VALIDATION OF A LIQUID CHROMATOGRAPHY METHOD FOR THE SIMULTANEOUS QUANTIFICATION OF OCHRATOXIN A AND ITS ANALOGUES IN RED WINES

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ABSTRACT

A validated high-performance liquid chromatography (HPLC) method with fluorescence detection for the simultaneous quantification of ochratoxin A (OTA) and its analogues (ochratoxin B (OTB), ochratoxin C (OTC) and methyl ochratoxin A (MeOTA)) in red wine at trace levels is described. Before their analysis by HPLC-FLD, ochratoxins were extracted and purified with immunoaffinity columns from 50 mL of red wine at pH = 7.2. Validation of the analytical method was based on the following parameters: selectivity, linearity, robustness, limits of detection and quantification, precision (within-day and between-day variability), recovery and stability. The limits of detection (LOD) in red wine were established at 0.16, 0.32, 0.27 and 0.17 ng L^{-1} for OTA, OTB, MeOTA and OTC, respectively. The limit of quantification (LOQ) was established as 0.50 ng L^{-1} for all of the ochratoxins. The LOD and LOQ obtained are the lowest found for OTA in the reference literature up to now. Recovery values were 93.5, 81.7, 76.0 and 73.4% for OTA, OTB, MeOTA and OTC, respectively. For the first time, this validated method permits the investigation of the co-occurrence of ochratoxins A, B, C and methyl ochratoxin A in 20 red wine samples from Spain.

Keywords: Ochratoxin A, ochratoxin analogues, wine, simultaneous validation, HPLC

1. Introduction

Ochratoxins are a family of toxic compounds produced by fungi of the genera *Aspergillus* and *Penicillium* that may occur as natural contaminants of different foods. Of these, the most important, due to its toxicity and occurrence, is ochratoxin A (OTA). Different *in vivo* studies regarding the chronic ingestion of this mycotoxin have demonstrated that it can cause nephrotoxic, hepatotoxic, carcinogenic, teratogenic and immunotoxic effects [1] in animals. In humans, it has been associated with Balkan Endemic Nephropathy and with urinary tract tumours [2]. Due to the fact that there is evidence of OTA carcinogenicity in experimental animals, although this has not been demonstrated in humans, this mycotoxin is classified by the International Agency for Research on Cancer (IARC) as a possible human carcinogen (Group 2B) [3].

OTA presence in wine and grape juice has been reported in different countries [4, 5, 6, 7, 8], including Spain [9, 10, 11], which is one of the main wine producers in the world (International Organisation of Vine and Wine (OIV); Food and Agriculture Organization (FAO)) [12, 13]. In Europe, wine represents the second largest source of OTA intake for humans identified by the SCOOP report [14] and by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) [15]. In order to minimize public health risk, the European Commission Regulation (EC) Nº 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs, established a maximum level for OTA in wine of 2 $\mu g\ L^{-1}$ [16].

It is known that fungi can also produce some analogues of this mycotoxin [17, 18], such as the dechlorinated derivative: ochratoxin B (OTB), and the ethyl and methyl esters: ochratoxin C (OTC) and methyl ochratoxin A (MeOTA) respectively. Their chemical structure is shown in figure 1. Under natural conditions, contamination of

foodstuffs with a mixture of fungal metabolites must be considered. The co-occurrence of different mycotoxins in one same foodstuff could origin additive or synergic effects on human or animal health; however, the knowledge regarding this aspect is still scarce.

The simultaneous appearance of OTA and OTC in wine was first described by Zimmerli and Dick in 1996 [19], although since then, no more studies have been carried out on the same topic. These authors indicated that in chromatograms of wine samples with OTA levels superior to 0.050 μg L^{-1}, an additional peak was detected and its retention time corresponded to that of the ethyl ester. The mean concentrations of OTC in wines were estimated to be approximately 10% of the corresponding OTA levels. The presence of OTC in wine may be due to fungal activity, but also to the chemical transformation of OTA into OTC. This second hypothesis is supported by the alcoholic and acidic nature of wine that permits transformation of the carboxylic acid into an ethanolic ester [19]. OTC is rapidly hydrolyzed to OTA after oral or intravenous administration, acting as a pro-OTA product [20].

Valero *et al.* (2008) found another unknown peak in all OTA positive wine samples and they suggested that OTA is sometimes accompanied by the non-chlorinated analogue, ochratoxin B (OTB) [21]. The methyl ester of ochratoxin A (MeOTA) is produced by the same family of fungi and is often synthesized from OTA as a confirmation procedure in OTA analysis [22], but its presence in red wine as a natural contaminant has not been reported.

