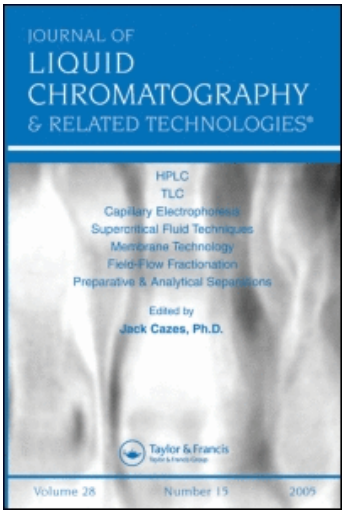

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HPLC DETERMINATION OF PESTICIDE RESIDUE ISOLATED FROM FOOD MATRICES

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ABSTRACT

Methods for the extraction, separation, detection, and quantification of ten herbicide, insecticide, and fungicide residues in fruits, vegetables, and cereal were evaluated. The extraction of the residues was achieved using liquid–liquid extraction (LLE), solid-phase extraction (SPE), and matrix solid-phase dispersion (MSPD). Determination was carried out by reversed-phase high performance liquid chromatography (RP-HPLC) with column switching and diode array detection (DAD). Recoveries, at spiked concentrations below the maximum acceptable residue levels established by the Polish Government, were between 68.7 and 105.0% with relative standard deviations ranging from

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0.5 to 6.2%. The limits of quantification varied from 0.02 to 0.25 μg/g.

Key Words: Pesticide residue; Fruits; Vegetables; Cereal; Sample preparation; HPLC; Column switching

INTRODUCTION

There are many preparation techniques in pesticide residue analysis that can be used individually or sequentially according to the complexity of sample, the nature of the matrix, the analytes, and the instrumental technique available.[1–4] The use of an extraction technique is common in the pre-treatment of the most types of sample.

Liquid–liquid extraction (LLE) is still quite popular because of the inherent simplicity, facility of operation procedure, and provided by the range of organic solvents, which are available.[3,5] This technique, apart from aspects, such as the labour intensity and the use of large volumes of often toxic organic solvents, has been used to extract some of the pesticides from different matrices.

LLE is rapidly being replaced by solid-phase extraction (SPE).[3,6–8] SPE has gained popularity for sample preparation of pesticides from biological, water and soil matrices. Because the choice of SPE column depends on the matrix and on the particular compound of interest, a wide range of solid-phase columns of different polarities have been used. For pesticide extraction from foods, biological samples, water, and soil, the phases include C_2, C_8, C_{18}, NH_2, CN, Diol, and other forms. Different mechanisms are involved in each solid support.[8,9]

Some of the most difficult samples to analyse are solid, semi-solid, or viscous, many of which are of biological origin. Adoption of techniques such as matrix solid-phase dispersion (MSPD) makes possible the preparation and extraction, as well as reduction, and especially the size of sample and the solvent consumption.[10–13] MSPD isolation technique involves blending a small amount of sample with a high-surface-area adsorbent, such as C_{18}, Florisil, aluminium oxide, and silica gel; the consistency changes to a highly dispersed powder. The mixture is placed into a syringe barrel with a filter in the bottom. The analyte is then eluted with an appropriate solvent and the tube is discarded.[14–17] The mechanisms of the MSPD include sample homogenisation, cellular disruption, exhaustive extraction, fractionation, and purification in a single process.[18,19]

In comparison to GC-based techniques, RP-HPLC with ultraviolet (UV) detection is more suitable for the determination of polar, non-volatile, and termolabile pesticides.[4,20] The wide application range, long-term stability, ease of use, low cost, and improved selectivity makes UV detection widely used in

residue analysis. However, UV detection does not deliver high sensitivity. It can be improved by coupling LC to LC, also called LC with column switching.[21] These coupled chromatographic techniques are the most sensitive and selective techniques available for the determination of pesticide residue in environmental and biological samples.[22] It is a powerful technique for the separation and clean up of multicomponent mixtures in which fractions from one chromatographic column are transferred selectively to secondary column for additional separation. Maximum resolution can be achieved by using different stationary and mobile phases.[23] The principles and details of this technique have often been discussed in literature, and will not be repeated here.[24–28]

In the current paper, comparison of three extractions techniques mentioned above (LLE, SPE, MSPD), and separating analytes by HPLC-DAD with column switching, has been the principal focus. The single-residue method (SRM) has been successfully applied in the determination of the class of the different pesticides in different matrices. Table 1 compares the extraction techniques for interesting pesticides chosen for this study.

