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using of ultra-performance liquid
chromatography-tandem mass spectrometry
(UPLC-MS/MS)**

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► **To cite this version:**

Tomas Kovalczuk, Ondrej Lacina, Martin Jech, Jan Poustka, Jana Hajslova. Novel approach to fast determination of 64 pesticides using of ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). *Food Additives and Contaminants*, 2008, 25 (04), pp.444-457. 10.1080/02652030701570156 . hal-00577414

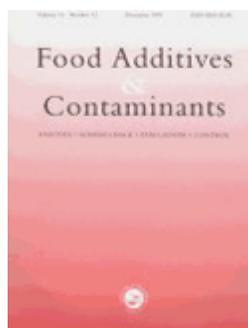
HAL Id: hal-00577414

<https://hal.science/hal-00577414>

Submitted on 17 Mar 2011

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Novel approach to fast determination of 64 pesticides using of ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS)

Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2007-065.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	07-Jul-2007
Complete List of Authors:	Kovalczuk, Tomas; Institute of Chemical Technology Prague, Department of Food Chemistry and Analysis Lacina, Ondrej; Institute of Chemical Technology Prague, Department of Food Chemistry and Analysis Jech, Martin; Institute of Chemical Technology Prague, Department of Food Chemistry and Analysis Poustka, Jan; Institute of Chemical Technology Prague, Department of Food Chemistry and Analysis Hajslova, Jana; Institute of Chemical Technology Prague, Department of Food Chemistry and Analysis
Methods/Techniques:	Chromatography - UPLC-MS/MS, QuEChERS Clean-up
Additives/Contaminants:	Pesticide residues
Food Types:	Baby food, Fruit

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3 **Novel approach to fast determination of pesticides using of ultra-**
4 **performance liquid chromatography - tandem mass spectrometry (UPLC-**
5 **MS/MS)**
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28 **Abstract**
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31 Rapid, high throughput method employing ultra-performance liquid chromatography
32 hyphenated with tandem quadrupole mass spectrometry (UPLC-MS/MS) has been developed
33 and optimized for simultaneous quantification and confirmation of 64 pesticide residues and
34 their toxic metabolites in fruit extracts prepared by buffered QuEChERS procedure. Total
35 time required for UPLC-MS/MS analysis was 8 min plus 2 min for re-equilibration to initial
36 UPLC conditions. Performance characteristics were determined for apple extracts spiked at
37 10 $\mu\text{g kg}^{-1}$. Repeatability of measurements expressed as relative standard deviations was in
38 the range 1.5-13% at this level for most analytes. Thanks to very low limits of quantification
39 ($< 10 \mu\text{g kg}^{-1}$ for majority of pesticides), optimized method allows reliable control of not only
40 common MRLs set in EU regulation for various pesticides/fruit combinations but also
41 uniform MRL 10 $\mu\text{g kg}^{-1}$ endorsed for baby food.
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Introduction

Pesticides applied at various stages of food crops cultivation and/or during their post-harvest storage, play an important role in intensification of agricultural production. The number of active ingredients intended for control of undesirable pests and weeds currently exceeds 800 (Tomlin 2002, Fernández-Alba 2005, Hertherton et al. 2004). Avoiding occurrence of some pesticide residues in food supply is obviously impossible to achieve and, therefore, not surprisingly, health risk associated with a dietary intake of these chemicals has become a safety issue, both for toxicologists and consumers. To address these concerns, reliable and cost effective analytical methods have to be available to control respective regulation requirements.

With regard to the high number of target analytes concerned, multiresidue methods represent the only practical solution that enables meeting the requirements of current extensive surveillance/compliance programs, both in terms of scope of analysis and the number of samples analyzed. 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 multiresidue methods are still widely used in control laboratories worldwide, nevertheless, it should be noted that their scope becomes more and more insufficient. Many registered pesticides are relatively polar compounds, not amenable to direct GC analysis, and on this account, development/validation of a 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.

Most of published LC-MS based methods involve either extensive, cost and labour demanding clean-up procedures for processing of crude extracts, what, unavoidably, results in a loss of some target analytes. Similarly, solvent-exchange step, which is in some cases carried out prior to introduction of extract onto LC column, makes the sample preparation process less effective (Hertherton et al. 2004). Challenges exists both in innovation of sample handling strategies and use of novel instrumentation applicable for determinative step. Regarding the first aspect, significant increase of sample throughput, reduction of labour demands and improvement of cost-effectiveness can be obviously achieved by implementation of so called QuEChERS method (Quick, Easy, Cheap, Effective, Rugged, Safe) originally developed for GC-based analysis of multiple pesticide residues in

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3 fruits/vegetables (Anastassiades et al. 2003). Acetonitrile extraction accompanied by
4 simultaneous liquid-liquid partitioning is followed by dispersive SPE clean-up. According to
5 recently modified version, either acetonitrile containing 1% acetic acid and acetate buffer
6 (Lehotay et al. a. 2005) or citrate buffer (www.quechers.com) are used in the first step.
7
8 Regarding the measurement tools, current LC-MS/MS instruments enable, thanks to novel
9 designs of ion sources and availability of fast electronics, determination of large range of
10 pesticides in complex matrices at (ultra)trace levels and, in addition, also an on-line
11 confirmation of residues identity (Hertherton et al. 2004, Leandro et al. 2006, Soler et al.
12 2006). In older studies there was a tendency to develop LC-MS method only for a single
13 residue or small multiresidue set of chemically related pesticides that were not amenable to
14 GC analysis because of their high polarity or low thermal stability (e.g. Fernández et al.
15 2006). Some of until now published LC-MS/MS-based methods (Hertherton et al. 2004,
16 Hernández et al. 2006, Lehotay et al. b. 2005, Hancock et al. 2004, Janson et al. 2004, Klein
17 et al. 2003, Alder et al. 2004, Ortelli et al. 2004) enable control of more than 50 pesticides in
18 one chromatographic run, however, only one of them (Hertherton et al. 2004) allows
19 simultaneous acquisition of two MS/MS transitions for all analytes in the method. It should be
20 noted that only one MS/MS transition monitored for each analyte does not provide absolute
21 confirmation of identity and further evidence may be required, especially in cases when
22 particular MRL is exceeded (Commission document No. SANCO/10232/2006).

