Application of a truly one-point calibration for pesticide residue control by liquid chromatography–mass spectrometry

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ABSTRACT

This paper presents the development of a simple fit-for-purpose Yes/No method for controlling pesticide residues in food by liquid chromatography tandem mass spectrometry (LC–MS/MS). A true one-point calibration ($y = C$, where C is a constant) was evaluated for its applicability, feasibility and performance in controlling pesticides in fruits. A process analytical technology approach was adopted. One-point calibrations equivalent to the maximum residual level (MRL) of a pesticide in a fruit were performed and used as the process and quality control parameters. The confidence level intervals were determined and used for controlling the pesticide residue levels in real fruit samples. Useful features of the proposed method, from practical point of view, include the easy access to historical data, their simple presentation, the simplicity of introducing new measurement data points, and these features make it an excellent diagnostic and analytical tool. This technique allows any method performance abnormality to be flagged early and reliable information on exceeding MRLs to be obtained quickly. This truly one-point calibration may find applications in any field where regulatory compliance requires that a measurand is shown to be within a particular limit.

1. Introduction

Control of pesticide residues in food and feed remains a challenge for analysts despite the enormous progress in developing new analytical techniques, procedures and equipment. To ensure food and feed safety and protect consumers from exposure to unacceptable levels of pesticide residues, the maximum residual levels (MRLs) allowed in a commodity are set by the authorities. In the EU, Regulation (EC) No. 396/2005 of the European Parliament and Council on Pesticide Residues regulates the quantity of pesticide residues allowed in food and feed. The number of compounds and food commodities that should be monitored is immense. Most modern methods for pesticide residue analysis are designed to determine as many pesticides as possible in a single run. The development and validation of multiresidue methods for each commodity of interest is a strain on laboratories that often prevents smaller laboratories from undertaking efforts to replace or improve their existing methods and manufacturers from starting in-house laboratories. A demand exists for new procedures that both improve and simplify current analytical methods to ensure the

timely and cost effective delivery of high quality data, and research in this field is of great practical importance.

Liquid chromatography–mass spectrometry has become one of the most popular analytical techniques for multiresidue pesticide determination in food. The number of multicomponent liquid chromatography–mass spectrometry (LC–MS) methods for the determination of over 50 pesticides in food has been increasing over the last five years [1–29]. This technique ensures the high precision, robustness, sensitivity and selectivity of the analysis. Quantification of the LC–MS determinations may be accomplished via multiple-point, one-point (through zero), statistically assisted and truly one-point calibrations. While multiple-point, one-point (through zero) and statistically assisted calibrations have been used in many applications, and their pros and cons have been discussed [30,31], no reports on truly one-point calibrations can be found. One-point through zero and truly one-point calibrations are valued for their efficiency with respect to time, workload and resources. Yet the former should not be used for the determinations with nonlinear response functions and when the y-intercept is not negligible, the latter is free of these limitations. Multiple-point calibration is well established, widespread used, and its theoretical backgrounds well understood. However, it is also associated with a high workload. Statistically assisted calibrations may reduce times and costs of the adopted multicomponent and multimatrix analytical method producing overall calibration curves for any analyte accounting for the

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uncertainty due to all the sources of uncertainty but they are also more complex than other calibration procedures. The external standard, internal standard and standard addition methods are used in LC–MS quantifications. Admittedly, the method with the greatest precision, robustness and selectivity is when isotopically labelled compound analogues (preferably using ^{13}C and ^{15}N as ^{2}H analogues have a different hydrophobicity and thus different retention time) are used as internal standards [32]. However, n-component methods require the use of n isotopically labelled analogues. Therefore, the analysis cost and workload increase immensely. For routine multicomponent determinations and pesticide residue analyses in various matrices, the analytical procedure adopted must offer the best compromise between the costs, workload and benefits.

The aim of this study is to develop a simple fit-for-purpose Yes/No method for controlling pesticide residues in food with LC–MS/MS; a truly one-point calibration ($y = C$, where C is constant) is proposed. An example of pesticide residue analysis in fruits is chosen to demonstrate the applicability, feasibility and performance of the method.

2. Experimental

2.1. Materials and samples

Pesticide analytical standards were purchased from Fluka, Sigma–Aldrich (Poznan, Poland). Individual pesticide stock solutions (1 mg mL^{-1}) were prepared in acetonitrile for each compound with the exception of ethirimol, carbendazim and propazine, which were prepared in methanol. Five multicompound working standard solutions for sample spiking were prepared by diluting the stock standard solutions with acetonitrile. All solutions were stored in a refrigerator at 4 °C in the dark. Triphenylphosphate (TPP) was obtained from Fluka, Sigma–Aldrich and used as an internal standard. Methanol and acetonitrile, both of LC–MS grade purity, were purchased from Merck (Warsaw, Poland). All other chemicals (e.g., ammonium formate) used in these analyses were obtained from Sigma–Aldrich (Poznan, Poland). Pre-packed Agilent Technologies QuEChERS (Quick Easy Cheap Effective Rugged Safe) kits for EN Method 15662 were obtained from Perlan Technologies (Warsaw, Poland). Strawberries, raspberries, black currants and red currants were obtained from local producers of frozen fruits and vegetables. All samples were stored in the dark at −20 °C.

2.2. Extraction

Pesticide extraction was performed using the QuEChERS method and carried out according to EN Method 15662. Pesticide-free samples were used as blanks for the validation studies and calibrations. Blank extracts were processed in the same manner as real samples. For the validation studies and matrix-matched calibrations, the samples were spiked with appropriate volumes of the working standard solutions before extraction.

