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Alternative calibration approaches to compensate the effect of co-extracted matrix components in liquid chromatography–electrospray ionisation tandem mass spectrometry analysis of pesticide residues in plant materials

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Abstract

The aim of this study was to evaluate the applicability of different calibration approaches in a multi- and single-residue analysis of modern pesticides in plant matrices using liquid chromatography–electrospray mass spectrometry (HPLC–ESI-MS). In the first set of experiments the determination of eight pesticides representing different groups of polar/unstable pesticides (carbamates, benzimidazoles, azoles, benzoylphenylurea) in apple samples was performed. The trueness and precision of data obtained by using: (i) external solvent standard calibration, (ii) external matrix-matched standard calibration and (iii) echo-peak internal standard calibration was compared. The last mentioned method is a novel technique providing the possibility to inject internal standard of the same identity as a target analyte, so that its retention time is close to the analyte from the sample. According to expectation, when using external standard solvent calibration the results were under- or overestimated due to suppression or enhancement of analyte's signal by matrix components. On the other hand with the use of matrix-matched calibration accurate data were obtained. With echo-peak technique accurate results comparable to those obtained by matrix calibration were obtained for six out of eight pesticides. In the second set of experiment we used the echo-peak technique to overcome the problem with the response instability in the analysis of chlormequat in pear concentrate samples. As an internal standard method the echo-peak technique provided the possibility of monitoring of signal decrease during the analytical sequence and to compensate this decrease by relating sample peak area relatively to this internal standard.
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1. Introduction

In recent years LC–MS has become a widely applied instrumental technique in many fields including pharmaceutical, environmental and food analysis. Modern LC–MS instruments employing atmospheric pressure ionisation (API) provide excellent sensitivity and selectivity, that enables determination of target analytes at (ultra) trace levels. When highly specific MS–MS analysis is applied almost no matrix

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interferences are recorded. Although they are 'invisible' in the chromatogram, coextracts present in the injected sample may cause poor accuracy of results. In quantitative analysis one of the major problems is the suppression/enhancement of the analyte signal in the presence of matrix components, which has been observed by many authors for analyses of complex samples [1–6]. This phenomenon was first discussed in greater detail by Kebarle et al. [7,8]. It was suggested, that organic compounds present in the sample in concentrations exceeding $\sim 10^{-5}$ *M* may compete with the analyte for access to the droplet surface for gas phase emission. In some instances the decrease of ion intensities of MH^+ ions of an analyte can be attributed to the gas-phase proton transfer between the electrosprayed gas-phase molecules and evaporated molecules of the stronger gas-phase base. Another hypothesis given in literature refers to the radius of droplets from which gas-phase ions are emitted. If samples are contaminated with non-volatile matrix components, droplets are prevented from reaching their critical radius and surface field, hence reduction of the ion signal for an analyte occurs [9].

As a result of the above described matrix effects the response of an analyte in pure solvent standard can differ significantly from that in matrix sample. If accurate results are to be obtained, matrix effects must be eliminated or compensated. One of the possible approaches to address this problem is the reduction of the amount of matrix components entering the MS detector at the same time as the analyte. This can be reached by a more selective extraction procedure [6] or more extensive sample clean-up [2] which is, however, time consuming and the risk of the loss of analytes during several consecutive clean-up steps is generally increased. Decreasing the amount of injected sample can also lead to matrix effects reduction [4], however, this is not a method of choice in trace level analysis. An improvement of HPLC separation efficiency, e.g. by means of two-dimensional LC [4] may result in decreased amount of sample components coeluted with the target analyte and thus reduction of matrix effects. Another alternative is the modification of the mobile phase composition. Choi et al. [5] observed a good correlation between the response of an analyte in the solvent standard and in the sample when low concentrations of mobile phase additives, such as formic acid, ammonium formate or ammonium hydroxide were used. However, the reduction of matrix effects appeared at concentrations, where the signal response from the standard was already significantly suppressed by the buffer.

If matrix suppression/enhancement phenomena cannot be eliminated by one of the above described ways, appropriate calibration technique compensating for matrix effects should be used. Calibration using external matrix-matched standards (standards with the same or similar matrix composition as the analysed sample) is often used. Unfortunately, a general prerequisite, i.e. the availability of appropriate blank (i.e. material free of residues of target analyte), may not always be accomplished. Another technique is the use of an internal standard. To compensate for matrix effects, the internal standard must have a retention time identical or very close to the retention time of the analyte. Isotopically labelled internal standards are well suited for this purpose. However, their use is rather expensive, especially in a multicomponent analysis, where a separate internal standard for each analyte is required. Moreover, for many compounds, such as modern pesticides, isotopically labelled standards are not commercially available. As a reliable and relatively cheap calibration technique appears a technique of postcolumn addition [3]. The internal standard—e.g. structure analoque of analysed compound(s)—is injected in a constant flow-rate into the effluent from LC separation column directly into MS detector. The response of analyte in the sample is then related to the response of the internal standard at the retention time of analyte. A technical obstacle to this method is that an additional pump is necessary for routine batch analyses.

Another very interesting calibration technique potentially compensating for matrix effects is so-called echo-peak technique [10,11]. Echo-peak technique is an internal standard method, however no isotopically labelled standards of the target analyte are necessary (for detailed description see Section 3.2).

In our study, we have evaluated the applicability of the echo-peak calibration technique for the quantitative analysis of multiple pesticides in apple. The accuracy of data obtained by calibration by both

external standard in solvent, and (external) matrix-matched standard has been compared with the results of echo-peak calibration. Another problem we have addressed in our experiments was a poor long-term stability of responses often encountered in LC–MS analysis of complex samples or in analyses where relatively high concentrations of mobile phase additives must be used. In the presented study we demonstrate the usefulness of echo-peak technique in solving this problem in the analysis of chlormequat in a pear concentrate.

