

Modification of Multiresidue QuEChERS Protocol to Minimize Matrix Effect and Improve Recoveries for Determination of Pesticide Residues in Dried Herbs Followed by GC-MS/MS

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Abstract An accurate, rapid, and reliable multiresidue QuEChERS method based on gas chromatography coupled with tandem mass spectrometry was developed for the determination of 235 pesticides in challenging, dry, complex herb matrices (*Centaurea cyanus* L., *Matricaria chamomilla* L., *Thymus vulgaris* L.). Sample mass and the type of cleanup sorbent used to estimate the procedure's effectiveness were optimized. Purification steps with ChloroFiltr, ENVI-Carb, GCB, octadecyl, PSA, and Z-Sep as cleanup sorbents and a step without purification were compared. To minimize the matrix effect and obtain acceptable recoveries for pesticides, 2 g of herb sample and 10 mL acetonitrile, followed by d-SPE cleanup step with a combination of 150 mg PSA/45 mg ENVI-Carb/900 mg MgSO₄, and additionally 50 µL of 5% formic acid and some droplets of dodecane, were needed. Matrix effects for the vast majority of pesticides were reduced (>20%), showing suppression or enhancement. Most recoveries were in the range of 70–120% (RSD < 18%), reaching the quantification limit of 0.001 to 0.002 mg kg⁻¹. There was excellent linearity within the range from 0.001 to 2.00 µg mL⁻¹, and a correlation coefficient higher than 0.999 was obtained. Expanded measurement uncertainty was estimated to be between 4 and 43%. Finally, the developed method was successfully employed

to identify and quantify pesticide residues in the analysis of 46 real herb samples.

Keywords Herbs · Multipesticide residues · Matrix effect · Gas chromatography

Introduction

Nowadays, herbs are used in several applications around the world, such as in pharmaceutical products, in food and cosmetic additives, as herbal tea, and in folk medicinal uses. Herbs may contain anthocyanidins, flavonoids, vitamins, and mineral salts (including manganese content) as well as substances which exhibit antioxidant effects. They have many health benefits, such as anti-inflammatory and cholesterol-lowering properties and antispasmodic and anxiolytic activities, and they improve appetite and digestion (Lozano et al. 2012).

Like other plants, herbs are sensitive to fungal diseases (e.g., gray mold), and they are attacked by harmful pests (e.g., aphids) and unwanted weeds. These factors diminish crop yields and its quality; consequently, chemical plant protection is indispensable (Matyjaszczyk 2011). However, pesticide residues may pose potential health risks. Moreover, they are accumulated at different stages of cultivation and during post-harvest storage (Hajou et al. 2004).

In order to ensure the safety of herbal products, depending on the intended purpose of the herbs (pharmaceutical products, food), maximum residue limits (MRLs) have been standardized by several international organizations, such as the European Union (EU) (EC Regulation No 396 2005), the European Pharmacopoeia/Pharmacopoeia Commission (EP 8.0 European Directorate for the Quality of Medicines and HealthCare 2014), and the Codex Alimentarius Commission (CA 2016). According to the European Food Safety Authority

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(EFSA) report (EFSA 2015), 65.1% of the analyzed herbs contained residues of which 9.4% exceeded MRLs established by the EU. This clearly shows that monitoring of pesticide residues in herbs is a very important issue.

Herbs, especially dry herbs, represent a challenge due to the high amount of matrix co-extractives which pose critical analytical problems. Herbs contain a broad spectrum of essential oils, flavonoids, phenolics, lipids, natural pigments, and sugars (Lozano et al. 2012; Ghani 2014). Co-extracted matrix ingredients exhibit both partitioning behavior and chromatographic characteristics similar to some pesticides, which may bring about serious matrix effects (ME) and significant interference in mass spectrometry analysis. According to European guidelines (SANTE 2015), the matrix effect is the influence of one or more undetected components in samples on the measurement of an analyte's concentration or mass. ME is one of the greatest difficulties in pesticide residue analysis, particularly in the analysis of complex dry samples.

Some methods to eliminate matrix effects are described in the literature, such as sample preparation, dilution of the extract samples, analytical protectants, addition of internal standards, use of different gas chromatography (GC) injection techniques, GC priming, and the matrix-matched standards method (Zrostlíková et al. 2001; Wang et al. 2011; Li et al. 2015; Liu et al. 2016; Nanita et al. 2017). The most common, relatively cheap, and simple method to compensate for matrix effects in gas chromatography is to use matrix-matched calibration standards (Kwon et al. 2012; Lehotay et al. 2010). Also, SANTE (2015) guidelines suggest matrix-matched calibration for pesticide residue analyses as a method of accounting for the matrix effect. Van de Steene et al. (2006) pointed out that due to variable matrix effect from sample to sample, a representative matrix should be used to prepare the calibration solutions. Unfortunately, it has been proved that finding a representative matrix may be very complicated or even impossible.

One of the methods of minimizing the matrix effect, mentioned above, is sample preparation which is required to isolate and quantify pesticide residues in troublesome matrices. Sample preparation is prone to errors and consumes about 80% of the analysis time (Gondo et al. 2016). On the one hand, recoveries for most pesticides need to be in the range of 70–120% (SANTE 2015). On the other hand, the amount of co-extractives needs to be as little as possible to minimize matrix effects and related instrumental problems.

Sample preparation for the analysis of pesticide residues in herbs commonly involves Soxhlet extraction (Rao et al. 2011), microwave-assisted extraction (MAE) (Wan et al. 2010), matrix solid-phase dispersion (MSPD) (Łozowicka et al. 2013), solid-phase microextraction (SPME) (Hwang and Lee 2000), ultrasonic extraction (Tong et al. 2014), and so on (Table 1). Some research works have reported pesticide residues in herbal material but were mainly based on surveying and monitoring market samples (Rao et al. 2011).

However, these methods are expensive, laborious, and time-consuming and require multiple steps, resulting in loss of analytes and errors in analysis. The need for a robust, inexpensive, and straightforward method that would provide high-quality results with a minimal number of steps and reduced reagent consumption for an extensive range of analytes and matrices at low detection limits led Anastassiades et al. (2003a, b) to develop the QuEChERS (quick, easy, cheap, effective, rugged, safe) sample preparation technique which provides a method fitting the aforementioned criteria.

A few papers related to pesticide detection in herbs based on QuEChERS methodology have been published (Table 1). Nguyen et al. (2010) developed the QuEChERS method for 234 pesticides in three kinds of Korean herbs using gas chromatography mass spectrometry (GC-MS). The procedure entailed extraction with acetonitrile containing 0.5% acetic acid (HAc) followed by the dispersive solid-phase extraction (d-SPE) cleanup step employing bulk sorbents: MgSO₄, primary secondary amine (PSA), and graphitized carbon black (GCB). Similarly, Dai et al. (2011) validated the QuEChERS method for determination of 23 pesticides in three medicinal herbs, entailing acetonitrile extraction and d-SPE cleanup with MgSO₄, PSA, and GCB sorbents and finally analysis by GC-MS. Liu et al. (2016) reported a QuEChERS method for the analysis of 74 pesticides in the Chinese materia medica. The pesticides were extracted with hexane and cleaned up with MgSO₄, octadecyl (C18), or Florisil, followed by determination by gas chromatography tandem mass spectrometry detection (GC-MS/MS). Despite the continuous appearance of many modifications of QuEChERS methods, there are very limited studies about the influence of these modifications on the matrix effect in scientific literature. Therefore, existing knowledge must be built upon.

This paper aims to improve the cleanup step to effectively minimize the matrix effect while maintaining excellent recovery in the analysis of 235 pesticides in various dried herbs using the QuEChERS method and detection by GC-MS/MS. Purification steps based on different d-SPE sorbent combinations and a step without cleanup were compared. Two grams of the sample and extraction with acetonitrile in combination with sorbents for d-SPE cleanup (PSA/ENVI-Carb/MgSO₄) may be used prior to GC-MS/MS. In addition, the resulting procedure was applied for quantification of pesticide residues in 46 herb samples.

