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Short communication

Reversed-phase liquid chromatographic method for the determination of ochratoxin A in wine

C. Dall'Asta, G. Galaverna, A. Dossena, R. Marchelli*

Dipartimento di Chimica Organica e Industriale, Università degli Studi di Parma, Parco Area delle Scienze 17/A, I-43100 Parma, Italy

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Abstract

In this paper, we propose a new, rapid, highly sensitive and reproducible RP-HPLC-FLD method for the detection of ochratoxin A (OTA) in wine, by directly injecting the liquid in the chromatographic system without any extraction or clean-up. An alkaline mobile phase (NH_4Cl:CH_3CN 85:15 (v/v), 20 mM, pH 9.8) was used to obtain a distinct fluorescence enhancement. This improvement allows to reach, without an immunoaffinity clean-up or concentration, a detection limit of 0.05 ng/ml, which is similar to those commonly obtained after immunoaffinity purification and acidic elution. The method was statistically validated and directly applied to a series of wine samples.
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Keywords: Wine; Mycotoxins; Ochratoxin A

1. Introduction

Ochratoxin A (OTA) is a mycotoxin produced by *Penicillium verrucosum*, *Aspergillus ochraceus* and other related species, which occurs in cereals (mainly wheat, barley, maize and oats), beans, groundnuts, spices, dried fruits and coffee beans. Moreover, OTA has been detected in beverages such as beer, wine and grape juices [1–8].

Ochratoxin A has a nephrotoxic, hepatotoxic, immunosuppressive, teratogenic and carcinogenic activity towards several animal species and has been classified as a possible carcinogen to human (Group 2B) by the International Agency for Research on Cancer (IARC) [1–4].

Currently, EC members have established a 0.2 ng/ml limit for OTA in beer, whereas no threshold for wine has yet been assessed, although recent surveys have shown a considerable level of contamination with high toxin concentrations (up to 7.0 ng/ml) and incidence (up to 92%) in red wine samples from southern regions of Europe and from northern Africa [1–3,8,9].

OTA chemical properties are related to its structure: it is a derivative of isocoumarinic acid linked to L-phenylalanine, so it shows an optical activity and a natural fluorescence (Fig. 1).

OTA detection methods are generally based on RP-HPLC with fluorimetric detection using C18 columns and an acid eluent at pH 5 or at slight alkaline pH using the ion-pair technique [2,3].

Some studies have been performed using an ammonia solution as a post-column fluorescence enhancer [5], reaching a detection limit of 0.003 ng/ml based on a standard solution. The most commonly used methods involve clean up and concentration steps by means of immunoaffinity columns.

In the present work, we propose a new rapid and simple method to detect OTA which involves the direct injection of a wine sample in a HPLC-FLD apparatus by using an alkaline mobile phase without any previous clean up procedures.

2. Experimental

2.1. Reagents

The ochratoxin A standard solution (benzene–acetonitrile, 98:2 (v/v); 50 μg/ml) was obtained from SIGMA (St. Louis, MO, USA). All solvents used (LC grade) were obtained from Carlo Erba (Milan, Italy); bidistilled water was

* Corresponding author: Tel.: +39-0521-905410; fax: +39-0521-905472.
E-mail address: rosangela.marchelli@unipr.it (R. Marchelli).

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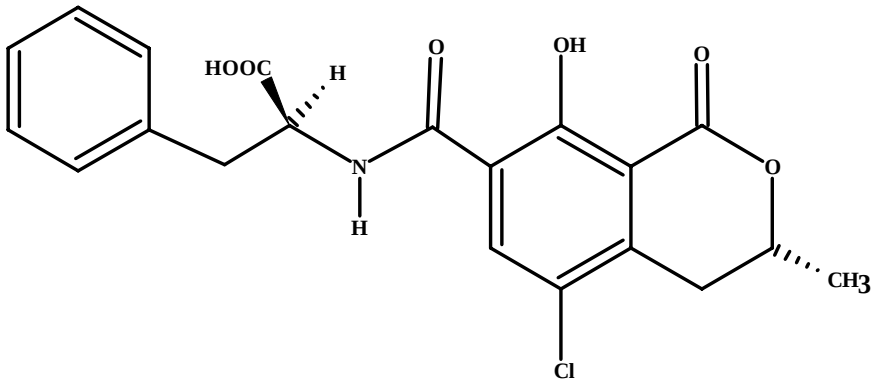

