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Ultra-performance liquid chromatography–tandem mass spectrometry: A novel challenge in multiresidue pesticide analysis in food

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Abstract

Potential of ultra-performance liquid chromatography (UPLC) separation strategy coupled with tandem (in space) mass spectrometric detection (MS/MS) in multiresidue pesticide analysis was critically assessed. Performance parameters such as number of theoretical plates, height of theoretical plate, peak symmetry and peak capacity were measured/calculated on the basis of data generated by analysis of apple extracts containing 17 (semi)polar pesticides representing various classes of active ingredients of widely used crop protective preparations. Ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) procedure provided improved chromatographic parameters resulting in significantly increased sample throughput including lower solvent consumption and lower limits of quantitation (LOQs) for most of target analytes compared to common method employing conventional high-performance liquid chromatography (HPLC) separation.
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Keywords: Ultra-performance liquid chromatography; High-performance liquid chromatography; Tandem quadrupole mass spectrometry; Multiresidue; Pesticides; Food analysis

1. Introduction

Pesticides applied at various stages of food crops cultivation and/or during their post-harvest storage play an important role in the agriculture intensification. Although modern (semi)polar pesticides that replaced classic organochlorine pesticides are fairly less persistent and do not bioaccumulate in biota, they are still regarded as hazardous for humans and environment.

To minimize dietary exposure of most vulnerable groups of consumers such as small children and infants uniform EU maximum residue limit (MRL) was set to 0.01 mg kg^{-1} for any pesticide residue [1,2]. To accomplish reliable control of such low levels of multiple analytes advanced instrumental techniques have to be used for separation/identification. High-performance liquid chromatography hyphenated with tandem mass spectrometric detection (HPLC–MS/MS) has become recently the technique of choice for analysis of wide range of (semi)polar pesticide residues in food crops [3].

The size parameters of conventional HPLC columns are typically as follows: (i) length in the range 10–25 cm, (ii) i.d. 2.1–4.6 mm and (iii) particle sizes 3–5 μm. Reverse phase systems with gradient elution represent the most common strategy in separation of multiple pesticide residues. Operating pressures under these conditions do not exceed 6000 psi (414 bar) that is the maximum upper limit achievable by common HPLC pumps integrated in routinely used instrumental systems.

With respect to the growing demand for high sample throughput, various strategies aimed at increasing the speed of chromatographic separation can be considered. Both advantages and limitations of approaches for this purpose conceivable are listed below:

(i) *Increase of mobile phase flow rate*: Although the LC separation run time is reduced [4], the height equivalent to theoretical plate (HETP) increases when using columns with particles in the most range (i.e. 3–5 μm). In addition to reduced separation power, high flow rates are not well compatible with optimal performance of electrospray (ES) MS source and therefore only part of effluent can be introduced usually into MS detector. On account of this splitting partial loss of detection sensitivity can occur.
(ii) *The use of short columns* with low back pressures might seem a conceivable solution for achieving fast run as long as modern tandem quadrupole mass spectrometers capable

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of very fast ion monitoring is coupled to the LC system [4–6]. Under such conditions, i.e. relying on the power of tandem mass spectrometry to deconvolute co-eluting peaks [7], high resolution LC system providing total (baseline) separation of sample components is not needed. However, under real life conditions low resolution power of short column results unavoidably in co-elution not only target analytes, but also co-extracted matrix components resulting in tendency to matrix effects (analyte response suppression/enhancement), i.e. when analysing such complex matrix as food. Although short columns with very small particles (1.8 μm) are available on the market [4], the increased resolution power is often still insufficient for food extracts.

(iii) *The use of normal size column filled with very small particles* (<2 μm) represents the solution for obtaining both high peak capacity and fast separation of even complex mixtures. However, due to very high backpressures at typical LC–MS flow rates such columns cannot be operated with by common HPLC pumps. The first instrument (Acquity, Waters, USA) capable to perform separation column under operational pressures as high as 15,000 psi (1025 bar) has been introduced at the market recently. These columns with porous 1.7 μm particles and common size parameters (100–150 mm, 2.1 mm) can be operated at high mobile phase flow rates without a loss of resolution under optimised conditions and as shown in several studies published until now [7,8] they can be used routinely for different purposes. It should be noted that since the end of 20th century several studies concerned with ultra-high pressure liquid chromatography (UHPLC) were realised. In the most of capillary columns with non-porous silica particles were employed [9–15].

Since applied experimental conditions such as lengths/internal diameters of separation columns as well as particle sizes vary largely in various studies, the comparison of various LC systems in analysis of pesticide residues aimed at selection of optimal one is a difficult task.

To assess the quality of chromatographic performance of LC system number of objective parameters of system such as peak efficiency, resolution and peak capacity can be measured/calculated [8]. Although, the peak efficiency factor derived from peak width and retention time is a concept more suitable for isocratic LC [8], the approach designed by Snyder et al. [16] allows its use for comparative purposes of gradient LC.

On the other hand, resolution and peak capacity are parameters suitable for characterisation of various set-ups of LC system. The former parameter is calculated for selected pair of analytes (in the most critical pair). The value of latter one, as shown by Wren [8], for multi component samples, is a function of column efficiency, gradient time, flow rate and analyte characteristics, hence enables comparison of LC systems.

The main aim of presented study was to introduce an UPLC–MS/MS method for determination of 17 pesticides and critically assess the possibility of replacement routinely used HPLC–MS/MS method with the novel UPLC–MS/MS strategy.

2. Experimental

2.1. Chemicals and material

The certified standards of bitertanol (purity 98%), carbaryl (99%), carbendazim (99%), carbofuran (99%), diflubenzuron (98%), epoxiconazole (99%), flufenoxuron (99%), flusilazole (99%), imazalil (96%), linuron (99%), methiocarb (99%), prochloraz (97%), propiconazole (97%), tebuconazole (98%), teflubenzuron (98%), thiabendazole (98%) and triflumuron (99%) for this study were obtained from Dr. Ehrenstorfer (Germany). Pesticide residue grade solvents (acetonitrile, methanol) were obtained from Merck (Germany). Deionised water for mixing of a mobile phase was produced by Milli-Q apparatus (Millipore, Germany). Apple samples (Golden Delicious) known to be free of pesticide residues were obtained in the frame of cooperation with an ecological farm.

