RESIDUES AND TRACE ELEMENTS

Determination of Seventeen Polar/Thermolabile Pesticides in Apples and Apricots by Liquid Chromatography/Mass Spectrometry

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A simple liquid chromatography/mass spectrometry (LC/MS) approach for the determination of widely used representatives of polar/thermolabile pesticides in fruits was developed and validated. The group of pesticides comprised benzimidazoles and azoles (carbendazim, thiabendazole, imazalil, propiconazole, prochloraz, epoxiconazole, flusilazole, tebuconazole, bitertanol); *N*-methylcarbamates (carbaryl, carbofuran, methiocarb); and phenylureas and benzoylphenylureas (linuron, diflubenzuron, triflumuron, teflubenzuron, flufenoxuron). Matrixes (apple, apricot) were extracted with acetonitrile and crude extracts were cleaned up by solid-phase extraction (SPE) using either mixed cation exchange or hydrophilic lipophilic balance cartridges. LC separation of pesticides was performed on a reversed-phase column, Discovery C_{18}. Electrospray ionization and ion trap MS/MS detection were applied. For most pesticides, overall recoveries ranged from 75 to 122%, and repeatability (as relative standard deviation) from 5 repetitive determinations of recovery ranged from 3 to 21%. Carbofuran was the only compound for which recovery was not satisfactory due to its loss in the SPE cleanup step. Limits of detection were 0.1–3 μg/kg for benzimidazole and azole fungicides and carbamate insecticides. For urea insecticides, detection limits were slightly higher (3–10 μg/kg).

Recently, a relatively strict hygienic limit has been adopted by the European Union (Directive 96/5/EC and subsequent revisions) for pesticide residues in baby and infant foods. Concentration of any pesticide in these products should not exceed 10 μg/kg. Regulations for common commodities and foodstuffs not intended for vulnerable groups of population, such as children, allow considerably higher residues, by more than 1 order of magnitude in some cases, for most pesticides. Accordingly, limits of quantitation (LOQs) of the analytical procedures routinely used for surveillance and compliance purposes often do not meet the requirements posed by this new legislation.

Generally, for many modern pesticides routinely applied gas chromatographic (GC) methods with element-selective detection (e.g., electron capture and nitrogen phosphorus) or mass spectrometric (MS) detection provide sufficient results even at low ppb concentration levels. However, because of their physicochemical properties, some pesticides are not amenable to GC and, therefore, liquid chromatography (LC) is the technique of choice. In recent years liquid chromatography/mass spectrometry (LC/MS) has become an important tool for sensitive determination of polar/thermolabile pesticides in fruits and vegetables (1). Although many LC/MS applications for various pesticides have been reported, only a limited number of these studies describe a multiresidue determination of pesticides belonging to different chemical classes.

We developed a sensitive LC/MS/MS approach for the determination of representatives of several important groups of pesticides in fruits. Compounds troublesome for GC analysis were selected as target analytes: thermolabile pesticides decomposing either in the injection port of a gas chromatograph or at a GC column, e.g., *N*-methylcarbamates (2), and phenyl- and benzoylphenylureas (3); and polar basic compounds, benzimidazoles or azoles, which are difficult to be sensitively determined by GC because their analysis is accompanied by peak tailing and relatively low responses. Apples and apricots are important raw fruits for baby food production; therefore, they were selected as target matrixes for the method validation.

Experimental

Chemicals and Materials

(a) *Certified pesticide standards.*—Obtained from Dr. Ehrenstorffer GmbH (Augsburg, Germany), purity 95–99%.

(b) *Pesticide residue grade solvents.*—Acetonitrile and methanol were obtained from Merck (Darmstadt, Germany).

(c) *Deionized water.*—Used for mixing of mobile phase; was produced in a Milli-Q apparatus (Millipore, Billerica, MA).

(d) *Ammonium acetate (purity 99.999%).*—Aldrich (Milwaukee, WI).

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Table 1. Concentrations of pesticides in stock and working solution

	Pesticide	CAS No.	Individual stock solutions in acetonitrile, μg/mL	Mixed stock solution (A and B separately) in methanol, μg/mL	Mixed calibration solution (A + B) in methanol–water (1 + 1, v/v)	Spiking level I	Spiking level II	LOD,[a] μg/kg	LOQ = LCL,[b] μg/kg
Group A	Carbendazim	10605-21-7	313	50.1	1002	0.25	0.025	0.1	2
	Thiabendazole	148-79-8	3000	48.0	960	0.24	0.024	0.1	4.8
	Epoxiconazole	106325-08-0	3000	48.0	960	0.24	0.024	1.5	4.8
	Flusilazole	85509-19-9	3000	48.0	960	0.24	0.024	0.6	1.9
	Imazalil	35554-44-0	3000	48.0	960	0.24	0.024	0.1	4.8
	Tebuconazole	107534-96-3	3000	48.0	960	0.24	0.024	3	9.6
	Propiconazole	262-104-4	3000	48.0	960	0.24	0.024	3	9.6
	Prochloraz	67747-09-5	3000	48.0	960	0.24	0.024	0.6	1.9
	Bitertanol	70585-38-5	3000	48.0	960	0.24	0.024	3	9.6
Group B	Carbofuran	1563-66-2	3000	9.0	1800	0.18	0.018	1	3.6
	Carbaryl	63-25-2	3000	9.0	1800	0.18	0.018	1	3.6
	Methiocarb	2032-65-7	3000	9.0	1800	0.18	0.018	1	3.6
	Linuron	330-55-2	3000	24.0	4800	0.48	0.048	8	24
	Triflumuron	64628-44-0	3000	48.0	9600	0.96	0.096	6	19
	Diflubenzuron	35367-38-5	3120	49.9	9980	1.00	0.1	6	20
	Teflubenzuron	83121-18-0	750	45.0	9000	0.90	0.09	3	18
	Flufenoxuron	101463-69-8	3000	48.0	9600	0.96	0.096		19

[a] LOD = Limit of detection.