With regard to their toxicity, researches on these analogues are limited. OTB is said to be about 10-fold less toxic than OTA [23, 24], but some studies mention its cytotoxic [25, 26, 27] and teratogenic effects [28]. OTC has been reported to have toxicity similar to that of OTA [22, 24, 29]. However, a subsequent *in vitro* study

indicated that OTC is more toxic than OTA, at least with respect to the impairment of immune cell functions [30, 31]. No studies have been made regarding methyl OTA toxicity.

An awareness of the presence of these toxins in wine is very important in order to avoid underestimating the total intake of ochratoxins. However, monitoring ochratoxins in wine depends on having reliable analytical methods available for use and their validation is essential for obtaining good data. Moreover, due to the low levels expected in wine, there is a need for sensitive methods that permit the detection and quantification of the ochratoxins.

The most frequently used technique for OTA quantification in wine is reversed phase liquid chromatography with fluorescence detection. With regard to its extraction and clean-up, the main procedure in OTA determination from different matrixes is the immunoaffinity column technique (IAC) [32]. The application of commercial IAC has been described for OTA purification, but its application to other OTA derivatives in wine has not been thoroughly investigated in the scientific literature.

In this work, a sensitive analytical method for the simultaneous determination of OTA, OTB, OTC and MeOTA in wine has been optimized and validated, using immunoaffinity columns and high-performance liquid chromatography with fluorescence detection. This method has been successfully applied to the analysis of 20 red wine samples from Navarra, a northern region of Spain. Confirmation of the presence of these analytes in the samples has been made using an LC/ion trap MS method developed for this purpose.

2. Experimental

2.1. Chemicals and materials

Ochratoxin A and B, ethyl acetate, acetonitrile and methanol CHROMASOLV® for HPLC were purchased from Sigma-Aldrich (St. Louis MO, USA). All of the reagents were of pro-analysis grade. Sodium hydroxide pellets, ethanol absolute, hexane and formic acid were purchased from Panreac (Barcelona, Spain). Dichloromethane (DCM) and hydrochloric acid fuming 37% were obtained from Merck (Darmstadt, Germany) and sodium hydrogen carbonate was obtained from Riedel-de-Haën (Seelza, Germany). Instamed Phosphate Buffered Saline (Dulbecco) w/o Ca^{2+}, Mg^{2+} was purchased from Biochrom AG (Berlin, Germany). Ochraprep® immunoaffinity columns (IACs) were obtained from R-Biopharm Rhône Ltd (Glasgow, UK). Millipore type I water was used to prepare all of the aqueous solutions and was obtained daily from a Milli-Q water purifying system. Syringe filters Millex®-HV, 0.45 μm, PVDF, 13 mm, non-sterile were purchased from Millipore Iberica S.A.U. (Madrid, Spain). Preassembled vial kit (amber screw top write-on, caps and septa) and deactivated glass inserts were acquired from Agilent Technologies (Madrid, Spain).

2.2. Safety precautions

Ochratoxins are toxic substances and should always be manipulated in solution, avoiding formation of dust and aerosols. Gloves, face shield and safety glasses should be used.

2.3. Synthesis of OTC and MeOTA

For validation purposes, the ethyl and methyl esters of ochratoxin A (OTC and Me-OTA, respectively) were synthesized according to the method of Li *et al*. (1998), based on an alcoholic esterification of OTA [22]. One hundred microliters of HCl 12N were added to two milliliters of two standard solutions of 25 mg L^{-1} of OTA in methanol

and ethanol, respectively; they were incubated for a period of 48 h at room temperature. Next, the solvent was evaporated under a stream of nitrogen and the residues were redissolved in 5 mL of an aqueous solution of $NaHCO_3$ (0.25%). 2.5 mL of dichloromethane were added and the mixtures were agitated in a rotatory shaker for 30 min. The organic layer was separated and the procedure was repeated with 2.5 mL of clean dichloromethane. Both organic layers were collected up and evaporated. The residue was redissolved in 700 µL of methanol. The presence of OTC and MeOTA were confirmed by LC/ion Trap MS, and the purity of these compounds (absence of OTA contamination) was confirmed by HPLC-FLD. Their concentrations in methanol were measured by spectrophotometry at 333 nm (MW = 431.8, ε = 7000 M^{-1} cm^{-1} for OTC [33]).

2.4. Standard solutions

A stock standard solution of approximately 100 mg L^{-1} was prepared by dissolving OTA powder (approximately 1 mg) in 10 mL of methanol. In the same way, OTB was prepared using ethanol as the dissolvent. Their concentrations were determined spectrophotometrically at 333 nm (MW = 403.8; ε = 5500 M^{-1} cm^{-1}) for OTA [34] and at 318 nm (MW = 369.4; ε = 6900 M^{-1} cm^{-1}) for OTB [33].

Three working standard solutions of 400, 40 and 4 µg L^{-1}, containing the four ochratoxins, were prepared in methanol from the stock solutions.