2. Experimental

2.1. Chemicals and materials

Certified pesticide standards were obtained from Dr. Ehrenstoffer (Germany) (purity 95–99%). Pesticide residues grade solvents were obtained from Scharlau (Italy) (ethyl acetate) and from Merck (Germany) (cyclohexane, methanol). Deionised water for mixing the mobile phase was produced in Milli-Q apparatus (Millipore, Germany). Ammonium acetate (purity 99.999%) was obtained from Aldrich (USA). Anhydrous sodium sulphate (Penta Chrudim, Czech Republic) was activated 5 h at 450 °C. Apples from a retail market were used for the preparation of matrix samples. Pear concentrate sample (Bayernwald, Germany) known to be blank was saved from previous analyses.

2.2. Apparatus

Apple samples were processed with a Waring blender homogeniser (Waring, USA) and tissumiser Turrax (IKA Werke, Germany). Extraction of pear concentrate was performed using a shaking machine (IKA Werke). All solvent reductions were performed on a Büchi rotary evaporator (Büchs, Switzerland).

An automated high-performance gel-permeation chromatographic (HPGPC) system (Gilson, France) equipped with a PL gel (600×7.5 mm, 50 Å) column (Polymer Laboratories, UK) was used for the clean-up of apple extracts.

2.3. HPLC conditions

LC separation was carried out with a HP1100 liquid chromatograph (Hewlett-Packard, USA) equipped with a switch valve in the column compartment. Following HPLC conditions were used:

(1) Experiment A: analysis of multiple pesticides in apple samples

Separations were performed on a reversed-phase Discovery C_{18} column (15 cm×3 mm, 5 μm). For echo-peak experiments another short column Discovery C_{18} (5 cm×2.1 mm, 5 μm) was used. The mobile phase was methanol–water with following gradient programmes: (i) normal separation: 0–11.5 min, linear from 20 to 80% methanol; 11.5–18 min, 80% methanol, 18–23 min, 100% methanol; (ii) echo-peak separation: 0–1 min, 5% methanol; 1–13 min, linear from 5 to 70% methanol; 13–15 min, 70% methanol; 15–20 min, 75% methanol; 20–26 min, 100% methanol. The dwell time between the first and second injection was 1 min (plus an additional 55 s necessary for the autosampler to complete the injection). Flow-rate was 0.5 ml/min, column temperature 25 °C and the injection volume was 10 μl in both experiments.

(2) Experiment B: analysis of chlormequat in pear concentrates

For the analysis of chlormequat a cation-exchange column Partisil SCX (15 cm×4 mm, 5 μm) was used and no additional precolumn was necessary for the echo-peak analysis. Dwell time between the first and second injection was 1 min (plus additional 55 s necessary for autosampler to complete injection). The mobile phase programme was isocratical 0.1 *M* ammonium acetate in water–methanol (60:40) for both normal and echo-peak analyses. A flow-rate of 0.7 ml/min, column temperature of 50 °C and injection volume 40 μl was used in all experiments. Total analysis time was 20 min and the divert valve was used so that in each analysis in only 4.5 min the mobile phase was flowing to the source.

2.4. MS–MS detection

Mass analysis was performed with LCQ Deca ion-trap instrument from Finnigan (USA). ESI ioni-

sation was applied in all experiments. Following experimental conditions were used:

(1) Experiment A: analysis of multiple pesticides in apple samples

Capillary temperature 280 °C, flow-rates of sheath gas and auxiliary gas 1.5 and 3 l/min, respectively, spray voltage 4–6 kV, capillary voltage was optimised individually for each analyte by automatic tune and ranged from −35 to +15 V. For MS–MS analysis time segments were set up, in each segment one or two pesticides were scanned. Five out of eight pesticides were monitored in the positive ion mode with following parent→daughter masses used: carbendazim, 192→160; thiabendazole 202→175; carbaryl 202→145; imazalil 297→201 and 255; prochloraz 376→308. Three benzoylurea pesticides were monitored in the negative ion mode with following parent→daughter masses used: triflumuron 357→154 and 321; teflubenzuron 379→339 and 359; flufenoxuron 487→467.

(2) Experiment B: analysis of chlormequat in pear concentrates

Capillary temperature 350 °C, flow-rates of sheath gas and auxiliary gas 1.5 l/min and 3 l/min, respectively, spray voltage 2 kV and capillary voltage 26 V. MS–MS was carried out in positive ion mode with parent m/z +122 and daughter ion m/z 58 and 59.

The acquired data were reprocessed using the XCALIBUR software (Finnigan).

2.5. Extraction and clean-up, preparation of blank extracts

2.5.1. Apples

A 25-g sample of blank apples was mixed with 125 ml ethyl acetate and 25 g sodium sulphate and homogenised for 2 min with a Turrax tissumiser. The suspension was filtered under vacuum, the volume of filtrate was reduced by evaporation to 12.5 ml and made-up with cyclohexane in a 25-ml volumetric flask. The crude extract was purified by HPGPC under the following conditions: mobile phase, cyclohexane–ethyl acetate (1:1, v/v); flow, 1 ml/min; injection volume, 2 ml; collected ('pesticide') fraction, 14–30 ml. This fraction was evaporated by the rotary evaporator and dried under a mild stream of nitrogen. The residue was redissolved in 2 ml of methanol–water (1:1, v/v) mixture and passed through a Millipore membrane filter prior to HPLC–MS analysis.

2.5.2. Pear concentrate

A 20-ml volume of methanol was added to 20 g of blank pear concentrate and the extraction was carried out by shaking the mixture for 2 h on a shaking machine. Since no solid particles were present, the extract was transfered without filtration to a 100-ml volumetric flask and made up with methanol–water (1:1, v/v) mixture. Before injection the extract was filtered through a Millipore filter.

2.6. Calibration standards and control samples

2.6.1. Analysis of eight pesticides (mixture A) in apple

Individual pesticide stock solutions (340–3000 μg/ml) were prepared by dissolving neat standards in acetonitrile, a small amount of acetone was added to improve solubility in case of teflubenzuron. Working and calibration solutions of mixture A were prepared by their further diluting with methanol or methanol–water (1:1, v/v). Apple matrix-matched standards were prepared by adding standard pesticide mixture A to purified blank extracts (see Section 2.5).

