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GC-MS Modified Quechers Method for Multiresidue Pesticide Determination in Red Wine

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Abstract: This work reports a new selective and accurate multiresidue procedure for determination of 25 pesticides in red wine by GC-MS. Proposed procedure uses an original approach in sample preparation technique based on QuEChERS theory. Main focus of method development was modification of salts thus increasing ionic strength of solution which improved pesticides partitioning and extraction efficiency. LOQs were in the range 0.01–250 μg L^{-1} with 56 % of target pesticides below or equal to 10 μg L^{-1}. RSD for most pesticides was < 20 % and recoveries were in the range 70–120 %. Matrix effect was found to be high for five pesticides confirming sample preparation procedure to be efficient. The proposed procedure was applied to 12 wine samples of different variety with determination of 40 % of target pesticides. Developed GC-MS methodology provides novel, selective and accurate approach for determination of 25 pesticide residues in red wine.

Keywords: multiresidue method, GC-MS, pesticide residues, red wine, QuEChERS.

INTRODUCTION

NOWADAYS, there is an essential need for pesticide residue analysis in food and environmental samples. Maximum levels for contaminants in food are decreasing, which has resulted in a growing need for more sensitive and reliable detection methodology. Such methods should not be oriented toward single pesticide analysis but toward development of multiresidue methods for simultaneous detection of a wide variety of pesticides. Furthermore, trace analysis of pesticides requires efficient sample preparation.[1] Determination techniques based on chromatographic separations are continuously improving; the use of mass spectrometry has enabled detection of analyte by the selection of ions of interest, reducing in this way the interferences. Matrix effects can have a great impact on detection systems; for instance detector noise, analyte response or ionization efficiency, which is directly related to the limits of detection and quantification.[2]

Nevertheless, sample treatment is still one of the key element of analytical procedure.[3] The choice of extraction technique depends on differences in physicochemical properties of analyte and complexity of the matrix. Sample preparation generally includes homogenization, extraction, clean up and concentration. Traditional sample preparation is time-consuming, expensive and in most cases includes the use of toxic organic solvents in large quantities. Compared to the classical liquid-liquid extraction (LLE), solid phase extraction (SPE) provides several advantages such as lower consumption of organic solvents, reduced time of analysis and efficient removal of matrix interferences, providing, in such way, lower limits of detection and quantification.[4–8]

A different sample preparation methodology with the acronym QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) has been first introduced by Wong and co-workers,[9] providing reliable results and reducing the number of sample preparation steps. Methodology has been successfully applied on food and vegetables matrices, ensuring selectivity and reproducibility with satisfactory recoveries of wide range of pesticides.[2–7] It is a method of choice which reduces the sample size and the quantity of

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laboratory glassware by combining several preparation steps. The QuEChERS methodology simplifies the analyte extraction and sample clean up, in principle without affecting the magnitude of analyte recoveries. Over the years methodology has been modified and enhanced depending on applied matrix.

Due to the high complexity of the matrix, analysis of wine is a challenge. In most cases, solid phase extraction has been method of choice for determination of pesticides residues in wine.[8–12] In last decade, QuEChERS methodology has been extended to various types of matrices. However, according to the literature QuEChERS has been poorly researched on wine samples.[13]

In this study a new multiresidue method for determination of 25 pesticides in wine samples by gas chromatography-mass spectrometry (GC-MS) is introduced. The focus of the method development was set on QuEChERS methodology, originally not proposed for wine samples.[9] With the aim of expanding a current knowledge about matrix influence, red wine had been chosen for method development. Influence of the wine matrix on identification and quantification of 25 pesticides was investigated by evaluation of extraction efficiency of the proposed methodology. Relevant statistical analysis of data obtained during validation was performed. For confirmation, proposed methodology was applied on real wine samples.

EXPERIMENTAL

Sample Collection

The wine varieties under study were Frankovka, Zweigelt, Plavac and Pinot noir, selected as the most widely consumed red wine varieties in Coatia. They were collected from three major wine-growing regions of Croatia: eastern contitnental Croatia, western continental Croatia and coastal Croatia. All analyzed samples were bottled after 1 year of aging in stainless steel tanks. Prior to the analyses, bottles were opened and aliquots containing 50 mL of wine samples were stored in plastic containers at 4 °C until analysis. Pesticide analyzes were conducted within the next 6 months.

Reagents and Chemicals

Pesticides included in this investigation include active compounds that were authorized for use in grapes in Croatia at the time of wine sample collection. 25 certified pesticide standards used in this work were purchased from Sigma-Aldrich (Germany) and Dr. Ehrenstorfer GmbH (Germany). Acetonitrile (HPLC grade) was purchased from J. T. Baker (Netherlands) and toluene from Sigma-Aldrich (Germany). Magnesium sulphate anhydrous was purchased from Acros Organics (USA), sodium chloride from Carlo Erba Reagents (Italy), sodium acetate and trisodium citrate dihydrate from Fischer Scientific (UK). Primary secondary amine (PSA) was purchased from Agilent (USA). Tetraphenylethylene (TEP) was used as an internal standard (Sigma-Aldrich, Germany). Cellulose acetate membrane filters (0.22 µm pore size) were purchased form Millipore (USA).