[b] LOQ = Limit of quantitation; LCL = lowest calibration level.

Table 2. MS/MS conditions

	Time window, min	Analyte	Ionization mode[a]	Parent ion *m/z*	Amp.,[b] %	Q value	Activation time, ms	Amu[c]	Daughter ion(s) *m/z*
Group A	0–8.35	Carbendazim		192	30	0.250	30	2	160
	8.35–9.60	Thiabendazole		202	40	0.300	40	2	175
	9.60–16.40	Epoxiconazole		330	40	0.250	30	2	121, 123, 141
	16.40–18.18	Flusilazole		316	34	0.250	30	2	187, 201
	18.18–20.80	Propiconazole		342	38	0.250	30	2	159, 205
		Tebuconazole		308	0	0.250	30	1	308[d]
		Imazalil		297	40	0.250	40	2	201, 255
	20.8–30.00	Prochloraz		376	32	0.250	30	2	308
		Bitertanol		338	28	0.250	30	2	269
Group B	0.00–7.11	Carbofuran		222	32	0.250	30	2	165
	7.11–8.47	Carbaryl		202	34	0.250	30	3	145
	8.47–10.00	Linuron		249	43	0.300	30	3	160, 182
		Methiocarb		226	26	0.300	30	3	169
	10.00–10.89	Diflubenzuron		309	50	0.300	40	2	289
	10.89–12.67	Triflumuron		357	40	0.300	40	2	321, 154
	12.67–14.19	Teflubenzuron		379	40	0.300	40	2	359, 339
	14.19–20.00	Flufenoxuron		487	40	0.250	30	2	467, 289

[a] + = Positive ion monitoring; – = negative ion monitoring.

[b] Activation amplitude.

[c] Amu = isolation width.

[d] Tebuconazole does not provide stable daughter ion spectrum when MS/MS in ion trap is performed. Therefore, collision energy parameter was set at zero and the nonfragmented molecular ion of tebuconazole was monitored.

(e) *Paper filters.*—Medium pore size 389 (yellow stripe), obtained from VEB Freiberger (Berlin, Germany), were used to filter sample suspensions.

(f) *Samples.*—Apple and apricot samples known to be free of pesticide residues were saved from previous analyses.

(g) *Solid-phase extraction (SPE) cartridges.*—Oasis hydrophilic lipophilic balance (HLB), 60 mg/3 mL, particle size 30 μm, product No. WAT094226, and Oasis MCX mixed cation exchange (MCX), 60 mg/3 mL, particle size 30 μm, product No. 186000254 from Waters Corp. (Milford, MA), were used for cleanup of crude extracts.

Pesticide Standards

Individual pesticide stock solutions (300–3120 μg/mL) were prepared by dissolving solid standard reference materials in acetonitrile. For benzoylurea pesticides, a small amount of acetone (ca 10%, v/v) was added to improve solubility. From these individual solutions, 2 mixed stock solutions, A and B (9–50 μg/mL) in methanol, were prepared. In each mixture, compounds of similar structure, hence of similar physicochemical properties, were included. In mixture A, *N*-containing heterocyclic compounds (benzimidazoles and azoles) were included, whereas mixture B comprised *N*-methylcarbamate, and phenyl- and benzoylphenylurea pesticides. For these 2 groups of pesticides, different LC/MS methods were created (*see LC Separation*, below). Because MS/MS provides specific detection, and thus the risk of interference from nontargeted pesticides present in the injected standards is low, working solutions in methanol–water (1 + 1, v/v) were prepared to contain all pesticides (A + B). The concentrations of individual pesticides in the highest calibration standard (standard 1) are given in Table 1. The other calibration standards, 2–7, were obtained by further dilution of standard 1 to obtain concentrations 2, 10, 20, 100, 200, and 500× lower than standard 1.

Matrix-matched standards for recovery determination, described below, were prepared at 2 different concentration levels from purified blank extracts by dissolving the residue after evaporation of SPE fraction in appropriate mixed (A + B) solvent standard corresponding to the given spiking level (Table 1). Nonpurified matrix-matched standards were prepared by evaporating an aliquot of crude extract and redissolving it in appropriate solvent standard.

Apparatus

Samples were processed with a Waring Blender homogenizer (Waring, New Hartford, CT) and Turrax tissumizer (IKA Werke, Staufen, Germany). Solvents were evaporated on a Büchi rotary evaporator (Büchi, Flawil, Switzerland).

SPE cleanup of samples was performed by using a Dorcus vacuum manifold (Tessek, Czech Republic).