Table 1. Extraction Techniques Used for Different Pesticide/Matrix Combinations

Extraction Technique	Pesticide/Matrix
Liquid–liquid extraction (LLE)	Flupoxam/wheat Linuron/carrot Rimsulfuron/potato Tetradifon/strawberry Benomyl/champinion Carbendazim/rye, wheat Thiophanate methyl/apple, black currant, cherry, cucumber, tomato
Solid-phase extraction (SPE)	Linuron/carrot Methomyl/cucumber, lettuce, tomato Tetradifon/strawberry Thiophanate methyl/apple
Matrix solid-phase dispersion (MSPD)	Linuron/broad bean, carrot, celery, green pea, leek, potato Diflubenzuron/champinion Fenoxycarb/apple Tetradifon/carrot, strawberry Thiophanate methyl/apple

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EXPERIMENTAL

Materials and Reagents

Acetonitrile and methanol were for HPLC grade from J.T. Baker (Deventer, The Netherlands). Deionized water was purified by Maxima water purification system (ELGA, High Wycombe, England). These solvents were filtered through 0.45 μm Nylon 66 Membranes (Supelco, Bellefonte, PA, USA) and degassed using helium sparging. *n*-Hexane, diethyl ether (not stabilised with ethanol), dichloromethane, methanol, acetone, and petroleum ether were residue analysis grade, and distilled-in-glass if necessary. Inorganic compounds were all reagent grade. Silica gel was Kieselgel 60 extrapure, particle size 0.063–0.200 mm (70–230 mesh ASTM) (Merck, Darmstadt, Germany) reactivated prior to use at 773K for 2 h, cooled in a dessicator, kept tightly closed. SPE columns were Florisil®, bore silica (SG), SG–NH_2 and SG–Diol; 500 mg, 6 mL (J. T. Baker).

Pesticide standards were obtained from Promochem (Wesel, Germany) and were used for fortification and quantification. Stock solutions (200 μg/mL) were prepared in for HPLC grade acetonitrile or methanol. The calibration and working standard solutions were prepared by diluting stock solutions with acetonitrile–deionized water or methanol–deionized water. These solutions were stored in the refrigerator at 277K.

The samples of fruits and vegetables were collected fresh from the market and private producers.

Apparatus

Food cutter (Hobart), high-speed blender, a commercial blender with a stainless steel jar (Waring Products Division, New Hartford, USA). Homogenisator was Ultra-Turrax (Janke & Kunkel, IKA-Labortechnik, Germany), SPE manifold was VISIPREP™ (Supelco). Rotary-vacuum-evaporator was Rotavapor-R type W (Büchi, Flawill, Switzerland) with 323K water bath. Shaker was type 358S (Elpan, Lubawa, Poland). Extraction columns were made from polypropylene cartridge, 130 × 25 mm id with a glass wool plug (Pharma-Plast A/S, Rodby, Denmark).

The HPLC system consisted of CM 3500 and 3200 pumps, autosampler Milton Roy type 713 (AS), UV-DAD detector type SM 5000 (TSP, Riviera Beach, FL, USA); programmable, six port column switching valve type WEC6WK (VICl, Valco Instruments, Houston, TX, USA); 100 μL injection loop (Supelco); Rheodyne Pneumatic Sample Injector Model 7126 (RH) (Rheodyne, Cotati, CA, USA). The data were collected and analysed with LCtalk computing system (TSP LCtalk™ HPLC software, version 2.03.02).

Separating columns, CC-1 clean up columns, and AC-2 analytical columns, appropriate concentration of mobile phases, and wavelengths are presented in Table 2. Flow-rates were 0.5 or 1 mL/min; injection volume was 100 μL. The set-up of the chromatographic system is presented in Fig. 1.

Sample Preparation

Representative portion of samples (200 g of whole fruits, vegetables, and cereal) were prepared using a blender, and mixed thoroughly. Samples were extracted using the LLE, SPE, or MSPD procedure.

LLE

Ten gram portions were sampled; 100 mL of acetone, methanol–hydrochloric acid, or acetone–hydrochloric acid were added, blended, and shook. After vacuum filtering, solvent was evaporated and the remaining phase was partitioned with dichloromethane or dichloromethane/*n*-hexane mixture. Solvent was evaporated to dryness, and residues were redissolved in the appropriate HPLC mobile phase before injection.

