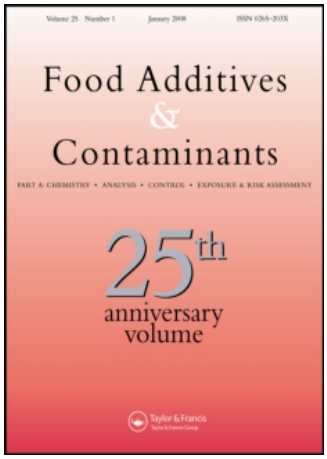

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Novel approach to fast determination of multiple pesticide residues using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS)

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Novel approach to fast determination of multiple pesticide residues using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS)

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Abstract
A rapid, high-throughput method employing ultra-performance liquid chromatography with tandem quadrupole mass spectrometry (UPLC-MS/MS) was developed and optimized for simultaneous quantification and confirmation of 64 pesticide residues and their toxic metabolites in fruit extracts prepared by a buffered QuEChERS procedure. The total time required for UPLC-MS/MS analysis was 8 min plus 2 min for re-equilibration to the initial UPLC conditions. Performance characteristics were determined for apple extracts spiked at 10 $\mu g\,kg^{-1}$. The repeatability of measurements expressed as relative standard deviations was in the range 1.5–13% at this level for most analytes. Thanks to very low limits of quantification (<10 $\mu g\,kg^{-1}$ for the majority of pesticides), an optimized method allows for the reliable control of not only common maximum residue limits (MRLs) set by European Union regulation for various pesticides/fruit combinations, but also of a uniform MRL of 10 $\mu g\,kg^{-1}$ endorsed for baby food.

Keywords: *Chromatography — ultra-performance liquid chromatography with tandem quadrupole mass spectrometry (UPLC-MS/MS), QuEChERS (quick, easy, cheap, effective, rugged, safe), clean-up, pesticide residues, baby food, fruit*

Introduction

Pesticides applied at various stages of food crops cultivation and/or during their post-harvest storage play an important role in the intensification of agricultural production. The number of active ingredients intended for the control of undesirable pests and weeds currently exceeds 800 (Tomlin 2002; Hertherton et al. 2004; Fernández-Alba 2005). Avoiding the occurrence of some pesticide residues in the food supply is obviously impossible to achieve and, therefore, unsurprisingly, the health risk associated with a dietary intake of these chemicals has become a safety issue for both toxicologists and consumers. To address these concerns, reliable and cost-effective analytical methods have to be available to control the respective regulation requirements.

With regard to the high number of target analytes concerned, multi-residue methods represent the only practical solution to meet the requirements of current extensive surveillance/compliance programmes, both in terms of the scope of analysis and the number of samples analysed. Historically, gas chromatography (GC) used to be the main technique employed for this purpose. As documented in recent reviews (Hertherton et al. 2004; Hernández et al. 2006), GC-based multi-residue methods are still widely used in control laboratories worldwide; nevertheless, it should be noted that their scope becomes increasingly more insufficient. Many registered pesticides are relatively polar compounds not amenable to direct GC analysis, and on this account the development/validation of broad-scope procedures employing high-performance liquid chromatography (HPLC) for sample separation and selective detection strategy, such as tandem mass spectrometry (MS/MS), has become an urgent task.

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Most published LC-MS-based methods involve extensive, cost and labour-demanding clean-up procedures for the processing of crude extracts, which unavoidably results in a loss of some target analytes. Similarly, the solvent-exchange step, which is in some cases carried out before introduction of the extract onto the LC column, makes the sample preparation process less effective (Hertherton et al. 2004). Challenges exist both in the innovation of sample-handling strategies and the use of novel instrumentation applicable for the determinative step. Regarding the first aspect, a significant increase of sample throughput, a reduction of labour demands and improvement in cost-effectiveness can obviously be achieved by the implementation of the so-called QuEChERS (quick, easy, cheap, effective, rugged, safe) method originally developed for GC-based analysis of multiple pesticide residues in fruits/vegetables (Anastassiades et al. 2003). Acetonitrile extraction accompanied by simultaneous liquid–liquid partitioning is followed by dispersive SPE clean-up. According to a recently modified version, either acetonitrile containing 1% acetic acid and acetate buffer (Lehotay et al. 2005a) or citrate buffer (http://www.quechers.com) are used in the first step. Regarding the measurement tools, thanks to novel designs of ion sources and availability of fast electronics, current LC-MS/MS instruments enable the determination of a large range of pesticides in complex matrices at (ultra)-trace levels and, in addition, also an on-line confirmation of residue identity (Hertherton et al. 2004; Leandro et al. 2006; Soler et al. 2006). In older studies there was a tendency to develop a LC-MS method only for a single residue or a small multi-residue set of chemically related pesticides that were not amenable to GC analysis because of their high polarity or low thermal stability (e.g. Fernández et al. 2006). Some of the until now published LC-MS/MS-based methods (Alder 2003; Alder et al. 2004; Hancock et al. 2004; Hertherton et al. 2004; Janson et al. 2004; Klein and Ortelli et al. 2004; Hernández et al. 2006; Lehotay et al. 2005b) enable control of more than 50 pesticides in one chromatographic run; however, only one of them (Hertherton et al. 2004) allows the simultaneous acquisition of two MS/MS transitions for all analytes in the method. It should be noted that only one MS/MS transition monitored for each analyte does not provide absolute confirmation of identity and further evidence may be required, especially in cases when particular a MRL is exceeded (European Commission, Directorate General Health and Consumer Protection 2006).

In general terms, several confirmative strategies could be employed. However, some of them, such as a change of LC separation system intended for confirmation based on retention times conformity and/or switching to a MS ionization mode employing a different principle (e.g. ESI versus APCI), are not convenient or feasible from a practical point of view. Medium and/or high-resolution MS detectors (European Commission, Directorate General Health and Consumer Protection 2006), e.g. those employing time-of-flight (TOF) mass analysers, represent another alternative for the detection of target analytes. It should be noted, however, that only hybrid instruments, such as quadrupole time-of-flight (Q-TOF), allow full confirmation of a particular analyte. Currently, most laboratories specialized in pesticide residue analysis prefer affordable low-resolution MS/MS for analysis of target compounds as an optimal option enabling both the quantification and the confirmation of target pesticides at trace levels (Fernández et al. 2000; Anastassiades et al. 2003; Klein and Alder 2003; Zrostlíková et al. 2003; Alder et al. 2004; Hancock et al. 2004; Hertherton et al. 2004; Janson et al. 2004; Lehotay et al. 2005a, 2005b; Díez et al. 2006; Ortelli et al. 2004; Hernández et al. 2006; Kovalczuk et al. 2006; Leandro et al. 2006; Mezcua et al. 2006). To confirm correctly detected pesticides, a minimum of two specific MS/MS transitions monitored for each analyte are then required (Hertherton et al. 2004; Leandro et al. 2006; Soler et al. 2006).