Materials and Methods

Materials and Reagents

Acetone, acetonitrile (AcN), and *n*-hexane were analytical grade and provided for pesticide residue analysis by J.T. Baker (Deventer, The Netherlands). Water was purified by a

Table 1 Application of the different methods to the analysis of pesticides in herb samples

Commodities (amount)	Pesticides	Sample preparation	Extraction	Cleanup step	Analytical technique	LOQs ($\mu\text{g L}^{-1}$)	Linear range ($\mu\text{g L}^{-1}$)	RSD (%)	Recovery (%)	Comments	Reference
Chinese herbal	19 (OCPs)	SPME	–	–	GC-MS	–	1–200	<11	–	–	Hwang and Lee (2000)
Peppermint anisum	32	AOAC 985.22/PAM.302	–	–	GC-ECD	–	–	<22	30–150	–	Hajjou et al. (2004)
Gastrodia tuber; Fructus corni, Spica prunellae (2 g)	4 (OCPs)	Ultrasonic extraction	10 mL petroleum ether	H ₂ SO ₄	GC-ECD	–	6–300	<2.84	77–102	–	Du et al. (2007)
<i>Acanthopanax senticosus</i> , <i>Morus alba</i> L., <i>Hovenia dulcis</i> (2.5 g)	234	QuEChERS	10 mL water, 10 mL ACN (HAc 0.5%), 4 g MgSO ₄ , 1 g NaCl	50 mg PSA, 300 mg GCB, 30 mg MgSO ₄	GC-MS	50	50–400	<21	62–119	–	Nguyen et al. (2010)
<i>Hiptis pectinata</i> (5 g)	20	Ultrasonic extraction	40 mL ACN/dichloromethane (2/1, v/v)	GPC-7.5 g silica gel, 20 mL petroleum ether/acetone (40/60, v/v)	GC-ECD	1–8.2	1–30	<10	74–115	–	Qian et al. (2010)
<i>Radix Gentianae</i> , Cortex Phellodendri, <i>Fritillaria ussuriensis</i> Maxim (2 g)	23 (OCPs, OPPs, PYR)	QuEChERS	8 mL water, 10 mL ACN, 1 g NaCl, 4 g MgSO ₄	300 mg PSA, 50 mg GCB, 50 mg MgSO ₄	GC-MS	<10	5–1	<9.5	78–119	–	Dai et al. (2011)
<i>Emblica officinalis</i> , <i>Terminalia chebula</i> , <i>Terminalia belerica</i> , <i>Withania somnifera</i> (2 g)	28 (OCPs, OPPs, PYR)	Soxhlet extraction	150 mL hexane	Cleanup column	GC-ECD	–	–	–	–	–	Rao et al. (2011)
<i>Yam</i> rhizome, Milkvetich, dried tangerine peel (10 g)	195	–	1 g NaCl, 100 mL acetone, 1 g Na ₂ SO ₄	GPC	GC-MS	–	–	–	–	Analyte protectants	Wang et al. (2011)
Melissa (2 g)	7	Ultrasonic extraction	15 mL ACN/HAc (9:1, v/v), 4 g Na ₂ SO ₄	SPE-C18 2000/6 mg mL ⁻¹ , 5 mL THF	HPLC-DAD	100–1350	100–100,000	–	–	–	Tuzimski (2011)
Caraway, dry mint, chamomile (3 g)	71	QuEChERS	15 mL water, 15 mL ACN	0.4 g PSA, 0.6 g MgSO ₄	GC-ECD/NPD	6–100	6–100	<22	70–120	Matrix-matched standards	Attallah et al. (2012)
Chinese materia medica (2 g)	39	QuEChERS	10 mL 1% HAc ACN/water (9:1, v/v), 4 g MgSO ₄ , 1 g NaCl, 0.5 g CH ₃ COONa	50 mg PSA, 25 mg GCB, 150 mg MgSO ₄	GC-MS	1–100	5–5000	<15	75–112	–	Hu et al. (2012)

Table 1 (continued)

Commodities (amount)	Pesticides	Sample preparation	Extraction	Cleanup step	Analytical technique	LOQs ($\mu\text{g L}^{-1}$)	Linear range ($\mu\text{g L}^{-1}$)	RSD (%)	Recovery (%)	Comments	Reference
Zataria, Matricaria chamomile, borage, spearmint, cumin seed	4 (OPPs)	SPME	Fiber coated with SWCNTs	–	GC-MS	–	–	–	–	–	Sarkhail et al. (2012)
TCM (1 g)	23 (OCs, OPPs, PYR)	Ultrasonic extraction	5 mL ethyl acetate, cyclohexane (1:1, v/v)	GPC-ethyl acetate, cyclohexane (1:1, v/v) at a flow rate of 5 mL min ⁻¹	GC-MS	0.67–10.81	10–1000	< 7.7	87.1–110.9	–	Zhuang and Gong (2012)
Radix Ginseng, Semenpersicae, Herba Lophatheri, Flos Loncerae, dogwood (2 g), Chamomile (2 g)	116	QuEChERS	10 mL water, 10 mL 0.1% HAAC/ACN, 4 g MgSO ₄ , 1 g NaCl	210 mg PSA, 70 mg GCB, 1050 mg MgSO ₄ , 200 mg C18	UHPLC-MS/MS	< 10	10–100	< 15	70–120	Matrix-matched standards	Chen et al. (2012)
Herbs (5 g)	52	SPLE	4 mL water, 10 mL ACN, 4 g MgSO ₄ , 1 g NaCl, 1 g Na ₂ C ₆ H ₅ O ₇ , 2H ₂ O, 0.5 g Na ₂ HC ₆ H ₅ O ₇ , 1.5H ₂ O	150 mg PSA, 30 μL 5% formic acid	LC-MS/MS	10–100	0.5–5	< 20	70–120	Matrix-matched standards	Lozano et al. (2012)
Basil, lovage, mint, oregano, parsley, rosemary, sage, tarragon (10 g)	27	QuEChERS	2.5 g diatomaceous earth, 5 mL water, 10 mL ACN, 4 g MgSO ₄ , 1 g NaCl, 1 g Na ₂ C ₆ H ₅ O ₇ , 2H ₂ O, 0.5 g Na ₂ HC ₆ H ₅ O ₇ , 1.5H ₂ O	5 g Florisil, 0.1 g GCB, ethyl acetate	GC-MS/MS	1–10	2–400	< 19	62–127	Matrix-matched standards	Du et al. (2012)
Chamomile, linden, lungwort, melissa, peppermint, thyme (2 g)	163	MSPD	4 g Florisil	150 mg PSA, 45 mg GCB, 900 mg MgSO ₄	GC-SIM-MS	1–12	0–400	< 15	71.6–116.9	Matrix-matched standards	Sadowska-Rociak et al. (2013)
Herbal medicines (0.1 g)	10	DLLME	1.2 mL ACN, add 80 μL CCl ₄ , 5 mL water	5 g sodium sulfate, 1 g C ₁₈ , 2.5 g silica gel, 25 mL acetone/methanol (9:1)	GC-ECD/NPD	5–40	5–2500	< 18	70–119	Matrix-matched standards	Łozowicka et al. (2013)
Rosemary, sage, laurel, oregano, spearmint (5 g)	5 (OCs)	LSE	<i>n</i> -Hexane/acetone (9:1)	8 g acid silica (H ₂ SO ₄ , 44% w/w), 50 mL hexane/	GC-MS	1–2	–	< 10	96–99	Matrix-matched standards	Storelli (2014)

Table 1 (continued)

