JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Determination of Ochratoxin A in Wine by Means of Immunoaffinity and Aminopropyl Solid-Phase Column Cleanup and Fluorometric Detection

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ABSTRACT: A new analytical method for the determination of ochratoxin A (OTA) in red wine has been developed by using a double-extract cleanup and a fluorometric measurement after spectral deconvolution. Wine samples were diluted with a solution containing 1% polyethylene glycol and 5% sodium hydrogencarbonate, filtered, and purified by immunoaffinity and aminopropyl solid-phase column. OTA contents in the purified extract were determined by a spectrofluorometer (excitation wavelength, 330 nm; emission wavelength, 470 nm) after deconvolution of fluorescence spectra. Average recoveries from wine samples spiked with OTA at levels ranging from 0.5 to 3.0 ng/mL were 94.5−105.4% with relative standard deviations (RSD) of <15% (n = 4). The limit of detection (LOD) was 0.2 ng/mL, and the total time of analysis was 30 min. The developed method was tested on 18 red wine samples (naturally contaminated and spiked with OTA at levels ranging from 0.4 to 3.0 ng/mL) and compared with AOAC Official Method 2001.01, based on immunoaffinity column cleanup and HPLC with fluorescence detector. A good correlation (r^2 = 0.9765) was observed between OTA levels obtained with the two methods, highlighting the reliability of the proposed method, the main advantage of which is the simple OTA determination by a benchtop fluorometer with evident reductions of cost and time of analysis.

KEYWORDS: *ochratoxin A, red wine, solid-phase extraction, immunoaffinity column, fluorometric method*

INTRODUCTION

Ochratoxin A (OTA), 7-(L-β-phenylalanylcarbonyl)-carboxyl-5-chloro-8-hydroxyl-3,4-dihydro-3*R*-methylisocumarin, is a widely distributed mycotoxin produced mainly by *Aspergillus ochraceus* and *Penicillium verrucosum* filamentous fungi. OTA has been shown to be nephrotoxic, hepatotoxic, teratogenic, and immunotoxic to several animal species and to cause kidney and liver tumors in mice and rats. The IARC (International Agency for Research on Cancer) has classified OTA as a possible carcinogen to humans (group 2B).[1] Moreover, OTA is suspected to be involved in Balkan Endemic Nephropathy (BEN), a fatal kidney disease occurring in some southeastern European countries (Bosnia, Serbia, Croatia, Bulgaria, and Romania), and to be associated with urinary tract tumors.[2]

OTA occurs in pig blood and kidney as well as in various plant products such as cereals, beans, groundnuts, spices, dried fruit, coffee, milk, beer, and wine.[1,3−8] OTA is more frequently detected in red wines than in rosé and white wines, suggesting that the reason for the different levels lies in different winemaking techniques. Moreover, wines from southern European grape-growing regions have been shown to be more contaminated than wines from northern areas. This is thought to be the consequence of different climatic conditions, especially humidity and temperature, which promote higher fungal growth in southern European countries than in northern ones.[8]

On the basis of the results of the EU SCOOP project, Scientific Cooperation Task 3.2.7, wine is the second major source of OTA dietary intake by the EU population, following cereals.[9] For this reason, to protect consumer health, the European Commission has established a maximum permitted level of OTA in wine of 2 ng/mL.[10] High-performance liquid chromatography with fluorescence detection and immunoaffinity column cleanup is the most widely used analytical method for OTA determination in wine.[11−16] With regard to wine sample preparation and cleanup, other methods have been reported, such as liquid−liquid extraction (LLE),[15,17−19] solid-phase extraction (SPE),[15,16,18,20−24] or SPE with molecular imprinted polymers (MISPE),[25] followed by chromatographic determination. Nevertheless, these methods are often expensive and require long procedures and skilled personnel, limiting their use for routine analysis. Alternatively, immuno-based assays appear to be promising techniques for rapid OTA detection in wine. However, they usually require a sample cleanup prior to the analysis due to matrix interferences, mainly with red wines, which have been proven to inactivate antibodies.[15,26−34] In addition, these immunoassays, even though rapid, easy to perform, and inexpensive, show poor analytical performances in term of accuracy and reproducibility compared with chromatographic techniques.

