ELSEVIER

Journal of Chromatography A, 676 (1994) 347–354

JOURNAL OF CHROMATOGRAPHY A

Ion-pair reversed-phase liquid chromatography with fluorimetric detection of pesticides

F. García Sánchez*, A. Navas Díaz, A. García Pareja

Departamento de Química Analítica, Facultad de Ciencias, Universidad de Málaga, 29071-Málaga, Spain

First received 6 December 1993; revised manuscript received 16 May 1994

Abstract

The pesticides asulam, propoxur, coumatetralyl, biphenyl-2-ol and thiabendazole were separated by ion-pair reversed-phase liquid chromatography with fluorescent detection. The mobile phase contained methanol, acetic acid and sodium cholate as ion-pair reagent. In addition, tetramethylammonium hydrogensulphate and triethylamine were added to elute the pesticides. Separations were accomplished in less than 20 min. Interferences from nineteen pesticides were studied and recoveries from synthetic mixtures ranging from 90 to 110%. Recoveries from spiked apples and wheat grains ranged from 94 to 105% with relative standard deviations between 1.4 and 9.2% and detection limits between 0.1 and 1.9 ng.

1. Introduction

The pesticides asulam, propoxur, coumatetralyl, biphenyl-2-ol and thiabendazole are a contact herbicide, a non-systemic insecticide, an anticoagulant rodenticide and two fungicides, respectively. Their mixtures can be found in fruits, vegetables, cereals and other types of crops as a consequence of the pre- and post-harvest treatment with a variety of chemicals such as herbicides and insecticides in the pre-harvest step and with fungicides and rodenticides in the storage step of the total harvest process. Although liquid chromatography (LC) [1–7] and gas chromatography (GC) [8–13] have been used for their separation and detection in several other mixtures, the above mixture has not been resolved.

Because the thermal lability of asulam and coumatetralyl and the high melting point of thiabendazole impede their GC investigation, LC is the technique of choice. Reversed-phase LC on a C_{18} column does not separate the mixture. However, as anionic, cationic and zwitterionic molecules can all potentially undergo ion-pair formation with appropriate counter-ionic reagents, the scope of LC can be extended. In ion-pair systems, a secondary chemical equilibrium is superimposed on the physical distribution of a solute between the mobile and stationary phases and consequently method development is generally more flexible and facilitates the determination of compounds using the reversed-phase mode [14].

Charged surfactants are used as the counter ions in the mobile phase. A great variety of surface-activity achiral compounds of different hydrophobicity are available as counter-ion re-

* Corresponding author.

0021-9673/94/$07.00
SSDI 0021-9673(94)00466-M

agents. Recently, new chiral surfactants such as bile salts have extended the scope of separations. Bile salts are used in micellar LC [15–19] but no data were found for their use as ion-pair reagents in LC. They differ from the typical long-chain alkyl micelle-forming surfactants previously employed as ion-pair reagents in that they have a rigid cholesterol-like steroidal ring structure and possess hydrophobic and hydrophilic faces.

We report in this paper a rapid, ion-pair reversed-phase LC method with a bile salt, sodium cholate, as organic counter ion for the separation and determination of pesticides. The separation was achieved by using gradient elution and the determination by using fluorimetric detection, with a total analysis time of 20 min.

2. Experimental

2.1. Instrumentation

The measurements were performed with a Merck–Hitachi (Darmstadt, Germany) liquid chromatograph consisting of an L-6200 pump, an AS-4000 autosampler, an L-4250 UV–visible detector and a D-6000 interface. Integration was carried out with a PC/AT computer and the instrumental parameters were controlled by Hitachi–Merck HM software. A Perkin-Elmer (Beaconsfield, UK) LS-50 fluorescence detector, placed in series with and after the UV–visible spectrophotometer, was equipped with a xenon discharge lamp and two monochromators. Fluorescence Data Manager (FLDM) software (LC program) and an RS232C interface were used to send information to an external computer. For graphical recording, an NEC Silenwriter2 S60P laser printer was connected to the spectrofluorimeter.

A Model KNK-3000 gas chromatograph (Konik Instruments, Barcelona, Spain) equipped with a flame ionization detector was used with the following conditions: splitless injector (240°C, 0.8 min); injection volume, 1 μl; detector temperature, 280°C; fused-silica column, BP5 (SGE, Victoria, Australia) stationary phase (5% diphenyl–dimethylsiloxane), 25 m × 0.22 mm I.D., 0.25-μm film thickness; temperature programme: 120°C for 2 min, increased to 240°C at 8°C/min and held at 240°C 40 min; carrier gas, helium at 11.6 ml/min; and make-up gas, nitrogen at 24 ml/min.