Commodities (amount)	Pesticides	Sample preparation	Extraction	Cleanup step	Analytical technique	LOQs ($\mu\text{g L}^{-1}$)	Linear range ($\mu\text{g L}^{-1}$)	RSD (%)	Recovery (%)	Comments	Reference
Chinese herbal medicines (2 g)	103	Ultrasonic extraction	2 g Na_2SO_4 , 40 mL ACN, ultrasonic bath	dichloromethane (1/1, v/v) Carb/NH ₂ SPE sorbent, 15 mL ACN/toluene (3:1, v/v)	GC-MS/MS	0.03–11.88	–	< 14.7	70–120	Matrix-matched standards	Tong et al. (2014)
Aniseeds (2 g)	10	QuEChERS	10 mL water, 10 mL ACN (1% HAc), 4 g MgSO_4 , 1 g NaCl	–	GC-ECD	50	25–250	< 13.94	82.29–116.3	Matrix-matched standards	Ghani (2014)
Peppermint (5 g)	46	QuEChERS	10 mL water, 10 mL ACN, 4 g MgSO_4 , 1 g NaCl, 1 g $\text{Na}_2\text{C}_2\text{H}_3\text{O}_7$, 2H ₂ O, 0.5 g $\text{Na}_2\text{HC}_6\text{H}_5\text{O}_7$, 1.5H ₂ O	150 mg PSA, 45 mg GCB, 900 mg MgSO_4	GC-ECD/NPD	4–10	4–5288	< 15	70–120	Matrix-matched standards	Slowik-Borowicz (2015)
Chinese materia medica (2 g)	74	QuEChERS	10 mL water, 20 mL <i>n</i> -hexane, 2 g NaCl, 3 g NaOAc	50 mg MgSO_4 , 50 mg C18, 50 mg Florisil	GC-MS/MS	10	5–250	< 10	70–120	Matrix-matched standards	Liu et al. (2016)
<i>Pterocarpus angolensis</i> , <i>Maerua angolensis</i> , <i>Terminalia sericea</i> , <i>Cassia abbreviata</i> , <i>Gymnosporia senegalensis</i>	14 (OCPs)	HS-SPME	65 μm PDMS/DVB fiber	–	GC-ECD	1.61–4.8	2–100	< 17.97	69.58–113	–	Gondo et al. (2016)
Basil, spearmint, chives, thyme, <i>Sabvia</i> (1 g)	28 (CAR)	QuEChERS	10 mL MeCN, 4 g MgSO_4 , 1 g NaCl	200 mg MgSO_4 , 20 mg C18	UHPLC-MS/MS	2	2–80	< 13	72	Matrix-matched standards	Nantia et al. (2017)

TCM traditional Chinese medicine, CAR carbamate, OCPs organochlorine, OPPs organophosphorus, PYR pyrethroid, ACN acetonitrile, HAc acetic acid, SPLE selective pressurized liquid extraction, SPME solid-phase microextraction, SWCNTs single-walled carbon nanotubes, $\text{Na}_2\text{C}_2\text{H}_3\text{O}_7 \cdot 2\text{H}_2\text{O}$ trisodium citrate dihydrate, $\text{Na}_2\text{HC}_6\text{H}_5\text{O}_7 \cdot 1.5\text{H}_2\text{O}$ disodium hydrogen citrate sesquihydrate, GPC gel permeation chromatography, SPE solid-phase extraction, DLLME dispersive liquid-liquid microextraction, LSE liquid solid extraction, MSPD matrix solid-phase extraction, UHPLC-MS/MS ultra-high pressure liquid chromatography tandem mass spectrometry, GC-MS/MS gas chromatography tandem mass spectrometry, GC-ECD/NPD gas chromatography, GC-MS gas chromatography mass spectrometry, GC-S/M-MS gas chromatography selected ion monitoring mass spectrometry, LC-MS/MS liquid chromatography tandem mass spectrometry, HPLC-DAD high-performance liquid chromatography with diode-array detection, HS-SPME head space solid-phase microextraction

Milli-Q (Millipore, Billerica, MA, USA) system. Water was cooled to a temperature of about 4 °C. The QuEChERS sorbent kits (1) Z-Sep+, 200 mg; (2) Z-Sep/C18, 12/300 mg; (3) Z-Sep+/PSA/ENVI-Carb/MgSO₄, 60/50/10/150 mg; (4) PSA/ENVI-Carb/MgSO₄, 150/45/900 mg; (5) PSA/DSC-18/ENVI-Carb/MgSO₄, 400/400/400/1200 mg; (6) GCB/PSA/MgSO₄, 25/2.5/150 mg; (7) ChloroFiltr/PSA/MgSO₄, 50/50/150 mg; and (8) ChloroFiltr/PSA/MgSO₄, 50/50/150 mg, were supplied by Agilent Technologies (Santa Clara, CA, USA), UCT (Bristol, UK), and Sigma-Aldrich (Steinheim, Germany).

Analytical reagent-grade magnesium sulfate, sodium chloride, trisodium citrate dihydrate (Na₃C₆H₅O₇·2H₂O), and disodium hydrogen citrate sesquihydrate (Na₂HC₆H₅O₇·1.5H₂O) salt were obtained from Agilent Technologies (Santa Clara, CA, USA). Formic acid (98% purity) was supplied by Merck (Darmstadt, Germany).

The standard pesticides (purity > 95%) were purchased from Dr. Ehrenstorfer Laboratory (Augsburg, Germany). The internal standard (IS), triphenyl phosphate (TPP), was obtained from Sigma-Aldrich (Steinheim, Germany).

Preparation of Standards

The stock standard solution was prepared separately in a concentration around 1000 µg mL⁻¹, using acetone as solvent. Intermediate stock standard mixtures in acetone, containing 10 µg mL⁻¹ of each compound, were prepared by mixing appropriate quantities of the individual stock solutions. A working standard mixture of 0.001/0.002–1.0/2.0 µg mL⁻¹ was prepared in an *n*-hexane/acetone mixture (9:1, v/v) from the above stock standard by serial dilution. The standard working solution was used to prepare matrix-matched standards and to spike samples in the validation study. The IS was prepared as described above. The stock, working standard solutions, and IS were stored in amber glassware under appropriate conditions, i.e., at –20 °C until the time of analysis.

Sample Preparation

Two grams of herb was weighed into a 50-mL centrifuge tube. Ten milliliters of cold purified water was added into the tube and shaken by hand for a few seconds to hydrate the samples. After standing for 10 min, 50 µL of 20 µg mL⁻¹ IS and 10 mL acetonitrile were added. The sample was shaken for 1 min. The tube was placed in a –20 °C freezer for 15 min. Next, 4 g MgSO₄, 1 g NaCl, 1 g Na₃C₆H₅O₇·2H₂O, and 0.5 g Na₂HC₆H₅O₇·1.5H₂O were added. The tube was shaken vigorously by hand for 1 min to prevent the formation of crystalline agglomerates during MgSO₄ hydration and then centrifuged for 5 min at 4500 rpm. Four-milliliter aliquots of the acetonitrile layer were transferred into a cleanup tube containing PSA/ENVI-Carb/MgSO₄ sorbent. The tube was shaken

for 1 min and centrifuged at 4500 rpm for 10 min. Two milliliters was downloaded and transferred into a flask and 50 µL of 5% formic acid and some droplets of dodecane were added. Afterwards, the supernatant was evaporated to near dryness using a rotary evaporator at a temperature of about 40 °C. The residue was redissolved in 2 mL of *n*-hexane/acetone (9:1, v/v) and filtered through a 0.45-µm nylon filter into an autosampler vial for GC analysis.

GC-MS/MS Conditions

Chromatographic analyses were performed in gas chromatograph coupled to triple quadrupole system MS/MS (Agilent Technologies, Palo Alto, CA, USA). The chromatograph was equipped with an Agilent 7693 autosampler. The GC system's specifications and the triple quadrupole system's parameters are summarized in Table 2. Data acquisition, control, and data processing were performed using MassHunter Workstation Software (B.06.00). Multiple reaction monitoring (MRM) transitions and other acquisition parameters can be found in the Supplementary Material (Table S1). Retention time locking (RTL) was used to eliminate the need for adjusting the time-segment windows of MRM groups, using chlorpyrifos methyl as the locking compound for a retention time of 16.593 min.

