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Comparison of different immunoaffinity clean-up procedures for high-performance liquid chromatographic analysis of ochratoxin A in wines

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Abstract

Three immunoaffinity clean-up procedures to analyse ochratoxin A (OTA) in wines were compared. The direct wine clean-up with Ochraprep and OchraTest columns gave equivalent results in terms of recovery and precision if compared with the reference procedure involving a preliminary extraction of OTA with chloroform. OTA quantification limit in wine ranged from 0.020 to 0.045 μg/l. The 'on-flow' OTA emission spectrum (excitation 333 nm) showed a maximum at 460 nm and could be used to confirm the quantitative results. The analysis of 11 red and white wines gave no significant quantitative differences between the three clean-up techniques.

Keywords: Wine; Food analysis; Sample preparation; Immunoaffinity precolumns; Ochratoxin A; Toxins

1. Introduction

Ochratoxin A (OTA) has been known as a toxic metabolite produced by several fungi and moulds as *Aspergillus ochraceus* and *Penicillium verrucosum* and related species [1]. OTA is a potent carcinogen and nephrotoxin of humans and animals and has been found in human blood serum and in a wide range of commodities, including cereals, coffee, dried fruits, meat, dairy products, nuts and beer [2–11].

HPLC with fluorimetric detection (FLD) has become the most popular method for OTA quantification in food and beverages. Chromatographic separations have been normally performed using RP-C_{18} columns and isocratic elution with diluted acidified acetonitrile [12]. However ion-pair chromatography, HPLC–tandem MS, time resolved luminescence or electrophoresis with laser induced fluorescence have been also proposed [13–17]. Immunochemical enzyme-linked immunosorbent assay (ELISA) methods were attractive [18,19], but sometimes caused systematic overestimates if compared to chromatographic methods [20].

Clean-up and concentration of the sample appeared generally necessary, when low detection limit was required [12]. Classic methods were liquid–liquid partition and solid-phase extraction (SPE), but sometimes the cleaning effect was inadequate for the complexity of the matrices. The immunoaffinity columns (IACs) specifically studied for OTA have simplified the clean-up methodology [21]. The main

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advantage of these columns seemed to be that OTA is bound specifically to the antibody and the matrix interference can be removed nearly completely. Many authors proposed their use to detect OTA in foods, beverages, human blood or serum [6,9–12,20–23].

More recently OTA has been also detected in wines [11,24–27] and wine was indicated as one of the possible source of OTA in human plasma [10].

In 1998 the Scientific Committee on Food of the European Union considered it would be prudent to reduce the tolerable daily OTA intakes below 5 ng/kg body mass (see [27]). The large wine consumption in Europe moved many researchers to develop simple, reliable and sensitive methods for monitoring the level of OTA occurrence in enological products.

Ospital et al. [28] obtained satisfactory results in terms of recovery and sensibility operating a sample clean-up with silica gel SPE cartridges. Zimmerli and Dick [11,26] proposed to extract OTA from acidified wines by chloroform and then to apply the residue to an IAC for a further purification.

It should be underlined that chloroform extraction after acidification was considered in many cases fundamental, because OTA binds to protein [12] and some food samples cannot be directly applied to an IAC. Nevertheless there is a trend towards minimising the amounts of halogenated and toxic solvents for environmental and sanitary reasons. Moreover a suitable clean-up procedure allowing the application of the sample directly onto the IACs could reduce the time of analysis improving the possibility of automation. Some authors [22,27] proposed the analysis of OTA passing directly diluted samples of beer and wine through the IAC, with apparently good results in terms of recovery and reproducibility.

In this study we compared the direct clean-up of wine sample on two different IAC columns with the procedure proposed by Zimmerli and Dick [11,26] involving a chloroform extraction and then the use of IACs. Recovery, repeatability, limit of detection, limit of quantification and comparison between results were in particular considered.

As the main aim of this study was to develop a rapid HPLC-FLD method to quantify OTA in wines, the use of 'on-flow' emission spectra was further evaluated as a tool to enhance the quantitative confirmation.

2. Experimental

2.1. Sample

A total of 11 red and white wines containing OTA were purchased in retail stores. Information about the origin of the commercial samples (Italy, France, Spain, USA) were taken from the bottle labels. Bottles were opened, kept sealed in the refrigerator (4°C) and analysed within a few days. The comparison between different clean-up procedures was carried out analysing each sample at the same time.