Calibration samples were prepared by evaporating adequate volumes of these working standard solutions in methanol under a stream of nitrogen; the residues were redissolved in 250 µL of mobile phase in the same way as in the preparation of the methanol extracts from red wine samples.

2.5. Apparatus and chromatographic conditions

Ochratoxins analysis was carried out in an Agilent Technologies 1100 liquid chromatographic system equipped with a fluorescence detector (model G1321A), controlled by Chemstation 3D software. The column used was a Zorbax Eclipse XDB-C18 column (15 x 0.46 cm; 5μm) from Agilent Technologies with a ODS precolumn from Teknokroma (Barcelona, Spain).

The mobile phase consisted of A (formic acid 0.4%) and B (acetonitrile). The elution program was: 10 min isocratic at 42% B, followed by a gradient to 60% B at 15 min, maintained until 25 min. After the analysis, the column was re-equilibrated during 5 min at 42% B. The injection volume was 100 μL and the flow rate was 1.0 mL min^{-1}. Chromatography was performed at 40ºC and the fluorescence conditions were: excitation at 318 nm from 0 to 7.5 min and 333 nm from 7.5 to 25 min, emission at 461 nm for the entire analysis. In these chromatographic conditions, the retention times were 5.6, 11.1, 18.5 and 21.3 min for OTB, OTA, MeOTA and OTC, respectively.

The chromatographic separation was evaluated using the following parameters: retention factor (k'), symmetry, peak width at half height (w_h), number of theoretical plates (N) and resolution (R_s).

Ochratoxins confirmation was made using an Agilent Technologies 1200 liquid chromatographic system coupled to a MSD Trap XCT Plus mass spectrometer (G2447A model) and equipped with an electrospray ionization source (ESI). Chromatography was performed at 40ºC on a Zorbax Extend-C18 column (5 x 0.21 cm; 3.5 μm) provided by Agilent Technologies. The mobile phase consisted of A (formic acid 0.4%) and B (acetonitrile). The elution program was: 2 min isocratic at 35% B, changed to 53% B, from 2.1 to 15 min. The column was re-equilibrated during 5 min at 35% B. The injection volume was 20 μL and the flow rate was 0.2 mL min^{-1}.

The mass spectrometer settings were: positive ion mode, nebulizer pressure 40 psi, drying gas flow 8.0 L min^{-1} and drying gas temperature 350ºC. Spectra were acquired in Manual MS(n) mode. The $[M+H]^+$ ion of each ochratoxin was isolated.

2.6. Ochratoxin extraction and immunoaffinity clean-up of the extracts

Ochratoxins were extracted using the method described in the instructions provided by the immunoaffinity columns supplier for its application in OTA analysis in beer, with some modifications for its use in wine matrix.

Approximately 60 mL of red wine were adjusted to pH 7.2 in a pHmeter BASIC 20 (Crison), using an aqueous solution of sodium hydroxide (2M). This solution was filtered by gravity. Fifty milliliters were passed through the IAC, previously conditioned with 10 mL of PBS. Next, the IAC was washed with 10 mL of PBS and 20 mL of water. Finally, it was dried by passing air with the use of a 20 mL syringe. Ochratoxins were eluted with 4 mL of methanol. During the elution, back flushing (or reversing the direction of flow with a syringe) ensures the complete denaturation of antibodies and it is recommended to achieve complete elution of ochratoxins. Air was pushed through the column to collect the last drops of eluate. The eluate was evaporated to dryness in a water bath at 40ºC under a stream of nitrogen, and the residue was redissolved in 250 µL of mobile phase before HPLC analysis (concentration factor = 200).

2.7. Validation of the analytical method

Validation of the quantitative analytical method for simultaneous determination of OTB, OTA, OTC and MeOTA in wine has been based on the following parameters: selectivity, linearity, precision of the instrumental system (within- and between-day variability), recovery, robustness, limit of detection and limit of quantification, and stability.

The selectivity of the method was improved by the use of immunoaffinity purification techniques and a selective fluorescence detector. In order to assess selectivity, and due to the fact that it was not possible to obtain wine samples in which the absence of ochratoxins was assured (they are natural contaminants), vial samples were reanalyzed after adding a volume of the adequate working solution of the four ochratoxins. The increase of the peak areas of the corresponding compounds was observed. Furthermore, retention time of each ochratoxin in the sample was the same as that found in calibration samples, with a tolerance of ± 1%.

The presence of ochratoxins in wine samples was confirmed using the previously described LC/ion trap MS method. The isolated m/z (370.2 for OTB, 404.4 for OTA, 418.4 for Me OTA and 432.4 for OTC), and the fragmentation ions of each peak obtained at the same retention times as in the standards, assured peak identity. Ions used in the confirmation were as follows: m/z 324.1, 352.2 and 307.1 for OTB; 358.1, 386.1 and 341.1 for OTA, MeOTA and OTC.