Extraction and Cleanup

A 12.5 g sample was mixed with 50 mL acetonitrile and homogenized for 2 min with a Turrax tissumizer. The suspension was filtered under vacuum, the filtration cake was washed 3 times with 10 mL acetonitrile, and filtrate volume was reduced by evaporation to 5–7 mL. The concentrated extract was transferred into a 50 mL volumetric flask, 2.5 mL acetonitrile was added, and the mixture was diluted with deionized water. A 4 mL amount of this crude extract was applied to SPE cartridge. Within the method validation, MCX cartridges were used for cleanup of pesticides of group A; for group B, HLB cartridges were used. HLB cartridges were conditioned with 4 mL acetonitrile, followed by 4 mL 20% (v/v) acetonitrile in water. After the sample loading, the HLB cartridge was washed with 8 mL 20% acetonitrile in water, and analytes were eluted with 4 mL acetonitrile. MCX cartridges were conditioned with 4 mL acetonitrile, 4 mL 1M ammonium acetate in methanol, and 4 mL 20% acetonitrile in water, respectively. After the sample loading, MCX column was washed with 8 mL 20% acetonitrile and analytes were eluted with 4 mL 1M ammonium acetate in methanol. Loading the sample, washing, and elution were performed at a flow rate 0.5 mL/min. Eluted fractions from SPE were evaporated on a rotary evaporator, dried with a mild stream of nitrogen, and the residue was redissolved in 1 mL methanol–water (1 + 1, v/v). A 1 mL amount of this purified extract corresponded to 1 g of original sample.

To evaluate the applicability of both SPE cartridges for both groups of pesticides, the resulting SPE purified samples from MCX and HLB cartridges were analyzed to determine pesticides from both A and B groups.

LC Separation

For LC/MS/MS determination, pesticides were analyzed in 2 groups (A and B), each group under specific separation and detection conditions. All LC separations were performed on an HP1100 liquid chromatograph (Hewlett Packard, Palo Alto, CA) using a reversed-phase Discovery C_{18} column (15 cm × 3 mm, 5 μm); (Supelco, Bellefonte, PA), product No. 504955-30. Mobile phase was methanol–water. LC conditions were as follows:

Group A: Gradient program was 0–5 min, linear from 20 to 63% methanol; 5–25 min, linear from 63 to 70% methanol; 25.5–30 min, isocratic 100% methanol. Total analysis time was 30 min; additional 7 min post run time was required to condition the column to starting conditions.

Group B: Gradient program was 0–5 min, linear from 40 to 75% methanol; 5–13 min, linear from 75 to 90% methanol; 13.1–20 min, 100% methanol. Total analysis time was 20 min, and additional 7 min post run time was required to condition the column to starting conditions.

Flow rate 0.5 mL/min, column temperature 25°C, and injection volume 20 μL were used in all experiments.

MS/MS Detection

MS detection was performed using LCQ Deca ion trap instrument from Finnigan (San Jose, CA). Electrospray ionization (ESI) was applied in all experiments. The following experimental conditions were used:

Group A: Capillary temperature 280°C, spray voltage 5 kV, positive ionization mode for all pesticides (Table 2).

Group B: Capillary temperature 230°C, spray voltage 6 kV for analytes monitored in positive ion mode and 5 kV for analytes monitored in negative ion mode (Table 2).

Flow rates of sheath gas and auxiliary gas were 1.5 and 3 L/min, respectively, in all experiments. Capillary voltage and ion optics parameters were tuned automatically for each compound. For MS/MS analysis, relevant time segments were set up. Where baseline separation of analytes could not be achieved, more than one analyte was scanned within one time segment by creating several scan events in the segment. To obtain sufficient number of data points (minimum 7) across the chromatographic peak, not more than 3 different analytes were scanned per time segment. Table 2 shows MS/MS parameters for pesticides in groups A and B. Automated gain control (AGC) was set to fill the ion trap to 2×10^7 ions. Three microscans were summed to obtain one data point. Maximum inject time was 250 ms. Data were processed with Xcalibur software (Finnigan).

Validation Procedure

The validation study was conducted for 2 matrixes (apple and apricot) spiked at 2 concentration levels. Five replicate determinations of recovery were performed for each matrix and concentration level. The higher concentration level (I) was 0.18–1 mg/kg (i.e., 0.18–1 μg/mL in the final sample); the lower concentration level (II) was 0.018–0.1 mg/kg (i.e., 0.018–0.1 μg/mL in the final sample; Table 1). Blank apricot and apple samples (12.5 g each) were spiked with 0.5 mL appropriate spiking solution and incubated ca 0.5 h before extraction. Extraction and cleanup were performed as described above. To compensate for signal suppression and enhancement phenomena in ESI interface caused by matrix components (4–7), matrix-matched standards were used for determination of method recovery.

Results and Discussion

Although more than 100 pesticide residues can be analyzed by GC within one run, with LC separation coupled to ion trap MS/MS, the analysis of multiple residues representing different chemical classes is rather more complicated. In our study, the whole set of 17 pesticides was not analyzed by a single procedure for several reasons: (*1*) In the cleanup step, different SPE cartridges were used for group A and B pesticides. (*2*) For LC/MS/MS analysis time, windows have to be created for scanning of individual analytes. To obtain sufficient density of data points per peak, not more than 3 analytes can be scanned in