Fig. 1. Chemical structure of ochratoxin A and protonation constants (carboxylic group: pK_a = 4.8; phenolic moiety: pK_a = 7.1).

produced in our laboratory utilising an Alpha-Q system from Millipore (Marlborough, MA, USA). The (33%) ammonia solution was from Riedel-de Haen (Seelze, Germany). The immunoaffinity columns OCHRAPREP™ were obtained from Rhone Diagnostic (Glasgow, UK).

2.2. Spectroscopic measurements

UV-Vis spectra were recorded on a Perkin-Elmer Lambda Bio 20 instrument (Perkin-Elmer, Beaconsfield, England). Fluorescence spectra were recorded on a Perkin-Elmer LS 50 instrument; both excitation and emission slits were set at 15 nm. All measurements were performed in triplicate in a 1 cm × 1 cm optical length quartz cell. The working solutions of ochratoxin A (10^{-6} M) were prepared by evaporating to dryness the appropriate amount of the standard solution (50 ng/μl) and dissolving the residue in bidistilled water or in the appropriate buffer. The measurements were performed using NaH_2PO_4/NaOH 20 mM buffers at pH 6.5 and 7.5, NH_4Cl/NH_3 20 mM buffers at pH 8.5, 9.5 and 10.5.

2.3. HPLC analysis

Chromatographic analyses were performed with an Alliance 2695 chromatographic system (Waters, Milford, MA, USA) equipped and a Model 474 fluorescence detector (λ_{ex} = 333 and 460 nm when using an acidic mobile phase; λ_{ex} = 380 and 440 nm when using an alkaline mobile phase). A X-Terra™ C18 Waters column (250 mm × 4.6 mm, 5 μm particles) was used, on account of its ability to resist in a wide pH range (2–12).

The acetonitrile–ammonia buffer (NH_4Cl/NH_3, 20 mM, pH 9.8) eluent was 15:85 (v/v); the acetonitrile–water eluent was 50:50 (v/v) added of 1% acetic acid. The flow-rate was 1 ml/min.

The calibration curve was obtained by analysing OTA standard solutions at six concentration levels (range: 0.1–10 ng/ml; three determinations at each level have been performed). The significance of linear regression and intercept (not significantly different from 0) were calculated by SPSS 8.0 statistical software with the ANOVA model and the Student's *t*-test, respectively (regression: $Y = (42503 \pm 543)X$, $r^2 = 0.993$).

The LC-MS system consisted of a 2695 Alliance, a 996 Photodiode Array (PDA) detector and a ZMD single quadrupole mass spectrometer (all from Waters). An electrospray probe operating in negative mode was used for the analyses. The electrospray parameters, optimized for the OTA analysis, are the following: the capillary and the cone voltages were 2.0 kV and 20 V, respectively; the total ion current scan range was 300–500 Da with an interscan delay of 4 s. The chromatographic conditions involved a microbore X-Terra™ C18 Waters column (150 mm × 1 mm, 3 μm particles) and an acetonitrile–ammonia buffer (CH_3COONH_4/NH_3, 20 mM, pH 9.8) solution (15:85 (v/v)) as mobile phase. The flow was set at 0.1 ml/min.

3. Results and discussion

3.1. Spectroscopic experiments and chromatographic studies

In order to develop a RP-HPLC method at the optimum fluorescence emission of OTA, we performed spectroscopic measurements at different pH values, using appropriate buffers from pH 6.5 to 10.5, the pK_a of the carboxylic group being 4.4 and the pK_a of the phenolic moiety being 7.1. The fluorescence enhancements of the OTA emission spectra performed at different pH values are shown in Fig. 2.