Regarding instrumental set-up, most of the current LC-MS/MS-based multi-residue methods employ conventional HPLC systems for the separation of sample components. (Klein and Alder 2003; Alder et al. 2004; Hancock et al. 2004; Hertherton et al. 2004; Janson et al. 2004; Ortelli et al. 2004; Lehotay et al. 2005b; Hernández et al. 2006; Soler et al. 2006). The sufficient separation of multiple residues (tens, even hundreds of analytes) in HPLC-MS/MS methods can be accomplished within 20–30 min; however, the need to carry out post-run column re-equilibration may increase the total analysis time up to 30–40 min. On this account, instrumental analysis becomes a limiting step in laboratory throughput. In theory, there are also other strategies that can increase the speed of chromatographic separation such as an increase of the mobile phase flow rate or the use of shorter columns (Kovalczuk et al. 2006); however, none is suitable for trace analysis of multiple pesticide residues in complex matrices such as food. The recent introduction of ultra-performance liquid chromatography (UPLC) system, such as Acquity^{TM}, capable of operating conventional 'HPLC size' columns with small (1.7 μm) porous particles at pressures as high as 15000 psi (1025 bar) has offered a new challenge to significantly increase the number of analysed samples per day. Under these conditions, the Van Deemter equation indicates that a significant

gain in efficiency is not diminished at increased flow rates of mobile phase (Leandro et al. 2006). As demonstrated in recently published studies (Kovalczuk et al. 2006; Leandro et al. 2006), the improvement in the performance parameters of several methods, including a decrease of limits of detection (LODs) and other characteristics, depends on a particular experimental set-up.

The aim of the present study was to develop a fast multi-residue UPLC-MS/MS method with performance characteristics fully complying with European Union legislation requirements (European Commission, Directorate General Health and Consumer Protection 2006) for both common fruit commodities and fruit-based baby food. The potential of the Acquity UPLC system with Quattro Premier XE tandem quadrupole mass spectrometer (Waters) is demonstrated in the determination of multiple pesticide residues in apple extract prepared by a QuEChERS procedure.

Materials and methods

Chemicals and material

Certified pesticide standards obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany) and/or Riedel de Haen (Seelze, Germany) were used for the preparation of individual stock standard solutions (concentrations in the range 0.3–3 mg ml^{-1}) in either methanol, acetonitrile or a acetone–acetonitrile mixture (1:9, v/v) depending on the solubility of the particular pesticide. These solutions were used for the preparation of: (1) individual stock standard solutions in methanol (1–5 μg ml^{-1}) for electrospray (ESI) source tuning and MS/MS transitions settings; and (2) the preparation of a mixed standard solution in acetonitrile (1 μg ml^{-1}). The working standard solutions (0.25–5000 ng ml^{-1}) used for calibration were then prepared from this solution by dilution with acetonitrile.

Deionized water for the preparation of a mobile phase was produced by a Milli-Q apparatus (Millipore, Billerica, USA). Ammonium acetate 99.99% obtained from Sigma-Aldrich (St. Louis, USA) and acetonitrile (Sigma-Aldrich) and methanol (Merck, Darmstadt, Germany) were HPLC gradient-grade solvents for pesticide residue analysis. The glacial acetic acid >99.99 was purchased from Sigma-Aldrich. Anhydrous magnesium sulfate, sodium acetate trihydrate and acetone were obtained from Penta (Praha-Dejvice, Czech Republic); Bondesil primary–secondary amine (PSA, 40 μm) sorbent was purchased from Varian (Palo Alto, USA). The magnesium sulfate was heated for 8 h at 520°C in a muffle furnace to remove any residual water. Apple samples free of pesticide residues were obtained from an ecological farm. Apple-based baby food was obtained from a retail market.

Sample preparation

The buffered QuEChERS procedure was employed within the pre-analytical step. Blank apples (obtained from organic farm) were used for the preparation of blank extracts. A representative apple sample (3 kg) was thoroughly homogenized using a 2094 Homogenizer (Foss Tecator, Höganäs, Sweden). A total of 10 g of homogenate were weighted into a polytetrafluoroethylene (PTFE) centrifugation tube (50 ml) and shaken vigorously for 1 min with 1% (v/v) acetic acid in acetonitrile. Anhydrous magnesium sulfate (4 g) and sodium acetate trihydrate (1.6 g) were added and the sample was then immediately vortexed (Ika-Werke, Staufen, Germany) to prevent the formation of coagulated magnesium sulfate. After shaking for 30 s, the samples were centrifuged (Hettich, Tuttlingen, Germany) at 11 000 RPM for 5 min. A total of 2 ml of supernatant were transferred into another PTFE tube (14 ml) containing anhydrous magnesium sulfate (300 mg) and primary–secondary amine (PSA) sorbent (100 mg). After shaking by Vortex and centrifugation (11 000 rpm, 5 min), the supernatant was filtered through a 0.2-μm PTFE filter (National Scientific, Rockwood, USA). All purified blank extracts were combined. A total of 950 μl of this extract were then mixed with 50 μl of the appropriate mixed working standard solution in acetonitrile and an aliquot (5 μl) of this spiked extract was then analysed by UPLC-MS/MS. The step following UPLC-MS/MS method optimization was its application to apple-based baby food analysis.

UPLC-MS/MS analysis

UPLC analyses were performed using an Acquity™ UPLC system (Waters, Milford, USA) equipped with an Acquity™ UPLC BEH C_{18} separation column (100 × 2.1 mm, 1.7 μm) (Waters). The sample temperature and column temperature were maintained at 25°C. The mobile phase contained 0.005 M ammonium acetate in deionized water (A) and methanol (B); the flow rate was 0.3 ml min^{-1}.

For UPLC separation, the gradient elution was employed with a starting composition of 20% B, rising linearly to 100% B over 6 min, and it was then held for 2 min at 100% B. A 2-min re-equilibration to the initial mobile phase composition followed. A sample injection volume of 5 μl was used in all experiments.

The identification/quantitation of target analytes was performed using the Quattro Premier XE tandem mass spectrometer (Waters). The detector

was operated in a positive electrospray (ESI+) ionization mode. Multiple reaction monitoring (MRM) conditions (collision energy and cone voltage) were optimized for each pesticide during infusion (5 μl min^{-1}) of individual pesticide solution (1–5 μg ml^{-1}) into the mobile phase flow (A:B = 50:50, v/v; 0.3 ml ml^{-1}). All MS experiments were realized by using the following parameters: Capillary voltage, 3.5 kV; extractor voltage, 4 V; source temperature, 120°C; desolvation temperature, 250°C; cone gas flow, 100 l h^{-1}; and desolvation gas flow, 700 l h^{-1} (both gases were nitrogen). Argon was used as a collision gas (3.3×10^{-3} mbar). Tuned and optimized MS/MS transitions as well as specific cone voltages and collision energies are summarized in Table I. Analytes were divided into MRM segments based on their elution characteristics (Table I). In each of these segments, the selected MS/MS transitions were monitored at the same dwell time of 5 ms, and inter-channel and inter-scan delays of 10 ms for all transitions. Generated experimental data were processed using MassLynx software version 4.0 Service Pack 4, Software Change Note #462 (Waters).

