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Article

Determination of Ochratoxin A in Wine by High-Performance Thin-Layer Chromatography using Charged Coupled Device

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Um método foi desenvolvido e validado para a determinação de ocratoxina A (OTA) em vinho. Foi utilizado um sistema contendo um detector de carga acoplada (CCD) para adquirir as imagens fluorescentes da micotoxina obtidas a partir das placas de cromatografia em camada delgada de alta eficiência (HPTLC) sob luz ultravioleta. O método desenvolvido mostrou recuperação média de 90,4%. O limite de quantificação e o limite de detecção foram de 0,1 μg L^{-1} e 0,016 μg L^{-1}, respectivamente. Os resultados da validação confirmaram a eficiência do método, que é suficientemente sensível para ser utilizado para quantificar OTA em vinho. A OTA foi encontrada em uma das amostras de vinho na concentração de 4,5 μg L^{-1}, que é superior ao limite de 2 μg L^{-1} considerado aceitável pelo Comitê Científico das Comunidades Européias. Este estudo demonstrou a aplicabilidade do sistema HPTLC-CCD para determinar OTA em vinho.

A method was developed and validated in-house for determination of ochratoxin A (OTA) in wine using charge coupled device (CCD) to acquire the fluorescence images of mycotoxin from high-performance thin-layer chromatography (HPTLC) under UV lamp. The method showed a mean recovery of 90.4%. The quantification and detection limist were 0.1 μg L^{-1} and 0.016 μg L^{-1} *per* spot, respectively. The results of validation confirmed the efficiency of the method, which is sensitive enough to be used to quantify OTA in wine. The occurrence of OTA in Brazilian wines was evaluated. OTA was found in one sample at a level of 4.5 μg L^{-1}, which is higher than the limit of 2 μg L^{-1} considered acceptable by the Scientific Commission of the European Communities. This study demonstrated the applicability of HPTLC using CCD as a tool to determine OTA in wine.

Keywords: ochratoxin A, wine, charge-coupled device imaging, high-performance thin-layer chromatography

Introduction

Ochratoxin A (OTA), 7-(L-b-phenylalanyl-carbonyl)-carboxyl-5-chloro-8-hydroxy-3,4-dihydro-3*R*-methyl-isocumarin, is a mycotoxin produced by *Penicillium verrucosum, Aspergillus ochraceus*,[1] *A. niger* and *A. carbonarius*.[2,3] OTA occurs in several food products such as in cereals,[4] coffee beans,[5] beer,[6] cacau,[7] wheat,[8] dried fruit,[9] cheese,[10] grape juice and wine.[11] Wine represents, after cereals, the major source (15%) of OTA intake for European population.[12]

OTA is receiving major attention for its nephrotoxic effects. This toxin is also known for its mutagenic,[13] teratogenic,[14] immunosuppressive[15] and carcinogenic proprieties.[16-19] OTA has been associated with Balkan Endemic Nephropathy and the development of urinary tract tumors in humans.[20,21] The International Agency for Research on Cancer classified OTA as a possible carcinogen to humans (group 2 B).[22] Thus, because of possible health effects, there is an increasing need to monitor this mycotoxin in food samples including wine. There are recommendations of Provisional Tolerable Weekly Intakes (PTWI) for OTA of 100 ng kg^{-1} body weight.[1] The Scientific Commission of the European Community established the maximum allowed level of 2 μg L^{-1} of OTA in wine.[23] There is yet no maximum permissible level established for this mycotoxin in wine in Brazil, but it is necessary to adopt limits for OTA so as to minimize the health hazard risk.