Individual standard pesticide stock solutions were prepared in acetone in concentration range 500–3125 mg L^{-1}. The standard working mixtures were prepared by appropriate dilution with acetone. Red wine without presence of target pesticide (*Babić*) was used for method development and for preparation of matrix-matched standard solutions. The internal standard solution was prepared in toluene at concentration 100 µg mL^{-1}. Standard and stock solutions were stored at 4 °C in the dark.

Sample Preparation

Red wine was filtered through cellulose acetate membrane filters and processed within 48 h. 10 mL of wine was transferred into a 50 mL centrifuge tube. Then, 10 mL of acetonitrile was added to the wine sample and vortexed (IKA Vortex Genius 3, Germany) for 1 min. Afterwards, 4 g of magnesium sulphate anhydrous, 1.5 g of sodium chloride, 2 g of sodium acetate and 2 g of trisodium citrate dihydrate were added to the sample. The centrifuge tube was mixed by hand, vortexed in turn for 10 min and centrifuged (Universal 320, Hettich, Germany) at 4000 rpm for 15 min. 8 mL of supernatant was transferred into a 15 mL centrifuge tube containing 400 mg of PSA and 1.2 g of magnesium sulphate anhydrous. The centrifuge tube was vortexed for 3 min and centrifuged at 4000 rpm for 15 min. The aliquot was evaporated to dryness by vacuum rotary evaporator (EZ-2 Plus, Genevac, UK) and diluted with 1 mL of acetone before GC-MS analysis.

Instrumentation Analysis

The analysis was performed on 6890N gas chromatograph with the mass spectrometer 5975 inert and 7863B autosampler (Agilent, USA). A HP-5MS capillary column (30 m x 0.25 mm, i.d. x 0.25 µm film thickness), (Agilent J&W Scientific Products, USA) was used for pesticide separation with helium as carrier gas (purity 6.0, Messer, Germany) and flow rate of 1.0 mL min^{-1}. 5 µL of sample was injected at 250 °C in pulsed splitless mode. The following oven temperature program was conducted: initial temperature 120 °C, held for 1 min; 30 °C per minute to 190°C, held for 1 min; 5 °C per minute to 205 °C, held for 1 min; 2 °C per minute to 217 °C, with no held; 6 °C per minute to 232 °C, held for 1 min; 10 °C per minute to 240 °C, with no held; 40 °C per minute to 320 °C, held for 3 min. The temperature of ion source was 230 °C, transfer line temperature was 280 °C and MS quad temperature was 150 °C. The electron

impact ionization was set to 70 eV. Total run time was 23.63 min with solvent delay 4.5 min. The analysis was performed in single ion monitoring (SIM) mode. Pesticides were identified according to their retention times, quantification ion (target ion) and three qualifier ions.

Validation and Matrix Effect

For validation studies red wine (*Babić*) with no traces of target pesticides was used as blank. Absolute recoveries were calculated as the difference between concentrations measured in spiked sample and non spiked sample, divided by theoretical concentration added to the sample, multiplied by 100. The recovery studies, expressed as method trueness and precision, were conducted at two concentration levels, 2 × LOQ and 10 × LOQ. For the reproducibility determination at each concentration level five replicates were evaluated per day for five days. Linearity was evaluated in the concentration range LOQ-2500 μg L^{-1}. The measure of matrix effect was estimated from the slopes ratio of the pesticides calibration curves in matrix and in solvent.

RESULTS AND DISCUSSION

Optimization of Extraction Procedure

Considering a wide range of pesticides physicochemical properties and their different chemical classes, extraction protocol often includes multistep procedure with additional sample clean up and usage of a large amount of chemicals. The QuEChERS method includes simple extraction with acetonitrile, partitioning with magnesium sulphate and other salts following additional clean up step using dispersive solid phase extraction (dSPE). As mentioned before, the proposed method was successfully applied on food and vegetables matrices.[9]

The main goal of this study was to develop and optimize new multiresidue method for determination of pesticides in wine based on QuEChERS methodology.[5,13,14] Original QuEChES methodology was applied (6 g of magnesium sulphate and 1.5 g of sodium acetate) but did not give satisfactory recoveries, < 20 % (Figure 1). An important factor that could affect the extraction efficiency is high ethanol content present in wine, ranging between 11–14 %.[5] In accordance with QuEChERS methodology, dilution with water did not improve extraction efficiency. These results are consistent with available literature reporting no improvement in recoveries for diluted samples with high water content (>80 %).[15]

One of the key elements in QuEChERS theory is salting out effect with magnesium sulphate and adjusting the polarity of organic phase.[3,16] Addition of salts induces phase separation and thus transition of analyte between two phases. It was observed that changing the amount of magnesium sulphate (2–8 g) improved recoveries, although not sufficiently. Addition of sodium chloride and trisodium citrate dihydrate to magnesium sulphate and sodium acetate had significant influence on recovery improvement. The purpose of sodium chloride is to increase ionic strength of the water, and thereby increasing its polarity.[16] The amount of sodium chloride is critical because the excess of sodium chloride reduces partitioning of polar pesticides to organic phase. Recoveries between 60–120 % were obtained with 1.5 g of sodium chloride, with exceptions for zoxamide (10 %) and captan (50 %). With higher amount of sodium chloride (3 g and 4 g) recoveries were lower for the most pesticides (< 70 %). An extended QuEChERS methodology includes the use of disodium citrate sesquihydrate along with magnesium sulphate, sodium chloride and sodium citrate.[21] However, addition of sodium citrate monobasic and disodium citrate sesquihydrate did not influence pesticide extraction.

