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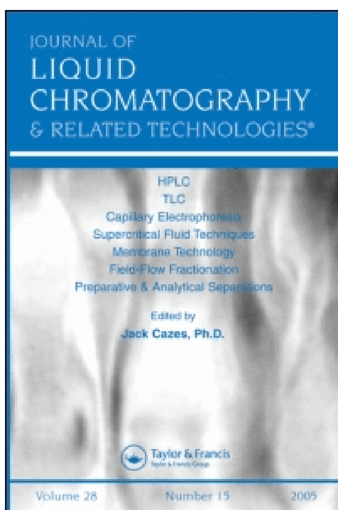

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 30 January 2003

To cite this Article Brera, Carlo , Grossi, Silvana , De Santis, Barbara and Miraglia, Marina(2003) 'Automated HPLC Method for the Determination of Ochratoxin A in Wine Samples', Journal of Liquid Chromatography & Related Technologies, 26: 1, 119 — 133

To link to this Article: DOI: 10.1081/JLC-120017157

URL: http://dx.doi.org/10.1081/JLC-120017157

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JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES®
Vol. 26, No. 1, pp. 119–133, 2003

Automated HPLC Method for the Determination of Ochratoxin A in Wine Samples

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ABSTRACT

Recently food safety issues received increasing attention and they will be more and more the matter of interest of risk assessors. Among the others, Ochratoxin A (OTA) contamination in wine resulted in an emerging risk for consumers. Recently, both monitoring programs and researches have been performed, aimed at individuating the status of contamination worldwide and critical control points in the wine-making chain. At the moment, all studies confirmed that red wines resulted in contamination more frequently and at higher levels than white wines. This paper describes a study to carry out an automated method of analysis for the determination of OTA in wine samples, in order to process a high number of samples for Hazard Analysis Critical Control Points (HACCP) purposes. Method

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DOI: 10.1081/JLC-120017157
1082-6076 (Print); 1520-572X (Online)
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performance characteristics, such as repeatability, internal reproducibility, and accuracy, showed good performance and reliability of the method, adequately matching with the criteria suggested by Comité Européen de Normalisation (CEN) for the analysis of mycotoxins. The advantages coming out from this method are, therefore, the saving of time of analysis, the possibility to analyze large amounts of samples, the reduction of the employment of personnel, and the obtaining of all the requirements requested by the national legislation dealing with the official control of foodstuffs.

Key Words: Wine; Ochratoxin A; Automated method; Mycotoxins.

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INTRODUCTION

It is well recognised that the occurrence of Ochratoxin A (OTA) in food can seriously impair human health.[1] According to surveys carried on in countries world-wide, OTA can contaminate many crops and food such as maize, wheat, barley, beans, coffee, beer, and cocoa.[2] Unfortunately, due to many possible sources of uncertainties or lack of information (reliability of sampling procedures and of analytical data, influence of technological procedures, consumption data), the main food sources of OTA intake for humans are still in debate. More recently, the evaluation of OTA in wine has been receiving increasing attention, since this commodity is considered an additional source of OTA intake for those countries in which this beverage is largely consumed. Recent studies showed that a considerable level of OTA contamination in wine samples from South Europe and North Africa areas exists.

Zimmerli and Dick[3] analysed 118 wine samples (24 white, 15 rosé, 79 red) from different European countries, with a level of contamination ranging from 0.003 to 0.18 ng mL^{-1}, 0.003 to 0.12 ng mL^{-1}, 0.003 to 0.39 ng mL^{-1} (limit of detection 0.003 ng mL^{-1}) respectively, and corresponding average levels of 0.01, 0.03, and 0.04 ng mL^{-1}. This study could establish a correlation between the level of contamination and the origin of wine samples to South Europe. Nevertheless, Burdaspal and Legarda[4] found a contamination level in South Europe countries lower than in other North Europe countries. In addition, data deriving from this study, confirmed that red wines resulted in contamination more frequently and at higher levels (92.3%; 0.054 ng mL^{-1}) than white wines (65.2%; 0.020 ng mL^{-1}).