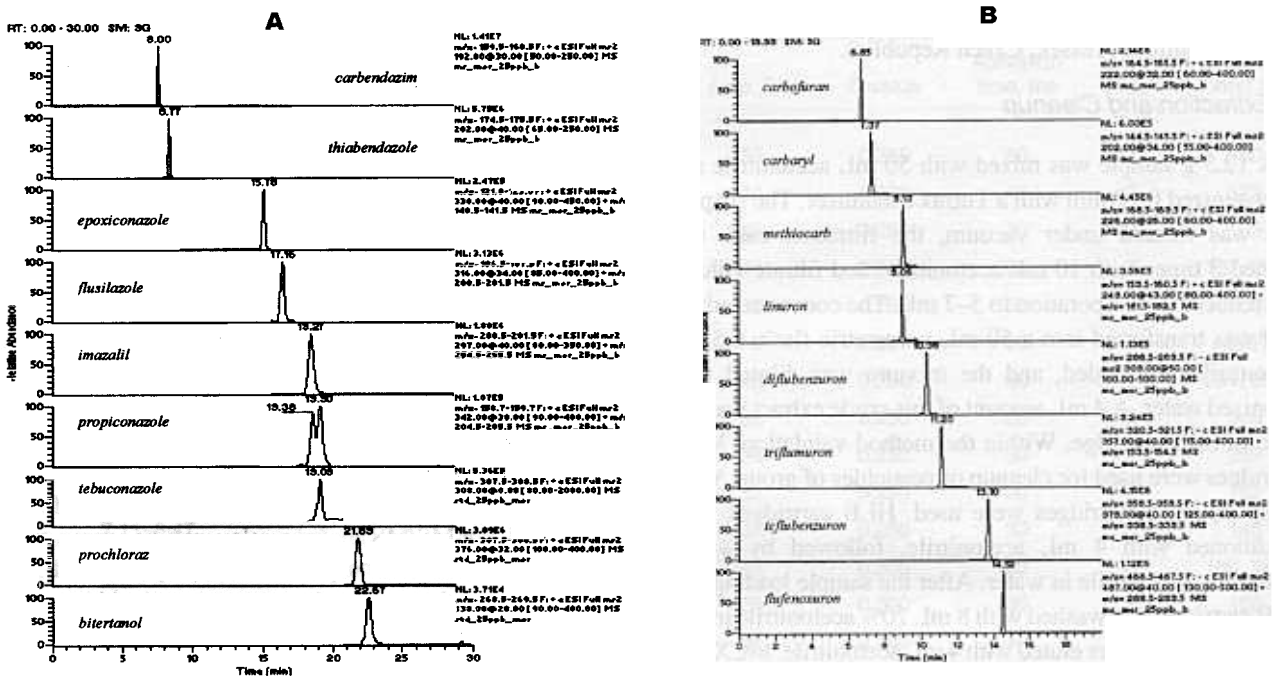

Figure 1. (A) LC/MS/MS chromatogram of mixture of pesticides in group A; matrix-matched standard prepared from MCX-purified apricot extract; concentration level of pesticides 0.025 μg/mL corresponds to lower spiking level used in validation experiments (*see* Table 1). For conditions, *see* text. (B) LC/MS/MS chromatogram of mixture of pesticides in group B; matrix-matched standard prepared from HLB-purified apricot extract; concentration level of pesticides 0.018–0.1 μg/mL corresponds to lower spiking level used in validation experiments (*see* Table 1). For conditions, *see* text.

one window; therefore, a good chromatographic separation is required. The separation of all 17 pesticides would require excessively long analysis time, resulting in an undesirable peak broadening in the case of late eluting compound. (*3*) From a practical point of view, the analysis of only one or several pesticides in the current sample is often required; in this case the quantitation method can be chosen according to the requirements.

LC/MS/MS

The Discovery C_{18} column provided good retention and peak shapes for all analytes. Most pesticides were eluted from the column before reaching 100% methanol; however, the gradient was set to rinse the LC column with 100% methanol at the end of the run to avoid possible retention of noneluted matrix components on the column.

As a potentially troublesome compound in terms of retention behavior, imazalil was identified. This basic azole pesticide tends to interact with free silanol groups of the column stationary phase; therefore its retention is dependent on the number of these groups present in the column. Although the Discovery C_{18} column is an endcapped column declared as suitable for analysis of basic compounds, we initially observed peak tailing of imazalil and significant retention time instability (systematic shift in long-term scale from one analysis batch to another). This problem was probably related to the age and sample load of the column, because it was largely reduced when a new Discovery C_{18} column was installed and conditioned. On the basis of this experience, we checked the retention time of imazalil before every sample sequence and, if necessary, modified the window for scanning imazalil.

The chromatogram of matrix-matched standard of apricot at the lower spiking level used in validation experiments is shown in Figures 1A and B. Under the conditions described above, both group A and B pesticides were determined within one analytical sequence, only by switching the instrument method in the software.

MS/MS analysis in ion trap was used to achieve good specificity of detection and to improve signal-to-noise (S/N) ratio of target analytes. For all compounds the (de)protonated molecular ion was the most abundant in full MS spectrum. Therefore, this molecular ion was selected for fragmentation in the ion trap, and characteristic secondary fragment ions were monitored. The only exception was tebuconazole, which did not provide stable MS/MS spectrum under fragmentation conditions. For mass analysis of tebuconazole, the collision energy was set to zero and the isolated ion m/z = 308, which corresponds to positive molecular ion, was monitored. Contrary to ion trap MS/MS spectra, in-source collision-induced dissociation (CID) spectra at 25 V were stable and provided specific secondary ions 125, 141, 151, 165, and 281. Therefore, in-source CID analysis can be used to confirm tebuconazole identity if this pesticide is found.

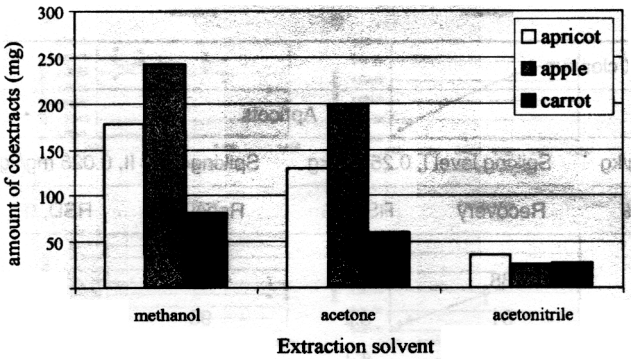

Figure 2. Amount of solid matrix components in crude extracts of selected fruits isolated by different extraction solvents (in 1 g original sample), measured by weighing evaporation residue of aliquot part of crude extract.

