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Validation and use of a QuEChERS-based gas chromatographic–tandem mass spectrometric method for multiresidue pesticide analysis in blackcurrants including studies of matrix effects and estimation of measurement uncertainty

Stanisław Walorczyk*

Institute of Plant Protection-National Research Institute, Władysława Węgorka 20, 60-318 Poznań, Poland

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ABSTRACT

A triple quadrupole GC–QqQ–MS/MS method was optimized for multiresidue analysis of over 180 pesticides in blackcurrants. The samples were prepared by using a modified quick, easy, cheap, effective, rugged and safe (QuEChERS) analytical protocol. To reduce matrix co-extractives in the final extract, the supernatant was cleaned up by dispersive-solid phase extraction (dispersive-SPE) with a mixture of sorbents: primary secondary amine (PSA), octadecyl (C18) and graphitized carbon black (GCB). The validation results demonstrated fitness for purpose of the streamlined method. The overall recoveries at the three spiking levels of 0.01, 0.05 and 0.2 mg kg^{-1} spanned between 70% and 116% (102% on average) with relative standard deviation (RSD) values between 3% and 19% except for chlorothalonil (23%). Response linearity was studied in the range between 0.005 and 0.5 mg kg^{-1}. The matrix effect for each individual compound was evaluated through the study of ratios of the slopes obtained in solvent and blackcurrant matrix. The optimized method provided small matrix effect ($<10\%$) for 77% of the compounds, whereas only for 14%, 5% and 4% compounds, the matrix effect was 10–20%, 20–30% and $>30\%$, respectively. Following the application of "top-down" approach, the expanded measurement uncertainty was estimated as being 21% on average (coverage factor $k=2$, confidence level 95%). If compared with samples of other crops, the analyses of blackcurrants revealed a high percentage of exceedance of the legislative maximum residue levels (MRLs), as well as some instances of the detection of pesticides unapproved on this crop.

1. Introduction

Blackcurrant (*Ribes nigrum* L.) is a species native to central and northern Europe and northern Asia and it is recognized as one of the major edible berries for fresh and processing market. Apart from blackcurrant, the cultivated *Ribes* crops include red and white currants (*Ribes rubrum* L.), as well as gooseberry (*Ribes uva-crispa* L.). Blackcurrant berries are prized for their strong aroma, flavor and high content of various bioactive compounds [1,2]. They contain high levels of ascorbic acid (vitamin C) and provide a rich source of phenolic compounds. Ascorbic acid is an antioxidant and acts as a cofactor for various enzymes in metabolic pathways whereas the phenolic compounds are strong antioxidants and display a diverse range of biological activities [3].

Although blackcurrant is suited for pesticide-free or organic farming cultivation [4], efficient crop protection most often depends on the use of synthetic pesticides [5]. As a crop, the blackcurrant can be affected by several pests and disease problems. The most serious blackcurrant pests are: blackcurrant gall mite (*Cecidophyopsis ribis)*, blackcurrant leaf curling midge (*Dasyneura tetensi* Rübs.), European leafroller (*Archips Rosana*), blackcurrant gall midge (*Resseliella ribis*), blackcurrant leaf midge (*Dasineura tetensi* Rübs.), blackcurrant flower midge (*Dasineura ribis)* two spotted spider mite (*Tetranychus urticae)*, currant shoot borer moth (*Lampronia capitella*), currant clearwing moth (*Synanthedon tipuliformis*), black currant sawfly (*Bacconematus pumilio)* and plant lice (Aphids). The major fungal diseases of blackcurrant include gray mold (caused by the pathogen *Botrytis cenerea* Pers), white pine blister rust (caused by the pathogen *Cronartium ribicola*) and anthracnose (caused by the pathogen *Drepanopeziza ribis*), Mycosphaerella leaf spot (caused by the pathogen *Mycosphaerella ribis*) and powdery mildew (caused by the pathogen *Sphaerotheca morsuvae*). Therefore chemical control of these pests and pathogens that cause the diseases represents a major part of the pest control measures necessary to protect blackcurrant orchards and maintain profitable crop yields [6].

In consequence, monitoring the pesticide residue levels in food commodities is of great interest in order to ensure food safety since the European Union regulates the use of agrochemicals to

* Tel.: +48 61 864 9181; fax: +48 61 867 9180.
E-mail addresses: s_walorczyk@tlen.pl, s.walorczyk@iorpib.poznan.pl

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control pests and pathogens in crops [7]. In general, many approaches were can be applied to carry out this challenge, as demonstrated in several reviews published recently [8–10]. Pesticide residue analysis in crops usually involves extraction, clean-up and chromatographic determination with the possibility of using various detection techniques [11]. Solvent extraction is the most widely employed extraction method in pesticide residue analysis in agricultural produce. It is typically combined with solid phase extraction-based (SPE) cleanup, including the quick, easy, cheap, effective, rugged and safe (QuEChERS) procedure [12]. Among the available sample extraction approaches are: matrix solid phase extraction (MSPD) [13], pressurized liquid extraction (PLE) or accelerated liquid extraction (ASE) [14], supercritical fluid extraction [15], solid-phase micro extraction (SPME) [16] and several other techniques [8,9]. Depending on the nature of matrix and the required level of detection, preconcentration may be needed to maximize the analytical sensitivity [17]. In some other cases, taking advantage of the high sensitivity of new generation instruments, sample is diluted to reduce interfering compounds [18].