2.2. Pesticide standards preparation

Individual pesticide stock solutions (0.3–3 mg mL^{-1}) were prepared by dissolving of solid substance in acetonitrile, in the case of benzoylureas in acetonitrile–acetone mixture (90:10, v/v) to improve their solubility. These individual stock solutions were used for preparation of pesticide mixture stock solution (50 μg mL^{-1}each) in methanol that was used for preparation of working standard solutions in methanol ranging between and 1 ng mL^{-1} and 5 μg mL^{-1}. For electrospray ionisation (ESI) source tuning and MS/MS transitions settings individual standard solutions (1 μg mL^{-1} in methanol) were prepared. To simulate real sample analysis matrix extract (apples) fortified by target compounds were analysed. These were prepared by addition of 100 μL of appropriate working standard solution into 1900 μL of blank matrix extract (prepared as described in next paragraph).

2.3. Extraction

Blank samples—approx. 1 kg of apples was thoroughly homogenized using 2094 Homogenizer (Foss Tecator, Denmark). 12.5 g of homogenate was mixed with 50 mL of acetonitrile and blended for 2 min with Ultra-Turrax tissumizer (IKA Werke, Germany). In the next step, the suspension was filtered under vacuum, the filtration cake was washed with 3× 10 mL of acetonitrile, and the volume of filtrate was then reduced using a rotary evaporator (Büchi, Switzerland) to 1–2 mL. Into the evaporation flask 15 mL of methanol was added and the volume was quantitatively transferred into 50 mL volumetric flask and made-up with methanol. The equivalent of matrix in our sample was 0.25 g mL^{-1}. Samples were filtered through PTFE filters (National Scientific, USA) prior to injection. While for HPLC–MS/MS analysis, sample was filtered through 5 μm filter, in the case of UPLC filtration through 0.2 μm filter was used.

Table 1
Optimised UPLC and HPLC gradient programs

UPLC		HPLC	
Time (min)	Methanol (%)	Time (min)	Methanol (%)
Initial	30.0	Initial	50.0
0.50	30.0	6.00	100.0
4.00	72.0	15.00	100.0
6.30	85.0	25.00	50.0
8.00	100.0		
10.00	100.0		
10.05	30.0		
12.00	30.0		

The composition of mobile phase was changed linearly.

2.4. LC–MS methods

2.4.1. UPLC

All UPLC separations were carried out on ACQUITY UPLC^{TM} system (Waters, USA) using a reversed phase column Acquity UPLC BEH C_{18} (100 mm × 2.1 mm) (Waters, USA), with 1.7 μm spherical porous particles. The elution was performed using gradient between water and methanol (Table 1). Separation time was 10 min and additional 2 min post run time was required for recondition of the column to initial conditions. Flow rate 0.3 mL min^{-1}, column and sample temperature 25 °C and the injection volume 5 μL were used in all experiments.

2.4.2. HPLC

HPLC separations were carried out on a 2695 Alliance module (Waters, USA) using a reversed phase column Discovery C_{18} (150 mm × 3 mm, 5 μm) (Supelco, USA). The elution was performed using gradient between methanol and water (Table 1). Separation time was 15 min and additional 10 min post run time was required for recondition of the column to initial conditions. Flow rate 0.3 mL min^{-1}, column temperature 25 °C and the injection volume 5 μL were used in all experiments.

2.5. MS/MS

Quattro Premier tandem quadrupole instrument (Waters, USA) was used in all UPLC–MS/MS and HPLC–MS/MS analysis. The instrument was operated in positive ESI mode. ESI parameters as well as selection and tuning of MS/MS transitions and analyte-dependent parameters (collision energy and cone voltage) were performed by direct infusion of individual pesticide standard solution (ca. 1 μg mL^{-1}) into the mobile phase flow (methanol–water; 50:50, v/v, 0.3 mL min^{-1}) for all analytes. In all experiments, following parameters were used: 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 gasses were nitrogen). Argon at pressure 3.3×10^{-3} mbar was used as collision gas. Tuned and optimised MS/MS transitions as well as specific cone voltages and collision energies are summarized in Table 2. Chromatographic elution zones were divided into appropriate number of time segments. In each time segment corresponding MS/MS transitions (i.e. channels) were monitored using the multiple reaction-monitoring (MRM) mode. Various values of dwell time: 5, 10, 15, 20 ms were tested. The interscan and inter-channel delay were 10 ms in all experiments. Table 3 summarizes pesticides divided into MRM groups for UPLC–MS/MS and HPLC–MS/MS, respectively. Data processing was done using MassLynx software Version 4.0 (Waters, USA). The limit of quantitation (LOQ) for each pesticide was estimated for both techniques as the lowest analyte concentration in calibration matrix-matched standard, at which signal to noise ratio of minimum 5 (S/N > 5) for primary MS/MS transition and S/N > 3 for secondary MS/MS transition was obtained.

