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# Determination of ochratoxin A in wine by means of immunoaffinity column clean-up and high-performance liquid chromatography

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**Abstract**

A new and accurate method to quantify ochratoxin A (OA) in table wine has been developed. The method uses commercial immunoaffinity columns for clean-up and high-performance liquid chromatography (HPLC) with fluorescence detection for quantification of the toxin. Wine was diluted with a solution containing 1% polyethylene glycol (PEG 8000) and 5% sodium hydrogencarbonate, filtered and applied to an OchraTest immunoaffinity column. The column was washed with a solution containing sodium chloride (2.5%) and sodium hydrogencarbonate (0.5%) followed by water. OA was eluted with methanol and quantified by reversed-phase HPLC with fluorometric detection (excitation wavelength 333 nm, emission wavelength 460 nm) using acetonitrile–water–acetic acid (99:99:2) as mobile phase. Average recoveries of OA from white, rosé and red wine samples spiked at levels from 0.04 to 10 ng/ml ranged from 88% to 103%, with relative standard deviations (RSDs) between 0.2 and 9.7%. Detection limit was 0.01 ng/ml based on a signal-to-noise ratio of 3:1. The method was applied successfully to 56 samples of red (38), rosé (8), white (9) and dessert (1) wine. The levels of OA ranged from $<0.01$ to 7.6 ng/ml with red wines more contaminated than rosé and white wines. A good correlation ($r=0.987$) was found by comparative analysis of 20 naturally contaminated samples using this method and the method of Zimmerli and Dick with better recoveries of OA and better performances for the new method. Several advantages of this method with respect to the actually available methods have been pointed out, with particular reference to red wine which appears to be the most difficult to analyze. 

*Keywords:* Wine; Food analysis; Ochratoxin A; Mycotoxins

## 1. Introduction

Ochratoxin A (OA), 7-(L-β-phenylalanyl-carbonyl) - carboxyl-5-chloro-8-hydroxy-3,4-dihydro-3*R*-methylisocumarin, is a widely distributed mycotoxin produced mainly by *Aspergillus ochraceus* and *Penicillium verrucosum* under diverse environmental conditions. OA occurs in various plant products such as cereals (mainly wheat, barley, maize and oats), beans, groundnuts, spices, dried fruits, coffee, milk, beer and wine, as well as in pig blood and kidney [1–6]. OA 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 [1,7]. The IARC (International Agency for Research on Cancer) has classified OA as a possible carcinogen to humans (Group 2B) [1]. OA is suspected to be involved in

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the Balkan Endemic Nephropathy (BEN), a fatal kidney disease occurring in some areas of south-eastern Europe (Bosnia, Serbia, Croatia, Bulgaria and Romania) and to be associated with urinary tract tumors [8]. Human exposure occurs mainly through the consumption of contaminated products and the toxin is frequently found in human blood due to the long elimination half-life (about 35 days in serum) [9]. OA has been found at relatively low levels in human blood collected in different countries from healthy individuals as well as from individuals suffering from different kidney diseases with 100% of positive samples in Italy and Switzerland [10,11]. High levels of the toxin have been found in human blood of nephropathic people from Tunisia [12] and of individuals suffering from BEN and/or urothelial urinary tract tumors in the Balkan area [13].

In recent years, scientific co-operations have been established by International Organizations in order to provide data on toxicity of OA and dietary exposure to the toxin. The Joint FAO/WHO Expert Committee on Food Additives (JECFA), after evaluation of the nephrotoxicity of OA, proposed for this mycotoxin a provisional tolerable weekly intake (PTWI) of 0.1 μg/kg body mass (equivalent to 14 ng/kg body mass/day) [7]. Based on carcinogenicity data, a working group of the Nordic Council of Ministers has proposed a maximum tolerable daily intake of toxin of 5 ng/kg body mass [14], similar to the provisional tolerable daily intake (PTDI) from 1.2 to 5.7 ng/kg body mass established by the Canadian authority [15]. In 1994, 13 European countries took part in a scientific collaboration (SCOOP) project in order to estimate OA intake in the European Union. Based on the SCOOP data, the Scientific Committee for Food of the European Commission established that it is prudent to reduce exposure to OA as much as possible, ensuring that exposures are ''towards the lower end of the range of tolerable daily intakes of 1.2–14 ng/kg body mass estimated by other bodies, e.g., below 5 ng/kg body mass'' [16].

Currently, nine countries have specific regulations for OA in one or more commodities at levels ranging from 1 to 50 μg/kg for foods and from 5 to 300 μg/kg for animal feeds [17], and no tolerance levels for OA in wine have been assessed. Recent surveys on the occurrence of OA in wine have showed a considerable level of contamination with higher toxin concentrations (up to 7.0 ng/ml) and incidence (up to 92%) in red wine samples originating from southern and warmer regions of Europe and from northern Africa [18–21]. Wine is a product widely consumed by adult individuals in both developed and developing countries and, due to its high frequency of contamination with OA, it may represents, after cereals, a major source of daily OA intake for these populations. Provisional estimates of Codex Alimentarius Commission, based on limited data, suggest that 15% of the total intake of this toxin is due to wine [22]. The availability of reliable methods for the determination of OA in wine is therefore highly desirable in order to fulfill the need to protect consumer health from the risk of exposure to the toxin. Rapid and accurate methods are necessary to ensure that the distributed wine products are safe and to allow public laboratories with national or regional responsibility for food quality control, wine producers, importers and exporters to analyze the highest number of samples in the shortest time.

During the past few years, the use of antibody-based immunoaffinity columns in the clean-up has given a strong impulse to the improvement of mycotoxin analysis [23]. The use of immunoaffinity chromatography in the clean-up step provides a number of advantages as compared to other methods, which include: (i) provision of clean extracts due to the specificity of the antibodies for the single toxin or a group of related toxins that can be easily separated by high-performance liquid chromatography (HPLC); (ii) optimal performances in terms of precision and accuracy within a wide range of concentrations which cover the field of practical interest; (iii) rapidity and, finally, (iv) a noticeable reduction of the use of dangerous solvents, highly desirable and necessary for the environmental protection.