After the first detection of OTA in wine,[24] several surveys were conducted in several countries to examine the true occurrence of this toxin in wine[25-27] as well

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as several methods have been developed for analysis of OTA in foods, including wine.[28-30] OTA is usually extracted with acidified organic solvents (e.g acidified chloroform). Clean-up is carried out by liquid-liquid or solid-phase extraction (SPE).[31] Monoclonal antibody based immunoaffinity columns (IACs) have also been developed to substitute the traditional solvent clean-up.[28-30] However, the relatively expensive and limited shelf-life of IACs are disadvantageous for use in large survey programs of OTA. Furthermore, because the antibodies used in IACs are fragile proteins, which can readily lose their binding affinity as a result of subtle changes in conditions, such as temperature and pH, experimental and storage conditions need to be carefully controlled and monitored.[32] Reversed-phase HPLC and enzyme-linked immunosorbent assays (ELISA) are used for OTA determination.[25,28,33,34] ELISA is a method expensive due to high price of specific mycotoxin antibodies and high-performance liquid chromatography (HPLC) requires sophisticated and costly equipment, extensive clean-up procedures and high purity solvents. Besides high recovery levels, low detection limit and repeatability, wineries and government in monitoring and survey programs, need inexpensive methods for routine analysis of ochratoxin A in wines. HPTLC is a fast, cheap and efficient method of separation and identification of many mycotoxins.

Charge-coupled devices (CCDs) are two-dimensional detectors containing an array of sensors that can image an area in fraction of seconds or real time.[35] The output from each sensor pixel on the CCD is a voltage, which is proportional to the intensity of light falling on the sensor and the exposure time. These series of voltages are digitized and transferred to a computer for storage and data processing.[36]

By coupling CCD detection with HPTLC, the entire HPTLC can be imaged in a single exposure yielding rapid quantification in shorter analysis time than of slit scanning densitometers.[37] CCD detectors have demonstrated extremely low dark current and read noise characteristics, high sensitivity and excellent linearity. These features have made the CCD an excellent detector for many imaging applications in chemical analysis, such as fluorescence detection.[38]

In this study a method using HPTLC quantification through the fluorescence images from UV lamp recorded by a CCD camera was developed for determination of ochratoxin A in wine. The potential of the method was demonstrated through the analysis of wine samples produced in Brazil.

Experimental

Ochratoxin A standard solution

A stock standard solution of OTA was prepared by dissolving 1 mg of pure crystalline OTA (Sigma-Aldrich) in toluene-acetic acid (99:1, v/v) at a concentration of 40 μg mL^{-1}. The standard solution was kept frozen (−18 °C). The concentration of the OTA stock solution was determined by measuring the UV absorbance at 366 nm and calculated by using the molar extinction coefficient ε of 5440 m^2 mol^{-1}. The concentration of working standard solution in toluene-acetic acid (99:1, v/v) was 8 μg mL^{-1}.[39]

Samples

Thirty-four red wines were purchased from retail stores. All samples were produced in Rio Grande do Sul state, Brazil. Bottles were stored at room temperature and opened only before analysis.

Sample preparation

Ochratoxin A extraction

A modified version of the method employed by Zimmerli and Dick.[24] A portion of 10 mL of wine was acidified to pH 2.0-2.2 using 85% orthophosphoric acid and intensively mixed for 1 min using a vortex mixer. After addition of 5 mL chloroform, the mixture was centrifuged at 2500 g for 5 min. The clear organic phase at the bottom was transferred to a pear-shaped flask and extracted twice with 5 mL of 1.25% $NaHCO_3$ solution. The $NaHCO_3$ phases were combined and adjusted to pH 2.5 with formic acid (approximately 0.5 mL formic acid was needed). This phase was extracted with 5 mL chloroform. The solvent was evaporated and redissolved in 100 μL of toluene-acetic acid (99:1, v/v). All samples were analyzed in triplicate.

Chromatography

Chromatographic separations were performed on 10 cm × 10 cm HPTLC glass plates coated with silica gel 60 (Merck). Four, eight and twelve microliter aliquots of sample extract and OTA standard solution (8 μg mL^{-1}) were spotted 1 cm apart on HPTLC plates. The spots were dried, and the plates developed in solvent system toluene:ethylacetate:formic acid (6:3:1 v/v/v). Calibration curve was done with OTA standard solution ranging of 0.8 to 32 μg L^{-1}. For confirmation of OTA, the plates were sprayed with alcoholic sodium bicarbonate solution (6g $NaHCO_3$, 100 mL water, 20 mL ethanol). The plates were dried at

room temperature and viewed under long wavelength UV light (366 nm). The fluorescence of OTA changed from greenish blue to blue and increased in intensity.