Extraction and Cleanup

Within the preliminary experiments, we attempted to use the same sample-handling strategy as that used in a multiresidue GC method routinely performed in our laboratory. This interlaboratory validated method (8) consists of ethyl acetate extraction followed by gel permeation chromatography (GPC) cleanup. However, we encountered several problems when using this sample preparation procedure for the LC/MS determination of target pesticides: (*1*) The extraction recoveries of basic benzimidazoles and azoles from acidic matrixes obtained by moderately polar solvent, such as ethyl acetate, are not satisfactory, although increasing the extraction pH may improve recoveries of some benzimidazole compounds; (*2*) during GPC, losses of imazalil and some other azole pesticides (e.g., prochloraz) occur probably because of irreversible sorption on gel structure; (*3*) for some matrixes, the evaporation residue left after GPC cleanup is not completely soluble in the methanol–water mixture (final solvent for LC/MS), because of the relatively low polarity of the coextracts isolated with ethyl acetate extraction. In a suspension formed in this way, there is a risk of loss of analytes by partition between the solid and liquid phases.

To improve recoveries of pesticides and to ensure better compatibility of the sample preparation step with LC/MS, water-miscible, polar solvents were tested for extraction of analytes. When comparing results obtained with acetone, methanol, and acetonitrile, the latter solvent provided best recoveries of target compounds; moreover, the amount of co-extracted sample components was significantly lower than with the other 2 extraction solvents (Figure 2). Therefore, acetonitrile was chosen as the extraction solvent for further experiments.

GPC cleanup was replaced by SPE, which is an effective approach widely applied for sample preparation before LC analysis of pesticides. After testing different SPE cartridges, we selected stationary phases Oasis HLB and MCX as the most suitable sorbents. The former phase, a polymer-based sorbent (*N*-vinylpyrrolidine divinylbenzene copolymer) displays reversed-phase properties, whereas the latter (the same polymeric sorbent substituted with sulfonyl groups) represents the combination of reversed-phase and cation exchange properties. Because of a very wide range of polarities and pKa values of pesticides included in the method, it was not possible to find a single, general SPE cleanup procedure. Polar, basic benzimidazoles and azoles (group A) were therefore separated from co-extracts by an MCX cartridge, whereas for nonionic and less polar compounds (group B), HLB cartridges were used for the cleanup.

For some pesticides, however, crude extract can be purified by both types of cartridges. To evaluate the potential of both types of SPE cartridges for separating the tested pesticides from co-extracts, within the method validation, pesticides of group A were determined in the extracts cleaned up by HLB cartridge, and pesticides of group B were analyzed in MCX cleanup samples. The overall recoveries were calculated by comparison with matrix-matched standards. Table 3 summarizes the applicability of both types of cartridges for different target analytes, showing that MCX cartridge can be used for all pesticides recovered in the study except 2 carbamates, carbofuran and carbaryl. Methiocarb, the third carbamate included in this study, is also less quantitatively recovered on an MCX sorbent than on HLB. In accordance with these results, the selection of a suitable SPE sorbent can be adjusted to the particular group of pesticides to be analyzed. Because a limited number of pesticides is often required for analysis, this method flexibility may be useful.

Reduction of matrix co-extracts, such as sugars and pigments with SPE cleanup, was significant. For instance, only about 4% of solids contained in crude extract of apples was

Table 3. Overview of applicability of HLB and MCX cartridges for SPE cleanup of target pesticides

Pesticide	HLB	MCX
Carbendazim		+
Thiabendazole		+
Epoxiconazole	+	+
Flusilazole	+	+
Imazalil	–	+
Tebuconazole	+	+
Propiconazole	+	+
Prochloraz	–	+
Bitertanol	+	+
Carbofuran	–	–
Carbaryl	+	
Methiocarb	+	+
Linuron	+	+
Diflubenzuron	+	+
Triflumuron	+	+
Teflubenzuron	+	+
Flufenoxuron		

Table 4. Recovery and repeatability of the method (n = 5)

	Group A–MCX cleanup							
	Apples				Apricots			
	Spiking level I, 0.25 mg/kg		Spiking level II, 0.025 mg/kg		Spiking level I, 0.25 mg/kg		Spiking level II, 0.025 mg/kg	
	Recovery	RSD, %	Recovery	RSD, %	Recovery	RSD, %	Recovery	RSD, %
Carbendazim	99	3.2	91	3.3	88	3.5	89	7.0
Thiabendazole	99	6.1	88	3.9	91	8.2	93	5.9
Epoxiconazole	87	6.6	92	6.1	93	4.1	81	4.2
Flusilazole	82	4.6	85	3.1	93	7.6	88	3.8
Imazalil	84	4.6	84	6.8	93	5.9	88	9.2
Tebuconazole	83	3.3	53	9.6	94	2.9	81	11.0
Propiconazole	81	5.5	87	6.4	100	4.0	87	9.9
Prochloraz	68	14.1	51	11.6	84	9.5	81	7.6
Bitertanol	84	8.7	84	20.7	96	2.0	91	9.9
	Group B–HLB cleanup							
	Spiking level I, 0.18–1 mg/kg		Spiking level II, 0.018–0.1 mg/kg		Spiking level I, 0.18–1 mg/kg		Spiking level II, 0.018–0.1 mg/kg	
Carbofuran	4	85.2	2	4.9	4	75.8	5	12.7
Carbaryl	84	3.1	91	5.2	85	4.3	75	11.0
Methiocarb	84	5.8	75	14.8	90	4.2	87	2.9
Linuron	92	7.7	85	13.4	93	3.2	97	2.7
Diflubenzuron	122	6.4	112	14.5	94	2.4	96	4.9
Triflumuron	78	14.7	105	4.0	88	9.4	92	4.4
Teflubenzuron	75	20.3	88	2.4	88	2.8	85	3.2
Flufenoxuron	61	36.1	95	47.0	83	2.1	50	20.2

left in the analyte fraction eluted from the HLB cartridge. In this way, LC/MS enhancement and suppression phenomena called matrix effects were effectively reduced.