2.2. Reagents and standard

Phosphate-buffered saline (PBS) was prepared adding potassium chloride (0.2 g), potassium dihydrogenphosphate (0.2 g), anhydrous disodium hydrogenphosphate (1.16 g) and sodium chloride (8.0 g) to 900 ml of distilled water. Then pH was adjusted to 7.4 and the solution made up to 1 l. All buffer salts were purchased from Carlo Erba (Milan, Italy). Acetonitrile, methanol, ethanol, water and chloroform HPLC-grade were furnished by Sigma–Aldrich (Milan, Italy).

A stock solution of OTA (1000 μg/l) dissolved in benzene–acetic acid (99:1, v/v) was furnished by Rhône Diagnostics Technologies (Glasgow, UK). The purity of this standard was checked by UV at 333 nm in benzene–acetic acid (99:1), considering a molar absorption coefficient (ε) of 5550 M^{-1} cm^{-1} [12]. The working standards (ranging from 0.05 to 20 μg/l) were prepared by evaporating under nitrogen the stock solution and dissolving the residue in an appropriate volume of mobile phase.

Two brands of commercial IACs, previously compared by other authors [23], i.e. Ochraprep (Rhône Diagnostics Technologies) and OchraTest (Vicam Science Technology, MA, USA) were used.

2.3. Direct clean-up on IAC

Working procedures for direct IAC clean-up procedures were optimised on the basis of the supplier

technical notes and taking into account the specific column capacity.

Each Ochraprep column was at first washed with 5 ml of PBS before use. Then 10 ml of wine adjusted to pH 7.8 using 2 *M* NaOH were diluted with 10 ml of PBS and directly applied with an adapter to the IAC, at a flow-rate of about 1–2 drops/s. Column was successively washed with 10 ml of PBS, 10 ml of HPLC-grade water at a flow-rate of 3–4 drops/s and then dried with air. Operating with the OchraTest columns the procedure was the same. In this case only 4 ml of wine adjusted to pH 7.8 using 2 *M* NaOH were diluted with 10 ml of PBS and directly applied with an adapter to the IAC. OTA was eluted by passing 2 ml HPLC-grade methanol through IAC at a flow-rate of 1 drop/s. Eluate was collected, and thoroughly mixed with 2 ml of mobile phase prior to HPLC analysis.

2.4. Clean-up with chloroform extraction

This procedure was strictly derived from that proposed by Zimmerli and Dick [11]. A sample of 5 ml of wine was mixed with 10 ml of saline acidic solution (H_3PO_4+NaCl), and then added with 5 ml of chloroform. The sample was intensively mixed for 2 min, centrifuged (4°C, 2500×*g*, 5 min) and then the clear organic phase collected. The extraction was repeated five times, each time with 5 ml of chloroform. The chloroform extracts were reunified, evaporated under nitrogen and the residue dissolved in 10 ml of PBS solution containing 10% (v/v) ethanol.

The extract was then passed through the Ochraprep column, which had been previously washed with 5 ml of PBS solution. The column was successively washed with 10 ml of PBS, 10 ml of HPLC grade water at a flow-rate of 3–4 drops/s and then dried with air. OTA was eluted by passing 2 ml HPLC-grade methanol through the IAC at a flow-rate of 1 drop/s. Eluate was collected, and thoroughly mixed with 2 ml of mobile phase prior to HPLC analysis.

2.5. HPLC analysis

The chromatographic system consisted of a PU-980 pump and a FP-1520 fluorescence detector (Jasco International, Tokyo, Japan). A Inertsil ODS-2 (GL Science, Japan) column (5 μm packing, 250× 4.0 mm I.D.) was used. The column was protected by an inline C_{18} Security Guard (5 μm, 4.0×3 mm I.D.) cartridge system (Phenomenex, Torrance, CA, USA).

The column was kept at 35°C using a heater 7980 (Jones Chromatography, Hengeod, UK). Chromatographic separation was performed using an isocratic elution (water–acetonitrile–acetic acid, 49:49:2, v/v/v) at a flow-rate of 0.75 ml/min. Eluent was freshly prepared and filtered (0.22 μm) before use. Sample injection was made with a 7725 valve (Rheodyne, Rohnert Park, CA, USA) equipped with a 100 μl loop. Detection was made working at an excitation wavelength of 333 nm and an emission wavelength of 460 nm. 'On-flow' emission spectra of OTA were taken both for the standard solution and for wine samples, using the scan option of the fluorescence detector. Data acquisition and handling were made with Borwin 1.5 software (JMBS Developpements, Le Fontanil, France) and a personal computer. For the quantitative analysis a calibration curve was created by injecting seven solutions containing known amounts of the pure standard ranging from 0.05 to 20 μg/l of OTA.

