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Determination of azolic fungicides in wine by solid-phase extraction and high-performance liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry☆

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Abstract

A method for simultaneous analysis of eight azolic fungicides: cyproconazole, diniconazole, tetraconazole, thiabendazole, flusilazole, triadimenol, triadimefon, carbendazim and the degradation product 2-aminobenzimidazole in wine samples is described. The compounds are isolated from the samples and concentrated by solid-phase extraction on polymeric cartridges. The determination is carried out by liquid chromatography with mass spectrometric detection in positive ionization and selected ion monitoring modes. The influence of parameters such as the mobile phase composition, column temperature, corona current and fragmentor voltage is studied and the proposed method is validated. Recoveries of the nine compounds added to wine samples range from 83 to 109%, with relative standard deviations below 10%. The quantitation limits are between 9 and 31 μg/L. Real wine samples are analyzed by the proposed method, also.

Keywords: Wine; Azolic fungicides; HPLC; Atmospheric pressure chemical ionization

1. Introduction

There are many pests and diseases that affect negatively to vineyards, influencing the vine physiology and, therefore, fruit yield and quality. The need for phytosanitary treatments in vineyards has the disadvantage of potential appearance of pesticide residues in grapes that can pass into must and may remain in wine, affecting not only the wine-making process but also the wine quality and, thereby, entering the food chain [1–3].

Among the chemical compounds that winegrowers can use to control pests in vine (*Vitis vinifera* L.) azolic pesticides are very usual, mainly as fungicides. Some of them have shown till now a good efficacy, but the apparition of resistance phenomena have led to the synthesis of new compounds, belonging to the same chemical family, but with better characteristics. For this reason, nowadays there are some well-known products in use, simultaneously with new ones, so their apparition altogether in some samples is possible. Because of that, it is necessary to have an analytical methodology able to determine the residues of these fungicides in wine samples.

Numerous analytical methods for determining pesticide residues in different fruits and vegetables have been reported, also in grape, must and wine. In these latter, some of the compounds here considered have been studied, mainly benomyl, carbendazim and thiabendazole, usually in conjunction with other compounds with different chemical moiety, but never the entire group of azolic compounds. The techniques usually employed to determine residues of azolic compounds are varied, although the chromatographic ones are the predominant. Gas chromatography with nitrogen–phosphorous, electron capture or mass spectrometric detection has been widely

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used [4–12], in many instances to develop multiresidue analysis methods where some azolic compounds are included [13–16]. However, taking into account that the target compounds usually have a low volatility and are thermally labile, liquid chromatography seems to be the best choice, so there is an increasing number of references [17–32] where this technique with ultraviolet (UV), diode array (DAD) and mass spectrometric (MS) detectors turns out to be an excellent solution. More recently, capillary electrophoresis [33–35] is showing good features to analyze compounds of this chemical family.

To prevent matrix-induced effects that give apparent recoveries [36] it is necessary to select adequately the extraction and clean-up procedures; although liquid–liquid extraction with different solvents is frequently recommended [5,19,37], nowadays, solid-phase extraction [5,12,13,23–28] or solid phase microextraction [9,30,38] are being preferred. Moreover, the last ones allow the simultaneous concentration of the analytes. In this paper, we report the optimization and validation of a method using solid-phase extraction on polymeric cartridges and a combined HPLC–APCI–MS technique that allows the sensitive determination of all possible residues of nine azolic fungicides, including a degradation product, in wine samples.

2. Experimental

2.1. Material and chemicals

The fungicides (cyproconazole, diniconazole, tetraconazole, flusilazole triadimenol, triadimefon, carbendazim and the degradation product: 2-aminobenzimidazole) were supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany) and thiabendazole standard by AccuStandard (New Haven, CT, USA) all of them with a minimum certified purity of 98%. Table 1 shows the chemical characteristics of the compounds.

HPLC-grade methanol, acetonitrile and 2-propanol were obtained from Labscan Ltd. (Dublin, Ireland). Ammonium acetate, glacial acetic acid and ammonium hydroxide were purchased from Scharlab (Barcelona, Spain). Ultrapure water was obtained in a Milli-RO plus system together with a Milli-Q system from Millipore (Bedford, MA, USA). All the solvents and solutions were passed through a 0.45 μm nylon filter from Phenomenex (Torrance, CA, USA) before use. For SPE, Oasis HLB (200 mg) cartridges were supplied by Waters (Milford, MA, USA).