In the assessment of linearity, three calibration curves were plotted in the ranges 0.1-1, 1-20 and 20-400 μg L^{-1}. Three replicates of five calibration samples were analyzed for each range. In order to obtain the equivalent concentration ranges in red wine (C_{wine}), the standard concentrations (C_{STD}) were corrected with the recovery value (*Rec*) and the concentration factor (*CF*) of the complete sample process, by means of the following expression: $C_{wine} = \left(\frac{C_{STD}}{CF}\right) \cdot \left(\frac{100}{Rec}\right)$.

Therefore, the ranges in wine were 0.583-5.83, 5.83-117 ng L^{-1} and 0.117-2.33 μg L^{-1} for OTB; 0.548-5.48, 5.48-110 ng L^{-1} and 0.110-2.19 μg L^{-1} for OTA; 0.675-6.75, 6.75-135 ng L^{-1} and 0.135-2.70 μg L^{-1} for MeOTA; 0.677-6.77, 6.77-135 ng L^{-1} and 0.135-2.71 μg L^{-1} for OTC.

Precision (as RSD %) and accuracy (as relative error of the mean, %) of the instrumental system were evaluated by analyzing three replicate calibration samples at the low, medium and high concentrations of each calibration curve (0.1, 0.4, 1, 8, 20, 120 and 400 μg L^{-1}), in one day (within-day precision) and on three days (between-day precision).

Recovery of ochratoxins was tested carrying out the complete sample process on one day (repeatability) and on three different days (intermediate precision) at five concentration levels: 0.0005, 0.003, 0.04, 0.6 and 2 μg L^{-1}. Adequate volumes of ochratoxin working solutions in methanol were added to red wine samples (200 mL) so as to reach these levels. Each concentration was prepared in triplicate. Recovery was determined comparing the absolute responses (peak area) of ochratoxins obtained from the wine spiked samples with the absolute responses (peak area) of calibration samples. Where relevant, measured ochratoxins levels were corrected in the case of any natural contamination, as indicated by the analysis of the non-spiked samples.

In the robustness study, the effect on recovery of the sample adjusted pH before IAC purification was tested. Samples of spiked red wine at 0.05 μg L^{-1} were adjusted in duplicate at pH 6.8, 7.2 and 7.6, respectively. They were extracted and analyzed.

The limit of detection (LOD) was established by analyzing three spiked wine samples at 0.3, 0.4, 0.5 ng L^{-1} in triplicate for the four ochratoxins and by using a method based on the calibration curve extrapolation at zero concentration. This method consists in plotting the mean peak areas versus the toxin concentration (curve 1), and the standard deviation of the areas obtained for each toxin level versus the concentration (curve 2). The following equation was used:

$$LOD = \frac{y + (k \cdot y')}{b \cdot \sqrt{n}}$$

with *y* and *b* being the values for y-intercept and slope, respectively, from curve 1, *y'* being the y-intercept from curve 2 and *n* being the number of replicates for each level (n = 3). The k value was 3 [35].

The LOQ corresponds to the minimum concentration assayed in red wine samples with adequate precision and recovery values. The LOQ value for each ochratoxin was included as the lowest level in the corresponding calibration curve.

Stability of the three working standard solutions stored at -20ºC was studied by comparing the initial concentration of ochratoxins with that obtained at one, three, six and twelve months after being prepared. Three replicates of each concentration were analyzed. In addition, stability of ochratoxins in the HPLC injector tray at two different concentrations was tested in both calibration (8 and 120 μg L^{-1}) and extracted wine samples (0.04 and 0.6 μg L^{-1}) for 48 h.

2.8. Samples

Twenty red wine bottles were purchased from different supermarkets within Navarra (Spain). All of the wines belonged to the Navarra Designation of Origin and they were from the 2006 and 2007 vintages. Their different alcoholic grade varied from 12.5 to 14.5% (v/v). Their measured pH was in the range of 3.3 to 3.8.

3. Results and discussion

3.1. Preparation of standards

Ochratoxin C and methyl ochratoxin A standards, not commercially available, were synthesized according to the method of Li *et al*. (1998). The conditions of

synthesis for obtaining the highest yield in the esterification reaction were 95% of methanol, HCl 12 N and 48 h [4]. However, after HPLC-FLD analysis of the obtained solutions, an OTA peak was observed in the chromatogram in both cases, showing that this method did not provide a total conversion of OTA in their esters. Therefore, a subsequent purification process was required. Due to the fact that aqueous solutions of K_2CO_3 and $NaHCO_3$ were used to decontaminate OTA from cocoa shells [36], these solvents were chosen as aqueous phases for purification. Combinations of K_2CO_3 (2%)-hexane, K_2CO_3 (2%)-ethyl acetate, water-dichloromethane (DCM) and $NaHCO_3$ (0.25, 0.5 and 1%)-DCM were assayed. The resulting OTC and MeOTA purified solutions in methanol were analysed with the HPLC-FLD method described in this paper. The use of dichloromethane-sodium hydrogen carbonate at 0.25% provided a simple and good purification of OTC and MeOTA, without the presence of the OTA peak above its LOD in the purified ester solution chromatograms ~~(figure 2).~~ The exact concentrations of the obtained solutions were evaluated by spectrophotometry at 333 nm. The molar absorption coefficient of the methyl ester of OTA was not found in the reference literature and it was assumed to be similar to that of OTC.