23
24 In general terms, several confirmative strategies could be employed. However, some of them,
25 such as a change of LC separation system intended for confirmation based on retention times
26 conformity and/or switching to a MS ionization mode employing different principle (e.g. ESI
27 vs. APCI) are not convenient or feasible from practical point of view. Medium and/or high
28 resolution MS detectors (Commission document SANCO/10232/2006), e.g. those employing
29 time-of-flight (TOF) mass analyzers, represent another alternative for detection of target
30 analytes. It should be noted, however, that only hybrid instruments, such as quadrupole-time-
31 of-flight (Q-TOF), allow full confirmation of particular analyte. Currently, most laboratories
32 specialized in pesticide residue analysis prefer affordable low resolution MS/MS for analysis
33 of target compounds, as an optimal option enabling both quantification and confirmation of
34 target pesticides at trace levels (Hertherton et al. 2004, Hernández et al. 2006, Anastassiades
35 et al. 2003, Lehotay et al. a,b 2005, Fernández et al. 2000, Hancock et al. 2004, Janson et al.
36 2004, Klein et al. 2003, Alder et al. 2004, Ortelli et al. 2004, Leandro et al. 2006, Kovalczuk
37 et al. 2006, Mezcuca et al. 2006, Díez et al. 2006, Zrostlíková et al. 2003). To correctly
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3 confirm detected pesticides minimally two specific MS/MS transitions monitored for each
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5 analyte are then required (Soler et al. 2006, Leandro et al. 2006, Hertherton et al. 2004).

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7 Regarding instrumental set-up, most of current LC-MS/MS based multiresidue methods
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9 employ conventional HPLC systems for separation of sample components. (Hertherton et al.
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11 2004, Hernández et al. 2006, Lehotay et al. b. 2005, Hancock et al. 2004, Janson et al. 2004,
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13 Klein et al. 2003, Alder et al. 2004, Ortelli et al. 2004, Soler 2006). The sufficient separation
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15 of multiple residues (tens, even hundreds of analytes) in HPLC-MS/MS methods can be
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17 accomplished as soon as within 20-30 min, however, the need to carry out post run column re-
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19 equilibration may increase the total analysis time up to 30-40 min. On this account,
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21 instrumental analysis becomes a limiting step in laboratory throughput. In theory, there are
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23 also other strategies that can increase the speed of chromatographic separation such as
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25 increase of mobile phase flow rate or the use of shorter columns (Kovalczuk et al. 2006),
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27 however none of them is suitable for trace analysis of multiple pesticide residues in complex
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29 matrices such as food. The recent introduction of ultra-performance liquid chromatography
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31 (UPLC) system, such as Acquity™, is capable to operate conventional “HPLC size” columns
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33 with small (1.7 µm) porous particles at pressures as high as 15000 psi (1025 bar) has offered a
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35 new challenge to increase significantly number of analyzed samples per day. Under these
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37 conditions, the van Deemter equation indicates that a significant gain in efficiency is not
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39 diminished at increased flow rates of mobile phase. (Leandro et al. 2006). As demonstrated in
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41 recently published studies (Leandro et al. 2006, Kovalczuk et al. 2006), the improvement of
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43 several methods performance parameters including decrease of limits of detection (LODs) and
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45 other characteristics depends on particular experimental set-up.

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47 The aim of presented study was to develop a fast multiresidue UPLC-MS/MS method with
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49 performance characteristics fully complying with EU legislation requirements (Commission
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51 document No SANCO/10232/2006), both for common fruit commodities and fruit-based baby
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53 food. The potential of Acquity UPLC system (Waters) hyphenated with Quattro Premier XE
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55 tandem quadrupole mass spectrometer (Waters) was demonstrated in determination of
56
57 multiple pesticide residues in apple extract prepared by QuEChERS procedure.

58 59 60 **Experimental**

Chemicals and material

Certified pesticide standards obtained from Dr. Ehrenstorfer GmbH (Germany) and/or Riedel
de Haen (Germany) were used for preparation of individual stock standard solutions

(concentrations in the range 0.3–3 mg mL⁻¹) in either methanol, acetonitrile or acetone:acetonitrile mixture (1:9, v/v) depending on the solubility of particular pesticide. These solutions were used for preparation of: (i) individual stock standard solutions in methanol (1–5 µg mL⁻¹) for electrospray (ESI) source tuning and MS/MS transitions settings and (ii) preparation of 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.

Deionised water for preparation of a mobile phase was produced by Milli-Q apparatus (Millipore, Germany). Ammonium acetate 99.99% was obtained from Sigma-Aldrich (Germany), acetonitrile (Sigma-Aldrich, Germany) and methanol (Merck, Germany) were HPLC gradient grade solvents for pesticide residue analysis. The glacial acetic acid 99.99+ was purchased from Sigma-Aldrich (Germany). Anhydrous magnesium sulfate, sodium acetate trihydrate and acetone were obtained from Penta (Czech Republic), and Bondesil-Primary-secondary amine (PSA, 40 µm) sorbent was purchased from Varian (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 retail market.