Validation Study

Two hundred and thirty-five pesticides were validated in three kinds of herb matrices, *Centaurea cyanus* L., *Matricaria chamomilla* L., and *Thymus vulgaris* L., using the QuEChERS method and GC-MS/MS analysis. The following parameters were determined according to European guidelines (SANTE 2015) during validation of the analytical method: linearity, recovery, precision, limit of quantification (LOQ), limit of detection (LOD), matrix effects (ME), and uncertainty (U).

Linearity was studied using pure solvent and matrix extracts of *Centaurea cyanus* L., *Matricaria chamomilla* L., and *Thymus vulgaris* L. in six different concentrations: 0.001/0.005, 0.005/0.01, 0.01/0.02, 0.05/0.1, 0.5/1.0, and 1.0/2.0 µg mL⁻¹. The LOQ was set at the minimum concentration that could be quantified with acceptable values of recovery (70–120%) and RSD (≤20%). In certain cases with multiresidue methods, recoveries outside this range may be accepted (60–130%) but demonstrate good precision (RSD ≤20%) (SANTE 2015). LOD was calculated using signal-to-noise ratio (*S/N*) criteria, in all cases; LOD = 3 *S/N*.

For recovery determination, the homogenized samples (2 ± 0.01 g) were spiked with working standard solution at three concentration levels: 0.005/0.01, 0.05/0.1, and 1.0/2.0 mg kg⁻¹. The spiked samples were set aside for 30 min and then processed according to the procedure described above. Precision

Table 2 Chromatographic conditions of GC-MS/MS pesticide residue analysis in dried herb samples

Agilent 7890B GC system		Agilent 7000B series MS/MS triple quadrupole system	
Column	HP-5MS 30 m × 0.25 mm ID and 0.25 μm	Ionization mode	Electron ionization mode (EI – 70 eV)
Column temperature	70 °C	Transfer line temperature	280 °C
Injection mode	Splitless	Ion source temperature	300 °C
Injection volume	2 μL	Quadrupole temperature	180 °C, 180 °C
Injection temperature	270 °C	Collision gases (flow)	Helium (2.25 mL min ⁻¹) Nitrogen (1.5 mL min ⁻¹)
Carrier gas (flow)	Helium (2.1 mL min ⁻¹)		
Oven temperature	70 °C (2 min hold) → 150 °C at 25 °C min ⁻¹ → 200 °C at 3 °C min ⁻¹ → 280 °C at 8 °C min ⁻¹ (10 min hold)		

Total running time 41.88 min

was expressed in terms of relative standard deviation (RSD %) and calculated for each spiking level ($n = 5$).

In order to evaluate the influence of matrix components in the detector's response, the peak area of standard in the matrix and the standard in the solvent at the same concentration level were used. Matrix effects were calculated as follows: $\%ME = ((\text{peak area}_{(\text{matrix standard})}/\text{peak area}_{(\text{solvent standard})}) - 1) \times 100$. The matrix effect could be negative or positive and would be classified in one of three categories: soft matrix effect, medium matrix effect (20–50% positive values and –20 to –50% negative values), and strong matrix effect (more than 50% positive and less than –50% negative). Soft matrix effect is considered as ME (%) values within repeatability range values (–20 to 20%) accepted in pesticide analysis (Ferrer et al. 2011).

The data from the validation study were used to estimate the measurement uncertainty associated with the analytical results. The major uncertainty sources in the uncertainty budget were the repeatability of recoveries from the spiked samples and uncertainty of the average recovery calculated for rectangular distribution. Finally, relative expanded uncertainty was calculated by using coverage factor $k = 2$ at a confidence level of 95% (Walorczyk et al. 2015).

Results and Discussion

Optimization of Extraction and Cleanup Method

This study presents a modification of one of the most widely described multiresidue methodologies—the QuEChERS approach, which has many advantages including speed, cost, ease of use, good performance characteristics, and wide applicability range (matrices and analytes). Over the years, since its first introduction by Anastassiades et al. (2003a, b), the method has undergone various modifications and enhancements to

ensure efficient extraction of pH-dependent compounds (e.g., phenoxyalcanoic acids) (Kaczyński et al. 2016), to minimize degradation of susceptible compounds (e.g., base and acid labile pesticides) (Lehotay et al. 2005b), and to expand the spectrum of food matrices amenable to the method (Łozowicka et al. 2016). Therefore, the QuEChERS method was optimized in order to achieve quick and effective extraction and a clean herbal extract.

In recent years, the significance of assessing matrix effects has increased relatively to recovery, which is a tool commonly used to estimate pesticide extraction efficiency. ME is described as one of the major sources of errors in gas chromatography (Kwon et al. 2012; Li et al. 2015) and may adversely affect the determination of pesticide residues in complex matrices.

In this study, recovery and matrix effect were chosen as criteria to estimate the method's effectiveness. Thus, the challenge was to assess both parameters for each pesticide (235 pesticides in total) in herb samples prepared by various procedures. Some factors that can influence the performance of QuEChERS, such as sample mass and cleanup step or no cleanup step, were investigated.

Firstly, to ensure adequate partitioning, interactions between pesticides and the matrix were weakened by the addition of distilled water (10 mL) and contact for 10 min before the extraction step. Some papers recommend the addition of water to low-moisture samples before extraction (Anastassiades et al. 2003a, b). Liu et al. (2016), Ghani (2014), and Chen et al. (2012) added water to their herb samples, which made it possible to increase extraction efficiency.

Secondly, the effect of sample mass (1, 2, 5 g) on pesticide recoveries was evaluated. This study indicated that recoveries were in the range of 60–128, 31–135, and 35–149% when the mass of samples was 2, 1, and 5 g, respectively, for herb matrices. Pesticides extracted from 1 g of dry sample showed the same values as extracted from 5 g, but less efficient

recovery than for the 2-g sample. However, more pesticides had recoveries below 40% when 1 g sample mass was used compared to 5 g. In the case of the highest sample mass (5 g), more pesticides had recoveries above 130%. Nevertheless, recoveries remained between 60 and 130% for most of the tested compounds (about 89%). Based on this experiment, 2 g sample mass was employed as the optimum amount in this study. Applications of different sample weights have been published (Nantia et al. 2017; Storelli 2014), and there are some reports where 2 g sample mass was chosen (Liu et al. 2016; Lozano et al. 2012) and gave satisfactory results.

Citrate-buffering salts were introduced in order to achieve a constant pH value during the extraction of herb matrices to improve the stability of base-sensitive compounds. These compounds undergo hydrolysis at a pH close to neutral. Under these conditions, the pH value of the matrix could be maintained within the range of 5.0–5.5, which was conducive to the stability of problematic pH-dependent pesticides. Even for troublesome (base-sensitive) pesticides, i.e., captan, chlorothalonil, or tolylfluanid, citrate-buffered QuEChERS enabled acceptable recoveries in all three matrices.

Herb samples contain many complex components, including essential oils, flavonoids, phenolic (Lozano et al. 2012), lipids, natural pigments, and sugars (Ghani 2014), which make analysis more complicated. Thus, the aim of this study was to reduce the co-extracted matrix components as much as possible. To achieve the best efficiency, the no cleanup step was compared to cleanup using eight different d-SPE sorbent combinations, which remove interferences and impact analyte recoveries (Fig. 1).

The step without cleanup showed that more than 10% of the analytes had recoveries higher than 130% (i.e., in the range of 130–148%) and over 30% exhibited significant ME (above 50%). It would seem that the procedure without the purification step was inherently associated with co-extractives. So, cleanup of the herbal extract was needed to minimize the matrix effect. Therefore, the next modification with a cleanup step was tested. Only Ghani (2014) applied the QuEChERS method for determination of 10 pesticides in aniseeds without a purification step (Table 1).