Special Issue: IX Italian Congress of Food Chemistry

Received: July 14, 2012
Revised: September 5, 2012
Accepted: September 6, 2012
Published: September 6, 2012

dx.doi.org/10.1021/jf303068m | *J. Agric. Food Chem.* 2013, 61, 1604−1608

This paper describes the development of a simple fluorometric method for the determination of OTA in red wine. The mycotoxin was extracted and purified by using a double cleanup procedure, consisting of an immunoaffinity column coupled to an aminopropyl (Bondesil-NH_2) SPE column, prior to direct fluorometric measurement. Validation parameters including accuracy (recovery percentage), repeatability (relative standard deviation), and limit of detection (LOD) were also reported. Finally, the OTA contents, obtained by analyzing naturally contaminated and spiked wine samples with the spectrofluorimetric method, were compared with those obtained by using AOAC Official Method 2001.01 for the determination of OTA in wine based on immunoaffinity column cleanup and HPLC with fluorescence detection.[35]

MATERIALS AND METHODS

Reagents and Samples. Ochratoxin A stock solution (1 mg/mL) was prepared by dissolving the solid standard purchased from Sigma-Aldrich (Milan, Italy) in toluene/acetic acid (99:1, v/v). The exact concentration of the stock solution was determined by UV spectrophotometer assuming the relative molar absorption coefficient of OTA in toluene/acetic acid (99:1, v/v) to be equal to 5.440 L mol^{-1} cm^{-1}.[12] Ochratoxin A standard solutions for calibration were prepared by dissolving adequate amounts of the stock solution, previously evaporated to dryness under nitrogen stream, in 0.75% (v/v) formic acid in ethyl acetate/cyclohexane (3:7, v/v) and in the HPLC mobile phase for fluorometric and chromatographic analysis, respectively. Acetonitrile, methanol, glacial acetic acid, formic acid, ethyl acetate, and cyclohexane were purchased from Mallinckrodt Baker (Milan, Italy). Sodium chloride (ACS grade), polyethylene glycol (PEG 8000), and sodium hydrogen carbonate ($NaHCO_3$, ACS grade) were purchased from Sigma-Aldrich. OchraTest immunoaffinity columns were purchased from VICAM, a Waters Business (Milford, MA, USA). Filter paper (no. 4) and glass microfiber filters (GF/A) were obtained from Whatman (Maidstone, U.K.). Bondesil-NH_2 sorbent was purchased from Agilent Technologies Italia S.p.A. (Milan, Italy). Ultrapure water was from Waters Milli-Q system (Waters, Milford, MA, USA).

Red wine samples (cv. Primitivo) were purchased from local retail outlets(bottled wine) or kindly provided by small farmers producing wine mainly for their family needs (homemade wine).

Sample Cleanup and Analysis. The HPLC determination of OTA was performed according to AOAC Official Method 2001.01.[37] Briefly, 10 mL of wine was diluted with 10 mL of a water solution containing PEG (1%) and $NaHCO_3$ (5%), mixed, and filtered through a Whatman GF/A glass microfibre filter. A 10 mL volume of diluted extract (equivalent to 5 mL of wine) was cleaned up through an OchraTest immunoaffinity column at a flow rate of about 1 drop per second. The column was washed with 5 mL of a solution containing NaCl (2.5%) and $NaHCO_3$ (0.5%) followed by 5 mL of distilled water at a flow rate of 1−2 drops per second. OTA was eluted with 2 mL of methanol. For HPLC analysis, the extract was dried under a nitrogen stream at ca. 50 °C and reconstituted with 500 μL of the HPLC mobile phase. The reconstituted extract was injected into the HPLC apparatus by a full loop injection system. For fluorometric determination, a further purification step was performed. The whole methanolic solution from the immunoaffinity column containing OTA was loaded at a flow rate of about 1 drop per second onto a Bondesil-NH_2 column (50 mg of stationary phase) preconditioned with 6 mL of 2% (v/v) formic acid in ethyl acetate/cyclohexane (3:7, v/v) followed by 2 mL of methanol. The column was washed with 2.5 mL of ethyl acetate, 2 mL of a solution containing 0.1% (v/v) formic acid in ethyl acetate/cyclohexane (3:7, v/v), and 0.5 mL of a solution containing 0.75% (v/v) formic acid in ethyl acetate/cyclohexane (3:7, v/v). OTA was eluted with 2 mL of 0.75% (v/v) of acid formic in ethyl acetate/cyclohexane (3:7, v/v), transferred to a quartz cuvette, and measured fluorometrically.