In order to prepare the working solutions for ochratoxins, methanol was used as solvent due to the fact that when using acetonitrile some adsorption processes of OTA and OTB on glass have been observed (data not shown).

3.2. Ochratoxin extraction and immunoaffinity clean-up of the extracts

Immunoaffinity purification of OTA for its single determination in foodstuffs has been widely studied. Also, the OIV ~~(International Organization of Vine and Wine)~~ specifies that an immunoaffinity column must be used for OTA pre-concentration and clean-up of the wine sample [37]. However, the use of these columns has not been

explored for the purification of other ochratoxins in wine, and the different IAC providers only mentioned their use for OTA analysis.

During the development phase of the analytical method, Ochratest® IACs were used because they had been previously used in our laboratory for OTA purification purposes [38]. They showed good recovery for OTA, OTC and MeOTA (data not shown) but OTB was not retained by these commercial columns and therefore could not be analyzed. Due to the aforementioned, Ochraprep® immunoaffinity columns were evaluated and chosen for carrying out the validation of the analytical method because they enabled the simultaneous extraction of OTB and the other ochratoxins. Even though these columns were described for the single determination of OTA, this study proves that they can be used for the simultaneous analysis of ochratoxin A, B, C and MeOTA, with excellent recoveries. The fact that the OTA recovery value obtained in this study is similar to that obtained in other studies, and the fact that the precision of the process is good at different concentrations, indicate that the presence of these analogues does not interfere with the capability of OTA to bind the antibodies of the column.

3.3. Development of the HPLC-FLD quantitative analytical method

Different chromatographic conditions were investigated in order to achieve the best separation and resolution of peaks so as to allow the quantification, especially for OTB, which appears at the start of chromatogram, close to other matrix compounds. Using the elution program described, no interference peaks appeared at the retention times of the peaks of interest. More apolar gradients and higher column temperatures were tested in order to improve the analysis time. Better width peak and less pressure were obtained by increasing the organic solvent, including up to 80%, or by using

higher column temperature (50 and 60ºC); however, they did not substantially decrease the analysis time, endangering the resolution and increasing the risk of interferences and false positives. Glass vials were used for sample analysis, due to the appearance in the chromatograms of unexpected peaks when plastic vials were used.

An emission wavelength of 461 nm was unvaryingly fixed for the four compounds. Although an increase of the peak area of OTA was observed when it was chromatographied using 225 nm as excitation wavelength [39], the high concentration factor of the method originated high baseline noise at 225 nm, resulting in higher LOD and LOQ values. The emission wavelength most often used in OTA determination is 333 nm, the maximum of its excitation spectrum, which coincides with the maximum for OTC and MeOTA spectra. OTB excitation spectrum showed a maximum at 318 nm. For these reasons, from 0 to 7.5 min an excitation wavelength of 318 nm was fixed, whereas after 7.5 min 333 nm was used.

Due to the high concentration factor applied to wine samples, interference of the matrix could be of some significance. Therefore, chromatograms obtained from wine samples containing low concentrations of analytes were studied. Table 1 shows the chromatographic parameters calculated for each peak; especially, the resolution of each compound from its preceding peak in the chromatogram.

Figure 2 shows HPLC-FLD chromatograms corresponding to non-spiked and spiked red wine samples and a calibration sample. Figure 3 shows LC/ion trap MS chromatograms, as well as the mass spectrum obtained for each mycotoxin, when analyzing a calibration sample and a wine sample.

3.4. Validation of the HPLC-FLD method

Linearity was assessed in a wide range of ochratoxin concentrations in order to include the levels that can be found in naturally contaminated samples. Suspecting that OTC concentrations would be very low, linearity was studied from the LOQ level to 2 μg L^{-1}, the maximum level permitted of OTA in red wines in the European Union (Commission Regulation Nº 1881/2006) [16].