Few methods have been proposed for the determination of OA in wine [18–20,24]. All these methods require the use of dangerous extraction solvent (aromatic or chlorinated solvents) and time consuming sample preparation procedures. With the exception of the method described by Ospital et al. [24] using silica gel cartridge, all of them use antibody-based immunoaffinity columns clean-up combined with HPLC. A time-saving sample prepa-

ration procedure has been recently reported by Ueno [21], but in our hands the method did not provide reliable results when applied to red wine (see below).

This paper describes a new, simple and accurate method for the determination of OA in wine (red, rosé and white wine) at ppt levels using commercially available immunoaffinity columns in the clean-up step combined with HPLC–fluorescence detection. The application of the method to naturally contaminated samples and the comparison with other published methods are reported.

## 2. Experimental

### 2.1. Chemicals and materials

OA ($M_r$ 403.1) stock solution (1.0 mg/ml) was prepared by dissolving in toluene–acetic acid (99:1, v/v) the solid standard purchased from Sigma–Aldrich (Milan, Italy). OA standard solutions for HPLC calibration or spiking purposes were prepared by dissolving in the mobile phase, adequate amounts of the stock solution, previously evaporated to dryness under nitrogen stream. Acetonitrile, methanol and water (HPLC grade) and glacial acetic acid were purchased from Mallinckrodt Baker (Milan, Italy). Sodium chloride (ACS), polyethylene glycol (PEG 8000), and sodium hydrogencarbonate (ACS) were obtained from Sigma–Aldrich. OchraTest immunoaffinity columns were obtained from Vicam (Watertown, MA, USA). Glass microfiber filters (Whatman GF/A) were obtained from Whatman (Maidstone, UK).

### 2.2. Wine samples

Wines were purchased from local retails (bottled wine) or kindly provided by small farmers producing wine mainly for their family needs (home-made wine). A total of 56 samples were analyzed: red (38), rosé (8), white (9) and dessert (1) wines.

### 2.3. Apparatus

The HPLC apparatus consisted of a LKB 2150 isocratic pump (LKB, Bromma, Sweden) equipped with a Rheodyne Model 7125 injection valve (Rheodyne, Cotati, CA, USA), a Perkin-Elmer LC 240 fluorometric detector ($\lambda_{ex}$=333 nm, $\lambda_{em}$=460 nm) and a Turbochrom 4.0 data system (Perkin-Elmer, Norwalk, CT, USA). The analytical column was a reversed-phase Discovery $C_{18}$ (15 cm×4.6 mm, 5 μm particles) (Supelco, Bellefonte, PA, USA) preceded by a Rheodyne guard filter (0.5 μm).

### 2.4. Sample preparation and immunoaffinity clean-up

A 10-ml volume of wine sample was diluted with 10 ml water solution containing PEG (1%) and $NaHCO_3$ (5%), mixed and filtered through Whatman GF/A glass microfibre filter (filtration was necessary for cloudy solutions or when solid residue was formed after dilution, while degassing was required for sparkling wines prior to dilution). A 10-ml volume of diluted extract (equivalent to 5 ml wine) was cleaned up through an OchraTest (Vicam) immunoaffinity column at a flow-rate of about 1 drop per second. The column was washed with 5 ml solution containing NaCl (2.5%) and $NaHCO_3$ (0.5%) followed by 5 ml distilled water at a flow-rate of 1–2 drops per second. OA was eluted with 2 ml methanol and collected in a silanized clean vial (Kimble Glass, USA) (the use of silanized vials is however not essential). The eluted extract was evaporated under nitrogen stream at ca. 50°C and reconstituted with 250 μl of the HPLC mobile phase.

### 2.5. HPLC determination and confirmation of ochratoxin A

A 100-μl volume of reconstituted extract (equivalent to 2 ml wine) was injected into the chromatographic apparatus by full loop injection system. The mobile phase consisted of a mixture of acetonitrile–water–acetic acid (99:99:2) eluted at a flow-rate of 1.0 ml/min. Quantification of OA was performed by measuring peak areas at OA retention time and comparing them with the relevant calibration curve. The identity of OA was confirmed in 10 selected positive samples (2 white, 2 rosé and 6 red wines) by methyl ester formation after derivatization of the extracts with 14% $BF_3$ in methanol as described by Nesheim et al. [25]. In addition, the identity of OA was confirmed in the most contaminated sample

(containing 7.6 ng/ml OA) by HPLC–mass spectrometry (MS) using a 1050-Ti chromatographic system (Hewlett-Packard, Palo Alto, CA, USA) equipped with a UV detector set at 254 nm (Hewlett-Packard) interfaced to an API 165 mass spectrometer equipped with a turbo-ionspray interface (Perkin-Elmer Sciex, Norwalk, CT, USA). Ten aliquots of this sample (10 ml per aliquot) were cleaned up through OchraTest columns and combined to concentrate OA in sufficient amount to obtain a good MS spectrum from the HPLC–MS analysis. A 100-μl volume of concentrated extract was injected via a 100-μl Rheodyne loop and eluted at a flow-rate of 1.0 ml/min through a Discovery $C_{18}$ (15 cm×4.6 mm, 5 μm particles) column in isocratic mode with acetonitrile–water–acetic acid (99:99:2) as mobile phase. Ammonium hydroxide solution (25%) was added via T-piece between the HPLC column and the ionization chamber at a flow-rate of 70 μl/min to eliminate acetic acid present in the mobile phase. Splitting of the HPLC flow was performed to allow just 200 μl to enter the turbo-ionspray interface. The mass spectrometric conditions (negative chemical ionization) were: nebulizer gas (air)=1.5 l/min, curtain gas (nitrogen) 14 l/min, desolvation gas (nitrogen) temperature=300°C, mass range=100–600 u, scan time=2 s, needle voltage=−4000 V, orifice voltage=−30 V, ring voltage=−180 V.

### 2.6. Column capacity

The capacity of the OchraTest columns was determined by comparing (duplicate measurements) the amount of OA added to the immunoaffinity column with the amount bound. Different amounts of OA, from 5 ng to 500 ng, were added to the immunoaffinity column by loading 10 ml (equivalent to 5 ml wine) of diluted red wine spiked with the corresponding amount of OA.

