, significant differences between grape-growing regions ($P < 0.05$) were found. Meanwhile, as noted, for the 2013/14 vintage, no differences between the OTA concentrations for the monitored regions were observed. Both ANOVA (parametric) and Kruskal–Wallis (non-parametric) tests were applied for data analysis and the same conclusions were observed. In addition, to evaluate the mean values of the data, the Games-Howell ($P < 0.05$) approach (ANOVA Welch's post-hoc test) was applied. From the results, the Mendoza South region was different from the others as a result of the lowest OTA concentration observed when considering the 2012/13 vintage. For the San Juan-Tulum Valley, La Rioja and Mendoza East locations, the highest values of OTA concentrations were found (Table 4). In addition, the same test was used for the vintage 2014/15. The lowest OTA concentrations were observed for the Mendoza South and Neuquén-La Pampa-Rio Negro regions,. Meanwhile, the highest levels of OTA contamination were observed in this vintage for the northern regions, Calchaquies Valley Salta-Catamarca and La Rioja.

OTA occurrence in the different grape-producing areas

The differences observed with respect to OTA contamination in the samples analyzed during the present study could be explained by considering the different climate conditions for the vineyards along the sampling areas. ANOVA was applied to evaluate differences between vintages for each region. Hence, Bartlett's method ($P < 0.05$) was applied for each grape-growing region and then, as result, equal variances were assumed. Similarly, both ANOVA and Kruskal–Wallis were applied and no significant differences were observed in general between the vintages in each region. However, in the Mendoza South region, a significant difference during the 2012/13 vintage was observed (Tuckey's test; $P < 0.05$). This might be explained considering that, for the periods 2013/14 and 2014/15, higher temperature (mean maximum and mean average) events were recorded, and then the OTA concentrations were slightly greater than those registered for the 2012/13 vintage. Also, a negative anomaly of rainfall (−10%) and low relative humidity for the period 2012/13 were observed. Furthermore, this might be related to what Chiotta *et al.*[15] has described with respect to the *A. niger* aggregate species having been isolated in most of the Argentinean grape-growing regions, with their optimal growth temperature of between 35 and 37 °C in a wide range of humidity levels favoring the occurrence of these fungi species in wine grapes, whereas *A. carbonarius* was observed in the northern regions where higher temperature and humidity levels were observed.[15]

Relationship between the OTA content and the wine variety assessed

Wine variety was also considered as a potential indicator of OTA concentration in the samples evaluated. As noted, two varieties

were tested, Malbec and Cabernet Sauvignon, both being distinctive in Argentinian wine production. ANOVA and Kruskal–Wallis were used for analysis of any significant differences. From the results obtained relating to a comparison of the different regions, significant statistical differences for the Malbec variety for the 2012/13 and the 2013/14 vintages were observed (Tukey's test; $P < 0.05$). Thus, the lowest OTA contamination was observed in the Mendoza-Uco Valley, Mendoza South and La Pampa-Neuquén-Rio Negro locations. Although no differences for the Malbec-type of samples were observed for the 2014/15 vintage. The intra-region relationships between varieties and vintages were evaluated. OTA concentrations in Malbec wine samples for each vintage tested were not different in each of the regions monitored. Although, for the Cabernet Sauvignon wine samples, the toxin concentrations were higher for the 2013/14 vintage in the Mendoza South region compared to other vintages in this same region, in general, no significant differences in OTA concentration levels between Cabernet Sauvignon and Malbec were observed. From these findings, it could be concluded that, for the samples analyzed, regions considered and vintages studied, the OTA concentration depends on the weather variables during grape-growing and harvest in each vintage, as well as the vitivinicultural practices employed.

Effect of weather on OTA concentration

The highest OTA levels of contamination were observed in the Calchaquies Valley Salta-Catamarca, La Rioja and Tulum Valley-San Juan regions. Likewise, in the vintage 2014/15 and during this grape-growing period, higher mean temperature anomaly (+0.7°C) and relative humidity and rainfall anomalies (+20%) values were observed compared to the other two vintages evaluated. These anomalies in the weather conditions could have affected the ochratoxigenic fungal growth and OTA contamination in the wine samples analyzed from these locations.