The calibration curves consisted of four concentration levels at 0.005, 0.05, 0.25 and 0.5 μg/ml (corresponding to 0.005, 0.05, 0.25 and 0.5 mg/kg of apples, respectively). As the control samples for the testing of the accuracy of different calibration approaches matrix-matched standards at two concentration levels, 0.01 and 0.1 μg/ml, (corresponding 0.01 and 0.1 mg/kg of apples, respectively) were used. The lower concentration level of samples was chosen with respect to the maximum residue limit (MRL) 0.01 mg/kg established by European legislation for pesticide residues in baby food. The higher concentration level approximately corresponds to common legislation limits given for fruits and vegetables. Standard at 0.05 μg/ml was used as a reference in the echo-peak experiments discussed in Section 3.4.

For the purpose of comparison of different cali-

bration approaches analysed sequence in all experiments was arranged as follows: (1) calibration set a: four injections of standards, (2) sample 0.01a, (3) sample 0.1a, (4) sample 0.01b, (5) sample 0.1b, (6) calibration set b: four injections of standards. Three conditioning injections of matrix sample were injected prior to this sequence.

2.6.2. Analysis of chlormequat in pear concentrate

Stock solution of chlormequat (1000 μg/ml) was prepared by dissolving the neat standard in methanol–water (1:1, v/v). Concentration levels used for calibration were set at 0.02, 0.1, 0.2 and 1 μg/ml (corresponding to 0.1, 0.5, 1 and 5 mg/kg of pear concentrate, respectively). This concentration range is derived from the detection limit of the method and also corresponds to common levels found in this type of matrix. Samples used for the testing of accuracy of different calibration approaches were at two different concentration levels 0.04 and 0.4 μg/ml (corresponding to 0.2 and 2 mg/kg of pear concentrate, respectively). Standard at 0.1 μg/ml was used as a reference in the echo-peak experiments discussed in Section 3.5.

The analysed sequence was arranged as follows: (1) three conditioning injections of standard at 0.02 μg/ml, (2) calibration set a: four injections of standards, (3) sample 0.04 a, (4) sample 0.4 a, (5) sample 0.04 b, (6) sample 0.4 b, (7) sample 0.04 c, (8) sample 0.4 c, (9) calibration set b: four injections of standards.

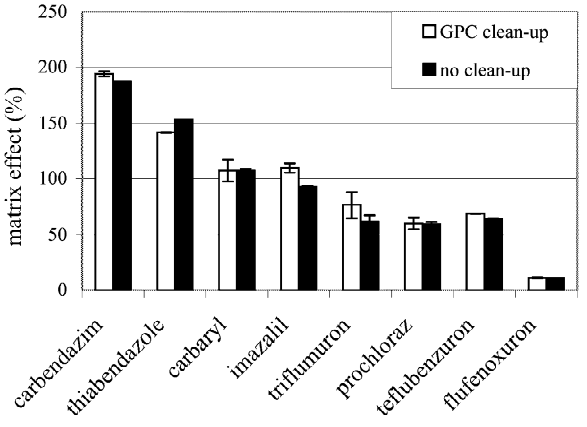

Fig. 1. Matrix effects in (A) GPC purified apple extracts, (B) crude apple extracts concentration level 0.005 μg/ml of pesticides, sample aliquot 1 g apple/ml of extract.

3. Results and discussion

3.1. Matrix effects in the analysis of pesticides in apple samples

For the pesticides of mixture A in apple matrix considerable differences in responses of an analyte in pure solvent standard and matrix-matched standard were observed. Response suppression caused by sample matrix components has been widely discussed in the literature (see above). In our experiments triflumuron, prochloraz, teflubenzuron and flufenoxuron showed response suppression in the presence of apple matrix (see Fig. 1). Less common behaviour was observed for carbendazim and thiabendazole, where the detector response was enhanced by matrix components. We assume that this phenomenon can be attributed to the gas-phase proton transfer. Both carbendazim and thiabendazole are pesticides of basic nature and the matrix components of acidic character could promote the formation of MH^+ ion of these analytes in the electrospray. It should be noted, that these analytes are eluted at low retention times and therefore coelution with such (polar) coextracts is quite probable. Rather surprisingly, almost no difference in matrix effects was recorded for GPC purified apple extracts and crude (non cleaned-up) samples (see Fig. 1). Gel-permeation chromatography (GPC), a widely used clean-up technique in a multiresidue analysis of GC amenable pesticides, was inefficient in removing sample components responsible for matrix effects in LC–MS.

3.2. Echo-peak calibration technique in multi- and single-residue analysis of pesticides

With the echo-peak technique each analysis comprises two injections into LC–MS system. An unknown sample and a standard solution are injected consecutively within a short time period, under the specific experimental conditions described below. As a result, the peak of analyte from the standard elutes in close proximity to the peak of analyte from the

sample, thus forming the 'echo peak'. Provided that retention times of these two peaks are close enough to be affected by the coeluted sample components in the same manner, matrix effects are compensated.

If multiple compounds are to be analysed in one run, switching of the mobile phase flow-rate to n additional precolumn or a short separation column during the analysis is required for echo peak analysis. In this case, the sample is injected into a separation column under isocratic conditions, while mobile phase flows directly into the separation column (column switch valve in position 1, see Fig. 2). Due to the low elution strength of a mobile phase analytes are retained in the front part of the HPLC column. After a short time period (~1 min), the column switch valve position is changed to direct the mobile phase through a precolumn into the chromatographic column. At this moment a reference, represented by a solvent standard of selected concentration level, is injected and gradient programme required for separation is initiated. As a result of above described set-up, the peak of an analyte from the reference standard elutes slightly behind the peak of analyte from the sample. The chromatogram of multiple pesticides (mixture A) obtained under optimised echo-peak separation conditions is shown in Fig. 3. The order of both injections can be reversed, obtaining the peak of a reference as a first and the peak of a sample as a second. For the purpose of quantification, a calibration plot is constructed from the peak area ratios of a standard and a reference standard. The concentration of analyte in unknown sample is calculated from the peak area ratio of sample and reference.

