Residue Determination of Some Insecticides and Fungicides on Grapes by Reversed-Phase High-Performance Liquid Chromatography

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Residues of some insecticides and fungicides were analyzed in different grapes (Monica, Nuragus, Nasco, and Bovale) in 1978 and 1979. Analyses were performed by HPLC on a reversed-phase column (RP-18) under isocratic elution conditions which allowed a simultaneous quantitative determination of eight chemically different pesticides. Extractions from grapes were carried out with light petroleum ether or benzene, with mean recoveries ranging between 70 and 118%. The found residues were lower than the legal limits admitted in Italy and in the European Economic Community on foodstuffs.

The major advantages of HPLC have been frequently pointed out; we want to emphasize, for our field of application, the reduced cleanup in comparison to GLC and the possibility of monitoring many chemically different pesticides (carbamate or organophosphorus or organochlorine) simultaneously (Seiber, 1974). Reversed-phase HPLC, moreover, is considered likely to give most efficient separation of pesticides when the mobile phase is water–acetonitrile or water–methanol (Seiber, 1974; Hoodless, 1978; Sparacino and Hines, 1976; Aten and Bourke, 1977). It has been successfully employed in the determination of residues of pesticides (Cabras et al., 1979a,b; Hoodless et al., 1978). So far only few HPLC simultaneous quantitative determinations of different pesticides, currently used on vineyards to control insects and fungine disease, have been reported (Lawrence and Turton, 1978). Here we describe a method which allows, after extraction from grapes, the separation and quantitative determination of eight pesticides representative of those employed against insects, aphides, and fungi.

Thus, we chose Dimethoate (I), Carbaryl (III), Tetrachlorvinphos (VI), and Phosalone (VIII) as representatives of organophosphorus and carbamate insecticides employed against *Polychrosis botrana*, Methylthiophanate (II), Folpet (V), and Vinchlozolin (VII) among fungicides used to control *Botrytis cinerea*, and Dinocap (IV) employed against *Uncinola necator*. The analyses were performed on extracts from grapes by using isocratic elution conditions, more fast and simple than gradient elution. By this way it is possible to achieve sensitivity levels 10–100 times lower than the legal limits admitted in EEC on foodstuff (EEC Council Directive, 1976).

EXPERIMENTAL SECTION

Apparatus. We have employed a Varian 5020 liquid chromatograph equipped with a variable-wavelength UV Varichrom detector, heater block, CDS 111 L data system, Varian 9176 recorder (1 mV/FS), and Valco AH 20 automatic injector (loop 50 μL). The data system was employed as an integrator with the external standard procedure.

Chromatography. A LiChrosorb RP-18 (250 × 4.0 i.d., 10 μm; Merck, Darmstadt, West Germany) column was employed. Mixtures of water–acetonitrile were employed in different ratios as the mobile phase, at the flow rate of 2.0 mL/min. The best wavelength for the simultaneous detection of all pesticides, except Phosalone, was found to be 221 nm, according to their UV spectra (Table I), the last one being detected at 240 nm.

Table I. UV Spectra of Pesticides in Acetonitrile Solution

pesticide	λ, nm	$10^3\epsilon_1$	λ, nm	$10^3\epsilon_2$	$10^3\epsilon_{221}$
Dimethoate	199.0	11.3	221.0	4.1	4.1
Methyl-thiophanate	272.5	17.0	210.0	26.0	18.0
Carbaryl	225.5	25.8	221.0	24.6	24.6
Dinocap	210.0	2185.0			1639.0
Folpet	228.0	28.4	221.0	25.8	25.8
Tetrachlor-vinphos	224.5	33.6	221.0	31.3	31.3
Vinchlozolin	280.0	0.8	230.0	5.0	4.6
Phosalone	277.0	8.3	240.0	17.7	12.0

The standard curve of each pesticide was constructed by plotting peaks areas (external standard method) vs. concentrations. A very good linearity, in the range 0–5 ppm, was achieved.