2.2. Chemicals and reagents

Sodium cholate, sodium dodecyl sulphate (SDS), cetyltrimethylammonium bromide (CTAB) and triethylamine (TEA) were obtained from Sigma (St. Louis, MO, USA) and tetramethylammonium hydrogensulphate (TMA) and acetic acid from Merck (Darmstadt, Germany). Methanol was of LiChrosolv gradient grade (Merck) and acetone of analytical-reagent grade (Merck). The pesticides asulam (purity 99.9%), propoxur (99.9%) and coumatetralyl (96%) were purchased from Dr. S. Ehrenstorfer (Augsburg, Germany), biphenyl-2-ol (>99%) from Merck and thiabendazole (Pestanal, 99%) from Riedel-de Haën (Hannover, Germany).

Stock standard solutions of asulam ($4.34 \cdot 10^{-3}$ M), propoxur ($4.78 \cdot 10^{-3}$ M), coumatetralyl ($3.42 \cdot 10^{-3}$ M), biphenyl-2-ol ($5.87 \cdot 10^{-3}$ M) and thiabendazole ($4.97 \cdot 10^{-3}$ M) were prepared by dissolving the compounds in methanol and stored at 4°C. Working standard solutions were prepared by dilution with methanol.

Solutions of sodium cholate ($7 \cdot 10^{-3}$ M) SDS ($3.5 \cdot 10^{-3}$ M), CTAB ($1 \cdot 10^{-4}$ M) and tetramethylammonium hydrogensulphate ($1 \cdot 10^{-2}$ M) were prepared in doubly deionized water. These solutions were filtered through 0.2-μm nylon membrane filters.

2.3. Extraction procedure for apples

A 250-g amount of apples was chopped in a food chopper and 15 g were transferred into a blender cup and blended with 50 ml of acetone containing 0.3 ml of orthophosphoric acid at high speed for 3 min. The homogenate was filtered through a fritted-glass Büchner funnel (coarse porosity) under reduced pressure and the filter cake washed with 5 ml of acetone. The filtrate was transferred into a 50-ml volumetric flask and

diluted to volume with acetone. An aliquot of 1 ml of the extract in acetone was diluted with methanol to a final volume of 5 ml. This solution was used for analysis.

2.4. Extraction procedure for wheat grain

Portions of 10 g of representative wheat grain samples were washed with 20 ml of acetone in order to eliminate any impurities present. The pesticides were extracted by adding 20 ml of acetone and sonicating for 5 min. The solution was filtered through a fritted-glass Büchner funnel (coarse porosity) under reduced pressure and the filter cake was washed with acetone. The filtrate was evaporated to dryness on a rotary evaporator. The residue was dissolved in methanol (5 ml) and this solution was used for analysis.

2.5. LC operating conditions

The pesticide samples were analysed using a Spherisorb S5 ODS-2 reversed-phase column (20 cm × 4.6 mm I.D.; 5-μm particle size) from Phase Separations (Deeside, UK). The injection volume was 20 μl for the standard methanolic solutions and samples and the flow-rate was 1 ml/min. The mobile phase composition and wavelength programme for fluorimetric detection are detailed in Table 1. The peak-area response was measured at the retention times of asulam (1.85 min), propoxur (3.74 min), coumatetralyl (6.19 min), biphenyl-2-ol (7.95 min) and thiabendazole (15.97 min). A calibration graph was constructed using the responses.

2.6. Recovery test

Apples samples were spiked with a solution of pesticides in methanol of composition asulam, coumatetralyl and propoxur 200 mg/l, biphenyl-2-ol 50 mg/l and thiabendazole 12.5 mg/l and left for 30 min before extraction. Wheat grains were washed with 20 ml of acetone in order to eliminate any impurities present and spiked with a solution of pesticides in methanol of composition asulam, coumatetralyl and propoxur 20 mg/l, biphenyl-2-ol 5 mg/l and thiabendazole 1.25 mg/l. After thorough mixing, the grains were left for 30 min before extraction. The apple and grain extracts in acetone were transferred into 50- and 10-ml volumetric flasks, respectively, and diluted to volume with acetone. Aliquots of these solutions were diluted with methanol to a final volume of 5 ml. These solutions were used for analysis.

