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Journal of Chromatography A, 882 (2000) 29–35

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Solid-phase extraction applied to the determination of ochratoxin A in wines by reversed-phase high-performance liquid chromatography

D. Jornet, O. Busto*, J. Guasch

Departament de Química Analítica i Química Orgànica (Unitat d'Enologia, CeRTA), Facultat d'Enologia de Tarragona, Universitat Rovira i Virgili, Avda. Ramón y Cajal 70, E-43005 Tarragona, Spain

Abstract

A reversed-phase high-performance liquid chromatographic method is described for the analysis of Ochratoxin A at low $\mu g\ l^{-1}$ levels in samples of artificially contaminated wines. The method involves solid-phase extraction of samples using octadecylsilane cartridges and an additional preconcentration step prior to chromatography with isocratic elution and fluorimetric detection. The method was evaluated for accuracy and precision with relative standard deviations lower than 10%. Recoveries of ochratoxin A added to commercial wines over the range 0.1–3.0 $\mu g\ l^{-1}$ were higher than 80% in the assays. The performance of the octadecylsilane cartridge method tested compared very favourably with results of other published studies of ochratoxin A which use immunoaffinity columns or solvent extraction techniques.

Keywords: Wine; Food analysis; Ochratoxin A; Mycotoxins

1. Introduction

Ochratoxin A (OTA) is a nephrotoxin and nephrocarcinogenic mycotoxin produced by several mould species belonging to genera *Aspergillus* and *Penicillium*.

Occurrence of OTA in humans is on account of the intake of many kinds of food (e.g. cereals, cocoa, dried fruits, nuts, spices, legumes, coffee beans) and beverages (e.g. wine, grape juice, beer, cow's milk) [1–4].

Although the provisional tolerable weekly intake (PTDI) proposed by the World Health Organisation is 100 ng OTA/kg bw/week for humans [5], only very few countries have regulation for OTA in feed, and, when controlled, the maximum level is related to few kinds of food [6].

Because of the low concentration level of OTA usually found in wines (lower than 1 $\mu g\ l^{-1}$), sensitive techniques should be applied for its analytical determination. The methods currently used are often based on reversed-phase high-performance liquid chromatography (RP-HPLC) with either isocratic [7–10] or gradient elution [11], but thin layer chromatography [12–14] and immunochemical methods based on an enzyme-linked immunosorbent assay (ELISA) [15,16] have also been applied. Although UV detection can be used after OTA derivatization [17,18], fluorescence detection is preferred.

The complexity of the samples requires a pretreatment step using techniques such as solvent extraction [19,20] or immunoaffinity columns [21–24], which enables isolation of OTA from the matrix,

*Corresponding author. Tel.: +34-977-22-9596; fax: +34-977-24-2321.

E-mail address: qaenol@fe.urv.es (O. Busto)

0021-9673/00/$ – see front matter
PII: S0021-9673(00)00237-5

thus increasing the selectivity of the method. Solvent extraction techniques are usually avoided because they involved long analytical times and the use of harmful solvents. The use of immunoaffinity columns, either combined or not with solvent extraction techniques, is commonly used in food analysis, but has shown low recoveries in the analysis of wines [5]. Furthermore, its use can be an inconvenient for routine analysis because they imply the use of specific stationary phases, which cannot be universally used and so increases expense.

Solid-phase extraction (SPE) represents a good alternative to the use of immunoaffinity columns, [25–27] especially if it can be used to apply the samples through ordinary stationary phases, such as octadecylsilane [28,29].

The aim of this work is to provide a rapid RP-HPLC method for the determination of OTA in wines, using a SPE process to increase the selectivity and the sensitivity of the method. The natural fluorescence of OTA is used to enhance the selectivity of the determination. This procedure has been shown to be reliable for red and white wines.

2. Experimental

2.1. Chemicals and reagents

The OTA standard was supplied by Aldrich-Chemie (Madrid, Spain). A working solution of 200 mg l^{-1} was prepared in HPLC-grade acetonitrile (Scharlau, Barcelona, Spain). More diluted solutions used in the different studies were prepared by dilution of this standard solution with mobile phase.