2.6. The void-time determination

Non-retained compound, in particular case uracil, was used for determination of void-time (t_0) under optimised LC condi-

Table 2
Optimised MS/MS transitions parameters

Compound		Primary transitions			Secondary transition		
No.	Name	MS/MS (*m/z*)	Cone voltage (V)	Collision energy (eV)	MS/MS (*m/z*)	Cone voltage (V)	Collision energy (eV)
1	Carbendazim	192 > 160	35	22	192 > 132	35	22
2	Thiabendazole	202 > 175	40	25	202 > 131	40	25
3	Carbofuran	222 > 165	23	12	222 > 123	26	22
4	Carbaryl	202 > 145	16	9	202 > 127	16	17
5	Linuron	249 > 159	33	17	249 > 182	33	17
6	Methiocarb	226 > 169	26	9	226 > 121	26	20
7	Epoxiconazole	330 > 121	30	20	330 > 141	30	20
8	Flusilazole	316 > 247	25	19	316 > 165	25	19
9	Diflubenzuron	311 > 158	25	13	311 > 141	25	29
10	Tebuconazole	308 > 70	34	20	308 > 125	34	30
11	Imazalil	297 > 255	35	20	297 > 201	33	17
12	Propiconazole	342 > 158	43	25	342 > 69	43	20
13	Triflumuron	359 > 156	29	16	359 > 139	29	30
14	Bitertanol	338 > 99	20	11	338 > 269	20	14
15	Prochloraz	376 > 308	24	11	378 > 310	24	11
16	Teflubenzuron	381 > 158	23	13	381 > 141	23	13
17	Flufenoxuron	489 > 158	25	19	489 > 141	25	19

Table 3
MRM time segments in analysis

Time segment	Compound	Rt (min)	R.S.D. Rt (%)	k'
UPLC–MS/MS (t_0 = 0.98 min)				
(1)	Carbendazim*	2.91	0.3	2.0
	Thiabendazole	3.40	1.0	2.5
	Carbofuran	3.83	0.3	2.9
	Carbaryl	4.06	0.2	3.2
(2)	Linuron	4.98	0.2	4.1
	Methiocarb	5.04	0.5	4.2
	Epoxiconazole	5.49	2.0	4.6
	Flusilazole	5.66	2.0	4.8
	Diflubenzuron	5.67	2.0	4.9
	Tebuconazole	5.95	1.0	5.1
	Imazalil	6.03	0.1	5.2
	Propiconazole	6.03	0.2	5.2
	Triflumuron	6.15	0.1	5.3
	Bitertanol	6.23	0.3	5.4
	Prochloraz	6.24	0.1	5.4
(3)	Teflubenzuron	7.12	0.1	6.3
	Flufenoxuron**	7.45	0.1	6.6
HPLC–MS/MS (t_0 = 2.97 min)				
(1)	Carbendazim*	6.07	0.2	1.0
	Thiabendazole	7.08	0.1	1.4
	Carbofuran	7.75	0.5	1.6
	Carbaryl	8.14	0.3	1.7
(2)	Linuron	9.64	0.3	2.2
	Methiocarb	9.68	0.3	2.2
(3)	Epoxiconazole	10.22	2.0	2.4
	Flusilazole	10.33	1.5	2.5
	Diflubenzuron	10.44	3.0	2.5
	Imazalil	10.63	2.2	2.6
	Tebuconazole	10.66	0.6	2.6
	Triflumuron	10.74	0.1	2.6
	Propiconazole	10.76	0.1	2.6
	Bitertanol	10.85	0.8	2.6
	Prochloraz	10.92	0.6	2.7
(4)	Teflubenzuron	11.62	0.2	2.9
	Flufenoxuron**	11.64	0.3	2.9

k' is the capacity factor was calculated as follows: $k' = (t_R - t_0)/t_0$, where t_R is a retention time of particular analyte, t_0 the void retention time. t_0 was determined using uracil as a non-retained compound as described in Section 2.6.
* Retention times of these compounds were used as t_a.
** Retention times of these compounds were used as t_z.

tions in both UPLC and HPLC systems (Table 1) as described by Claesens et al. [17].

3. Results and discussion

As discussed in Section 1, the increased speed of chromatographic analysis could be achieved using several strategies. The use of short columns providing only small resolution in some applications was possible in recent decade thanks to availability of modern fast scanning MS instruments. Although high selectivity can be obtained when detector is operated in MS/MS mode, nevertheless, this approach is not fully compatible with multiresidue methods since the number of operable MS/MS transitions in a single MRM segment is limited. Fast separation under low resolution conditions may also lead to overlap of target analytes with matrix components contained unavoidably in even purified extract. Ionisation process taking place in atmospheric pressure ionisation (API) interface can be largely influenced. Mostly suppression or occasionally enhancement of analyte responses can occur. (The character of these phenomena is in detail described in several papers [3,18–22].) This problem is most pronounced in the front part of chromatogram, where quantitation (LOD, accuracy) process of analyte with low capacity factors can be most impaired. With regards to these facts the use of HPLC columns with higher separation power (higher N) is preferred for multi component analysis. Ideally, analytes should be eluted over the whole LC run also because possibility to optimise MRM set-up with as low as possible number of analytes in one segment.

Multiresidue pesticide analysis puts a special demands on optimisation of LC–MS system set-up. Both good LC resolution of target analytes enhancing specificity of detection systems and spectral clarity and the ability of the MS instrumentation to collect sufficient number of data points across the peak (condition for reliable integration) have to be considered within the method development process. This effort is focused not only on the meeting of the required performance characteristics specified in EU legislation [23] but also on the achievement of other practical aspects such as high sample throughput.

Routinely used HPLC–MS/MS method was validated and accredited for wide scope of fruit matrices. For the comparison of original HPLC–MS/MS and novel UPLC–MS/MS was used commonly analysed fruit—apples, which was previously verified as a representative matrix for other fruit analysis [3,18,24]. Moreover apples are the main commodity used for baby food production, and achieving low LOQ for target pesticides is most critical.

In paragraphs below implementation of method for fast analysis of multiple pesticide residues in fruit employing fast chromatography using common size column with very small particles is discussed and critically assessed employing relevant theoretical assumption.

