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HPLC Method for Simultaneous Determination of Fungicides: Carbendazim, Metalaxyl, Folpet, and Propiconazole in Must and Wine

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A simple high-performance liquid chromatography method for simultaneous determination of fungicides, carbendazim, metalaxyl, folpet, and propiconazole, in must and wine has been developed. Analyses are carried out on a reversed-phase column (Spherisorb ODS-1 C_{18}) with an acetonitrile–water gradient as mobile phase. Absorbance at 220 nm is recorded as compounds are separated. The method provides minimum detectable levels, between 4 and 8 μg/L, for the four above-mentioned fungicides.

Botrytis cinerea, *Uncinula necator*, and *Plasmopara viticola* are the most common fungi encountered in viticultural pests. Several different fungicides are widely used to control them including carbendazim (methyl 1*H*-benzimidazol-2-ylcarbamate), metalaxyl (methyl *N*-(2-methoxyacetyl)-*N*-2,6-xylyl-DL-alaninate), folpet (2-[(trichloromethyl)thio]-1*H*-isoindole-1,3(2*H*)-dione), and propiconazole ((±)-1-[[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl]-1*H*-1,2,4-triazole).

Studies on individual determinations of some pesticides in grape must and wine at residue level have been published (Austin et al., 1976; Cabras et al., 1982; Chiba and Sing, 1986). Other studies on the effect of folpet and metalaxyl fungicides on the yeast flora during must fermentation and on yeast metabolism have been published in recent years (Benda, 1978; Minarik and Ragala, 1979; Guerzoni et al., 1982; Gnaegy et al., 1983; Conner, 1983).

Our method will permit the simultaneous determination of the four fungicides in must and wine and, thus, we will carry out studies on the influence of vinification technology on pesticide degradation.

EXPERIMENTAL SECTION

Chemicals. Water was distilled twice and filtered through a Millipore apparatus before use. Solvents (benzene, acetonitrile) were high-performance liquid chromatography grade (Chrom. HPLC; Mallinckrodt Inc.). Analytical samples of propiconazole (≥99.0%) and metalaxyl (≥99.6%) were donated by Ciba Geigy S. A. (Barcelona, Spain); and carbendazim (≥97.5%) and folpet (≥99.5%), by Basf Española S. A. (Barcelona, Spain) and Ind. Quim. Valles S. A. (Barcelona, Spain), respectively.

Preparation of Standard Solutions. Standard solutions of carbendazim, metalaxyl, folpet, and propiconazole were prepared at concentrations between 0.2 and 4 mg/L in methanol. These solutions can be stored at low temperature (−20 °C) for 1 week without appreciable degradation.

HPLC Apparatus. A Waters Associates model equipped with a TCM-002011 temperature control system, a Waters U6K injector (loop 200 μL), a Waters 490 variable-wavelength UV/vis detector, and a Data Module 730 reporting integrator was used.

HPLC Chromatography. The column employed was Spherisorb ODS-1 (250 × 4 mm (i.d.), 10 μm; Teknokroma SCL, Barcelona, Spain). A linear gradient from 20 to 80% acetonitrile in water in 20 min was applied at a flow rate of 2 mL/min. The best wavelength for the simultaneous determination of the studied fungicides was 220 nm according to their UV spectrum. The analyses were carried out at 25 °C.

A good linear response in the range 0–4 mg/L was achieved for all four fungicides. The minimum detectable levels for each were between 0.2 and 0.05 mg/L according to the results shown in Table I.

Preparation of Samples. Water and hydroalcoholic solution (10:100, v/v) were fortified with about 1 mg/mL of the four fungicides.

Must and white wine samples were obtained from Chardonnay vineyards and red wine samples from Cabernet Sauvignon.