CCD Imaging system

The quantification of the fluorescence intensities from UV lamp were recorded by a CCD camera (Sony, Tokyo, Japan). The HPTLC plate was positioned in system and CCD camera was aligned for optimal pixel resolution of CCD images. The image was analyzed using Image Processing and Analysis in Java package (Image J, http://rsbweb.nih.gov/ij/). The package is freely available and it is very appropriate for the current application. Image J is one of the many image processing tools available and is required for the analysis of images generated by the CCD camera and to carry out the necessary operations. It is a versatile software, which offers a wide array of functionalities that can be customized to meet specific requirements.[40]

The simplest way to measure the spots brightness is called diaphragm photometry and we adopted circular diaphragms due to the OTA spots symmetry. To discount the background, several backgrounds around every spot were measures with the same diaphragm.

Method validation

Validation of the analytical method was based on the following parameters: linearity, recovery percentage, precision, limit of detection (LOD) and limit of quantification (LOQ). The sensitivity of the HPTLC-CCD technique was evaluated by determining LOD for OTA, which was derived from the fluorescence of OTA standard with the lowest detectable signal. The result was compared with that obtained for the minimum concentration of OTA that the human eye is capable of detecting in TLC plates under UV light. The LOQ was obtained as the least amount of OTA in samples that can be quantitatively determined with accuracy and precision. Linearity was determined by analysis of seven point calibration curves using the intensity of OTA fluorescence versus OTA concentration. Ten calibration curves were done. The precision of OTA determination on HPTLC plates using CCD camera to take images and Image J software was evaluated by taking five successive HPTLC images by CCD camera of ten plates containing 0.8, 1.6, 3.2, 4.8, 6.4, 8.0 and 12.8 μg L^{-1} of OTA. Then, the precision was calculated by the relative standard deviation (RSD) for intra- and inter-plates determinations. Repeatability of the OTA extraction method and the accuracy of the HPTLC-CCD technique were assessed by measuring the percentage recovery of OTA-free wine samples spiked with the toxin. Recovery was determined by analyzing of ten wine samples spiked with 0.3, 0.5, 1.5, 2.0, 3.5, 5.0 and 8.0 μg L^{-1} of OTA. This procedure was done in triplicate. Within-day precision was determined by analyzing ten replicates of spiked wines at levels of 0.3, 0.5, 1.5, 2.0 and 5.0 μg L^{-1} of OTA, and between-day precision was determined by analyzing ten replicates over a 10-day period at the same levels.

Results and Discussion

This technique is based in use of CCD to acquire the fluorescence images of OTA from HPTLC plate under UV lamp. Under acid conditions OTA generates blue fluorescence at 366 nm. The ability of the CCD array detector to take analytical information is demonstrated in Figure 1a. A chromatogram can be generated by positioning of cursors on fluorescent spots which correspond to different OTA concentrations of each analyzed wine sample (Figure 1b). Each peak corresponds to a concentration of extract of wine spotted on HPTLC plate. The CCD camera system is sufficiently sensitive to see small changes in fluorescence intensity caused by illumination gradients from the UV light source. The change in fluorescence signal intensity corresponds to the different concentrations of OTA standard. Seven OTA levels ranging from 0.8 to 12.8 μg L^{-1} were spotted on the same HPTLC plate to evaluate intra-plate precision (Table 1). In addition, this procedure was repeated using ten plates to evaluate inter-plate precision. The RSD of measurements for all OTA levels was lower than 9.9% and 11.5% for the intra and inter-plate assay, respectively.