3.1. LC–MS/MS optimisation

The HPLC separation involved in accredited method routinely used before UPLC testing was designed not only with regard to performance criteria but also the demand to obtain minimal run time was considered. On this account, fast gradient (Table 1) starting at time 0 min at 50% of methanol and rising linearly to 100% of methanol over 6 min and then analytes were eluted under isocratic conditions (100% methanol) enabling relatively fast elution of analytes with high k' (Fig. 1; Table 3). It should be noted that when selective tandem MS is used as detector, some co-elutions of analytes in total ion current (TIC) can be tolerated hence total (baseline) separation of eluted analytes is not necessary anymore. Actually, under real-life conditions in multiresidue screening it is mostly not achievable. In this case analytes are scanned in MRM segments. In each MRM segment, only a certain number of selective MS/MS transitions of target analytes is monitored (Table 3). It should be noted that in all

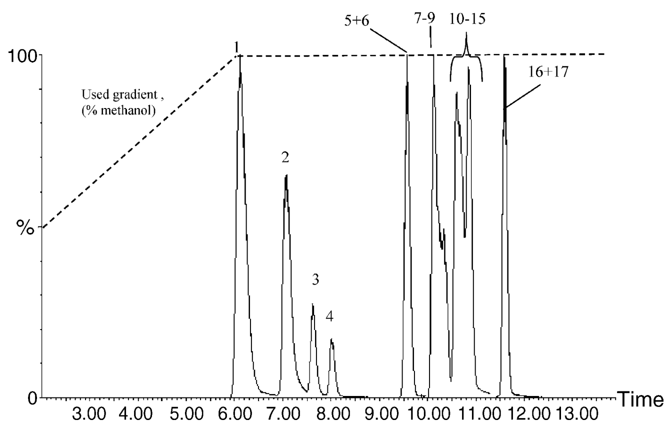

Fig. 1. Combined HPLC–MS/MS chromatogram of apple crude extract spiked with 17 (semi)polar pesticides (conc. 0.02 mg kg^{-1} of each) based on the quantifying MS/MS transitions (Table 2). The peak numbering is shown in Table 3. For illustration, used gradient (% of methanol) is also shown. Experimental conditions are described in Section 2.4.

experiments, no co-elution in any selective MS/MS transitions occurred.

When using UPLC column the application of HPLC conditions (described in Section 2.4) resulted in a very fast elution of all analytes within 6 min (Fig. 2). Under these conditions, only a few critical pairs (i.e. co-elutions in TIC) were present in chromatogram. Regarding Quattro Premier potential for relatively fast scanning the overlapping of time segments could be employed, however the attainable number of data points per peak might be critical for some compounds. Setting of robust MRM segments was almost impossible, also because of poor repeatability of k' values for some analytes (e.g. imazalil).

In following experiments, we therefore searched for elution conditions enabling optimal MRM setting. The result of UPLC separation tuning is shown in Fig. 3. Under these conditions, three well separated peak clusters can be accomodated (with respect to robust MRM setting).

Various mobile phase modifiers were tested within the validation study using HPLC separation. In general, it is almost impossible to find the modifier that would be optimal for multianalyte method. For instance, while LODs of some analytes were decreased by addition of 5 mM formic or acetic acid, the response of many pesticides remained unchanged or even decreased (e.g. tebuconazole). Therefore, only methanol–water was used as a mobile phase in both LC–MS systems that were subjected to comparison.

A band broadening may occur when injection of sample is carried out in “stronger” solvent that is the mobile phase at the time of injection. However, to our experience injection of methanolic extract up to volume 7 μL (considering the size of column we used) does not influence the peak shapes.

For both HPLC and UPLC system an appropriate number of time segments (MRM) was used to meet optimal detection conditions. In the case of UPLC–MS/MS the number of MRM segments was changed from four (for HPLC) to three because of higher peaks time compression. In accordance with changes in elution bands character (width, height) different dwell times were tested to find the best detection parameters (sufficient number of data point across the peak).

Comparison is summarized in Fig. 4 and as apparent only the negligible differences were found. Therefore, for further comparisons dwell 20 ms was chosen. Chromatograms illustrating optimised HPLC–MS/MS and UPLC–MS/MS analysis of 17 (semi)polar pesticides are shown in Figs. 1 and 3, respectively. The total run time was reduced by more than 50% when using UPLC. The reduced analysis time consequently resulted into significantly lower consumption of organic solvents.

3.2. Separation efficiency

For calculation of column efficiency—expressed as theoretical column plate number (N) following Eq. (1) is mostly used in case of isocratic separation [25,26]

$$N_{\text{Isocratic}} = 5.54\left(\frac{t_r}{W_{0.5}}\right)^2. \quad (1)$$

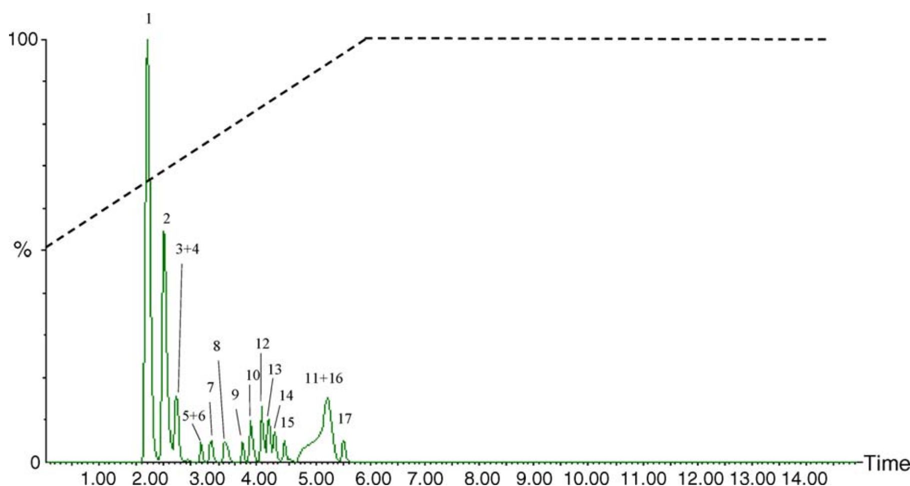

Fig. 2. Combined UPLC–MS/MS chromatogram of apple crude extract spiked with 17 (semi)polar pesticides (conc. 0.05 mg^{-1} of each) based on the quantifying MS/MS transitions (all in one MRM segment) when using same gradient as in HPLC. The peak numbering is shown in Table 3. For illustration, used gradient (% of methanol) is shown. Experimental conditions were described in Section 2.4.