Approximately 1 kg of the fruit samples was chopped and homogenised using a Braun MR 6550 M blender. Ten grams of the homogenised samples was transferred to 50 mL centrifuge tubes, and 20 μL of 1 μg mL^{-1} triphenyl phosphate (TPP) was added. 10 mL of acetonitrile was pipetted into the tubes and the tubes were shaken for 1 min by hand. Next, a buffer–salt mixture was added and the tubes were shaken vigorously for another 1 min by hand. To adjust the pH to 5–5.5, a 5 mol dm^{-3} NaOH solution was added to the acid-rich samples (100 μL for strawberries, 400 μL for raspberries and 800 μL for black currants). The extract was then centrifuged using an Eppendorf centrifuge model 5804 at 3000 rpm for 5 min, and 1 mL of the organic supernatant (upper layer) was transferred to dispersive solid phase extraction (DSPE) tubes containing 150 mg

of MgSO$_4$, 25 of primary secondary amine (PSA) and 2.5 mg of GCB (graphitised carbon black C18); no GCB was present in DSPE tubes used for strawberries. The tubes were manually shaken for 2 min and then centrifuged in an Eppendorf centrifuge model 5415R at 8000 rpm for 5 min. An aliquot of the supernatant (600 μL) was transferred to an autosampler vial for LC–MS/MS analysis.

2.3. LC–MS method

High-performance liquid chromatography (HPLC) was performed using an Agilent Technologies 1290 Infinity series liquid chromatograph equipped with a binary pump (G 4220A), autosampler (G 4226A), thermostat TCC (G 1316C) and DAD detector (G 4212A). The chromatographic separation was performed using an Agilent Zorbax Plus C18 analytical column, 2.1 mm × 100 mm, 1.8 μm particle size. The column temperature was set to 60 °C, and the mobile phase consisted of 5 mM ammonium formate and 0.01% formic acid in deionised water (A) and 5 mM ammonium formate and 0.01% formic acid in methanol (B). The elution gradient was from 6% to 98% B over 15 min with a subsequent 3 min hold at 98% B. A 4 min post run using initial mobile phase composition was performed after each analysis. The flow rate was 0.5 mL min^{-1}, and the injection volume was 5 μL.

The mass spectrometric analyses were performed using an Agilent Technologies 6460 triple quad LC/MS spectrometer equipped with a Jet Stream ion source (G 1958-65138) operating in the positive ion mode and the following operation parameters: gas temperature of 325 °C, gas flow rate of 8 L min^{-1}, nebuliser gas pressure of 35 psi and capillary voltage of 4500 V, nitrogen was used in the ion source and the collision cell. Ion acquisition was accomplished in the dynamic multiple reaction monitoring (DMRM) mode. Agilent Mass Hunter software version B.03.01 was used for data acquisition, instrument control and data analysis. The retention times and DMRM transitions are presented in Supplementary material (Table S1).

2.4. Statistical analysis

Statistical analysis was carried out using Statistica 8.0 (StatSoft Inc.).

2.5. Method validation

The method was validated with respect to the recovery matrix effect, process efficiency, accuracy, precision, selectivity, processed sample stability, standard solution stability and limit of detection as recommended [33].

2.5.1. Selectivity

The selectivity was examined by monitoring the pesticide retention times and two ion transitions for a quantifier and qualifier ion and their ratio. The relative standard deviation (RSD) of the retention times and qualifier/quantifier ratios were used as the acceptance criterion.

2.5.2. Recovery, matrix effect and process efficiencies

The pesticide recovery, matrix effects and process efficiencies were examined by comparing the pesticide MS signals (absolute pesticide peak areas) for three sets of samples: (a) pesticide solutions in acetonitrile, (b) pesticide solutions in blank sample extracts and (c) blank sample extracts spiked with pesticides before extraction (nine replications). These solutions were prepared at concentrations equal to the pesticide maximum residual levels. The recovery, matrix effect and process efficiency results were obtained

by comparing the pesticide peak areas for the samples in sets c and b, b and a, and c and a, respectively.

2.5.3. Accuracy, precision and calibration

Homogenised fruit samples (10 g) were spiked for calibration with an appropriate amount of the pesticide standards to obtain concentrations equal to the pesticide maximum residual levels. The internal standard solution, containing TPP, was then added. Nine replicates, three on each of three different days, were prepared and analysed as described in Sections 2.2 and 2.3. The relative peak areas, i.e., the ratio of the peak areas for the pesticide to the internal standard ($A_P/A_{i.s.}$), were determined. ANOVA was employed to estimate the intra- and inter-day mean squares for the calculation of the within-day precision (repeatability) and total between-day precision (intermediate precision) as well as the calibration confidence intervals [34]. The precision was expressed in terms of imprecision and computed as the relative standard deviation of the measurements. To determine the accuracy, the concentrations were calculated via a five-point linear calibration for the pesticides over a concentration range of 0.5–3 times the MRL ($R^2 > 0.99$). The accuracy was calculated for each pesticide in terms of the percent deviation of the calculated mean concentration from the corresponding theoretical concentration.

2.5.4. Limit of detection

The limit of detection (LOD) was defined as the concentration where a signal-to-noise ratio of three to one for the quantifier and qualifier was obtained, and the peaks could be clearly identified using the identification criteria.

2.5.5. Stability of the standard mixtures and processed samples

The stability of the multicomponent standard solutions and calibrating extracts was tested over 4 weeks by analysing samples in triplicate each week. The RSD of the pesticide peak areas was calculated for the stability assessment.

2.5.6. Measurement uncertainty

The measurement uncertainty was estimated via the top-down approach using data derived during the validation of this method [34].

3. Theoretical considerations

A true one-point calibration was proposed as a simple Yes/No method for controlling pesticide residues in food by LC–MS/MS. The theoretical foundations of this technique are discussed below.

3.1. Concept of a true one-point calibration

A truly one-point calibration can be described by the simple function $y = C$, where y is an analytical signal (e.g., UV/vis absorbance, peak area or peak area ratio) obtained for the analyte and C is a constant. This calibration may be applicable to every field where regulatory compliance requires that a measurand, such as the concentration of a toxic substance, be shown to be within particular limits. Let us assume that C is equal to the analytical signal obtained for a compound concentration equal to the limit. Thus, in the case of compliance with an upper control limit, all samples with concentrations that generate a signal greater than C (above the function line) are non-compliant and those yielding signals less than C (below the function line) are compliant. The opposite applies in the case of compliance with a lower limit. However, one must be aware that such a statement is largely a simplification. First, each analytical result is characterised by a measurement uncertainty and, unless otherwise required, should be given with an expanded uncertainty (U) calculated using the coverage factor $k = 2$ to obtain a
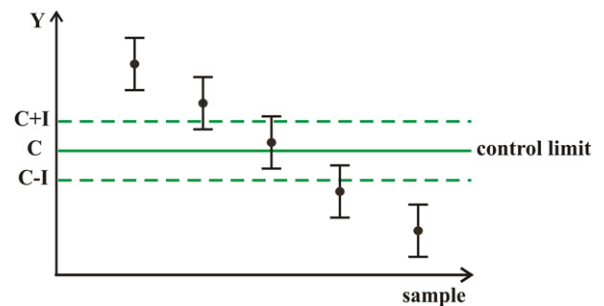