In order to prove linearity, a statistical study was performed (see table 2). The assays exhibited linearity between the response (y = area of peak) and the respective concentration of ochratoxins (*x*) over the three ranges assayed, and all of the criteria used to verify linearity were achieved. The correlation and determination coefficients were higher than 0.999 in the twelve calibration lines. The representation of residuals versus the estimated values gave rise to a distribution of the points at random and did not reflect any trend. The coefficient of variation between response factors was lower than 10%, considered to be a good value due to the lower levels of concentration used. The Student's t-test indicated that the slopes were statistically different from zero and the confidence interval of the slopes did not include 0. In contrast, the confidence interval of the intercepts included zero. The Cochran's test indicated that the concentration factor had not any influence on the variability of results (the variances of the concentrations were homogeneous). The value for G_{tables} ($\alpha = 0.05$, k = 5, n = 3) was 0.68 and all values were below this value, except for OTA at 0.1-1 μg L^{-1} range. This could be due to the fact that 0.1 μg L^{-1} in calibration samples could be considered to be the limit of quantification.

Precision (RSD %) and accuracy (as relative error of the mean, %) of the instrumental system were adequate: within and between-day precision were less than 15% (see table 3).

Recovery results displayed in table 4 show that the extraction method used allowed for a recovery of ochratoxins between 73.4 and 93.5% over the concentration range 0.0005-2 μg L^{-1}, and that recovery remained homogeneous even when the concentration or the days were varied (RSD under reproducibility conditions < 20% in all cases). All of the results were within the performance criteria range for OTA, established in the Commission Regulation (EC) Nº 401/2006 of February 23, 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs [40]. This states a recovery value between 50 and 120% for samples with a concentration of OTA < 1 μg L^{-1} and a RSD under repeatability conditions and under reproducibility conditions less than 40 and 60%, respectively. The mean recovery value of each mycotoxin was used to correct the results found in the wine samples.

With regard to robustness, there were no differences in recovery values or in method precision when adjusting spiked wine at pH 6.8, 7.2 or 7.6 (see table 5). In fact, RSD of the concentrations obtained and recoveries were less than 10%.

The limits of detection (LOD) in red wine were established at 0.16, 0.32, 0.27 and 0.17 ng L^{-1} for OTA, OTB, MeOTA and OTC, respectively. The limit of quantification (LOQ) was established as 0.50 ng L^{-1} for all of the ochratoxins. The LOD and LOQ obtained are the lowest found for OTA in the reference literature up to now. The sensitivity of the method is a very important parameter for achieving quantification of OTA analogues. Zimmerli and Dick hypothesised that concentration of OTC was 10% of the naturally contaminated OTA levels in wine, and this was probably below the limit of detection of the methods described so far. In addition, in a previous study in our

laboratory (data not published) OTC was not found in any of the 20 red wine samples analysed, possibly due to the fact that the LOD for OTA was 0.01 μg L^{-1}.

Stability of the three working standard solutions in methanol stored at -20ºC was proved for 12 months. The relative error between the initial and the last concentrations measured was < 5%. Calibration samples of 8 and 120 μg L^{-1} were stable for 24 h in the HPLC injector tray at room temperature. After this time, there was an increase in the initial concentration of more than 5% for one of the mycotoxins. Fortified and extracted red wine samples showed the same behaviour. Therefore, samples were analyzed in a time period of less than 24 h after having been processed in order to assure stability.

3.5. Application to real samples

This method was successfully used in the study of ochratoxins levels in 20 red wine samples and results are shown in table 6. Median values were calculated taking into account all the levels encountered, including those below LOQ. Mean values were calculated for the >LOQ levels. In the study of the presence of mycotoxins in samples, the samples taken into consideration were those which had mycotoxin levels above the LOD.

Co-occurrence of ochratoxins in red wine has been confirmed. 100% of the samples had detectable levels for at least two ochratoxins (OTA and OTB), although at very low levels, and none of them exceeded the maximum level permitted by legislation for OTA (2 μg L^{-1}), with 44.8 ng L^{-1} being the maximum level found for this toxin. 60% of the samples presented levels of three ochratoxins (8 samples OTA + OTB + OTC and 4 samples OTA + OTB + MeOTA). 30% of the samples presented the four ochratoxins. Moreover, the presence of MeOTA as natural contaminant is described for first time in red wine.

4. Conclusions

In this paper an analytical method which permits the study of the co-occurrence of ochratoxin A, B, C and MeOTA in wine has been validated and successfully applied to 20 red wine samples. The analytical method meets all of the preestablished validation parameters, being robust, selective, linear, precise and accurate in the three intervals studied for the 4 ochratoxins.

The limits of detection and quantification obtained for OTA are the lowest found in the literature up to now. This is the reason for having found the presence of ochratoxins in 100% of the wine samples and for having found OTC and MeOTA in red wine. None of the samples exceeded the maximum level permitted by legislation for OTA (2 μg L^{-1}), with 44.8 ng L^{-1} being the maximum level found for this toxic compound. However, the co-occurrence of several ochratoxins, and therefore synergic or additive effects, should be taking into account when determining permitted levels or risk assessment.