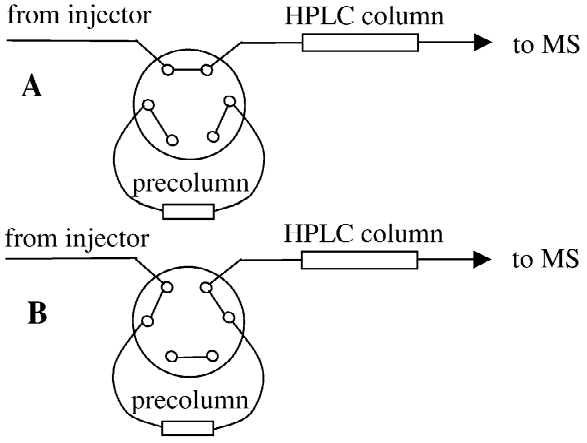

Fig. 2. Instrumental set-up for the echo-peak technique in a multiresidue analysis. (A) Column switch valve position 1. Sample is injected, mobile phase flows directly into the separation column, mobile phase composition, methanol–water (5:95); duration; 1 min. (B) Column switch valve position 2. Reference standard is injected, mobile phase flows through a precolumn into the separation column, gradient required for separation starts.

As demonstrated in Section 3.5, in a single residue method whole set-up can be significantly simplified. No additional precolumn is used and isocratical conditions throughout the whole run can be applied.

3.3. Compensating for matrix effects for pesticides in apple samples

In this set of experiments the potential of the echo-peak technique to compensate matrix effects at a single concentration level was tested. The analysed sequence consisted of two analyses of standard (0.5 μg/ml) in pure solvent, followed by five analyses of apple sample (0.5 μg/ml) and completed by two analyses of solvent standard (0.5 μg/ml). To obtain an intense matrix effect, a relatively high equivalent of original sample (5 g/ml) was contained in the matrix-matched standards. As a reference standard, a pesticide mixture at 0.5 μg/ml was injected in each analysis. Both conceivable orders of injections were tested, i.e. (i) set-up I: reference as a first, sample as a second, (ii) set-up II: sample as a first, reference as a second.

In Table 1, calculation equations are shown, while the results of the experiment are summarised in Table 2. Matrix effects, i.e. the ratio of the responses in matrix-matched standard and solvent standard, were calculated for both peak 1 and peak 2 (line 5 in Table 2). Matrix effects calculated by using the peak of reference as the internal standard are given in line 6. From these data it can be seen that compensation occurred in those cases where both the peak of sample and reference were influenced by matrix to the same extent, in other words their matrix effects were similar (see line 5). In set-up I, matrix effects were totally compensated for imazalil, triflumuron and prochloraz and somewhat reduced for carbendazim. In set-up II, good trueness of results was obtained for all compounds with exceptions of teflubenzuron and flufenoxuron. As seen from Table

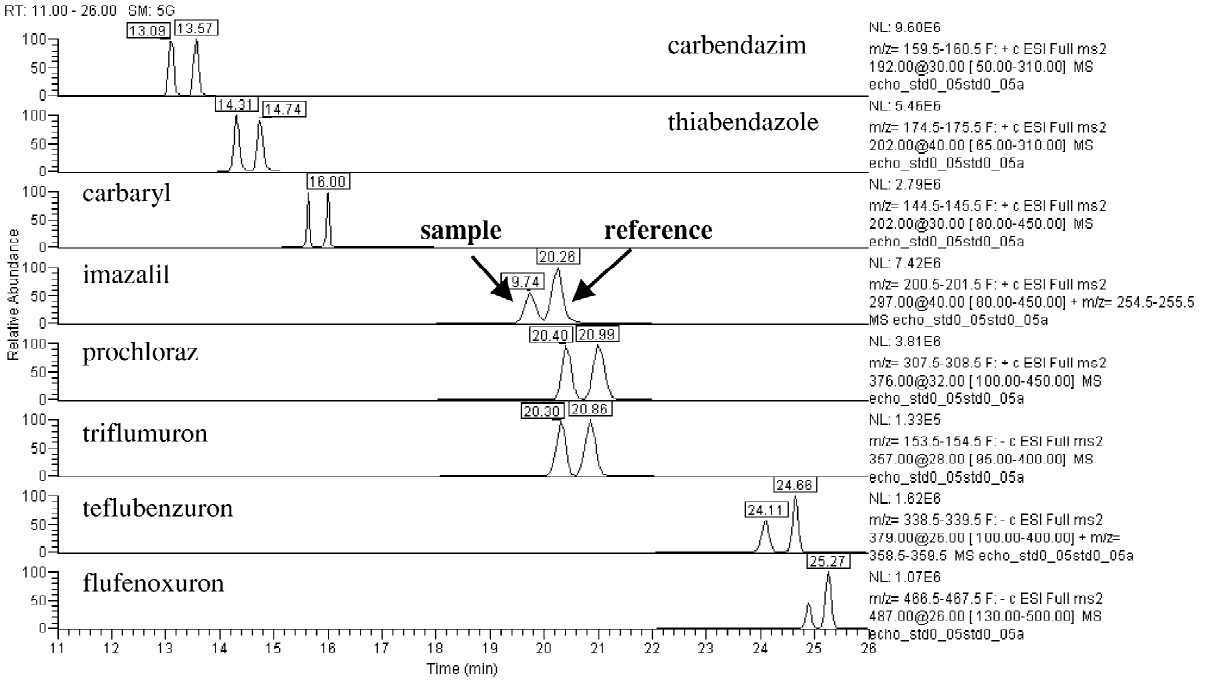

Fig. 3. LC–MS chromatogram of pesticide mixture—application of echo-peak injection 10 μl injection. 1st peak, sample: standard of pesticides (0.5 μg/ml) in methanol–water (1:1, v/v). 2nd peak, reference: standard of pesticides (0.5 μg/ml) in methanol–water (1:1, v/v).