In Italy, as a leading country in wine production, several studies have been so far performed. A study performed in our Institute (60 red wine samples)

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showed levels of OTA contamination higher in South Italy than in other geographical areas of the country ($0.60\,ng\,mL^{-1}$, $0.28\,ng\,mL^{-1}$, and $0.52\,ng\,mL^{-1}$ in South, Central, and North Italy respectively) with a frequency of contamination of 85% and an average level of $0.40\,ng\,mL^{-1}$.[5]

Another study performed on 47 samples, showed the same trend of results with higher frequency (97% and 44%) and average levels of OTA contamination ($1.23\,ng\,mL^{-1}$ and $0.29\,ng\,mL^{-1}$) for red and white wine samples, respectively.[6] Consequently, the interest that has been devoted by the wine-making area in the last five years to OTA contamination, has stressed the need of implementing several initiatives, either as monitoring programs or prevention measures. The setting of maximum tolerable limits for table wine, that will mainly be based upon the available occurrence data and information within each member state, with particular regard to red wine is currently under discussion. It is well known, in fact, that the prolonged contact time between the peel and the must for red wine can play a major role in the occurrence of OTA contamination in wine as a carry over.

In this respect, The European Commission invited each member state to provide as much possible data and information addressed to the individuation of the critical control points in the wine-making chain, and to the evaluation of the actual status of OTA contamination in wine.[7]

With this aim, a large number of surveys are expected to be performed with the need of handling large amounts of data corresponding to the analyses of wine samples.

Even if some analytical methods are available, none of them are based on an automatic analysis able to reduce times of analysis, improve the precision and accuracy of the results, and to save costs of analysis.

In addition, it should be considered the involvement of the wine industry in handling a large amount of samples necessary for the implementation of preventive and control activities on the basis of HACCP principles.

The aim of this study has been, therefore, devoted to carry out an automated method of analysis for OTA determination in wine samples, in order to process a high number of samples in an analytical cycle, according to the principles of Quality Assurance. This method has successively been used for a monitoring program aimed at establishing the status of contamination by OTA in national and imported white and red wine samples in consideration of several variables, among them different geographical areas, costs, consumption rates, closeness to the sea of production areas, and grape varieties.

The method basically consisted of a dilution step of samples with a solution of phosphate buffer saline, and in a cleanup step by immunoaffinity columns. The final quantification was made by high performance liquid chromatography (HPLC) by fluorescence detection.

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EXPERIMENTAL

Precautions

This method requires the use of stock solutions of OTA. Ochratoxin A is a potent nephrotoxin and liver toxin; it has been reported to have immunosuppressant properties and to cause kidney and liver tumours in mice and rats.

Gloves and safety glasses should be worn at all times, and all standard and sample preparation stages should be carried out in a fume cupboard.

Ochratoxin A is subject to light degradation. Therefore, it is necessary to protect analytical work adequately from the daylight, and keep OTA standard solutions protected from light by using amber vials or aluminium foil.

The use of non-acid glassware (vials, tubes) for OTA aqueous solutions may cause a loss of mycotoxin. Special attention should be taken with new glassware. Therefore, before use, soak the glassware in diluted acid (sulphuric acid, 2 mol L^{-1}) for several hours; then rinse with distilled water to remove all traces of acid (this can be checked by using a pH-paper).

Decontamination procedures for laboratory wastes[8,9] were developed and validated by the International Agency for Research on Cancer (WHO).

Toluene is highly flammable and harmful. Standard preparation involving this solvent must be performed in a fume cupboard.

Reagents

During the analysis, unless otherwise stated, only reagents of recognised analytical grade and only distilled water, or water of grade 1 according to EN ISO 3696 have been used. All solvents were HPLC grade.