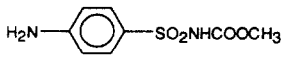
3. Results and discussion

The structures of asulam, propoxur, coumatetralyl, biphenyl-2-ol and thiabendazole are shown in Fig. 1 [20]. These compounds possess

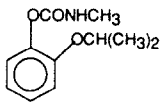
Table 1
Mobile phase composition and wavelength programme

Time (min)	Methanol (%)	Ion-pair mixture 1[a] (%)	Ion-pair mixture 2[b] (%)	$\lambda_{excitation}$ (nm)	$\lambda_{emission}$ (nm)
0.0	60	40	0	255	342
2.5				272	303
4.5				310	385
6.8				247	340
8.0	60	40	0		
9.0	60	0	40		
10.0				298	342
18.0	60	0	40	298	342

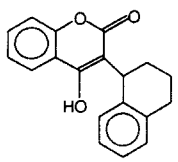
[a] Ion-pair mixture 1: aqueous solution of 7 m*M* sodium cholate, 25 m*M* acetic acid and 5 m*M* tetramethylammonium.
[b] Ion-pair mixture 2: aqueous solution of 7 m*M* sodium cholate, 2.5 *M* acetic acid and 84 m*M* triethylamine.



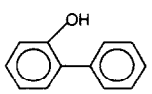
ASULAM
Methyl sulphanilylcarbamate



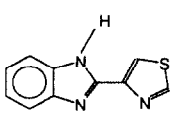
PROPOXUR
2-Isopropoxyphenylmethylcarbamate



COUMATETRALYL
4-Hydroxy-3-(1,2,3,4-tetrahydro-1-naphthyl) coumarin



BIPHENYL-2-OL
(1,1'-Biphenyl-2-ol)



THIABENDAZOLE
2-(thiazol-4-yl) benzimidazole

Fig. 1. Structures of the pesticides.

sufficient natural fluorescence to permit their detection without pre- or postcolumn derivatization steps and so fluorimetric detection was selected.

The retention behaviour was studied on a C_{18} reversed-phase column under a variety of mobile phase conditions including concentrations of organic modifier, ion-pairing reagent and acetic acid, pH and concentrations of TMA and TEA in the mobile phase.

Because of the quenching effect on the fluorescence of the solutes exerted by acetonitrile, the latter was discarded as an organic modifier and methanol was selected. Methanol concentrations below 50% cause turbidity of the mobile phase; concentrations between 50 and 70% give the greatest differences in capacity factor and 60% methanol in the mobile phase was selected as the optimum concentration.

Different surfactants of cationic and anionic character were studied to determine the best ion-pairing reagent. The results indicated that in absence of surfactants asulam and propoxur overlap completely. Alternatively, the use of ion-pairing reagents in the mobile phase separates the compounds. The peak heights obtained with the anionic surfactants sodium cholate and SDS are higher than those with cationic surfactants such as CTAB whereas the retention times with anionic surfactants are similar or shorter than those with cationic surfactants or without surfactants. The greater efficiency was achieved with sodium cholate and this was selected as the ion-pair reagent for the separation. Fig. 2A shows a plot of the capacity factor vs. sodium cholate concentration in the mobile phase, from which it is deduced that all the pesticides can be separated satisfactorily with 2.8 m*M* sodium cholate.

The effect of acetic acid on the capacity factor is summarized in Fig. 2B. Whereas an acetic acid concentration of 10 m*M* in the mobile phase gives the greatest efficiency for the separation of asulam, propoxur, coumatetralyl and biphenyl-2-ol, thiabendazole is strongly retained into the column and 1 m*M* acetic acid in the mobile phase is needed to elute it. This behaviour of thiabendazole is consistent with the behaviour of a weaker acid. As the pH decreases, the retention increases rapidly for coumatetralyl, thiabendazole and asulam and remains constant for propoxur and biphenyl-2-ol. The last two compounds form ion pairs with sodium cholate in a wide pH range, but coumatetralyl requires $pH < 7$ and thiabendazole and asulam $pH < 5$.