### 2.7. Recovery experiments

Recovery experiments were performed in triplicate by spiking OA-free samples of white and rosé wine with OA at levels of 0.04, 0.1, 0.2, 0.5, 1.0 and 2.0 ng/ml and of red wine at levels of 0.04, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 ng/ml.

## 3. Results and discussion

### 3.1. Development of the analytical method

The method developed by Scott and Kanhere [26] for beer and by Ueno [21] for wine have been tested as a start point for the improvement of analytical methods for the determination of OA in wine. These methods were selected because they are based on immunoaffinity column clean-up and do not require any preliminary liquid–liquid extraction but a simple dilution of the sample with a basic solution (2% $NaHCO_3$–15% NaCl and 2.5% $Na_2CO_3$–1.5% NaCl, respectively). Results obtained with the method of Scott and Kanhere [26] applied to rosé and white wines were satisfactory giving recoveries higher than 90% and relative standard deviations (RSDs) less than 6% ($n$=3) at spiking level of 0.5 ng/ml OA. Recoveries dropped down to about 30% when the method was applied to red wine (see Fig. 1b for a sample containing 4.7 ng/ml OA). Similarly, the application of the method described by Ueno [21] failed in the analysis of red wine, as no OA was recovered even from a sample containing 4.7 ng/ml OA (see Fig. 1a).

Problems encountered with OA analysis in red wine may be attributed to the presence of several compounds, such as anthocyanins and other pigments, that could interfere, for example by physical occlusion, with OA binding to the antibody. Attempts to eliminate or reduce anthocyanins from red wine by treatment with polyvinylpyrrolidone (PVP) (15 ml wine were loaded on a minicolumn containing 2.5 g PVP) failed because OA was adsorbed together with pigments by the resin and could not be detected in the clarified solution of contaminated wine samples.

Poor recoveries (<30%) were obtained by applying to red wine the dilution procedure recommended by Vicam [extract dilution with different phosphate-buffered saline (PBS)–Tween-20 solutions prior to loading on the immunoaffinity column] for the analysis of OA in different matrices, such as green coffee and roasted coffee, currants, figs and raisins [27]. Recoveries were improved by avoiding the use of PBS and increasing the Tween-20 concentration, or increasing the pH of wine with a solution of sodium hydrogencarbonate. However the maximum

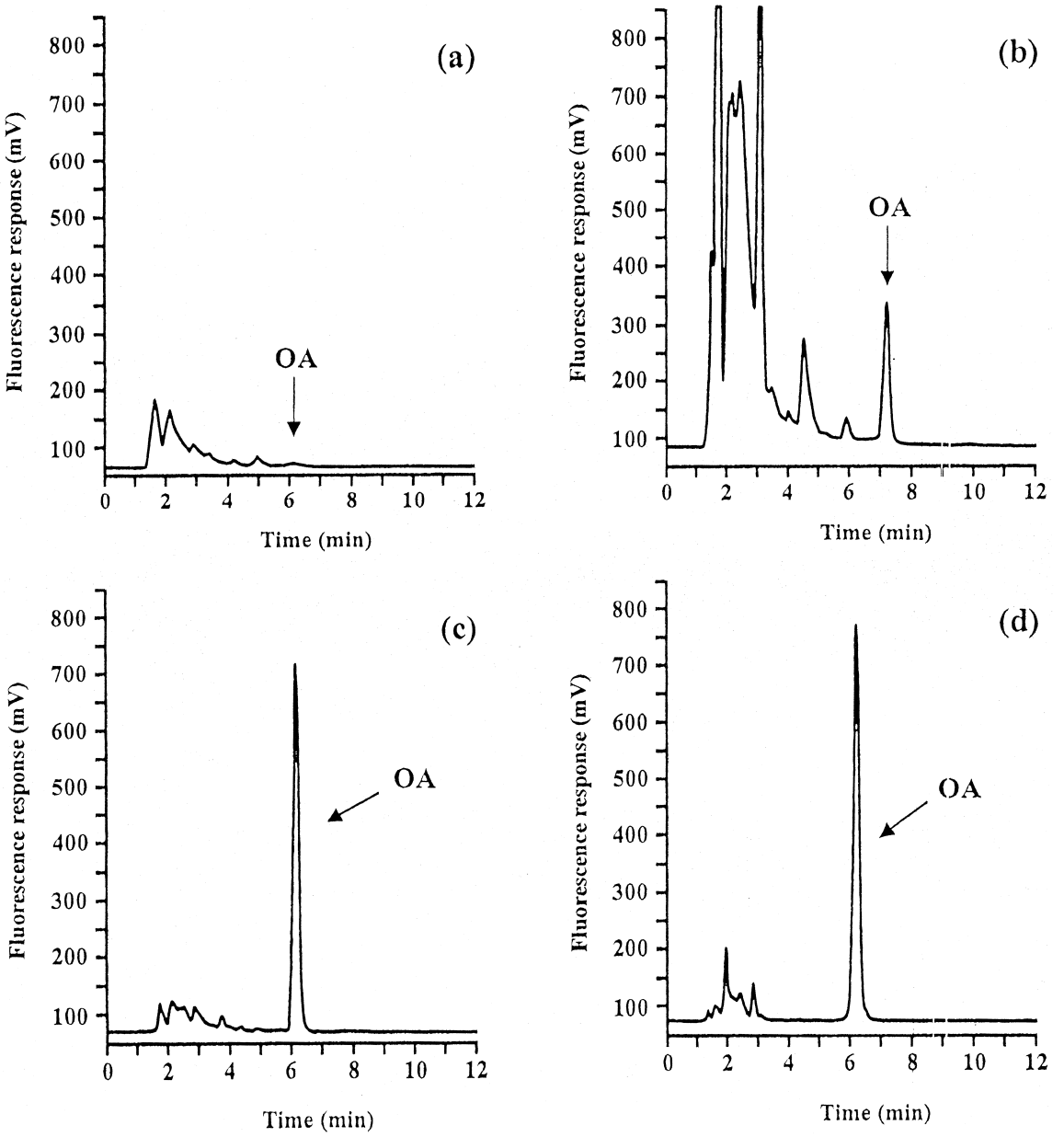


Fig. 1. Chromatograms relevant to a red wine sample (sample 28, injected amount equivalent to 2 ml wine) naturally contaminated with 4.72 ng/ml ochratoxin A (OA) using different sample preparation (see Table 2 for details) prior to immunoaffinity clean-up: (a) dilution with $Na_2CO_3$+NaCl solution [21]; (b) dilution with $NaHCO_3$+NaCl solution [26]; (c) liquid–liquid extraction [18] and (d) present paper. Chromatographic conditions are reported in Sections 2.3 and 2.5.

achievable recovery (60%) was obtained by diluting wine with 0.1% Tween-20 (1:1, v/v) and adjusting the pH of wine at about 7 prior to the immunoaffinity clean-up. Under these experimental conditions, a precipitate was formed after addition of Tween-20 and the resulting residue, after filtration, accounted for the amount of OA not found in the filtered solution. The use of other surfactants, i.e., Triton

X-100 (*tert*.-octylphenoxypolyethoxyethanol) or sodium cholate, while improved considerably the extract clean-up, had no effect on OA recovery.