2.2. Preparation of standards and spiked samples

Stock solutions of diniconazole, cyproconazole, flusilazole, tetraconazole, triadimenol, triadimefon, thiabendazole and 2-aminobenzimidazole were prepared in acetonitrile at a concentration of 100 mg L^{-1} and the carbendazim in methanol at 100 mg L^{-1}. Working solutions of pertinent concentrations (10–1000 μg L^{-1}) to prepare a calibration graph were made daily by an appropriate combination and a serial dilution of standard solutions with water; coefficients of correlation, r^2, were 0.990, at least. All standards and stock solutions were stored in glass-stopper bottles at +4 °C.

Recovery tests were carried out by adding known volumes of a mixed standard solution to the wine samples. The concentrations of the fungicides in wine were 0.05, 0.50 and 2 mg L^{-1}. Rose, red and white wines were spiked.

2.3. Solid-phase extraction procedure

The extraction procedure was studied on samples of 10 mL of rose wine spiked with 50 μg L^{-1} of each compound. The influence of the following aspects was evaluated. The volume of wine sample was changed from 10 to 75 mL, considering an 1:1 dilution of the sample with water, also. The extract retained on the stationary phase was eluted with 6 mL of water, water/methanol, water/2-propanol, water/acetonitrile and water/ethanol; the latter in a proportion 90/10. The necessity of drying the cartridges was also investigated; cartridges were dried by passing nitrogen through them during 5, 15 and 30 min. Finally, the volume of methanol to elute the retained compounds was varied between 1 and 6 mL. The type of wine: white, rose or red was also considered. All the assays were made in triplicate.

The final method for the sample preparation consisted of the extraction of 50 mL of wine samples (red, rose or white) diluted with water (1:1, v/v) which were loaded on a SPE polymeric cartridge (previously conditioned with 6 mL of methanol and 6 mL of water) at about 5 mL/min using a suction system. Then, the analytes were eluted with 3 mL of methanol by gravity; an aliquot of the collected liquid, whose final volume was not verified, was injected into the HPLC system.

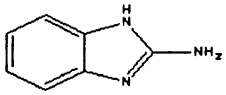
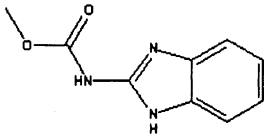
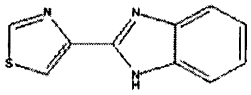
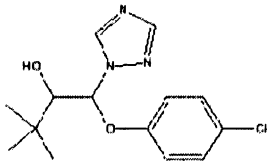
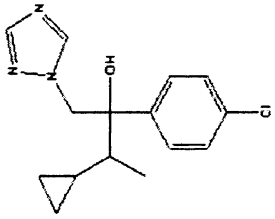
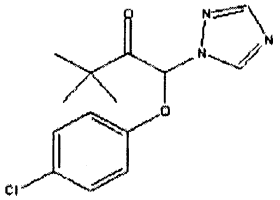
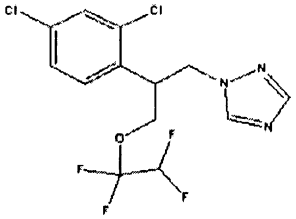
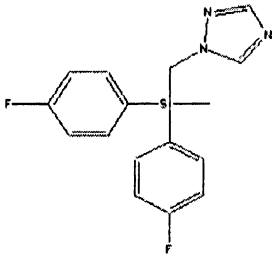
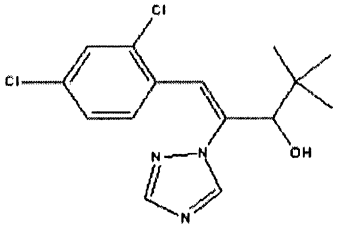
2.4. Instrument: MS and HPLC conditions

An Agilent Technologies (Palo Alto, CA, USA) 1100 Series LC//MSD system consisting of a vacuum degasser, a quaternary solvent pump, an autosampler with a column oven and a MSD coupled with an analytical workstation was used. The mass detection system was equipped with a standard APCI source.

A Synergy Max-RP C12 (250 mm × 4.6 mm, 4 μm) column and a guard-column Security Guard Max-RP C12 (4.0 mm × 3.0 mm), both from Phenomenex (Torrance, CA, USA) were used to separate the analytes.