Variation in the sodium cholate content of the mobile phase alone does not allow adequate control over the column selectivities for pesticides and an ion-pair mixture of counter ions of different hydrophobicities is used. Therefore, low concentrations of a cationic species such as TMA or TEA were added; this addition to a mobile phase of low pH tends to lower the capacity factors of positively charged solute molecules owing to co-ion repulsive interaction. A 2 m*M* TMA concentration in the mobile phase was selected as the optimum to elute asulam,

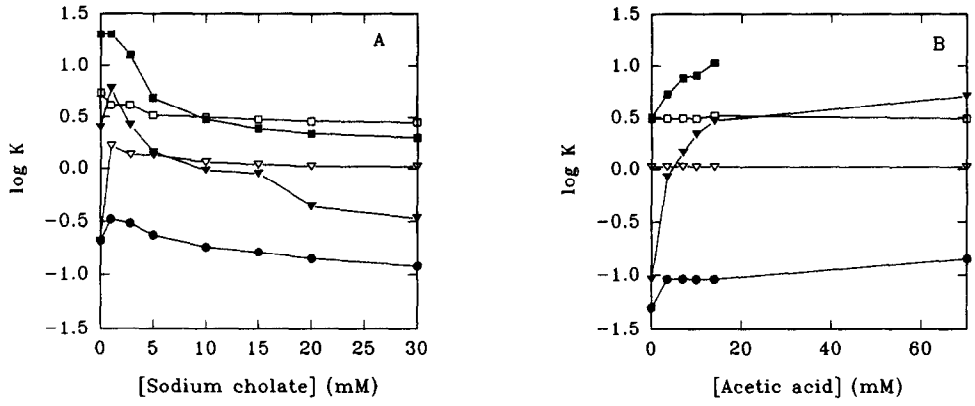


Fig. 2. Effect of mobile phase parameters on the retention of the pesticides: (A) sodium cholate concentration in the mobile phase; conditions, methanol–water (60:40) solution of 25 m*M* acetic acid and 5 m*M* TMA; (B) acetic acid; conditions, methanol–water (60:40) solution of 2.5 m*M* acetic acid and 85 m*M* TEA. ● = Asulam; ▼ = coumatetralyl; □ = biphenyl-2-ol; ▽ = propoxur; ■ = thiabendazole.

propoxur, coumatetralyl and biphenyl-2-ol. However, the ion-pair mixture of sodium cholate, acetic acid and TMA does not lower the capacity factor of thiabendazole sufficiently and the addition of another hydrophobic reagent, TEA, instead of TMA is needed to elute thiabendazole.

3.1. Calibration graphs and comparison of results with those of GC–FID

The calibration graphs are linear between 1.8 and 200 ng for asulam, 3 and 200 ng for propoxur, 0.6 and 200 ng for coumatetralyl, 0.6 and 100 ng for biphenyl-2-ol and 0.2 and 40 ng for thiabendazole. The lower limit of the linear dynamic range is determined by the detection limit. Typical relative standard deviations (R.S.D.s) are between 0.7 and 5.8%.

The results obtained for propoxur and biphenyl-2-ol by LC were compared with those given by GC. Coumatetralyl, asulam and thiabendazole decomposed on-column and they cannot be determined by GC without a previous derivatization step. Universal flame ionization detection (FID) was used with GC because propoxur and biphenyl-2-ol do not give response with the more commonly used detectors in pesticide residue analysis, such as electron-capture or nitrogen–phosphorus detectors.

The correlation between the LC and GC methods was $r = 0.9998$; the slope of the regression line was 1.0218 and the intercept −4.7792 for propoxur and $r = 0.9999$, slope = 0.9911 and intercept = 2.3364 for biphenyl-2-ol. The detection limits with the LC and GC methods are 2.4 and 2.6 ng, respectively, for propoxur and 0.4 and 2.4 ng, respectively, for biphenyl-2-ol. The R.S.D.s ($n = 3$) using the LC and the GC methods are 2.2 and 1.3%, respectively, for propoxur and 2.6 and 18.5%, respectively, for biphenyl 2-ol. These results indicate that both methods correlated well.

3.2. Application to food samples

Prior to application to real samples, the method was evaluated with synthetic mixtures of the most commonly used pesticides in pre- or post-harvest treatment. Nineteen potential interferents were selected among insecticides, fungicides, rodenticides and herbicides usually found in cereals, fruits, vegetables and other types of crops [7,13]. The synthetic mixtures were prepared using a fixed concentration of the pesticide to be recovered, namely asulam 0.2 μg/ml, coumatetralyl 0.2 μg/ml, biphenyl 2-ol 0.2 μg/ml, propoxur 0.5 μg/ml and thiabendazole 0.1 μg/ml, and adding the potential interferents at several levels. The organophos-

