Liquid Chromatography Determination of Simazine and Antimycin A in Must

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A method for extraction and determination of trace amounts of simazine and four antimycins A in must is described. A 5.0 mL sample was extracted in two portions of 3.0 mL of diethyl ether at pH 5.0. After removal of the solvent, the extracted sample was dissolved in 1.0 mL of 20 mM acetic acid/sodium acetate aqueous solution (pH 5)–methanol (20:80) and analyzed by liquid chromatography with UV detection at 230 nm. Recoveries of simazine and antimycin A at the 0.45–3.0 mg L^{-1} level were between 79 and 106% with standard deviation between 2.3 and 4.5% ($n = 8$). Detection limits for analytes in must were 20, 90, 115, 50, and 45 $\mu g\ L^{-1}$ for simazine, antimycin A_1, antimycin A_2, antimycin A_3, and antimycin A_4, respectively.

Keywords: *Must; simazine; antimycin; liquid chromatography*

INTRODUCTION

Simazine [2-chloro-4,6-bis(ethylamino)-1,3,5-triazine] is one of the most widely used chlorotriazine herbicides. It is applied as a pre- and post-emergent weed control agent to improve crop yields. This pesticide is very persistent in soils, around 18 months, due to its low solubility in water and high chemical stability (Barceló, 1988). Consequently, simazine is usually included in multiresidue analysis of pesticides in food and beverages, including wines and derivatives (Yess, 1992; Holland et al., 1994). Currently used methods for the analysis of simazine involve sample enrichment by liquid–liquid (Ambrus et al., 1981) or solid phase extraction (Battista et al., 1989; Durand et al., 1989; Holland et al., 1994) followed by gas chromatography (Bagheri et al., 1992; Bernal et al., 1992), gas chromatography/mass spectrometry (Pereira et al., 1990; Durand and Barceló, 1991),, and liquid chromatography (Battista et al., 1989; Owens and Sturrock, 1986; Schüssler, 1989).

Antimycin A is the antibiotic responsible for the antifungal activity of certain *Streptomyces* species. This antibiotic is a mixture of at least four active components (Figure 1). Component A_3 has an antibiotic potency several times higher than that of A_1, but both are equally effective as inhibitor systems of mammalian tissue. Antimycin A is active against fungi and certain insects. It has been established that antimycin A is a very active inhibitor of respiration, and it is known that antimycin A blocks the reoxidation of cytochrome *b* by cytochrome *c* (Chance, 1956). Because of the low volatility of antimycin A, derivatization is necessary for its determination by gas–liquid chromatography; however, sensitivity limitations still remain. Consequently, HPLC techniques are currently applied, using several detectors such as UV (Abidi, 1988), chemical ionization MS (Abidi, 1982), or nuclear magnetic resonance (NMR) (Ha et al., 1989).

On the other hand, herbicide and fungicide residues can be present at low levels in must and fruit juice (Cabras et al., 1987) as a result of extensive treatment for weed control and to avoid fungi attacks. Moreover, some fungicides were used to avoid juice fermentation. EC Regulation 822 (1987) bans the use of antimycin A and other antiseptics for must preservation in the European community, but they can be used for sterilization of corks for bottles of wine and must (Konrad, 1982).