It was observed that recoveries fluctuated significantly with changes in the amount of sodium acetate and trisodium citrate dihydrate. This could be related to pH of the sample. According to the original methodology, pH should be between 5 and 5.5.[9] However, pH at which extraction is performed can influence the coextraction of matrix compounds. Lower and higher pH values result in inefficient pesticide partitioning between two layers, depending on pesticide polarity. With addition of citrate salt and sodium acetate pH of the sample can be controlled. The acceptable recoveries (80–120 %) were achieved with 2 g of sodium acetate and 2 g of trisodium citrate dihydrate for all pesticides with exceptions for zoxamide (30 %) and captan (55 %). pH of the sample was 5.5 as in original methodology. The lower amount of these salts (1 g) resulted in lower recoveries (< 80 %). Addition of different amounts of salt resulted in variation of the red colour of the organic phase what could indicate pH change.

Above mentioned reddish colour indicates the presence of anthocyanins.[6,22] At natural pH of wine (3–3.5) anthocyanins are present in ionized form. Due to their reactivity, anthocyanins are expected to be extracted with pesticide thus pronouncing the matrix effect. With pH increasing anthocyanins start to deprotonate. The result of these reactions is colour change from red to bluish red.

Sample cleanup is essential for matrix interferences removal. The obtained results showed extremely low recoveries (< 20 %) without sample clean up. Due to its high ion exchange ability, PSA is the most popular solid phase extraction (SPE) sorbent for pesticide multiresidue analysis with successful matrix interference removal.[23] Considering that wine is water based sample without presence of fat, carotenoids or chlorophyll, dispersive SPE is performed using PSA according to the original methodology. The effect

of different amounts of PSA was investigated (200 mg, 400 mg and 600 mg). At higher amount of PSA (600 mg) recoveries were lower compared to medium amount (400 mg). This could be related to immediate pH change to 8 after the contact with PS.[19] At higher pH many pesticides become unstable. The degradation of captan and chlorothalonil can occur at higher amount of PSA which has been confirmed by recovery loss and chromatogram appearances. At 200 mg of PSA recoveries were equal or lower to those obtained at 600 mg of PSA. Compared to colourless extracts obtained with 400 mg and 600 mg of PSA, extraction with 200 mg of PSA yielded reddish extracts indicating inefficient anthocyanins removal and causing low pesticide recoveries. Based on obtained results 400 mg of PSA was chosen as the amount of sorbent needed for efficient matrix interference removal. For the removal of excess water and additional improvement of extraction, magnesium sulphate has been added to PSA. It has been noted that the amount of magnesium sulphate did not have significant influence at this step of sample preparation. Therefore, 1.2 g of magnesium sulphate was chosen according to the original methodology.

Our results indicate that original QuEChERS methodology is not applicable for determination of pesticide residues in red wine. Obtained recoveries were not acceptable without further development and optimization of original methodology. For pesticide extraction pH should be 5–5.5.[9] However, pesticide extraction is inefficient without additional combination of salts which was not proposed by original methodology.

Method Validation

Validation of the proposed analytical methodology was performed according to the guidelines for pesticides residues monitoring[17] for which linearity, selectivity, limit of detection (LOD) and quantification (LOQ), precision and accuracy were determined. In order to reduce established matrix interference described in details in subsection 3.4., calibration solutions were prepared in matrix. Analytical

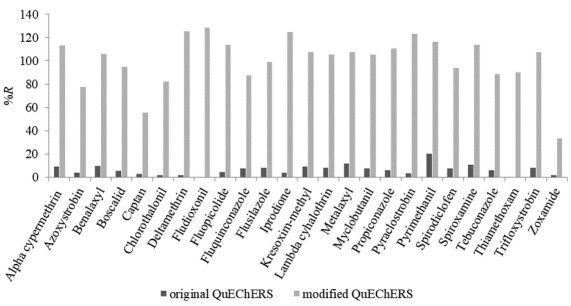

Figure 1. Comparison of original and modified QuEChERS methodology.

procedure was developed and validated in red wine with no presence of target pesticides.

The determination of *selectivity* was based on comparison of chromatograms of blank red wine and pesticide standard prepared in solvent. No interference on target peaks was observed.

LOD and *LOQ* were in the range 0.01–50 μg L^{-1} and 0.25–250 μg L^{-1} respectively. Values were determined based on signal-to-noise ratio (S/N), three times ratio for LOD and ten times ratio for LOQ, estimated from blank wine sample (Table 1).

Linearity was determined by calculating determination coefficient (R^2) of the linear regression equations based on minimum five point calibration curve. Concentration ranges and determination coefficient are shown in Table 1. Determination coefficient for all pesticide was > 0.99.

