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

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Abstract

Rapid, high throughput method employing ultra-performance liquid chromatography hyphenated with tandem quadrupole mass spectrometry (UPLC-MS/MS) has been developed and optimized for simultaneous quantification and confirmation of 64 pesticide residues and their toxic metabolites in fruit extracts prepared by buffered QuEChERS procedure. Total time required for UPLC-MS/MS analysis was 8 min plus 2 min for re-equilibration to initial UPLC conditions. Performance characteristics were determined for apple extracts spiked at $10 \mu\text{g kg}^{-1}$. 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 ($< 10 \mu\text{g kg}^{-1}$ for majority of pesticides), optimized method allows reliable control of not only common MRLs set in EU regulation for various pesticides/fruit combinations but also uniform MRL $10 \mu\text{g kg}^{-1}$ endorsed for baby food.

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

fruits/vegetables (Anastassiades et al. 2003). Acetonitrile extraction accompanied by simultaneous liquid-liquid partitioning is followed by dispersive SPE clean-up. According to recently modified version, either acetonitrile containing 1% acetic acid and acetate buffer (Lehotay et al. a. 2005) or citrate buffer (www.quechers.com) are used in the first step. Regarding the measurement tools, current LC-MS/MS instruments enable, thanks to novel designs of ion sources and availability of fast electronics, determination of large range of pesticides in complex matrices at (ultra)trace levels and, in addition, also an on-line confirmation of residues identity (Hertherton et al. 2004, Leandro et al. 2006, Soler et al. 2006). In older studies there was a tendency to develop LC-MS method only for a single residue or small multiresidue 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 until now published LC-MS/MS-based methods (Hertherton et al. 2004, Hernández et al. 2006, Lehotay et al. b. 2005, Hancock et al. 2004, Janson et al. 2004, Klein et al. 2003, Alder et al. 2004, Ortelli et al. 2004) enable control of more than 50 pesticides in one chromatographic run, however, only one of them (Hertherton et al. 2004) allows 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 MRL is exceeded (Commission document No. SANCO/10232/2006).

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 different principle (e.g. ESI vs. APCI) are not convenient or feasible from practical point of view. Medium and/or high resolution MS detectors (Commission document SANCO/10232/2006), e.g. those employing time-of-flight (TOF) mass analyzers, represent another alternative for 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 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 quantification and confirmation of target pesticides at trace levels (Hertherton et al. 2004, Hernández et al. 2006, Anastassiades et al. 2003, Lehotay et al. a,b 2005, Fernández et al. 2000, Hancock et al. 2004, Janson et al. 2004, Klein et al. 2003, Alder et al. 2004, Ortelli et al. 2004, Leandro et al. 2006, Kovalczuk et al. 2006, Mezcuca et al. 2006, Díez et al. 2006, Zrostlíková et al. 2003). To correctly

confirm detected pesticides minimally two specific MS/MS transitions monitored for each analyte are then required (Soler et al. 2006, Leandro et al. 2006, Hertherton et al. 2004).

Regarding instrumental set-up, most of current LC-MS/MS based multiresidue methods employ conventional HPLC systems for separation of sample components. (Hertherton et al. 2004, Hernández et al. 2006, Lehotay et al. b. 2005, Hancock et al. 2004, Janson et al. 2004, Klein et al. 2003, Alder et al. 2004, Ortelli et al. 2004, Soler 2006). The sufficient separation of multiple residues (tens, even hundreds of analytes) in HPLC-MS/MS methods can be accomplished as soon as 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 increase of mobile phase flow rate or the use of shorter columns (Kovalczuk et al. 2006), however none of them 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™, is capable to operate 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 increase significantly number of analyzed 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 (Leandro et al. 2006, Kovalczuk et al. 2006), the improvement of several methods performance parameters including decrease of limits of detection (LODs) and other characteristics depends on particular experimental set-up.