As shown, going from neutral to alkaline solutions a 10-fold fluorescence enhancement is obtained; moreover, the increased OTA conjugation of the phenate ring in alkaline solution causes a red shift of the absorbtion spectra (λ_{abs} from 333 to 380 nm) and a blue shift of the emission spectra (λ_{em} from 450 to 440 nm).

Systematic studies were carried out in order to establish the chromatographic conditions compatible with the variation of the mobile phase pH value. The classical

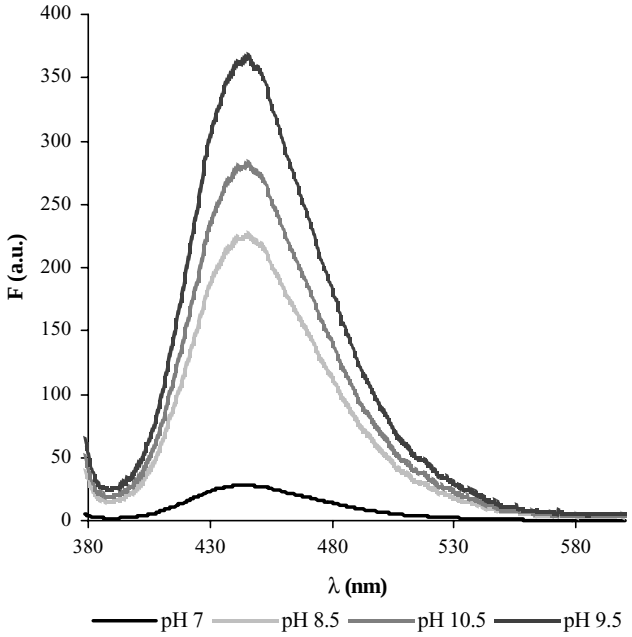

Fig. 2. OTA fluorescence enhancements at different pH values.

mobile phase CH_3CN–H_2O 1:1 (v/v), acidified with 1% CH_3COOH, was compared with the new one CH_3CN–ammonia buffer (NH_4Cl/NH_3 20 mM, pH 9.8). These studies show that the use of an alkaline mobile phase in HPLC determination allows an 10-fold fluorescence enhancement. The retention time of OTA was longer (Rt = 18 min) than with the previous method at lower pH (Rt = 9 min), but generally stable to slight changes in the composition of the eluent. The OTA calibration curve is linear in the range studied (10–0.05 ng/ml), showing correlation coefficients ≥0.99. The inter-day precision of the method was proved by five repeated injections of OTA solutions at three concentration levels: the relative standard deviations were 3.3% for 5 ng/ml, 4.7% for 1 ng/ml and 5.9% for 0.2 ng/ml.

3.2. Application to wine samples

The direct injection method here proposed was applied to the analysis of several samples of wine and compared with the Visconti's method (IAC clean-up phase) [10,11].

Wine samples (2 ml) were filtered on a 0.45 μm filters and directly injected in the HPLC system (5 μl). The OTA peak was well separated from interfering peaks in less then 25 min, as shown in Fig. 3a and b.

Recovery experiments were performed on OTA free samples (three determinations at each concentration level have been performed). With our method, within the spiking range of 0.2–10.0 ng/ml, the mean recovery for OTA of 96 ± 1, 95 ± 5 and 93 ± 6% was obtained, while the average R.S.D. was 3%. No matrix effect was observed. The limit of detection (S/N = 3:1) was 0.05 ng/ml (5 μl injected), the same