Recovery studies were performed at two concentration levels; 2 x LOQ and 10 x LOQ. Precision and accuracy were determined based on five replicates on each level for five consecutive days. At lower concentration level the intra-day recoveries were within acceptable range 70–120 % with the lowest recovery for deltamethrin and spirodiclofen (77 %) and the highest for chlorothalonil (120 %). For all pesticides relative standard deviations (RSD) at given level were < 20 % with highest value for boscalid and spiroxamine (7 %). At higher concentration level for 20 pesticide recoveries were between 70–120% with exception for spirodiclofen (67 %). RSD values for these pesticides were acceptable (< 20 %) with exception for fludioxonil (24 %). Recoveries were significantly lower for 5 pesticides: azoxystrobin (55 %), pyraclostrobin (43 %), boscalid (60 %), zoxamide (51 %) and captan (40 %) in comparison with lower level where recoveries were 115 %, 120 %, 94 %, 101 % and 89 % respectively. RSD values for these 5 pesticides were similar to those at lower level. For zoxamide RSD value were slightly higher (16 %) in comparison with lower level (3 %). Results obtained for these 5 pesticides indicate the presence of matrix which has been described in subsection 3.4. In addition, recovery loss is expected for these pesticides as mentioned in subsection 3.1.These pesticides are base sensitive so they start to degrade in contact with PSA where pH changes to 8, especially at higher concentrations. The average recoveries were calculated based on the results collected during five consecutive days. Recoveries at both concentration levels were in the range 70–120 % with RSD < 20 %. Exceptions were azoxystrobin (57 %), pyraclostrobin (42 %), boscalid (55 %), zoxamide (49 %) and captan (46 %) at higher concentration level. For fludioxonil average RSD was 18 % which is lower than intra-day value (24 %) in comparison with zoxamide whose intra-day RSD were higher (25 %) than inter-day value (16 %). The precision and accuracy data are demonstrated in the Table 1.

Table 1. LOD, LOQ, linearity, accuracy and precision at two concentration levels (2 × LOQ and 10 × LOQ). Recovery (%*R*) and relative standard deviation (RSD%).

Compound	LOD / μg L^{-1}	LOQ / μg L^{-1}	Linearity		Repeatibility[(a)]		Reproducibitily[(b)]	
					%*R* (RSD / %)		%*R* (RSD / %)	
			R^2	Range / μg L^{-1}	2 × LOQ	10 × LOQ	2 × LOQ	10 × LOQ
Azoxystrobin	50	250	0.9970	LOQ-2500	114.7 (2)	55.3 (6)	111.2 (4	56.5 (11)
Benalaxyl	0.01	0.25	0.9923	LOQ-1500	82.1 (3)	79.4 (6)	77.0 (4)	96.6 (6)
Boscalid	10	50	0.9917	LOQ-1000	94.1 (7)	60.1 (6)	94.7 (4)	54.9 (5)
Captan	2.5	50	0.9917	LOQ-2500	88.9 (1)	40.5 (3)	82.9 (3)	45.9 (4)
Chlorothalonil	1	10	0.9941	LOQ-2500	120.1 (3)	93.6 (3)	117.0 (7)	104.2 (4)
Lambda cyhalothrin	0.025	5	0.9954	LOQ-1500	81.6 (2)	74.7 (4)	75.8 (3)	79.4 (5)
Alpha cypemethrin	0.5	5	0.9955	LOQ-1500	80.3 (4)	73.2 (2)	78.5 (4)	83.5 (4)
Deltamethrin	5	50	0.9921	LOQ-2500	76.7 (2)	75.5 (3)	74.4 (3)	87.3 (3)
Fludioxonil	50	250	0.9916	LOQ-2500	109.9 (3)	73.4 (24)	103.9 (3)	74.4 (18)
Fluopicolide	0.25	5	0.9931	LOQ-1500	110.0 (3)	81.5 (4)	109.4 (3)	85.5 (4)
Fluquinconazole	0.01	5	0.9903	LOQ-1000	83.1 (1)	70.0 (4)	75.0 (3)	70.5 (4)
Flusilazole	0.5	5	0.9928	LOQ-1500	117.6 (2)	76.6 (9)	111.2 (3)	79.0 (5)
Iprodione	0.01	1	0.9979	LOQ-1500	93.0 (2)	106.2 (3)	94.7 (4)	118.0 (4)
Kresoxim methyl	0.5	5	0.9919	LOQ-1500	102.0 (2)	81.1 (2)	95.0 (3)	90.5 (3)
Metalaxyl	0.01	10	0.9993	LOQ-400	119.3 (0.4)	96.9 (2)	112.0 (3)	105.4 (3)
Myclobutanil	2.5	10	0.9928	LOQ-1500	119.2 (5)	90.2 (2)	121.1 (4)	102.2 (4)
Propiconazole	2.5	5	0.9984	LOQ-400	99.5 (1)	100.1 (5)	98.4 (3)	90.6 (6)
Pyraclostrobin	50	250	0.9944	LOQ-2500	120.0 (1)	43.5 (7)	118.9 (3)	41.5 (10)
Pyrimethanil	0.01	5	0.9923	LOQ-1500	104.9 (1)	116.1 (2)	114.4 (3)	121.1 (4)
Spirodiclofen	5	50	0.9921	LOQ-2500	76.7 (3)	67.1 (2)	78.1 (3)	69.2 (4)
Spiroxamine	10	50	0.9906	LOQ-1500	110.7 (7)	91.3 (2)	112.5 (4)	102.3 (3)
Tebuconazole	25	50	0.9981	LOQ-1000	115.4 (2)	87.3 (22)	107.9 (3)	81.1 (8)
Thiamethoxam	25	50	0.9953	LOQ-2000	113.4 (2)	81.9 (5)	107.8 (3)	86.3 (5)
Trifloxystrobin	0.025	5	0.9912	LOQ-1500	117.9 (4)	71.3 (3)	114.5 (3)	81.7 (3)
Zoxamide	5	50	0.9901	LOQ-1500	100.8 (3)	50.5 (16)	88.1 (4)	49.3 (25)