3. Results and discussion

Fig. 1 shows the chromatogram of a standard solution of OTA at a concentration of 0.025 μg/l (signal/noise, $S/N=5$). The average baseline noise (35 μV) was calculated from three injections of the standard solution, measuring a 1 min range around the OTA retention time. Under our conditions the absolute limit of detection was estimated at approximately 0.015 μg/l ($S/N=3$) corresponding to 0.0015 ng OTA.

Considering the response of reagent blanks (Fig. 2a and b) and the concentration factors of the sample, the limit of quantification (LQ) for OTA in wine ($S/N=9$) was estimated to be about 0.020, 0.045 and 0.040 μg/l for direct clean-up with Ochraprep or OchraTest and chloroform extraction with subsequent clean-up with Ochraprep, respectively. These results may be considered satisfactory, considering that IACs eluates containing OTA were not evaporated to dryness and no post-column addi-

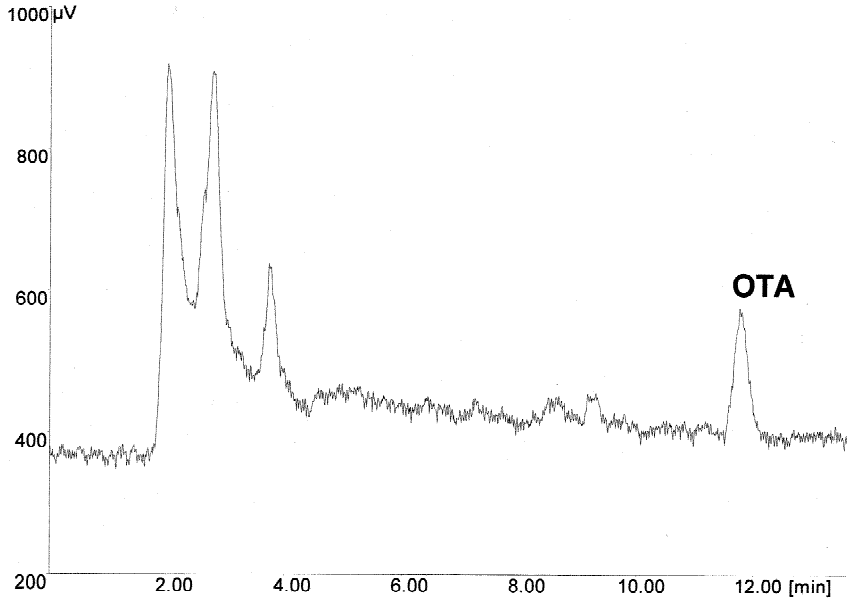

Fig. 1. Chromatogram of a standard solution containing 0.025 μg/l of OTA (100 μl of solution injected, corresponding to 0.0025 ng of OTA).

tion of ammonia was made [11] to improve sensitivity.

The different LQ between the two brands of IACs was the consequence of the loading sample adjustment in function of the specific column capacity. A decrease of recovery was observed when higher sample volumes where loaded onto the columns, especially when wines containing high levels of phenolics were analysed.

The average retention time for OTA (11.77 min–relative standard deviation RSD=1.2%; within 3 days, five injections a day) was significantly higher than those reported by other authors working on wine extracts [11,21,26,27]. The eluent composition and/or the high carbon load of our stationary phase may explain this result. On the other hand the increase in retention time contributed to enhance the chromatographic resolution of OTA peak from other interfering compounds.

Moreover the use of a ultra purified silica stationary phase ensured a limited peak asymmetry (1.13).

Recovery performance was obtained using six different red and white wines (naturally containing 0.67±4.12 μg/l OTA) spiked at 2.5 μg/l. The recovery averaged 90.2% (RSD 4.2%, n=6), 86.9% (RSD 5.4%, n=6), 88.9% (RSD 7.2%, n=6) using the direct clean-up with Ochraprep or OchraTest and with chloroform extraction plus Ochraprep, respectively. The values obtained with the chloroform extraction are in good accordance with those obtained by Zimmerli and Dick [26] on wine samples, while both the two direct clean-up procedures gave comparable results.

Visconti et al. [27] observed lower OTA recovery performance using PBS, but they operated under non-comparable conditions (i.e. the dilution rate of wine in buffer).