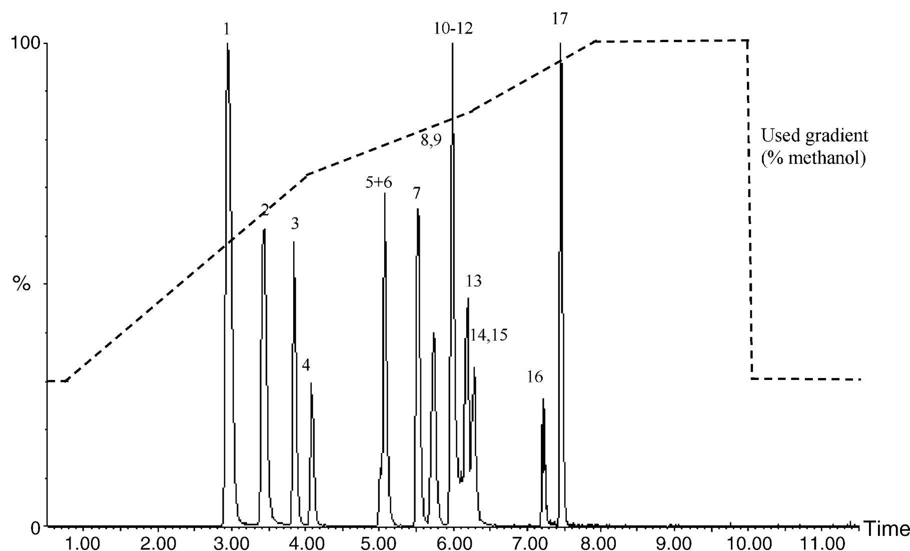

Fig. 3. Combined UPLC–MS/MS chromatogram of apple crude extract spiked with 17 (semi)polar pesticides (conc. 0.02 mg^{-1} of each) based on the quantifying MS/MS transitions (Table 2). The peak numbering is shown in Table 3. For illustration, used gradient (% of methanol) is shown. Experimental conditions are described in Section 2.4.

To avoid overestimation of N that can occur when Eq. (1) is applied for data obtained in systems employing gradient separation, corrected Eq. (2) published by Snyder et al. [16] can be used:

$$N_{\text{Gradient}} = \left[\frac{(2.3b+1)Gt_0}{2.3b \cdot 1.699 W_{0.5}}\right]^2, \qquad (2)$$

where G is compression factor—constant, which can be estimated from experimental curve published in Snyder's study, experimental quantities t_0 and $W_{0.5}$ are determined from chromatogram, b is the gradient steepness, which is calculated as follows:

$$b = \Phi' S t_0, \qquad (3)$$

where Φ' is a change (%/min) in concentration of B component (organic solvent in water–organic mixtures as the mobile phase), S the solvent strength of the pure solvent ($S = 3$ for methanol [16]) and t_0 is the column void-time. In our study, three gradients rather differing in their steepness were applied within the UPLC run. Unfortunately, it is almost impossible to find a simple mathematical description of this situation. With regard to this fact we interpolated the mobile phase composition using the starting and final concentration of methanol. Under these simplifications assumption the average Φ' value was 8.75 % min^{-1}. Therefore, only one b value was calculated using Eq. (3).

Employing this concept (Eq. (2)) N was calculated for both systems, see Fig. 5. Higher N values were obtained for almost all analytes when using conventional HPLC system, obviously due to higher lengths of separation column (15 cm versus 10 cm in UPLC). The only exception was flufenoxuron, analyte that was eluted within HPLC isocratic plateau (100% methanol).

Less unequivocal results were obtained when height of theoretical plate (HETP, see Eq. (4) [26]) was applied as criterion indicating separation efficiency.

$$\text{HETP} = \frac{L}{N}, \qquad (4)$$

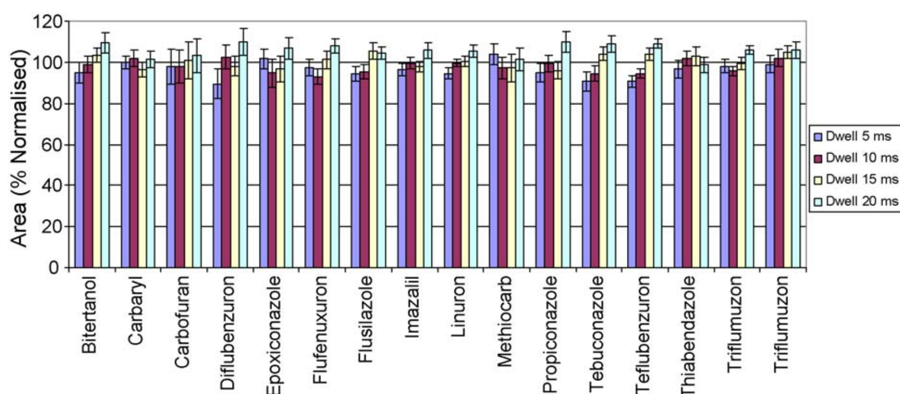

Fig. 4. Peak areas obtained for individual sampling rate. The relationship between dwell time period and peak area was estimated for UPLC–MS/MS within six sequences. In each sequence the sample fortified with target pesticides (at 10 $\mu g\,kg^{-1}$) was analysed using MRM approach with 5, 10, 15 and 20 ms dwell time, respectively.