LOD, LOQ, linearity, accuracy and precision at two concentration levels (2 × LOQ and 10 × LOQ). Recovery (%*R*) and relative standard deviation (RSD%).

[(a)] Intra-day (5 replicates in one day).

[(b)] Inter-day (5 replicates in five consecutive days).

R^2 – determination coefficient, %*R* – recovery, RSD – relative standard deviation.

Measurement Uncertainty

Measurement uncertainty was determined using validation data obtained from five replicates on two concentration levels (2 × LOQ and 10 × LOQ) for five consecutive days. As uncertainty (u) of the measurement (Y) can be expressed as: $u(Y)^2 = s^2_{WL} + u(\delta)^2 + s^2_{other}$, where s_{WL} stands for within-laboratory standard deviation, $u(\delta)$ is uncertainty associated with the bias of the method and s_{other} stands for all other contribution to overall uncertainty.[11] Since the major contributions to uncertainty are from within-laboratory standard deviation and uncertainty of the method, overall uncertainty can be expressed in simplified form as $u(Y)^2 = s^2_{WL} + u(\delta)^2$. It is possible to estimate bias uncertainty of the method $u(\delta)$ and within-laboratory variance from within day s^2_{WD} and between-days s^2_{BD} variances, thus analysis of variance procedure (ANOVA) was conducted over validation dataset. Assuming that all measurements for each pesticide on each day on both concentration levels are independent, assuming normality

of the measurements and considering that all variances were similar, ANOVA procedure was used to retrieve within-day s^2_{WD} and between-days s^2_{BD} variances. Uncertainty of the measurements $u(Y)$ were afterword expanded into expanded uncertainty $U(Y)$ with coverage factor k = 2 to account for 95 % confidence interval and results are given in Table 2.

Based on uncertainty values from Table 2 it can be concluded that relative uncertainties are generally similar on two concentration levels with tendency for slightly higher uncertainty on higher concentration level (10 × LOQ). Noticeable exceptions are pyraclostrobin and azoxystrobin since relative expanded uncertainties $U(Y)$r for them are lower on higher concentration level (could be related to high LOQ values). Chlorothalonil (on two concentration levels), together with kresoxim-methyl, lambda-cyhalothrin and benalaxyl (only on higher concentration level) have relative expended uncertainties around or higher than 50 %, while it was found for the rest of the pesticides that relative expended uncertainties are lower than 50 %, that is, below acceptable value.

Matrix Effect

The matrix effect is still one of the major problems in pesticides analysis. Although determination techniques like liquid and gas chromatography coupled with tandem mass spectrometry are powerful tools for identification and quantification of pesticides residues, in many cases they are not sufficient for reducing influence of matrix. Source of errors can be both, matrix and analyte dependent. Active

Table 2. Measurement uncertainty at two concentration levels (2 × LOQ and 10 × LOQ) for 25 pesticides estimated from validation data over five days.