As regards the separation, the influence of some working conditions was studied. Thus, the percentage of organic modifier in the mobile phase consisting of 10 mM ammonium acetate and acetonitrile was varied between 40 and 50% for isocratic assays, the ionic strength of the mobile phase was modified through the concentration of ammonium acetate: 10, 30 or 50 mM, the pH was varied from 3 to 9 by adding

Table 1
Chemical characteristics of the studied compounds

Name	Chemical structure	Molecular weight	Molecular formula
2-Aminobenzimidazole		133	$C_7H_7N_3$
Carbendazim		191	$C_9H_9N_3O_2$
Thiabendazole		201	$C_{10}H_7N_3S$
Triadimenol		295	$C_{14}H_{18}ClN_3O_2$
Cyproconazole		291	$C_{15}H_{18}ClN_3O$
Triadimefon		293	$C_{14}H_{16}ClN_3O_2$
Tetraconazole		371	$C_{13}H_{11}Cl_2F_4N_3O$
Flusilazole		315	$C_{16}H_{15}F_2N_3Si$
Diniconazole		325	$C_{15}H_{17}Cl_2N_3O$

acetic acid or ammonium hydroxide, the column temperature was increased from 25 to 55 °C in 10 °C increments and the assayed injection volumes were comprised 20 and 100 μL.

In the same way, the influence of all those parameters of the APCI interface that could affect to the detector response was studied by flow injection analysis with a mobile phase made up of 10 mM ammonium acetate and acetonitrile in proportion 70:30, respectively. It was verified that only the corona current and fragmentor voltage affected the response. The first one was varied from 1 to 10 μA and the second one from 50 to 300 V. Full-scan spectra were obtained by scanning from *m*/*z* 100 to *m*/*z* 400.

The final conditions of the HPLC–APCI–MS system for the analyses are as follows. The column temperature was set at 35 °C. The solvents used in the mobile phase were acetonitrile and a solution of 10 mM ammonium acetate in water, at a flow rate of 1.0 mL min^{-1}. In gradient-elution analysis, the first mobile phase was 15% acetonitrile, increased linearly to 45% in 15 min and held at 45% for 35 min. A return to the initial conditions was carried out in 8 min. The injection volume was 80 μL. Operating conditions of the APCI interface in positive ionization mode were: vaporizing temperature, 300 °C; nebulizer gas (N_2) at a pressure of 50 psi; drying gas (N_2) at a flow rate of 6 L min^{-1} and temperature of 350 °C; capillary voltage, 2500 V; fragmentor voltage, 150 V and corona current, 4 μA. Electron multiplier voltage was set 300 V-units above the autotune value. Time scheduled SIM of the most abundant ions of each compound was used for quantitation.

3. Results and discussion

3.1. HPLC conditions

The results of the HPLC optimization are summarized. Mobile phases were prepared with acetonitrile instead of methanol because the first one enhanced notably the peak symmetry. A linear gradient of mobile phase was necessary to reach the whole elution of the analytes in a reasonable time. The ionic strength did not alter practically the retention of the analytes and the symmetry of the chromatographic peaks while the acidic pHs decreased the retention of 2-aminobenzimidazole and diniconazole. Moreover, some compounds were degradated at pH below 4. As regards the influence of the temperature, a loss of symmetry was observed at middle and high temperatures for the three lowest retained compounds: 2-aminobenzimidazole, carbendazim and thiabendazole. Finally, an injection volume of 80 μL was used because for higher injection volumes the linear capacity of the column was surpassed: the chromatographic peaks began to be slightly deformed and the retention times varied. Ionization and corona needle dirtiness problems were not observed as a consequence of the use of high percentages of acetonitrile in the mobile phase; ammonium acetate was always added to mobile phase to improve the ionization of the analytes.

3.2. Mass spectrometry optimization

The first experiments to select the optimum MS parameters and the appropriate ions were carried out by flow injection analysis (FIA) of the individual solutions of the fungicides. It could be observed that fragmentor voltage and, to a lesser extent, corona current were the parameters that had the greatest influence on the sensitivity of the detection. In general, the signal was higher in the range from 4 to 8 μA, so a value of 4 μA was finally selected. Moreover, the signals of the compounds increased when the fragmentor voltage increased up to 150 V, for higher fragmentor voltages the signals decreased. A voltage of 150 V supplied the highest signals for most of the fungicides and was selected as suitable for their analysis.