The HPLC apparatus was an Agilent 1100 series equipped with a G1312A binary pump, a G1313A autosampler, a G1316A column thermostat set at 25 °C, a G1321A spectrofluorometric detector set at 333 nm (λ_{ex}) and 460 nm (λ_{em}), and an Agilent Chemstation G2170AA Windows 2000 operating system (Agilent, Waldbronn, Germany). The separation were performed with an Xterra C18 column (150 × 4.6 mm, 5 μm) (Waters) preceded by a Rheodyne guard filter (3 mm, 0.45 μm pore size). The mobile phase was an isocratic mixture of acetonitrile/water/acetic acid (99:99:2, v/v/v) eluted at a flow rate of 1.0 mL/min. Quantification of OTA was performed by measuring peak areas at OTA retention time and comparing them with the relevant linear calibration curve (concentration range covered 0.5−40.0 ng/mL corresponding to 0.05−4.00 ng/mL OTA in wine).

Fluorescence measurements were performed by a Varian Cary Eclipse spectrofluorometer equipped with a xenon flash lamp and a photomultiplier tube as detector. Quartz cuvettes with an optical path of 1 cm were used. OTA content in the sample extract was determined by excitation at 330 nm and recording the maximum emission at 470 nm and comparing the measured fluorescence value with a calibration curve obtained by linear interpolation of fluorescence values of OTA standards (concentration range covered 1.0−10.0 ng/mL corresponding to 0.40−4.00 ng/mL OTA in wine). The fluorescence data were calculated after spectra deconvolution performed by Peak Fit v 4.12 software (SeaSolve Software Inc., San Jose, CA, USA).

Recovery Experiments. Recovery experiments were performed in quadruplicate. Because of the unavailability of an ochratoxin A-free sample of red wine, a naturally contaminated sample was used for recovery experiments at spiking levels of 0.5, 1.0, 2.0, and 3.0 ng/mL. Endogenous toxin concentration (0.24 μg/mL) was determined by HPLC prior to spiking. The spiking solution was prepared by dissolving adequate amounts of the stock solution, previously evaporated to dryness under nitrogen stream, in methanol.

RESULTS AND DISCUSSION

Preliminary experiments showed a direct fluorometric measurement (λ_{ex} = 379 nm λ_{em} = 426 nm) of OTA in methanol extracts, obtained following the immunoaffinity column cleanup reported in AOAC Official Method 2001.01,[35] did not permit accurate determinations of the mycotoxin in wine due to the presence of coeluting interferences, which led to an overestimation of OTA contents. In particular, analyzing the extracts of red wine sample (spiked at OTA levels of 0.5, 1.0, 2.0, and 3.0 ng/mL), even if acceptable relative standard deviations (RSDs, n = 4) were calculated, that is, ≤15%, unsuitable average recoveries were obtained ranging from 161.6 to 415.0%. Recently, aminopropyl silica sorbent has been used for the purification of red wine prior to OTA determination by HPLC or fluorescence polarization immunoassay.[24,30] In the study reported herein, an aminopropyl solid-phase material (Bondesil-NH_2) was used in combination with an immunoaffinity column to carry out a double cleanup of wine with the aim to reduce matrix interferences and perform a simple OTA quantification in wine by spectrofluorometric measurement. In particular, the methanol extract, obtained from the immunoaffinity column, was further purified on the Bondesil-NH_2 SPE column following the protocol reported by Varelis et al.[24] with some modifications in the column preconditioning and washing steps.

Because the solvent used to elute OTA from the Bondesil-NH_2 was 0.75% (v/v) of acid formic in ethyl acetate/cyclohexane (3:7, v/v), a spectrofluorometric characterization of the mycotoxin in this solvent was necessary as a preliminary step. The proper excitation wavelengths (λ) were selected by scanning the excitation spectrum of a standard solution of OTA (2.0 ng/mL), finding two maximum absorptions at 330 and 379

nm. The emission scans were performed in the wavelength range of 390−600 nm. With excitation at 330 nm, the toxin had an emission peak at 470 nm; otherwise, with excitation at 379 nm, the peak obtained at 472 nm was only due to the solvent fluorescence. In fact, this peak was also observed in the absence of OTA. Therefore, subsequent experiments were performed using excitation λ of 330 nm.