Sample preparation

The buffered QuEChERS procedure was employed within the pre-analytical step. Blank apples (obtained from organic farm) were used for preparation of blank extracts. Representative apple sample (3 kg) was thoroughly homogenized using 2094 Homogenizer (Foss Tecator, Sweden). 10 g of homogenate were weighted into 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, Germany) to prevent formation of coagulated magnesium sulfate. After shaking for 30 s, the samples were centrifuged (Hettich, Germany) at 11 000 RPM for 5 min. 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 0.2 µm PTFE filter (National Scientific, USA). All purified blank extracts were combined. 950 µL of this extract were then mixed with 50 µL of appropriate mixed working standard solution in acetonitrile and aliquot (5 µL) of this spiked

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3 extract was then analyzed by UPLC-MS/MS. The step following UPLC-MS/MS method
4 optimization was its application to apple-based baby food analysis.
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7 ***UPLC-MS/MS Analysis***

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9 UPLC analyses were performed using an Acquity™ UPLC system (Waters, USA) equipped
10 with Acquity™ UPLC BEH C₁₈ separation column (100 x 2.1 mm, 1.7 μm) (Waters, USA).
11 The sample temperature as well as column temperature was maintained at 25 °C. The mobile
12 phase contained 0.005 M ammonium acetate in deionised water (A) and methanol (B), the
13 flow rate was 0.3 mL min⁻¹.
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16 For UPLC separation the gradient elution was employed, starting composition of 20% B,
17 rising linearly to 100% B over 6 min, and then held for 2 min at 100% B. 2 min re-
18 equilibration to initial mobile phase composition followed. Sample injection volume 5 μL was
19 used in all experiments.
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22 Identification/quantitation of target analytes was performed using the Quattro Premier XE
23 tandem mass spectrometer (Waters, USA). The detector was operated in a positive
24 electrospray (ESI+) ionization mode. Multiple reaction monitoring (MRM) conditions
25 (collision energy and cone voltage) were optimized for each pesticide during infusion (5 μL
26 min⁻¹) of individual pesticide solution (1-5 μg mL⁻¹) into the mobile phase flow (A:B 50:50,
27 (v/v); 0.3 mL min⁻¹). All MS experiments were realized employing following parameters:
28 capillary voltage 3.5 kV, extractor voltage 4 V, source temperature 120°C, desolvation
29 temperature 250°C, cone gas flow 100 L h⁻¹ and desolvation gas flow 700 L h⁻¹ (both gases
30 were nitrogen). Argon was used as a collision gas (3.3 10⁻³ mbar). Tuned and optimized
31 MS/MS transitions as well as specific cone voltages and collision energies are summarized in
32 Table 1. Analytes were divided into MRM segments based on their elution characteristics
33 (Table 1). In each of these segments selected MS/MS transitions were monitored at the same
34 dwell time 5 ms, inter-channel and inter-scan delays of 10 ms for all transitions. Generated
35 experimental data were processed using MassLynx software version 4.0 Service Pack 4,
36 Software Change Note #462 (Waters, USA).
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52 [Insert Table 1 about here]
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55 ***Method performance***

56 The analytes mixture in pure solvent was repeatedly analyzed within the
57 optimization/development of UPLC-MS/MS to tune chromatographic and MS/MS
58 parameters. Repeatability and LODs of UPLC-MS/MS method were obtained on the basis of
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3 data generated through six replicate sequences comprising set of matrix-matched standards
4 consisting of 10 purified apple extracts spiked by target analytes mixture at levels: 250; 150;
5 50; 20; 10; 5; 2; 1; 0.5 and 0.25 $\mu\text{g kg}^{-1}$, respectively. Prior to running the sequences, the
6 electrospray (ESI) source chamber was cleaned and then twelve repeated injections of blank
7 sample extract were performed to minimize the changes of analytes responses within the
8 initial injections of test samples. The LOD for each pesticide was estimated as an analyte
9 concentration, at which signal to noise ratio value for primary MS/MS at least 3 ($S/N \geq 3$) was
10 obtained within all six sequences (described above). For S/N estimation Peak-to-peak (PtP)
11 strategy integrated in the MassLynx software was employed. The limit of quantitation (LOQ),
12 (fixed as lowest calibration level - LCL) was defined as an analyte concentration at which
13 following requirements were met: (i) primary MS/MS transition - $S/N \geq 5$ and (ii) secondary
14 MS/MS transition - $S/N \geq 3$ obtained in all six sequences. Within the study also the matrix
15 effect for each analyte was estimated. The calculation resulted from replicated analyses of six
16 sequences comprising: (i) set of spiked purified apple extracts M_1 - M_3 (prepared as described
17 in chapter 3.3) at levels 10, 20 and 50 $\mu\text{g kg}^{-1}$ (ii) standards in pure solvent (S_1 - S_3) at levels:
18 10, 20 and 50 ng mL^{-1} (prepared as described in section "Chemicals and material") and (iii)
19 blank apple extracts (B); (prepared as described in section "Sample preparation"). The
20 injection order within the sequence for ME investigation is shown in Table 2. The blank apple
21 extract was analyzed to check the cross-contamination within the sample injections. The value
22 of matrix effect was calculated as a ratio of average area of matrix-matched standards and
23 average area of solvent standards for each analyte.
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45 **Results and discussion**

46 ***Sample preparation***

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48 Depending on a sample preparation strategy, various co-extracts are contained in analyzed
49 samples (Díez et al. 2006, Hercegová et al. 2004, Zrostlíková et al. 2003, Alder et al.2004).
50 Their amount and character may influence, in a different extent, the overall performance of
51 respective analytical method. From a wide range of isolation/purification approaches
52 conceivable in pesticide residue analysis (e.g. described by Fernández-Alba 2005, Lehotay et
53 al. a. 2005, Janson et al. 2004, Leandro et al. 2006) recently introduced QuEChERS strategy
54 (Anastassiades et al. 2003, Lehotay et al. b. 2005) was chosen for our study. As demonstrated
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3 within international collaborative studies (Lehotay at al. a. 2005, Díez et al. 2006) for low fat
4 matrices such as fruits/vegetables, QuEChERS currently represents the most challenging pre-
5 analytical option for analysis of a wide range of pesticides representing various polarity
6 classes. Based on the list of target analytes involved in these studies, representatives of
7 various pesticide classes were selected for our experiments. Another option we had to decide
8 was the choice of optimal solvent strength for introduction of QuEChERS extract (acetonitrile
9 solution) onto UPLC column. Theoretically, dilution of sample by water should be carried out
10 to obtain a solvent strength similar to initial mobile phase composition (in particular case A:B
11 8:2, v/v). Such approach was employed in most of LC-MS based studies employing various
12 “classic” sample preparation techniques (e.g. Hertherton 2004, Hernández et al. 2006,
13 Hancock at al. 2004, Klein and Alder 2003, Alder et al. 2004). However, addition of water to
14 QuEChERS acetonitrile extract may result in the matrix precipitation and, consequently, in
15 some loss of target analytes. With regards to these problems, undiluted sample was injected.
16 Thanks to its low volume (5 μ l), a distortion of early eluting peaks was negligible. Although
17 some sensitivity was sacrificed by reduction of sample equivalent to 5 mg, the LODs were
18 still low enough, to enable reliable control of “baby food limit” (10 μ g kg⁻¹) for most of
19 analytes. Moreover, decreasing of sample load also increases column life-time and reduces
20 the demands for MS source maintenance.
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37 UPLC-MS/MS