The best results, both in terms of recoveries and extract clean-up, were obtained by using PEG as diluting solution prior to the immunoaffinity clean-up. PEG is a compound with low toxicity that has been used successfully for the analysis of deoxynivalenol (DON) in wheat based on immunoaffinity clean-up [28]. Results obtained with a naturally contaminated red wine at different pH and PEG concentration are reported in Table 1. OA recoveries higher than 90% were obtained by diluting wine with 1% PEG solution (1:1, v/v) and adjusting pH at 5.5–8.5 with 5% $NaHCO_3$ solution. Recoveries increased from 7.5% to 97.2% with pH changing from 3.5 to 7.4 and decreased drastically at pH higher than 8.5 (Table 1). The presence of sodium chloride in the pH correcting solution did not effect recoveries. Finally, optimal experimental conditions were obtained by diluting (1:1, v/v) wine with a solution containing both 1% PEG and 5% $NaHCO_3$ (pH 8.5) before the immunoaffinity column clean-up. In this way the pH of the diluted wine was always within the optimal range (pH 7.0–8.5). A comparison of performances of different methods using immunoaffinity column clean-up for the determination of OA in wine or beer is summarized in Table 2. Typical chromatograms relevant to the same wine sample analyzed with these methods are reported in Fig. 1. The presence of PEG in the diluting solution was a determining factor to increase recoveries of OA while drastically reduced the number and intensity of additional chromatographic peaks unrelated to OA (see Fig. 2). The use of PEGs with different molecular masses, i.e., PEG 3350 (Sigma), PEG 6000 (BDH, UK) and PEG 8000 (Sigma) did not affect recoveries.

### *3.2. Performance of the analytical method*

Results of the recovery experiments (triplicate measurements) of the full analytical procedure carried out on wine samples (white, rosé and red wine) spiked with OA at different levels are reported in Table 3. Within the spiking range 0.04–10.0 ng/ml the overall average recovery (mean of means) of OA from red wine was 92.8%, with minimum value at 88.1%, and the average RSD was 3.8%. Average recovery for white and rosé wine spiked with OA at levels between 0.04 and 2.0 ng/ml were 94.5% for both types of wine with average RSDs of 5.5% and 4.3%, respectively (see Table 3). RSDs were lower than 3% for all spiking levels but the lowest one

Table 1
Effect of PEG concentration and pH on the extraction factor (recovery) of ochratoxin A from wine

| Raw | Diluting solution[a] | pH correcting solution | Final pH of solution | OA found (ng/ml) | Recovery[b] (%) |
|---|---|---|---|---|---|
| 1 | PEG 0% | 2% $NaHCO_3$+15% NaCl | 8.2 | 1.25 | 26.5 |
| 2 | PEG 0.01% | 2% $NaHCO_3$+15% NaCl | 7.2 | 2.37 | 50.2 |
| 3 | PEG 0.05% | 2% $NaHCO_3$+15% NaCl | 7.3 | 2.56 | 54.2 |
| 4 | PEG 0.1% | 2% $NaHCO_3$+15% NaCl | 7.0 | 2.95 | 62.5 |
| 5 | PEG 1% | 2% $NaHCO_3$+15% NaCl | 7.1 | 3.48 | 73.7 |
| 6 | PEG 2% | 2% $NaHCO_3$+15% NaCl | 7.2 | 3.34 | 70.8 |
| 7 | PEG 5% | 2% $NaHCO_3$+15% NaCl | 7.0 | 3.48 | 73.7 |
| 8 | PEG 1% | 5% $NaHCO_3$ | 3.5 | 0.35 | 7.5 |
| 9 | PEG 1% | 5% $NaHCO_3$ | 4.5 | 2.07 | 43.8 |
| 10 | PEG 1% | 5% $NaHCO_3$ | 5.5 | 4.24 | 89.9 |
| 11 | PEG 1% | 5% $NaHCO_3$ | 6.4 | 4.25 | 90.0 |
| 12 | PEG 1% | 5% $NaHCO_3$ | 7.4 | 4.59 | 97.2 |
| 13 | PEG 1% | 5% $NaHCO_3$ | 8.5 | 4.72 | 100 |
| 14 | PEG 1% | 1 *M* NaOH | 10.5 | 0.19 | 4.0 |

[a] Wine:diluting solution ratio (1:1, v/v).
[b] All recoveries are referred to the highest value found after method optimization (raw 13), assumed as 100%.

Table 2
Comparison of different methods of ochratoxin A analysis based on immunoaffinity column (IAC) clean-up relevant to the same red wine sample

| Method[a] | Diluting solution | Wine:diluting solution (v/v) | OA found (ng/ml) | Recovery[b] (%) | Ref. |
|---|---|---|---|---|---|
| a | $Na_2CO_3$ (2.5%)+NaCl (1.5%) (IAC washing: 0.5% $Na_2C0_3$–2.5% NaCl solution+PBS) | 5:1 | 0.02 | 0.4 | [21] |
| b | $NaHCO_3$ (2%)+NaCl (15%) (IAC washing: 0.5% $NaHC0_3$–2.5% NaCl solution+water) | 5:1 | 1.47 | 31.1 | [26] |
| c | Extraction with: $CHCl_3$+$H_3PO_4$+NaCl (IAC washing: 15% $CH_3OH$/PBS+water) | – | 3.32 | 70.3 | [18] |
| d | PEG (1%)+$NaHCO_3$ (5%) (IAC washing: 0.5% $NaHC0_3$–2.5% NaCl solution+water) | 1:1 | 4.72 | 100 | This study |

[a] Chromatograms are reported in Fig. 1.
[b] All recoveries are referred to the highest value found after method optimization (raw 4), assumed as 100%.