Fig. 1. Truly one point calibration graph.

confidence level of approximately 95%; the results should be given as $x \pm U$ (unit). Second, C is also determined experimentally and thus burdened with a measurement error, which should be stated as the confidence interval (I) obtained from the precision study in the form $C \pm I$ (unit). Therefore, both the uncertainty of the analytical result and the confidence interval of the calibration signal should be taken into account when assessing compliance (Fig. 1). However, depending on the regulation, these uncertainty results may or may not need to be considered. In the case of official food regulations, compliance with the pesticide MRL must assume the lower limit of the uncertainty interval ($x - U$) to be the highest complying analyte concentration for the sample. Therefore, the MRL is exceeded when $x - U > $ MRL, which means that only the samples represented by the first bar in Fig. 1 exceed the MRL limit and demonstrate non-compliance.

The advantages of a truly one-point calibration are its high efficiency with respect to time, workload and resources, especially when the number of samples in one batch is low or when high throughput is required. This method is free of bias and easily and quickly delivers Yes/No results useful for analyte compliance/non-compliance determination. Its simple presentation makes this technique a useful diagnostic tool for method performance because it may easily flag any abnormalities. Therefore, appropriate acceptance criteria should be set, and analyte samples with concentrations equal to the MRL should be used as quality control samples. The easy access to historical data makes adjustment of the confidence intervals of this method possible on a regular basis, and the more data that are available, the more trustworthy the confidence intervals become. A truly one-point calibration also has drawbacks. For example, it does not provide the absolute component concentration but only information on sample compliance or non-compliance with the control limit. Therefore, this technique cannot be used when the control limit is the sum of an analyte and its isomers, different forms or metabolites, e.g., the MRL of dimethoate is the sum of dimethoate and omethoate expressed as dimethoate, the MRL of spinosad is the sum of spinosad A and spinosad D, the MRL of 2,4-D is the sum of 2,4-D, and its esters are expressed as 2,4-D. Similar to multiple-point calibrations, a truly one-point calibration is sensitive to changes in the LC/MS system; for example, cleaning the MS source and capillary, making changes to the solvent and its impurity profile, and tuning the MS will influence the MS signals, and re-calibration will be required.

4. Results and discussion

To develop a simple Yes/No method for pesticide residue control in food by LC–MS/MS, the use of a truly one-point calibration was proposed. The dynamic multiple reaction monitoring (DMRM) method was developed, validated and used for the analysis of 53 pesticides in fruits (raspberries, strawberries, and black and red currants) by LC–MS/MS. A general internal standard (TPP) was

used to minimise any possible analytical variation from the sample preparation.

Retention times and MRM transitions for the examined pesticides are presented along with their MRL values for examined fruits in Supplementary material (Table S1). Method selectivity was accomplished by monitoring both the pesticide retention times and two ion transitions for a quantifier and qualifier ion as well as their ratio. The relative standard deviation (RSD) of the retention time and qualifier/quantifier ratio for each pesticide did not exceed 1% and 8%, respectively, for any matrix. The stability of the multicomponent standard solutions and processed samples (extracts) was tested over 4 weeks. No significant changes in the pesticide concentrations were observed over this period of time (RSD < 1.5%). The instrumental variability, i.e., the variability ascribed to the instrumental precision, was found to be less than 1%.

4.1. Recovery study

Pesticide recovery from four tested fruit matrices was examined. The results of this study are presented in Table 1. Over 98% of the pesticides in red currants and 80% of the pesticides in the other matrices had between 80% and 120% recovery. A few pesticides had recoveries in the range of 120–135%. The thiabendazole recovery was approximately 70%, except for that of strawberries, which had a 90% recovery. The metosulam recoveries were very low (20–30%) in all matrices; however, they were reproducible (RDS < 13%) and the MS signal was strong. Therefore, it was possible to include this pesticide in this study. The relative standard deviations of the recoveries were less than 10% for 95% of the pesticides from all matrices, and less than 5% for 83% of the pesticides from strawberries, 75% from black currants, 66% from red currants and 57% from raspberries. The RSD of recoveries was below 4.50%, 3.87%, 3.61% and 3.94% for half of the pesticides from raspberries, strawberries, black currants and redcurrants, respectively. The recovery of the internal standard (TPP) was 101.4–104.2% (RSD < 6%). The recoveries and their reproducibility indicate that the method performed well.

4.2. Matrix effect

The matrix effects, i.e., ionisation suppression or enhancement, caused by the co-elution of matrix components with the pesticides were studied by comparing the MS signal for pesticides from a matrix to those from a solvent to assess the reliability and selectivity of the developed HPLC–MS/MS method and are shown in Table 1. The ionisation of 42% of the examined pesticides was suppressed in all matrices. An enhancement was observed for 13% of the pesticides in all of the matrices. The signal was either enhanced or suppressed depending on the matrix for 45% of the pesticides. Soft (<20%) and medium (20–50%) matrix effects were observed. Fig. 2 shows the distribution of the matrix effects. None of the examined pesticides was insensitive to the matrix effect, i.e., none of them had negligible matrix effects in all of the matrices studied. Soft matrix effects are dominant and observed in 86% of the cases (89% in raspberries and red currants, 81% in strawberries and 85% in black currants). The majority of pesticides experienced soft suppression. Suppression of pesticide ionisation occurred in more cases than enhancement (65% vs. 34%). The internal standard, TPP, experiences soft suppression in all matrices (7–15.5%). Matrix effects were found highly reproducible between different batches of samples (RSD < 5%).