Ochratoxin A Calibrant Solution

A 10 μg mL^{-1} OTA calibrant in toluene : acetic acid, (98 : 2; V : V) has been prepared. The concentration of this calibrant has been checked using a UV spectrophotometer.[10] Standard solutions have been stored at 4°C. All the solvents used for quantitative analyses was HPLC grade.

Phosphate Buffered Saline (PBS) (pH 7.4)

PBS can be prepared from potassium chloride (0.20 g), potassium dihydrogen phosphate (0.20 g), anhydrous disodium hydrogen phosphate

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(1.16 g), and sodium chloride (8.00 g) added to 900 mL purified water. After dissolution, pH must be adjusted to 7.4 (with 0.1 mol L^{-1} HCl or 0.1 mol L^{-1} NaOH as appropriate) and the solution made to 1.0 L.

HPLC Mobile Phase Solvent

Acetonitrile : water : acetic acid (50 : 49 : 1 : V : V : V).

Principle of the Method

Samples have been diluted with a solution of PBS, filtered, and cleaned up by an immunoaffinity column (IAC) containing antibodies specific to OTA. After washing the immunoaffinity column, OTA is eluted with methanol, separated by reversed-phase HPLC, and quantified by fluorescence.

Manual Method

Apparatus

Silanised glass vials.

Vacuum manifold to accommodate immunoaffinity columns.

Reservoirs and attachments to fit to immunoaffinity columns.

Glass microfibre filters.

IAC specific for OTA clean up (Ochratest Vicam).

HPLC Apparatus Comprising the Following

Injection system with 200 μL injection loop.

Mobile phase pump (isocratic) capable of delivering 1.0 mL min^{-1} constant flow rate.

HPLC analytical column—C 18 reverse phase, (150 × 4.6 mm) packed with 3.5 μm.

Fluorescence detector fitted with flow cell and set at 333 nm (excitation) and 460 nm (emission).

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Chromatographic data handling software (Unipoint—Gilson).

Spectrophotometer (optional for checking concentration of calibrant solutions).

Automated Method

Apparatus

Silanised glass vials.

Glass microfibre filters.

Immunoaffinity columns specific for OTA clean up (Ochratest—Vicam).

Automatic solid-phase extraction (ASPEC—Gilson).

Containers glass (180 mL and 22 mL).

Software for the management of ASPEC (Software 735—Gilson).

HPLC Apparatus Comprising the Following:

The apparatus used was the same as for the manual method.

Procedure

Sample Preparation (Manual and Automated Method)

Add 5 mL of sample to 60 mL of PBS. Mix vigorously. Filter through glass microfibre filter. Filtration is necessary for cloudy solutions or when solid residues are formed after dilution.

For the automated method a portion (22 mL) of this solution was transferred into a glass vial and placed in the rack of the workstation.

Immunoaffinity Column Clean-Up (Manual Method)

Immunoaffinity columns (IAC) should be kept at room temperature prior to conditioning. Connect the immunoaffinity column to the vacuum manifold and attach the reservoir to the immunoaffinity column. For conditioning, apply 4 mL of PBS on the top of the column and let it pass at a speed of 2–3 mL min^{-1} through the column, avoiding letting the column run dry. Add 20 mL diluted sample (equivalent to 1.54 mL of sample) to the reservoir

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and pass through the immunoaffinity column at a flow rate of about 1–2 drops/second (gravity). Flow rate should not exceed 5 mL min^{-1}. The immunoaffinity column must not be allowed to run dry. Wash the immunoaffinity column with 9 mL PBS and 8 mL de-ionised water at a flow rate of about 1–2 drops/second. Dry the column by applying a light vacuum for 5–10 s or pass air through the immunoaffinity column by means of a syringe for 10 s. Remove the immunoaffinity column from the vacuum manifold and place it over a silanised vial.