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

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

extract was then analyzed 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, USA) equipped with Acquity™ UPLC BEH C₁₈ separation column (100 x 2.1 mm, 1.7 μm) (Waters, USA). The sample temperature as well as column temperature was maintained at 25 °C. The mobile phase contained 0.005 M ammonium acetate in deionised water (A) and methanol (B), the flow rate was 0.3 mL min⁻¹.

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

Identification/quantitation of target analytes was performed using the Quattro Premier XE tandem mass spectrometer (Waters, USA). 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 min⁻¹). All MS experiments were realized employing 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 1. Analytes were divided into MRM segments based on their elution characteristics (Table 1). In each of these segments selected MS/MS transitions were monitored at the same dwell time 5 ms, 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, USA).

[Insert Table 1 about here]

Method performance

The analytes mixture in pure solvent was repeatedly analyzed 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 10 purified apple extracts spiked by target analytes mixture at levels: 250; 150; 50; 20; 10; 5; 2; 1; 0.5 and 0.25 $\mu\text{g kg}^{-1}$, respectively. Prior to running the sequences, the electrospray (ESI) source chamber was cleaned and then twelve 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 signal to noise ratio value for primary MS/MS at least 3 ($S/N \geq 3$) was obtained within all six sequences (described above). For S/N estimation Peak-to-peak (PtP) strategy integrated in the MassLynx software was employed. The limit of quantitation (LOQ), (fixed as lowest calibration level - LCL) was defined as an analyte concentration at which following requirements were met: (i) primary MS/MS transition - $S/N \geq 5$ and (ii) secondary MS/MS transition - $S/N \geq 3$ obtained in all six sequences. Within the study also the matrix effect for each analyte was estimated. The calculation resulted from replicated analyses of six sequences comprising: (i) set of spiked purified apple extracts M_1 - M_3 (prepared as described 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: 10, 20 and 50 ng mL^{-1} (prepared as described in section "Chemicals and material") and (iii) blank apple extracts (B); (prepared as described in section "Sample preparation"). The injection order within the sequence for ME investigation is shown in Table 2. The blank apple extract was analyzed to check the cross-contamination within the sample injections. The value of matrix effect was calculated as a ratio of average area of matrix-matched standards and average area of solvent standards for each analyte.

[Insert Table 2 about here]

Results and discussion

Sample preparation

Depending on a sample preparation strategy, various co-extracts are contained in analyzed samples (Díez et al. 2006, Hercegová et al. 2004, Zrostlíková et al. 2003, Alder et al.2004). Their amount and character may influence, in a different extent, the overall performance of respective analytical method. From a wide range of isolation/purification approaches conceivable in pesticide residue analysis (e.g. described by Fernández-Alba 2005, Lehotay et al. a. 2005, Janson et al. 2004, Leandro et al. 2006) recently introduced QuEChERS strategy (Anastassiades et al. 2003, Lehotay et al. b. 2005) was chosen for our study. As demonstrated

within international collaborative studies (Lehotay et al. 2005, Díez et al. 2006) for low fat matrices such as fruits/vegetables, QuEChERS currently represents the most challenging 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 our experiments. Another option we had to decide was the choice of optimal solvent strength for introduction of QuEChERS extract (acetonitrile solution) onto UPLC column. Theoretically, dilution of sample by water should be carried out to obtain a solvent strength similar to initial mobile phase composition (in particular case A:B 8:2, v/v). Such approach was employed in most of LC-MS based studies employing various “classic” sample preparation techniques (e.g. Hertherton 2004, Hernández et al. 2006, Hancock et al. 2004, Klein and Alder 2003, Alder et al. 2004). However, addition of water to QuEChERS acetonitrile extract may result in the matrix precipitation and, consequently, in some loss of target analytes. With regards to these problems, undiluted sample was injected. Thanks to its low volume (5 μ l), a distortion of early eluting peaks was negligible. Although some sensitivity was sacrificed by reduction of sample equivalent to 5 mg, the LODs were still low enough, to enable reliable control of “baby food limit” (10 μ g kg⁻¹) for most of analytes. Moreover, decreasing of sample load also increases column life-time 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, overwhelming evidence that sample actually contains particular pesticide, i.e. proof of its identity, has to be provided by respective analytical procedure. To meet this requirement and avoid the need of positive samples re-analysis, we aimed to incorporate two MS/MS transitions to get a required specificity of detection process for all analytes. Contrary to the majority of existing multiresidue LC-MS/MS methods in which (as a compromise enabling to achieve low enough LODs for a wide range of analytes) only one MS/MS transitions was monitored, two selective transitions with the highest abundance were selected for detection in our 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 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 suitable for analysis of residues in plant materials by other authors (Leandro et al. 2006, Hertherton et al. 2004, Soler 2006).