4.3. Process efficiency

The process efficiencies, also known as the absolute recoveries because they combine both the recovery and matrix effects, were
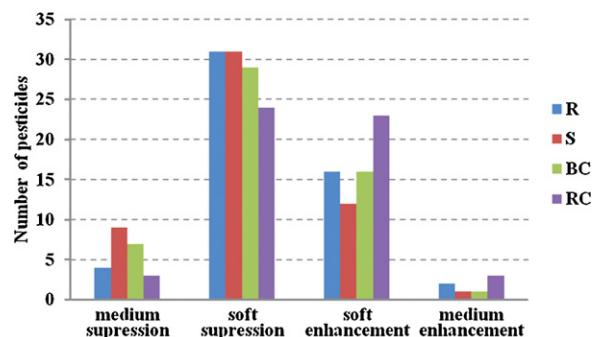
Fig. 2. Distribution of the matrix effects in case of raspberries (R), strawberries (S), black currants (BC) and red currants (RC).

determined and are presented in Table 1. Fig. 3 shows the distribution of the process efficiencies for the four matrices. Over 80% of the pesticides from raspberries and strawberries (83% and 81%, respectively) and over 70% of those from both black and red currents (73% and 79%, respectively) have absolute recoveries in the range of 80–120%. 4% of the pesticides from strawberries and approximately 10% of the pesticides from other matrices had absolute recoveries higher than 120%. Two pesticides from raspberries and four from the other three matrices have process efficiencies less than 70%. Methamidophos and metosulam have low process efficiencies for all four matrices (approximately 50 and 20%, respectively). The low process efficiency of the latter is related to its low recoveries, whereas that of the former is a result of ionisation suppression (approximately 40%) from the matrix effect. Aminocarb has low process efficiencies for all matrices except raspberries (57%, 62% and 69% in strawberries, black currants and red currants, respectively). Similar results are observed for thiabendazole from black and red currants (56% and 58%, respectively) and metamitron from strawberries (68%). These results come from both the low recoveries (approximately 80% and 65%, respectively) and ionisation suppression (in the range of 16–32% and 12–20%, respectively) of aminocarb and thiabendazole, whereas the low absolute recovery of metamitron in strawberries is a result of its medium suppression (27%). The process efficiency for TPP is in the acceptable range, 88–94%.

4.4. Precision, accuracy and limit of detection

The precision of the method was expressed in terms of imprecision and computed as the relative standard deviations of the
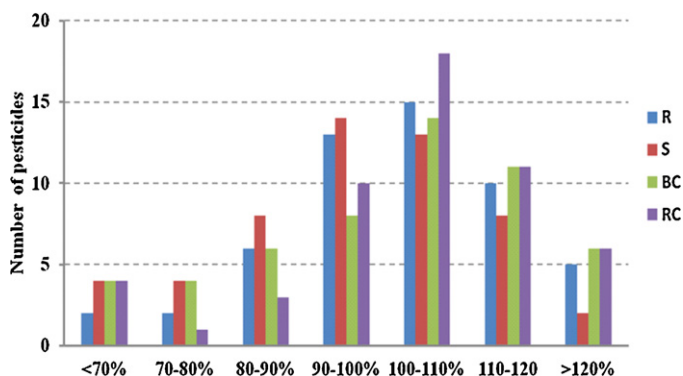
Fig. 3. Distribution of the process efficiencies in case of raspberries (R), strawberries (S), black currants (BC) and red currants (RC).

Table 1
Mean recoveries with percent relative standard deviations in parentheses, matrix effects and process efficiencies for raspberries (R), strawberries (S), black currants (BC) and red currants (RC).