Eluting the Ochratoxin A in a Two Steps Procedure

Apply 0.50 mL of methanol to the column and let it pass through by gravity.

Wait for 1 min and apply a second portion of 0.750 mL methanol. Collect most of the applied elution solvent by passing air through, after most has passed through by gravity.

Add 500 µL de-ionised water to eluate, mix vigorously, and store at 4°C until HPLC analysis.

HPLC Analysis

Inject 150 µL of dilute extract (equivalent to 0.132 mL of sample) into the chromatographic apparatus by a partial loop injection system.

Automatic Clean-up and Delivery Analysis with HPLC

The automated HPLC system consisted of a Gilson 321 pump, a Gilson ASPEC XL as Sample Processor for Solid Phase Extraction system, fitted with a Rheodyne 7010 injector, a Gilson 402 Diluitor, and a Jasco FP1520 fluorescence detector. The detector, the Gilson pump, and the ASPEC XL system were linked with a Gilson 506 C system interface module.

The control of the HPLC pump and the chromatographic data acquisition were performed by Gilson Unipoint System Software. The control of the ASPEC XL system was performed by Gilson 735 System Software.

The analytical column (Symmetry Waters ODS 3.5 µm particle size, 150 × 4.6 mm I.D.) was maintained at 40°C.

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The ASPEC XL Gilson System (Fig. 1) was equipped with

A rack (Code 61) containing four 180-mL glass bottles of water (one bottle), PBS (two bottles) and methanol (one bottle).

Two racks (Code 24) fitted for up to 14 glass containers (containing up to 22 mL of diluted sample as maximum volume).

A rack (Code 21) fitted for up to 60 glass amber vials (containing up to 2 mL of solution standard as maximum volume).

A DEC rack (Code 101): mobile DEC (polypropylene) fitted for up to 36 immunoaffinity columns, and collect rack (aluminium) fitted for up to 36 glass tubes for the collection of the eluate from the column.

A schematic view of the workstation is shown in Fig. 1.

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Sequence of Operations for the Automated Clean-up of Samples Using the ASPEC System

Conditioning of immunoaffinity column with PBS (4 mL at 1.5 mL min^{-1}).

Loading of IAC with diluted sample (20 mL) at 1.3 mL min^{-1} as flow rate.

Washing of IAC with PBS (8 mL at 1.5 mL min^{-1}).

Washing of IAC with water (9 mL at 1.5 mL min^{-1}).

Elution from the column of OTA with methanol (0.50 and 0.75 mL at 0.4 mLmin^{-1}) and by flushing air through the column (0.5 mL at 6 mL min^{-1}).

Dilution of the eluate with water (0.50 mL at 3 mL min^{-1}).

Mixing of the diluted eluate in two steps: by flushing twice the eluate before and air afterwards.

Injection of diluted eluate (150 μL) using partial loop fill into a 200 μL-loop.

The cycle-sequence by ASPEC included both the sample analysis and the standard injection. During the acquisition of the last sample (or standard) the