Milli-Q quality water (Millipore, Bedford, MA, USA), methanol, acetonitrile and tetrahydrofuran (all from Scharlau) used in chromatographic and extraction methods were of HPLC grade. The buffer solution of sodium acetate (0.05 *M*) was also supplied by Scharlau.

2.2. Equipment

Chromatographic experiments were performed using a Hewlett-Packard 1050 liquid chromatograph with a fluorescence detector HP model 1046A. The injection of the samples was carried out through an automatic injector HP Series 1050. Separation was performed using a Waters Spherisorb S5 ODS2 cartridge (250×4.6 mm) supplied by Waters (Barcelona, Spain).

2.3. High-performance liquid chromatographic method

A mixture of 52% 4 m*M* sodium acetate–acetic acid (19:1) and 48% acetonitrile was used to separate 20 μl of the samples, following HPLC isocratic elution.

Determination was performed at 30°C with a flow-rate of 1 ml min^{-1} and the OTA was detected by monitoring its fluorescence at 247 nm and 480 nm as wavelengths of excitation and emission, respectively. Under these conditions OTA was eluted in less than 15 min.

2.4. Calibration experiences

The standard solutions needed to obtain the calibration lines were prepared in the same eluent as the one used in the mobile phase. Two sets of solutions were prepared. The first one was used to quantify the most concentrated samples and ranged from 0.1 to 1 mg l^{-1}. The other set was used to determine the lowest contents of OTA and ranged from 10 to 50 μg l^{-1}.

2.5. Solid-phase extraction

C_{18} cartridges (Varian, Harbor City, USA) were activated and conditioned by rinsing them with two volumes of acetonitrile and two volumes of water. Aliquots of 100 ml of wine, either fortified or not, were applied through the cartridge. For the analysis of the highest contents of OTA (3 μg l^{-1}), the sorbent mass of the cartridges was 1 g for the red wines and 500 mg for the white ones. The elution was carried out with 2 ml of acetonitrile, which were then evaporated up to 0.4 ml under nitrogen steam.

The 0.1 μg l^{-1} spiked samples of red wines were applied through cartridges with a sorbent mass of 5 g and eluted with six aliquots of 1 ml of acetonitrile. The last five aliquots were collected in a vial where they were concentrated to 0.4 ml under nitrogen steam.

2.6. Precision

The study of precision was developed at two concentration levels and in three steps. In the first step, five samples of red wines were spiked with suitable volumes of OTA to reach a concentration level of 3 μg l^{-1}. Each spiked wine was divided into aliquots of 100 ml and six of the replicates obtained were analysed by the same analyst, in the same way, to evaluate the within-day repeatability.

In the second step, ten samples of different wines (five red, five white) were spiked with OTA to reach the same concentration level as in the previous study. Each spiked wine was divided into aliquots of 100 ml and stored in darkness at 4°C. Every aliquot was analysed in the same way, by the same analyst, in alternate days. As in the previous study, six replicates were used to calculate the between-day standard deviation of the method proposed.

The final step consisted on spiking five more wines (all red) with OTA to reach a concentration level of 0.1 μg l^{-1}. These samples were analysed under conditions of within-day repeatability.

3. Results and discussion

After suitable adjustment of the chromatographic conditions found in the literature, good resolution was obtained between the OTA peak and other peaks that exhibit natural fluorescence in wines.

In order to verify the linearity of the response of the OTA at the previously specified wavelengths for the working concentration, standard solutions of OTA were prepared and injected. First of all, the influence of the matrix in the response was evaluated. Different standards of OTA were prepared in water, mobile phase, synthetic wine (hydro–alcoholic solution of tartaric acid set at pH 3.5), red wines and white wines. There were no significant differences when the standards were prepared in organic solvents, but any chromatographic response was obtained when they were prepared in water. So, to reduce to the maximum the analytical time, the OTA standards were prepared in the eluent used in the mobile phase.

Calibration graphs of OTA were built at concentration levels which ranged from 0.1 to 1 mg l^{-1} for the 3 μg l^{-1} repeatability study, and from 10 to 50 μg l^{-1} for the 0.1 μg l^{-1} one. The graphs were constructed by plotting the OTA peak area against the OTA concentration. Linear least-squares regression was used to calculate the slope, intercept and correlation coefficient. The goodness of the regression line was further assessed by an analysis of the residuals and an analysis of the variance (ANOVA) to check for linearity [30].