SPE

The samples were homogenised in acetone or acetone/dichloromethane/*n*-hexane mixture, and the products of homogenisation were partitioned with dichloromethane. After evaporating dichloromethane and redissolving in dichloromethane/*n*-hexane mixture, the residues were percolated through preconditioned SPE tubes containing Florisil, SG, SG–NH_2 or SG–Diol. The compounds of interest were selectively eluted with methanol/dichloromethane, *n*-hexane/diethyl ether, or methanol/dichloromethane mixture. The solvent was evaporated to dryness, the dry residue dissolved in HPLC mobile phase, and analysed.

MSPD

Sample material and an adequate quantity of water were disintegrated by high speed blending in order to obtain the homogenous pulp. Pulp subsamples, representing a 5 g sample, were weighed into a mortar; 10 g silica gel was added and ground to obtain the consistency of the free-flowing powder. The extraction

Table 2. Separating Columns and Mobile Phases for HPLC with Column Switching Analysis

Analyte	Wavelength (nm)	CC-1 Clean Up Column Mobile Phase	AC-2 Analytical Column Mobile Phase
Flupoxam	240	Adsorbosphere Phenyl, 150 × 4.6 mm, 5 μm ACN/H_2O (60 + 40, v/v)	Alltima C18, 250 × 4.6 mm, 5 μm ACN/H_2O (75 + 25, v/v)
Linuron	248	Supelcosil LC-8-DB, 150 × 4.6 mm, 5 μm ACN/H_2O (60 + 40, v/v)	Alltima C18, 250 × 4.6 mm, 5 μm ACN/H_2O (75 + 25, v/v)
Rimsulfuron	254	Zorbax Phenyl, 80 × 4 mm, 5 μm MeOH/H_2O (44 + 56, v/v)	Zorbax Rx C8, 250 × 4.6 mm, 5 μm MeOH/H_2O (22 + 78, v/v)
Diflubenzuron	254	Supelcosil LC-8-DB, 150 × 4.6 mm, 5 μm ACN/H_2O (60 + 40, v/v)	Alltima C18, 250 × 4.6 mm, 5 μm ACN/H_2O (75 + 25, v/v)
Fenoxycarb	228	Supelcosil LC-8-DB, 150 × 4.6 mm, 5 μm ACN/H_2O (50 + 50, v/v)	Zorbax Rx C8, 250 × 4.6 mm, 5 μm ACN/H_2O (75 + 25, v/v)
Methomyl	232	Zorbax Phenyl, 80 × 4 mm, 5 μm ACN/H_2O (20 + 80, v/v)	Zorbax Rx C8, 250 × 4.6 mm, 5 μm ACN/H_2O (30 + 70, v/v)
Tetradifon	214	Alltima C18, 250 × 4.6 mm, 5 μm ACN/H_2O (60 + 40, v/v)	Zorbax Rx C8, 250 × 4.6 mm, 5 μm ACN/H_2O (30 + 70, v/v)
Benomyl Carbendazim Thiophanate Methyl	279	Zorbax Rx C8, 250 × 4.6 mm, 5 μm MeOH/H_2O (45 + 55, v/v)	Alltima C18, 250 × 4.6 mm, 5 μm MeOH/H_2O (60 + 40, v/v)

Columns manufacturers: Adsorbosphere, Alltima—Alltech Associates, Inc., Deerfield, IL, USA. Supelcosil—Supelco, Bellefonte, PA, USA. Zorbax—Rockland Technologies, Inc., Nuenen, The Netherlands.