Compound	Recovery (%)								Matrix effect (%)				Process efficiency (%)			
	R		S		BC		RC		R	S	BC	RC	R	S	BC	RC
Acetamiprid	103.47	(2.46)	99.78	(4.55)	98.61	(2.31)	103.59	(2.76)	−3.69	−12.24	−1.83	−3.16	99.65	87.57	96.81	100.32
Alachlor	91.50	(6.28)	97.33	(6.61)	108.33	(4.90)	104.49	(7.02)	26.55	13.92	4.33	10.30	115.80	110.88	113.02	115.25
Aminocarb	88.36	(1.78)	84.39	(9.05)	84.29	(3.48)	82.00	(2.50)	−4.05	−32.76	−26.25	−16.15	84.78	56.75	62.16	68.76
Atrazine	107.97	(4.33)	111.02	(3.86)	111.89	(2.50)	105.72	(2.86)	−14.45	−11.83	−7.78	−0.15	92.38	97.88	103.18	105.56
Bendiocarb	112.91	(9.45)	114.05	(5.27)	111.19	(9.79)	130.34	(11.9)	−20.85	−22.72	−18.33	−22.34	89.37	88.14	90.80	101.23
Bupirimate	110.72	(3.39)	104.00	(4.17)	105.37	(2.19)	105.19	(2.02)	4.55	9.19	4.10	−2.70	115.75	113.56	109.69	102.35
Carbendazim	84.22	(5.28)	86.78	(4.41)	80.92	(1.97)	82.09	(2.50)	−2.34	−2.53	−6.28	−3.86	82.25	84.59	75.84	78.92
Chlorotoluron	129.69	(5.40)	124.97	(4.01)	125.99	(2.26)	102.47	(2.93)	−10.57	−17.18	−2.52	19.00	115.98	103.50	122.82	121.94
Cyanazine	124.02	(6.89)	124.65	(4.25)	128.17	(3.24)	108.33	(3.26)	−0.70	1.71	3.70	21.90	123.16	126.78	132.91	132.05
Cyprodinil	103.36	(5.94)	105.93	(3.81)	97.87	(2.99)	93.93	(3.99)	5.27	8.45	12.57	8.73	108.80	114.88	110.17	102.13
Diazinon	92.85	(4.66)	98.88	(3.93)	105.89	(2.91)	101.57	(3.88)	14.79	3.98	0.98	2.49	106.59	102.82	106.93	104.10
Dichlorvos	105.78	(3.92)	103.97	(5.04)	109.18	(5.71)	116.39	(4.54)	−8.37	−7.27	−17.06	−17.60	96.93	96.41	90.56	95.91
Difenoconazol	106.98	(3.74)	103.10	(2.06)	106.20	(4.36)	98.72	(5.62)	4.38	6.34	7.45	17.94	111.67	109.64	114.11	116.43
Diuron	119.45	(3.94)	118.46	(4.38)	120.33	(5.01)	93.90	(8.18)	−11.90	−4.46	2.49	23.57	105.24	113.17	123.32	116.03
Ethirimol	85.08	(4.40)	89.44	(2.02)	82.49	(3.13)	86.62	(2.85)	−16.93	−15.88	−12.48	−6.68	70.67	75.24	72.20	80.84
Fenazaquin	93.59	(5.14)	99.27	(3.56)	98.87	(4.31)	95.03	(3.06)	−5.60	−7.03	−3.17	−5.26	88.35	92.29	95.73	90.04
Fenhexamid	86.03	(6.06)	88.03	(3.88)	87.82	(4.63)	90.32	(5.29)	27.88	15.09	21.10	26.87	110.02	101.31	106.35	114.59
Fenpropidin	99.28	(4.83)	98.88	(2.79)	103.87	(6.61)	102.83	(5.17)	3.57	−0.15	6.32	1.77	102.82	98.73	110.44	104.64
Fludioxonil	111.00	(3.99)	108.74	(3.78)	109.09	(3.66)	106.79	(3.48)	9.17	−6.05	−0.13	16.67	121.18	102.16	108.94	124.60
Flusilazole	110.67	(3.85)	107.73	(3.25)	105.43	(3.25)	114.26	(2.45)	−14.71	−14.72	−21.00	−21.55	94.39	91.87	83.29	89.64
Hexazinone	124.01	(5.43)	122.94	(3.63)	123.43	(3.37)	102.44	(3.20)	−7.89	−8.38	−5.96	14.23	114.23	112.64	116.07	117.02
Imazalil	98.72	(6.99)	93.41	(3.47)	111.05	(8.54)	100.02	(6.22)	−2.84	−4.01	−19.70	−3.61	95.91	89.66	89.18	96.41
Iprovalicarb	105.69	(4.55)	104.70	(2.06)	107.58	(5.49)	109.40	(4.73)	−2.94	−5.04	−6.93	−7.89	102.59	99.42	100.12	100.77
Isoproturon	129.27	(5.31)	125.53	(3.69)	128.62	(2.99)	103.70	(4.28)	−23.06	−14.05	−9.73	12.82	99.46	107.90	116.10	117.00
Linuron	123.93	(7.00)	124.88	(6.19)	135.88	(6.68)	112.85	(6.14)	−0.73	−4.17	−6.31	11.98	123.02	119.67	127.30	126.38
Malathion	99.20	(3.98)	104.58	(1.91)	104.04	(4.31)	106.96	(5.75)	6.83	−0.97	2.96	−0.85	105.98	103.56	107.13	106.06
Metalaxyl	105.86	(4.41)	100.88	(3.50)	105.92	(5.40)	109.93	(5.02)	−8.28	−11.13	−8.13	−10.49	97.09	89.65	97.31	98.40