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Figure captions.

Fig. 1. Structure of ochratoxins.

Fig. 2. HPLC-FLD chromatograms corresponding to a) ochratoxin calibration sample of 10 μg L^{-1}, b) wine sample naturally contaminated with OTB and OTA and c) wine sample, fortified at 0.05 μg L^{-1} with the four ochratoxins.

Fig 3. LC/ion trap MS chromatograms and spectrum corresponding to a) ochratoxin calibration sample of 10 μg L^{-1}, b) wine sample naturally contaminated with OTB and OTA and OTC.

Table 1. Chromatography parameters

	OTB	OTA	MeOTA	OTC
Retention time (t_R) (min)	5.7	11.2	18.5	21.4
Retention factor (k')	4.7	10.2	17.5	20.4
Symmetry	0.78	0.89	0.91	0.88
Peak width at half height (w_h)	0.32	0.52	0.27	0.36
Number of theoretical plates (N)	5125	7496	77829	88456
Resolution (R_s)	1.4	2.3	1.4	9.2

Table 2. Linearity data

ratoxins	Range (µg L^{-1})	Curve equation	Correlation coefficient (r)	Determination coefficient (r^2)	Slope limits	Intercept limits	RSD (%) of response factors (n=15)	G_{exp}
	0.1-1	$y = 24.2\,x - 0.233$	0.9998	0.9997	(23.4; 24.9)	(-0.649; 0.184)	7.3	0.37
OTB	1-20	$y = 23.0\,x + 1.66$	0.9997	0.9995	(22.0; 23.9)	(-9.13; 12.4)	2.6	0.44
	20-400	$y = 20.6\,x + 92.8$	0.9996	0.9991	(19.5; 21.7)	(-140; 326)	3.8	0.36
	0.1-1	$y = 25.4\,x - 0.348$	0.9998	0.9996	(24.5; 26.4)	(-0.885; 0.189)	9.8	0.86
OTA	1-20	$y = 23.7\,x + 1.99$	0.9997	0.9994	(22.7; 24.8)	(-9.74; 13.7)	2.7	0.53
	20-400	$y = 21.5\,x + 89.1$	0.9996	0.9992	(20.4; 22.6)	(-147; 325)	3.5	0.33
	0.1-1	$y = 26.1\,x - 0.140$	0.9998	0.9996	(25.1; 27.1)	(-0.696; 0.416)	5.9	0.40
eOTA	1-20	$y = 24.7\,x + 1.74$	0.9997	0.9995	(23.7; 25.7)	(-9.65; 13.1)	2.6	0.51
	20-400	$y = 22.2\,x + 102$	0.9995	0.9991	(21.0; 23.4)	(-156; 361)	3.8	0.35
	0.1-1	$y = 25.1\,x - 0.029$	0.9996	0.9991	(23.8; 26.5)	(-0.785; 0.726)	6.4	0.28
OTC	1-20	$y = 24.1\,x + 1.71$	0.9998	0.9995	(23.1; 25.1)	(-9.27; 12.7)	2.6	0.42
	20-400	$y = 21.8\,x + 93.2$	0.9996	0.9992	(20.6; 22.9)	(-149; 336)	3.6	0.34

Table 3. Precision and accuracy of the instrumental system

		Within-day (n = 3)			Between-day (n = 9)		
ratoxins	**C (µg L^{-1})**	**Mean**	**RSD (%)**	**RE (%)**	**Mean**	**RSD (%)**	**RE (%)**
	0.1	0.095	7.2	5.4	0.091	8.6	8.5
	0.4	0.400	5.9	0.01	0.393	4.1	1.9
	1	0.995	3.0	0.5	1.01	3.3	1.4
OTB	**8**	7.97	3.6	0.4	7.82	2.9	2.3
	20	19.8	1.2	0.7	19.8	1.6	1.1
	120	122	1.8	1.4	122	2.2	1.7
	400	396	1.5	0.9	398	2.0	0.4
	0.1	0.092	11.1	8.3	0.092	9.8	6.6
	0.4	0.400	1.7	0.05	0.392	3.9	2.1
	1	0.994	1.9	0.6	1.01	3.0	0.7
OTA	**8**	7.96	3.3	0.5	7.81	2.8	2.3
	20	19.8	1.1	0.8	19.8	1.5	1.0
	120	122	2.0	1.3	121	2.1	1.0
	400	397	1.5	0.8	397	2.0	0.8
	0.1	0.092	3.1	7.9	0.091	3.3	9.2
	0.4	0.403	2.2	0.8	0.395	2.4	1.4
	1	0.993	2.4	0.7	1.01	2.4	1.1
eOTA	**8**	7.96	3.4	0.5	7.80	2.8	2.5
	20	19.9	1.0	0.7	19.8	1.7	1.2
	120	122	2.0	1.5	121	2.3	1.0
	400	396	1.5	0.9	396	2.1	0.9
	0.1	0.088	3.8	11.8	0.087	4.9	12.5
	0.4	0.403	3.3	0.7	0.395	3.6	1.3
	1	0.991	3.5	0.9	1.02	3.0	1.6
OTC	**8**	7.98	3.4	0.3	7.82	2.8	2.3
	20	19.8	1.0	0.7	19.8	1.6	1.1
	120	122	2.0	1.4	121	2.2	1.7
	400	396	1.4	0.9	397	2.1	0.7