For the final determination, capillary gas chromatography–mass spectrometry (GC–MS) is widely used for the detection, identification and quantification of pesticide residues in produce samples. The full scan mode is an inherent feature of all MS detectors and provides identification of all eluted compounds unless covered by the co-extracted matrix [19]. In complex matrix, the selectivity of full scan can be improved by the use of comprehensive two-dimensional gas chromatography (GC × GC) [20] or high resolution mass spectrometry (HRMS) [21]. When using unit resolution mass spectrometry, selected ion monitoring (SIM) is used to enhance the detection due to lower number of scans [22] whereas tandem mass spectrometry (MS/MS) makes identification and quantification even more reliable at the low $\mu g\,kg^{-1}$ concentrations [23]. GC–MS operated in electron impact ionization (EI) is a common analytical tool in the field of pesticide residue analysis but negative chemical ionization (NCI) [24] or atmospheric pressure chemical ionization (APCI) can be also useful for the improvement of sensitivity and selectivity of some pesticides [25]. Some new generation GC–MS instruments are capable of the simultaneous full scan/SIM or full scan/MS/MS data acquisition but the potential of these techniques must be proved in real-world applications in analysis of microcontaminants including pesticides [26].

This work employs a streamlined multiresidue method based on the application of a modified QuEChERS method followed by gas chromatography–tandem mass spectrometry (GC–MS/MS) for the multiresidue analysis of over 180 pesticides in blackurrants. Method validation in terms of recovery, precision, linearity, as well as assessment of matrix effects and estimation of measurement uncertainty is presented. In order to prove fitness for purpose of the validated method, it was applied to analysis of real samples.

To our knowledge, pesticide residues can be detected in blackcurrants more frequently than in other types of agricultural produce [27]. Therefore, it was considered desirable to device an improved analytical procedure in order to achieve low detection of pesticide residues in blackcurrants in a rugged, reliable, inexpensive and uncomplicated way.

2. Material and methods

2.1. Chemicals and reagents

Acetonitrile and acetone (for residue analysis) were purchased from Witko (Łódź, Poland). Toluene (for residue analysis) and formic acid (ACS grade) were purchased from Merck Sp. z o.o. (Warszawa, Poland). Anhydrous magnesium sulfate (reagent grade) and Supel Que Citrate (EN) tubes containing 4 g magnesium sulfate, 1 g sodium chloride, 0.5 g sodium citrate dibasic sesquihydrate, 1 g sodium citrate tribasic dehydrate, and EnviCarb bulk sorbent (120/400 sieved fraction) were purchased from Sigma-Aldrich Sp. z o.o. (Poznań, Poland). Bondesil PSA (40 μm) bulk sorbent was purchased from Perlan Technologies (Warsaw, Poland) and C18 (50 μm) bulk sorbent was purchased from Witko (Łódź, Poland).

2.2. Pesticide analytical standards

All high purity certified pesticide standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Stock solutions of approximately 1000 $\mu g\,mL^{-1}$ concentrations were prepared in acetone, taking into account the purity of the standard when calculating the concentration of each stock solution. A single composite stock solution at a concentration of 4 $\mu g\,mL^{-1}$ was prepared in acetone, and the working standards of 0.005, 0.01, 0.02, 0.05, 0.2, 0.5 and 1.0 $\mu g\,mL^{-1}$ concentrations were prepared by diluting the single composite stock solution with acetone. The single composite mixtures at the appropriate concentrations were used to calibrate the GC–QqQ–MS/MS instrument and to spike the blackcurrant samples in the validation experiments. The matrix-matched standards were obtained by evaporating 1.5 mL of the standards at the appropriate concentrations in acetone and reconstituting the residue after evaporation in toluene extract of blackcurrant containing 1 g sample per mL^{-1} solvent.

2.3. GC–QqQ–MS/MS conditions

The analysis of the pesticides was performed using a Varian CP-3800 gas chromatograph coupled with a Varian 1200 triple quadrupole mass spectrometer (Varian Inc., Middelburg, The Netherlands). The separation was achieved on a DB-5 30 m × 0.25 mm × 0.5 μm capillary column, protected by a deactivated guard column (2 m × 0.53 mm). Helium of 99.9999% purity at a flow rate of 1.2 mL min^{-1} was used as the carrier gas. The column oven temperature was programmed as follows: 80°C held for 3 min, ramped at 30°C min^{-1} –150°C, then ramped at 10°C min^{-1}–300°C and held for 10 min. Injector temperature was programmed from 250°C held for 1 min then increased to 300°C at 200°C min^{-1} and held for 20 min. The injection volume was 5 μL splitless.

The mass spectrometer was operated in electron impact ionization mode (EI, 70 eV). The filament current was 50 μA. Electron multiplier voltage was set at 200 V above the voltage determined by automatic tuning with perfluorotribulylamine (PFTBA). The manifold ion source and transfer line temperatures were 40, 270 and 290°C, respectively. The collision gas for MS/MS experiments was argon of 99.9998% purity, and the pressure in the collision cell was set at 1.7 m Torr. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode. MRM transitions and other acquisition parameters can be found in Table S1 of supplementary data included with this article. For the instrument control, data acquisition and processing, Varian MS Workstation software, version 6.6, was used.