The acquisition data in positive ionization mode supplied higher chromatographic peaks for most of the analytes in comparison with those obtained in the negative ionization mode. This behaviour was expected because the studied compounds have a basic character. Only the monitoring of carbendazim, 2-aminobenzimidazole and thiabendazol in negative ionization mode supplied slightly higher signals.

Table 2 shows the relative abundances of the main ions observed in the spectra for two fragmentor voltages. The protonated molecular ions and the corresponding isotopic signals were the predominant ions in the spectra except for carbendazim. In a previous manuscript [29], a neutral loss from the molecule that corresponds to the triazol ring is reported for some triazolic compounds: the abundances of the generated ions are about 50%. We have also observed these losses for three compounds: triadimefon ($[M + H\text{-}C_2HN_3]^+$, ion 225), flusilazole ($[M + H\text{-}C_2HN_3]^+$, ion 247) and triadimenol ($[M - C_2HN_3]^+$, ion 228) but the abundances were below 4%. Perhaps, the different abundances could be attributed to the different mobile phase compositions: methanol–water was used as mobile phase in the above-mentioned manuscript.

Only for carbendazim and its degradation product 2-aminobenzimidazole some notable ions-fragments were observed. For carbendazim, the loss of methanol from the methyl ester (ion 160) is a minor fragmentation while a satisfactory explanation for the ion at *m*/*z* 134 was not found; the mass differences between the pseudomolecular ion and these fragments are similar to those observed in the electron impact ionization spectrum of carbendazim recorded by using a particle beam interface [39].

SIM mode was used to obtain the maximum sensitivity in the quantitative analysis. Table 2 also indicates the mass-to-charge (*m*/*z*) ratios chosen for each compound and the time scheduling.

3.3. Fungicide residue extraction

3.3.1. Volume sample and dilution with water

When spiked wine samples were analyzed, carbendazim and 2-aminobenzimidazole recoveries were notably lower in comparison with the other fungicides which was attributed

Table 2
Main ions and their relative abundances (in %) obtained by FIA-APCI-MS at the working conditions

Group start time (min)	Compound		Tentative ion (*m*/*z*)	Relative abundance, fragmentor voltage (V)	
				50	150
5	1	2-Aminobenzimidazole	134 $[M+H]^+$ [a]	100	100
			175 $[M+H+CH_3CN]^+$	30	–
10	2	Carbendazim	192 $[M+H]^+$	15	3
			160 $[M+H]^+$–CH_3OH	27	36
			134 $[M+H]^+$–HCN–CH_3O or $[M+H]^+$–NCO–CH_3–H or –CO–CH_2O [a]	100	100
	3	Thiabendazole	202 $[M+H]^+$ [a]	100	100
24	4	Triadimenol	298 $[M+H]^+$ *	38	37
			296 $[M+H]^+$ [a]	100	100
			294 $[M-H]^+$	8	7
	5	Cyproconazole	294 $[M+H]^+$ *	32	35
			292 $[M+H]^+$ [a]	100	100
34	6	Triadimefon	296 $[M+H]^+$ *	37	36
			294 $[M+H]^+$ [a]	100	100
	7	Tetraconazole	376 $[M+H]^+$ *	14	16
			374 $[M+H]^+$ *	67	74
			372 $[M+H]^+$ [a]	100	100
	8	Flusilazole	316 $[M+H]^+$ [a]	100	100
47	9	Diniconazole	330 $[M+H]^+$ *	13	15
			328 $[M+H]^+$ *	67	75
			326 $[M+H]^+$ [a]	100	100

[a] Ion used in SIM mode.
* Isotopic signal; M: molecular ion, –: not detected.

to the ethanol content of the wine sample. So, experiments involving a dilution 1:1 (v/v) of the sample with water were devised. Table 3 shows the recoveries after diluting a wine sample of 10 mL. As it can be observed, the recoveries improved for all the compounds, particularly for carbendazim and 2-aminobenzimidazole.

The sample volume was increased up to 75 mL, keeping constant the dilution-rate, to enhance the detection limits. Results are shown in Table 3, also. The recoveries were almost constant up to a volume of 50 mL decreasing for higher volumes. A volume of 50 mL followed by a dilution 1:1 was clearly the best option. The coefficients of variation were always comprised between 2 and 5% ($n = 3$).