Metamitron	86.72	(3.25)	93.29	(4.01)	97.52	(1.81)	96.20	(2.30)	1.71	−26.98	−15.84	−1.39	88.21	68.12	82.07	94.85
Metazachlor	107.67	(4.45)	110.70	(2.16)	112.76	(3.19)	102.21	(2.75)	0.49	−1.56	−1.18	9.67	108.19	108.97	111.42	112.09
Methamidophos	81.76	(2.01)	93.51	(1.95)	86.39	(4.93)	91.81	(2.14)	−33.49	−33.61	−42.64	−39.79	54.37	62.08	49.56	55.28
Metobromuron	134.62	(6.05)	131.30	(4.18)	119.20	(2.40)	104.07	(5.96)	−15.06	−23.96	−0.89	13.73	114.35	99.84	118.15	118.36
Metolachlor	111.31	(3.99)	114.60	(2.47)	116.56	(3.79)	103.30	(3.83)	−1.31	−5.20	−5.45	6.03	109.85	108.64	110.21	109.52
Metosulam	19.67	(13.07)	32.54	(12.52)	25.27	(9.85)	25.78	(6.66)	6.76	3.67	7.19	2.50	21.00	33.74	27.08	26.42
Metoxuron	109.96	(3.76)	108.07	(3.74)	108.01	(3.17)	99.43	(3.33)	−8.62	−10.38	−25.60	0.81	100.49	96.85	80.36	100.24
Metribuzin	96.70	(7.26)	100.18	(3.98)	107.92	(7.03)	112.77	(6.66)	−0.23	−13.27	−29.24	−17.28	96.48	86.89	76.36	93.28
Molinate	107.58	(8.41)	91.84	(7.48)	123.83	(5.51)	115.21	(8.27)	17.12	36.78	0.71	6.90	126.00	125.62	124.71	123.16
Monolinuron	121.79	(4.57)	126.44	(5.45)	126.94	(2.55)	106.69	(2.75)	0.36	−5.53	1.46	19.02	122.23	119.45	128.79	126.99
Oxamyl	98.55	(2.44)	97.67	(2.03)	98.46	(3.87)	104.60	(2.69)	−8.00	−19.57	−19.73	−7.70	90.66	78.55	79.03	96.55
Pencycuron	104.98	(2.79)	102.50	(2.84)	104.26	(3.32)	102.39	(3.26)	−7.33	−6.57	−4.63	−6.38	97.29	95.77	99.43	95.86
Pendimethalin	100.98	(5.15)	110.37	(4.52)	110.04	(4.58)	99.76	(3.59)	9.42	−0.67	1.81	8.38	110.49	109.63	112.03	108.12
Pirimicarb	108.95	(2.98)	101.33	(2.96)	96.55	(2.07)	95.39	(4.23)	−0.99	3.70	9.14	13.17	107.88	105.08	105.38	107.95
Propanil	111.82	(5.36)	103.76	(4.98)	106.29	(4.82)	110.59	(4.02)	−10.11	−6.13	−1.64	−7.87	100.51	97.40	104.55	101.89
Propargite	109.25	(3.68)	105.15	(1.59)	104.05	(3.97)	105.42	(5.45)	−7.06	−5.74	−0.84	−5.09	101.54	99.12	103.18	100.05
Propazine	109.40	(3.30)	105.46	(2.74)	104.65	(2.53)	106.79	(3.76)	−11.32	−28.26	−22.75	−8.52	97.02	75.65	80.84	97.69
Pyraclostrobin	94.81	(5.30)	100.46	(4.12)	109.94	(6.18)	108.46	(6.80)	−12.83	−26.26	−14.03	−17.50	82.65	74.07	94.52	89.48
Pyrimethanil	105.94	(10.33)	105.93	(4.63)	108.79	(5.21)	103.91	(4.80)	12.51	11.71	−6.82	11.06	119.19	118.33	101.37	115.41
Quinalphos	111.13	(3.98)	106.02	(1.87)	106.80	(3.57)	104.89	(5.47)	−10.29	−9.86	−7.69	0.32	99.69	95.57	98.58	105.22
Simazine	122.89	(5.48)	130.33	(5.52)	124.80	(1.63)	99.61	(4.20)	−22.25	−27.62	−30.49	0.52	95.55	94.34	86.75	100.13
Terbuthylazin	130.42	(6.49)	130.28	(4.98)	129.13	(2.77)	111.18	(4.70)	−17.07	−25.55	−19.92	−13.19	108.16	97.00	103.40	96.52
Terbutryn	96.91	(3.18)	100.53	(3.86)	107.42	(3.73)	98.48	(2.93)	9.05	3.97	−3.42	5.38	105.69	104.52	103.74	103.77
Thiabendazole	73.88	(8.54)	89.65	(4.47)	69.38	(4.30)	66.09	(3.59)	−2.44	−10.41	−19.91	−12.07	72.07	80.32	55.56	58.11
Thiacloprid	107.62	(3.13)	102.34	(4.85)	100.14	(1.94)	104.85	(3.43)	7.34	−17.83	3.35	5.05	115.52	84.09	103.50	110.15
TPP	101.35	(3.48)	104.15	(3.39)	104.06	(3.07)	102.32	(5.92)	−6.98	−15.42	−11.24	−10.46	94.27	88.10	92.36	91.61
Trifloxystrobin	110.54	(1.89)	105.61	(1.48)	105.60	(1.96)	103.37	(3.43)	−2.95	3.75	9.64	10.04	107.27	109.57	115.78	113.75