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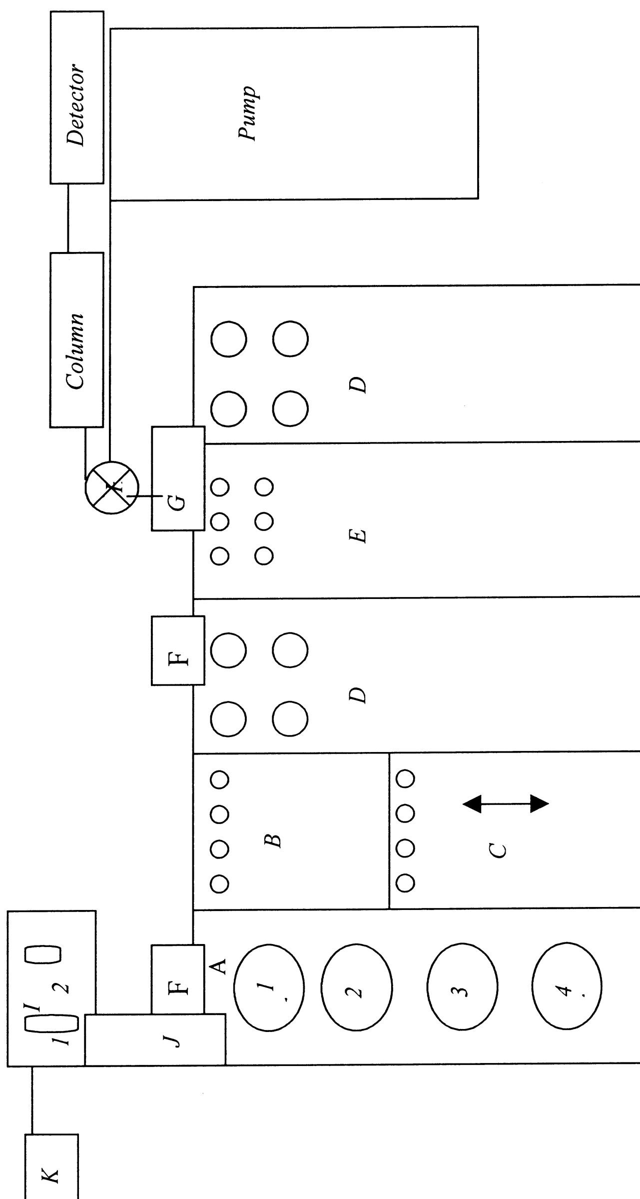

Figure 1. ASPEC System Scheme: (A) rack containing bottles for solvent; (B) collect rack; (C) mobil DEC; (D) rack containing containers glass for diluted sample; (E) rack containing amber glass vials for standard solutions; (F) washings ports (inside and outside the needle); (G) inject port; (H) rheodyne valve; (I) dilutor (1-syringe 10 mL, 2-syringe 500 mL); (J) vertical arm with needle; (K) reservoir: washings solvent for cleaning system.

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Table 1. Comparison of automated and manual methods for naturally contaminated wine samples at different OTA contamination levels.

Level of contamination	Number of replicates	Average (ngmL^{-1}) ± SD, automated method	Average (ngml^{-1}) ± SD, manual method
Low level	10	0.090 ± 0.01	0.086 ± 0.01
High level	10	2.13 ± 0.05	2.12 ± 0.04

subsequent sample was processed. Each sample was purified, injected, and quantified within 45 min.

The automated and manual methods were compared with regard to recovery (Tables 1 and 2).

HPLC Operating Conditions (Manual and Automated Method)

Flow rate: 1.00 mL · min^{-1}. Mobile Phase: acetonitrile : water : acetic acid (50 : 49 : 1 : V : V : V). Fluorescence detection: Excitation wavelength:333 nm, Emission wavelength: 470 nm. Limit of detection: 0.01 ngm L^{-1}.

Calibration Curve

Six appropriate working calibrant solutions were prepared. Each concentration was injected in triplicate. The corresponding graph of calibration curves is shown in Fig. 2. The resulting values showed good linearity with a correlation coefficient of 1.000 and a coefficient of determination of 0.999.

Table 2. Average recovery factors (%), for spiked samples with OTA at different levels, with automated and manual methods.