Compound	2 × LOQ / µg L^{-1}	s_{WL} / µg L^{-1}	$u(\delta)$ / µg L^{-1}	$u(Y)$ / µg L^{-1}	$U(Y)$ / µg L^{-1}	$U(Y)$r / %	10 × LOQ / µg L^{-1}	s_{WL} / µg L^{-1}	$u(\delta)$ / µg L^{-1}	$u(Y)$ / µg L^{-1}	$U(Y)$ / µg L^{-1}	$U(Y)$r / %
Alpha cypermethrin	10.12	1.016	0.38	1.085	2.169	21 %	50.6	8.847	3.679	9.582	19.163	38 %
Azoxystrobin	407.59	55.697	22.199	59.958	119.916	29 %	2499.5	177.739	50.543	184.786	369.572	15 %
Benalaxyl	0.5	0.059	0.027	0.065	0.129	26 %	2.51	0.889	0.276	0.931	1.862	74 %
Boscalid	101.4	7.86	2.335	8.2	16.399	16 %	405.59	36.381	13.342	38.75	77.499	19 %
Captan	102.09	11.041	4.534	11.935	23.87	23 %	408.36	55.622	23.627	60.432	120.863	30 %
Chlorothalonil	24.83	6.477	2.247	6.856	13.712	55 %	99.3	44.229	15.475	46.859	93.717	94 %
Deltamethrin	101.09	8.307	3.329	8.949	17.898	18 %	404.38	64.219	26.611	69.514	139.028	34 %
Fludioxonil	409.86	47.48	18.98	51.134	102.267	25 %	2486.88	428.564	179.09	464.479	928.958	37 %
Fluopicolide	9.7	1.372	0.56	1.482	2.964	31 %	48.51	5.874	1.884	6.169	12.337	25 %
Fluquinconazole	10.05	1.155	0.486	1.253	2.506	25 %	50.24	3.556	0.792	3.643	7.286	15 %
Flusilazole	9.65	1.166	0.473	1.259	2.518	26 %	48.27	8.344	2.911	8.837	17.674	37 %
Iprodione	2.54	0.391	0.166	0.425	0.849	33 %	10.18	2.261	0.802	2.399	4.799	47 %
Kresoxim methyl	9.71	1.411	0.591	1.53	3.06	32 %	48.54	12.162	4.718	13.045	26.091	54 %
Lambda cyhalothrin	9.93	1.043	0.43	1.128	2.257	23 %	49.63	11.764	4.383	12.554	25.109	51 %
Metalaxyl	25.35	3.617	1.443	3.894	7.788	31 %	101.4	20.293	8.123	21.858	43.717	43 %
Myclobutanil	25.22	2.435	0.743	2.546	5.092	20 %	100.89	17.871	7.619	19.427	38.855	39 %
Propiconazole	9.9	1.034	0.394	1.106	2.212	22 %	49.48	7.852	3.186	8.474	16.948	34 %
Pyraclostrobin	403.6	47.197	17.562	50.359	100.717	25 %	2497.5	199.451	64.763	209.702	419.405	17 %
Pyrimethanil	10.2	1.626	0.664	1.756	3.512	34 %	50.99	6.075	2.224	6.469	12.938	25 %
Spirodiclofen	99.12	9.199	3.703	9.917	19.833	20 %	396.47	25.965	7.716	27.087	54.175	14 %
Spiroxamine	99	8.861	2.291	9.152	18.305	18 %	396	76.292	33.358	83.266	166.532	42 %
Tebuconazole	98.01	14.712	6.054	15.909	31.817	32 %	392.03	42.262	16.372	45.322	90.645	23 %
Thiamethoxam	100.47	12.773	4.833	13.656	27.313	27 %	401.88	48.439	19.626	52.264	104.528	26 %
Trifloxystrobin	10.07	1.387	0.534	1.486	2.973	30 %	50.34	5.513	2.26	5.958	11.916	24 %
Zoxamide	103.09	8.800	3.909	9.629	19.257	19 %	412.34	40.747	15.065	43.443	86.886	21 %

s_{WL} – within-laboratory standard deviation, $u(\delta)$ – bias uncertainty of the method, $u(Y)$ – measurement uncertainty, $U(Y)$ – expended measurement uncertainty (k = 2), $U(Y)$r – relative measurement uncertainty.

sites in GC systems (injector, column, detector) are often responsible for enhancement of signal causing degradation or retention of analytes.[18] These active sites are filled only with analytes in the case of pesticides solvent solutions. However, in matrix samples these sites are filled mainly with matrix component which results in signal enhancement and/or suppression. There are several approaches aiming at reduction of matrix influence. The optimization of sample preparation is the most efficient way for reducing the amount of matrix components that are often coextracted together with analyte. The use of analyte protectants or isotopically labeled internal standards is a good choice, but very expensive and as such not applicable for routine analysis. The standard addition method is option for precise reduction of matrix components but it is time consuming and as such not practical for multiresidue analysis. Matrix-matched calibration is the most common approach due to its simplicity.

The measure of matrix effect can be estimated from signal responses in matrix and signal responses in solvent (for identical concentration of pesticide).[12,19] Equivalent measure of matrix effect (*ME*) can be deduced from the ratio of the slopes of the pesticide calibration curves in matrix and in solvent[5,21]. Constructed in this way, measure *ME* provides information on matrix effect in the whole linearity range. Matrix effect has been estimated using the latter approach, calculating *ME* using the formula: $ME(\%) = (1 - S_m / S_s) \times 100$, where S_m is the slope of the calibration curve in matrix while S_s is the slope of the calibration curve in pure solvent for same pesticide. Results of matrix effect estimation are presented in Table 3. Impact of the matrix was proclaimed as low for values of *ME* in range –20 % < *ME* <

Table 3. Physico-chemical properties of 25 pesticides, matrix effect (*ME*) and extraction efficiency (*EE*%).