The dilution pH value seemed to influence the OTA recovery [27]. We also obtained similar indications; that is why our procedures were optimised to ensure a precise pH control in the diluted sample (values ranging from 7.4 to 7.8). This may account for the differences in recovery performances.

Precision, expressed as RSD, of 2.0% (Ochraprep), 2.5% (OchraTest) and 3.1% (chloroform extraction plus Ochraprep) were obtained on repeated analysis (n=5) of a red wine sample naturally containing 2.28 μg/l OTA.

The emission wavelength of the fluorescence detector was optimised considering the ‘on-flow’

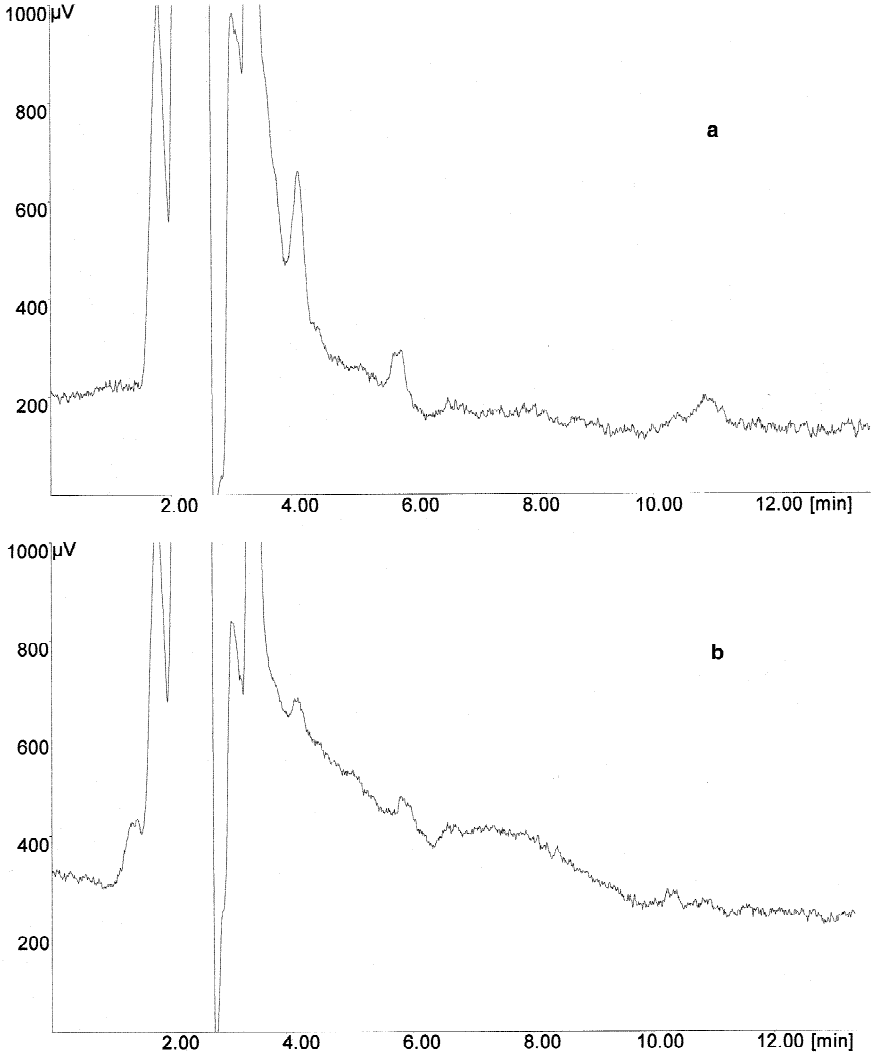

Fig. 2. Chromatograms of reagent blanks after a clean-up with Ochraprep (a) and OchraTest (b) IACs, respectively.

emission spectrum of a standard solution. With an excitation wavelength of 333 nm the emission spectrum exhibited a maximum at 460 nm. Fig. 3 shows the OTA emission spectra corresponding to a standard solution (a) and to a sample of red wine naturally contaminated with OTA (b).

Some authors have underlined the problems connected to the OTA confirmation via its methyl or ethyl esters for the occurrence of natural ethyl ester in wine and the difficulty to obtain an acceptable blank value [11,26]. The use of FLD excitation spectra was also proposed [12]. We have observed that the use of 'on-flow' emission spectra seems to be a further interesting tool to improve and speed up the OTA confirmation. Under our conditions significant peak emission spectra could be acquired until an approximate concentration of OTA in wine sample ranging from 0.200 to 0.450 μg/l (LQ×10). For lower levels of OTA, spectra quality could be improved operating a concentration step of the sample.