The instrumental detection limit was calculated from the amount of OTA required to give a signal-to-noise ratio of 3, by injecting a spiked wine, and it was 5 μg l^{-1}.

To perform the solid-phase extraction of OTA with commercially available cartridges, several variables were studied: volume of sample, organic solvent used to desorb OTA, volume of this solvent, volume of concentrated sample, solvent used to prepare standards, storage of samples at low temperature and influence of the percentage of ethanol in wines.

The extraction procedure was optimised at two concentration levels, one close to the maximum level established in the *Codex Alimentarius* for cereals (5 μg l^{-1}) [31] and one close to the level of OTA which is more common in wines (less than 1 μg l^{-1}) [32].

Octadecylsilane (C_{18}) was the stationary phase used to isolate the analyte from wines. Several sorbent masses of the cartridges used were tested and compared. While 500 mg was enough for white wines, higher capacities were needed for the red ones: 1 g for the 3 μg l^{-1} spiking level and 5 g for the 0.1 μg l^{-1}. Tentative studies were done with other kinds of cartridges, such as strong anion-exchangers with slight modification of the pH of samples, but no satisfying result was obtained, even varying the eluent used. So, the study of the between-day repeatability was done with 500 mg C_{18} cartridges for white wines and 1 g C_{18} cartridges for red wines, while the within-day repeatability was done with 5 g C_{18} cartridges. It can be quite surprising that the least concentrated samples require the highest cartridge capacity. This is caused by the great concentration of compounds that show affinity to the C_{18} phase, thus competing with OTA for the active retention sites. When the OTA concentration is relatively high, no retention problems are found, but

when the concentration is as low as 0.1 μg l^{-1}, the ratio between the interferences (major compounds) and OTA concentrations increases, and OTA co-elutes with the wine matrix. So, higher capacities are needed in order to retain these compounds with no exclusion of the analyte of interest. Aliquots of 100 ml of spiked wines were analysed in order to achieve good limits of determination for the method proposed. Lower volumes did not enable the desired level to be arrived at and higher volumes resulted in poor recoveries because of the capacity of the stationary phase, which was saturated with other organic compounds, mainly polyphenolic substances.

Since acetonitrile showed the best results in the elution of the OTA standard, it was used as the eluent in further studies. The percentage of ethanol in samples was also tested, because there are some differences in the percentage of ethanol in wines that can influence the recovery of the extraction. So, synthetic wines with a different percentage of ethanol (9–14%) were applied through the cartridge and no differences related to the peak area of the OTA were observed.

The next step consisted of observing whether the storage temperature of the spiked samples showed influence on the extraction results. No differences between the results of the assays performed were observed when samples were stored at room temperature, 4°C or frozen, but it was detected that OTA decomposes if samples are applied trough the cartridge and stored — with the analyte adsorbed in the stationary phase — at 4°C. So, the samples were prepared at room temperature and conserved at 4°C.

Finally, the volume of eluent was also tested. Several fractions of acetonitrile were passed trough the cartridge to achieve the maximum recovery of OTA in the minimum volume of eluate. For the 3 μg l^{-1} level, four fractions of 0.5 ml were applied and collected in a specially graduated vial. These 2 ml were concentrated up to 0.4 ml and injected into the chromatograph. Fig. 1 shows the chromatograms that resulted from the injection of two of the fortified wines analysed under the conditions described.

For the 0.1 μg l^{-1} addition level, five fractions of 1 ml were applied trough the cartridge of 5 g. These fractions were collected and concentrated as described above. Fig. 2 shows an example of the chromatogram that resulted from the analysis of a red wine.