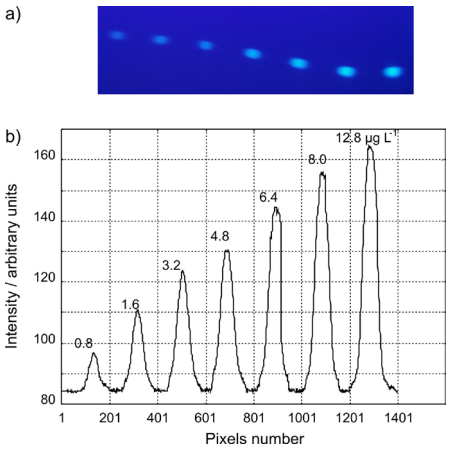

Figure 1. Image of HPTLC plate acquired by the CCD camera with 0.8, 1.6, 3.2, 4.8, 6.4, 8 and 12.8 μg L^{-1} of OTA standard solution. (b) Chromatogram constructed from HPTLC plate image by Image J software.

The procedure used to extract OTA from wine was simple and practical. Reduced amounts of chloroform were used to extract OTA, considering a current trend towards minimizing the amounts of toxic solvents for

Table 1. Precision of OTA determination on HPTLC plates using CCD camera to take images and Image J software to quantify OTA.

OTA spot / (μg L^{-1})	Intra-plate (n = 5) RSD[a] / (%)	Inter-plate (n = 50) RSD[a] / (%)
0.8	7.6	11.5
1.6	5.2	10.9
3.2	8.8	9.3
4.8	6.2	8.5
6.4	8.5	8.3
8.0	9.9	9.0
12.8	8.7	9.6

[a]RSD = Relative standard deviation.

environmental and sanitary reasons. One common problem in chromatographic determination of OTA is the interferences from matrix components (such as flavonoids) that are usually present in red wines.[41] These components were selectively removed from red wine samples before HPTLC-CCD analysis (Figure 2). Figure 3 shows a HPTLC plate that contains spots of wine extract without

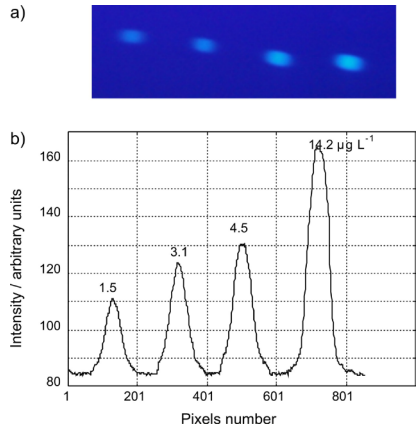

Figure 2. (a) HPTLC image of OTA with 5, 8 and 10 μL of sample extract with $NaHCO_3$ treatment, which contain 1.5, 3.1 and 4.5 μg L^{-1} of OTA; the last spot corresponds to 14.2 μg L^{-1} of OTA standard solution. (b) Chromatogram constructed from HPTLC plate image by Image J software.

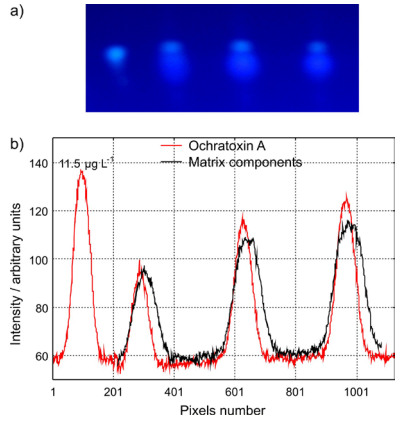

Figure 3. (a) HPTLC image of OTA with 11.5 μg L^{-1} of OTA standard solution and 5, 8 and 10 μL of sample extract without $NaHCO_3$ treatment. (b) Chromatogram constructed from HPTLC plate image by Image J software showing matrix components interfering in OTA quantification.

the $NaHCO_3$ treatment. Some matrix components difficult OTA quantification.

Sample clean-up was done using $NaHCO_3$ solution. Then, the use of immunoaffinity columns or other clean-up method was not necessary. Techniques based on immunoaffinity columns for clean-up sample are frequently used but they have a high cost.[25,29,30]

The validated in-house method showed good linearity. The linear regression coefficient of standard solution curve (y = 5.067x + 1.6786) for OTA within the concentration ranging from 0.8 to 32 μg L^{-1} was 0.996. The mean recovery of OTA was 90.4% with mean standard deviation of 7.7% (Table 2). The LOD value obtained was 0.016 μg L^{-1} *per* spot and the LOQ was 0.1 μg L^{-1}. The results of the within and between-day precision of the assay (Table 3) show that the method has good precision, with RSDs ≤ 9.0.