To improve the cleanup procedure, several experimental conditions were carried out. An investigation was conducted to verify if the cleanup columns released interfering substances in the elute solution that could interfere with the fluorescence measurements. For this purpose, a hydroalcoholic solution (12% ethyl alcohol) was prepared and subjected to the procedure of double purification. The relevant fluorescence spectrum was recorded showing the presence of a broad band, and additional experiments demonstrated that this phenomenon was due to substances deriving from the Bondesil-NH_2 column. To reduce this effect, preconditioning steps of the aminopropyl cartridges were tested with different volumes (2, 3, 4, and 6 mL) and acid percentages (1, 2, and 5%) of a solution of formic acid in ethyl acetate/cyclohexane (3:7, v/v). Subsequently, to reduce coextracted materials from wine, other experiments were carried out in which different wash steps were tested prior to OTA elution by using various volumes (1, 2, 5, 10, and 15 mL) of ethyl acetate/cyclohexane (3:7, v/v) containing 0.05 and 0.1% of formic acid (v/v). These lower formic acid concentrations, with respect to those used in the eluting solution, avoided OTA losses. Finally, different amounts of Bondesil-NH_2 stationary phase (50, 75, and 100 mg) were also evaluated. Figure 1 shows fluorometric spectra of a wine

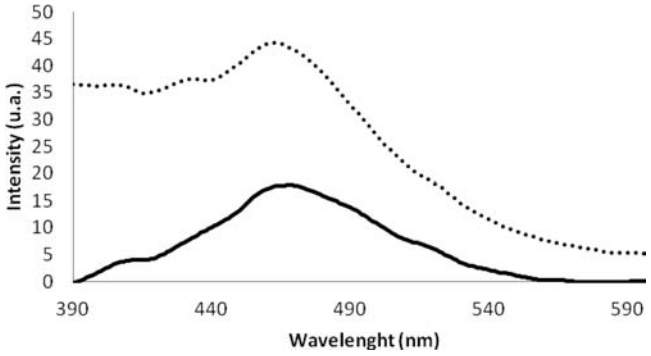

Figure 1. Emission spectra relevant to a sample of wine spiked with OTA at a level of 2.0 ng/mL purified with single (dotted line) and double (solid line) cleanup procedures.

sample contaminated with OTA at 2.0 ng/mL obtained with single- and double-cleanup procedures, demonstrating that the double cleanup drastically reduced the interfering substances. Optimal double-cleanup conditions are reported under Materials and Methods.

To assess the accuracy and precision of the full analytical procedure (double purification plus fluorometric determination), recovery experiments were carried out analyzing in quadruplicate a red wine spiked with OTA at four levels, that is, 0.5, 1.0, 2.0, and 3.0 ng/mL. As shown in Table 1, average recoveries ranged from 114.2 to 181.2% with RSD values of <15%, showing that a considerable improvement of accuracy was obtained by using the double-cleanup procedure. However, the method still does not meet the recovery parameters set by the European Commission, which establishes performance

Table 1. Recovery and Repeatability Results from the Analysis of a Sample of Wine Contaminated with Ochratoxin A at Different Spiking Levels Obtained without and with Spectra Deconvolution

	no spectra deconvolution		spectra deconvolution	
spiking level (ng/mL)	recovery (%)	RSD[a] (%)	recovery (%)	RSD (%)
0.5	181.2	12	98.0	15
1.0	138.6	10	105.4	12
2.0	118.1	11	95.2	12
3.0	114.2	9	94.5	4

[a]RSD, relative standard deviation (n = 4).

criteria for acceptability of an analytical method for the determination of OTA in food matrices (Regulation EC No. 401/2006).[36] In particular for wine, OTA recoveries should be in the ranges of 50−120 and 70−110% at levels of <1.0 ng/g and from 1.0 to 10.0 ng/g, respectively.

To overcome this problem, a deconvolution program was applied to the fluorescence spectra aiming at mathematically removing the contributions arising from interfering substances eluted with OTA.

Preliminarily, different functions were tested to find the most appropriate for the study of the OTA fluorescence by fitting a spectrum of an OTA standard solution (2 ng/mL). A good fitting was obtained with an asymmetric log-normal function (r^2 = 0.999), which has been already reported in the literature for analogous studies,[37−41] and therefore it was used in the subsequent experiments. To investigate the ability of the deconvolution technique to isolate the OTA fluorescence, the emission spectra of wine samples and of a OTA standard, having both the same concentration of OTA (2.0 ng/mL), were compared before (Figure 2a) and after the deconvolution analysis (Figure 2b). The results showed that a good overlap of OTA peaks was achieved after deconvolution, demonstrating the goodness of such an approach.

After application of the deconvolution, the mean recoveries were improved and fell within the parameters defined by the European Commission at all concentrations investigated (average recovery of 98.3%), with RSDs ranging from 4 to 15% (n = 4). The value of the LOD, calculated as $3s_a/b$, where s_a is the standard deviation of the response (estimated by the standard deviation of y-intercept of the regression line) and b is the slope of the linear calibration curve, was equal to 0.20 ng/mL.