Table I. Chromatographic Characteristics of Carbendazim, Metalaxyl, Folpet, and Propiconazole under Experimental Conditions

fungicide	retention time, min	correln[a]	MDL,[b] mg/L
carbendazim	7.50 ± 0.50	0.9912	0.2
metalaxyl	13.25 ± 0.05	0.9996	0.2
folpet	17.45 ± 0.55	0.9998	0.05
propiconazole	19.45 ± 0.10	0.9996	0.2

[a] Concentrations of corresponding fungicides were prepared in methanol at 4, 2, 1, 0.4, and 0.2 μg/mL levels. Results are the mean of three injections. [b] MDL were those concentrations giving a signal 2 times higher than the block signal at the studied pesticides retention time when 80 μL of sample was injected.

Table II. Recoveries of Fungicides in Water and HAS

fungicide	fortificn level, μg/mL	% recovery ± RSD[a] water	% recovery ± RSD[a] HAS
carbendazim	1.0	63.1 ± 7.1	54.9 ± 4.3
methalaxyl	1.0	85.1 ± 9.7	80.0 ± 9.6
folpet	1.0	94.9 ± 5.1	93.6 ± 8.4
propiconazole	1.0	99.0 ± 6.0	96.0 ± 2.2

[a] Results are the mean of four extractions.

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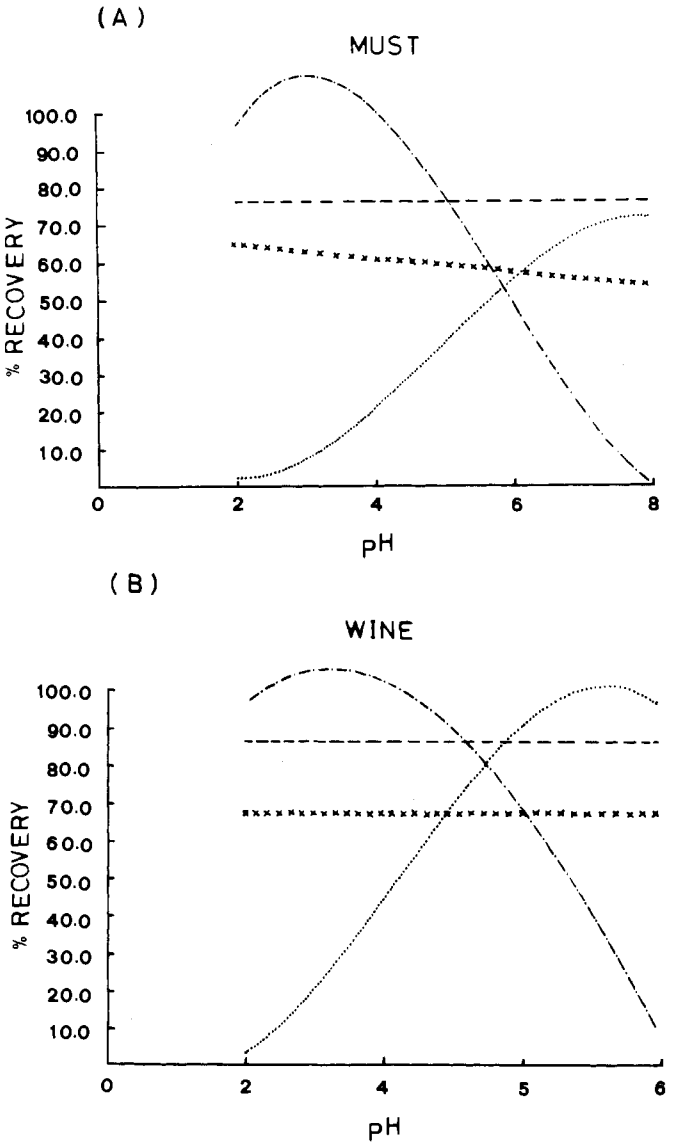

Figure 1. Recoveries of fungicides from 1 mg/L fortified must (A) and white wine (B) at several pH values [fungicide, must, wine]: (···) carbendazim, $r^2 = 0.94$, $r^2 = 0.98$; (- - -) metalaxil, $r^2 = 0.89$, $r^2 = 0.98$; (-·-) folpet, $r^2 = 0.96$, $r^2 = 0.97$; (×××) propiconazole, $r^2 = 0.82$, $r^2 = 0.99$.