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Table 2

Mean relative peak areas with their 95% confidence intervals, and intermediate precisions (%RSD) for raspberries (R), strawberries (S), black currants (BC) and red currants (RC).

Compound	Relative peak area ($A_P/A_{i.s.}$)								Intermediate precision (%)			
	R[a]		S[a]		BC[a]		RC[a]		R	S	BC	RC
Acetamiprid	0.40	0.01	0.38	0.01	0.40	0.01	0.42	0.01	3.15	2.57	2.12	3.90
Alachlor	0.72	0.02	0.74	0.04	0.72	0.03	0.74	0.04	4.43	6.22	4.65	7.77
Aminocarb	0.58	0.02	0.42	0.04	0.43	0.01	0.48	0.02	3.37	11.72	2.56	6.36
Atrazine	1.64	0.03	1.86	0.05	1.87	0.04	1.93	0.06	2.50	3.17	2.93	3.83
Bendiocarb	0.21	0.01	0.23	0.005	0.22	0.02	0.25	0.02	7.07	2.72	10.80	9.14
Bupirimate	49.07	1.08	51.52	1.87	237.3	3.44	223.7	10.2	2.87	4.73	1.88	5.90
Carbendazim	2.46	0.09	2.70	0.06	2.31	0.04	2.43	0.15	4.90	2.84	2.11	7.97
Chlorotoluron	1.91	0.06	1.82	0.05	2.07	0.05	2.07	0.08	4.27	3.68	3.04	5.07
Cyanazine	0.10	0.004	0.11	0.003	0.11	0.003	0.11	0.004	5.57	3.50	3.85	4.71
Cyprodinil	151.1	6.89	85.41	2.25	78.12	1.41	73.27	5.10	5.92	3.41	2.35	9.04
Diazinon	0.26	0.01	0.27	0.01	0.26	0.003	0.26	0.01	2.78	3.90	1.66	3.39
Dichlorvos	0.04	0.001	0.05	0.001	0.04	0.002	0.04	0.002	2.35	2.34	5.86	6.73
Difenoconazol	10.88	0.17	15.24	0.33	7.56	0.12	7.78	0.15	2.05	2.78	2.08	2.50
Diuron	1.25	0.07	1.43	0.03	1.49	0.10	1.42	0.15	7.27	2.61	8.44	14.03
Ethirimol	1.18	0.03	5.38	0.08	49.26	0.99	55.72	2.28	3.74	1.81	2.61	5.32
Fenazaquin	0.57	0.02	63.84	2.41	0.63	0.01	0.60	0.02	3.93	4.90	2.48	4.48
Fenhexamid	60.46	1.68	29.83	0.99	29.84	0.71	32.44	0.65	3.60	4.33	3.09	2.59
Fenpropidin	2.88	0.08	2.96	0.05	3.16	0.11	3.02	0.07	3.69	2.09	4.67	3.12
Fludioxonil	59.95	0.98	32.46	0.79	33.00	0.45	38.11	0.91	2.13	3.16	1.78	3.10
Flusilazole	0.60	0.01	0.63	0.02	0.54	0.01	0.59	0.03	1.39	3.41	3.15	5.81
Hexazinone	1.09	0.03	1.16	0.02	1.14	0.03	1.16	0.04	3.46	2.22	3.09	4.50
Imazalil	0.58	0.02	0.58	0.02	0.55	0.03	0.60	0.03	4.39	4.14	7.88	6.65
Iprovalicarb	6.85	0.09	7.11	0.10	6.83	0.20	6.94	0.14	1.65	1.79	3.73	2.54
Isoproturon	2.75	0.08	3.20	0.06	3.28	0.10	3.34	0.17	3.59	2.25	3.82	6.76
Linuron	0.37	0.02	0.38	0.02	0.39	0.03	0.39	0.03	5.77	5.48	9.01	10.15
Malathion	0.82	0.01	0.86	0.02	0.85	0.03	0.84	0.01	2.01	2.50	4.78	2.00
Metalaxyl	2.80	0.03	27.71	0.89	2.87	0.12	2.93	0.14	1.42	4.17	5.33	6.34
Metamitron	0.77	0.02	0.64	0.02	0.73	0.01	0.85	0.04	3.23	3.75	2.58	6.31
Metazachlor	11.12	0.21	11.99	0.19	11.69	0.26	11.88	0.46	2.45	2.05	2.89	4.99
Methamidophos	0.10	0.003	0.12	0.005	0.09	0.003	0.11	0.01	4.11	4.97	4.67	7.99
Metobromuron	0.06	0.002	0.06	0.001	0.07	0.002	0.07	0.003	4.15	2.59	3.42	5.34
Metolachlor	4.53	0.09	4.79	0.11	4.64	0.09	4.65	0.15	2.71	3.11	2.65	4.29
Metosulam	0.02	0.002	0.04	0.004	0.03	0.003	0.03	0.002	13.60	14.03	12.09	9.80
Metoxuron	0.37	0.01	0.38	0.004	0.30	0.01	0.38	0.01	2.46	1.34	2.88	4.65
Metribuzin	0.65	0.02	0.63	0.02	0.53	0.03	0.65	0.02	4.64	4.14	7.67	4.41
Molinate	0.05	0.003	0.06	0.003	0.05	0.002	0.05	0.003	8.83	5.44	5.98	7.65
Monolinuron	0.45	0.01	0.47	0.01	0.48	0.01	0.48	0.02	3.81	3.93	2.44	5.14
Oxamyl	0.31	0.01	0.29	0.01	0.27	0.01	0.34	0.01	2.52	4.06	2.95	5.03
Pencycuron	5.08	0.07	5.35	0.10	5.30	0.08	5.16	0.16	1.76	2.33	1.85	4.08
Pendimethalin	0.41	0.01	0.44	0.02	0.43	0.02	0.42	0.02	2.61	6.60	4.58	5.30
Pirimicarb	162.2	2.14	253.6	6.05	80.86	1.23	83.56	1.65	1.72	3.10	1.98	2.57
Propanil	0.67	0.01	0.69	0.03	0.71	0.02	0.70	0.02	2.44	4.72	4.15	3.70
Propargite	0.48	0.01	0.50	0.01	0.50	0.01	0.49	0.01	1.68	2.51	2.62	3.71
Propazine	0.35	0.01	0.29	0.01	0.30	0.01	0.36	0.01	2.55	3.11	2.70	4.61
Pyraclostrobin	68.36	1.41	32.79	0.46	119.71	4.95	114.30	3.59	2.58	1.81	5.37	4.08
Pyrimethanil	61.77	3.89	32.87	0.86	26.85	0.86	30.86	0.82	8.19	3.39	4.15	3.45
Quinalphos	0.83	0.01	0.85	0.02	0.84	0.02	0.90	0.02	1.87	2.34	3.62	3.40
Simazine	0.91	0.03	0.96	0.03	0.84	0.03	0.98	0.02	3.78	4.21	4.56	3.02
Terbuthylazin	3.54	0.12	3.40	0.10	3.46	0.12	3.26	0.13	4.30	3.76	4.53	5.23
Terbutryn	1.43	0.03	1.52	0.04	1.44	0.02	1.45	0.05	2.38	3.62	2.09	4.35
Thiabendazole	2.03	0.13	2.43	0.05	1.60	0.04	1.69	0.12	8.21	2.50	3.21	9.43
Thiacloprid	189.7	3.33	49.27	1.80	57.84	0.86	62.12	1.45	2.28	4.75	1.93	3.62
Trifloxystrobin	2.23	0.04	48.68	1.62	98.05	1.29	97.23	2.73	2.42	4.32	1.71	3.65