Method performance

The analytes mixture in pure solvent was repeatedly analysed within the optimization/development of UPLC-MS/MS to tune chromatographic and MS/MS parameters. Repeatability and LODs of UPLC-MS/MS method were obtained on the basis of data generated through six replicate sequences comprising set of matrix-matched standards consisting of ten purified apple extracts spiked by target analytes mixture at levels: 250, 150, 50, 20, 10, 5, 2, 1, 0.5 and 0.25 μg kg^{-1}, respectively. Before running the sequences, the electrospray (ESI) source chamber was cleaned and then 12 repeated injections of blank sample extract were performed to minimize the changes of analytes responses within the initial injections of test samples. The LOD for each pesticide was estimated as an analyte concentration, at which a signal-to-noise ratio for primary MS/MS was at least 3 ($S/N \geq 3$) was obtained within all six sequences (described above). For S/N estimation a peak-to-peak (PtP) strategy integrated in the MassLynx software was employed. The limit of quantitation (LOQ) (fixed as the lowest calibration level, LCL) was defined as an analyte concentration at which the following requirements were met: (1) a primary MS/MS transition — $S/N \geq 5$; and (2) a secondary MS/MS transition — $S/N \geq 3$ obtained in all six sequences. Within the study the matrix effect for each analyte was also estimated. The calculation resulted from replicated analyses of six sequences comprising: (1) a set of spiked purified apple extracts M_1–M_3 (prepared as described in below) at levels of 10, 20 and 50 μg kg^{-1}; (2) standards in pure solvent (S_1–S_3) at levels of 10, 20 and 50 ng ml^{-1} (prepared as described above); and (3) blank apple extracts (B) (prepared as described above). The injection order within the sequence for matrix effect investigation is shown in Table II. The blank apple extract was analysed to check the cross-contamination within the sample injections. The value of a matrix effect was calculated as a ratio of the average area of matrix-matched standards and the average area of solvent standards for each analyte.

Results and discussion

Sample preparation

Depending on a sample preparation strategy, various co-extracts are contained in analysed samples (Zrostlíková et al. 2003; Alder et al. 2004; Hercegová et al. 2005; Díez et al. 2006). Their amount and character might influence, in a different extent, the overall performance of the respective analytical method. From a wide range of isolation/purification approaches conceivable in pesticide residue analysis (e.g. described by Janson et al. 2004; Fernández-Alba 2005; Lehotay et al. 2005a; Leandro et al. 2006) recently introduced QuEChERS strategy (Anastassiades et al. 2003; Lehotay et al. 2005b) was chosen for the present study. As demonstrated within international collaborative studies (Lehotay et al. 2005a; Díez et al. 2006) for low-fat matrices such as fruits/vegetables, QuEChERS currently represents the most challeng ing pre-analytical option for analysis of a wide range of pesticides representing various polarity classes. Based on the list of target analytes involved in these studies, representatives of various pesticide classes were selected for the experiments. Another option that had to be decided upon was the choice of an optimal solvent strength for the introduction of the QuEChERS extract (acetonitrile solution) onto the UPLC column. Theoretically, the dilution of the sample by water should be carried out to obtain a solvent strength similar to the initial mobile phase composition (in particular case A:B = 8:2, v/v). Such an approach was employed in most of the LC-MS-based studies employing various 'classic' sample preparation techniques (e.g. Klein and Alder 2003; Alder et al. 2004; Hancock et al. 2004; Hertherton 2004; Hernández et al. 2006). However, the addition of water to the QuEChERS acetonitrile extract may result in matrix precipitation and, consequently, in some loss of target analytes. With regards to these problems, an undiluted sample was injected. Thanks to its low volume (5 μl), a distortion of the early eluting peaks was negligible. Although some

Table I. Selected chromatographic and MS/MS (in ESI, positive mode) parameters for the analysis of 64 target pesticides.

	Pesticide	Retention time (RT) (min)	Relative standard deviation (RSD) (RT) (%)	MS/MS transitions (*m/z*)	Cone voltage (V)	Collision energy (eV)	Multiple reaction monitoring (MRM) segment
1	Methamidophos	1.27	0.34	**142 > 94**	40	13	1
				142 > 125	40	13	
2	Omethoate	1.50	0.27	**214 > 183**	20	22	
				214 > 125	20	11	
3	Aldicarb sulfoxide	1.59	0.31	**224 > 150**	22	16	
				224 > 187	22	8	
4	Aldicarb sulfone	1.76	0.35	**240 > 223**	33	8	
				240 > 148	33	13	
5	Oxydemeton methyl	1.94	0.28	**247 > 169**	35	15	
				247 > 105	35	15	
6	Methomyl	2.05	0.35	**163 > 88**	20	10	
				163 > 106	20	10	
7	Demeton *S*-methyl sulfone	2.08	0.27	**263 > 169**	40	20	
				263 > 109	40	20	
8	Imidacloprid	2.60	0.30	**256 > 209**	29	16	2
				256 > 175	29	15	
9	Methiocarb sulfoxide	2.71	0.29	**242 > 185**	39	13	
				242 > 170	30	14	
10	Methiocarb sulfone	2.86	0.24	**218 > 122**	29	15	
				218 > 201	29	15	
11	Carbofuran 3-hydroxy	2.89	0.27	**238 > 163**	29	14	
				238 > 181	29	14	
12	Bentazone	2.88	0.34	**238 > 135**	40	25	3
				238 > 137	40	25	
13	Acetamiprid	2.92	0.29	**223 > 126**	31	14	
				223 > 56	31	14	
14	Dimethoate	2.95	0.18	**230 > 125**	30	30	
				230 > 79	30	30	
15	Thiacloprid	3.24	0.20	**253 > 126**	35	25	
				253 > 186	35	14	
16	Carbendazim	3.32	0.11	**192 > 160**	35	22	4
				192 > 132	35	22	
17	Aldicarb	3.61	0.13	**116 > 89**	43	8	5
				116 > 70	43	8	
18	Dichlofluanid	3.66	0.27	**201 > 92**	40	30	4
				201 > 137	40	30	
19	Thiabendazole	3.76	0.15	**202 > 175**	40	25	
				202 > 131	40	25	
20	Thiophanate-methyl	3.98	0.14	**343 > 151**	41	19	
				192 > 160	41	17	
21	Carbofuran	4.07	0.10	***222 > 165***	26	12	6
				222 > 123	26	22	
22	Malaoxon	4.17	0.09	**315 > 127**	30	30	
				315 > 99	30	11	
23	Carbaryl	4.28	0.21	**202 > 145**	16	9	
				202 > 127	16	27	
24	Thiodicarb	4.33	0.17	**355 > 88**	30	10	
				355 > 108	30	12	
25	Tolylfluanid	4.42	0.18	**347 > 238**	40	15	7
				347 > 137	40	15	
26	Phorate sulfoxide	4.50	0.13	**277 > 97**	39	13	
				277 > 143	39	27	
27	Pirimicarb	4.51	0.10	**239 > 72**	30	20	
				239 > 182	30	18	
28	Phorate sulfon	4.58	0.21	**293 > 97**	37	30	
				293 > 115	37	35	
29	Metalaxyl	4.71	0.12	**280 > 220**	23	13	8
				280 > 248	23	10	
30	Phorate oxon	4.86	0.22	**245 > 75**	30	12	
				245 > 48	30	25	

(*continued*)

Table I. Continued.