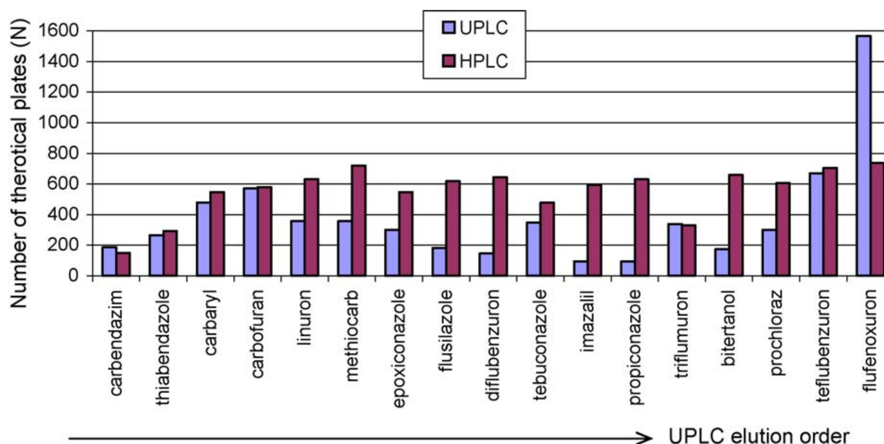

Fig. 5. The number of theoretical plates (*N*) calculated according to Eq. (2); pesticides were separated under HPLC and UPLC conditions described in Section 2.4.

where L is the length of column (cm) and N is the number of theoretical plates. In particular case, N_{Gradient} parameter was used for calculation.

For some analytes such as imazalil or propiconazole even several times lower HETP was obtained in HPLC system, distinctly higher values as compared to UPLC were calculated only for early eluting peak (Fig. 6). It should be emphasized again that the selectivity of stationary phases in HPLC and UPLC columns were not equal. On this account, the elution order of target analytes was not identical (see Table 3) what makes comparison of experimental LC rather complicated.

3.3. Peak capacity

One of important parameters characterising LC systems involved in our study is the peak capacity (PC) defined as the maximum number of elution bands that fit within a chromatogram (or part of it) with a resolution $R = 1$. Following Eqs. (6) and (7) should be used for PC calculation [8,27,28]:

$$\text{PC}_{\text{MAX}} = 1 + \frac{t_{\text{G}}}{W}, \quad \text{or} \tag{6}$$

$$\text{PC}_{\text{MAX}} = 1 + \frac{t_{\text{G}}}{1.699 W_{0.5}}, \quad \text{respectively,} \tag{7}$$

where t_{G} is a gradient time, W the peak width measured at the baseline and $W_{0.5}$ is the peak width measured at the half of peak height. Both these equations correspond to a full-range gradient (0–100% of organic component). For elution with partial gradient ($\Delta\varphi$) is then corresponding PC* estimated using equation [27]:

$$\text{PC}^{*} = \Delta\varphi \text{PC}. \tag{8}$$

where $\Delta\varphi$ is the difference in content of organic component (%) in mobile phase between the end and start of gradient elution: $\Delta\varphi = (\%_{\text{final}} - \%_{\text{start}})$.

For real-life situations (e.g. when some analytes with high k' value are eluted under isocratic conditions) peak capacity for set of sample components separated under particular LC conditions can be defined as the *sample peak capacity*—Eq. (9) [27] or (10), depending whether W or $W_{0.5}$ is applied for PC estimation.

$$\text{PC}_{\text{sample}} = \frac{t_{\text{z}} - t_{\text{a}}}{W}, \tag{9}$$

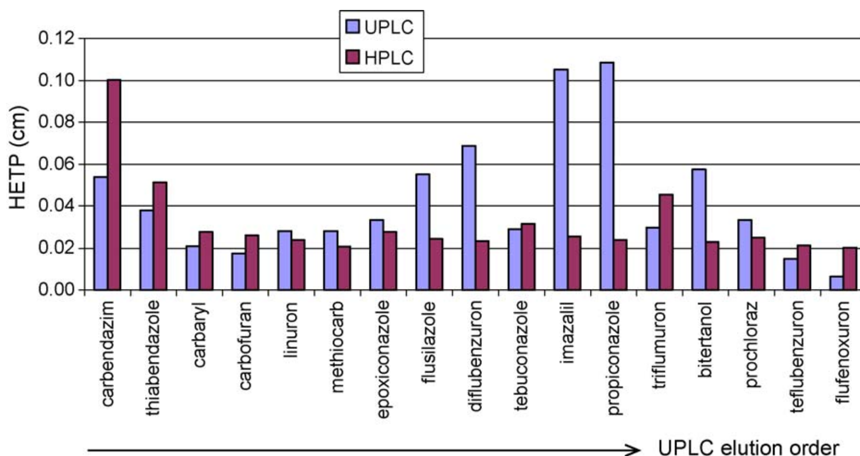

Fig. 6. The heights of theoretical plates (HETP). For HETPs calculation Eq. (4) and N_{Gradient} parameters were used. Pesticides were separated under HPLC and UPLC conditions described in Section 2.4.

Table 4
Peak widths measured at half of the peak height ($W_{0.5}$) obtained for both systems under optimised conditions (see Table 1)

Pesticide	Peak widths $W_{0.5}$ (min)	
	UPLC	HPLC
Carbendazim	0.093	0.193
Thiabendazole	0.078	0.138
Carbofuran	0.058	0.100
Carbaryl	0.053	0.098
Linuron	0.067	0.094
Methiocarb	0.067	0.088
Epoxiconazole	0.073	0.101
Flusilazole	0.094	0.095
Diflubenzuron	0.105	0.093
Tebuconazole	0.068	0.097
Imazalil	0.130	0.108
Propiconazole	0.132	0.130
Triflumuron	0.069	0.094
Bitertanol	0.096	0.092
Prochloraz	0.073	0.096
Teflubenzuron	0.049	0.098
Flufenoxuron	0.032	0.087