Compound	molecular formula	molecular weight / g mol^{-1}	solubility in water / mg L^{-1}	K_{ow} (20°C)	*EE* / %	*ME* effect
Alpha cypermethrin	$C_{22}H_{19}Cl_2NO_3$	416.3	0.004	6.6	60	medium suppression
Azoxystrobin	$C_{22}H_{17}N_3O_5$	403.4	6	2.5	61	medium enhancement
Benalaxyl	$C_{20}H_{23}NO_3$	325.4	28.6	3.5	64	low effect
Boscalid	$C_{18}H_{12}Cl_2N_2O$	343.2	4.64	2.9	67	high suppression
Captan	$C_9H_8Cl_3NO_2S$	300.6	3.3	2.8	103	high suppression
Chlorothalonil	$C_8Cl_4N_2$	265.9	0.81	2.9	191	high suppression
Deltamethrin	$C_{22}H_{19}Br_2NO_3$	505.2	0.0002	4.6	58	medium suppression
Fludioxonil	$C_{12}H_6F_2N_2O_2$	248.2	1.8	4.1	72	medium suppression
Fluopicolide	$C_{14}H_8Cl_3F_3N_2O$	383.59	2.8	3.3	87	low effect
Fluquinconazole	$C_{16}H_8Cl_2FN_5O$	376.2	1	3.2	73	low effect
Flusilazole	$C_{16}H_{15}F_2N_3Si$	315.4	42	3.9	87	low effect
Iprodione	$C_{13}H_{13}Cl_2N_3O_3$	330.2	13	3.0	56	medium suppression
Kresoxim methyl	$C_{18}H_{19}NO_4$	313.8	2	3.4	92	low effect
Lambda cyhalothrin	$C_{22}H_{19}ClF_3NO_3$	449.9	0.005	7.0	63	medium suppression
Metalaxyl	$C_{15}H_{21}NO_4$	279.3	8.4	1.7	85	low effect
Myclobutanil	$C_{15}H_{17}ClN_4$	288.8	142	2.9	111	low effect
Propiconazole	$C_{15}H_{17}Cl_2N_3O_2$	342.2	100	3.7	88	low effect
Pyraclostrobin	$C_{19}H_{18}ClN_3O_4$	387.8	1.9	4.0	41	medium enhancement
Pyrimethanil	$C_{12}H_{13}N_3$	199.3	0.12	2.8	66	low effect
Spirodiclofen	$C_{21}H_{24}Cl_2O_4$	411.3	0.05	5.8	64	high suppression
Spiroxamine	$C_{18}H_{35}NO_2$	297.5	470	2.8	83	low effect
Tebuconazole	$C_{16}H_{22}ClN_3O$	307.8	32	3.7	86	medium enhancement
Thiamethoxam	$C_8H_{10}ClN_5O_3S$	291.7	4100	–0.1	68	medium enhancement
Trifloxystrobin	$C_{20}H_{19}F_3N_2O_4$	408.4	0.61	4.5	105	low effect
Zoxamide	$C_{14}H_{16}Cl_3NO_2$	336.7	0.681	3.8	67	high suppression

K_{ow} – distribution coefficient between n-octanol and water, EE – extraction efficiency, ME – matrix effect.

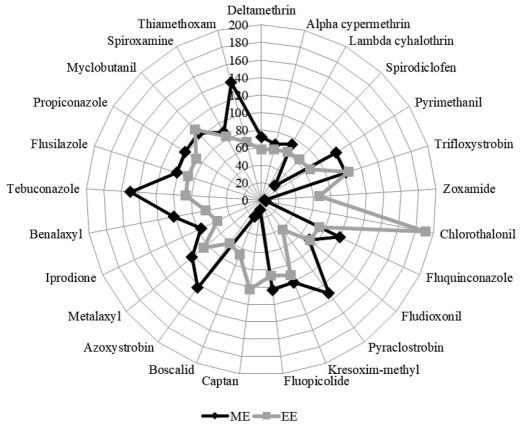

Figure 2. Matrix effect (ME) and extraction efficiency (EE) sorted by pesticides solubility in water.

20 %, medium suppression for –50 % < *ME* < –20 %, or high suppression for *ME* < –50 %. Positive values indicate analytical signal enhancement therefore values of *ME* in range 20 % < *ME* < 50 % were considered as medium enhancement while 50 % < *ME* was proclaimed as high enhancement. Based on this criteria out of 25 pesticides 11 of them demonstrate low matrix effect, 4 medium suppression, 5 medium enhancement and 5 high enhancement. Since matrix effect was established on 40 % of the pesticides of interest, use of matrix-matched calibration was justified.

Extraction efficiency (*EE*) of the procedure was calculated at two concentration levels (2 × LOQ and 10 × LOQ) as: $EE(\%) = [(A_{ss} - A_{ns}) / (A_{se} - A_{ne})] \times 100$, where A_{ss} is signal of wine sample spiked before extraction and A_{ns} is signal of non-spiked wine sample, A_{se} is signal of wine sample spiked after extraction and A_{ne} is signal of the non-spiked aliquot of the same wine sample. The average of two concentration levels for *EE* values are presented in Table 3. Extraction efficiency of proposed methodology was higher on 10 × LOQ concentration level for 68 % of the pesticides. In 76 % of the cases (pesticides), presence of matrix effect coincides with more significant extraction efficiency break from the norm which is considered to be around 100 %. This is another indicator of already assumed strong influence of the matrix.

In Figure 2 pesticides are ordered according to their solubility in water (Table 3) in a clockwise direction in increasing order starting with deltamethrin (0.0002 mg L^{-1}), for each pesticide average *EE* value and modified *ME* value are plotted. *ME* values for the purpose of demonstration are shifted by a factor of 100 so that 100 % represents low matrix effect and the rest of the criteria are shifted accordingly.

ME are generally low or medium, while *EE* values are between 60 and 120 %, with tendency for higher values of *EE* and more prominent *ME* for pesticides with higher solubility. Noticeable exceptions are spirodiclofen, chlorothalonil, pyraclostrobin and boscalid. In case of chlorothalonil high signal suppression found in *ME* together with extremely high *EE* could indicate high influence of matrix, not related to the extraction procedure.