3.3.2. Washing of the cartridges after eluting the sample

The cartridges were washed with different solvent mixtures to test the influence of this step. So, 50 mL of wine diluted with water in proportion 1:1 were eluted through the cartridges. The recoveries with the mixtures of water with methanol, ethanol and 2-propanol, always in proportion 90:10, were similar, only with the water/acetonitrile solution the recoveries were somewhat lower. It was verified that the inclusion of the washing step did not supply simpler chromatograms, so this step was removed from the sample preparation procedure. Finally, it was verified that the quantitative data and chromatograms were also similar after drying or not the cartridges before eluting the analytes.

Table 3
Recoveries and coefficients of variation (both in percentage) obtained in the solid-phase extraction after conditioning the cartridges with 6 mL of methanol and 6 mL of water and loading different sample volumes diluted with water ($n = 3$)

	Recovery (coefficient of variation) in percentage				
Volume of wine (mL):	10	10	20	50	75
Volume of water (mL):	0	10	20	50	75
2-Aminobenzomidazole	36.8 (4.2)	59.6 (2.8)	59.5 (3.2)	59.3 (2.9)	43.2 (4.8)
Carbendazim	54.4 (3.6)	69.2 (3.1)	70.1 (2.8)	68.7 (2.9)	53.1 (3.6)
Thiabendazole	83.4 (3.2)	85.9 (3.3)	86.5 (3.1)	85.2 (2.7)	75.5 (3.5)
Triadimenol	85.3 (3.0)	90.3 (2.5)	88.9 (2.9)	90.6 (2.6)	75.7 (3.6)
Cyproconazole	91.6 (3.8)	98.7 (3.4)	96.1 (2.4)	97.9 (2.8)	85.4 (2.5)
Triadimefon	92.4 (2.4)	96.4 (2.9)	98.4 (2.8)	98.6 (2.7)	79.4 (4.6)
Tetraconazole	95.0 (3.2)	98.9 (2.2)	97.2 (3.1)	98.4 (3.2)	87.1 (3.4)
Flusilazole	95.4 (2.6)	97.7 (3.2)	96.1 (2.3)	98.0 (2.4)	90.0 (3.0)
Diniconazole	100.9 (2.5)	102.8 (2.1)	98.9 (3.1)	100.6 (2.9)	88.9 (2.8)

3.3.3. Elution of the extract

Methanol was the best solvent to elute the analytes from the cartridges. The use of solvents such as acetonitrile, acetone and ethyl acetate was initially considered. A volume of 3 mL of methanol was chosen to elute the compounds because from a volume equal or higher than 3 mL the recoveries were similar.

In conclusion, the solid-phase extraction involves the use of Oasis cartridges conditioned with 6 mL of methanol and water, the elution of 50 mL of sample diluted with water and the subsequent elution of the extract with 3 mL of methanol without any washing or drying of the cartridges.

3.4. Validation of the procedure for HPLC–APCI–MS

Validation was carried out following the ICH guidelines [40] and IUPAC technical report of 2002 [41], determining selectivity, limits of quantitation and detection, linearity, precision and trueness. Selectivity was checked by injecting extracts of non-spiked wine samples; it can be deduced from Fig. 1 that there are not interferences in the extracts of a red wine. The same happened for white and rose wines. The proposed conditions generated narrow and reproducible chromatographic peaks, except for cyproconazole due to the coelution of two isomers. A *m*/*z* ratio characteristic of each fungicide was used to monitor them because flusilazole and thiabendazole had not another high ions in the spectrum: their isotopic signals were low. Obviously, the monitoring of two or three ions for each analyte increases the selectivity.

The detection limit (LOD) and quantitation limit (LOQ) were determined injecting a number of extracts of non-spiked wine samples ($n = 6$) and measuring the magnitude of the background analytical response. We estimated the LOD and LOQ as three or ten times the signal-to-noise ratio, respectively. Noise was similar regardless the wine matrix: white, red or rose. The values obtained, for each compound, are listed in Table 4.