Spiking level (ng · mL^{-1})	Number of replicates	Recovery (%) ± SD, automated method	Recovery (%) ± SD, manual method
0.1	3	84 ± 3	90 ± 2
0.5	3	85 ± 2	86 ± 3
1	3	90 ± 1	88 ± 2
5	3	95 ± 1	94 ± 2

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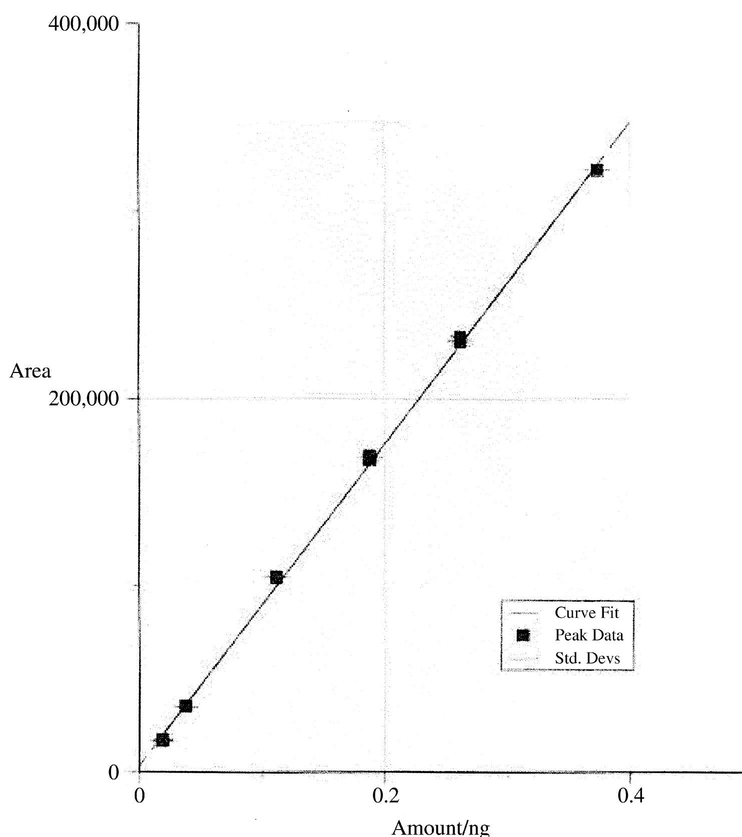

Figure 2. Calibration curve. Linear fit: correlation coefficent: 1.000. Coefficient of Determination: 0.999. Standard Error of Estimate: 3306.461. Fit Coefficients: 0 = 1754.425, 1 = 864756.625.

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Evaluation

Quantification of OTA has been performed by interpolation with the calibration curve. The concentration (C_{OTA}) of OTA was calculated in nanogram per milliliter (ngm L^{-1}) using the equation:

$$C_{\text{OTA}} = M_A \times 3.25 \times V_3/(V_1 \times V_2)$$

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Where:

M_A is the amount of OTA (in ng) in the aliquot of matrix injected on column and determined by calibration curve. 3.25 is the dilution factor. V_1 is the volume of sample analysed (5 mL). V_2 is the volume of test solution injected on column (0.15 mL). V_3 is the volume of dilute extract solution (1.750 mL).

If the method is exactly followed as described, the equation to be used for the calculation of concentration is simplified as follows:

$$C_{\mathrm{OTA}} = 7.58\ M_A$$

Spiking Procedure

All the spiked samples are left in a fume cupboard for two hours to allow the interaction of OTA with the matrix.

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RESULTS AND DISCUSSION

The aim of this study was to carry out an automated HPLC method for OTA detection in wine samples. In order to test the performance of the automated method, two naturally contaminated red wine samples, one at low level (approx. 0.1 ngm L^{-1}) and the second at high level (approx. 2 ngm L^{-1}) were analysed, both with the automated and manual method. The manual method acts currently as the reference method, used by the laboratory, in the performance of the activities related to the official control of analyses for food as requested at national level by the current legislation.[11] The precision (repeatability) of the automated method was tested by comparing the results of ten replicate analyses from both methods (Table 1) and the accuracy by calculating the recovery factors at four levels of contamination (Table 2).