[a] The first value is the mean relative peak area, the second is 95% confidence interval.

relative peak area. The expected mean squares were obtained from the mean squares of the ANOVA (grouping by pesticide and matrix) and used for the calculation of the repeatability, between-day and intermediate precision variance. The inter-day precision results are shown in Table 2. The RSD values for both the intra- and inter-day precision have right-handed lognormal distributions. Excellent method precision is observed with 92% of the intra-day and 76% of inter-day RSDs being less than 5%. No significant difference in method precision was observed between the matrices. The intra-day precision median was 1.93%, 1.86%, 2.06% and 2.20% for raspberries, strawberries, black currant and red currant, respectively. The median of the inter-day precision was approximately 3% for all of the matrices except red currant,

for which it was 4.7%. The run-to-run variability (precision) of the method meets the acceptance criteria for an analytical method, which proves the efficacy of the method.

The method accuracy was evaluated in terms of the percent deviation of the calculated mean concentration from the corresponding theoretical concentration. No significant differences in method accuracy were observed for the various matrices, and good method accuracies were obtained, with mean biases of 1.5%, 0.7%, 0.3% and 2.5% for raspberries, strawberries, black and red currants, respectively.

The method detection limits, expressed as the pesticide concentration required to obtain a signal-to-noise ratio of three-to-one for both the quantifier and qualifier, are listed in Supplementary
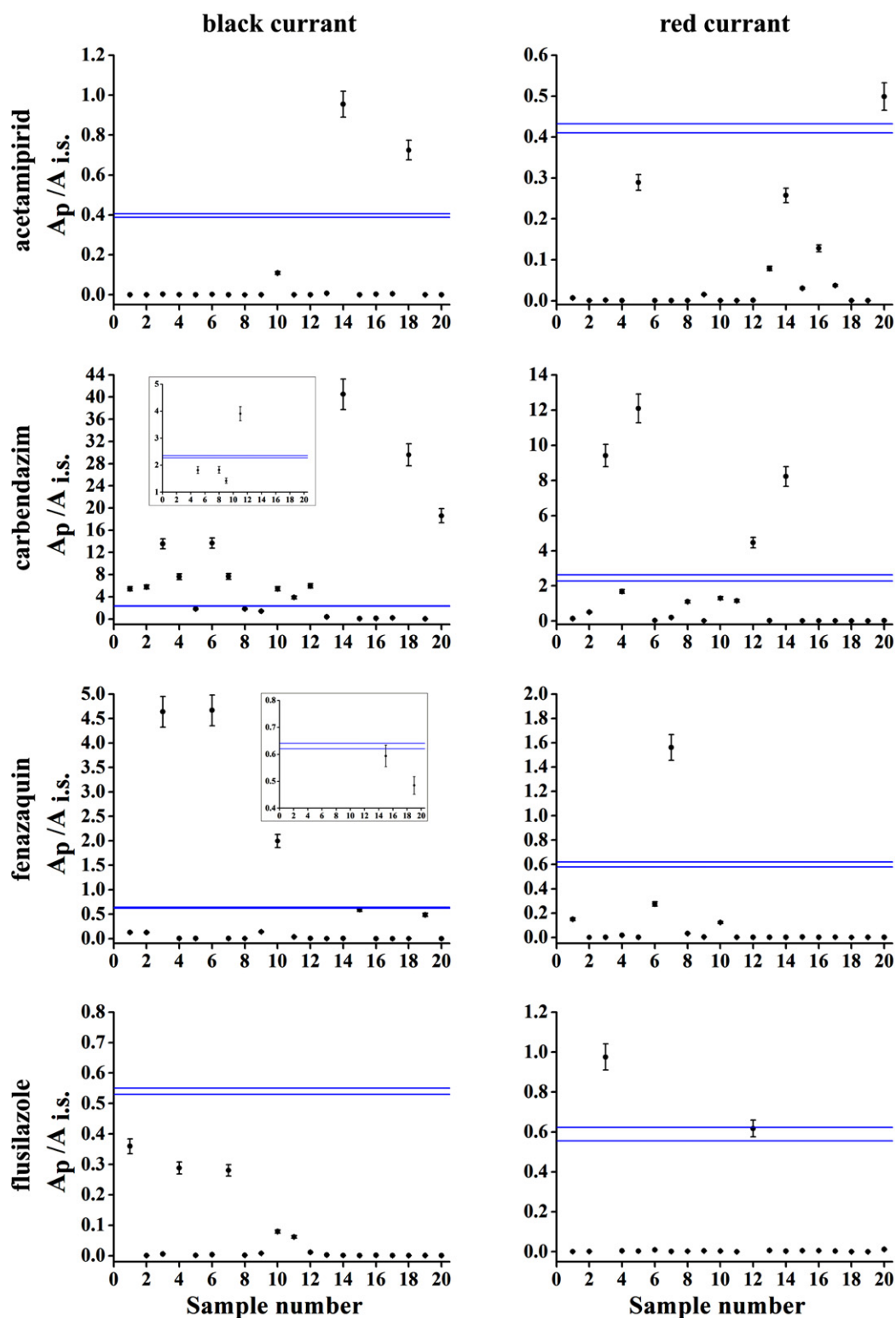
Fig. 4. Truly one-point calibration curves for acetamipirid, carbendazim, fenazaquin and flusilazole from black and red currants with the measurement points of the real samples shown; relative peak area vs. sample number. The insets show the expanded, narrower y-axis range calibration graphs.

material (Table S1). The LOD was in the range of 0.3–22.7 μg kg^{-1}, which indicated good method sensitivity.

4.5. Estimation of the measurement uncertainty

The measurement uncertainty was estimated via the top-down approach using the data collected during the method validation and only considered the analytical process. These data cover sample preparation, standard dilution and both chromatographic and MS detection variabilities. The relative peak area, i.e., the ratio of the pesticide peak area to the internal standard peak area, was the evaluated measurand. The overall run to run variation in the analytical procedure (method precision) was studied for the 53 pesticides in the four matrices over three repetitions on three different days.

Multifactor ANOVA was performed, and the overall variance was calculated as sum of the model error, between-day variance and between-matrix variance. The overall relative standard variation from the intra-laboratory study was found to be 3.38%. Using the overall RSD and a coverage factor $k = 2$, which gives approximately 95% confidence level, the expanded uncertainty of the analytical process was estimated as 6.76%.

4.6. Application to real samples

The results of the validation studies indicated that all of the examined pesticides could be reliably and accurately determined by the developed method. Although in the case of a few pesticides the acceptance criteria were exceeded, the high method repeatability ensured the reliable determination of each pesticide. To prepare truly one-point calibration graphs, the ratios of the pesticide peak areas to the internal standard peak areas were calculated. The mean relative peak areas are shown in Table 2. As the standard deviation of the population is unknown and must be estimated from the samples, the confidence intervals (I) are calculated from the t-distribution. For a population with an unknown mean and standard deviation, the confidence interval, based on a simple random sample of size n, is $I = \pm t \cdot s \cdot n^{-1/2}$; where t is the critical value for the t-distribution with $n-1$ degrees of freedom, and s is the standard deviation of the sample. To calculate the confidence intervals of the calibration graphs, the standard deviations were derived from the intermediate precisions (variances) and Student's t value at 95% confidence for eight degrees of freedom was used. The 95% confidence intervals are listed in Table 2.

To demonstrate the applicability of the truly one-point calibration method, real raspberry, strawberry, black currant and red currant samples were selected from the frozen homogenised fruits available in the laboratory, which were part of the routine pesticide residue analysis performed by the laboratory. Samples that exceeded the pesticide MRLs were intentionally chosen first, and then the sample sets were complemented with the remaining fruits to a total of 20 samples for each matrix. The selected samples were thawed and subjected to both extraction and analysis. Fig. 4 shows the example results, the results of the determination for acetamipirid, carbendazim, fenazaquin and flusilazole from black and red currant. It was found that the acetamipirid concentration exceeded the MRL in two black and one red currant samples. The carbendazim content exceeded the MRL in twelve and four of the black and red currant samples, respectively. The fenazaquin concentration exceeded the MRL in three black and one red currant samples. In one red currant sample, the flusilazole content exceeded its MRL. In the case of raspberries, the acetamipirid content was higher than its MRL in two samples, while flusilazole and propargite each exceeded their MRLs in one sample. Propargite also exceeded its MRL in six black and two red currant samples. All of the pesticides detected from strawberries were below their MRLs. These findings and conclusions, derived using a truly one-point calibration, were consistent with those obtained from determinations based on five-point calibration curves.

5. Conclusions

A truly one-point calibration method, which delivers Yes/No information, was developed, and its applicability to pesticide residue control in fruits was demonstrated. The measurement

uncertainty was only estimated for the analytical process. To determine the uncertainty of the laboratory measurements, the uncertainty associated with sampling procedure also needs to be included in this evaluation. This method may be used as a simple fit-for-purpose tool to determine compliance with regulatory limits.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.06.014.

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