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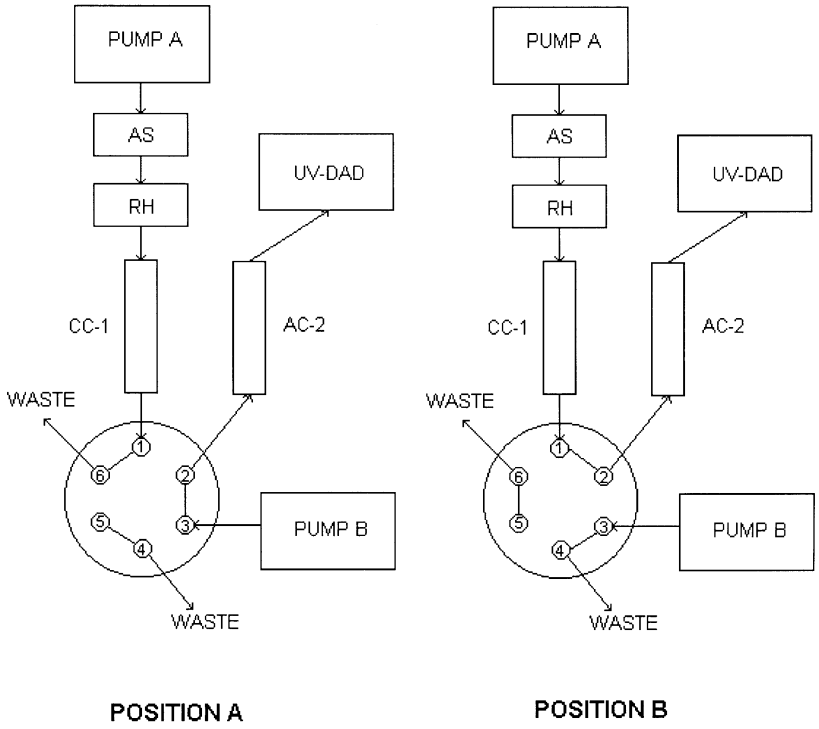

Figure 1. Schematic diagram of the column switching system.

column was plugged with glass wool; the powdery samples were transferred through a widemouth polypropylene funnel. The residues were extracted with eluent (*n*-hexane/diethyl ether or methanol/dichloromethane mixture) and collected in round-bottomed flasks. The solvent was evaporated to dryness using a rotary evaporator, and the dry residue was dissolved in HPLC mobile phase.

Column Switching Procedure

The sample extract was automatically injected (Fig. 1) by AS via Rheodyne valve (RH), fitted with a 100 μL loop, into clean-up column 1 (CC-1), through which pump A was pumping mobile phase A. At the same time, pump B was pumping mobile phase B through analytical column 2 (AC-2) (Position A). At a predetermined time, the switching valve changed into Position B, at which time

a portion of effluent from CC-1 was transferred into AC-2. The valve was then automatically switched back into Position A.

Determination

The compositions of both mobile phases were chosen in such a manner that the analysis time was kept to a reasonable minimum, and peak broadening was avoided through analyte preconcentration on column heads. The retention time of interesting pesticide on CC-1 was determined by connecting CC-1 directly to the UV-DAD detector. The chromatogram, obtained in this way, allowed the determination of the width at the base of peak and, thus, the required switching valve opening window. This window had to be experimentally checked each time the new mobile phases were prepared.

RESULTS AND DISCUSSION

Recovery Study

To determine extraction efficiency, control samples were fortified with pesticide stock solutions. Recovery studies were made, so as to reproduce as far as possible, the natural incorporation of the residue in a sample matrix. Materials such as fruits and vegetables do not generally exhibit binding of residues, but disruption of tissue during homogenisation releases hydrolytic enzymes, which may cause breakdown of some pesticides. Therefore, control samples of fruits and vegetables were homogenised, applying the spiking solution over the weighted sample as widely as possible, rehomogenised thoroughly, and then were extracted (compiled by R. B. Maybury in Laboratory Manual for Pesticide Residue Analysis in Agricultural Products, Laboratory Services Division, Ottawa, Canada, 1984). Physical binding and the formation of conjugates is more likely in grains, therefore, a very through mixing with the spiking solution, followed by standing overnight for equilibration was done. Recoveries were calculated from seven replicates of each sample by comparing the peak height of spiked solution of the same concentration injected directly onto the HPLC system.

In this study, linuron in carrot, tetradifon in strawberry, and thiophanate methyl in apple were analysed using these three extraction procedures, LLE, SPE, and MSPD. Recovery rates and RSDs obtained from the optimisation assay are presented in Table 3 and on the graph in Fig. 2. These results show, clearly, the best efficiency for MSPD procedure and less clearly when LLE and SPE are

Table 3. Recovery Rates and Relative Standard Deviations (RSD) of Pesticides According to Peaks Height