Fortification of the must and white wine samples was carried out at several levels in the range 0.2–10 mg/L with the four fungicides at several pH levels by adding 0.5 N HCl or 0.5 N NaOH solutions. Unfortified must and wine samples were raised to pH 6 and 5, respectively, by adding 0.5 N NaOH solution.

Extraction Procedures. Benzene (2 × 25 mL) was added to the corresponding sample (25 mL) in a glass screw-capped tube, and the mixture was shaken twice for 30 min. After the phase separation stage, organic layers were collected and evaporated to dryness under reduced pressure (15 mmHg, ≃40 °C). The residue was dissolved in 2 mL of methanol.

RESULTS AND DISCUSSION

Analytical Method. During preliminary investigations using reversed-phase chromatography and water–methanol mixtures as mobile phase, carbendazim failed to chromatograph reproducibly. By means of replacing water in the solvent mixture by a weak aqueous buffer ($NaHPO_4$–NaH_2PO_4, pH 7, ionic strength 0.05), it is possible to obtain reproducible results, independent of the way of presentation of ionizable solutions (Austin et al., 1976; Chiba and Singh, 1986). Using an acetonitrile–water gradient as mobile phase, we found reproducibility for the four fungicides (see Table I) without the use of buffer solutions.

Table III. Recoveries of Fungicides in Must and White Wine

fungicide	fortifcn level, μg/mL	% recovery ± RDS[a,b] must	wine
carbendazim	0.2	56.0 ± 5.1	70.0 ± 6.5
	1.0	54.7 ±5.0	81.0 ± 6.2
	5.0	61.7 ± 7.0	74.0 ± 6.8
	10.0	60.6 ± 4.3	79.0 ± 7.1
folpet	0.2	57.2 ± 6.1	77.0 ± 6.8
	1.0	54.7 ± 5.4	81.0 ± 7.7
	5.0	65.2 ± 9.0	82.0 ± 7.3
	10.0	66.0 ± 2.0	82.9 ± 7.4
metalaxyl	0.2	77.5 ± 5.6	88.4 ± 5.2
	1.0	80.4 ± 10.2	86.9 ± 8.7
	5.0	75.2 ± 8.9	83.2 ± 6.5
	10.0	83.1 ± 8.7	85.1 ± 9.9
propiconazole	0.2	63.0 ± 8.3	66.2 ± 7.3
	1.0	59.0 ± 5.0	69.2 ± 5.3
	5.0	62.7 ± 7.9	70.4 ± 7.0
	10.0	62.5 ± 6.5	69.6 ± 6.6

[a] Results are the mean of three extractions. [b] pH 6 and 4.8 were used for must and wine extractions, respectively.

Table IV. Treatment Conditions of Vineyards (Chardonnay v.v.)

trademark	active compd	dose	treatment period
Kemdazim	carbendazim	500 mL/ha	one before flowering, another 14 days later
Tilt 10EC	propiconazole	100 mL/ha	one when buds were 15–30 cm long
Ridomill	folpet, metalaxyl	1 kg/ha	one when buds were 15–30 cm long

Table V. Concentration Levels (mg/L) of Fungicides Found in Must and Wine[a]

fungicide	must[a]	white wine[b]	red wine[c]
carbendazim	0.33 ± 0.05	0.22 ± 0.03	1.12 ± 0.40
metalaxyl	0.04 ± 0.01	0.01 ± 0.00	0.58 ± 0.11
propiconazole	0.01 ± 0.00	0.01 ± 0.03	0.70 ± 0.35

[a] Results are the mean of four extractions. [b] From Chardonnary v.v. [c] From Cabernet Sauvignon v.v.

Recoveries in Fortified Samples. The recoveries from water and hydroalcoholic solution (HAS) fortified with about 1 mg/L of fungicides are shown in Table II. Extraction yields are 80% for metalaxyl, folpet, and propiconazole and >55% for carbendazim.