2 for these two compounds, in neither of the set-ups was the reference affected by matrix in the same way as the peak from sample, therefore matrix effect compensation could not be achieved. We assume that the possibility of compensation of matrix effects by the echo-peak technique is related to the width of the elution zone of coextracted matrix. If the band of the matrix components causing matrix suppression/enhancement is sufficiently broad in relation to the retention time difference of peaks of the sample and reference, then the matrix will overlap with both of these peaks and the compensation of matrix effects will be achieved. On the other hand, in the case of relatively narrow zones of matrix coextracts, the overlap with both peaks is not possible and therefore no compensation occurs. Similarly, the different results obtained by using both set-ups I and II in echo-peak experiments can be explained by the differences in the elution profile of matrix in both set-ups. It should be noted, that there is unfortunately no simple way to observe elution profiles of components responsible for matrix effects. By inspecting

Table 1
Calculation of matrix effects using echo-peak calibration—example for set-up I

	Peak 1 reference	Peak 2
Peak area of standard	X1	X2
Peak area of standard related to reference		X2/X1
Peak area of sample	Y1	Y2
Peak area of sample related to reference		Y2/Y1
Matrix effect	(Y1/X1)×100	(Y2/X2)×100
Matrix effect after echo echo-peak compensation	–	[(Y2/Y1)/(X2/X1)]×100

Table 2
Matrix effects obtained by echo-peak technique for apple samples at concentration level 0.5 μg/ml

	Carbendazim		Thiabendazole		Carbaryl		Imazalil		Triflumuron		Prochloraz		Teflubenzuron		Flufenoxuron	
	Peak 1 reference	Peak 2	Peak 1 reference	Peak 2	Peak 1 reference	Peak 2	Peak 1 reference	Peak 2	Peak 1 reference	Peak 2	Peak 1 reference	Peak 2	Peak 1 reference	Peak 2	Peak 1 reference	Peak 2
(a) Set-up I, reference injected as a first, sample injected as a second																
1. Peak area of standard ($\times 10^6$)	255	247	201	195	132	132	463	552	10	11	217	296	42	49	16	34
2. Peak area of standard related to reference		0.966		0.972		0.998		1.191		1.156		1.364		1.159		2.155
3. Peak area of sample ($\times 10^6$)	371	431	187	233	63	117	414	486	6	6	130	178	39	28	19	17
4. Peak area of sample related to reference		1.160		1.246		1.870		1.173		1.108		1.369		0.731		0.917
5. Matrix effect (%)	145	175	93	120	47	89	89	88	57	54	60	60	93	58	119	51
6. Matrix effect after echo-peak compensation (%)		120		128		187		99		96		100		63		43
(b) Set-up II. sample injected as a first, reference injected as a second																
1. Peak area of standard ($\times 10^6$)	261	252	201	196	131	129	459	564	11	12	211	292	42	48	16	35
2. Peak area of standard related to reference	1.036		1.024		1.011		0.815		0.840		0.723		0.878		0.455	
3. Peak area of sample ($\times 10^6$)	274	251	146	159	105	98	415	473	6	7	141	210	22	45	5	24
4. Peak area of sample related to reference	1.092		0.917		1.068		0.878		0.800		0.672		0.497		0.188	
5. Matrix effect (%)	105	100	73	81	80	76	90	84	54	56	67	72	53	94	29	70
6. Matrix effect after echo-peak compensation (%)	105		90		106		108		95		93		57		41	

the background signal obtained by either full MS detection or spectroscopic DAD detection, we were not able to find any relation between the detected background peaks and the matrix suppression/enhancement phenomena observed for our analytes. This is probably due to the fact that matrix components responsible for matrix effects may not be detectable neither by MS (e.g. for their low ionisation efficiency) nor by DAD (they do not absorb UV light).

3.4. Accuracy of data obtained by different calibration approaches for multiple pesticides in apple samples

The accuracy of data obtained by the use of three different calibration techniques: (i) external solvent standard calibration, (ii) external matrix-matched standard calibration and (iii) echo peak calibration, was compared.

Two control apple samples (0.01 and 0.1 μg/ml) were analysed (for more detail see Experimental). Both samples were treated as unknowns, i.e. they were analysed in the LC sequence, enveloped by two calibration sets, and their concentrations were calculated from calibration curves. For the whole calibration range, the linear calibration curve fitted poorly to the measured calibration points. Accordingly, distorted results at lower concentrations were obtained. The quadratic curve fitted better to the standard points, nevertheless, the improvement in data trueness was still insufficient. The best results were obtained when linear interpolation using only the two calibration points [12] surrounding the current sample was applied. For this reason, the two points linear interpolation was used in all subsequent experiments.

In Fig. 4 concentrations of analytes in samples calculated by the use of different calibration techniques are shown. In agreement with the character of matrix effects (see Fig. 1), overestimated (carbendazim, thiabendazole) or underestimated (triflumuron, prochloraz, teflubenzuron, flufenoxuron) results were obtained, when external solvent standards were used for quantification. On the other hand, good accuracy of results was achieved for most compounds when using matrix-matched calibration. In particular cases, poor results were obtained even when using two-pint matrix-matched calibration. For carbaryl at the lower concentration level the result was ~60% of the correct value. By carefully inspecting the data we found that carbaryl shows considerable nonlinearity of response in the lower range of concentrations (from 5×LOD to 50×LOD). In our experiment, the linear interpolation between the calibration points 0.005 and 0.05 μg/ml was used for the quantification of carbaryl in the sample at 0.01 μg/ml. We assume that such a density of calibration points is not sufficient with respect to the observed nonlinearity. In the follow-up study we managed to quantify carbaryl precisely (max. bias ±10%) at the level of 10 ppb using more calibration points in the lower part of the calibration graph. For flufenoxuron the results were underestimated by 40–60% when using matrix calibration. Unfortunately we are unable to explain the biased results for this compound. The only possible explanation is that slightly different matrix composition was present in matrix standards, since they were prepared within other extraction and clean-up batches 2 days before the preparation of spiked control samples. Compared to other target compounds ionisation of flufenoxuron is strongly influenced by present coextracts left after GPC (see Section 3.1). In any case some further experiments and method modification will be necessary to address the low ruggedness of the method for flufenoxuron.