	Pesticide	Retention time (RT) (min)	Relative standard deviation (RSD) (RT) (%)	MS/MS transitions (*m/z*)	Cone voltage (V)	Collision energy (eV)	Multiple reaction monitoring (MRM) segment
31	Triforine	4.81	0.23	**390 > 98**	46	28	9
				390 > 215	46	28	
32	Azoxystrobin	4.97	0.17	**404 > 344**	25	10	8
				404 > 329	25	10	
33	Linuron	5.13	0.16	**249 > 160**	33	17	8
				249 > 182	33	17	
34	Pyrimethanil	5.18	0.09	**200 > 82**	54	24	10
				200 > 107	54	24	
35	Azinphos-methyl	4.88	0.30	**318 > 160**	25	10	
				318 > 132	25	10	
36	Methiocarb	5.18	0.08	**226 > 169**	26	9	
				226 > 121	26	20	
37	Malathion	5.25	0.36	**331 > 99**	50	30	
				331 > 125	50	33	
38	Triadimefon	5.32	0.13	**294 > 69**	40	15	11
				294 > 197	40	18	
39	Myclobutanil	5.36	0.15	**289 > 70**	26	25	
				289 > 125	25	13	
40	Fenhexamid	5.44	0.13	**302 > 97**	40	30	
				302 > 55	40	20	
41	Diphenylamine	5.48	0.17	**170 > 93**	36	20	
				170 > 92	36	20	
42	Epoxiconazole	5.53	0.12	**330 > 121**	30	19	12
				330 > 141	30	19	
43	Flusilazole	5.63	0.14	**316 > 247**	25	19	
				316 > 165	25	19	
44	Bentazone-8-hydroxy	5.87	0.16	**300 > 258**	35	20	
				300 > 179	35	20	
45	Cyprodinil	5.90	0.11	**226 > 93**	40	35	
				226 > 77	40	28	
46	Triadimenol	5.45	0.18	**294 > 69**	40	15	
				294 > 197	35	18	
47	Diflubenzuron	5.66	0.19	**311 > 158**	25	10	13
				311 > 141	25	29	
48	Bupirimate	5.67	0.21	**317 > 166**	35	25	
				317 > 108	35	25	
49	Kresoxim methyl	5.75	0.19	**314 > 222**	30	9	13
				314 > 267	30	14	
50	Penconazole	5.83	0.11	**284 > 159**	35	30	
				284 > 70	35	32	
51	Tebuconazole	5.83	0.16	**308 > 70**	34	14	14
				308 > 125	34	30	
52	Imazalil	5.86	0.14	**297 > 255**	35	20	
				297 > 201	35	20	
53	Propiconazole	5.90	0.23	**342 > 159**	43	25	
				342 > 69	43	20	
54	Triflumuron	5.91	0.10	**357 > 154**	29	11	
				357 > 177	29	11	
55	Bitertanol	5.98	0.33	**338 > 269**	20	9	15
				338 > 99	20	14	
56	Prochloraz	6.00	0.08	**376 > 308**	24	11	
				378 > 310	24	11	
57	Phorate	6.02	0.12	**261 > 75**	40	11	
				X X	×	×	
58	Difenoconazole	6.08	0.26	**406 > 251**	50	20	
				406 > 337	50	20	
59	Dodine	6.43	0.69	**228 > 57**	45	22	
				228 > 186	45	18	
60	Teflubenzuron	6.46	0.13	**381 > 158**	23	13	16
				381 > 141	23	13	

(*continued*)

Table I. Continued.

	Pesticide	Retention time (RT) (min)	Relative standard deviation (RSD) (RT) (%)	MS/MS transitions (*m/z*)	Cone voltage (V)	Collision energy (eV)	Multiple reaction monitoring (MRM) segment
61	Flufenoxuron	6.54	0.10	**487 > 467**	35	11	
				489 > 469	35	11	
62	Fenazaquin	6.95	0.16	**307 > 53**	21	24	
				307 > 161	21	16	
63	Etofenprox	7.10	0.32	**394 > 177**	20	14	
				394 > 135	20	26	
64	Spiroxamin	7.41	0.13	**298 > 101**	40	25	
				298 > 144	40	25	

Quantitation MS/MS transitions are emboldened. ×, Second transition was not established.

Table II. Sequence used for estimation of matrix effect values.

Injection order	1	2	3	4	5	6	7	8	9	10	11	12
Sample code	S_3	M_3	S_3	B	S_2	M_2	S_2	B	S_1	M_1	S_1	B
Concentration levels ($ng\,ml^{-1}$)		50		–		20		–		10		–

For apple extracts (samples B and M) the concentration corresponded to $\mu g\,kg^{-1}$ in the sample. M_1–M_3, spiked purified apple extracts; S_1–S_3, standards in pure solvent; B, blank apple extracts. For more details, see the Materials and methods section.

sensitivity was sacrificed by reduction of the sample equivalent to 5 mg, the LODs were still low enough to enable the reliable control of the 'baby food limit' ($10\,\mu g\,kg^{-1}$) for most of the analytes. Moreover, decreasing sample load also increases column lifetime and reduces the demands for MS source maintenance.

UPLC-MS/MS

MS/MS parameters. With regards to the principles of confirmation defined in recent Commission document No. SANCO/10232/2006 (European Commission, Directorate General Health and Consumer Protection 2006), overwhelming evidence that the sample actually contains a particular pesticide, i.e. proof of its identity, has to be provided by the respective analytical procedure. To meet this requirement and avoid the need of the re-analysis of positive samples, we aimed to incorporate two MS/MS transitions to obtain a required specificity for the detection process for all analytes. Contrary to the majority of the existing multi-residue LC-MS/MS methods in which (as a compromise enabling one to achieve low enough LODs for a wide range of analytes), only one MS/MS transition was monitored; two selective transitions with a highest abundance were selected for detection in the study. The confirmation of identity was based on the ion ratio statistics for two MS/MS transitions monitored, as recommended by Commission document No. SANCO/10232/2006 (European Commission, Directorate General Health and Consumer Protection 2006) and as usually applied in the analysis of veterinary drugs residues (Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, 2002/657/EC). This strategy has been demonstrated as being suitable for the analysis of residues in plant materials by other authors (Hertherton et al. 2004; Leandro et al. 2006; Soler et al. 2006).