2.4. Sample preparation procedures

A 10 g of homogenized sample was weighted into a polypropylene centrifuge tube (50 mL), 50 μL internal standard solution (TPP at 150 $\mu g\,mL^{-1}$) and 10 mL acetonitrile were added, and the contents were mixed using a Multi Reax vortexing device for 5 min. Hereafter, 0.5 g disodium hydrogencitrate sesquehydrate, 1 g trisodium citrate dihydrate, 4 g anhydrous magnesium sulfate, and 1 g sodium chloride were added, immediately shaken for 1 min, then centrifuged at 4500 rpm for 2.5 min. A 5 mL aliquot of the supernatant was transferred to a polypropylene centrifuge

tube (15 mL) containing 0.5 g anhydrous magnesium sulfate, 0.125 g PSA, 0.250 g C18 and0.0375 g GCB. The contents of the tube were vortexed for 2 min and centrifuged at 4500 rpm for 2.5 min. A 1.5 mL aliquot of the supernatant was transferred into an autosampler vial and acidified with 50 μL of 5% formic acid in acetonitrile (v/v) to stabilize the base-sensitive pesticides. The extract was evaporated to dryness under a gentle stream of nitrogen and reconstituted in 1.5 mL toluene prior to GC–QqQ–MS/MS analysis.

At the stage of optimization of sample preparation method, two other sorbent mixtures for dispersive-SPE cleanup were tested: (i) 0.5 g magnesium sulfate and 0.125 g PSA, and (ii) 0.5 g magnesium sulfate, 0.125 g PSA and 0.250 g C18, per 5 mL aliquot of the acetonitrile extract.

2.5. Method validation

The validation study was carried out using the blackcurrant samples that were previously checked to be free of the pesticides of interest. The recoveries were determined in six repetitions at the three spiking levels: 0.01, 0.05 and 0.2 mg kg^{-1}. The samples were spiked before proceeding with the sample preparation. Average recovery and relative standard deviation (RSD) values per spiking level and the overall value were calculated for each pesticide. The results were assessed for compliance with the European Union guidelines SANCO/12495/2011, according to which the average recovery should be in the range 70–120% with RSD less or equal 20% [28]. The limit of quantification (LOQ) was set at the lowest spiking concentration that has been validated with satisfactory recovery and precision parameters.

The calibration was carried out by internal standard method with reference to TPP which served as the internal standard to all the target analytes. Linearity of calibration curves was studied by GC–QqQ–MS/MS analysis of six calibration solutions at the pesticides concentrations of 0.005, 0.01, 0.02, 0.05, 0.2 and 0.5 μg mL^{-1} ($n=2$), both in pure solvent (toluene) and in blackcurrant extracts. These concentrations corresponded to the pesticide concentrations in real samples in the range between 0.005 and 0.5 mg kg^{-1}. The obtained slopes of the calibration curves were used to evaluate the percentage of matrix effects (%ME) for each analyte, which were determined by comparing solvent and matrix-matched calibration curves in terms of slope ratios: $\%ME = 100\% \times (1 - slope_{toluene}/slope_{blackcurrant})$ [29].

The measurement uncertainty was estimated according to the "top down" approach using the data obtained in the validation study [30,31]. The major uncertainty sources in the uncertainty budget were the repeatability of recoveries from the spiked samples and uncertainty of the average recovery calculated from rectangular distribution, see Eq. (1). Combined uncertainty was calculated following the rules for propagation of uncertainty with the data obtained at the LOQ and the high spiking level, by using Eq. (2). Finally, the relative expanded uncertainty was calculated by using the coverage factor $k=2$ at the confidence level of 95%.

$$u(\%) = \sqrt{RSD^2 + \left(\frac{100 - R}{2\sqrt{3}}\right)^2} \quad (1)$$

$$uc(\%) = \sqrt{{}^{u}LOQ(\%)^2 + u_{0.2}(\%)^2} \quad (2)$$

where $u(\%)$ represents measurement uncertainty, RSD relative standard deviation (%), R recovery (%), $u_C(\%)$ combined measurement uncertainty, $u_{LOQ}(\%)$ measurement uncertainty at the LOQ and $u_{0.2}(\%)$ measurement uncertainty at 0.2 mg kg^{-1}.

3. Results and discussion

3.1. Optimization of GC–QqQ–MS/MS conditions

Optimization of the MS/MS multiple reactions monitoring (MRM) conditions for each analyte involved selecting candidate precursor ions in full scan spectra, running product ion scans with several collision energy voltages (ranged between 5 and 35 V with a step of 5 V), selecting the most promising transitions of precursor ions fragmenting to product ions at optimized collision energies and running them in MRM mode, and finally selecting MRM transitions for each analyte (typically two MRMs per analyte).