During the experiments, it was found that various d-SPE sorbent combinations had a significant influence on the purification and recovery rates of analytes and many studies considered the use of different d-SPE sorbents in herb samples

(Nguyen et al. 2010; Chen et al. 2012; Liu et al. 2016). Mostly, PSA, GCB, octadecyl (C18), and Florisil were described (Table 1). PSA can act as both a polar phase and weak anion exchanger with the ability to remove fatty acids, sugars, and other matrix co-extractives (organic acids, anthocyan pigments) which might adversely affect chromatographic performance (Kinsella et al. 2009; Anastassiades et al. 2003a, b). GCB has been reported to be a highly effective sorbent for sample cleanup (Kinsella et al. 2009). This is a nonporous reversed-phase sorbent and removes planar molecules such as natural pigments (e.g., chlorophyll, hemoglobin, and carotenoids), sterols, and nonpolar interferences (Anastassiades et al. 2003a, b). On the other hand, the use of high amounts of GCB (> 10 mg per 1 mL of acetonitrile extracts) may lead to unacceptable losses of some planar pesticides (Walorczyk et al. 2015). In this study, when the combination of GCB/PSA/MgSO₄ was used, relatively weak recoveries (for about 20% of pesticides) were obtained, probably because of being subjected to strong GCB adsorption. Some nonpolar pesticides, especially those with a planar structure (chlorothalonil, cyprodinil, fenazaquin, hexachlorobenzene, mepanipirim, pirymetamil, prochloraz, quinoxifen, thiabendazole) had poor recoveries. About 25% of the tested compounds had ME above 50%. Similarly, Liu et al. (2016), during optimization of the QuEChERS method, established that using PSA/GCB/MgSO₄ as a sorbent for simultaneous determination of 135 pesticides in Chinese herbal medicines gave substantially lower recoveries (40.3–60.2%) for some particular pesticides (methamidophos, aldicarb, bifentazate, and bitertanol).

Other sorbents considered for use in this study are zirconium dioxide-based sorbents (Z-Sep and Z-Sep+), ChloroFiltr, and ENVI-Carb, and there is a lack of reports on the subject of pesticide determination in herbs using these kinds of sorbents. The retention mechanism of Z-Sep sorbents involves Lewis acid/base interactions. Due to its properties, it can reduce the amount of lipids and some pigments (Lozano et al. 2014) and provide an optimum balance between planar pesticide recovery and color removal. ChloroFiltr is a polymeric-based sorbent designed for the removal of chlorophyll without sacrificing the recovery of planar analytes (Walorczyk et al. 2015). ENVI-Carb is used to remove trace chemical compounds such as pigments, polyphenols, and other polar compounds. ENVI-Carb is a strong sorbent with a carbon surface comprised of hexagonal ring structures, interconnected and layered into

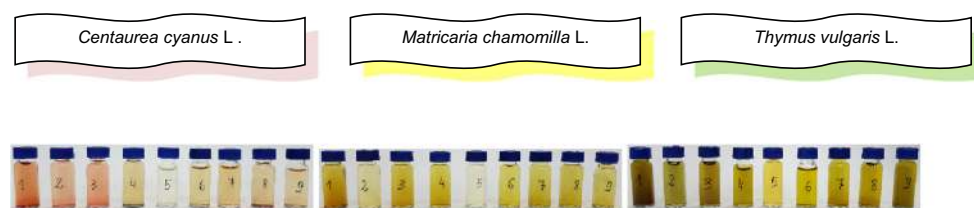


Fig. 1 Visual comparison of extracts: (1) without and with cleanup, (2) Z-Sep+, (3) Z-Sep/C18, (4) Z-Sep+/PSA/ENVI-Carb/MgSO₄, (5) PSA/ENVI-Carb/MgSO₄, (6) PSA/DSC-18/ENVI-Carb/MgSO₄, (7) ChloroFiltr/PSA/MgSO₄, (8) ChloroFiltr/PSA/MgSO₄/C18, and (9) GCB/PSA/MgSO₄

graphitic sheets, and the nonporous nature of the carbon phase allows for rapid processing; adsorption does not require analyte diffusion into pores. The combination of ChloroFiltr/PSA/MgSO₄ exhibits a wider spectrum of pigment adsorption. Over 80% of pesticides had acceptable recoveries, and 20% showed ME above 50%. Unfortunately, they were not applicable to this research. Similar results were achieved for combinations with sorbents based on zirconium dioxide. Recoveries in the acceptable range of 60–130% were achieved for about 75% of the analytes. In addition, the matrix effects were not sufficiently reduced (over 20% of pesticides above 50% ME). Excellent results were achieved using the PSA/ENVI-Carb/MgSO₄ combination. This combination provided satisfactory recoveries (60–128%), and over 65–72% exhibited soft ME (<±20%) (Figs. 2 and 3).

In addition, octadecyl like PSA and GCB is the sorbent most commonly used to remove co-extractives from herbs (Table 1). C18 is a hydrophobic reversed-phase sorbent due to its extreme retentive nature toward nonpolar compounds such as lipids and pigments, and it also has stronger adsorption to nonpolar pesticides (Lehotay et al. 2005a). Octadecyl with a combination of ChloroFiltr, ENVI-Carb, and Z-Sep was checked, and the results showed decreased recovery for some pesticides (Fig. 2). Some nonpolar pesticides (alpha-HCH, beta-HCH, lindane, *p,p'*-DDE, *p,p'*-DDD, *o,p'*-DDT, *p,p'*-DDT) were retained on this sorbent, leading to lower analyte recoveries. About 20% of compounds showed strong matrix effects in all cases. These results indicated a higher presence of co-extractives compared with the extracts after cleanup with C18, which resulted in the occurrence of strong matrix effects. Thus, there is no need to employ C18 in the cleanup step.

As shown in this study, in such a complex matrix like herbs, 2 g of the sample and extraction with acetonitrile in combination with cleanup sorbents (PSA/ENVI-Carb/MgSO₄) may be used prior to GC-MS/MS analysis to provide sufficient pigment removal (Fig. 1) while maintaining excellent recovery (60–130%—according to SANTE (2015) guidelines) and reducing matrix effects for the majority of pesticides tested in herb matrices (Figs. 2 and 3).

After d-SPE cleanup, formic acid in acetonitrile (5% v/v, 50 µL) was added to stabilize base-sensitive pesticides, including captan, chlorotalonil, dichlofluanid, dicofol, folpet, and tolyfluanid, which are susceptible to degradation in contact with the basic PSA sorbent. The pH of the final extracts measured after PSA cleanup reaches values in the range from 8 to 9, which endanger the stability of base-sensitive pesticides. The addition of formic acid brings the extract's pH to a value of about 5 and seems to be the easiest solution to this problem.

Validation Study

The optimized analytical method for determination of 235 pesticides in herb samples using GC-MS/MS was evaluated. The

investigated pesticides represent various chemical classes and a wide range of physicochemical properties, including volatility, polarity, chemical and thermal stability, etc. Their polarity ranges from the nonpolar esfenvalerate (logP 6.22), to the polar acephate (logP -0.89), their solubility from the least soluble deltamethrin (logS -3.7) to the most soluble acephate (logS 5.9), and their volatility from the least volatile fluquinconazole (logV.P. -5.19) (V.P., vapor pressure) to the most volatile dichlorvos (logV.P. 3.32). Their molecular weights ranged from 141.13 (methamidophos) to 541.44 (acrinathrin) (PPDB 2016). The pesticides belong to over 50 different classes, i.e., chloronitrile, organochlorine, organophosphate, and pyrethroid (Supplementary Table S1). A series of experiments, covering linearity, precision, recovery, LOQ, LOD, and uncertainty, were performed to validate the modified QuEChERS method under optimized conditions using *Centaurea cyanus* L., *Matricaria chamomilla* L., and *Thymus vulgaris* L.