Analyte	Matrix	Extraction Technique	Recovery (%)	RSD (%)
Flupoxam	Wheat	LLE	96.1	5.9
Linuron	Broad bean	MSPD	72.3	2.3
		LLE	75.4	4.2
	Carrot	SPE	80.2	3.7
		MSPD	94.5	0.5
	Celery	MSPD	92.9	1.2
	Green pea	MSPD	78.5	2.8
	Leek	MSPD	94.8	4.0
	Potato	MSPD	85.1	6.2
Rimsulfuron	Potato	LLE	82.4	4.6
Diflubenzuron	Champinion	MSPD	92.2	1.8
Fenoxycarb	Apple	MSPD	81.3	3.2
Methomyl	Cucumber	SPE	89.5	1.8
	Lettuce	SPE	84.3	5.7
	Tomato	SPE	81.7	4.4
Tetradifon	Carrot	SPE	105.0	2.0
		LLE	72.3	5.8
	Strawberry	SPE	79.3	3.8
		MSPD	83.9	3.1
Benomyl	Champinion	LLE	75.2	2.5
Carbendazim	Rye	LLE	84.7	3.4
	Wheat	LLE	82.1	3.7
Thiophanate Methyl		LLE	73.1	3.4
	Apple	SPE	82.0	4.2
		MSPD	81.2	3.0
	Black currant	LLE	82.0	2.7
	Cherry	LLE	68.7	4.3
	Cucumber	LLE	90.4	3.3
	Tomato	LLE	92.6	4.5

employed. Results are obtained for the samples analysed and compared between themselves:

94.5%, 80.2% and 75.4% for linuron in carrot using MSPD, SPE and LLE, respectively,
83.9%, 79.3% and 72.3% for tetradifon in strawberry, and
81.2%, 82.0% and 73.1% for thiophanate methyl in apple.

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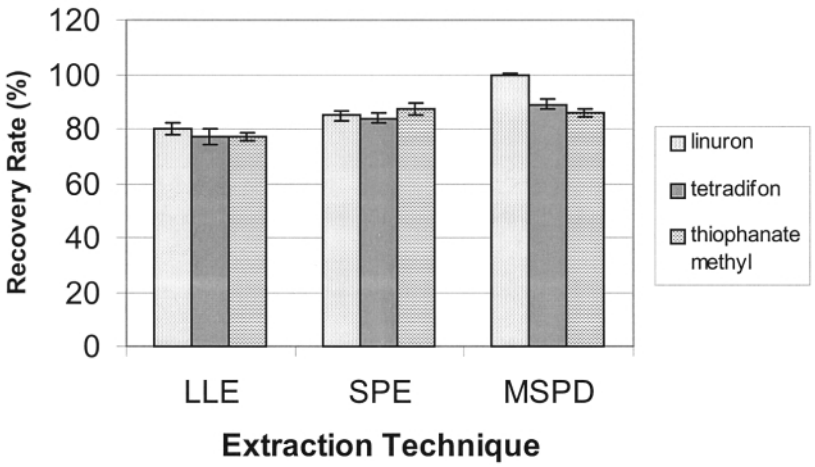

Figure 2. Comparison of recovery rates and RSD of examined pesticide residues (linuron in carrot, tetradifon in strawberry, and thiophanate methyl in apple) obtained from optimisation parameters of LLE, SPE, and MSPD extraction techniques.

Furthermore, the reproducibility of MSPD is also better than the LLE and SPE procedures:

0.5%, 3.7% and 4.2% for linuron in carrot using MSPD, SPE and LLE, respectively,
3.1%, 3.8% and 5.8% for tetradifon in strawberry and
3.0%, 4.2% and 3.4% for thiophanate methyl in apple.

These extraction procedures were tried for comparison between the remaining analyses, and the results are presented generally below. Different parameters were studied to optimise the extraction technique:

Time required for extraction: lowest when MSPD are used; highest for LLE.
Best recovery averages for MSPD (94.5, 83.9, 81.2%), next for SPE (80.2, 79.3, 82.0%), and LLE (75.4, 72.3, 73.1%).
Lowest average RSD values for MSPD (0.5, 3.1, 3.0%), next for SPE (3.7, 3.8, 4.2%), and LLE (4.2, 5.8, 3.4%).
Use of disposable and non-expensive equipment: lowest when MSPD are used, more expensive for SPE.
Consumption of organic solvents; minor amounts of it are used with MSPD compared to SPE and LLE, which involves large amounts of dichloromethane and *n*-hexane.

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Facility of operation: superior for MSPD.
Possibility for automation: highest for SPE, next for MSPD.

Several another parameters are: emulsion formation can cause problems during LLE, less time and less organic solvents are needed for cleaning glass equipment because disposable SPE cartridges and MSPD columns are used for extraction.