OTA concentration levels were lowest in Mendoza South and Mendoza Uco Valley regions, both with a cold steppe (BSk) climate. The same occurred for the Neuquén–La Pampa–Rio Negro region, where the average mean temperature is also low and the climate can be classified as Bwk. These grape-growing locations were less susceptible to OTA contamination for both Malbec and Cabernet Sauvignon varieties as a result of the influence of low temperatures and the amount and frequency of rainfall.

In recent grape-growing regions in Argentina that have not been reported previously, such as Buenos Aires South, Cordoba and Entre Rios, the OTA concentration profiles in wine was evaluated for first time. Although having a mild climate, and generally being warm and temperate, with a significant amount of rainfall during the year, which might comprise potential conditions favoring the ochratoxigenic fungus occurrence and growth in grape and hence OTA contamination, the OTA levels were low and relatively similar to those for the other regions monitored with respect to the tree vintages assayed and for both Malbec and Cabernet Sauvignon varieties.

Therefore, the OTA concentration levels in Argentine red wine samples from the grape-growing regions mentioned are comparable to those reported in previous studies,[15,26] with the largest OTA concentration levels in samples from San Juan and La Rioja. This might be explained by the fact that *A. niger* aggregate species were dominant in all grape-growing regions in Argentine, whereas *A. carbonarius* (the most ochratoxigenic strain) was relevant in La Rioja and San Juan regions.[15]

Correlation between soil texture and OTA concentration

It has been proposed that the air movement deposits the fungi spores (ochratoxigenic species) from the soil on the grape-wine berry surfaces. Thus, the risk of contamination with OTA in wines might be related to the presence of toxigenic strains in the soil.[45] Therefore, soil texture in the vineyard might be a potential factor of movement of the fungi spores. Thus, the relationship between soil texture and OTA concentration on the wine samples monitored was also evaluated. No correlation was observed between the soil texture and the OTA levels in each vintage ($P < 0.01$). This might be a result of the practices stipulated by the OIV that were performed during the grape ripening and harvesting period. To limit the transfer of soil particles and associated fungi to the grapes, the use of vegetation or an organic cover of soils is recommended, in addition to avoiding working the soil between the beginning of the grape ripening and harvesting period.[22]

During the vintages surveyed (2013–2015), OTA levels were relatively lower than the levels reported previously. This is because some vitivinicultural practices have been improved over the years to comply with the related regulations established by the OIV.[22] Also, the low levels of OTA concentrations found in wine could be explained on basis of grape's good health status, having been harvested with optimal hydration over the different vintages evaluated, and also by considering that the sampling was performed randomly and there was no selection of damaged and undamaged grapes. The application of these practices in the grape-growing regions surveyed might have reduced OTA contamination and decreased OTA level concentrations. This is consistent with the actions of a cropping system that improved grapevine wellness, mainly preventing pest and disease damage on berry, including the control of black aspergilli, as well as the optimization of postharvest logistics and of wine making.[46]

CONCLUSIONS

A QuEChERS-SPE sample extraction/clean-up procedure prior to the UHPLC-MS/MS determination of OTA in red wine samples at low $\mu g\,L^{-1}$ levels has been developed. The critical variables influencing the extraction/clean-up procedure were optimized to reduce the matrix effect and therefore improve the OTA signal in the UHPLC-MS/MS determination. Under the proposed conditions, the initially observed matrix effects were substantially reduced and the OTA quantification levels obtained were considerably below the maximum allowable limits. The developed methodology allowed determination of OTA with high efficiency, sensitivity, accuracy, as well as a marked diminishing of matrix effects, which are commonly observed when using mass spectrometry and are detrimental for any substance determination, particularly for those at trace levels.

The proposed methodology was successfully applied to the monitoring of OTA in Argentinean wines for diverse producer regions and vintages. Thus, the results obtained in the present study provide relevant information on OTA profiles and contamination. Moreover, the data might be used to evaluate the application of appropriate management strategies with respect to reducing or preventing the development of ochratoxigenic species and OTA occurrence in wine.

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