Chemicals. Water was distilled twice and filtered through a Millipore apparatus; benzene and acetonitrile were HPLC-grade solvents (LiChrosolv, Merck), light petroleum ether was suitable for pesticide analysis (Carlo Erba, Milan, Italy). Analytical samples of Dimethoate (≥98.5%), Phosalone (≥98.5%), Methylthiophanate (≥97.5%), and Vinchlozolin (≥99.0%) were kindly donated by Montedison (Milan, Italy), Ravit (Rome, Italy), SIPCAM (Milan, Italy) and BASF Agritalia (Milan, Italy), respectively. Analytical standards of other pesticides were purchased from Hoechst (Pestanal, ≥99.0%; Milan, Italy).

Sampling and Extraction Procedure. Grapes were collected in 1978 and 1979 in 12 different vineyards occupying some 6 ha each, according to a statistic method (Huglin and Julliard, 1959). In these vineyards the studied pesticides were applied under authority of the Tecnical Assistence Centers of Consorzio di Bonifica della Sardegna Meridionale by using airblast sprayers and following the recommendation of manufacturers.

Field-sprayed grapes wer milled, and 25 g of homogenate was shaken for 5 min with 25 mL of the organic solvent. After centrifugation (5 minutes at 3000 rpm) the organic layer was separated, 1.0 mL evaporated to dryness under reduced pressure (T = 40 °C), and the residue dissolved with 1.0 mL of eluting mixture.

Table II shows the percent recovery obtained by extraction with the organic solvents from grapes fortified with known amounts of the studied pesticides. Fortification volumes were 12.5, 25.0, and 50.0 μL of the eluting mixture solution containing each pesticide, except Folpet, in concentrations 1000 times the legal limits. Since Folpet

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Table II. Recoveries of Pesticides: Simultaneous Determination[a]

pesticide	fortification level, ppm	% recovery: petroleum ether	% recovery: benzene
Dimethoate (I)	0.75	n.d.[b]	119
	1.50	n.d.	103
	3.00	n.d.	117
Methylthiophanate (II)	0.25	n.d.	96
	0.50	n.d.	71
	1.00	n.d.	116
Carbaryl (III)	1.00	70	77
	2.00	85	97
	4.00	61	115
Dinocap (IV)	0.50	103	n.d.
	1.00	76	n.d.
	2.00	82	n.d.
Folpet (V)	0.75	103	91
	1.50	100	91
	3.00	84	91
Tetrachlorvinphos (VI)	0.75	91	112
	1.50	103	118
	3.00	102	110
Vinchlozolin (VII)	0.75	95	113
	1.50	116	87
	3.00	103	120
Phosalone (VIII)	0.50	104	101
	1.00	112	98
	2.00	96	92

[a] Results are the means of duplicate extractions. [b] Not detectable.

has a very high ϵ and legal limit (15.0 ppm), we used a solution only 100 times the legal limit.

RESULTS AND DISCUSSION

The chromatographic parameters which allowed the separation of the above-mentioned pesticides are reported in Table III.

The separation could be carried out with the different conditions of experiments 1–3. Elution of Dimethoate was poorly affected by the water content in the mobile phase, according to its good solubility in aqueous media (Worthing, 1979), while the retention times of pesticides II–VIII were linearly related to the increasing water content.

However, we used the no. 2 experimental conditions for the routine determinations of grape extracts since they gave the best separation of the pesticides under study; only Phosalone analysis were performed under no. 4 experimental conditions.

The extractions from grapes could be carried out with light petroleum ether or benzene. A very similar percent recovery for all pesticides was obtained with both solvents, except for Dimethoate, Methylthiophanate, and Dinocap. The first two pesticides are not extracted by light petroleum ether, according to their solubility in organic media (Worthing, 1979); the latter was partially lost with both solvents during evaporation procedure. Therefore, Dinocap is determined directly in the petroleum ether extract, while it is not detectable in the benzenic one, because the solvent peak is so large that it covers it.