Table 5
LOQs achieved for both technique and improved S/N for UPLC

Pesticide	LOQ (μg kg^{-1})		[(S/N$_{UPLC}$)/(S/N$_{HPLC}$)]
	UPLC	HPLC	
Carbendazim	0.5	2.0	5
Thiabendazole	0.5	2.0	4
Carbofuran	0.5	2.0	5
Carbaryl	0.5	2.0	9
Methiocarb	1.0	2.0	7
Linuron	1.0	2.0	6
Imazalil	8.0	8.0	1
Tebuconazole	2.0	4.0	10
Diflubenzuron	8.0	8.0	1
Flusilazole	4.0	4.0	1
Epoxiconazole	1.0	4.0	7
Bitertanol	8.0	8.0	1
Propiconazole	4.0	4.0	2
Triflumuron	2.0	4.0	7
Prochloraz	4.0	8.0	5
Teflubenzuron	4.0	8.0	4
Flufenoxuron	1.0	4.0	6

$$PC_{sample} = \frac{t_z - t_a}{1.699 W_{0.5}}, \qquad (10)$$

where t_a, t_z are retention times corresponding to first and last eluted compound, respectively.

In any case, PC is well adequate for comparison of various LC systems. Different approaches could be used for PC calculation: (A) Eqs. (6) and (7) or (8) involving $\Delta\varphi$, or alternatively (B) Eqs. (9) and (10) can be used. The latter approach (Eq. (10)) was employed in our study for PC calculation, because it provides also good applicability under real-life conditions. Peak widths measured at the half of peak height ($W_{0.5}$) were determined from chromatograms and are summarized in Table 4. The values of t_a and t_z are referred in Table 3.

In Fig. 7, PC values for both experimental systems (optimised conditions) are shown. Using UPLC for 60% of analytes PC was higher, however compared to conventional system routinely used for determination of (semi)polar pesticides the calculated PC values varied largely among the analytes. Although the character of interactions of analytes with UPLC stationary phase was apparently different than that observed in case of HPLC used in our experiments, the indicative value of PC remains practically unaffected.

3.4. Peak symmetry

Not only N and PC are useful measures of column quality, also peak shape (T) is very important parameter closely linked to quality of generated data (e.g. peak tailing may result in increased LODs and higher R.S.D.s).

T was estimated according to Eq. (11), where $a_{0.1}$ is the distance between the peak front and the peak maximum measured

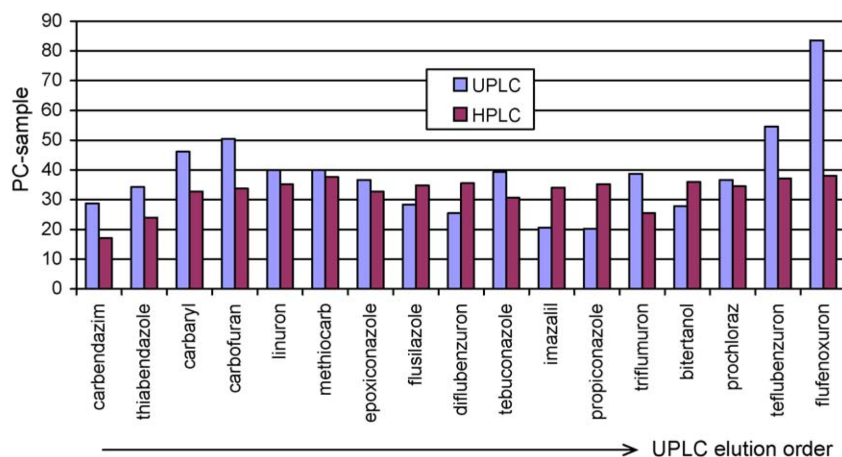

Fig. 7. Peak capacity using PC$_{Sample}$—Eq. (10); pesticides separated under HPLC and UPLC conditions described in Section 2.4. The retention time of first-eluted compound (in both systems carbendazim) was used as t_a. The retention time of last-eluted compound (flufenoxuron) was used as t_z. t_a, t_z values are shown in Table 3. Values of $W_{0.5}$ parameters are summarized in Table 4.

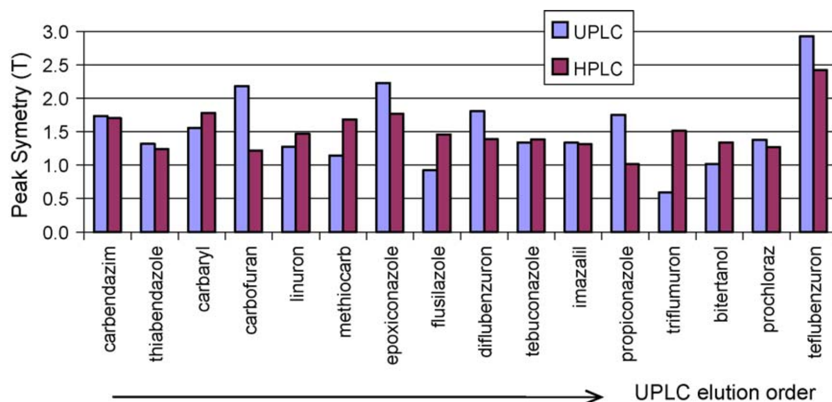

Fig. 8. Peak symmetry (T) for UPLC and HPLC columns.

at 10% of height. Likewise, $b_{0.1}$ is the distance between the peak maximum and peak end [24]

$$T = \frac{b_{0.1}}{a_{0.1}}. \tag{11}$$

As shown in Fig. 8, best values of T were obtained for methiocarb (1.1), flusilazole (0.9) and bitertanol (1.0) in the case of UPLC and for propiconazole (1.0) in the case of HPLC. Thanks to different selectivity of used columns distinct T values for triflumuron were achieved using UPLC (0.6) and HPLC (1.5), respectively.

3.5. S/N and LOQ values

Signal to noise ratio (S/N) is one of the most important performance characteristics considered in assessment of multiresidue method potential to measure low analyte levels. In routine practise, the S/N value is used for prediction of LOQ.