Summarising, the high efficiency of MSPD for studied pesticide/matrix combinations can be clearly observed, comparing the recovery data with those obtained with LLE and SPE. In order to obtain the highest efficiency, MSPD is the method of choice. The results showed good performance of the analytical protocol with fruits and vegetables.

Figure 3 illustrates typical chromatograms of the linuron standard, and unfortified and fortified carrot sample extracts using MSPD technique. No interfering peaks were observed on the chromatogram of the unspiked extracts obtained under the selected conditions.

Linearity and Quantification Limits

The instrument was calibrated by measuring the peak height of a series of standard solutions of varying concentrations of each pesticide, in order to find the linearity range of the UV-DAD detector response, and to ensure that the valve

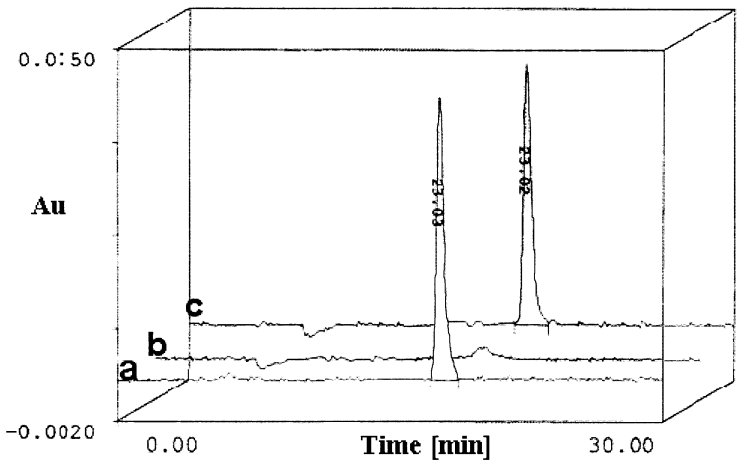

Figure 3. Typical chromatograms: a—linuron standard 0.26 µg/mL; b—5 g control carrot; c—5 g fortified carrot over the level 0.1 µg/g.

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Table 4. Wavelengths, Correlation Coefficients, and Quantification Limits of Determined Pesticides

Analyte	Wavelength (nm)	Correlation Coefficient R^2	Matrix	Quantification Limit (μg/mL)
Flupoxam	240	0.9994	Wheat	0.07
Linuron	248	0.9999	Broad bean	0.05
			Carrot	0.03
			Celery	0.08
			Green pea	0.05
			Leek	0.07
			Potato	0.02
Rimsulfuron	254	0.9999	Potato	0.04
Diflubenzuron	254	0.9999	Champinion	0.03
Fenoxycarb	228	0.9999	Apple	0.05
Methomyl	232	0.9999	Cucumber	0.03
			Lettuce	0.25
			Tomato	0.1
Tetradifon	214	0.9999	Carrot	0.07
			Strawberry	0.03
Benomyl carbendazim	279	0.9996	Champinion	0.1
			Rye	0.02
			Wheat	0.03
Thiophanate methyl			Apple	0.1
			Black currant	0.09
			Cherry	0.07
			Cucumber	0.1
			Tomato	0.1

opening window was sufficiently wide for complete transfer of the analyte at maximum concentrations from the calibration curve. We found, that over the concentration range of 0.01–2.65 μg/mL, the response of the detector was linear, and the correlation coefficient was the same or better than 0.999. The detection wavelengths, correlation coefficients, and quantification limits are given in Table 4.

CONCLUSION

This study has shown that several extracting procedures, LLE, SPE, and MSPD, and HPLC with column switching, make possible determination of

pesticide residue in fruits, vegetables, and cereal in single-residue methods. The obtained results indicate, that the best extraction technique is MSPD. This technique, modification of that reported by Kadenczki et al.[29] constitutes a significant advance in efficiency, provides good recoveries, requires small amounts of samples and solvents, involves few steps, and sample manipulation is simple. This technique is, at present, being tested by our laboratory on more biological and food matrices.

Column switching HPLC with UV-DAD detection is a versatile tool for the determination of these organic compounds in difficult foods and biological matrices. The main advantages of multidimensional chromatographic techniques are the enhanced selectivity, improved sensitivity, and automation potential: *on-line* system with no manual operation. As demonstrated by the results obtained, the MSPD extraction technique and HPLC with column switching determination can be used as a routine technique in the laboratory.

A systematic approach to method development renders the technique to be an important means in the operation of a flexible monitoring program. Its application to real samples proves, unmistakably, the importance of the inclusion to protect the consumer's health.

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