Table 4. Recovery

		Repeatability				Intermediate precision			
	C (µg L^{-1})	**Recovery (%) (n = 3)**	**RSD (%) (n = 3)**	**Global recovery (%) (n = 15)**	**RSD (%) (n = 15)**	**Recovery (%) (n = 9)**	**RSD (%) (n = 9)**	**Global recovery (%) (n = 45)**	**RSD (% (n = 45)**
OTB	0.0005	71.7	7.7	87.0	13.2	63.8	13.0	81.7	16.2
	0.003	100.6	13.8			83.9	19.9		
	0.04	83.4	6.4			84.9	4.2		
	0.6	93.5	2.0			92.7	3.3		
	2	85.7	5.8			83.1	8.4		
OTA	0.0005	93.3	4.7	91.1	4.8	103.7	11.2	93.5	10.5
	0.003	92.9	2.3			95.7	8.3		
	0.04	89.6	5.3			90.8	3.7		
	0.6	93.8	2.7			93.8	3.1		
	2	85.7	5.5			83.7	8.3		
MeOTA	0.0005	80.0	9.5	76.7	8.6	80.7	19.1	76.0	13.1
	0.003	76.6	2.1			75.6	5.6		
	0.04	77.2	5.2			76.3	4.5		
	0.6	82.7	4.2			82.4	3.9		
	2	66.7	5.2			64.8	8.7		
OTC	0.0005	65.7	18.8	74.2	11.4	65.0	18.8	73.4	13.4
	0.003	77.3	4.7			77.1	6.4		
	0.04	78.0	3.5			77.3	4.0		
	0.6	83.2	4.5			82.7	3.8		
	2	67.0	4.8			64.9	8.9		

Table 5. Robustness

OTB		OTA		MeOTA		OTC	
C (µg L^{-1})	**Recovery (%)**	**C (µg L^{-1})**	**Recovery (%)**	**C (µg L^{-1})**	**Recovery (%)**	**C (µg L^{-1})**	**Recover (%)**
0.045	90.4	0.045	89.1	0.038	76.3	0.037	73.9
0.049	97.7	0.049	97.1	0.041	83.0	0.041	81.9
0.043	85.6	0.045	89.5	0.041	81.6	0.041	81.1
0.046	92.9	0.048	96.6	0.041	82.0	0.041	81.5
0.041	82.8	0.044	88.2	0.038	76.7	0.039	77.2
0.046	92.7	0.050	100.0	0.042	84.3	0.043	85.7
0.045	90.3	0.047	93.4	0.040	80.7	0.040	80.2
6.1	6.0	5.3	5.4	4.3	4.2	5.1	5.1

Table 6. Concentration of ochratoxins found in 20 red wines samples.

ample number	OTB (ng L^{-1})	OTA (ng L^{-1})	MeOTA (ng L^{-1})	OTC (ng L^{-1})
1	13.5	0.5	0.4*	< LOD
2	7.2	6.6	<LOD	0.4*
3	4.6	3.4	<LOD	0.3*
4	19.5	44.8	<LOD	3.7
5	8.0	19.0	<LOD	2.7
6	12.8	15.2	<LOD	0.8
7	7.2	6.0	0.4*	2.8
8	10.8	15.9	0.5	0.7
9	22.8	3.6	0.8	<LOD
10	4.9	3.8	0.3*	<LOD
11	15.2	6.7	0.6	0.6
12	3.9	3.3	0.3*	2.8
13	7.1	5.3	0.3*	0.4*
14	10.1	10.8	< LOD	1.1
15	7.2	13.5	< LOD	1.4
16	11.5	3.8	< LOD	< LOD
17	10.1	14.6	< LOD	1.7
18	3.3	5.2	< LOD	< LOD
19	5.5	3.2	0.4*	< LOD
20	6.0	12.2	0.3*	1.2
Range (ng L^{-1})	3.3 - 22.8	0.5 - 44.8	< LOD – 0.8	< LOD - 3.7
Median (ng L^{-1})	7.6	6.3	0.2	0.6
samples > LOD	100%	100%	50%	70%
an of values > LOQ (ng L^{-1})	9.6	9.9	0.6	1.8

D: below LOD
LOQ