Application of the Method

Developed method was successfully applied to 12 Croatian red wine samples of different variety. The five point matrix-matched calibration solutions (LOQ, 2 × LOQ, 5 x LOQ, 10 × LOQ and 25 × LOQ) were used for pesticide quantification. As demonstrated in Table 4, 10 pesticides were detected

Table 2. Concentration of target pesticides in 12 red wine samples (given as mean together with uncertainty in µg L^{-1}).

Wine code	Type of grape	Pyrimethanil	Metalaxyl	Benalaxyl	Trifloxystrobin	Tebuconazole	Cyhalothrin lambda	Cypermethrin alpha	Fluquinconazole	Myclobutanil	Flusilazole
45	Frankovka	61.1 ± 0.2	27±0.3	n.d.	n.d.	n.d.	6.0±0.2	n.d.	n.d.	n.d.	n.d.
46	Frankovka	16.9 ± 0.3	24.1 ± 0.3	1.6 ± 0.5	n.d.	n.d.	6.7 ± 0.2	n.d.	n.d.	n.d.	n.d.
47	Frankovka	17.3 ± 0.3	13.3 ± 0.3	19 ± 5	n.d.	79.8 ± 0.3	5.9 ± 0.2	n.d.	n.d.	n.d.	n.d.
48	Frankovka	61.8 ± 0.2	15.6 ± 0.3	1.9 ± 0.6	n.d.	n.d.	6.1 ± 0.2	n.d.	n.d.	n.d.	n.d.
49	Frankovka	21.8 ± 0.3	17.3 ± 0.3	1.5 ± 0.5	n.d.	n.d.	6.0 ± 0.2	10.4 ± 0.2	n.d.	n.d.	n.d.
50	Frankovka	22.1 ± 0.3	17. ± 0.3	2.3 ± 0.7	n.d.	n.d.	6.1 ± 0.2	n.d.	n.d.	n.d.	n.d.
51	Frankovka	19.5 ± 0.3	18.2 ± 0.3	1.7 ± 0.5	n.d.	n.d.	6.2 ± 0.2	6.3 ± 0.2	n.d.	n.d.	n.d.
52	Frankovka	17.4 ± 0.3	15.9 ± 0.3	1.4 ± 0.5	5.7 ± 0.3	n.d.	6.0 ± 0.2	6.3 ± 0.2	7.3 ± 0.3	20.1 ± 0.2	8.7 ± 0.3
53	Zweigelt	9.5 ± 0.4	19.0 ± 0.3	0.4 ± 0.2	n.d.	n.d.	6.6 ± 0.2	n.d.	n.d.	n.d.	n.d.
54	Zweigelt	n.d.	19.2 ± 0.3	0.6 ± 0.3	10.6 ± 0.3	n.d.	15.8 ± 0.3	22.1 ± 0.3	n.d.	n.d.	n.d.
55	Plavac	n.d.	14.3 ± 0.3	0.9 ± 0.4	5.8 ± 0.3	n.d.	n.d.	6.0 ± 0.2	n.d.	n.d.	n.d.
56	Pinot noir	n.d.	28.9 ± 0.3	1.2 ± 0.4	5.8 ± 0.3	n.d.	n.d.	6.4 ± 0.2	n.d.	n.d.	n.d.

n.d.- not detected

above LOQ value. Pyrimethanil and metalaxyl were detected in concentrations above 10 μg L^{-1}. Metalaxyl, lambda-cyhalothrin, pyrimethanil, benalaxyl and alpha-cypermethrin were most frequently found in red wines. At least 3 pesticides were found in each red wine.

Obtained results are consistent with findings of other authors. For example, Fontana[12] reported significant levels of metalaxyl, myclobutanil, benalaxyl and tebuconazole, while Economou[5] detected metalaxyl and pyrimethanil. Globally, the presence of these pesticides was confirmed in many European wines.[10,20,21] According to the recent study, a 30 % of overall analysed pesticides in grapes are transferred to wines.[22] Overall results indicate significant concentrations of pesticides residues in wine with raising concern for consumer intake.

CONCLUSION

Developed GC-MS methodology provides novel, selective and accurate approach for determination of 25 pesticide residues in red wine. At the same time, major findings of this research significantly expand current knowledge about the QuEChERS theory. Originally developed for vegetables products, QuEChERS method was proved to be inadequate for analysis of selected pesticides in red wine producing recoveries under 20 %. With the major goal of modifying the QuEChERS, as very simple extraction technique, and making it suitable for red wine analysis, a new multiresidue method has been developed and successfully applied on complex matrix of red wine. Limits of detection and quantification of new method were satisfactory (0.01–250 μg L^{-1}) and well below maximum residue levels prescribed by European Union. Presence of matrix effect has been investigated and confirmed. Matrix-matched calibration was used for quantification of pesticides due to established matrix effect. Quality of the method has been investigated and confirmed during the validation procedure. Satisfactory recoveries (70–120 %) and expanded relative uncertainties (< 50 %) were found for 80 % and 90 % of the pesticides respectively. The proposed analytical procedure has been applied to red wine samples of different origin. Results indicated significant levels of wine contamination what is in accordance with recent studies conducted in Europe.

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Author Contribution Statement. OP designed experiments, performed chemical analyses and wrote manuscript; AV performed biological assays; IC collected plant materials and assisted during the experiments.

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

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