Method Recovery and Repeatability

As shown in Table 4 recoveries exceeding 80% and a good repeatability [calculated as relative standard deviation (RSD_r) from 5 repetitive determinations of recovery] ranging from 2 to 11% have been achieved for benzimidazole and azole compounds comprised in group A. Within group A, slightly lower recoveries were obtained only for prochloraz and tebuconazole in apple matrix (especially at lower spiking levels). As regards compounds of group B, most pesticides gave mean recoveries ≥75% with RSD_r ranging from 3–20%.

Very low mean recoveries and insufficient repeatability of results were obtained for carbofuran, which was due to the loss of this compound during the SPE cleanup on an HLB cartridge. Using this sorbent, quantitative elution was achieved neither by acetonitrile nor ethyl acetate as an elution solvent. Different behavior of carbofuran during SPE cleanup was observed when using MCX cartridges; only part of the loaded amount of the compound was retained on the sorbent, and the rest was lost in the washing step. To improve the performance of SPE cleanup step for carbofuran, further adjustment of SPE conditions will be necessary. However, omitting the cleanup step, sufficiently high recoveries (>90%) and good repeatability were obtained. It should be, however, noted that the analysis of larger batches of nonpurified samples might result in adverse effects occurring in LC/MS interface such as high extent of matrix effects and/or low long-term stability of LC/MS responses (*see also Method Ruggedness*).

Slightly low recoveries (<60% at the lower spiking level) were observed for tebuconazole and prochloraz in apple matrix. In apricots, however, recoveries (>80%) were satisfactory. No breakthrough during the loading and washing step of SPE and no instability of these pesticides were observed during the performance experiments. Although we cannot fully explain these results, we assume they are related to the different nature of apple and apricot co-extracts potentially affecting extraction and cleanup efficiency.

Another rather troublesome compound was flufenoxuron, for which repeatability of the analysis was very low, especially in apple matrix. According to our previous experience, this compound is prone to matrix-induced suppression in the