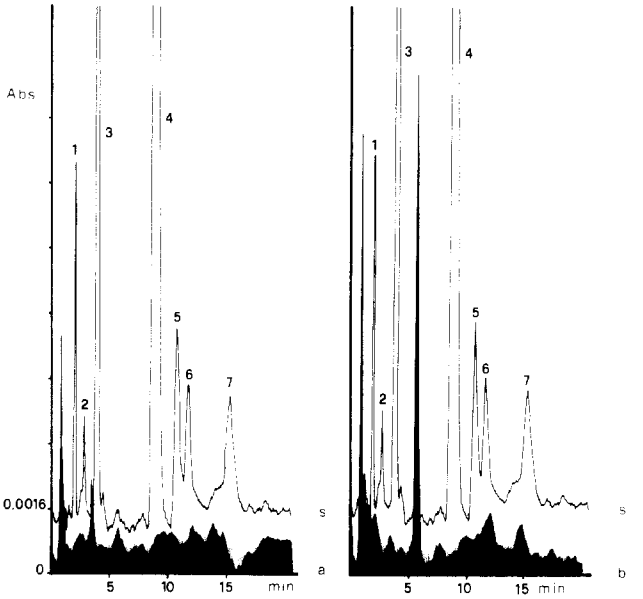

Figure 1. High-performance liquid chromatograms: [left (a)] petroleum ether extract of untreated white grape; [right (b)] benzene extract of untreated white grape. Standards (s): 1, Dimethoate (3.0 ppm); 2, Methylthiophanate (0.5 ppm); 3, Carbaryl (2.0 ppm); 4, Dinocap (1.0 ppm); 5, Folpet (1.5 ppm); 6, Tetrachlorvinphos (1.5 ppm); 7, Vinchlozolin (1.5 ppm). HPLC conditions: column, RP-18 (250 × 4.0 mm i.d.); mobile phase, water–acetonitrile (55:45); flow rate, 2.0 mL/min; sample size, 50 μL; UV detector at 221 nm, 0.016 AUFS.

Figure 1 shows blanks of petroleum ether (a) and benzene (b) extracts from Nuragus white grape. The main differences were found in the content of interfering materials (perhaps colorants), the benzenic extract being richer. This fact was found to be constant in both white and red grapes; of course, the content of interfering materials was higher in the red grape than in the white one.

We think to be a reasonable assumption that the content of interfering materials could be directly related to the increasing polarity of the solvent employed for the extraction.

Thus, on going from petroleum ether to benzene to ethyl acetate (ϵ^0 = 0.00, 0.32, and 0.58 respectively), we found an increase of interfering materials in the extracts (unpublished data). However, as shown in the graphs of blanks, the extraction performed with low polarity solvents gave a clear blank, in which the interfering materials did not overlap the studied pesticides and made possible a quantitative determination with the minimal detectables values (MDV) reported in Table IV. MDV were considered as those concentrations giving a signal 2 times higher than the blank signal, at the studied pesticide retention times, when 50 μL of extract was injected.

It is remarkable that extractions performed after longer maceration times gave extracts showing an higher content of interfering materials, probably due to the formation of oxidation products.

Table III. Retention Times of Pesticides on RP-18 Column (Minutes)

expt no.	λ, nm	mobile phase, H_2O–CH_3CN	flow rate, mL/min	I	II	III	IV	V	VI	VII	VIII
1	221	60:40	2.0	2.00	3.22	4.64	11.60	17.15	19.90	27.07	
2	221	55:45	2.0	1.79	2.62	3.58	8.39	10.78	11.90	15.93	
3	221	50:50	2.0	1.63	2.16	2.86	6.18	7.11	7.54	9.70	
4	240	35:65	2.0	1.26	1.39	1.39	2.35	2.85	2.85	3.20	4.40