38 *MS/MS parameters*

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40 With regards to the principles of confirmation defined in recent Commission document No.
41 SANCO/10232/2006, overwhelming evidence that sample actually contains particular
42 pesticide, i.e. proof of its identity, has to be provided by respective analytical procedure. To
43 meet this requirement and avoid the need of positive samples re-analysis, we aimed to
44 incorporate two MS/MS transitions to get a required specificity of detection process for all
45 analytes. Contrary to the majority of existing multiresidue LC-MS/MS methods in which (as a
46 compromise enabling to achieve low enough LODs for a wide range of analytes) only one
47 MS/MS transitions was monitored, two selective transitions with the highest abundance were
48 selected for detection in our study. The confirmation of identity was based on the ion ratio
49 statistics for two MS/MS transitions monitored, as recommended by Commission document
50 No. SANCO/10232/2006 and as usually applied in the analysis of veterinary drugs residues
51 (Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC
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3 concerning the performance of analytical methods and the interpretation of results
4 (2002/657/EC). This strategy has been demonstrated as suitable for analysis of residues in
5 plant materials by other authors (Leandro et al. 2006, Hertherton et al. 2004, Soler 2006).
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8 Analytes detection and fragmentation parameters (cone voltages and collision energies) were
9 optimized, under ESI+ conditions by infusing a standard solution by a syringe pump into the
10 mobile phase. Alike in similar studies (Hertherton et al. 2004, Hernández et al. 2006, Leandro
11 et al. 2006), maximum permitted tolerances for relative ion ratios (Commission document
12 SANCO/10232/2006) were considered for confirmation in presented procedure employing
13 MRM mode. Phorate was only pesticide for which secondary (qualifying) MS/MS transition
14 was not established. Confirmation of this compound, based on ion ratio, was therefore
15 impossible.
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23 **MRM optimization**

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25 In theory, the replacement of conventional HPLC column (particles in the range 3-5 μm) with
26 novel UPLC columns with sorbent consisting of small particles (2 μm and less) results in
27 reduced heights of theoretical plate (HETP) and increased peak capacity (Leandro et al. 2006,
28 Kovalczuk et al. 2006). As far as narrow peaks such as those generated in UPLC analysis are
29 to be accurately integrated, MS scanning frequency has to be adjusted properly. For meeting
30 the sufficient data density, i.e. at least 10 data points (Hill et al. 2002) across the peak ,even
31 for analytes at the lowest calibration levels, the dwell time period was set down to 5 ms, what
32 is a minimal setting attainable by Quattro Premier XE instrument, inter-scan and inter-channel
33 delays were then 10 ms (under this setting the density for peaks at 150 $\mu\text{g kg}^{-1}$ was rather
34 excessive, 20-30). With regard to high number of analytes eluted within a short time, and
35 considering strong requirement for low LODs in pesticide residue analysis, several time
36 windows, within which only a limited number of MS/MS transitions is monitored, have to be
37 established across the chromatogram. Unfortunately, under real-life conditions, slight
38 fluctuation of analytes retention times may occur, namely during analysis of large series of
39 samples. Therefore, to avoid running of analytes out of their elution slot, some overlap of
40 these time windows has to be established for reliable acquisition of respective data. Increased
41 number of MS/MS transitions is then monitored in the area of overlap and, consequently,
42 acquisition frequency may significantly decrease. As far as acquisition of data points for
43 particular analyte occurs under largely differing scanning speeds, as shown for aldicarb in
44 Fig. 1A, poor repeatability of generated data (relative standard deviation (RSD) 23% in
45 particular case) is typically encountered.
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9 One of conceivable strategies to minimize the above unfavourable effects is illustrated in
10 Fig. 1B. In this case, some peaks in chromatogram are eluted within the overlap of adjacent
11 windows, in which, both the scanning speed and dwell time are equal or very similar.
12 Repeatability of areas of peaks eluted at the segment “borderline” is fairly improved due to
13 only small differences in acquisition set-up across the analyte elution band. Using this
14 approach repeatability of aldicarb measurement (expressed as RSD) was as low as 6%. It
15 should be noted, however, that optimization of windows setting is a very demanding task and
16 any the expanding method scope (adding of a new analyte), is rather complicated.