The detection of analytes and fragmentation parameters (cone voltages and collision energies) were optimized under ESI+ conditions by infusing a standard solution by a syringe pump into the mobile phase. As in similar studies (Hertherton et al. 2004; Hernández et al. 2006; Leandro et al. 2006), the maximum permitted tolerances for relative ion ratios (European Commission, Directorate General Health and Consumer Protection 2006) were considered for confirmation in the presented procedure employing the MRM mode. Phorate was the only pesticide for which a secondary (qualifying) MS/MS transition was not established. Confirmation of this compound, based on ion ratio, was therefore impossible.

MRM optimization. In theory, the replacement of a conventional HPLC column (particles in the range of 3–5 μm) with novel UPLC columns with sorbent consisting of small particles (2 μm and less) results in reduced heights of theoretical plate (HETP) and increased peak capacity (Kovalczuk et al. 2006; Leandro et al. 2006). As far as narrow peaks such as those generated in UPLC analysis are to be

accurately integrated, MS scanning frequency has to be adjusted properly. To meet sufficient data density, i.e. at least ten datum points (Hill et al. 2004) across the peak, even for analytes at the lowest calibration levels the dwell time period was reduced to 5 ms, which is a minimal setting attainable by the Quattro Premier XE instrument; inter-scan and inter-channel delays were then 10 ms (under this setting the density for peaks at $150\,\mu g\,kg^{-1}$ was rather excessive, 20–30). With regard to a high number of analytes eluted within a short time, and considering a strong requirement for low LODs in pesticide residue analysis, several time windows, within which only a limited number of MS/MS transitions is monitored, have to be established across the chromatogram. Unfortunately, under real-life conditions, a slight fluctuation of analytes retention times might occur, namely during the analysis of a large series of samples. Therefore, to avoid analytes the running out of their elution slot, some overlap of these time windows has to be established for the reliable acquisition of respective data. An increased number of MS/MS transitions is then monitored in the area of overlap and, consequently, the acquisition frequency may decrease significantly. As far as the acquisition of data points for a particular analyte occurs under largely differing scanning speeds, as shown for aldicarb in Figure 1A, poor repeatability of generated data (with a relative standard deviation (RSD) 23% in particular case) is typically encountered.

One of the conceivable strategies to minimize the above unfavourable effects is illustrated in Figure 1B. In this case, some peaks in the chromatogram are eluted within the overlap of adjacent windows in which both the scanning speed and dwell time are equal or very similar. The repeatability of areas of peaks eluted at the segment 'borderline' is fairly improved due to only small differences in acquisition set-up across the analyte elution band. By using this approach the repeatability of aldicarb measurement (expressed as RSD) was as low as 6%. It should be noted, however, that the optimization of the windows setting is a very demanding task and any expanding method scope (adding of a new analyte) is rather complicated.

In Figure 1C, a further improved, obviously simpler, approach is presented. Contrary to the previous strategy, the elimination of problems associated with the overlap of time windows characterized by different scanning conditions is achieved by separating analytes into many time segments within which only a small number of MS/MS transitions is monitored. This set-up allows a large flexibility in the method optimization since modification of windows overlap (when needed) does not result in significant changes in scanning frequencies. In addition, for some peaks a special MRM segment including respective two characteristics MS/MS transitions can be easily added without a significant change of the scanning frequency. The example in Figure 1C shows flexible settings of 'tailor-made' windows for aldicarb. The later strategy was employed in the comprehensive optimization of the current UPLC-MS/MS procedure.

Method performance characteristics

Considering a large number of registered pesticide/fruit combinations involved in various surveillance/compliance studies, it was obviously impossible to carry out the validation of UPLC-MS/MS procedure for all of them. On this account, a generic approach was adopted for the set-up of this study. The choice of test matrice was based on assumptions on the comparable nature of matrix effects occurring in LC-MS/MS analysis of commodities characterized by a similar composition (Hercegová et al. 2005; European Commission, Directorate General Health and Consumer Protection 2006), i.e. a similar nature of co-extracts interfering with the ionization process. In a particular case apples were selected for the study as being representative of a high moisture, low-fat commodity category. The applicability of a new method in the final phase of study was demonstrated with the analyses of apple-based fruit baby foods.

For most of the residues, an optimized method allowed one to obtain LODs and LOQs (lower than $10\,\mu g\,kg^{-1}$; Table III) that enabled it to be used for the control of a uniform baby food MRL set at this level. For two 'priority' pesticides specified in European Commission Directive 2003/13/EC (European Commission 2003) included in the present study (oxydemethon methyl and demethon *S*-methyl sulfone), $LODs = 0.5\,\mu g\,kg^{-1}$ were obtained, which allows their reliable control of even lower level. (The MRL for demethon *S*-methyl is $6\,\mu g\,kg^{-1}$, expressed as the sum of demethon *S*-methyl, oxydemethon-methyl and demethon *S*-methyl sulfone.) The calibration curves were realized by six calibration sets of spiked QuEChERS apple extracts over a wide concentration range of 0.25–250 ng ml^{-1} (equivalent to a contamination level of 0.25–250 $\mu g\,kg^{-1}$, prepared as described above). The correlation coefficient (R^2) was calculated by MassLynx software for the concentration range LOQ–250 $\mu g\,kg^{-1}$ (which corresponds to LOQ–250 ng ml^{-1}). For the majority of the tested pesticides the calibration curves were linear (correlation coefficient $R^2 > 0.98$) over the tested range. As shown in Table III, for acetamiprid, carbendazim, carbofuran, pirimicarb and thiacloprid