Table 2
Analytical characteristics and recovery of pesticides from spiked foods

Compound	Apples[a]					Wheat grains[a]				
	D_L^b (ng)	C_Q^c (ng)	Concentration range (μg/ml)	Mean recovery (%)	R.S.D. (%)	D_L^b (ng)	C_Q^c (ng)	Concentration range (μg/ml)	Mean recovery (%)	R.S.D. (%)
Asulam	0.8	2.7	0.5–8.0	101.0	5.1	1.9	6.5	0.5–8.0	104.9	2.5
Propoxur	0.9	2.9	0.5–8.0	97.3	3.9	1.4	4.7	0.5–8.0	97.0	1.4
Coumatetralyl	0.6	2.0	0.5–8.0	98.6	3.5	1.0	3.5	0.5–8.0	96.0	2.8
Biphenyl-2-ol	0.1	0.30	0.05–2.0	99.4	6.8	0.3	1.1	0.06–2.0	93.6	2.6
Thiabendazole	0.2	0.6	0.05–0.5	103.1	9.2	0.1	0.4	0.03–0.5	95.9	3.7

[a] $n = 3$.
[b] Detection limit (signal-to-noise ratio = 3).
[c] Quantification limit (signal-to-noise ratio = 10).

phates azinphos-ethyl, chlorpyrifos, dialifos, fenamifos and parathion-methyl were added together, as were the organochlorides chlorfenson, chlorobenzilate, dicofol and tetradifon. Recoveries from these synthetic mixtures ranged from 90 to 110% at a pesticide-to-interferent ratio of 50 for the most of pesticides; organochlorines are the best tolerated and analysis for thiabendazole is least subject to interference from the other pesticides.

To check the usefulness of the procedure, recoveries of the pesticides in apples and wheat grain were determined. These samples were chosen because of their high consumption and to demonstrate the applicability of the procedure to different product matrices.

A number of solvent extraction systems have been used for residue screening procedures. The use of acetone and acetone–water mixtures is the most widely used [13,21–24], simplest and most efficient method to remove organic chemical residues quantitatively from foodstuffs because high concentrations of residues can be extracted.

Apples and wheat grain samples were spiked prior to extraction with a methanolic solution of the pesticides, after checking for the absence of the pesticides under study. After extraction, the samples were subjected to the LC procedure. The chromatograms of apple and wheat grain extracts are reported in Fig. 3. Table 2 gives the results obtained with recoveries ranging from 97 to 103% in apples and from 94 to 105% in wheat grains. The accuracy is excellent. The precision deduced from the R.S.D. values is consistently good; these results were affected by the particularly high R.S.D. for repeatability for low levels of biphenyl-2-ol and thiabendazole in apples. The detection limits, defined as the lowest amount that gave a signal three times higher than the baseline noise, ranged from 0.1 ng for thiabendazole to 1.9 ng for asulam and are higher than those obtained for apples because the wheat grain blanks are affected by a high R.S.D.

4. Conclusions

A mixture of the pesticides asulam, propoxur, coumatetralyl, biphenyl-2-ol and thiabendazole was resolved by ion-pair reversed-phase liquid chromatography. The chiral surfactant sodium