Analytes detection 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. Alike in similar studies (Hertherton et al. 2004, Hernández et al. 2006, Leandro et al. 2006), maximum permitted tolerances for relative ion ratios (Commission document SANCO/10232/2006) were considered for confirmation in presented procedure employing MRM mode. Phorate was only pesticide for which 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 conventional HPLC column (particles in the range 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 (Leandro et al. 2006, Kovalczuk 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. For meeting the sufficient data density, i.e. at least 10 data points (Hill et al. 2002) across the peak, even for analytes at the lowest calibration levels, the dwell time period was set down to 5 ms, what is a minimal setting attainable by Quattro Premier XE instrument, inter-scan and inter-channel delays were then 10 ms (under this setting the density for peaks at 150 $\mu\text{g kg}^{-1}$ was rather excessive, 20-30). With regard to high number of analytes eluted within a short time, and considering 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, slight fluctuation of analytes retention times may occur, namely during analysis of large series of samples. Therefore, to avoid running of analytes out of their elution slot, some overlap of these time windows has to be established for reliable acquisition of respective data. Increased number of MS/MS transitions is then monitored in the area of overlap and, consequently, acquisition frequency may significantly decrease. As far as acquisition of data points for particular analyte occurs under largely differing scanning speeds, as shown for aldicarb in Fig. 1A, poor repeatability of generated data (relative standard deviation (RSD) 23% in particular case) is typically encountered.

[Insert Figure 1 about here]

One of conceivable strategies to minimize the above unfavourable effects is illustrated in Fig. 1B. In this case, some peaks in chromatogram are eluted within the overlap of adjacent windows, in which, both the scanning speed and dwell time are equal or very similar. 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. Using this approach repeatability of aldicarb measurement (expressed as RSD) was as low as 6%. It should be noted, however, that optimization of windows setting is a very demanding task and any the expanding method scope (adding of a new analyte), is rather complicated.

In Fig. 1C, further improved, obviously simpler approach is presented. Contrary to the previous strategy, 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. Example in Fig. 1C shows flexible settings of “tailor-made” windows for aldicarb. The later strategy was employed in 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, generic approach was adopted for set-up of this study. The choice of test matrices was based on assumptions on comparable nature of matrix effects occurring in LC-MS/MS analysis of commodities characterized by similar composition (Commission document No. SANCO/10232/2006, Hercegová et al. 2004), i.e. similar nature of co-extracts interfering with ionization process. In particular case, apples were selected for our study as a representative of high moisture low fat commodity category. The applicability of a new method in the final phase of study was demonstrated on analyses of apple-based fruit baby foods.