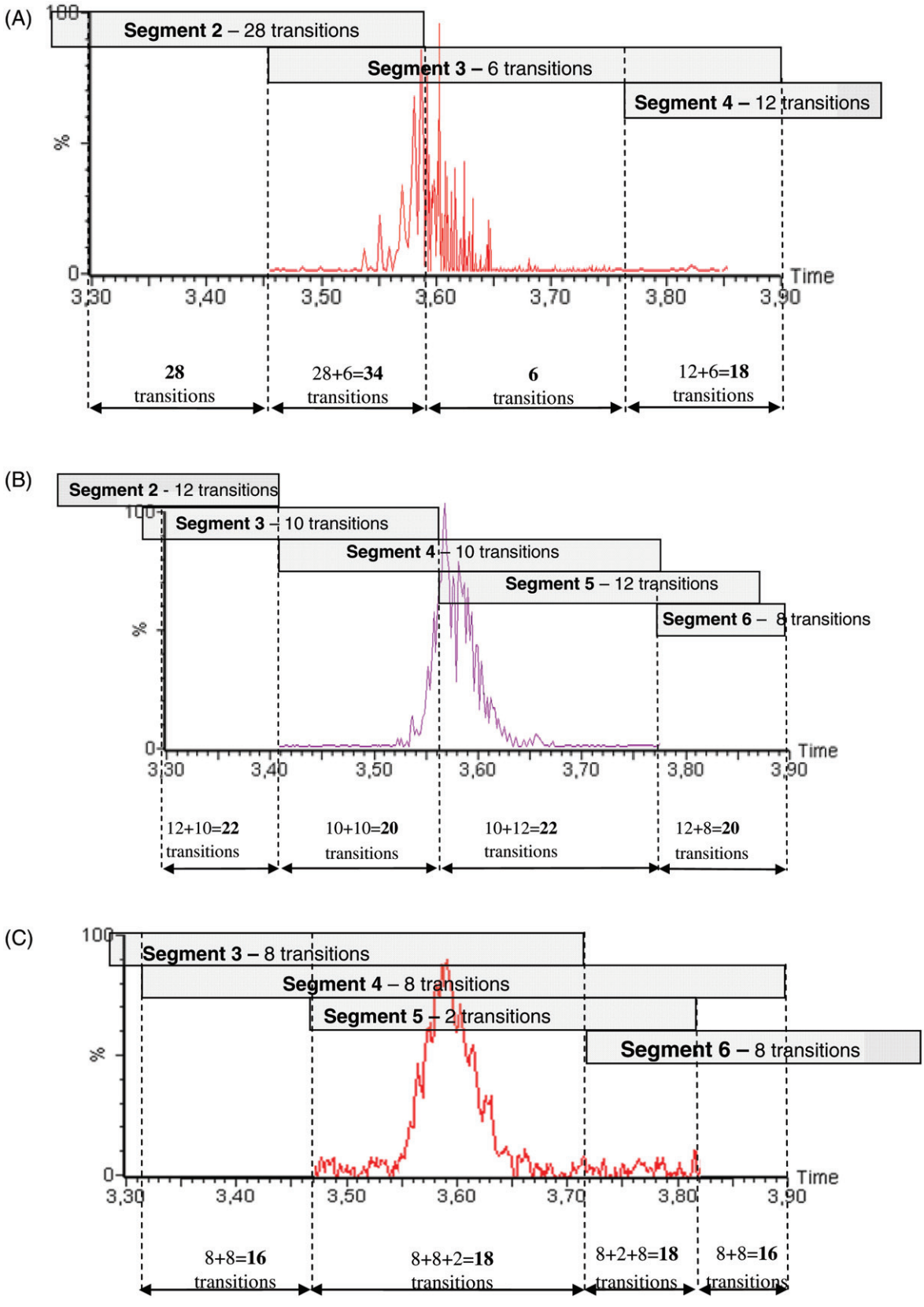

Figure 1. Impact of data acquisition setting in UPLC-MS/MS (changes of scanning frequency) on the recording of analyte elution profile; aldicarb was shown as an example. In each segment a certain number of MS/MS transitions were monitored according to the particular MRM method. The total number of MS/MS transitions monitored in each moment, expressed as the sum of all overlapped segments, is shown below each chromatogram. The phenomenon of poor repeatability based on largely changed scanning frequencies is demonstrated on peak of aldicarb. For details, see 'MRM optimization'.

Table III. Performance characteristics obtained by repeated UPLC-MS/MS analyses ($n = 6$) of spiked apple extracts (concentrations of target analytes corresponded to contamination level 10 μg kg^{-1}).

	Pesticide	Relative standard deviation (RSD) (%)	Matrix effect (%)‡	Limit of quantitation (LOQ) (μg kg^{-1})$^{+}$	(R^2)†	Ion ratio (quantity/quality)	Ion ratio RSD (%)	Acceptable limit‖ (%)	Met criteria
1	Acetamiprid	2.8	91	0.25	0.99	1.3	11	20	yes
2	Aldicarb	5.8	101	5	0.99	2.0	18	25	yes
3	Aldicarb sulfone	5.2	90	5	0.98	1.5	8	20	yes
4	Aldicarb sulfoxide	3.7	77	5	0.99	6.7	16	30	yes
5	Azinphos-methyl	6.8	137	5	0.98	1.3	12	20	yes
6	Azoxystrobin	4.3	167	5	0.99	1.5	3	20	yes
7	Bentazone	6.2	85	5	0.99	5.8	14	30	yes
8	Bentazone-8-hydroxy∗	9.9	100	20	0.98	1.6	5	20	yes
9	Bitertanol	5.8	91	5	0.98	1.8	4	20	yes
10	Bupirimate	4.7	154	1	0.98	1.1	8	20	yes
11	Carbaryl	2.8	95	1	0.99	2.2	17	25	yes
12	Carbendazim	1.5	87	0.25	0.98	12.1	18	50	yes
13	Carbofuran	2.9	93	0.5	0.98	1.2	13	20	yes
14	Carbofuran 3-hydroxy	5.6	101	2	0.99	3.5	5	25	yes
15	Cyprodinil	4.1	85	5	0.98	2.6	6	25	yes
16	Demeton *S*-methyl sulfone	4.8	108	1	0.99	1.4	19	20	yes
17	Dichlofluanid	5.0	84	5	0.98	8.1	15	30	yes
18	Difenoconazole	5.2	124	2	0.98	1.1	14	20	yes
19	Diflubenzuron	3.0	76	5	0.98	1.1	7	20	yes
20	Dimethoate	4.3	92	2	0.99	2.4	6	25	yes
21	Diphenylamine	2.8	83	10	0.99	3.3	14	25	yes
22	Dodine	21.2	136	5	0.94	5.1	18	30	yes
23	Epoxiconazole	2.8	85	5	0.98	8.4	16	30	yes
24	Etofenprox	2.2	82	5	0.98	2.2	5	25	yes
25	Fenazaquin	4.9	100	5	0.98	6.5	13	30	yes
26	Fenhexamid	4.4	88	2	0.99	3.0	20	25	yes
27	Flufenoxuron	3.0	120	5	0.99	14.2	30	50	yes
28	Flusilazole	2.1	107	5	0.96	1.4	18	20	yes
29	Imazalil	3.4	79	5	0.99	1.4	10	20	yes
30	Imidacloprid	3.7	83	2	0.99	1.5	5	20	yes
31	Kresoxim methyl	9.7	90	5	0.98	1.2	15	20	yes
32	Linuron	4.3	85	1	0.98	1.8	3	20	yes
33	Malaoxon	4.5	116	1	0.99	4.4	14	25	yes
34	Malathion	5.5	106	1	0.98	3.2	12	25	yes
35	Metalaxyl	5.9	95	1	0.98	2.3	8	25	yes
36	Methamidophos	4.4	80	2	0.99	2.5	19	25	yes
37	Methiocarb	3.4	119	1	0.99	1.5	14	20	yes
38	Methiocarb sulfone	8.7	92	5	0.99	179	28	50	yes
39	Methiocarb sulfoxide	9.2	148	5	0.98	2.8	19	25	yes
40	Methomyl	5.5	77	1	0.99	1.1	17	20	yes
41	Myclobutanil	2.2	100	1	0.99	2.8	20	25	yes
42	Omethoate	3.8	96	1	0.99	6.5	15	30	yes
43	Oxydemeton methyl	4.6	97	1	0.99	3.3	4	25	yes
44	Penconazole	4.6	85	2	0.99	1.3	10	20	yes
45	Phorate	6.1	92	5	0.98	X		X	no
46	Phorate oxon∗	4.5	103	20	0.98	33.8	20	50	yes
47	Phorate sulfon	9.9	92	1	0.99	1.9	12	20	yes
48	Phorate sulfoxide	7.8	93	1	0.99	1.2	15	20	yes
49	Pirimicarb	4.0	86	0.5	0.99	3.7	17	25	yes
50	Prochloraz	2.8	85	5	0.98	4.6	5	20	yes
51	Propiconazole	3.6	93	5	0.98	1.4	14	20	yes
52	Pyrimethanil	5.3	100	2	0.99	1.1	13	20	yes
53	Spiroxamin	9.2	55	2	0.98	2.5	5	25	yes
54	Tebuconazole	4.1	95	2	0.98	5.6	22	30	yes
55	Teflubenzuron	6.8	87	5	0.98	6.6	12	30	yes
56	Thiabendazole	3.3	78	1	0.99	6.6	5	20	yes
57	Thiacloprid	4.2	94	0.5	0.99	1.4	16	50	yes