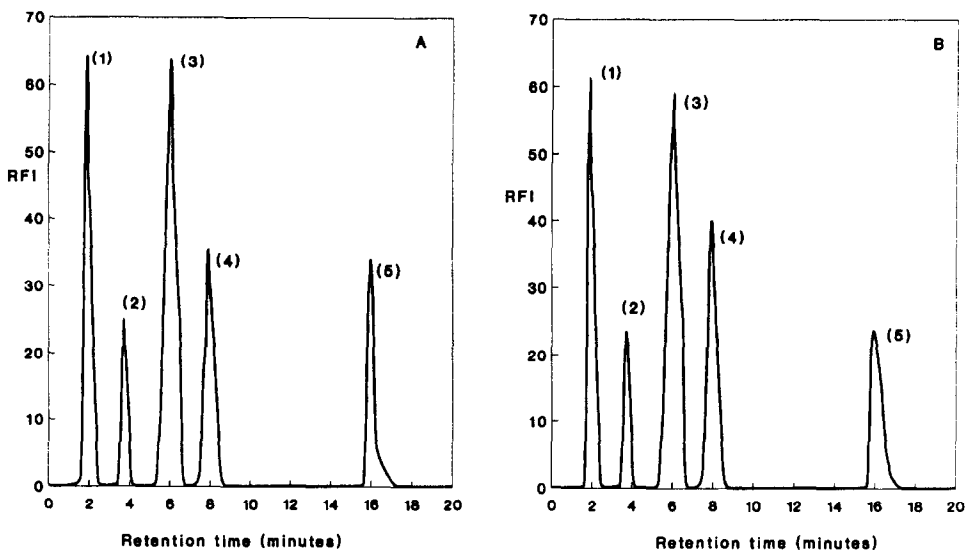


Fig. 3. Chromatograms of (A) apples spiked with (1) 2 μg/ml of asulam, (2) 2 μg/ml of coumatetralyl, (3) 0.5 μg/ml of biphenyl-2-ol, (4) 2 μg/ml of propoxur and (5) 0.10 μg/ml of thiabendazole and (B) wheat grains spiked with (1) 2 μg/ml of asulam, (2) 2 μg/ml of coumatetralyl, (3) 0.5 μg/ml of biphenyl-2-ol, (4) 2 μg/ml of propoxur and (5) 0.125 μg/ml of thiabendazole.

cholate proved its utility as a counter ion in the mobile phase. The method compares well with GC. As the analytical characteristics (ease of applicability, detection limits, precision) show the method is suitable for the analysis of food samples.

Acknowledgement

The authors thank the Comisión Interministerial de Ciencia y Technología (Project PB89-0583) for supporting this study.

References

[1] R.T. Kon, L. Geissel and R.A. Leavit, *Food Addit. Contam.*, 1 (1984) 67.
[2] C.H. Marvin, I.D. Bridle, C.D. Hall and M. Chiba, *Anal. Chem.*, 62 (1990) 1495.
[3] D.M. Gilvydis and S.M. Walters, *J. Assoc. Off. Anal. Chem.*, 73 (1990) 753.
[4] A.R. Long, L.C. Hsieh, C.R. Short and S.A. Barker, *J. Chromatogr.*, 475 (1989) 753.
[5] Y. Kitada, M. Sasaki and K. Tanigawa, *J. Assoc. Off. Anal. Chem.*, 65 (1982) 1302.
[6] A.M. Marti, A.E. Mooser and H. Kock, *J. Chromatogr.*, 498 (1990) 145.
[7] B. Ohlin, *Var Föda, Suppl.*, 38 (1986) 111.
[8] K. Isshiki, S. Tsumura and T. Watanabe, *Agric. Biol. Chem.*, 42 (1978) 2375.
[9] N. Motohashi, H. Nagashima and R. Meyer, *J. Liq. Chromatogr.*, 13 (1990) 345.
[10] S.Y. Szeto and K.M.S. Sundaram, *J. Chromatogr.*, 200 (1980) 179.
[11] R.E. Cline, L.W. Yert and L.L. Needham, *J. Chromatogr.*, 32 (1984) 420.
[12] L. Ogierman, *J. Chromatogr.*, 210 (1981) 83.
[13] A. Anderson and B. Ohlin, *Var Föda, Suppl.*, 38 (1986) 79.
[14] M.T.W. Hearn (Editor), *Ion-Pair Chromatography. Theory and Biological and Pharmaceutical Applications*, Marcel Dekker, New York, 1985.
[15] S. Terabe, M. Shibata and Y. Miyashita, *J. Chromatogr.*, 480 (1989) 403.
[16] R.O. Cole, M.J. Sepaniak and W.L. Hinze, *J. High Resolut. Chromatogr.*, 13 (1990) 579.
[17] R.W. Williams, Jr., Z. Fu and W.L. Hinze, *J. Chromatogr. Sci.*, 28 (1990) 292.
[18] H. Nishi, T. Fukuyama, M. Matsuo and S. Terabe, *Anal. Chim. Acta*, 236 (1990) 281.
[19] A. Dobashi, T. Ono, S. Hara and J. Yamaguchi, *Anal. Chem.*, 61 (1989) 1984.
[20] *The Pesticide Manual —A World Compendium*, British Crop Protection Council, Croydon, 1983.
[21] Y. Aoki, M. Takeda and M. Uchiyama, *J. Assoc. Off. Anal. Chem.*, 50 (1975) 1286.
[22] M.C. Bowman and M. Beroza, *J. Assoc. Off. Anal. Chem.*, 50 (1967) 1228.
[23] M.A. Luke and G.M. Doose, *Bull. Environ. Contam. Toxicol.*, 30 (1983) 110.
[24] L.D. Sawyer, *J. Assoc. Off. Anal. Chem.*, 68 (1985) 64.