(0.04 ng/ml OA). When the method was applied to a sample of dessert wine (Marsala wine containing 0.29 ng/ml OA) the recovery of OA spiked at 0.5 ng/ml levels was 92.5±2.1% ($n$=3).

The limit of detection of the method was 0.01 ng/ml, based on a signal/noise of 3:1. Chromatograms of a blank red wine sample and the same sample spiked with OA at levels close to the detection limit are shown in Fig. 3. Considering the high sensitivity of the method, no post-column addition of ammonia to enhance fluorescence detection of OA was necessary [11].

The OchraTest column capacity was found to be about 160 ng of OA. Above this level no increase of the fluorescence response was observed, indicating the saturation of ochratoxin binding sites. Considering the column capacity, the range of applicability of the method is very wide, from 0.01 ng/ml to 30.0 ng/ml of OA in wine.

The method was compared with the method described by Zimmerli and Dick [18] using HPLC analysis and immunoaffinity clean-up, which in our hands resulted the most effective of the published methods. Results of comparative analyses of 20 naturally contaminated samples are reported in Table 4. In particular, by assuming a 100% hypothetical

Table 3
Recoveries from blank wine spiked with ochratoxin A at different levels

| Spiking level (ng/ml) | Red wine | | Rosé wine | | White wine | |
|---|---|---|---|---|---|---|
| | Recovery±SD[a] (%) | RSD[b] (%) | Recovery±SD[a] (%) | RSD[b] (%) | Recovery±SD[a] (%) | RSD[b] (%) |
| 0.04 | 96.7±2.2 | 2.3 | 94.1±6.1 | 6.5 | 91.6±8.9 | 9.7 |
| 0.1 | 90.8±2.6 | 2.9 | 89.9±1.0 | 1.1 | 88.4±0.2 | 0.2 |
| 0.2 | 91.3±0.6 | 0.7 | 88.9±2.1 | 2.4 | 95.1±2.4 | 2.5 |
| 0.5 | 92.3±0.4 | 0.5 | 91.6±0.4 | 0.4 | 93.0±0.2 | 0.2 |
| 1.0 | 97.8±2.6 | 2.6 | 103.6±2.5 | 2.5 | 100.7±1.0 | 1.0 |
| 2.0 | 96.5±1.6 | 1.7 | 98.6±1.8 | 1.8 | 98.0±1.5 | 1.5 |
| 5.0 | 88.1±1.3 | 1.5 | – | – | – | – |
| 10.0 | 88.9±0.6 | 0.7 | – | – | – | – |
| Mean of means | 92.8±3.5 | 3.8 | 94.5±5.2 | 5.5 | 94.5±4.1 | 4.3 |

[a] SD=Standard deviation ($n$=3 replicates).
[b] RSD=Relative standard deviation.

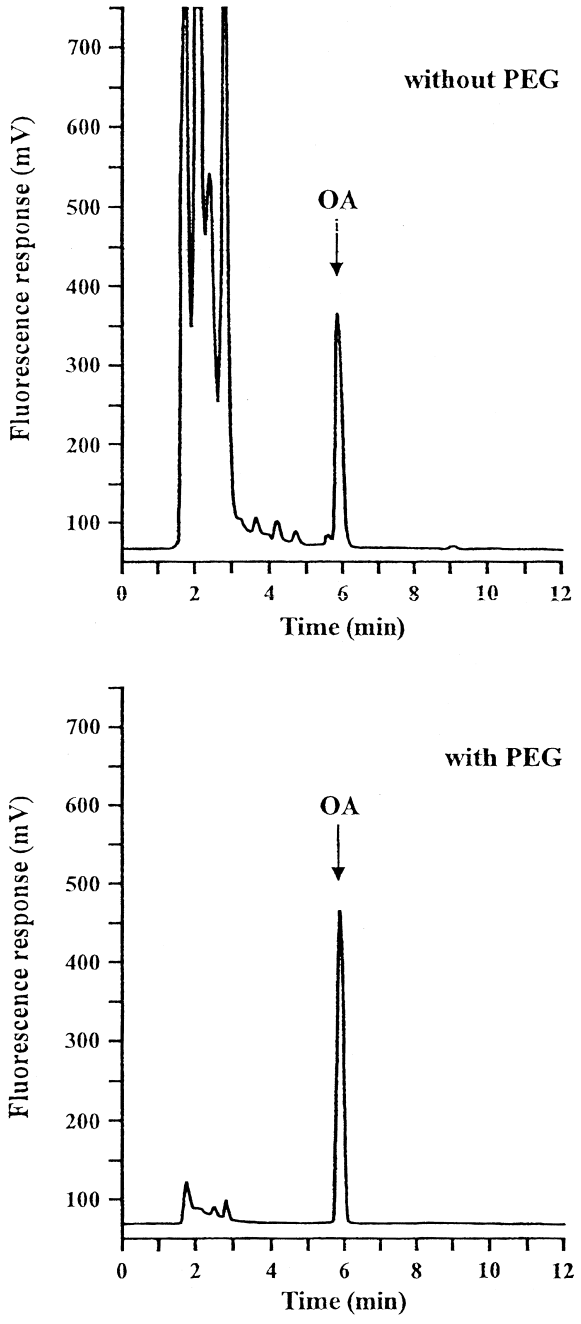


Fig. 2. Effect of PEG in the sample preparation prior immunoaffinity clean-up: chromatograms of red wine sample spiked with ochratoxin A (OA) at level of 2.0 ng/ml. Wine diluted with PEG solution (1% PEG+5% $NaHCO_3$) (lower; OA found=1.97 ng/ml) and without PEG (5% $NaHCO_3$) (upper, OA found=1.43 ng/ml).