(*continued*)

Table III. Continued.

	Pesticide	Relative standard deviation (RSD) (%)	Matrix effect (%)‡	Limit of quantitation (LOQ) ($\mu g\,kg^{-1}$)+	(R^2)†	Ion ratio (quantity/quality)	Ion ratio RSD (%)	Acceptable limit‖ (%)	Met criteria
58	Thiodicarb	6.4	129	2	0.99	3.2	18	25	yes
59	Thiophanate-methyl	6.8	104	1	0.99	9.6	19	30	yes
60	Tolylfluanid	12.5	102	2	0.71	1.2	11	20	yes
61	Triadimefon	6.0	78	2	0.99	1.6	8	20	yes
62	Triadimenol	5.4	108	2	0.99	8.7	4	30	yes
63	Triflumuron	4.9	86	5	0.99	1.9	6	20	yes
64	Triforine*	9.5	96	20	0.98	18.1	33	50	yes

*Repeatability was calculated on the LOQ.
‡Matrix effect was calculated as described in section 3.
§Correlation coefficient was calculated for the calibration range LOQ–250 $\mu g\,kg^{-1}$.
×, Qualifying MS/MS transitions were not established.
+$\mu g\,kg^{-1}$ corresponds to $ng\,ml^{-1}$.
‖Specified in European Commission, Directorate General Health and Consumer Protection (2006).

excellent linearity was achieved over the whole calibration range (0.25–250 $\mu g\,kg^{-1}$).

It should be noted that the applicability of QuEChERS pre-analytical procedure for isolation of a wide range of pesticides from apples and similar matrices was reported in earlier published studies (Anastassiades et al. 2003; Lehotay et al. 2005a, 2005b; Díez et al. 2006) and, therefore, the recoveries of target analytes were not examined in the present experiments. The current study was mainly focused on the critical assessment of performance characteristics of the UPLC-MS/MS determinative step, and on this account only spiked extracts prepared from blank apples were analysed. Regarding random errors of six repeated injections (as described above), relatively low RSDs of 6% on average were found for all the tested pesticides at a concentration level of 10 $\mu g\,kg^{-1}$. For only a few analytes with an LOQ > 10 $\mu g\,kg^{-1}$ the repeatability was calculated at their LOQ (Table III). Dodine was the only pesticide for which the RSD exceeded 20% (Table III). The uncertainty of measurement of this troublesome analyte (Figure 2), as documented in our long-term records, increases with a growing number of samples analysed on an Acquity BEH C18 separation column. Although the column with different stationary phase (Discovery C_{18}) was used in our routine multi-residue HPLC-MS/MS method, similar problems were encountered.

The suppression/enhancement of individual analytes signal measured in the presence of matrix components, i.e. matrix effect, was estimated on the basis of comparison with pure solvent standard. Within this study, matrix effects ranging from 55 to 160% were observed, with the highest enhancement of response obtained for azoxystrobin, and the most intensive signal suppression found for spiroxamine. Although for most of analytes matrix effects were in an acceptable range of 80–120%, the matrix-matched standards were used for quantification thorough the study to achieve the good accuracy of generated data.

The possibility to obtain a significant reduction of analysis time by the use of UPLC-MS/MS system, even in case of such complex sample as multiple residues in plant matrices, was documented. The UPLC-MS/MS examination of apple extract was completed within 8 min (Figure 3). Compared with similar procedures employing conventional HPLC-MS/MS systems, sample throughput was approximately three to four times higher, as summarized in Figure 4.

The time necessary for re-equilibration the separation column was set to 2 min, and as documented by negligible RSDs of retention times (0.11–0.36%), this time was sufficient for their good repeatability.

Conclusions

The UPLC Acquity separation system (Waters) coupled with a Premier Quattro XE (Waters) tandem quadrupole mass spectrometric detector, used for the analysis of multiple pesticide residues, enabled an overall improvement of method performance characteristics when QuEChERS apple extract was injected:

- Due to the reduced band broadening on a high-resolution UPLC column, narrower analyte peaks, and hence increased signal-to-noise ratios, were obtained. This resulted in limits of quantification (LOQs) being achieved