Once the procedures were optimised, the study of the repeatability was undertaken. The within-day and between-day precisions were studied for the 3 μg l^{-1} concentration level. Since OTA is more common in red than in white wines, the first study — within-day repeatability — was performed with five different red wines. Each sample was spiked with a known amount of OTA to reach a concentration within the range of the linear response (3 μg l^{-1}). Aliquots of

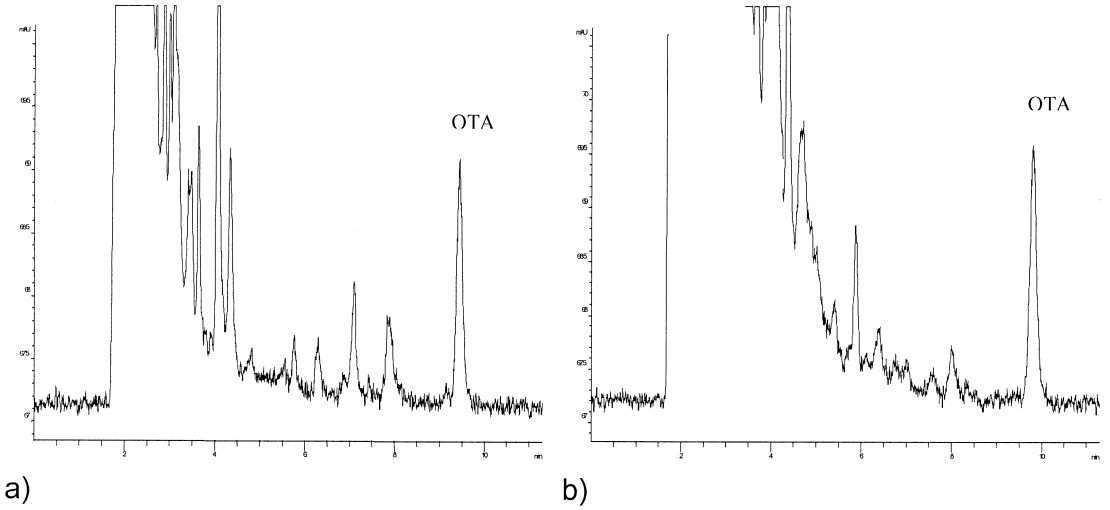

Fig. 1. Chromatograms showing the OTA peak after the treatment of (a) red wine and (b) white wine spiked at 3 μg l^{-1}.

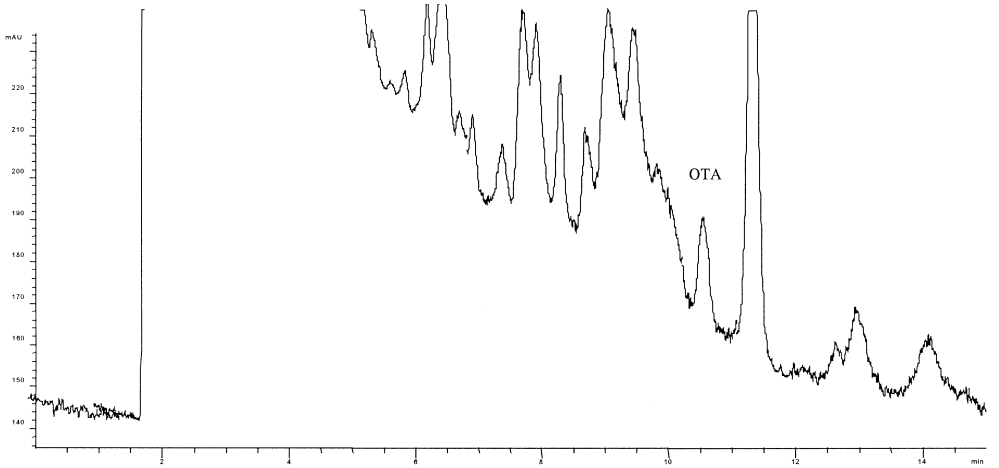

Fig. 2. Chromatograms showing the OTA peak after the treatment of a red wine spiked at 0.1 μg l^{-1}.

100 ml were analysed each day, by the same analyst, under the same conditions. The recoveries obtained are shown in Table 1. As it can be seen, the mean recovery is almost 90% and the relative standard deviations (RSDs) are satisfying (5%). Only one of the wines analysed showed recoveries lower than 80%. It was the only wine in which a precipitate appeared at the bottom of the vial where concentration was set up. It was observed that, when a solid appears during the preconcentration step, the recovery of OTA diminishes. This wine was the one with the highest content of polyphenolic substances.