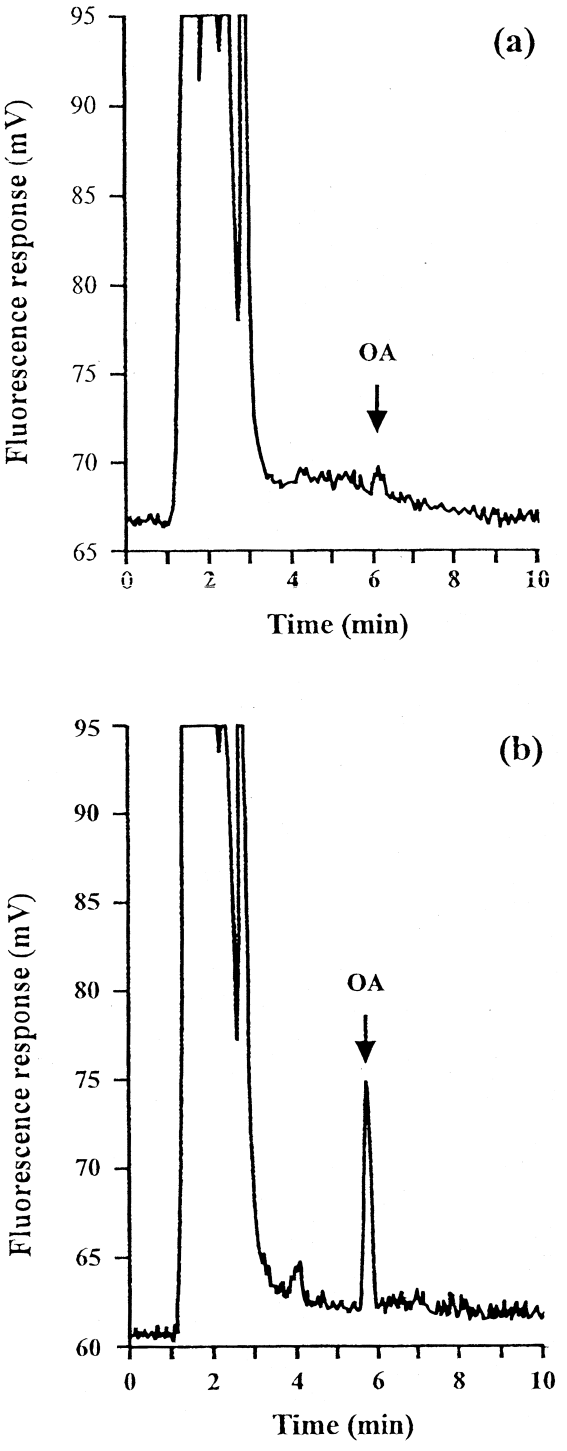


Fig. 3. Chromatograms of red wine sample: (a) blank (<0.01 ng/ml) and (b) blank sample spiked with ochratoxin A (OA) at levels of 0.04 ng/ml (injected amount equivalent to 2 ml wine).

recovery for the new method, average recoveries obtained with the method of Zimmerli and Dick for white, rosé and red wines corresponded to 82, 75 and 76%, respectively, of those obtained with the new method (Table 4). The overall mean recovery of OA with the Zimmerli and Dick method corresponds to 77.1% of the one obtained with the new method. The regression curve comparing the two methods (Fig. 4) clearly shows a good correlation ($r$=0.987) and better recoveries in favor of the method proposed herein. Another important advantage of the method described herein with respect to that of Zimmerli and Dick consists in the drastically reduced time of analysis as no liquid–liquid extraction procedure is required. In particular, the average time for sample preparation (extraction and clean-up) prior to HPLC analysis was about 10 min (excluding evaporation step) with the new method and about 70 min with the one of Zimmerli and Dick. When a high frequency of analysis is required, up to 48 samples/day can be worked out by a single operator (using a 12-port solid phase extraction vacuum manifold) with the new method vs. 12 sample/day with the previous one. In addition, the proposed method presents a number of advantages with respect to the only method tested in a collaborative interlaboratory study for determining OA in wine which is based on silica cartridge clean-up [29]. In particular the new method avoids (i) the high volumes of hazardous solvents used, (ii) tedious liquid–liquid extraction with possibility of forming emulsion mainly with red wine and (iii) long time of analysis and poor repeatability particularly for red wines.

In conclusion, both the results of the analysis of naturally contaminated samples and the recoveries obtained with spiked materials are indicative of a better accuracy of the proposed immunoaffinity method as compared to both the method of Zimmerli