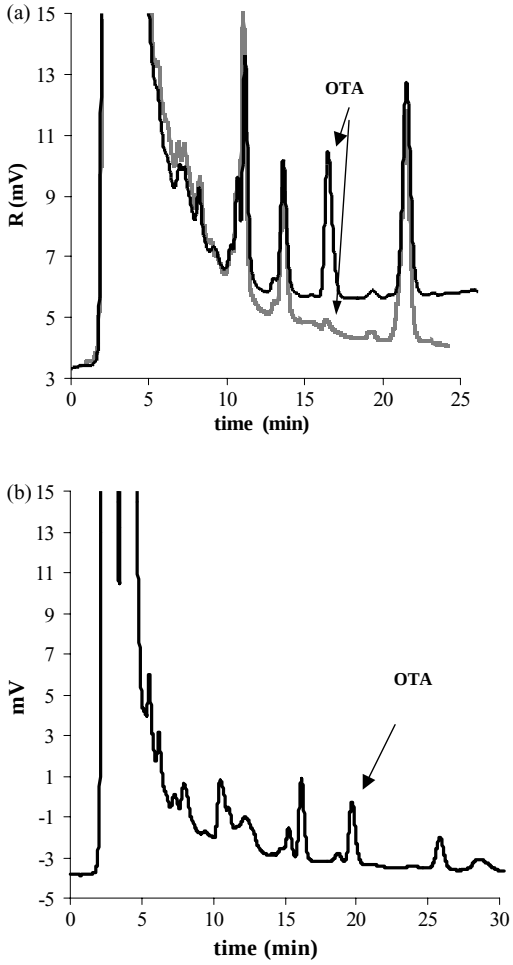

Fig. 3. (a) Chromatograms of a natural wine sample (grey line) and of a spiked wine sample (black line), (b) chromatogram of a naturally contaminated commercial red wine.

order of magnitude as the LC-MS method and one order higher than the IAC-concentrated samples. Thus, the present method is very easy, less time-consuming, reproducible, very cheap and it does not requires clean up or concentration procedures.

The OTA identification by LC-ESI-MS involved the analysis of a spiked wine sample (2 ng/ml of OTA) using an alkaline mobile phase similar to that used for the HPLC-FLD analysis: ammonium chloride was substituted with the more volatile ammonium acetate. In order to achieve the sensitivity necessary to detect OTA at the ng/ml level, a microbore C18 column (1 mm × 150 mm, 3 μm) was used. The analysis was performed using the negative ionization mode and the extracted ion technique: the m/z = 402 and 358 fragments, corresponding to the $[M - H]^-$ and the $[M - H–CO_2]^-$ species, respectively, were monitored. The LC-MS chromatogram of a spiked wine sample (2 ng/ml of OTA) is reported in Fig. 4.

The direct injection method was applied to 82 commercial wines from Italy, as a rapid screening for detection of OTA. In agreement with the literature, white (14 samples)