17
18 In Fig. 1C, further improved, obviously simpler approach is presented. Contrary to the
19 previous strategy, elimination of problems associated with the overlap of time windows
20 characterized by different scanning conditions is achieved by separating analytes into many
21 time segments within which only a small number of MS/MS transitions is monitored. This set-
22 up allows a large flexibility in the method optimization, since modification of windows
23 overlap (when needed), does not result in significant changes in scanning frequencies. In
24 addition, for some peaks a special MRM segment including respective two characteristics
25 MS/MS transitions can be easily added without a significant change of the scanning
26 frequency. Example in Fig. 1C shows flexible settings of “tailor-made” windows for aldicarb.
27 The later strategy was employed in comprehensive optimization of the current UPLC-MS/MS
28 procedure.
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43 ***Method performance characteristics***

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45 Considering a large number of registered pesticide/fruit combinations involved in various
46 surveillance/compliance studies, it was obviously impossible to carry out the validation of
47 UPLC-MS/MS procedure for all of them. On this account, generic approach was adopted for
48 set-up of this study. The choice of test matrices was based on assumptions on comparable
49 nature of matrix effects occurring in LC-MS/MS analysis of commodities characterized by
50 similar composition (Commission document No. SANCO/10232/2006, Hercegová et al.
51 2004), i.e. similar nature of co-extracts interfering with ionization process. In particular case,
52 apples were selected for our study as a representative of high moisture low fat commodity
53 category. The applicability of a new method in the final phase of study was demonstrated on
54 analyses of apple-based fruit baby foods.
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3 For most of residues, optimized method allowed to obtain LODs and LOQs (lower than 10
4 $\mu\text{g kg}^{-1}$, see Table 3) what enables to use it also for control of uniform baby food MRL set at
5 this level. For two “priority” pesticides specified in EU Commission Directive 2003/13/EC
6 included into our study (oxydemeton methyl and demeton-S-methyl sulfone) LODs 0.5
7 $\mu\text{g kg}^{-1}$ were obtained, what allows their reliable control of even lower level. (MRL for
8 demeton-S-methyl is 6 $\mu\text{g kg}^{-1}$, expressed as sum of demeton-S-methyl, oxydemeton-
9 methyl and demeton -S-methyl sulfone). The calibration curves were realized by six
10 calibration sets of spiked QuEChERS apple extracts over a wide concentration range 0.25-250
11 ng mL^{-1} (equivalent to contamination level 0.25-250 $\mu\text{g kg}^{-1}$, prepared as described in 3.4).
12 The correlation coefficient (R^2) was calculated by MassLynx software for the concentration
13 range LOQ-250 $\mu\text{g kg}^{-1}$, (corresponds to LOQ-250 ng mL^{-1}). For the majority of tested
14 pesticides the calibration curves were linear (correlation coefficient $R^2 > 0.98$) over the tested
15 range. As shown in Table 3, for acetamiprid, carbendazim, carbofuran, pirimicarb and
16 thiacloprid, excellent linearity was achieved over the whole calibration range (0.25-250 $\mu\text{g kg}^{-1}$).
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30 It should be noted that the applicability of QuEChERS pre-analytical procedure for isolation
31 of a wide range of pesticides from apples and similar matrices was reported in earlier
32 published studies (Anastassiades et al. 2003, Lehotay et al. a,b 2005, Díez et al. 2006) and,
33 therefore, recoveries of target analytes were not examined in our experiments. The current
34 study was mainly focused on critical assessment of performance characteristics of UPLC-
35 MS/MS determinative step, and on this account, only spiked extracts prepared from blank
36 apples were analyzed. Regarding random errors of six repeated injections (as described in
37 3.4), relatively low RSDs 6 % in average were found for all the tested pesticides at
38 concentration level 10 $\mu\text{g kg}^{-1}$. For only few analytes with LOQ > 10 $\mu\text{g kg}^{-1}$ the repeatability
39 was calculated at their LOQ (see Table 3). Dodine was the only pesticide for which the RSD
40 exceeded 20% (see Table 3). The uncertainty of measurement of this troublesome analyte (see
41 Fig. 2), as documented in our long-term records, increases with growing number of samples
42 analyzed on Acquity BEH C18 separation column. Although column with different stationary
43 phase (Discovery C₁₈) was used in our routine multiresidue HPLC-MS/MS method, similar
44 problems were encountered.
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58 [Insert Table 3 about here], [Insert Figure 2 about here]
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3 The suppression/enhancement of individual analytes signal measured in the presence of
4 matrix components, i.e. matrix effect, was estimated on the basis of comparison with pure
5 solvent standard. Within this study, matrix effects ranging from 55 to 160% were observed,
6
7 with the highest enhancement of response obtained for azoxystrobin, and the most intensive
8 signal suppression found for spiroxamine. Although for most of analytes matrix effects were
9 in acceptable range 80-120%, the matrix-matched standards were used for quantification
10 thorough the study to achieve good accuracy of generated data.

11
12 The possibility to obtain significant reduction of analysis time by the use of UPLC-MS/MS
13 system, even in case of such complex sample as multiple residues in plant matrices, was
14 documented. The UPLC-MS/MS examination of apple extract was completed within 8 min
15 (see Fig. 3). Compared to similar procedures employing conventional HPLC-MS/MS systems
16 sample throughput was approx. 3-4 times higher, as summarized in Fig. 4.

17
18 The time necessary for re-equilibration the separation column was set to 2 min, and as
19 documented by negligible RSDs of retention times (0.11-0.36%), this time was sufficient
20 enough for their good repeatability.

21
22 [Insert Figure 3 and 4 about here]

23 24 25 26 27 28 29 30 31 32 33 34 35 36 **Conclusions**

37
38 The UPLC Acquity separation system (Waters) coupled with Premier Quattro XE (Waters)
39 tandem quadrupole mass spectrometric detector, used for analysis of multiple pesticide
40 residues, enabled an overall improvement of method performance characteristics when
41 QUEChERS apple extract was injected:

- 42
43 • Due to the reduced band broadening on a high resolution UPLC column, narrower
44 analyte peaks, hence increased signal to noise ratios, were obtained. This resulted in
45 achieving limits of quantification (LOQs) for most pesticides fairly below $10 \mu\text{g kg}^{-1}$.
46 Under these conditions, not only MRLs established by EU regulation for common fruit
47 commodities, but also uniform baby food limit ($10 \mu\text{g kg}^{-1}$) can be reliably controlled.
- 48
49 • Thanks to the high detection sensitivity, simultaneous acquisition of two characteristic
50 MS/MS transitions was possible for all the target analytes. In this way, contrary to
51 most of currently existing LC-MS based methods, on-line confirmation of residues
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identity was obtained for any positive signal. Confirmation was also supported by highly stable retention times, even within 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 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 particular case) attainable thanks to possibility to operate 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 maximum residue limits (MRLs), enforcement actions, or assessment of consumer exposure to pesticides as laid down in Commission Document N° SANCO/10476/2003.