LOD and LOQ are important validation parameters and were used to evaluate the sensitivity of the analytical methods. LOQs ranged from 0.001 to 0.002 mg kg⁻¹ and were equal to or lower than the European MRLs (EC Regulation No. 396/2005 with amendments). Obtained LOD values ranged from 0.0003 to 0.0007 mg kg⁻¹ (Supplementary Table S2).

Good linearity results, with satisfactory correlation coefficients, were obtained for all pesticides, ranging from 0.99000 to 0.99999 (Supplementary Table S2).

Recoveries obtained for 235 pesticides were satisfactory and ranged from 60 to 127% with RSD 1–15% for *Centaurea cyanus* L., 60 to 128% with RSD 1–18% for *Matricaria chamomilla* L., and 60 to 128% with RSD 1–16% for *Thymus vulgaris* L. (Supplementary Table S3). In all three matrices, a similar group of problematic compounds was observed to have recovery values in the 60–130% range. Since consistent results (≤20% RSD) were achieved for these pesticides, the results could be corrected according to the recovery factors known from analyses. According to SANTE (2015) guiding criteria, pesticides resulting in the recovery range 60–130% can be used in routine multiresidue analysis. The fact that the same compounds presented problems in each of the different matrices suggests that the extraction conditions, not matrix components, were responsible for the low recoveries of these compounds. Among these compounds, dichlorvos, dichlobenil, and methamidophos are volatile and have the shortest retention times, and captan, chlorotalonil, dichlofluanid, dicofol, folpet, and tolyfluanid easily degrade during sample preparation and/or GC injection. Consistent recoveries of these problematic pesticides were typically below 70% but above 60%. Only 6% of tested pesticides showed recovery values above 120%, but below 130%.

Almost all pesticides had similar recoveries at three levels for all three matrices. Moreover, the recoveries of some pesticides in *Matricaria chamomilla* L. and in *Thymus vulgaris* L. are higher than in *Centaurea cyanus* L., i.e., bifenthrin, clomazone,

Fig. 2 Recoveries of 235 pesticides for the modified QuEChERS procedures with and without cleanup

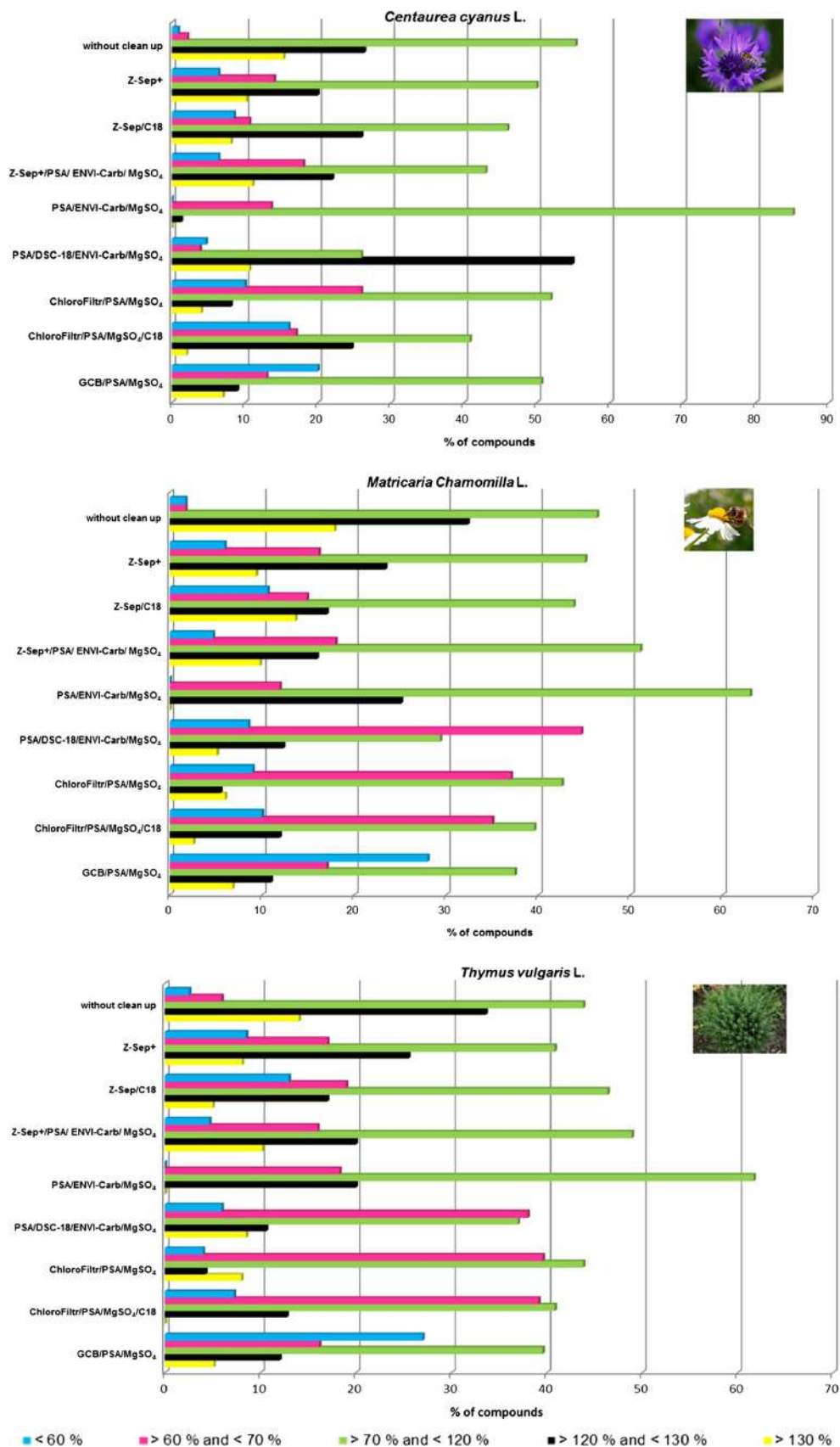
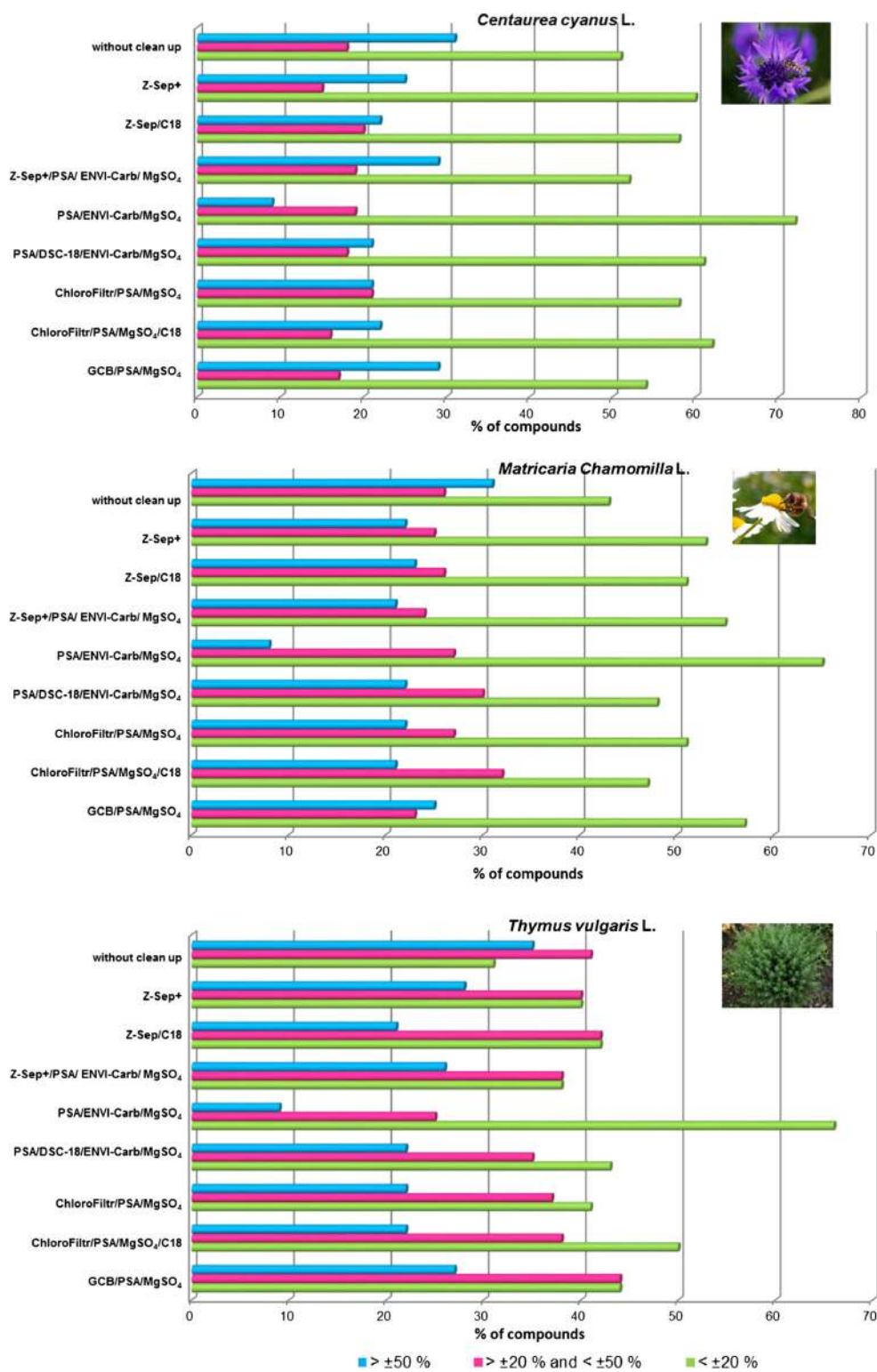