Once selected the best MRMs (in terms of required sensitivity and selectivity), a time-scheduled MRM data acquisition method was developed. The distribution of time-windows including MRM transitions was dependent on the distribution of the analytes' retention times throughout a chromatographic run, and is shown schematically in Fig. 1, whereas the specific GC–QqQ–MS/MS acquisition conditions including precursor and product ions, collision energies and retention times are detailed in Supplementary information included with this article (Table S1). With the triple quadrupole instrument used in this work, the dwell time, and thereby sensitivity and repeatability of the response, were dependent on the number of recorded MRM transitions. For this reason, data acquisition involved a rather large number of time-windows (i.e., 31) which was necessary to obtain sufficiently high response. However, it was reported that newer models of triple quadrupoles may maintain these parameters unaffected by the dwell time used during data acquisition [25], as well as allow for automatic set of MRM time-windows [32].

To meet the quantification and identification requirements for regulatory monitoring, the identification criteria included the retention time and two product ions with a proper abundance ratio between the MRM transitions selected for the purpose of quantitation and identification [28]. Two MRM transitions were monitored for all the pesticides with the exception of acephate, benalaxyl, captan, dimethoate, fenhexamid, heptenofos, methidation, tolylfluanid and TPP (I.S.), for which either the second transition was not adequately selective or just one abundant transition was obtained. It must be emphasized that arbitrary criteria must be used with caution to minimize the potential for false negatives and false positives, especially when applied with automatic data evaluation. Preliminary software findings were always assessed by an experienced analyst to avoid false negative and/or false positive findings. However, the compounds commonly found in real samples of blackcurrants could always be identified with required selectivity as recommended by the EU guidelines (see Application to real samples).

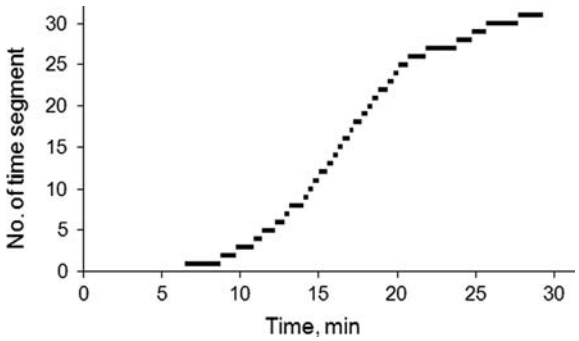

Fig. 1. Schematic of the time-scheduled GC–MS/MS data acquisition method. Detailed GC–MS/MS conditions for the target pesticides can be found in Supplementary information included with this article (Table S1).

3.2. Choice and optimization of the sample preparation method

The QuEChERS technique, entailing solvent extraction (typically acetonitrile), salting out (typically magnesium sulfate), liquid–liquid partitioning, and dispersive-SPE cleanup, in the past several years has become a generic sample preparation technique for a variety of applications in pesticide residue analysis. Its application yields excellent results for a wide range of compounds, at the same time being less expensive and less complicated than previously used methods, and such improving laboratory efficiency and throughput [33]. A significant advantage of the QuEChERS is its susceptibility to accommodate modifications depending on the analyte properties, matrix composition, laboratory resources and required analytical performance [34]. Published articles deal with modifications including: addition of hexane [35], addition of chloroform [36], addition of dry ice [37], application of freezing out cleanup [31], application of column SPE instead of dispersive-SPE [38] and application of different dispersive-SPE sorbents, e.g., circonium-based Z-sep and Z-sep Plus [39].

Proper choice of sorbent(s) is critical to remove matrix interferences, while assuring consistently good recoveries of the target analytes. Therefore, for the choice of sorbent(s), matrix composition and target analytes properties must be considered. Primary and secondary amine sorbent (PSA) is the base sorbent used for the QuEChERS cleanup of fruit and vegetable extracts because it removes organic acids and sugars that might adversely affect chromatographic performance. In addition, C18 may be used to remove lipids but it also can help the dispersive-SPE performance [33–40]. Whereas graphitized carbon black (GCB) may be very helpful in cleaning up pigmented matrices [33].

Various sorbents mixtures were evaluated for the efficiency of cleanup, then blackcurrant extracts were run in GC–MS full scan mode (*m/z* 95–600) to compare the background remaining after cleanup. These Total Ion Chromatograms (TICs) indicated that mixed sorbents (PSA+C18+GCB) provided better cleanup in the form of reduced background and lower level of co-extracted interferences visible on the TIC chromatogram whereas more matrix remained behind after the cleanup by PSA alone (Fig. 2). More efficient removal of matrix co-extractives that may obscure the target analytes will translate to greater sensitivity, improved peak integration and mass spectral matches. In Fig. 3, the sum of areas of chromatographic peaks in TIC chromatograms, as normalized to that obtained after the PSA cleanup, is graphically presented showing better removal of matrix co-extractives by the application of mixed sorbents. Therefore, this method was subjected to validation study in terms of evaluation of linearity and matrix effects, recovery, precision, as well as estimation of measurement uncertainty.