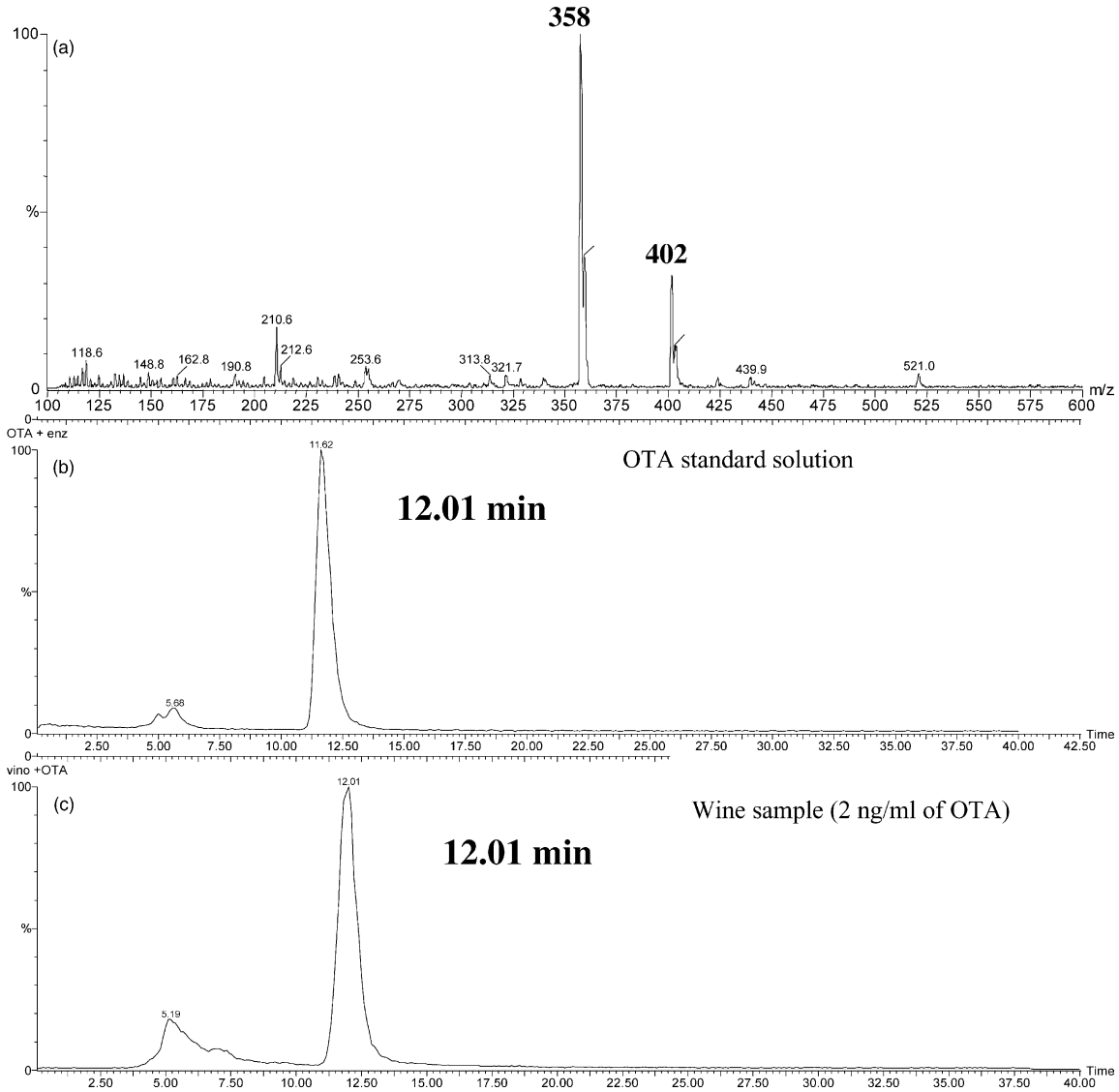

Fig. 4. The LC-ESI-MS analysis of OTA in a naturally contaminated red wine (2 ng/ml): mass spectrum of the peak at Rt = 12.01 min (a); comparison between the single ion recording (SIR) chromatograms of the $[M - H]^- = 402$ and $[M - H–CO_2]^- = 358$ fragments of an OTA standard solution (b) and the contaminated wine sample (c).

and roseé wines (4 samples) are generally not contaminated, whereas the liqueur-like (4 samples) and the red ones (60 samples) often contain OTA: the 50% of the red wines turned out to be contaminated (>0.2 ng/ml) and in the 32% the contamination level was quite high (>0.5 ng/ml).

4. Conclusions

In conclusion, the 10-fold OTA fluorescence enhancement obtained by using an alkaline eluent allows the detection of OTA by direct injection of wine samples at the same level as the LC-MS analysis (0.05 ng/ml). Thus, the present method is very easy, less time-consuming, reproducible, very cheap and it gives the same detection limit as the much more costly LC-MS. Therefore, the method here proposed is very useful not only for the rapid determination of OTA at the probable EC legal limits, but also, if combined with immunoaffinity clean up procedures, may furtherly lower the LOD of the commonly used procedure.

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