Table IV. Residues of Pesticides on Grapes

expt no.	grape	pesticide	spraying date	spraying amount, g/ha	sampling date	residue concn, ppm	legal limit, ppm	safety time, days	MDV
1	Nuragus	Carbaryl	5/22/78	1000					
			8/4/78	1000					
			8/10/78	1000					
			8/28/78	1000	9/25/78	0.02	2.0	7	0.005
2	Nuragus	Carbaryl	5/25/78	1000	9/25/78	n.d.	2.0	7	0.005
3	Nuragus	Carbaryl	5/25/78	1000		n.d.	2.0	7	0.005
		Dimethoate	7/4/78	750					
			8/10/78	750					
			8/28/78	750	9/25/78	0.56	1.5	20	0.200
4	Nuragus	Carbaryl	5/25/78	1000		n.d.	2.0	7	0.005
		Tetrachlorvinphos	7/4/78	750					
			8/10/78	750					
			8/28/78	750	9/25/78	0.66	1.5	14	0.040
5	Monica	Dimethoate	6/6/78	750					
			8/17/78	750					
			9/5/78	750	9/21/78	1.27	1.5	20	0.200
6	Nuragus	Methylthiophanate	6/22/78	750		0.19	0.5	15	0.100
		Tetrachlorvinphos	8/13/78	750					
			8/21/78	750					
			9/4/78	750	9/25/78	0.50	1.5	14	0.040
7	Nuragus	Vinchlozolin	6/22/78	1500					
			9/4/78	1500		0.24	1.5	21	0.050
		Tetrachlorvinphos	8/21/78	750					
			9/4/78	750	9/25/78	0.20	1.5	14	0.040
8	Monica	Carbaryl	5/17/79	1500					
			7/7/79	1500					
			8/8/79	1500		0.01	2.0	7	0.005
		Phosalone	6/17/79	1500					
			7/28/79	1500	9/26/79	n.d.	1.0	21	0.050
9	Nuragus	Dinocap	5/20/79	1200		0.36	1.0	20	0.001
		Carbaryl	7/1/79	2100	9/26/79	n.d.	2.0	7	0.005
10	Nuragus	Carbaryl	6/24/79	1600		0.01	2.0	7	0.005
		Tetrachlorvinphos	7/2/79	1300					
			8/12/79	1300					
			8/29/79	1300	9/28/79	0.03	1.5	14	0.040
11	Nasco	Dinocap	5/5/79	250					
			7/13/79	250		0.23	1.0	20	0.001
		Folpet	5/18/79	750		n.d.	15.0	40	0.020
		Tetrachlorvinphos	8/22/79	2000	9/20/79	0.08	1.5	14	0.040
12	Bovale	Dinocap	5/10/79	1500					
			5/17/79	1800					
			5/24/79	1800					
			6/2/79	2100					
			6/14/79	2100					
			6/29/79	2100	9/28/79	0.60	1.0	20	0.001

Therefore, it has been possible, with the above-described procedure, to choose the extraction solvent according to the pesticides sprayed on grapes. For example, grapes sprayed in the field with Carbaryl and Folpet can be extracted with light petroleum ether, while samples containing Dimethoate and/or Methylthiophanate have to be carried out with benzene, even if their quantiative determination may be affected in excess. This is because MDV are very little and the signals of interfering materials relatively high, when working at levels lower than half of the legal limits.

The pesticide residue concentrations found on 12 samples of grapes, 7 collected in 1978 and 5 in 1979 and treated with the above procedure, are summarized in Table IV.