3.3. Linearity and matrix effects

Linearity was assessed by studying six-level calibration curves constructed of a set of pesticide standards prepared in solvent (toluene) as well as in blackcurrant extracts (matrix-matched), over a concentration range of 0.005–0.5 mg kg^{-1}. The response was characterized by highly satisfactory linearity with coefficients of determination (R^2) ≥ 0.99 for all the tested analytes with the exception of captan, cyhalothrin-lambda, desmedipham, flonicamid, omethoate and phenmedipham in toluene, and captan, cyhalothrin-lambda, heptachlor exo-epoxide, imazalil and thiabendazole in the extracts of blackcurrants. For some pesticides, the linearity was evaluated over a narrower concentration range as a result of their poorer response (higher level of detection), e.g., buprofezin from 0.01 to 0.5 mg kg^{-1}, azinphos-methyl from 0.02 to 0.5 mg kg^{-1} and captan from 0.05 to 0.5 mg kg^{-1}. All the R^2 values were >0.97 and >0.96 when studied in solvent and in matrix, respectively.

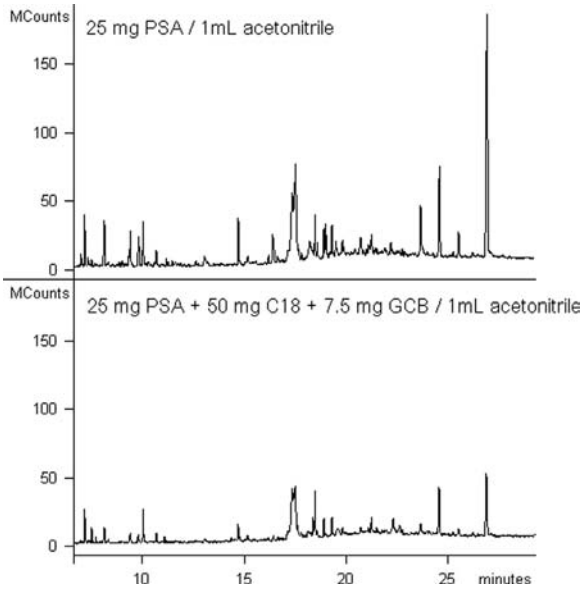

Fig. 2. GC–MS total ion chromatograms (TICs) of blackcurrant extracts after cleanup using PSA (25 mg per 1 mL of acetonitrile extract) and PSA+C18+GCB (25 mg+50 mg+7.5 mg per 1 mL of acetonitrile extract), *m/z* 95–600.

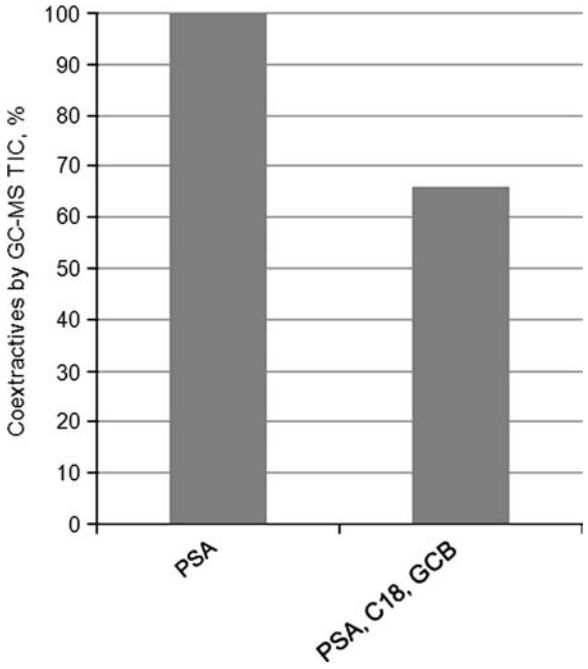

Fig. 3. Relative amounts of co-extractives from blackcurrants as determined by GC–MS total ion chromatograms (TICs) after cleanup by PSA (25 mg per 1 mL of acetonitrile extract) and PSA+C18+GCB (25 mg+50 mg+7.5 mg per 1 mL of acetonitrile extract). The total peaks area obtained when using dispersive-SPE with PSA gave 100%.

The linearity data are detailed in Supplementary information included with this article (Table S2).

The linearity data were used to assess the percentage of matrix effect (%ME), which was calculated as the difference between the slope of the matrix-matched and solvent-only calibration curves divided by the slope of solvent-only calibration curve. Calculated %MEs for the studied compounds are listed in Table S2, Supplementary information as well as their absolute values are graphically presented in Fig. 4. The %ME values were in the range between −75% (thiabendazol) and 31% (pyraclostrobin), of which

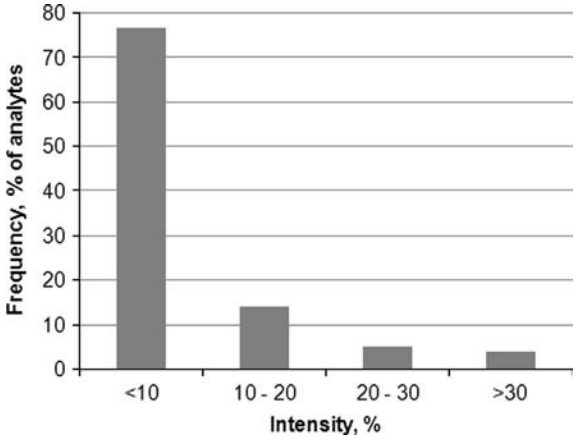

Fig. 4. Distribution of the occurrence of matrix effects.