Acknowledgement

This study was a part of research project MSM 6046137505 granted by Ministry of Education, Youth and Sports of the Czech Republic. UPLC system Acquity loan, before its purchase is acknowledged to WATERS Gesellschaft m.b.H, Prague, Czech Republic.

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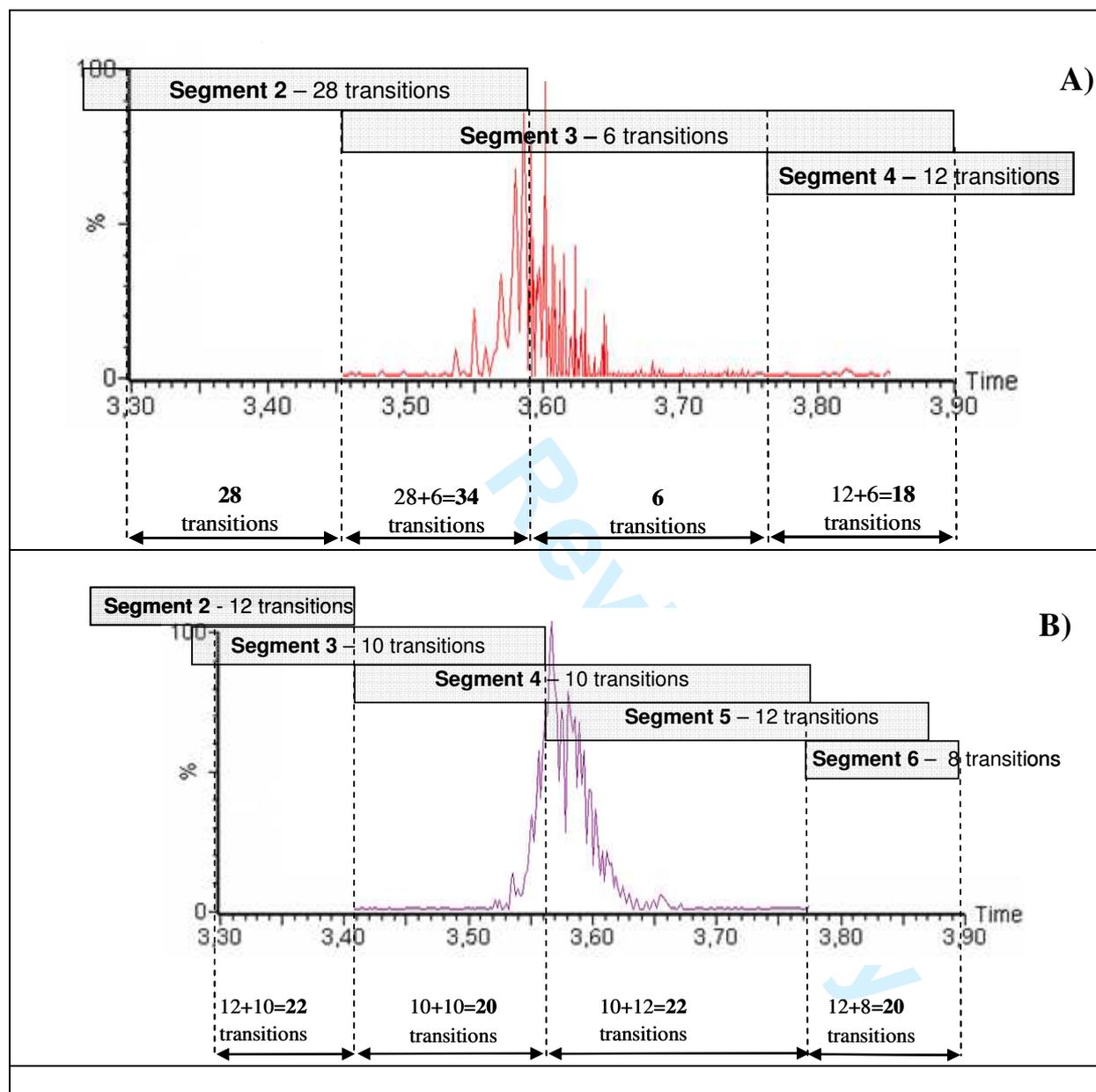
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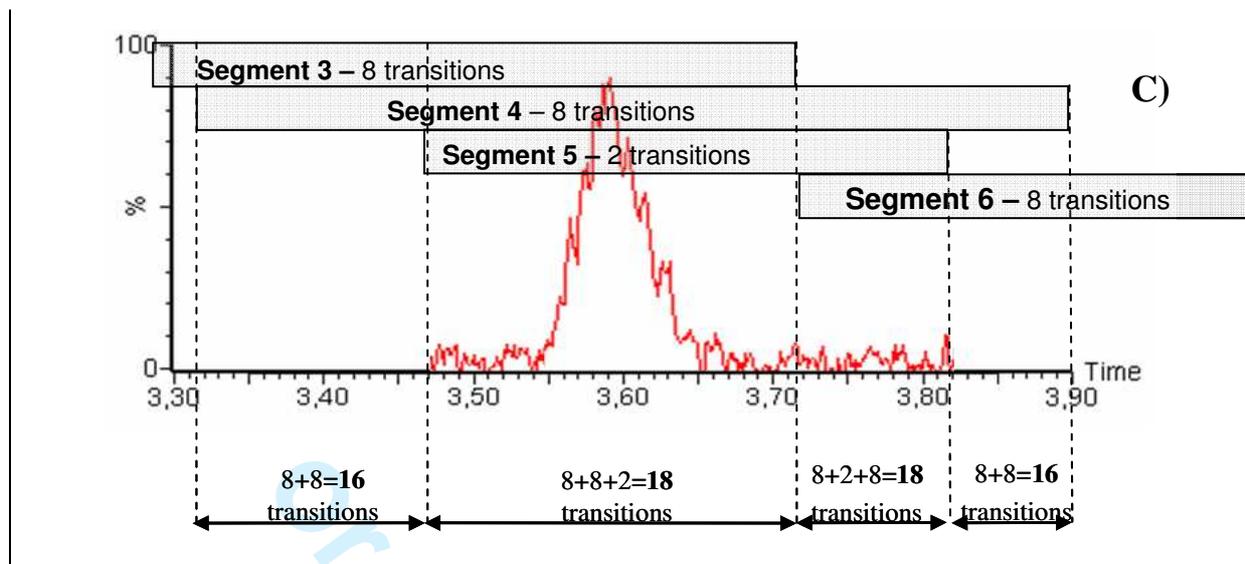
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Figure 1: The impact of data acquisition setting in UPLC-MS/MS (changes of scanning frequency) on recording of analyte elution profile; aldicarb was shown as an example: In each segment a certain number of MS/MS transitions was monitored according to 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 paragraph “MRM optimization”.