From Table IV the following considerations can be made. (1) The concentrations of all pesticides are very low and always lower than the legal limits. (2) This fact also occurs in the no. 7 sample, which has been collected on just the last day of Vinchlozolin safety time; only the no. 5 sample shows a concentration of Dimethoate close to the legal limit. This finding could be related to the pesticides's slow degradation. Furthermore, the sample was collected 4 days before the safety time. (3) It is remarkable that the samples sprayed with longer safety time pesticides show the highest residue concentrations when the grapes were treated several times with the same formulation (e.g., Tetrachlorvinphos no. 4 and Dinocap no. 12). This finding could be tentatively explained with a small "accumulation effect" of these products in the grape that is probably due to their low water solubility. (4) The concentration of Dinocap in samples no. 8, 10, and 12 has been found to be on the same order as those reported by Ripley et al. (1978).

In conclusion, we think our analyses provide a sensitive and rapid means for a quantitative routine determination of chemically different pesticides in grapes without any complicated cleanup. The concentrations of residues found in grapes are remarkably low and they have no toxicological importance. It would be expected that also in wines

even the relatively high concentrations found in the no. 4 and no. 12 samples will be lower.

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Effects of Hapten Structure and Bridging Groups on Antisera Specificity in Parathion Immunoassay Development

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Five conjugates of parathion to bovine serum albumin (antigens) were prepared wherein bridging groups of varying length and structure were used between the hapten and the protein carrier. In three of these conjugates, the bridging group was attached at either the 2- or 3-phenyl position of parathion, allowing the preservation of all parathion determinant groups. Several immunization modes and schedules were also investigated. Antisera obtained from the use of these antigens contained no antibody specificity for free parathion or 4-nitrophenol in radioimmunoassay binding tests. Enzyme-linked immunosorbent assay binding and inhibition experiments demonstrated the presence of antibodies in antisera associated with three of the conjugates. These antibodies preferentially bind the parathion derivatives that contain the bridging group structures and the various precursors of these derivatives. Of four immunization regimens used, only the combination of intradermal and intravenous modes produced any anti-hapten antibody activity.

The important advantage of immunoassays for pesticide residues lies in the minimal requirement for sample cleanup. The specificity of the antibody reaction allows the deletion of a good portion of the rigorous sample purification required by more conventional methods such as GLC, HPLC, and colorimetry. The promise of immunoassays for pesticides was first envisioned by Ercegovich (1971) and further detailed by Hammock and Mumma (1980). Concrete evidence of this potential has been presented in radioimmunoassay (RIA) procedures successfully developed for parathion (Ercegovich et al., 1977, 1981; Vallejo, 1981), dieldrin (Langone and Van Vunakis, 1975), *S*-bioallethrin (Wing et al., 1978; Wing and Hammock, 1980), and benomyl (Newsome and Shields, 1981), an enzyme-linked immunosorbent assay (ELISA) for parathion (Al-Rubae, 1978), and a fluorescence immunoassay for the degradation product of benomyl (Lukens et al., 1977).

The parathion RIA was successfully developed by employing an antigen wherein parathion, by reducing its nitro group, was directly conjugated to a carrier protein via diazo coupling. The assay allowed detection of parathion residues in plant and plasma samples to the 10-ng level, equivalent to 0.1 ppm, without any sample cleanup.

Improvement of the sensitivity of the assay was thereafter sought by enhancing the specificity of the antibody response. Bridging groups of varying length and structure were employed between the hapten and carrier protein to render the hapten more immunogenically visible. The structure of the hapten moiety was also manipulated with a particular view to preserving all determinant groups. In this regard, antigens were prepared wherein parathion was conjugated to the carrier protein via a bridging group attached to an unsubstituted aryl position, thus avoiding alteration of the nitro or any other functional group. Finally, the relative efficacies of different immunization modes and schedules were investigated. The specificities of the antisera obtained from these experiments were characterized by RIA and ELISA binding and inhibition tests.

MATERIALS AND METHODS

Reagents and Equipment. Parathion, 98.5%, was obtained from American Cyanamid Co., Princeton, NJ. Bovine serum albumin (BSA) (crystallized, purified bovine albumin fraction V), rabbit serum albumin (RSA), Freund's complete and incomplete adjuvant, and peroxidase–anti-rabbit IgG were purchased from Miles Lab-

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