62%, 22% and 16% compounds showed matrix effects <0, >0 and equal 0 (as rounded). It is noteworthy to mention that matrix effects for the vast majority of the pesticides (91%) were small showing suppression or enhancement in the range 0–20%. %ME in the range between -20% and 20% can be considered as insignificant because such variability is close to the repeatability RSD values. Compared with previously published data, relatively few pesticides showed strong matrix effects [40]. This may be explained by a more efficient reduction of the co-extracted matrix components as indicated by the high occurrence of rather small suppression effect. Because this is not typical for the GC where it is more common to observe enhancement effect resulting from blocking of the column active sites by matrix components [41]. Hence, calibration standards in solvent can be used for quantification of pesticide residue results without the need for matrix-matching. Nevertheless, when the MRL concentration of a pesticide in a sample is approached or an unapproved pesticide detected, matrix-matching may be beneficial for more accurate quantification.

3.4. Recovery study

The recovery experiments with the three spiking levels of 0.01, 0.05 and 0.2 mg kg^{-1} were used to validate the final method using mixed sorbents in dispersive-SPE cleanup (PSA, C18 and GCB), and each level was tested in six replicates. At the lowest spiking level of 0.01 mg kg^{-1}, 95% of the analytes satisfied the EU criteria of SANCO/12495/2011 (i.e., showed the recoveries in the range 70–120% with RSDs <20%) [28]. The recoveries of the validated compounds ranged from 72 to 120% and RSDs from 1 to 20%. At the intermediate spiking level of 0.05 mg kg^{-1}, except for captan, dimethoate and fenpiroximate, all the analytes were recovered showing the recoveries ranging from 68 to 120% with RSDs from 2 to 20%. The recovery of 68% (slightly below the lower limit of 70%) was obtained for both chlorothalonil and dichlofluanid but these compounds, as well as tolylfluanid and captan, are well known for being problematic in the most of the pesticide multiresidue methods [31–33,42]. Whereas at the highest spiking level of 0.2 mg kg^{-1}, all the analytes were recovered showing the recoveries ranging from 68 (isoxaflutole) to 123% (carbofuran) with RSDs from 1 to 21%. The overall recoveries at the three spiking levels were in the range between 70% and 116% (102% on average) with relative standard deviation (RSD) values between 3% and 19% except for chlorothalonil (23%), thus indicating good accuracy (recovery and precision) of the method. The performance characteristics obtained from the validation study are detailed in Table S3, Supplementary information.

To determine the limit of quantification (LOQ) for each analyte, we followed the fitness for purpose approach. Hence, the LOQ was defined as the minimum concentration that has been demonstrated to be accurately quantified by the method, and practically it was the lowest spiking level at which the validation criteria were satisfied with the average recovery 70–120% and RSD <20%. Nearly all the analytes were able to be quantified and identified at 0.01 mg kg^{-1}, except for nine and three pesticides which were able to be quantified and identified at 0.05 mg kg^{-1} and 0.2 mg kg^{-1}, respectively (Table S3) Supplementary information. For the three pesticides (captan, dimethoate and fenpiroximate) that failed validation at 0.05 mg kg^{-1}, the LOQs were established as the lowest calibrated level through injection of matrix-matched standards. For these three pesticides, the concentration of 0.1 mg kg^{-1} yielded the signal-to-noise apparently higher than 10 (S/N >10) and it was accepted as the practical LOQ.

3.5. Measurement uncertainty

The "top down" approach that comprehensively took into account the uncertainties due to precision, recoveries, matrix effects, and concentration variability was applied to the data resulting from the validation study [30]. Although, precision was identified as the main contribution to the uncertainty, the uncertainty associated with the recovery was also included in the uncertainty budget of the method to avoid underestimation of the total uncertainty. Combined standard uncertainty was calculated according to the law of uncertainty propagation, see Eqs. 1 and 2. This combined standard uncertainty basically covered uncertainties arising from RSD (intermediate precision) and recovery (trueness) of the method including matrix effects.

Table 1
Summary of pesticide residues detected in the analyzed samples of blackcurrants ($n=136$).

Pesticide	Range, mg kg^{-1}	MRL, mg kg^{-1}	Frequency,	
			% total samplesc	Samples > MRL[a]
Difenoconazole	0.010–0.189	0.2	33.1	–
Cypermethrin	0.010–0.124	0.05	22.1	1.5
Bifenthrin	0.010–0.173	0.5	19.1	–
Propargite	0.010–0.995	0.01	14.0	12.5
Fenazaquin	0.013–0.327	0.01	11.0	9.6
Boscalid	0.010–2.04	10	8.8	
Cyhalothrin-lambda	0.018–0.136	0.2	7.4	–
Pyraclostrobin	0.019–0.824	3	6.6	–
Chlorpyrifos	0.010–0.070	1	5.9	–
Flusilazole	0.012–0.032	0.02	5.1	–
Pirimicarb	0.010–0.267	1	4.4	–
Bupirimate	0.026–0.255	5	3.7	–
Endosulfan[b]	0.044–0.598	0.05	2.9	2.2
Pyrimethanil	0.013–0.031	5	2.2	–
Deltamethrin	0.010–0.015	0.5	1.5	–
Esfenvalerate[c]	0.046–0.048	0.02	1.5	1.5
Cyprodinil	0.188	5	0.78	–
Diclorvos	0.011	0.01	0.7	–
Propiconazole	0.052	0.05	0.7	–
Symazine	0.065	0.01	0.7	0.7
Tebuconazole	0.022	2	0.7	–
Triadimenol	0.012	1	0.7	–

[a] – uncertainty of 50% was taken into account [28].
[b] – sum of alpha- and beta-isomers, and endosulfan-sulfate expressed as endosulfan.
[c] – sum of fenvalerate and esfenvalerate.