As regards echo-peak technique, very accurate results comparable to those obtained by matrix calibration were achieved for carbendazim, thiabendazole, triflumuron and prochloraz at both concentration levels, regardless the order of injections of reference and sample into the LC–MS system. Fig. 4 shows that distorted results were generated for carbaryl when echo-peak calibration in set-up I was applied. This is in agreement with matrix effects obtained at a single concentration level discussed in Section 3.3 (see Table 2). The problem is most probably related to the elution profile of matrix components. In set-up I, the matrix coelutes with peak 1 (reference), while it leaves the second peak unaffected. On the contrary, in set-up II the elution zone of matrix components overlaps with both sample and reference peak, thus matrix effects are compensated. Another kind of problem was observed for imazalil in set-up II. Since it is a relatively basic

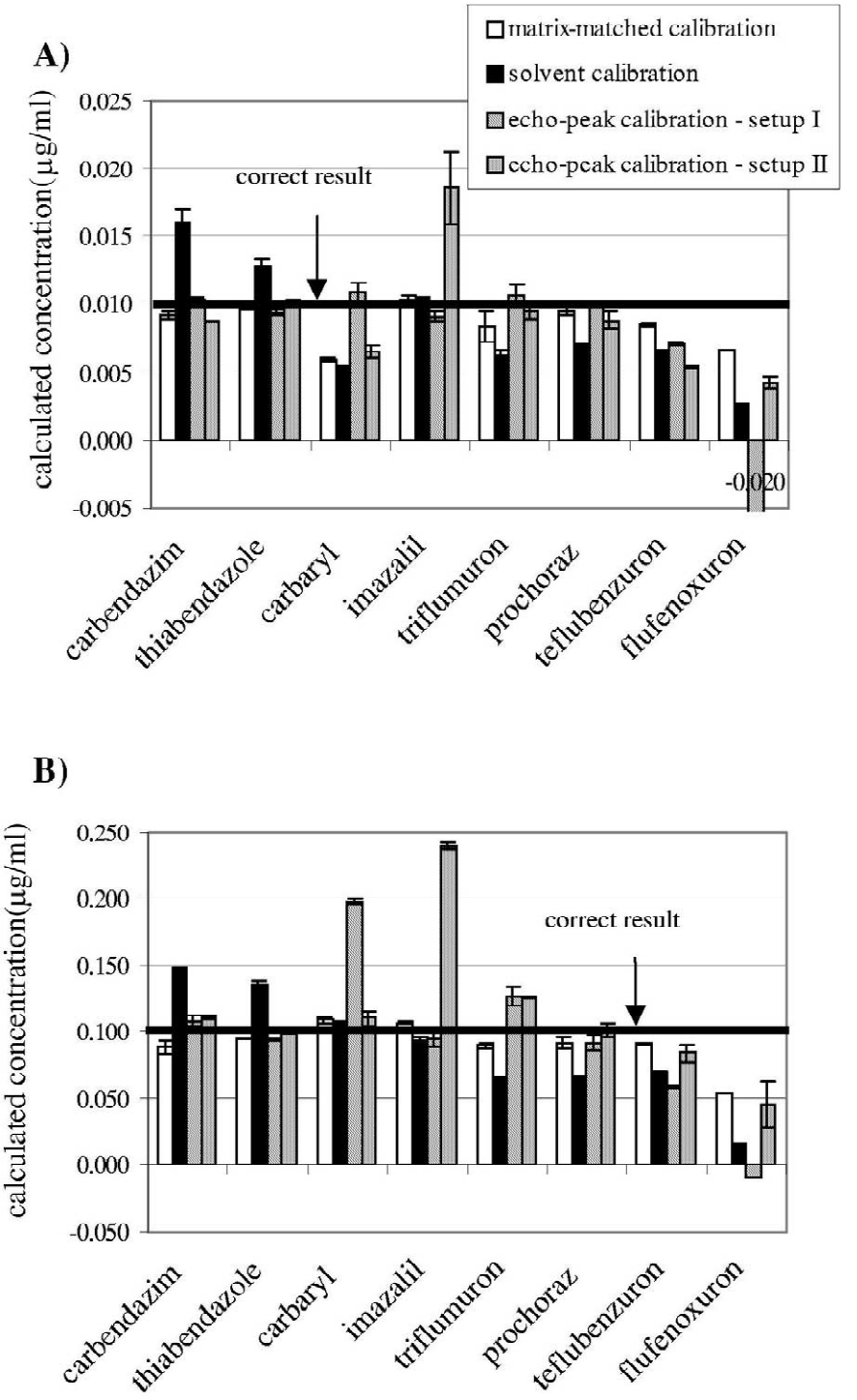

Fig. 4. Accuracy of data obtained by different calibration techniques, GPC purified apple extracts (1 g/ml) spiked with pesticides (mixture A): (A) sample at 0.01 μg/ml; (B) sample at 0.1 μg/ml.

compound, the peak of imazalil characteristically tails on a C_{18} column. Although, at the first sight, the separation of the sample and reference peaks seems to be satisfactory, the peak area of a reference (injected as a second) can be increased by a tail of the first peak. This is, of course, the more significant, the higher is the concentration of the sample injected as a first. This theory is supported by the fact, that in set-up II the peak area of a reference throughout the calibration set was not constant, however, it was rising as the concentration of the first injected standard increased. In set-up I no such problem arose, the peak area of a reference was constant over the whole calibration range and also the calculated sample concentrations were not overestimated (see Fig. 4).

For teflubenzuron and flufenoxuron no considerable compensation of matrix effects was achieved in either of the tested set-ups of the echo-peak technique. This corresponds to the results of previous experiment (see Table 2). Since the peaks of both sample and reference were not influenced by matrix to the same extent, complete compensation of matrix effects could not occur.