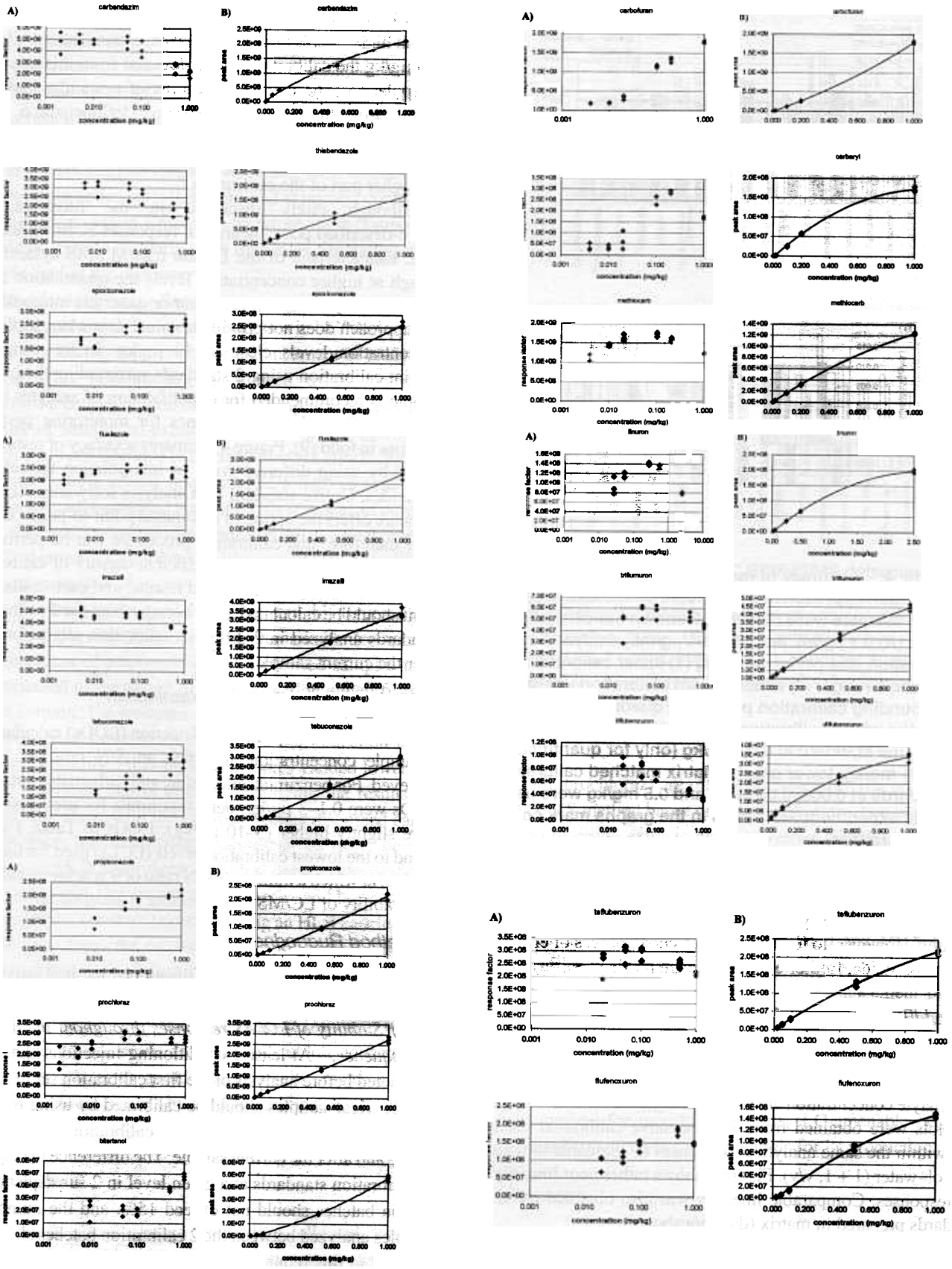

Figure 3. LC/MS/MS calibration curves of tested pesticides; data from 3 consecutive calibration batches of standards in pure solvent. For calibration levels, *see* Table 1. (A): Response factors (peak area/concentration) vs concentration (in μg/mL corresponding to mg/kg pesticides in original sample); (B): peak areas vs concentration (plotted in logarithmic scale).

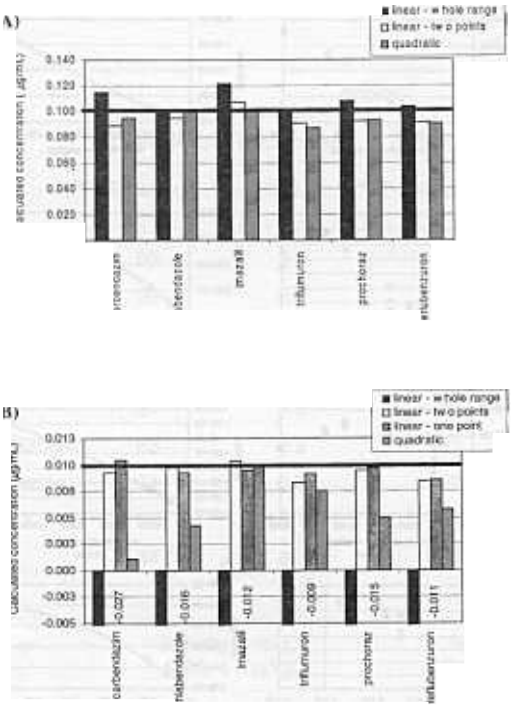

Figure 4. Accuracy of results obtained by different calibration approaches for selected pesticides in apples. Apple samples were spiked at 2 known concentration levels (A) 0.1 μg/mL and (B) 0.01 μg/mL, analyzed and calibration was perfrmed using (1) linear calibration over the whole range; (2) linear 2-point calibration using surrounding calibration points; (3) quadratic calibration over the whole calibration range; (4) 1-point calibration, using the standard at 0.02 mg/kg (only for quantification at 0.01 mg/kg; lower graph). Matrix matched calibration standards at 0.005, 0.05, 0.25, and 0.5 mg/kg were used in this experiment. Bold lines in the graphs mark correct concentrations.

LC/MS interface, which may cause greater variability of results. However, to identify the particular sources of errors for this pesticide, further experiments are needed.

Linearity of Calibration

Calibraton curves (peak area versus concentration) and response factors (peak area/concentration) plotted against analyte concentration are shown in Figure 3. The data in these plots were obtained from 3 consecutive calibration batches (within the same analytical sequence) of standards in methanol–water (1 + 1, v/v). Figure 3 shows rather poor linearity of responses. Comparable linearity was also obtained for standards prepared in matrix (data not shown).

Linearity of response over the whole calibration range was good only for flusilazole. For carbendazim and imazalil linear ranges of 0.002–0.05 and 0.005–0.1 mg/kg, respectively, were achieved. The remaining pesticides provided no linear response at all. For thiabendazole, epoxiconazole, tebuconazole, carbofuran, and diflubenzuron, the calibrations could be interpolated by quadratic curve, which fitted very well at higher concentration; however, at concentration levels <10 μg/kg the calibration points deviated from this quadratic interpolation. The calibration curves of pesticides carbaryl, methiocarb, linuron, flufenoxuron, teflubenzuron, and triflumuron are characterized by an increase of response factors in the lower part of the calibration graph and a decrease in the higher part of the calibration.

Although rarely discussed in the literature, the above-described poor linearity of responses is, in our experience, characteristic of the LC/ion trap MS/MS system. Although at higher concentration levels the quantitation accuracy is usually sufficient for linear or quadratic interpolation, this approach does not provide accurate data when applied at concentration levels close to 10 μg/kg. In this situation, 2-point calibration using 2 standards surrounding the current sample is recommended for quantification of analytes in the European Commission guidelines for monitoring pesticide residues in food (9). Figure 4 compares accuracy of results obtained by using different types of interpolation for selected pesticides. Unlike standard data analysis softwares, Xcalibur software offers the option of automated point-to-point calibration; therefore, this calibration procedure can be performed easily in routine practice. A sufficient density of calibration points is needed to avoid biased results, and each calibration point should be calculated as an average from the 2 calibration standards analyzed in 2 different calibration sets before and after the current sample.

Limits of Detection and Quantitation

Table 1 shows the limits of detection (LODs) calculated as a sample concentration at which an S/N ratio of 3 was achieved. For benzimidazole, azole, and carbamate pesticides, LODs were 0.1–3 μg/kg; detection limits for urea pesticides were slightly higher (3–10 μg/kg). LOQs in Table 1 correspond to the lowest calibration levels (LCLs) used for the calibration, at which a minimum S/N ratio of 9 is achieved and the repeatability of LC/MS measurement is acceptable.