The validation of the developed fluorometric method was carried out by comparing the results obtained from the analysis of 15 samples of wine (5 naturally contaminated samples of wine and 10 spiked samples in a concentration range between 0.41 and 3.00 ng/mL) by the fluorometric technique and by using the chromatographic AOAC Official Method 2001.01.[35] Linear regression of data showed a good correlation (r^2 = 0.9765; Figure 3); moreover, the slope and the intercept of the regression line were not significantly different (p = 0.01) from 1 and 0, respectively, demonstrating the good accuracy of the proposed method.

In conclusion, the fluorometric method developed herein allows an accurate and precise quantification of OTA in red wine, in accordance with the parameters defined by EC Regulation No. 401/2006.[36] In addition, it is sensitive enough to detect OTA in wine at levels lower than the EU limit of 2.0 ng/mL. The method is simple, fast, inexpensive, and safe, and, therefore, it should be considered as an interesting alternative

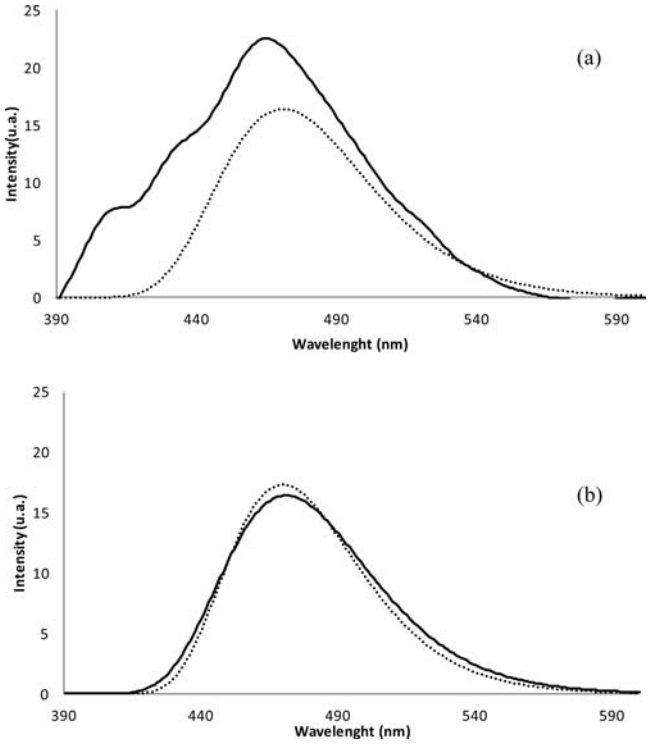

Figure 2. Emission spectra relevant to a sample of wine spiked with OTA (solid line) and of a OTA standard solution (dotted line), having both the same toxin concentration of 2.0 ng/mL, before (a) and after (b) the deconvolution analysis.

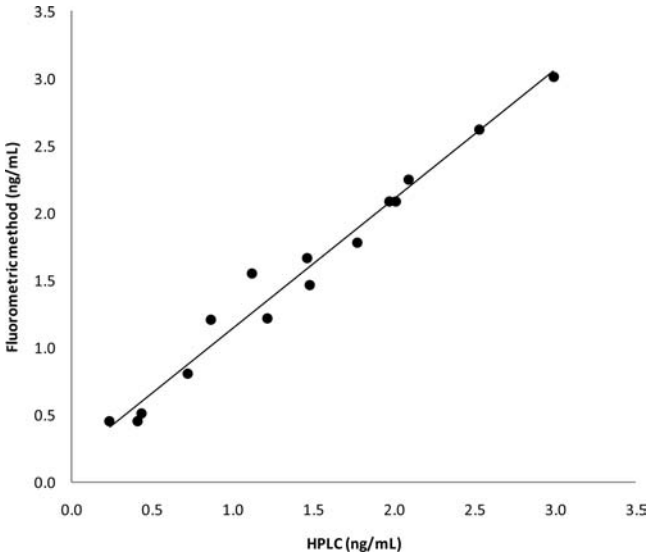

Figure 3. OTA content in naturally contaminated and spiked red wine samples analyzed by fluorometric method compared to the expected values (determined by HPLC).

to HPLC/immunoaffinity cleanup methodology to quantify OTA in wine. Finally, the method could be very useful for wine producers because it does not need either special equipment or skilled operators.

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Notes

The authors declare no competing financial interest.

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