Fig. 4 provides chromatograms of the same naturally contaminated (4.6 μg/l OTA) wine sample, injected after a direct clean-up on Ochraprep (a) or

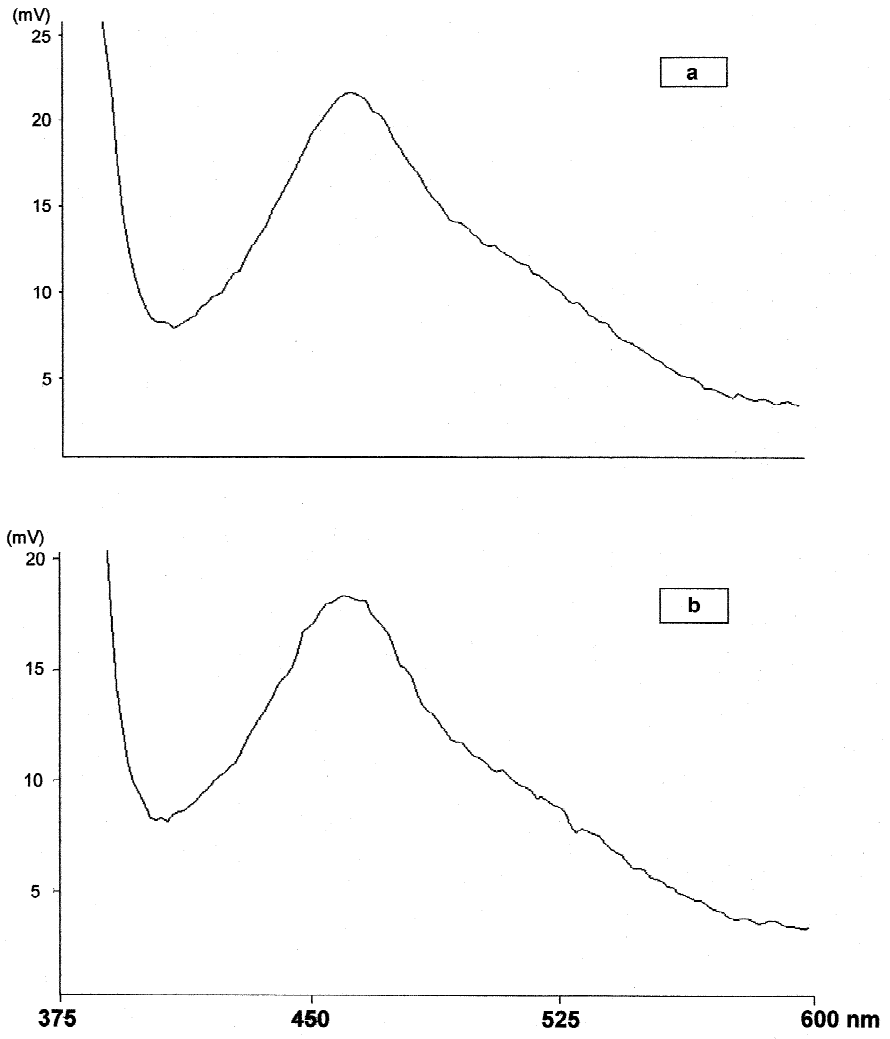

Fig. 3. OTA emission spectra from 375 to 600 nm (excitation 333 nm) for a standard solution (a) (0.25 ng OTA) and a red wine extract (b) (0.21 ng OTA).

OchraTest (b) and with a previous chloroform extraction (c). Each procedure gave chromatograms essentially free of interference, confirming the high specificity of the direct clean-up on IACs at least at the LQ level.

Table 1 reports the results of analysis with the three clean-up procedures for 11 wines naturally contaminated with OTA. The *t*-paired tests indicated no significant difference between the direct clean-up procedures vs. the pre-extraction with chloroform.

4. Conclusions

The direct wine clean-up with two commercial IAC columns for OTA analysis gave comparable results in terms of recovery and precision to those obtained with the reference procedure involving a previous extraction with chloroform. The direct clean-up showed satisfactory limit of quantification for OTA in wine even if the sample and the methanol extracts were minimally manipulated (i.e.