As regards the repeatability of results, this parameter did not depend on the calibration technique used, see confidence intervals plotted in Fig. 4.

To sum up, it has been demonstrated, that in case of relatively broad peaks of coextracts, that matrix effects can be successfully compensated by echo peak calibration. According to observations made by other authors [4,5], sample components responsible for matrix effects are often not present in the chromatogram as narrow distinct peaks, but they tail throughout the whole chromatogram as a result of overloading of the column by these components. In this particular situation, the potential of the echo peak technique for solving a problem with matrix effects is very high.

3.5. Compensation of instability of responses in the analysis of chlormequat in pear concentrate

Instability of responses during large sample sequences is another problem affecting the data accuracy in LC–MS. In the analysis of growth regulator chlormequat in pear concentrate we have encountered a problem with significant decrease of peak areas during analyses of longer sequences. It should be noted, that LC separation of chlormequat requires relatively high concentrations of buffers, regardless of whether the separation is performed on a reversed-phase [13] or cation-exchange column [14]. If, moreover, samples with high content of polar coextracts (e.g. sugars) are injected, the response drop during a sample sequence becomes dramatic due to the successive blocking of a heated transfer capillary.

In our study we attempted to solve the problem with poor response stability by the use of echo-peak calibration technique. We assumed that using this internal standard technique would enable compensation of the signal decrease during the sequence and thus improve data accuracy. As already mentioned in Section 3.2, in a single residue method the implementation of echo peak technique is very simple. This was demonstrated in our study for the analysis of chlormequat, where almost no adaptation of the original analytical method was necessary. Echo-peak analysis was performed on a single separation column Partisil SCX under isocratic conditions (0.1 *M* ammonium acetate–methanol, 60:40, v/v). When optimising the echo-peak separation conditions it was found that a good shape of the peaks of sample and reference was achieved only if the matrix sample was injected prior to reference standard (see Fig. 5), while in reversed injection order the first peak (reference) was distorted and inadequately small. Unfortunately we were not able to fully explain this phenomenon, however it occurred reproducibly in our experiments.

We analysed pear concentrate samples spiked with chlormequat at two known concentration levels, 0.2 and 2 mg/kg (see Experimental). Similarly to experiments discussed in Section 3.4, these samples were treated as unknowns, i.e. they were analysed in the LC sequence, enveloped by two calibration sets, and their concentration was calculated from the calibration curves. The data accuracy using (i) external standard calibration and (ii) echo-peak calibration was compared. Since no matrix suppression/enhancement was observed in preliminary experiments, standards in pure solvent were used for external calibration in experiment (i).

For echo-peak calibration, standard at 0.1 μg/ml was injected as a reference in each analysis. Before each new sequence, the bore of the heated capillary

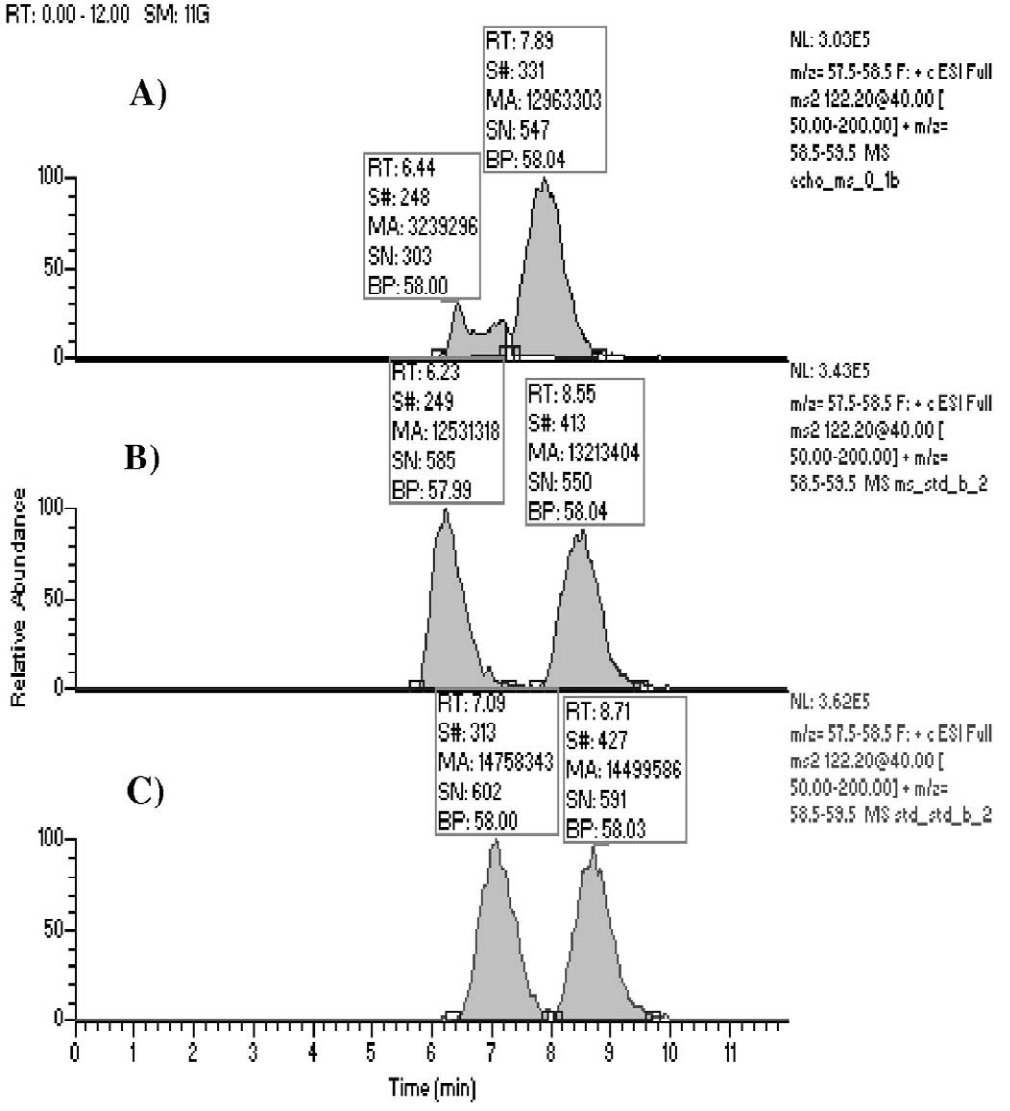

Fig. 5. Chromatograms of chlormequat obtained by echo-peak technique; (A) injection 1, standard (0.1 μg/ml); injection 2, sample of pear concentrate (0.1 μg/ml). (B) Injection 1, sample of pear concentrate (0.1 μg/ml); injection 2, standard (0.1 μg/ml). (C) Injection 1, standard (0.1 μg/ml); injection 2, standard (0.1 μg/ml).

was cleaned with a hypodermic cleaning tube to ensure identical 'starting' conditions. The time period for the eluent to enter the source in one run was set identical in both experiments.