Finally, the expanded measurement uncertainty was obtained by multiplying the combined standard uncertainty by the accepted coverage factor (k) of 2 which corresponded to the 95% confidence level. The results are listed in Table S3, Supplementary information. As seen, the pesticides had uncertainties between 7% (cyprodinil) and 53% (folpet) with the overall average uncertainty of 21%. The majority of the compounds had uncertainties $<30\%$. The highest uncertainty value of 53% was obtained for folpet in consequence of poor recovery and high RSD of this problematic (base-sensitive) compound.

The designed scheme appears to be practical for estimating the measurement uncertainty based on recovery and precision data resulting from the validation study. It can be concluded that the method selected for sample preparation and chromatographic analysis is efficient and suitable for the determination of the target pesticides in blackcurrant samples.

3.6. Application to real samples

In order to demonstrate the applicability of the validated method and its fitness for the purpose of routine pesticide residue analysis in blackcurrant, it was applied to analysis of 136 real samples. The results obtained by GC–MS/MS are summarized in Table 1. In the 100 samples (74%), the residues of at least one pesticide were found. But most of the positive samples, contained two or more pesticides (up to five in one samples) and a total of 22 different pesticide residues were detected in the samples of blackcurrants. In terms of co-occurrence of pesticide residues, 72 samples (53% of the total samples) contained more than one residue, 32 samples (24%) contained more than two pesticide residues, 11 samples (8%) contained more than three pesticide residues and 4 samples (3%) contained five pesticide residues.