In our study for all analytes S/N (at concentration level 0.02 mg kg^{-1}) and LOQ were evaluated for both LC–MS/MS systems. As shown in Table 5, S/N values were mostly significantly higher in UPLC system. In the case of UPLC, improved values of S/N were mostly followed by significantly decreased LOQs (2–4×). Though for most analytes UPLC provided narrowed peaks, for imazalil, bitertanol, flusilazole and propiconazole similar peak widths were obtained, which resulted into similar S/N ratios and consequently into identical LOQs for these analytes.

Based on theoretical assumptions, the compression of peak width is bound up with the increase of peak height and S/N ratio. Consequently, the sensitivity (expressed as LOD or LOQ) is improved. In our study comparing UPLC and HPLC chromatograms on the concentration level close to LOQ the significant differences in peak shape namely width and height were observed as documents Fig. 9. In any case, the peak compression caused appropriate S/N increase and LOQ improvement.

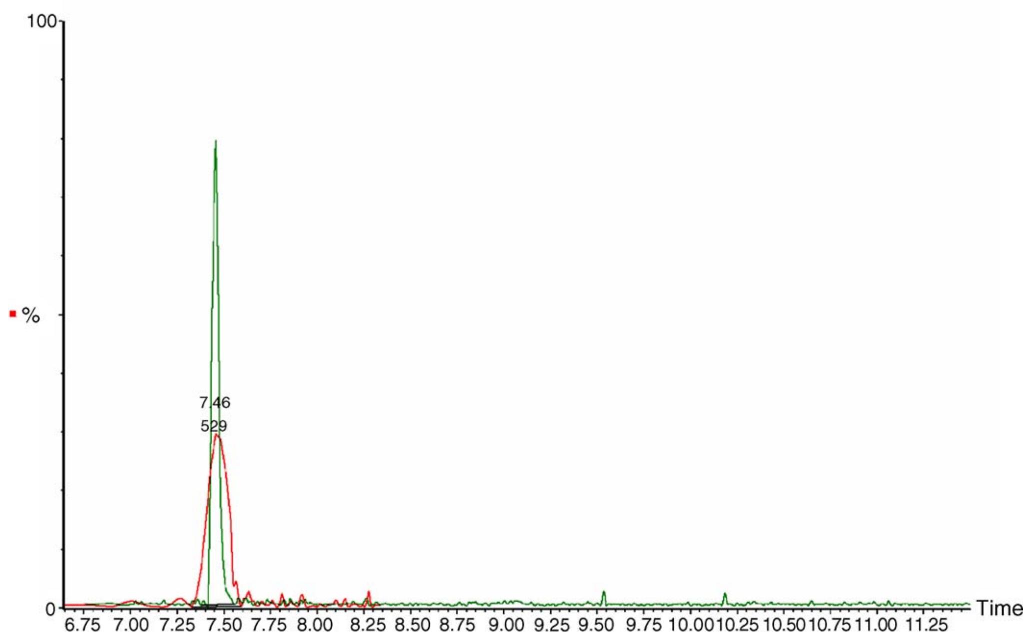

Fig. 9. Comparison of peak shape in UPLC and HPLC. The character of flufenoxuron peak was compared. In the case of UPLC the peak width was reduced and peak height was increased about approx. 100%. In this case, the sensitivity (LOQ) was decreased down to 1 μg kg^{-1} (for HPLC 4 μg kg^{-1}).

The values of LOQs reported in our study may, seem to be rather high as compared to other publications. However, described (accredited) method uses only simple acetonitrile extraction followed by direct injection of crude extract into LC–MS system (the equivalent of sample matrix was as low as $0.25\,g\,mL^{-1}$). Under these conditions when analysing $5\,\mu L$ of extract the injected amount of matrix is 1.25 mg. Although, due to analysis of very low amount of analysed sample equivalent, the matrix effects are minimized and good long term stability of analyte responses is achieved, extremely low LODs are partly sacrificed in this approach. Since the purpose of study was to investigate the outcome obtained by replacing HPLC separation by UPLC, the same volume of sample were injected into both experimental systems.

4. Conclusions

Alike reported in other studies [29–31], also our experience shows that the use of "classic" HPLC separation strategy followed by detection employing tandem quadrupole mass spectrometric detector such as Quattro Premier (Waters) allows robust analysis of low levels of multiple pesticide residues in such complex matrices as foodstuffs. Introduction of UPLC lead us to considerations of attaining not only reduction of LC separation step but also further improvement in performance characteristics of determinative step.

Comparative analyses of 17 (semi)polar pesticide residues in crude apple extract that were realised in two alternative LC–MS/MS systems documented the potential of UPLC to replace "classic" HPLC separation strategy and thus enable substantial increase of quantification step throughput. Based on the data generated in optimised systems employing either (i) Acquity UPLC (Waters) or (ii) Alliance HPLC (Waters) hyphenated with Quattro Premier (Waters) MS detector (tandem quadrupole) calculated performance criteria can be summarized as follows:

- The number of theoretical plates illustrating separation efficiency was for most analytes higher in system employing HPLC and with lower variability compared to UPLC.
- The values of HETP obtained in UPLC were mostly higher, however their variability was rather high.
- Similar PC and peak symmetry values were obtained in both LC–MS/MS systems under optimised conditions; nevertheless the analysis time in system employing UPLC was reduced by more than 50% with similar analytical output. In addition, very important is also significantly lower solvent consumption for UPLC.
- UPLC provided significantly improved S/N followed by decreased LOQs for majority of compounds. LOQs of imazalil, bitertanol, flusilazole and propiconazole were not affected by using UPLC thanks to their similar peaks width in both separation systems.

In any case, UPLC is a challenging technique that undoubtedly will find a wide range of applications in analysis of multiple pesticide residues potentially occurring in food crops.

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