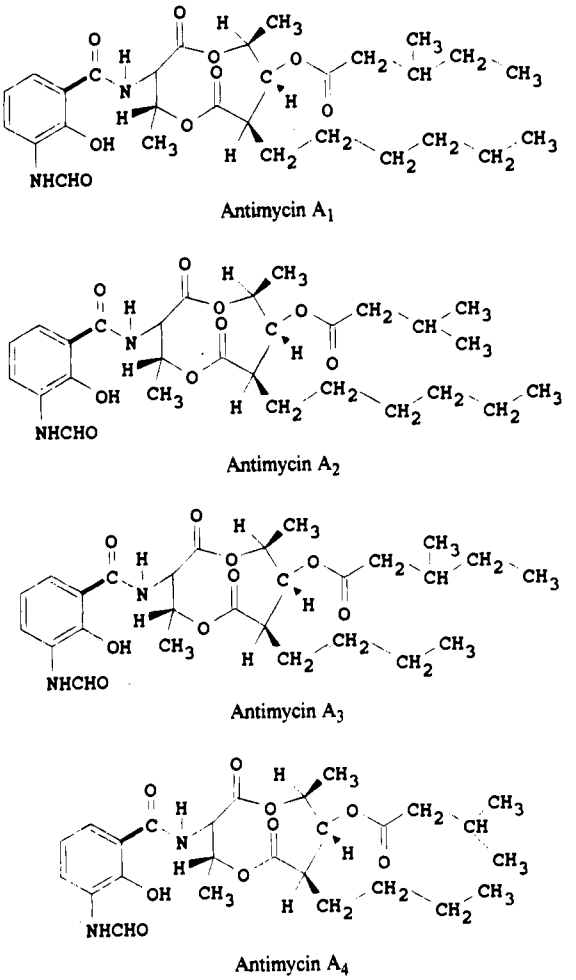

Figure 1. Structural formulas of antimycin A.

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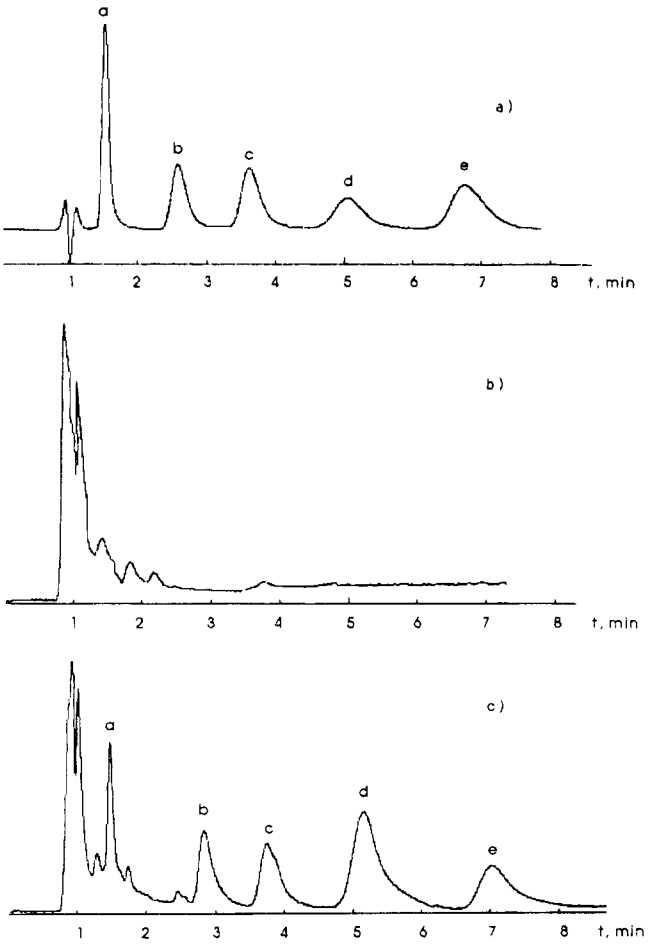

Figure 2. (a) HPLC chromatogram of simazine and antimycin A (simazine 0.72 μg mL^{-1}, four antimycin 1.6 μg mL^{-1}). (b) HPLC chromatogram of the unspiked must. (c) HPLC chromatogram of the spiked must (simazine 0.50 μg mL^{-1}; antimycin A_4, A_3, A_1 1.0 μg mL^{-1}; antimycin A_2 2.9 μg mL^{-1}). All chromatograms are performed at 20 °C: flow rate, 1.5 mL min^{-1}; chromatographic column, Spherisorb 5 ODS(2); mobile phase, 20 mM acetic acid/sodium acetate aqueous solution (pH 5)–methanol (20:80); UV detection at 230 nm. Peaks: (a) simazine; (b) antimycin A_4; (c) antimycin A_3; (d) antimycin A_2; (e) antimycin A_1.

A new method has been developed to determine simazine and antimycins A_1, A_2, A_3, and A_4 in bottled white must from Airen grapes, a white cultivar, by using liquid–liquid extraction and HPLC-UV determination.