Method Ruggedness

Several critical steps of the developed method have been identified within the validation process.

(a) *Stability of LC/MS responses throughout the analytical sequence.*—At least 2 conditioning injections should be completed before analysis of the first calibration set in the sequence. Real samples should be calibrated by using bracketing standards, i.e., standards from 2 calibration sets analyzed before and after the current sample. The difference in response of calibration standards at a given level in 2 subsequent calibration batches should not exceed 15%, and the number of samples analyzed between the 2 calibration batches should be adjusted to this requirement. For SPE cleaned up apple and apricot samples, about 10 samples could be analyzed between the 2 bracketing calibration sets. Quality control samples (e.g., standard at concentration level close to the expected level of pesticides) should be analyzed repetitively in each sequence to

monitor the detector response. As soon as the response drop during the sequence becomes unacceptable, i.e., when the difference in response between the 2 calibration batches becomes unacceptable, or the LODs of the method are increased to the unacceptable level (depending on the purpose of analysis), the sequence should be interrupted and the bore of heated capillary in the MS detector cleaned. With long-term use, contamination of ion optics parts (tube lens and skimmer) contributes to lowering of system sensitivity; therefore, these components should be cleaned regularly, with frequency depending on the sample load of the instrument.

(b) *Matrix effects.*—Sample components present in the injected sample can influence the processes in LC/MS interface, which can lead to suppression or enhancement of analyte response. This phenomenon is referred to as matrix effects (4–7). By application of SPE cleanup in our method, the amount of sample co-extracts was decreased significantly, which led to considerable reduction of matrix effects. Figure 5 compares matrix effects (expressed as a ratio of analyte response in matrix-matched standard to its response in pure solvent standard) measured in nonpurified, HLB cleaned up and MCX cleaned up samples. Results are shown only for the cleanup procedure that provides reasonable recoveries for a particular analyte. As can be be seen from this figure, the effect of sample cleanup is especially remarkable for apricots. Enhancement of responses in MCX-purified samples might be attributed to the nature of matrix fraction eluted from the MCX column. The ionizable matrix compounds present in the MCX-purified samples might support the ionization in ESI source. Generally, HLB-purified samples yielded lower (i.e., closer to 100%) matrix effect. Considering the results presented here along with the recovery results discussed earlier, the decision for a particular analyte can be made between HLB and MCX cartridges, e.g., for some azole pesticides (epoxiconazole, flusilazole, tebuconazole, and bitertanol) the matrix effects are more favorable using an HLB cartridge, and at the same time, the recoveries of these compounds on an HLB cartridge are satisfactory (Table 4). Although SPE sample cleanup was an efficient way to suppress matrix effects, the effect of sample components on analyte responses was still observed for some pesticides, especially at low concentration levels. Therefore, to avoid biased results, matrix-matched standards should always be used for calibration of unknown samples.

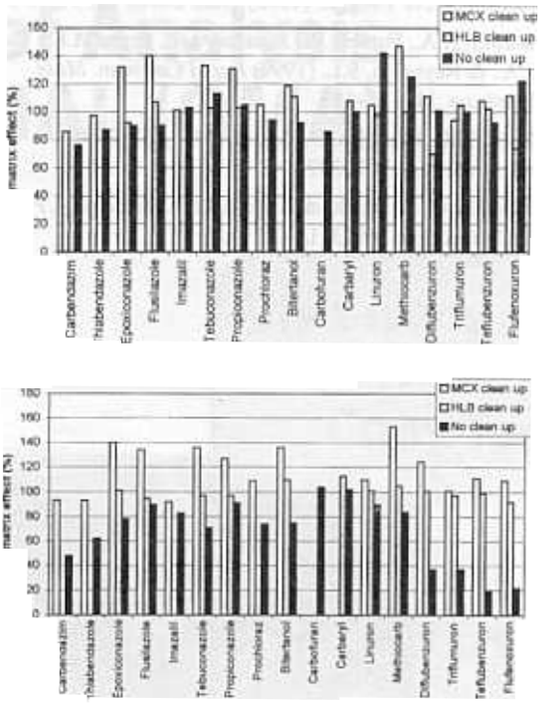

Figure 5. Matrix effects in LC/ESI/MS/MS determination of tested pesticides. Matrix effect (%) = peak area of matrix-matched standard/peak area of solvent standard × 100. Sample aliquot 1 g/mL; concentration level of analytes 0.25–1 μg/mL corresponds to higher spiking level used in validation experiments. A = Apples, B = apricots.

Conclusions

Although ethyl acetate extraction followed by GPC cleanup represents a sample preparation strategy that enables determination of a wide range of modern pesticides, its extraction efficiency for some LC-amenable polar pesticides, such as benzimidazoles and azoles, is not satisfactory unless the extraction pH is adjusted. GPC recoveries of these pesticides (imazalil, prochloraz) are often unsatisfactory. The proposed LC/MS/MS method based on acetonitrile extraction and SPE cleanup of crude extracts gave recoveries >75% and RSDs from 5 replicate determinations <21% for most of the 17 included pesticides. The only compound for which overall recovery was not satisfactory due to its loss in the SPE cleanup step was carbofuran. SPE cleanup on HLB and MCX cartridges efficiently removed co-extracted sugars and pigments. In these clean extracts, suppression and enhancement phenomena in LC/MS were much lower than in crude extracts. Overall LODs of the method were ≤3 μg/kg for benzimdazole, azole, and carbamate pesticides and ≤10 μg/kg for urea pesticides. These low detection limits allow application of the presented method for monitoring priority LC-amenable pesticides in baby food and/or raw materials for its production.

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