When we tried to apply the extraction procedure to fortified must and white wine we found that carbendazim was poorly extracted. The basicity of this fungicide (pK_a 4.48) explains its stability in acid medium as water-soluble salt and justifies the poor recoveries found at low pH values (pH ≃3). In Figure 1 the dependence of recovery yields of the four fungicides with respect to pH of the medium is presented. These studies were carried out in must and white wine fortified with 1 mg/L. Three samples each were allowed at pH 2, 3, 4, 5, 6, 7, and 8, and after the corresponding extraction procedures the analyses were carried out and the graphics monitored.

Although we did not find significant dependence for metalaxyl and propiconazole, recoveries of carbendazim and folpet were dependent on the pH of the medium. High recoveries were found at high pH values for carbendazim (maximum at pH 7–8) and at low pH values for folpet (maximum at pH 3). Folpet is rapidly hydrolyzed under alkaline conditions, which could explain the low extraction yields observed when we allowed the sample to reach neutral or basic conditions with use of KOH solutions.

These results have led us to conclude that the optimal conditions for the simultaneous extraction of the above-

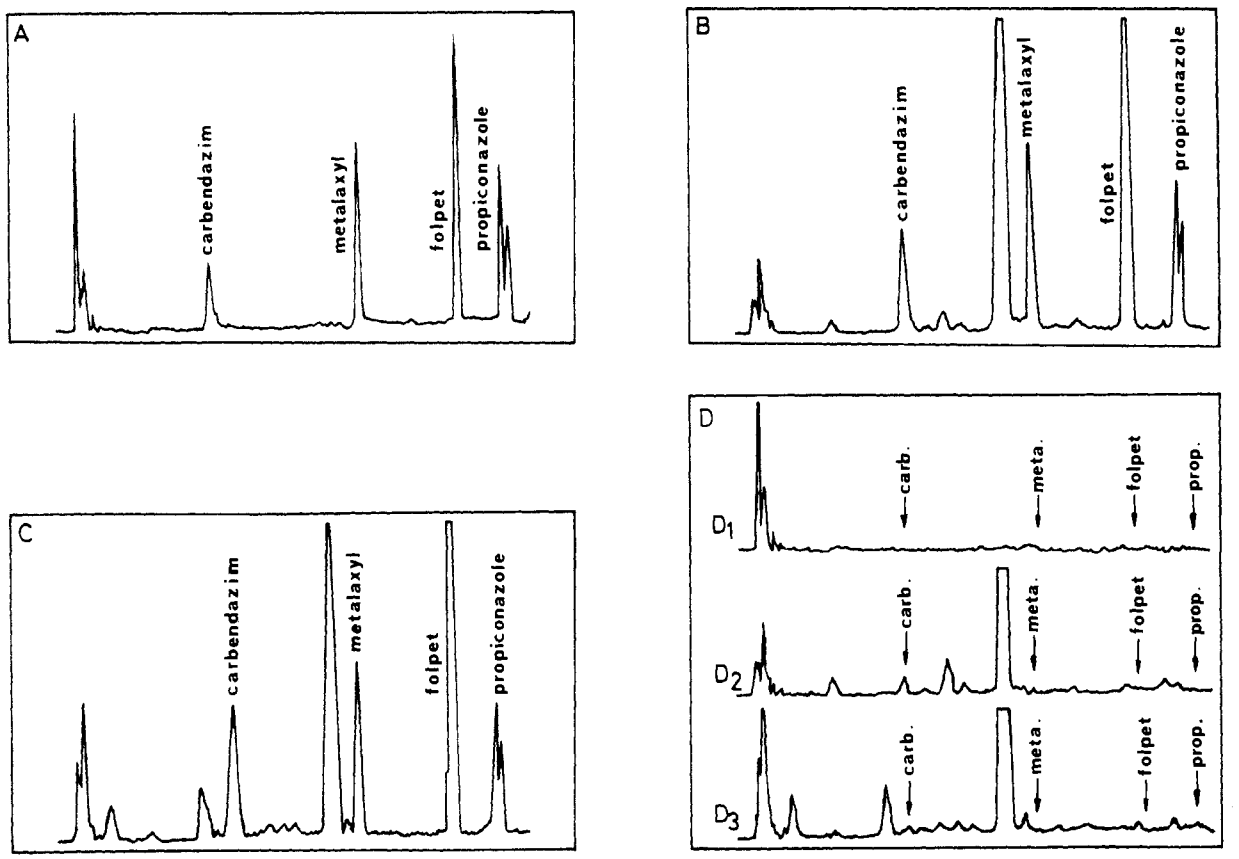

Figure 2. Chromatograms of fungicides extracted from fortified samples (AUFS 0.2). Injected sample 30 μL. Extracts: (A) fortified water; (B) fortified must (Chardonnay v.v.); (C) fortified wine (Chardonnay v.v.); (D) nonfortified samples of water (D1), must (D2), and wine (D3).

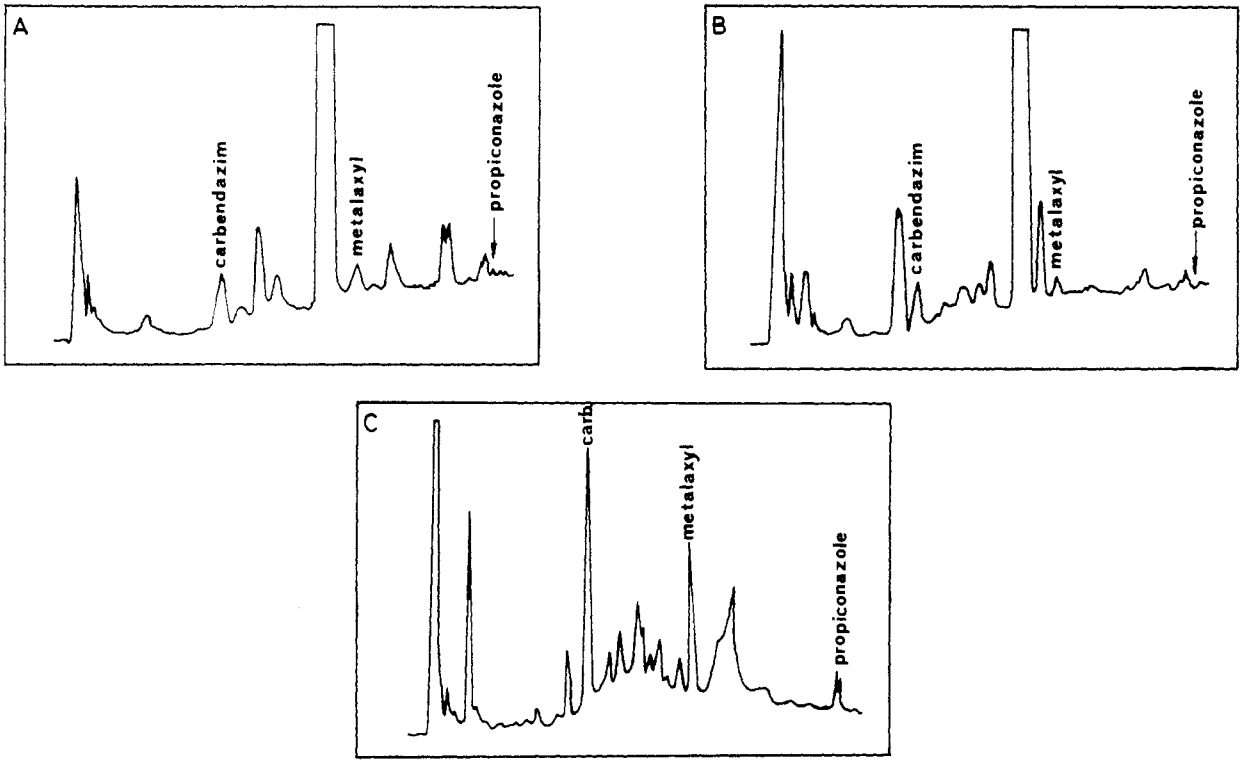

Figure 3. Chromatograms of fungicides extracted from nonfortified samples (AUFS 0.05). Injected sample 80 μL. Extracts: (A) nonfortified must (Chardonnay v.v.); (B) nonfortified white wine (Chardonnay v.v.); (C) nonfortified red wine (Cabernet Sauvignon v.v.).