Fig. 3 Matrix effects of 235 pesticides for the modified QuEChERS procedures with and without cleanup



dimoxystrobin, etoxazol, imazalil, iprodione, and picoxystrobin but still within the acceptable range. The reason for these differences is associated with the presence of varying proportions of chlorophyll in herbal matrices. On the other hand, some pesticides, i.e., malaoxon, paraoxon ethyl, and methyl, had

considerably lower recoveries (below 70%) in *Matricaria chamomilla* L. and *Thymus vulgaris* L. compared to recoveries in *Centaurea cyanus* L. (Supplementary Table S3).

Expanded measurement uncertainties were estimated employing a “top-down” empirical model to be between 5

and 35% for *Centaurea cyanus* L., 4 and 39% for *Matricaria chamomilla* L., and 7 and 43% for *Thymus vulgaris* L. (coverage factor $k = 2$, confidence level 95%). This is less than the maximum threshold value of 50% recommended by European Union guidelines (SANTE 2015) (Supplementary Table S3).

Matrix Effects

In multiresidue analysis of pesticides in plant products by gas chromatography, co-extracted matrix components often increase the response, resulting in inaccurate quantification. Co-extracted matrix ingredients can compete with target pesticides for access to the active site during the injection process, which may result in enhancement of the detector's signal. Meanwhile, (semi) polar or thermal-sensitive analytes may decompose at active sites in the liners, column, and detector, giving losses and distorted peak shapes, which may result in suppression of the chromatographic signal (Hajšlová et al. 1998).

The variability of matrix effects was estimated for the validated and optimized QuEChERS method. In the present study, interactions of pesticides with the matrix were observed, and matrix effect values are summarized in Supplementary Table S3, ranging from -59 to 69% for *Centaurea cyanus* L., -69 to 63% for *Matricaria chamomilla* L., and -59 to 65% for *Thymus vulgaris* L. Among the 235 pesticides analyzed, 72% for *Centaurea cyanus* L., 65% for *Matricaria chamomilla* L., and 66% for *Thymus vulgaris* L. exhibited soft matrix effects. About 9% of the tested pesticides had strong matrix effects in all three matrices. ME was comparable in all three selected dried herbs but was more significant when they were in lower concentrations. Analogously, Nantia et al. (2017) showed that ME obtained for five fresh herbal products were quite similar. Therefore, we can confirm that matrix effects are not only associated with pesticides but also the concentration level.

Pesticides with amino ($-R-NH-$), azole ($-N=$), benzimidazole, carbamate ($-O-CO-NH-$), carboxyl ($-COOH$), hydroxyl ($-OH$), imidazole, organophosphate ($-P=O$), and urea ($-NH-CO-NH-$) groups are the analytes most susceptible to the matrix effect (Anastassiades et al. 2003a). These pesticides have functional groups which interact with silanol groups or metal ions on glass surfaces (liners, glass wool, etc.), so they usually show high matrix effects (Anastassiades et al. 2003a). For example, diniconazole (azole group), imibenconazole (benzimidazole), carbaryl (carbamate), acephate (organophosphate), and other classes like amitraz, chloridazon, and fenhexamid exhibited strong matrix-induced enhancement/suppression effects in all three herb matrices. On the other hand, some pesticides from the aforementioned groups susceptible to ME had a low matrix effect. In addition, compounds with high polarity are among the pesticides having the functional groups listed above as well as acephate, dicrotophos,

dimethoate, methamidophos, and mevinphos. Other pesticides with high molecular mass (over 400 g mol^{-1}), such as imibenconazole, propaquizafop, and tau fluvalinate, were also susceptible to ME (Supplementary Table S3). Compounds with high molecular mass used as neat standards generally gave poor peak shapes, which made it more difficult to integrate them and generate a response increase in matrix-matched standards (Sánchez-Brunete et al. 2005).

Signal suppression is less common in GC analysis; in this study, only 7% of pesticides showed signal suppression in all tested matrices. The majority of pesticides presented a similar tendency (enhancement or suppression) and intensity in three matrices. The exceptions are fenamiphos and propham which showed signal suppression in *Matricaria chamomilla* L. and *Thymus vulgaris* L. and enhancement in *Centaurea cyanus* L. *Matricaria chamomilla* L. and *Thymus vulgaris* L. gave slightly higher ME results than *Centaurea cyanus* L. This could be explained by a greater amount of interfering substances extracted from cornflower than from chamomile, causing signal enhancement.

In summary, variation of ME depends on several factors, such as the physical-chemical properties of pesticides, the matrix itself, and the interactions between them.

GC-MS/MS Optimization

The use of MRM mode based on QqQ mass spectrometry allows low analyte detectability and is, at present, one of the most selective approaches for trace analysis. However, for multiclass, multiresidue methods, a precise optimization of MS/MS parameters is needed in order to maximize the signal for each pesticide. The first step was to select the parent ions (precursor ions), which were chosen after examination of the full scan spectra of each pesticide. The base peak was not always preferred (highest intensity), and a more selective one was chosen for several pesticides. For some pesticides, three parent ions were selected, one for each transition.

Secondly, four transitions were taken for each pesticide after the fragmentation of each parent ion was checked in product scan mode. Finally, MRM collision energy was optimized for each selected transition using matrix-matched solutions, and the three most selective transitions were chosen. Supplementary Table S1 provides the selected conditions implemented in the MRM program used for GC-MS/MS analysis.