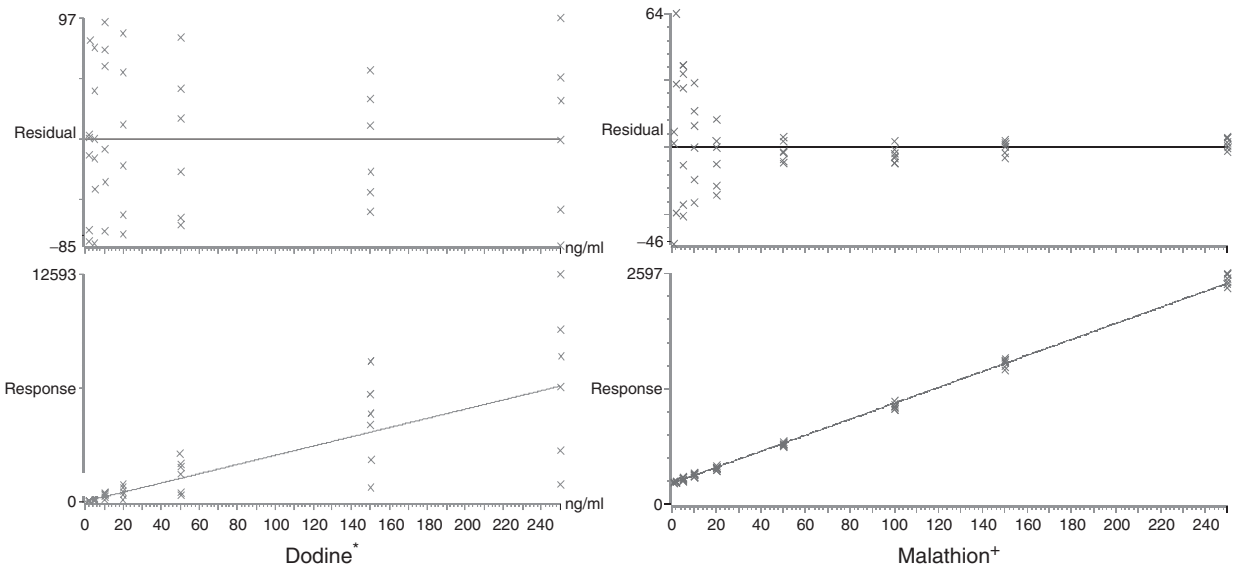

Figure 2. Calibration curves. Rather poor repeatability of dodine over the whole calibration range is compared with the calibration curve of malathion. In case of malathion as well as most pesticides, calibration curves were linear ($R^2 > 0.98$). Spiked apple extracts were used for calibration within one sequence containing six calibration sets (each had ten points ranging from 0.25 to 250 $\mu g\,kg^{-1}$). *Calibration range 5–250 $\mu g\,kg^{-1}$ (equivalent to 5–250 $ng\,ml^{-1}$); $^{+}$calibration range 1–250 $\mu g\,kg^{-1}$ (equivalent to 1–250 $ng\,ml^{-1}$).

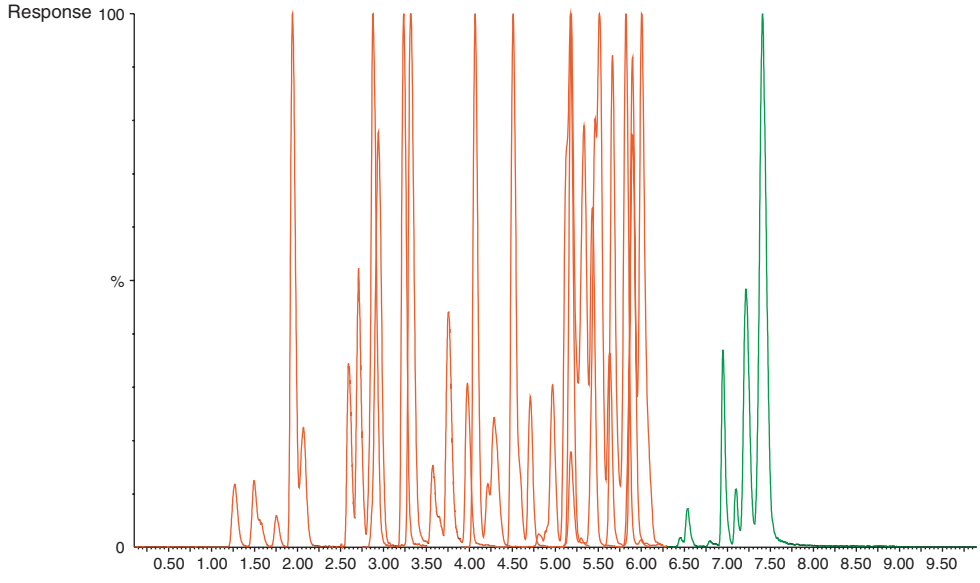

Figure 3. Combined UPLC-MS/MS MRM chromatogram based on the quantifying MS/MS transitions (Table I) of spiked apple extracts at 0.05 $mg\,kg^{-1}$.

for most pesticides fairly below 10 $\mu g\,kg^{-1}$. Under these conditions, not only MRLs established by the European Union regulation for common fruit commodities, but also a uniform baby food limit (10 $\mu g\,kg^{-1}$) can be reliably controlled.

- Thanks to the high detection sensitivity, simultaneous acquisition of two characteristic MS/MS transitions was possible for all the target analytes. In this way, contrary to most currently existing LC-MS based methods, on-line confirmation of residues identity was obtained for any positive signal. Confirmation was also supported by highly stable retention times, even within a large series of injected samples.
- Distinct improvement of respectabilities of peak areas measurement was obtained by increasing the number of (partly overlapping) time windows adjusted throughout the chromatographic run, and, at the same time, decreasing the number of MS/MS transitions set within each of them. Large changes in acquisition frequencies across the eluting

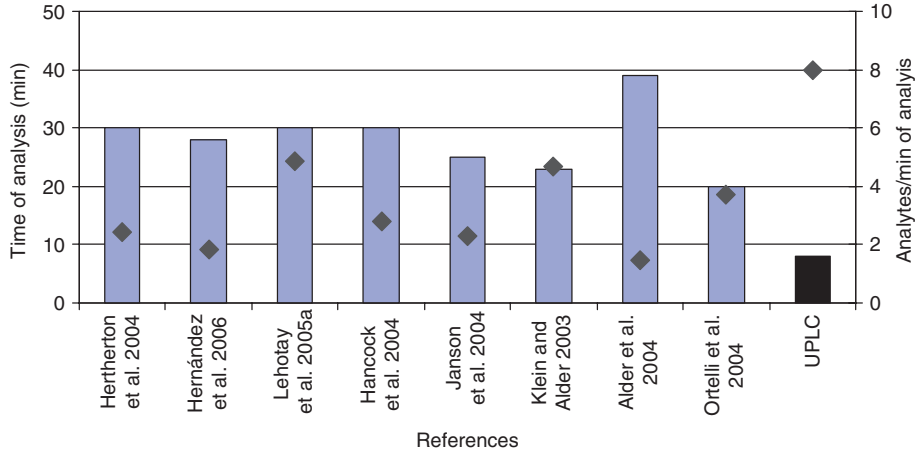

Figure 4. Comparison of different LC-MS/MS methods.

analyte band that may cause unacceptable variations in recorded signals (too high standard deviations, RSDs) can be eliminated by this data-acquisition strategy.

- Matrix effects (peak suppression/enhancement) were not too intensive (only small sample equivalent inject), generally in the range 80–120%. In spite of that, the use of matrix-matched standards for accurate quantification is recommended.
- Distinctly reduced analysis time (10 min in this particular case) attainable thanks to the possibility of operating the UPLC column at high mobile phase flow rates without any loss of resolution enabled significantly increased sample throughput.

In conclusion, the newly developed procedure fully meets the analytical quality control (AQC) requirements to support the validity of data used for checking compliance with MRLs, enforcement actions, or the assessment of consumer exposure to pesticides as laid down in Commission Document No. SANCO/10232/2006.

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