For most of residues, optimized method allowed to obtain LODs and LOQs (lower than 10 $\mu\text{g kg}^{-1}$, see Table 3) what enables to use it also for control of uniform baby food MRL set at this level. For two “priority” pesticides specified in EU Commission Directive 2003/13/EC included into our study (oxydemeton methyl and demeton-S-methyl sulfone) LODs 0.5 $\mu\text{g kg}^{-1}$ were obtained, what allows their reliable control of even lower level. (MRL for demeton-S-methyl is 6 $\mu\text{g kg}^{-1}$, expressed as sum of demeton-S-methyl, oxydemeton-methyl and demeton -S-methyl sulfone). The calibration curves were realized by six calibration sets of spiked QuEChERS apple extracts over a wide concentration range 0.25-250 ng mL^{-1} (equivalent to contamination level 0.25-250 $\mu\text{g kg}^{-1}$, prepared as described in 3.4). The correlation coefficient (R^2) was calculated by MassLynx software for the concentration range LOQ-250 $\mu\text{g kg}^{-1}$, (corresponds to LOQ-250 ng mL^{-1}). For the majority of tested pesticides the calibration curves were linear (correlation coefficient $R^2 > 0.98$) over the tested range. As shown in Table 3, for acetamiprid, carbendazim, carbofuran, pirimicarb and thiacloprid, excellent linearity was achieved over the whole calibration range (0.25-250 $\mu\text{g kg}^{-1}$).

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. a,b 2005, Díez et al. 2006) and, therefore, recoveries of target analytes were not examined in our experiments. The current study was mainly focused on critical assessment of performance characteristics of UPLC-MS/MS determinative step, and on this account, only spiked extracts prepared from blank apples were analyzed. Regarding random errors of six repeated injections (as described in 3.4), relatively low RSDs 6 % in average were found for all the tested pesticides at concentration level 10 $\mu\text{g kg}^{-1}$. For only few analytes with LOQ > 10 $\mu\text{g kg}^{-1}$ the repeatability was calculated at their LOQ (see Table 3). Dodine was the only pesticide for which the RSD exceeded 20% (see Table 3). The uncertainty of measurement of this troublesome analyte (see Fig. 2), as documented in our long-term records, increases with growing number of samples analyzed on Acquity BEH C18 separation column. Although column with different stationary phase (Discovery C₁₈) was used in our routine multiresidue HPLC-MS/MS method, similar problems were encountered.

[Insert Table 3 about here], [Insert Figure 2 about here]

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 acceptable range 80-120%, the matrix-matched standards were used for quantification thorough the study to achieve good accuracy of generated data.

The possibility to obtain 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 (see Fig. 3). Compared to similar procedures employing conventional HPLC-MS/MS systems sample throughput was approx. 3-4 times higher, as summarized in Fig. 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 enough for their good repeatability.

[Insert Figure 3 and 4 about here]

Conclusions

The UPLC Acquity separation system (Waters) coupled with Premier Quattro XE (Waters) tandem quadrupole mass spectrometric detector, used for 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, hence increased signal to noise ratios, were obtained. This resulted in achieving limits of quantification (LOQs) for most pesticides fairly below $10 \mu\text{g kg}^{-1}$. Under these conditions, not only MRLs established by EU regulation for common fruit commodities, but also uniform baby food limit ($10 \mu\text{g kg}^{-1}$) 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 of 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 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.

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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”.

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})

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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

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	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	1
			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	1
			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	1
			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	2
			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	2
			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	3
			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	3
			253 > 186	35	14	

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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	Tolylfluanid	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	Metaxyl	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	
				318	>	132	25	10	
36	Methiocarb	5.18	0.08	226	>	169	26	9	10
				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	
				289	>	125	25	13	
40	Fenhexamid	5.44	0.13	302	>	97	40	30	11
				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	
				311	>	141	25	29	
48	Bupirimate	5.67	0.21	317	>	166	35	25	13
				317	>	108	35	25	

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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	
53	Propiconazole	5.90	0.23	342	>	159	43	25	
				342	>	69	43	20	14
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	
				x		x	x	X	15
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	
				307	>	161	21	16	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 (ng mL ⁻¹)	50			-	20			-	10			-

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For apple extracts (sample B and M) the concentration correspond to µg 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 µg kg⁻¹)

Pesticide	RSD (%)	ME ‡ (%)	LOQ (µg 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 Difenconazole	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	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 (µg 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 µg kg⁻¹

x - Qualifying MS/MS transitions was not established

+ - µg kg⁻¹ correspond to ng mL⁻¹

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