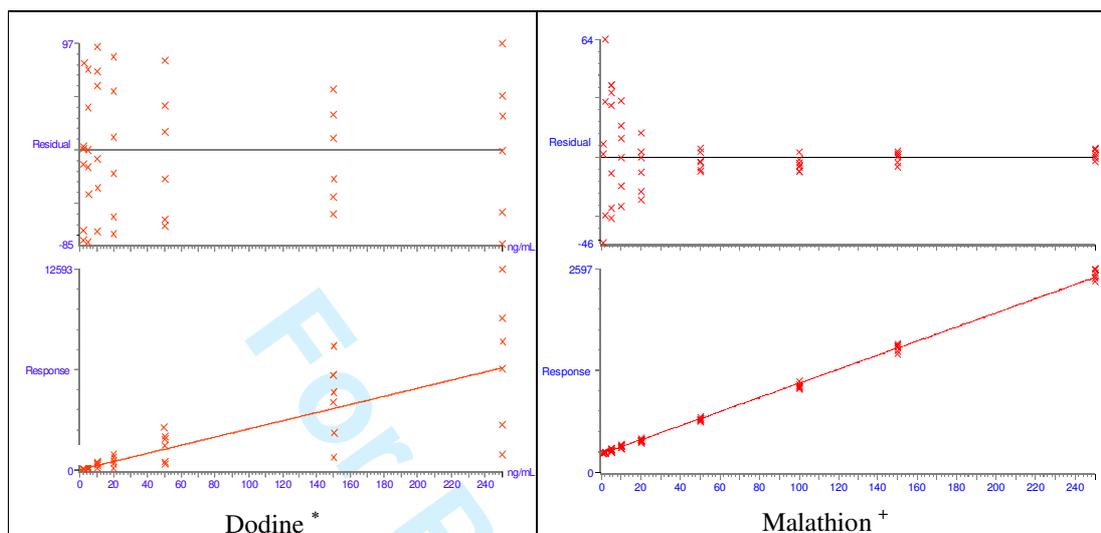


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Figure 2: Calibration curves.



Rather poor repeatability of dodine over the whole calibration range is compared with 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 10 points ranging from 0.25-250 $\mu\text{g kg}^{-1}$).

* – calibration range 5-250 $\mu\text{g kg}^{-1}$ (equivalent to 5-250 ng mL^{-1})

+ – calibration range 1-250 $\mu\text{g kg}^{-1}$ (equivalent to 1-250 ng mL^{-1})

Deleted: 5-250 ng mL^{-1} Deleted: 5-250 $\mu\text{g kg}^{-1}$. Within one sequence six calibration sets (each had 10 points ranging from 0.25-250 ng mL^{-1}).Deleted: ng mL^{-1} Deleted: $\mu\text{g kg}^{-1}$