Application to Real Samples

Real samples were applied in the analysis in order to prove the suitability of the optimized method. Forty-six samples of herbs were randomly analyzed, namely *Centaurea cyanus* L. (4 samples), *Chelidonium majus* (1), *Cirsium* Mill. (1), *Echinacea purpurea* L. (1), *Epilobium* L. (2), *Equisetum* L. (2), *Filipendula ulmaria* (1), *Glechoma hederacea* L. (2),

Table 3 Pesticide residue analysis of real herb samples

Commodity	Pesticide (category)	Concentration (mg kg ⁻¹)	LOQ (mg kg ⁻¹)	MRL (mg kg ⁻¹)
<i>Centaurea cyanus</i> L.	Chlorpyrifos (I)	0.030	0.005	0.010
	Tebuconazole (F)	0.510	0.005	0.010
<i>Epilobium</i> L.	Permethrin (I)	0.020	0.010	0.100
<i>Equisetum</i> L.	Permethrin (I)	0.060	0.010	0.100
<i>Hypericum perforatum</i>	Chlorpyrifos (I)	0.040	0.005	0.010
	Diazinon (I)	0.005	0.005	0.010
<i>Malva</i> L.	Chlorpyrifos (I)	0.589	0.005	0.500
<i>Origanum vulgare</i>	Chlorpyrifos (I)	0.040	0.005	0.010
	Chlorothalonil (F)	0.270	0.005	0.040
	Cypermethrin (I)	0.420	0.005	0.100
	Heptachlor (I)	0.040	0.005	0.010
<i>Pulmonaria</i> L.	Chlorpyrifos (I)	0.016	0.005	0.500
<i>Sambucus</i> L.	DEET (I)	0.067	0.005	0.010
<i>Tilia</i>	Acetamiprid (I)	1.103	0.010	0.050
<i>Tilia</i>	Acetamiprid (I)	0.016	0.010	0.050

LOQ limit of quantification, MRL maximum residue level., F fungicide, I insecticide

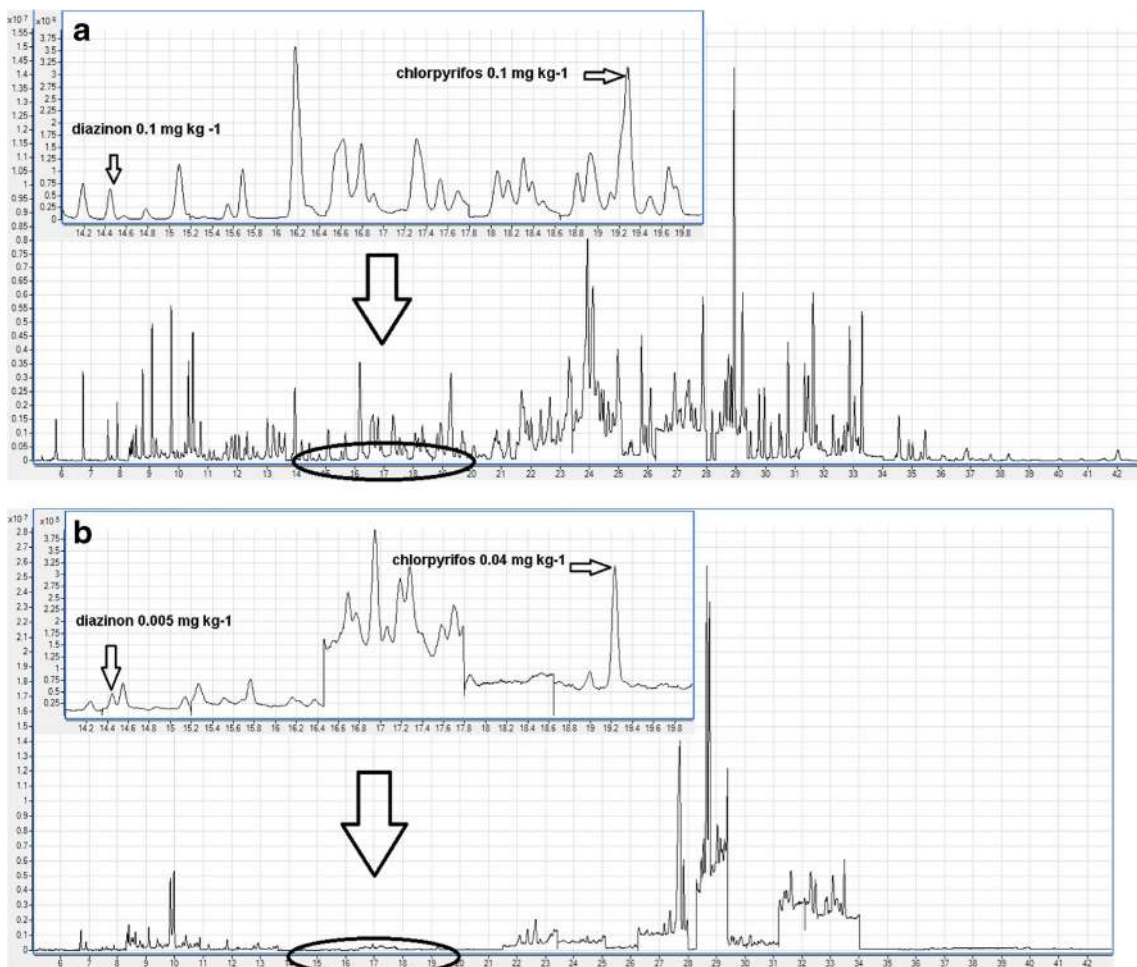


Fig. 4 Chromatograms of **a** matrix *Hypericum perforatum*-matched standards of 235 pesticides and **b** real sample of *Hypericum perforatum* containing diazinon (0.005 mg kg⁻¹) and chlorpyrifos (0.04 mg kg⁻¹)

Hypericum perforatum (1), *Malva* L. (1), *Marrubium vulgare* L. (1), *Matricaria chamomilla* L. (1), *Origanum vulgare* (2), *Pulmonaria* L. (5), *Sambucus* L. (1), *Taraxacum officinale* (2), *Thymus vulgaris* (1), *Tilia* (6), *Urtica dioica* L. (1), *Valeriana officinalis* L. (1), and *Veronica* L. (9). Ten samples were positive and nine of 235 pesticides were found (Table 3) in the range 0.005–1.103 mg kg⁻¹. In five samples, the detected levels were above the MRLs specified by the European Union (EC Regulation No. 396/2005 with amendments). Recovery factors were used to calculate pesticide concentration only in the case of pesticides indicating recoveries outside the range of 70–120% (within the range of 60–69 and 121–130%). Figure 4 shows chromatograms of matrix-matched standards and real samples containing diazinon 0.005 mg kg⁻¹ and chlorpyrifos 0.04 mg kg⁻¹ detected in *Hypericum perforatum*.

Conclusion

A modified QuEChERS method coupled with GC-MS/MS was developed for the simultaneous determination of 235 pesticides in various kinds of herbs: *Centaurea cyanus* L., *Matricaria chamomilla* L., and *Thymus vulgaris* L. This study discusses the impact of certain parameters, such as sample mass (1, 2, 5 g) and type of cleanup sorbent on recoveries and matrix effects, with the aim of choosing suitable methods for multiresidue analyses. Finally, the modified QuEChERS method based on acetonitrile extraction followed by PSA/ENVI-Carb/MgSO₄ cleanup was validated in terms of linearity, recovery, precision, LOQ, ME, and U. The application of PSA/ENVI-Carb/MgSO₄ did not have a negative influence on recoveries and was efficient in the removal of pigments and other co-extracted compounds. Recoveries of the tested pesticides were between 60 and 128% and RSDs were consistently < 18%. It should be noted that this combination ensured the best matrix effect values. Low ME was observed for the majority of pesticides showing suppression or enhancement (> 20%). The proposed method meets EU criteria and MRLs and is thus useful for routine analyses of pesticide residues in herbs. This method was applied for monitoring of pesticides in 46 samples of herbs.

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Compliance with Ethical Standards

Conflict of Interest Ewa Rutkowska declares that she has no conflict of interest. Bożena Łozowicka declares that she has no conflict of interest. Piotr Kaczyński declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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