Table 2. Recovery results of the method for determination of ochratoxin A in wine

OTA spiked / (μg L^{-1})	Recovery / (μg L^{-1})	Recovery / (%)	RSD / (%)[a]
0.3	0.25	83	5.0
0.5	0.4	84	5.7
1.5	1.4	87	6.3
2	1.8	90	11
3.5	3.1	89	6.5
5	5.1	102	10.0
8.0	7.4	93	9.5

[a]RSD = Relative standard deviation.

Table 3. Precision of the method for determination of ochratoxin A in wine using HPTLC-CCD technique

OTA spiked / (μg L^{-1})	Within-day		Between-day	
	Recovery / (μg L^{-1})	RSD[a] / (%)	Recovery / (μg L^{-1})	RSD[a] / (%)
0.3	0.25	6.3	0.26	6.5
0.5	0.4	7.6	0.4	6.1
1.5	1.4	8.6	1.3	9.0
2.0	1.8	6.0	1.9	6.3
5.0	4.7	7.2	5.2	8.6

[a]RSD = Relative standard deviation.

These results of validation were similar to those of the official method used to determine ochratoxin in wine, which found average recoveries from wine samples spiked at levels from 0.04 to 10 μg L^{-1} ranging from 88% to 103% and the detection limit was 0.01 μg L^{-1} using immunoaffinity column clean-up and HPLC.[6] Noba *et al.*[42] developed a method based in the use of immunoaffinity column for clean-up

and liquid chromatography-tandem mass spectrometry for quantification of this mycotoxin. The average recoveries of OTA from white and red wine were 95 and 96.7% to spiked OTA level of 0.05 μg L^{-1}. The repeatabilities expressed as relative standard deviation were 3.8 and 2.4%, respectively.

The applicability of the developed method in this work was tested analyzing 34 red wine samples. This survey utilized red wine to study OTA occurrence considering that red wines are the most consumed in Brazil, and previous reports from other countries have indicated that this wine is more susceptible to OTA contamination.[24,43] The analyzed wines were produced in Rio Grande do Sul, the southernmost state of Brazil, where are produced approximately 90% of Brazilian wines. OTA was found in only one sample at level of 4.5 μg L^{-1}, which is higher than the limit of 2 μg L^{-1} considered acceptable by The Commission of the European Communities.

During the last decade, the occurrence of OTA in different wines originating from various countries has been reported.[25,27,42-46] In Brazil, Rosa *et al.*[47] analyzed 80 samples of national and imported wines. OTA were detected in 28.75% of samples, at concentrations ranging from 0.021 to 0.071 μg L^{-1}, with an average of 0.037 μg L^{-1}. Shundo *et al.*[48] found OTA contamination in nine of 29 Brazilian red wines with levels ranging from 0.10 to 1.33 μg L^{-1}.

More studies must be conducted on OTA occurrence in wines and grape juices produced in Brazil. Since the wine consumption in this country is increasing, the controlling the levels of OTA is necessary. Moreover, knowledge of the extent of OTA contamination in Brazilian products is important to establish national regulations. This is the first report of use of HPTLC-CCD system to quantify OTA. This system was used to quantify patulin in apple juice[49-51] and aflatoxin in peanuts.[52] The HPTLC-CCD method is useful mainly for producers and governments to have a rapid and inexpensive method for quantification of this mycotoxin, including producers which intend to export their products. This method is important for analysis where the cost is often the first factor considered before the method is adopted.

This study demonstrated the applicability of HPTLC-CCD technique as a tool to determinate OTA in wine. The method is fast, simple and economical. The use of a single HPTLC plate allows the separation, identification and quantification of OTA. The CCD imaging system provides good sensitivity, precision and linearity for the quantitative determination of OTA. The results of validation confirmed the efficiency of the method, which is sensitive enough to be used in studies required to quantify OTA in red wines. The method can be immediately available in laboratories without the necessity of acquiring expensive equipment.

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