EXPERIMENTAL PROCEDURES

Equipment. Analyses were performed with a Perkin-Elmer isocratic LC pump coupled with a Perkin-Elmer Model LC 290 UV–vis detector. Chromatographic separation of simazine and antimycins was performed with a reversed-phase Spherisorb 5 ODS(2) column (150 × 4.6 mm). The analyte quantitation was carried out with the Nelson software package.

Chemicals. All chemicals were of analytical grade, solvents were of HPLC grade, and purified water was obtained using a Milli-Q apparatus system (Millipore). Simazine (99% pure), supplied by Riedel-De-Häen, and antimycins A_1 (>90% pure), A_2 (>90% pure), A_3 (>90% pure), and A_4 (>90% pure), by Sigma, were used for preparing 60 mg mL^{-1} stock standards in methanol. Antimycin solutions were stored below −10 °C and their stability tested periodically by spectrophotometric measurement.

Sample. Bottled white must from the Airen grape variety was analyzed.

Table 1. Analytical Characteristics[a]

compd	LOD (μg L^{-1})	s_r[b], %	calibration equations[c]
simazine	30	1.3	$A = 10.6 \times 10^3 + (8.6 \times 10^4)c$
antimycin A_4	130	1.4	$A = -4.8 \times 10^3 + (4.7 \times 10^4)c$
antimycin A_3	90	1.2	$A = -1.5 \times 10^3 + (2.7 \times 10^4)c$
antimycin A_2	100	0.7	$A = 3.1 \times 10^3 + (3.0 \times 10^4)c$
antimycin A_1	60	1.3	$A = 1.5 \times 10^3 + (2.8 \times 10^4)c$

[a] Mobile phase, 20 mM acetic acid/sodium acetate aqueous solution (pH 5)–methanol (20:80); chromatographic column, Spherisorb 5 ODS(2); injection volume, 20 μL; wavelength, 230 nm. [b] n = 6 (20 ng). [c] A = peak area; c = concentration (μg mL^{-1}).

Table 2. Percent Recoveries of Simazine and Antimycin A as a Function of pH and the Extractant Solvent[a]

	hexane		diethyl ether	
compd	pH 2[b]	pH 5[b]	pH 2[b]	pH 5[b]
simazine	51.2	25.8	48.9	79.0
antimycin A_4	98.9	80.9	69.6	91.5
antimycin A_3	112	99.3	87.4	90.4
antimycin A_2	148	123	96.2	96.5
antimycin A_1	191	161	110	101

[a] Spiked level 1.0 μg mL^{-1}. [b] n = 3.

Procedure. *HPLC Determination.* Twenty microliters of sample was injected into the chromatographic systems. Retention times were measured with the temperature maintained at 20 °C and a flow rate of 1.5 mL min^{-1} using a mobile phase of 20 mM acetic acid/sodium acetate aqueous solution (pH 5)–methanol (20:80). The best wavelength for the determination of the analytes studied was 230 nm according to their UV spectra.

Preparation of Samples. Must samples were fortified with amounts between 0.45 and 3.0 mg L^{-1} of each compound studied, and the pH was adjusted to 5.0 with a 60 mM acetic acid/sodium acetate buffer solution. Two sequential extractions were carried out with 3 mL of diethyl ether on 5.0 mL of the sample in a glass screw-capped tube, and the mixture was shaken for 2 min each time and centrifuged for 1.5 min at 3000 rpm. After the phase separation stage, the two organic extracts were collected and evaporated first under reduced pressure in a rotary evaporator, and then the residue was dried in a nitrogen stream and dissolved in 1.0 mL of the chromatographic mobile phase (direct nitrogen evaporation is also possible). Before injection, the extract was filtered through a nylon microfilter (0.45 μm). Fortified samples were analyzed by following the above procedure just after fortification.

RESULTS AND DISCUSSION

Optimization of Chromatographic Conditions. Since antimycin A is labile at high pH values, the studies were carried out in 63 mM acetic acid (pH 3.0)–methanol and in 20 mM acetic acid/sodium acetate buffer (pH 5)–methanol (20:80) as mobile phases. During preliminary investigations, several water–methanol mixtures were studied as mobile phases. In general, the capacity factors (k) decreased when the percentage of methanol was increased, as expected. At the same water–methanol ratio, capacity factors at pH 5.0 were lower than those at pH 3.0. From these results, a 20 mM acetic acid/sodium acetate (pH 5) aqueous solution–methanol (20:80) mobile phase was chosen for further studies.