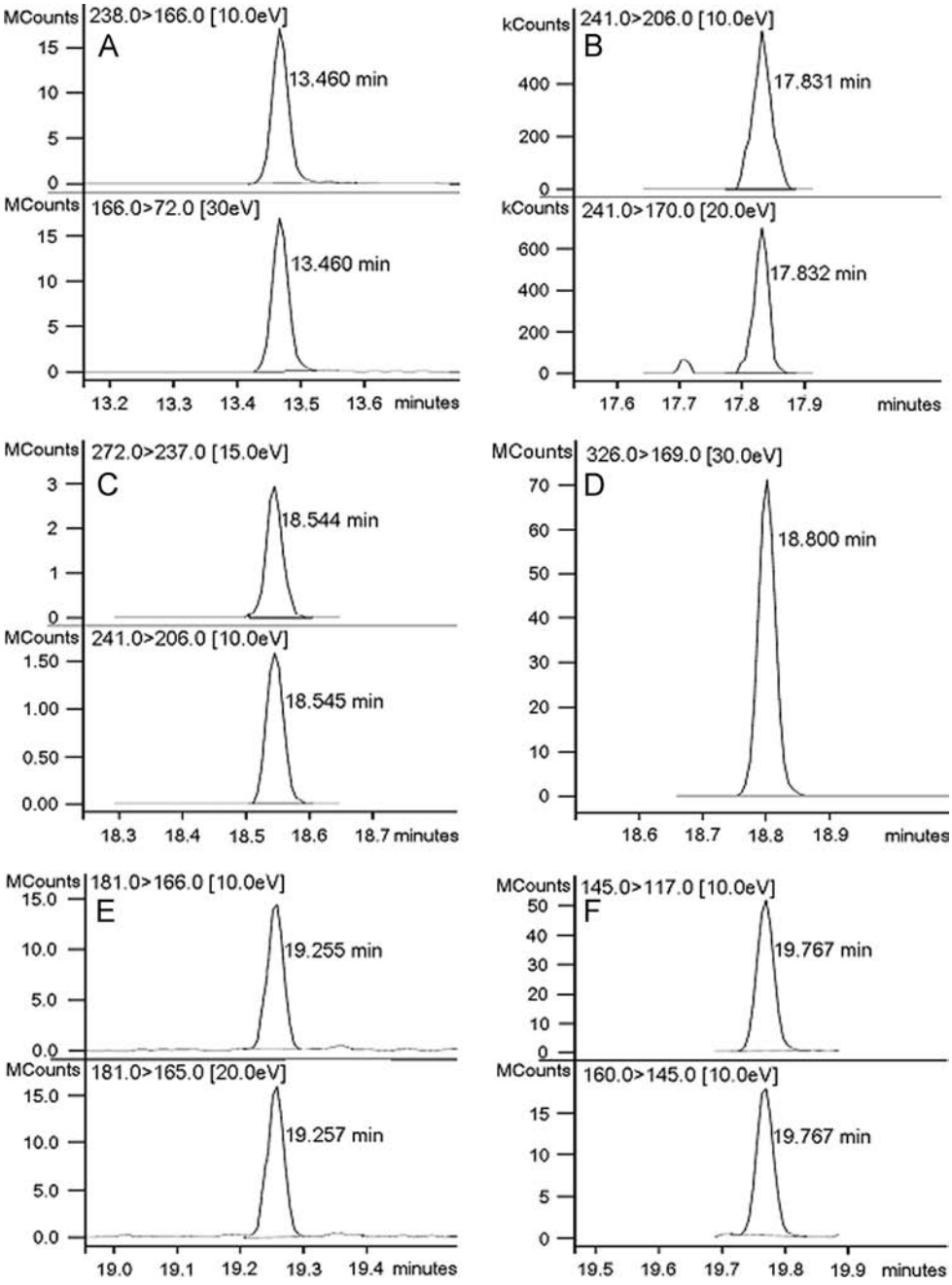

Fig. 5. GC–MS/MS MRM chromatograms of a sample of blackcurrants containing residues of multiple pesticides: (A) pirimicarb (0.153 mg kg^{-1}), (B) endosulfan-beta (0.045 mg kg^{-1}), (C) endosulfan-sulfate (0.065 mg kg^{-1}), (D) TPP I.S., (E) bifenthrin (0.031 mg kg^{-1}), and fenazaquin (0.054 mg kg^{-1}).

Blackcurrant is highly susceptible to pests and pathogens and needs numerous applications of pesticides, leaving in consequence more residues than occur in less intensively treated plants. The most serious blackcurrant pests include blackcurrant gall mite, two spotted spider mite, European leafroller, sawfly, clearwing moths, midges and plant lice, whereas the major fungal diseases include anthracnose, leaf spot, powdery mildew, white pine blister rust and gray mold. Therefore, the combination of insecticide and fungicide residues in one sample was frequent. As an example, Fig. 5 shows GC–MS/MS MRM chromatograms of a sample of blackcurrants containing residues of multiple pesticides: pirimicarb (0.153 mg kg^{-1}), endosulfan-beta (0.045 mg kg^{-1}), endosulfan-sulfate (0.065 mg kg^{-1}), (E) bifenthrin (0.031 mg kg^{-1}) and fenazaquin (0.054 mg kg^{-1}).

In 33 samples (24%), the concentration of at least one pesticide exceeded the statutory maximum residue levels set by the Regulation (EC) No. 396/2005 [43]. The pesticides exceeding the MRL were: propargite, fenazaquin, endosulfan, cypermethrin, esfenvalerate and simazine (in the order of frequency of detection). This was evaluated by taking into account the default expanded uncertainty value of 50% as recommended by the EU guidelines SANCO/12495/2011. This effectively means that the measured concentration in a sample is above the MRL with a confidence level of 95% (i.e., result – uncertainty > MRL) [28]. However, for the purpose of trade, measurement uncertainty is typically not employed in determining if an MRL exceedance has occurred. In that case, 45 samples (33%) did not satisfy the requirements for international trade.

Some of the blackcurrant samples contained residues of pesticides which were not approved for use on blackcurrant or crops in general, or were not registered for the use in Poland (bifenthrin). According to the current legislation four of the detected pesticides are no longer approved in the EU according to the Regulation (EC) No. 1107/2009 (dichlorvos, endosulfan, propargite and simazine) [44]. Dichlorvos was a biocide compound used against crawling and flying insects and its occurrence was probably due to storage contamination. A more complicated situation was found for esfenvalerate and fenvalerate, of which only esfenvalerate is approved for the use on crops. But as being stereoisomers, these compounds are difficult to be distinguished by GC analysis (typically two peaks are always present on chromatograms). Nevertheless, the MRL is set for the sum of esfenvalerate and fenvalerate and for the two samples of blackcurrants, the MRL of 0.02 mg kg^{-1} was apparently exceeded (Table 1).

In must be emphasized that the MRL values represents the maximum concentration of the pesticide residue which is legally permitted in food commodities. They have been set by the public authorities to safeguard consumers' health and promote good agricultural practices in the use of pesticides [45]. Compliance with the MRLs is an essential prerequisite of international trade with food and agricultural products [46]. In this work, the analysis of blackcurrant real samples revealed a higher rate of MRL exceedances than is typically observed for other crops, as well as more incidents of the detection of unapproved pesticides.

4. Conclusions

A method for multiresidue pesticide analysis in blackcurrants (over 180 target pesticides) was streamlined to improve performance characteristics in terms of recovery and precision, which was achieved through reduction of the amount of co-extractives and selective and reliable identification and quantification by triple quadrupole GC–MS/MS. A notable advantage of the proposed approach is that application of mixed sorbents cleanup (PSA, C18, GCB) in a modified QuEChERS method has led to high occurrence of negligible matrix effects (−20 to 20%), i.e., for over 90% of the compounds.

Analyses of real samples were carried out and revealed a high frequency of the pesticide residues presence above their legislative MRLs, as well as the presence of pesticides unapproved for the use on blackcurrants. Thus, the proposed method helped to cover some of the most important needs in the area of pesticide residue analysis in blackcurrants, and assess the state of MRL exceedances in order to separate the samples which are not suitable for the international trade from those being compliant with the acceptance criteria.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.11.087.

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