mentioned fungicides are achieved in weak acid medium. According to Figure 1 we propose use of pH 6 for must extractions and pH 5 for wine extractions.

Concentration levels of fungicides in must and white wine did not substantially affect their corresponding recoveries in the assayed concentration range (0.2–10 μg/mL)

according to results shown in Table III.

In Figure 2 some of the chromatograms of the extracts obtained from water, must, and white wine are presented. No significant interferences of the background were found under the working chromatographic conditions (see Figure 2D).

Analyses of Must and Wine. Vineyards (Chardonnay and Cabernet Sauvignon v.vs.) were treated with the above-mentioned fungicides in the periods and at doses shown in Table IV.

Must and wine from the Chardonnay grapes were allowed at pH 6 and 5, respectively, prior to being subjected to the analyses described above.

Wine from Cabernet Sauvignon grapes was allowed at pH 5 and then analyzed.

In Table V some of our earlier results are shown. Residues are very low, and at these levels we found some interferences in the area of carbendazim and metalaxyl, especially with red wine (see Figure 3). Evidence of the presence of the corresponding compounds in the chromatogram was obtained by using spiked samples. Fungicide residues are at higher levels in the red wine than in the white, which is a logical result considering the conditions of the vinification process. In white vinification, must residues are quite similar to the wine ones with a little decrease of carbendazim and metalaxyl levels during alcoholic fermentation.

Carbendazim was the fungicide found at higher levels (about 0.4 mg/L in must, 0.25 mg/L in white wine, and 1.5 mg/L in red wine). Residue levels of propiconazole and metalaxyl are lower (less than 0.05 mg/L in must and white wine and 0.7 mg/L in red wine). Folpet was not detectable at quantifiable levels in our analyses.

Further studies on the influence of vinification technology on residue levels of some of these fungicides are under way.

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Registry No. Carbendazim, 10605-21-7; metalaxyl, 57837-19-1; folpet, 133-07-3; propiconazole, 60207-90-1.

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Microbiological Screening of Mevalonate-Suppressive Minor Plant Constituents[1]

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Halobacterium halobium is an extremely halophilic bacterium whose survival in a high-salt environment rests on its capacity to synthesize via the mevalonate pathway the diether phytanyl phosphatidyl glycerol phosphate constituent of its cell membranes. We report that a variety of monoterpenes exert a dose-dependent, mevalonate-reversible suppression of *H. halobium* growth. In companion feeding trials, the monoterpenes suppressed hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase activity. These results suggest that the mevalonate-suppression action of plant metabolites can be screened by monitoring their effects on *H. halobium* growth.

Halobacterium halobium is an extremely halophilic bacterium whose survival is due to its capacity to synthesize, via the mevalonate pathway, the diether phytanyl phosphatidyl glycerol phosphate constituent of its cell membranes (Kates, 1978). Mevalonate metabolism in *H. halobium* appears to be regulated through the modulation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) concentration rather than of HMG-CoA reductase activity (Watson et al., 1983; Cabrera et al., 1986).

The inverse association between the intake of plant products and plasma cholesterol levels is strongly supported by epidemiological studies. Early evidence pointed to the cholesterol-lowering action of a diet rich in plant constituents including linoleic acid, various types of fiber, and phytosterols and poor in cholesterol and saturated fatty acids (Anderson et al., 1973). Another prospective cholesterol-lowering action of this diet, consistent with the Brown and Goldstein (1980) concept of the multivalent

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