Analytical Characteristics for Standards. The instrumental characteristics of the method have been studied. Resolution for the closest peaks (antimycin A_2 and antimycin A_3, peaks c and d in Figure 2) was 1.7, indicating resolution to the baseline. A good linear response in the range 0.4–60 mg L^{-1} (linear regression

Table 3. Recoveries in Must

compd	amount added (mg mL^{-1})	recoveries, $n = 3$ (%)
simazine	0.72	83
	0.96	79
	1.2	77
	1.4	78
	1.7	78
	1.9	79
	2.2	79
	2.4	80
		mean = 79
		s_r, % = 2.3
antimycin A_4	0.45	93
	0.56	91
	1.0	89
	1.1	89
	1.5	89
	1.7	91
	2.2	96
	2.9	94
		mean = 92
		s_r, % = 2.6
antimycin A_3	0.64	88
	0.90	89
	1.1	88
	1.4	90
	1.7	94
	2.2	93
	2.6	89
	3.1	92
		mean = 90
		s_r, % = 2.8
antimycin A_2	0.52	92
	0.83	92
	1.0	97
	1.3	101
	1.5	95
	1.9	96
	2.1	96
	2.5	105
		mean = 97
		s_r, % = 4.5
antimycin A_1	0.52	110
	0.83	105
	1.0	108
	1.3	103
	1.5	106
	1.9	105
	2.1	102
	2.5	106
		mean = 106
		s_r, % = 2.6

coefficient between 0.9995 and 0.9999) was achieved for simazine and the four antimycins. The limits of detection (3/1 signal-to-noise ratio), calibration equations for each component, and reproducibility of the chromatographic method for 20 ng of each compound are shown in Table 1.

Figure 2a shows the chromatogram obtained under the proposed experimental conditions.

Determination of Simazine and Antimycin A in Must. Several solvents such as hexane, toluene, and diethyl ether have been used for extraction of simazine (Durand et al., 1991; Steinwandter, 1990) in soil, fruit, and juice. In this study, these solvents were tested for extraction of simazine and antimycin A from must. Toluene did not show satisfactory results due to coextraction of other compounds; although it was possible to quantify simazine, antimycin A_1, antimycin A_2, and antimycin A_3, toluene did not allow the quantitation of antimycin A_4 due to its serious overlapping with endogenous compound peaks. Better results were obtained with *n*-hexane or diethyl ether. Since must is a natural product with a great variety of organic compounds (i.e. polyphenols, aldehydes, and esters) whose extractabilities change with the pH, it became necessary to perform first a pH study. The pH of the commercial must studied was 3.5, and assays made with *n*-hexane and diethyl ether as extractants showed erratic results with recoveries between 18 and 400%. More reproducible results were obtained when the pH was adjusted to between 2.0 and 5.0 with buffered solutions. Table 2 shows the results of recoveries obtained at both pH values and with the two solvents used. High recoveries for antimycin A_1 and antimycin A_2 were obtained with hexane, due to the presence of interfering peaks, not well resolved, from the coextracted endogenous compounds. From these results, diethyl ether as extractant and pH 5.0 were chosen as the optimum conditions.

The study of recoveries and reproducibility was carried out by spiking eight subsamples of must with various amounts of simazine and antimycin A. Chromatograms of the unspiked and spiked must are shown in Figure 2b,c. The amount added, recovery, and standard deviation are shown in Table 3. High recoveries, between 79 and 106%, with standard deviations between 2.3 and 4.5% (eight determinations) were obtained for spike levels in the range 0.45–3.0 μg mL^{-1}. Limits of detection of the analytical method were determined on the basis of three extracts of the control must sample and the measurements of the 0.50 μg mL^{-1} spiked. Experimental detection limits, taking into account that the procedure involves a preconcentration factor of 5, were 20, 90, 115, 50, and 45 μg L^{-1} for simazine, antimycin A_1, antimycin A_2, antimycin A_3, and antimycin A_4, respectively.

Conclusions. The HPLC procedure described is suitable to simultaneously determine potential residues of different nature, as simazine and antimycins in must samples. This simple procedure could be useful for other similar samples.

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