Once within-day repeatability was known and the results showed that the method was quite simple and well-established, the study of between-day precision was performed. For this study, ten wines were used (five red, five white). Proceeding as in the previous study, each wine was spiked and subdivided in replicates of 100 ml which were analysed, in alternate days, for the same analyst under the same conditions. The recoveries obtained are shown in Table 2. As it can be seen, the recoveries were higher in red (mean=87.6%) than in white (mean= 83.2%) wines, although all the results are satisfying from the analytical point of view. The RSDs were also acceptable.

From the recoveries obtained in both studies, in which fifteen different wines were analysed, it can be assumed that solid-phase extraction, using C_{18} cartridges is a suitable procedure to determine the OTA content in wines.

The study of the within-day repeatability at a concentration level of 0.1 μg l^{-1} of OTA was considered, because several workers have found levels of OTA lower than 1 μg l^{-1} in wines. Five red wines, different from the ones analysed in the previous studies, were analysed using 5 g of C_{18} cartridges. Aliquots of each wine were analysed using the procedure described above. The results are shown in Table 3. As it can be seen, although more

Table 1
Within-day repeatability of the determination of OTA at 3 μg l^{-1} according to the procedure described in text

Red wine sample	Replicates						Recovery (%)	RSD (%)
	1	2	3	4	5	6		
1	94.5	85.3	97.4	95.6	97.9	96.8	94.5	4.9
2	76.4	76.2	78.2	74.8	76.1	68.5	75.1	4.5
3	93.9	92.5	92.5	100.3	98.3	99.1	96.1	3.7
4	91.9	80.4	87.2	87.0	80.3	84.8	85.3	5.2
5	96.7	86.8	94.4	88.2	89.3	85.2	90.1	5.0

Table 2
Between-day precision of the determination of OTA at 3 μg l^{-1} according to the procedure described in text

	Wine sample	Replicates						Recovery (%)	RSD (%)
		1	2	3	4	5	6		
Red wines	1	78.0	83.2	83.5	96.0	78.3	87.7	84.5	8.0
	2	74.1	88.0	81.9	92.9	79.3	84.7	83.5	7.9
	3	83.5	93.9	91.6	94.5	91.5	84.0	89.8	5.4
	4	95.7	93.9	93.8	93.4	94.2	88.8	93.3	2.5
	5	84.4	88.9	82.3	92.6	88.2	83.6	86.7	4.5
White wines	6	88.8	86.6	84.2	89.9	87.4	73.9	85.1	6.8
	7	81.1	88.6	76.2	82.6	77.5	78.4	80.8	5.6
	8	86.9	78.2	87.1	83.6	97.5	84.4	86.3	7.3
	9	83.2	78.2	79.3	82.7	83.8	86.0	82.2	3.6
	10	81.7	85.7	80.5	73.5	73.9	92.9	81.4	9.0

Table 3
Within-day repeatability of the determination of OTA at 0.1 μg l^{-1} according to the procedure described in text

Red wine sample	Replicates						Recovery (%)	RSD (%)
	1	2	3	4	5	6		
1	88.8	89.4	92.3	92.1	78.3	89.3	89.3	8.1
2	92.9	95.0	94.5	101.0	102.0	96.8	96.8	5.5
3	97.7	82.7	92.6	78.6	93.4	89.6	89.6	7.5
4	80.8	84.4	86.7	89.7	84.3	86.8	86.8	7.4
5	98.6	97.1	108.3	93.5	92.0	96.3	96.3	6.8

interferences appear at this concentration level, the repeatability is quite good (mean recovery=91.8%), so we can conclude that the method is adequate to reach concentrations under the 1 μg l^{-1} level.

4. Conclusions

The method proposed appears to be suitable for the determination of OTA in wines. It involves clean-up and concentration by SPE in order to determine the low OTA concentrations present in these samples. These are the main advantages over other existing methods that include very long isolation procedures, which normally comprise solvent extraction techniques. The recoveries are also good when compared to other analytical methods in which solvent extraction is used.

The method was successfully applied to the analysis of different wines. Solid-phase extraction seems to be an adequate alternative to the immunoaffinity columns and the solvent extraction techniques as it reduces the analytical time and provides good recovery for OTA in red and white wines. The method seems also be adequate for oenology laboratory because of the simplicity of the equipment employed.

Acknowledgements

The authors wish to thank CICYT (project ALI97-0765) for the financial support given.

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