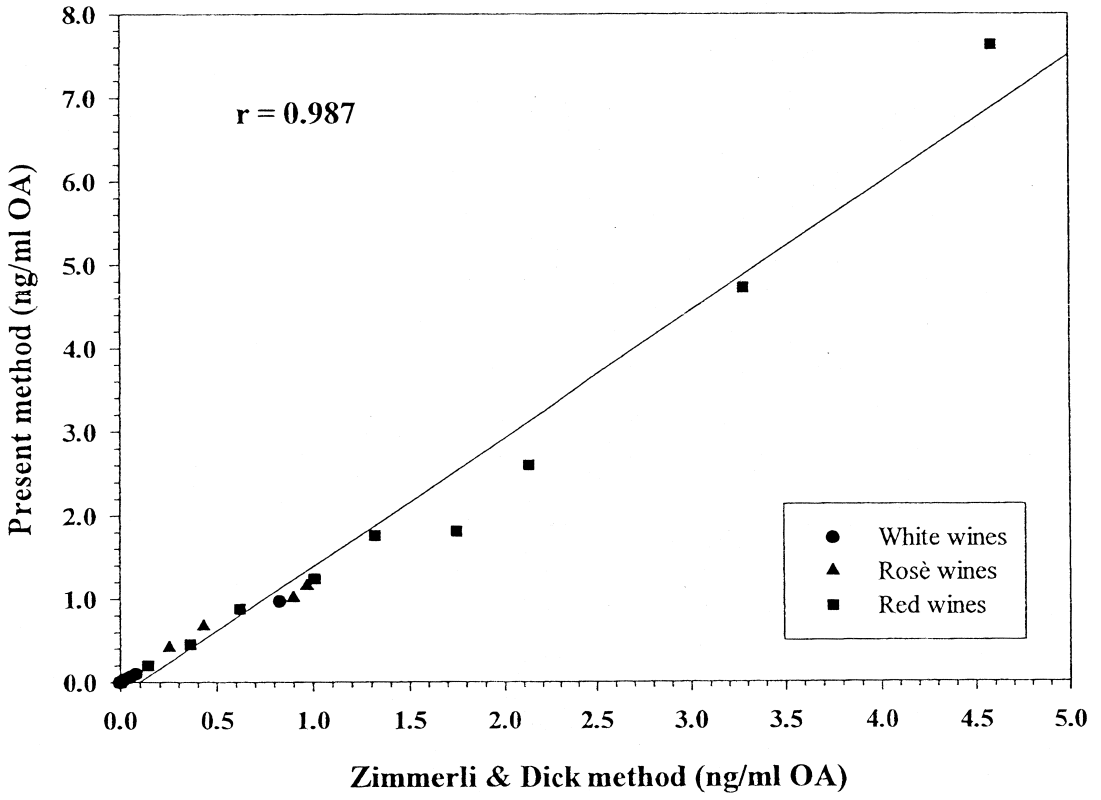


Fig. 4. Regression curve of the present method vs. Zimmerli and Dick method [18] for the determination of ochratoxin A in 20 wine samples naturally contaminated with ochratoxin A (OA).

Table 4
Comparison of ochratoxin A determination in wine by the present method (A) and the method of Zimmerli and Dick (B)

| Sample | (A) Present method (ng/ml) | (B) Zimmerli and Dick method (ng/ml) | (B)/(A) |
|---|---|---|---|
| White wine | | | |
| 49 | n.d.[a] | n.d. | – |
| 19 | 0.025 | 0.02 | 0.80 |
| 51 | 0.06 | 0.05 | 0.83 |
| 9 | 0.10 | 0.08 | 0.80 |
| 15 | 0.97 | 0.83 | 0.86 |
| Average B/A ratio | | | 0.82 |
| Rosé wine | | | |
| 46 | n.d. | n.d. | – |
| 8 | 0.41 | 0.25 | 0.61 |
| 24 | 0.67 | 0.43 | 0.64 |
| 10 | 1.01 | 0.90 | 0.89 |
| 23 | 1.15 | 0.97 | 0.84 |
| Average B/A ratio | | | 0.75 |
| Red wine | | | |
| 55 | n.d. | n.d. | – |
| 47 | 0.20 | 0.14 | 0.70 |
| 36 | 0.45 | 0.36 | 0.80 |
| 43 | 0.88 | 0.62 | 0.70 |
| 35 | 1.24 | 1.01 | 0.81 |
| 1 | 1.81 | 1.75 | 0.97 |
| 32 | 1.76 | 1.32 | 0.75 |
| 20 | 2.60 | 2.13 | 0.82 |
| 2 | 4.72 | 3.28 | 0.69 |
| 28 | 7.63 | 4.59 | 0.60 |
| Average B/A ratio | | | 0.76 |
| Overall average B/A ratio | | | 0.77 |

[a] n.d.=Not detected (<0.01 ng/ml).

and Dick [18], which is presently considered the most effective one for the determination of OA in wine and the method of Ospital et al. [24] which is the only one validated by a collaborative trial.

### *3.3. Analysis of naturally contaminated samples*

The method was applied to 56 samples of red (38), rosé (8), white (9) and dessert (1) wine (bottled and home-made wine) originating from Italy. Most of these samples were contaminated and the levels of OA ranged from <0.01 to 7.6 ng/ml, with red and rosé wines being more contaminated than white wines. Fig. 5 shows typical chromatograms of three naturally contaminated wine samples (white, rosé and red wine) containing levels of OA commonly found in wine. Chromatograms clearly show the absence of interfering signals at the OA retention time for the different types of wine. The identity of OA was confirmed in 10 selected positive samples (2 white, 2 rosé and 6 red wines) by esterification with $BF_3$ in methanol which caused the disappearance in the chromatogram of peak relative to OA ($t_R$=6 min) and the appearance of a new peak at 12.5 min relative to OA methyl ester. The identity of OA was further confirmed by HPLC–MS in the sample with the highest OA contamination (7.6 ng/ml) after combining the extracts deriving from 10× 10 ml aliquots of wine cleaned up individually (see Section 2.5). OA was confirmed by the presence in