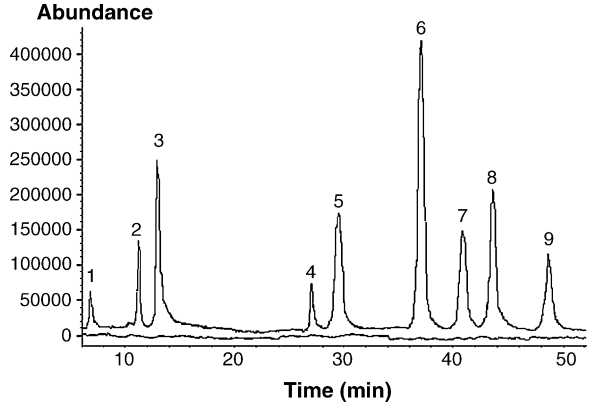


Fig. 1. HPLC–APCI–MS (in SIM mode) chromatograms of an extract of rose wine spiked with 50 μg L^{-1} of each compound and a non-spiked red wine. See Table 2 for peak identification.

Table 4
Slope, linearity, detection limit (LOD) and quantitation limit (LOQ) obtained after applying the proposed method and using a calibration graph made from extracts of spiked samples

Compound	Slope	Linearity (%)	LOD (μg/L)	LOQ (μg/L)
2-Aminobenzoimidazole	826	99.36	6	23
Tiabendazole	1066	99.79	8	27
Carbendazim	4981	99.76	4	14
Triadimenol	1190	99.70	7	31
Cyproconazole	4033	99.71	7	28
Triadimefon	13485	99.78	3	12
Tetraconazole	3405	99.72	7	28
Flusilazole	6690	99.79	6	21
Diniconazole	5195	99.79	2	9

With the aim of estimating the linearity of the calibration graph we used now a matrix-standard calibration: wine samples were spiked with variable amounts of fungicides, between 20 and 1500 μg L^{-1}, and subjected to the same treatment as the samples. The extracts obtained were considered as standards to obtain the calibration graphs. Plotting the peak areas versus the concentration for six standards, the graphs obtained were straight lines of intercept not significantly ($p < 0.05$) different from zero, which confirmed the linearity through the range studied and the lack of bias. The slope and linearity obtained for each compound are listed in Table 4.

The precision (repetitivity) was evaluated by the same analyst; six determinations were made on a same spiked wine sample and for three different concentrations: 0.05, 0.5 and 2 mg L^{-1}. Results showed that the coefficients of variation were lower than 10%. Recovery and trueness were determined on spiked samples of red, white or rose wines at the three above-mentioned concentration levels. The mean recoveries ranged from 83.3 to 108.8%. Table 5 shows the mean results obtained for the three types of wine after the matrix-standard calibration. An analysis of variance made with the raw data revealed the absence of significant differences ($p < 0.05$) between the assayed levels of spiking and between the three types of wine.

The validated method was applied to the determination of possible fungicide residues in wine. A set of 60 wine samples

Table 5
Mean recoveries and precisions (both in percentage) for the concentrations 0.05, 0.5 and 2 mg L^{-1} after spiking the wine samples ($n = 6$)

	Recovery (coefficient of variation) (%)		
	White wine	Red wine	Rose wine
2-Aminobenzoimidazole	89.3 (7.8)	83.3 (7.9)	91.5 (8.6)
Thiabendazole	102.4 (6.5)	94.5 (5.7)	96.7 (5.9)
Carbendazim	102.5 (9.1)	108.8 (8.4)	102.6 (8.4)
Triadimenol	99.8 (5.6)	99.4 (7.0)	99.0 (6.7)
Cyproconazole	99.9 (8.0)	94.8 (8.4)	94.8 (7.5)
Triadimefon	100.2 (5.9)	102.3 (4.4)	101.9 (6.8)
Tetraconazole	101.2 (4.6)	104.5 (6.6)	97.7 (7.1)
Flusilazole	100.0 (7.7)	99.5 (6.0)	98.4 (6.5)
Diniconazole	97.2 (7.1)	94.6 (8.0)	99.3 (8.1)

collected in three Apellations of Origin of our Country were selected to analyze the residues of these fungicides, none of them gave results over the detection limits.

4. Conclusions

A sensitive method for the simultaneous determination of azolic fungicides in wine samples was developed. A simple solid-phase extraction on polymeric cartridges of the samples diluted with water (1:1, v/v) was enough to obtain satisfactory recoveries. A clean-up step was not required. A mass spectrometric detection in positive chemical ionization mode allowed to determine all the analytes.

The benzimidazolic compounds: carbendazim, 2-aminobenzimidazole and thiabendazole were the least retained analytes on the cartridges. The sample preparation and determination techniques were in compliance with the maximum residue limits usually accepted for the compounds in wine samples.

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