In Fig. 6 the response of a reference peak throughout the whole sequence is shown. It is obvious that a drop in the response of chlormequat is very dramatic. However, the echo peak technique enabled us to monitor the response decrease and to compensate it by using the peak of reference as an internal standard. In Fig. 7 concentrations calculated for samples are shown. When using external calibration, the sample concentrations calculated from the calibration set a were ~50% lower than the correct value, on the other hand, when calculated from the calibration set b cca 60% higher values than correct

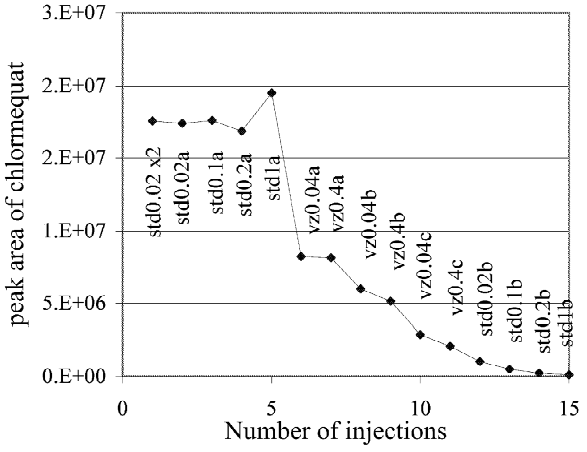

Fig. 6. Peak area of a reference standard (0.1 μg/ml) during 15 injections of solvent standards and pear concentrate samples.

were obtained. Moreover, the results from three triplicate analyses of the same sample showed a remarkable trend, i.e. the result for a particular sample was strongly dependent on its actual position in a sequence. With the use of echo-peak technique the best result was 5% higher and the worst result 35% lower than the correct value for both concentration levels tested. Besides, the data from triplicate injections did not show any dependence on their placement in the analysed sequence. A systematic bias of ~15% giving consistently lower values than a correct value was observed with the echo peak technique.

It should be emphasised that the echo-peak technique cannot solve a problem with worsening of analyte detectability due to response decrease. In any case a cleaning of the source must be performed, when considerable drop affecting the limits of detection of a method occurs. On the other hand, the monitoring of a reference response can be useful, especially where the response stability cannot be predicted (e.g. different types of matrices are analysed).

4. Conclusions

In quantitative LC–MS analysis, the presence of matrix sample components may cause problems due to response suppression/enhancement phenomena or

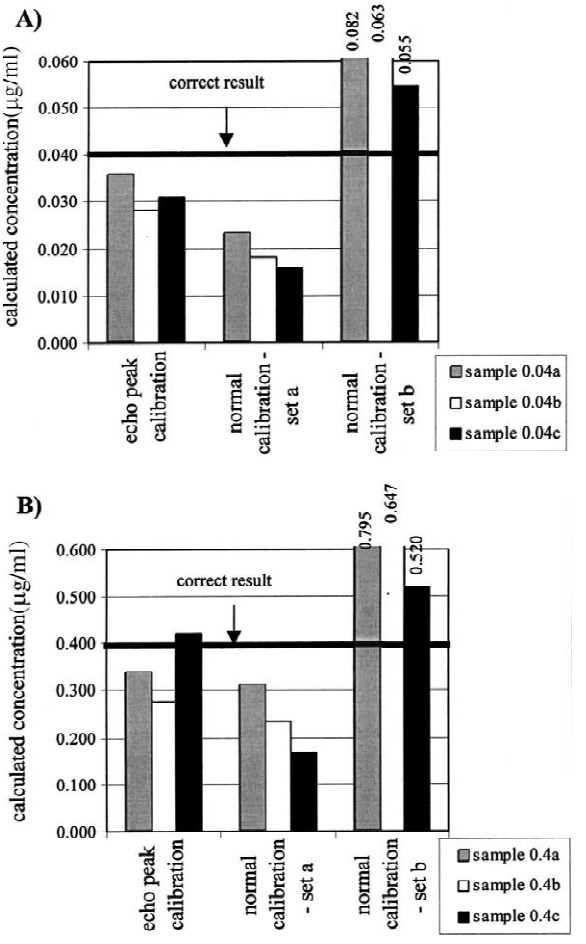

Fig. 7. Accuracy of data obtained by (i) echo peak calibration, (ii) external standard calibration pear concentrate extract spiked with chlormequat. (A) Sample at 0.04 μg/ml; (B) sample at 0.4 μg/ml.

because of detector response instability during larger sample sequences. These problems should be solved by using appropriate calibration method. Matrix-matched calibration showed to be effective in compensation of matrix effects. Another interesting calibration approach, the echo peak technique, simulates the use of internal standard, without the demands for isotopically labelled analogues of target analyte. After optimisation of parameters of echo peak technique for the analysis of multiple pesticides in apple extracts, we were able to compensate for matrix suppression/enhancement phenomena for six out of eight pesticides. Also as an internal standard method the echo peak calibration can be used to

overcome the problem with response instability during large sample sequences, which was demonstrated in the analysis of chlormequat. In this single residue method the implementation of echo peak technique is very easy and does not require significant adaptation of the original analytical method.

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