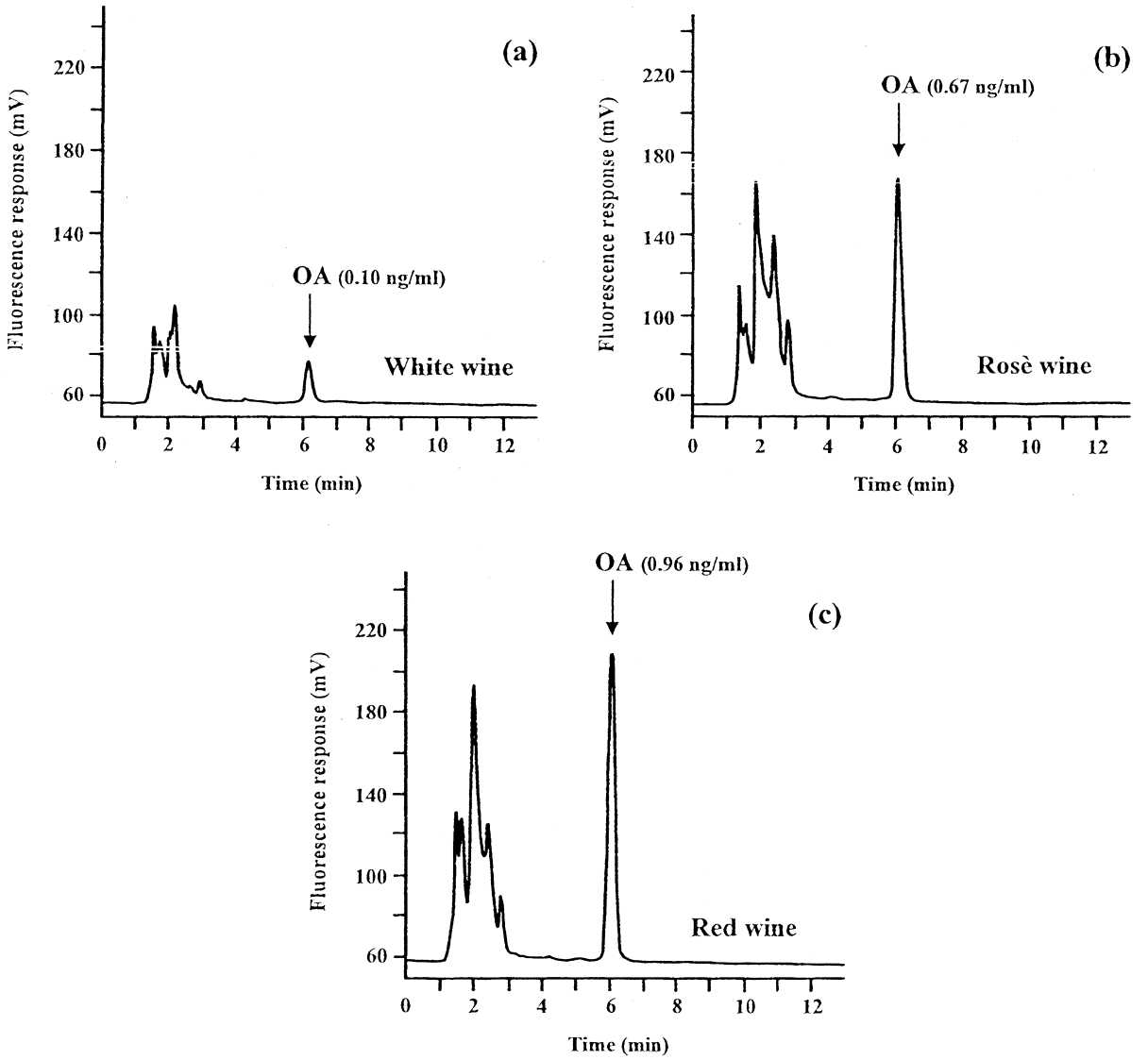


Fig. 5. Chromatograms relevant to three naturally contaminated wine samples containing levels of ochratoxin A commonly found in wines: (a) white wine, (b) rosé wine, (c) red wine (injected amount equivalent to 2 ml wine).

the MS spectrum of the two major ions with $m/z$ 402.2 $[M-H]^-$ (base peak) and $m/z$ 357.9 $[M-H-CO_2]^-$ similar to those found in the MS spectrum of OA standard. Table 5 summarizes the results of samples grouped in commercial (bottled wine) and home-made wine. The OA mean (median) concentration of the positive samples of red, rosé and white wine were 1.24 ng/ml (0.76 ng/ml, n=37), 0.72 ng/ml (0.67 ng/ml, $n=7$) and 0.29 ng/ml (0.08 ng/ml, $n=4$). Except for white wines, commercial wines showed levels of contamination generally higher than home-made wines. The only sample of special wine (Marsala) analyzed resulted contaminated with OA at 0.29 ng/ml.

The distribution of OA contamination levels in white, rosé and red wine samples is presented in Table 6. Most of rosé (75%) and red (95%) wine samples were contaminated with OA at level higher

Table 5
Ochratoxin A concentrations in commercial (bottled wines) and home-made wines

| Samples | Positives/total | Mean of positives (ng/ml) | Median (ng/ml) | Range (ng/ml) |
|---|---|---|---|---|
| Red wines | | | | |
| Commercial | 26/27 | 1.269 | 0.895 | n.d.[a]–7.63 |
| Home-made | 11/11 | 1.185 | 0.660 | 0.46–4.72 |
| Rosé wines | | | | |
| Commercial | 5/6 | 0.804 | 1.010 | n.d.–1.15 |
| Home-made | 2/2 | 0.525 | 0.525 | 0.41–0.64 |
| White wines | | | | |
| Commercial | 2/7 | 0.045 | 0.045 | n.d.–0.06 |
| Home-made | 2/2 | 0.535 | 0.535 | 0.10–0.97 |
| Special wines | | | | |
| Marsala | 1/1 | 0.29 | – | 0.29 |

[a] n.d.=Not detected (<0.01 ng/ml).

than 0.2 ng/ml and about one third of these types of wine contained more than 1.0 ng/ml OA. The high incidence of OA contamination of Italian wines is not surprising, as similar results have been previously reported [18–21]; nevertheless the levels of contamination found in this study are generally higher than those found in similar investigations on wines from different countries [18–21]. A tentative explanation could be that most of wine samples came from southern Italy where the climatic condition could favor the growth of OA producing fungi.

## 4. Conclusion

A number of advantages have been shown by using the method presented herein with respect to the other available methods for determining OA in wine. In particular the use of hazardous solvents (such as chlorinated or aromatic solvents) is avoided, clean extracts are obtained from the immunoaffinity column due to the specificity of the antibody, rapid sample preparation and clean-up procedure bring to convenient analysis time-saving. Although no collaborative interlaboratory validation of the proposed method has been performed, the laboratory performances in terms of accuracy (recovery) and precision (repeatability) are very significant and superior to other published methods. Validation of the method by interlaboratory trial is highly desirable, particularly in consideration of the fact that the only validated method for determining OA in wine [29] presents a number of drawbacks that can be easily overcome by using the method presented herein.

Although a limited number of wine samples were analyzed, the results suggest a real risk of OA

Table 6
Distribution of ochratoxin A contamination levels in white, rosé and red wine samples analyzed in this study

| Type | *n* samples | Incidence of OA contamination (%) | | | | |
|---|---|---|---|---|---|---|
| | | <0.01 (ng/ml) | 0.01–0.20 (ng/ml) | 0.21–1.00 (ng/ml) | 1.01–2.00 (ng/ml) | >2.00 (ng/ml) |
| White | 9 | 55.6 | 33.3 | 11.1 | – | – |
| Rosé | 8 | 12.5 | 12.5 | 37.5 | 37.5 | – |
| Red | 38 | 2.6 | 2.6 | 63.2 | 15.8 | 15.8 |
| Overall | 55 | 12.7 | 9.1 | 50.9 | 16.4 | 10.9 |

ingestion, especially for red and rosé wine drinkers. Research is urgently needed to identify the major factors leading to the development of ochratoxigenic fungi and the consequent ochratoxin contamination of wine in order to prevent the problem.

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