Average levels, standard deviation, and recovery factors satisfactorily fitted each other, with the pre-set values chosen as target values, i.e., 10% for repeatability, 15% for reproducibility at μg kg^{-1} levels, and 70–110% for accuracy evaluation. In addition, all values abundantly fell within the performance characteristics for OTA suggested by CEN[12] that are $\leq 40\%$ as RSD_r at levels of concentration of less than 1 μg/L and $\leq 20\%$ as RSD_r at levels of concentration within 1–10 μg/L. Furthermore, in order to test the internal reproducibility for only the automated method, the same sample with a high level of concentration (approximately 2 μg/L) was analysed ten times in two different days. The results showed good performance, as shown also by the RSD_R value (4.9%) and Horrat values 0.12 (Table 3).

Horrat values were considered as a reference parameter, even if no interlaboratory study was performed.

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Table 3. Performance characteristics of automated method as calculated in a naturally contaminated wine analysed in two different days.

	Average[a] level ($ngmL^{-1}$)	s_r[b]	s_R[c]	r[d]	Internal reproducibility R[e]	RSD_r[f], %	RSD_R[g], %	Horrat[h]
1st Day	2.09	±0.05		0.15		2.6		
2nd Day	2.13	±0.08		0.25		4.1		
Combined	2.11		±0.07		0.29		4.9	0.12

[a]number of replicates for each day = 10.
[b]s_r defined as the standard deviation, calculated from results generated under repeatability conditions.
[c]s_R defined as the Standard deviation, calculated from results under reproducibility conditions.
[d]r defined as the value below which the absolute difference between two single test results obtained under repeatability conditions (i.e., same sample, same operator, same apparatus, same laboratory, and short interval of time) may be expected to lie within a specific probability (typically 95%) and calculated as $r = 2.8 \times s_r$.
[e]R defined as the value below which the absolute difference between single test results obtained under reproducibility conditions (i.e., on identical material obtained by operators in different laboratories, using the standardised test method) may be expected to lie within a certain probability (typically 95%) and calculated as $R = 2.8 \times s_R$.
[f]Relative standard deviation, calculated from results generated under repeatability conditions $[(s_r/\bar{x}) \times 100]$.
[g]Relative standard deviation calculated from results generated under reproducibility conditions $[(s_R/\bar{x}) \times 100]$.
[h]Horrat: RSD_R(observed)/RSD_R(predicted). The RSD_R predicted was derived by the Horwitz equation.

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In order to test the accuracy of the methods, four different spiking levels were chosen, i.e., low (0.1 ngm L^{-1}), medium (0.5 ngm L^{-1}), and high (1 and 5 ngm L^{-1}) levels of contamination. The spiking levels were chosen on the basis of the considerations that should lead to set maximum tolerable legal limits.

All the obtained values fell within 80–95% showing good performance of both methods (Table 2).

As far as time of analysis is concerned, a meaningful saving was obtained with the automated method in comparison with the manual one. Specifically, on the basis of a whole analytical cycle of 20 samples in a day, each sample was analysed within 45 min in the case of automated method vs approximately 70 min with the manual method. A relevant matter is to consider the extremely advantageous rationalisation of the personnel and the daily work. In fact, with the automated method the employment of personnel is strongly reduced from a full employment of the operator estimated in 8 hours of daily work with the manual method, down to 90 min for giving the start up to the whole analytical cycle of 20 samples using the automated method. This method can be satisfactorily applied for both white and red wines.

CONCLUSIONS

From the above, it can be concluded that the automated method tested for accuracy and precision showed good performance and reliability when compared to the reference manual method. The advantages coming out from this method are, therefore, the saving of time of analysis, the possibility to analyse a large amount of samples, the reduction of the employment of personnel, and the obtaining of all the requirements requested by the national legislation dealing with the official control of foodstuffs. For this reason, this method is predominantly addressed both to public laboratories involved in this activity, and to private laboratories dealing with the HACCP principles implementation, and it can be also used for monitoring studies involving a large number of samples to be analysed.

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Received July 25, 2002
Accepted August 30, 2002
Manuscript 5908

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