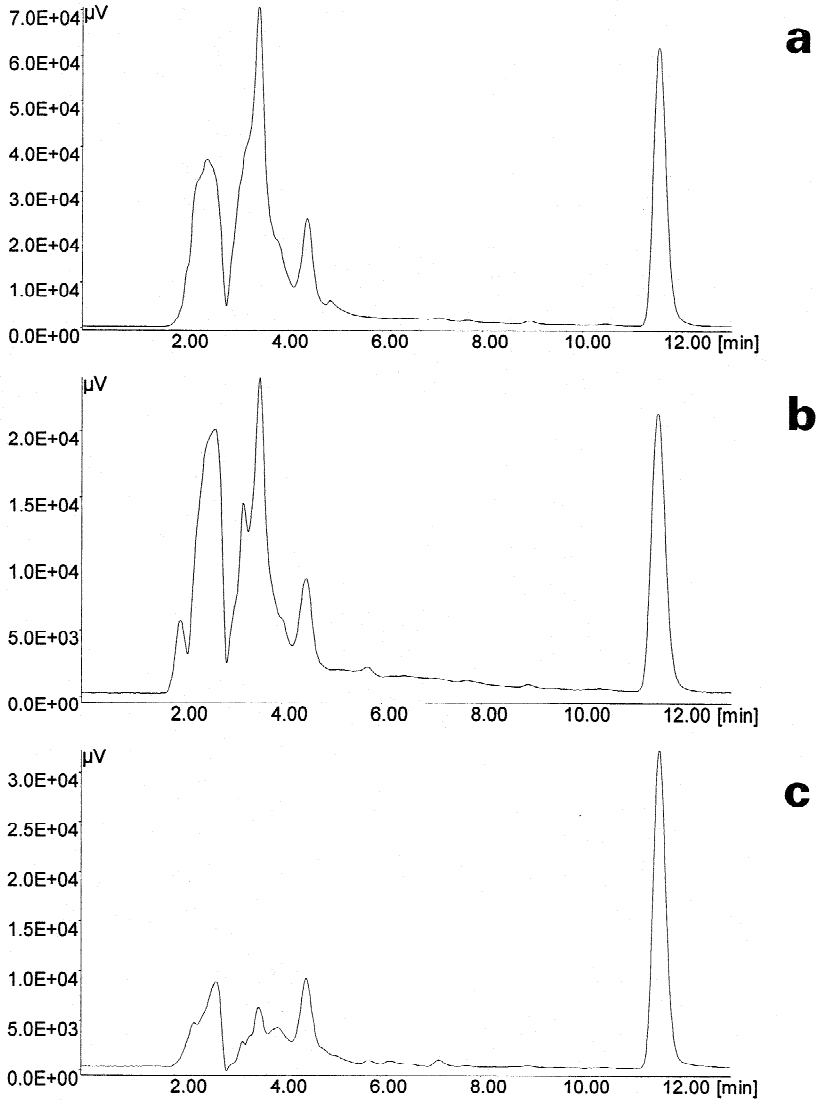

Fig. 4. Chromatograms of a red wine naturally contaminated (4.6 μg/l OTA) purified with a direct clean-up on Ochraprep (a) or OchraTest (b) and with a previous chloroform extraction (c).

no use of chloroform to extract OTA from the wine sample, no concentration steps to improve the detection limit).

On the other hand repeatability was improved and the time of analysis was reduced if compared with the reference procedure. The study of the 'on-flow' emission spectrum of OTA may be useful to confirm the results. The proposed direct clean-up procedures appeared suitable to improve the degree of automation for the determination of OTA in wine samples.

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Table 1
Results of analysis for 11 wine samples treated with the three different clean-up procedures

Wine			Direct clean-up		Pre-extraction with $CHCl_3$:
Sample	Origin	Colour	Ochraprep, OTA (μg/l)	Ochraprep, OTA (μg/l)	Ochraprep, OTA (μg/l)
1	Italy	Red	0.06	0.06	0.03
2	Italy	Red	3.53	3.49	3.65
3	Italy	White	0.12	0.15	0.10
4	France	White	0.11	0.14	0.10
5	Italy	Red	3.05	3.26	3.35
6	Spain	Red	0.74	0.74	0.55
7	Italy	Red	5.00	4.65	4.90
8	Italy	Red	0.15	0.15	0.18
9	Italy	Red	2.37	2.27	2.22
10	Italy	Red	4.01	4.24	4.14
11	USA	Red	2.14	2.09	2.08

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