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

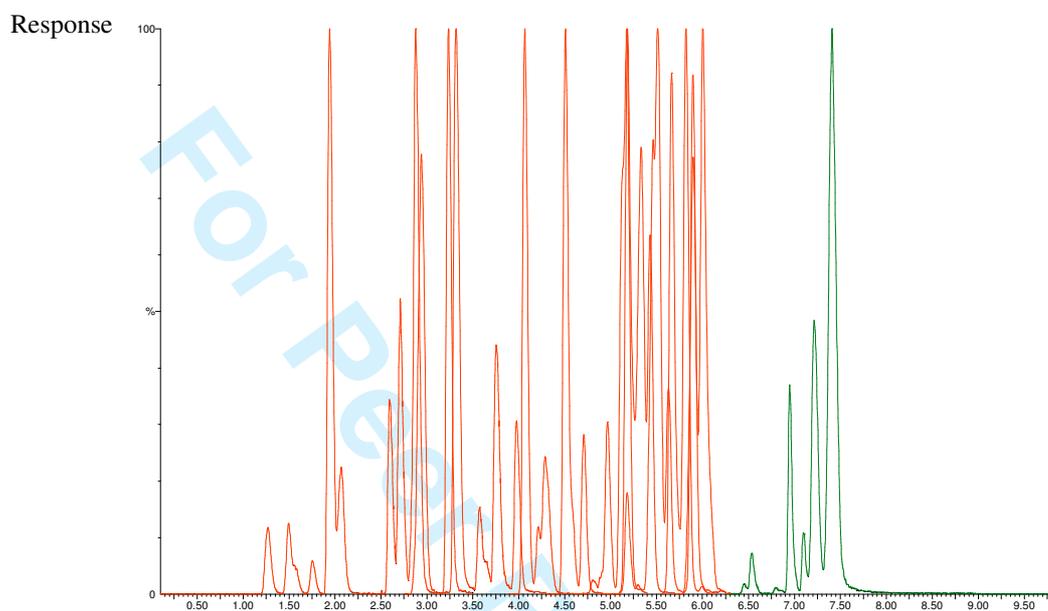
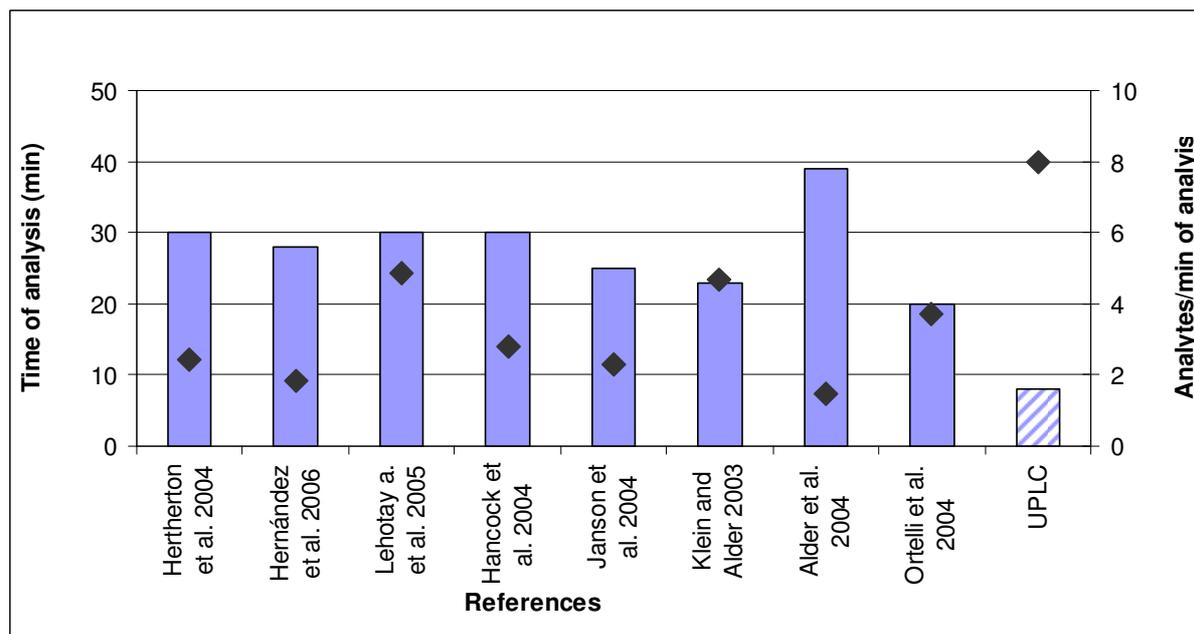


Figure 4: Comparison of different LC-MS/MS methods



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Table 1: Selected chromatographic and MS/MS (in ESI, positive mode) parameters for analysis of 64 target pesticides

Pesticide	RT (min)	RSD RT (%)	MS/MS transitions (m/z)	Cone voltage (V)	Collision energy (eV)	MRM segment
1 Methamidophos	1.27	0.34	142 > 94	40	13	
			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	1
			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	
			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	2
			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	
			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	3
			230 > 79	30	30	
15 Thiacloprid	3.24	0.20	253 > 126	35	25	
			253 > 186	35	14	

Table 1 (continued)

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	
				201 > 137	40	30	
19	Thiabendazole	3.76	0.15	202 > 175	40	25	4
				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	
				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	6
				202 > 127	16	27	
24	Thiodicarb	4.33	0.17	355 > 88	30	10	
				355 > 108	30	12	
25	Tolyfluanid	4.42	0.18	347 > 238	40	15	
				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	7
				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	
				280 > 248	23	10	
30	Phorate oxon	4.86	0.22	245 > 75	30	12	8
				245 > 48	30	25	
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	

Table 1 (continued)

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	
				200 > 107	54	24	
35	Azinphos-methyl	4.88	0.30	318 > 160	25	10	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	
				294 > 197	40	18	
39	Myclobutanil	5.36	0.15	289 > 70	26	25	11
				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	
				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	12
				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	

Table 1 (continued)

49	Kresoxim methyl	5.75	0.19	314 > 222	30	9	
				314 > 267	30	14	13
50	Penconazole	5.83	0.11	284 > 159	35	30	
				284 > 70	35	32	
51	Tebuconazole	5.83	0.16	308 > 70	34	14	
				308 > 125	34	30	
52	Imazalil	5.86	0.14	297 > 255	35	20	
				297 > 201	35	20	14
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	
				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	15
				x x x	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	
				381 > 141	23	13	
61	Flufenoxuron	6.54	0.10	487 > 467	35	11	
				489 > 469	35	11	
62	Fenazaquin	6.95	0.16	307 > 53	21	24	16
				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 highlighted by bold

x – second transition was not established.

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Table 2: 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 ($\mu\text{g mL}^{-1}$)	50		-		20		-		10		-	

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For apple extracts (sample B and M) the concentration correspond to $\mu\text{g kg}^{-1}$ in the sample

M1-M3 - spiked purified apple extracts; *S1-S3* - standards in pure solvent; *B* - blank apple extracts. For more details see Experimental.

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Table 3

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

Pesticide	RSD (%)	ME ‡ (%)	LOQ ($\mu\text{g kg}^{-1}$) ⁺	(R ²) [†]	Ion ratio (quan./qual.)	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

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Table 3 (continued)

Pesticide	RSD (%)	ME‡ (%)	LOQ (µg kg ⁻¹) ⁺	(R ²) [†]	Ion ratio (quan./qual.)	Ion ratio RSD (%)	Acceptable limit [‡]	
							(%)	Met criteria
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	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

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Pesticide	RSD (%)	ME (%)	LOQ ($\mu\text{g kg}^{-1}$) ⁺	R ² †	Ion ratio (quan./qual.)	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 Tolyfluanid	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

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* - repeatability was calculated on LOQ

† - Matrix effect calculated as described in 3.4.

§ - Correlation coefficient calculated for calibration range LOQ-250 $\mu\text{g kg}^{-1}$

x - Qualifying MS/MS transitions was not established

+ - $\mu\text{g kg}^{-1}$ correspond to ng mL^{-1}

|| Specified in Commission document No. SANCO/10232/2006