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Stir bar sorptive extraction methods for determination of pesticide residues in wine and a study of storage wines from Slovenia

Metodi za določanje ostankov fitofarmacevtskih sredstev v vinu s sorpcijsko ekstrakcijo z mešalom ter študija slovenskih vin s trgovskih polic

Helena BAŠA ČESNIK (✉), Špela VELIKONJA BOLTA

Agricultural Institute of Slovenia, Hacquetova ulica 17, SI-1000 Ljubljana, Slovenia

✉ Corresponding author: helena.basa@kis.si

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ABSTRACT

New analytical methods for the determination of pesticide residues in wine were introduced and validated. Extraction was performed with sorption of the active substances on the stir bar and determination by gas chromatography-mass spectrometry and liquid chromatography-tandem mass spectrometry. The methods were applied in practice. A total of 77 active substances were searched for in 42 wine samples from all 3 wine-growing regions of Slovenia. The fungicides dimethomorph, fludioxonil and pyraclostrobin were found in 78.6% of all analysed samples. The risk assessment concluded that the analysed wine samples do not pose an unacceptable risk to consumers. The results were compared with previous studies.

Keywords: wine, GC-MS, LC-MS/MS, SBSE, pesticide residues, multiresidual method

IZVLEČEK

Uvedli in validirali smo novi analizni metodi za določanje ostankov fitofarmacevtskih sredstev v vinu. Ekstrakcijo smo izvedli s sorpcijo na vrteče se magnetne paličice in določitev s plinsko kromatografijo, sklopljeno z masno spektrometrijo, in s tekočinsko kromatografijo, sklopljeno s tandemsko masno spektrometrijo. Metodi smo uporabili v praksi. V 42 vzorcih vina, zbranih iz vseh treh vinorodnih območij Slovenije, smo določali skupno 77 aktivnih spojin. Fungicide dimetomorf, fludioksonil in piraklostrobin smo določili v 78,6 % vseh analiziranih vzorcev. Z oceno tveganja smo ugotovili, da analizirani vzorci vina ne predstavljajo nesprejemljivega tveganja za potrošnika. Rezultate smo primerjali s predhodnimi raziskavami.

Ključne besede: vino, GC-MS, LC-MS/MS, SBSE, ostanki pesticidov, multirezidualna metoda

INTRODUCTION

Wine is one of the most consumed beverages in the world (Ravelo-Pérez et al., 2008). Unfortunately, the grapes from which wine is made are attacked by various diseases and insects during their growth. The most common in Slovenia are powdery mildew, downy mildew, grey mould, and vine moth (Baša Česnik et al., 2012; Čuš et al., 2010). In order to achieve a healthy and abundant harvest, plant protection products (PPPs) are often used. The disadvantage of their application is residues of active substances, which PPPs contain, on the grapes. These substances can be toxic and therefore have a major impact on consumer health. Modern-day consumers are demanding and require not only high quality wine but also wine that is safe for consumption. Multiresidual analytical methods, capable of measuring numerous active substance residues simultaneously, are therefore needed.

There are two main ways of analysing pesticide residues in wine. The first is the use of organic solvents. Liquid-solid extraction, followed by dispersive solid phase extraction is nowadays mainly performed by the QuEChERS method, using acetonitrile as the organic solvent (Cunha et al., 2009; Hou et al., 2020). Solid phase extraction is performed with cartridges containing C-18 sorbent (Doulia et al., 2017) or hydrophilically modified styrene polymer (Castro et al., 2020; Vitali Čepo et al., 2018), using ethyl acetate (Doulia et. al., 2017), acetonitrile and methanol mixture (Castro et al., 2020) or n-hexane and acetonitrile (Vitali Čepo et al., 2018) as eluent. The second approach is the so-called Green Analytical Chemistry supported extraction methodology, where no organic solvents are used. Solid phase microextraction (SPME), introduced by Arthur and Pawliszyn (1990), can be used, where fibres with different coated sorbents such as polydimethylsiloxane (PDMS) (Martins et al., 2011), polydimethylsiloxane-divinylbenzene (PDMS-DVB) (Millá et al., 2003) or polyacrilate (PA) (Zambonin et al., 2004) can be used. In the SPME technique, the fibre is usually immersed in the wine sample and the active substances are subsequently desorbed from their coating. Another option is the stir bar sorptive extraction (SBSE), technique introduced by Baltussen et al. (1999). In the SBSE technique, a stir bar coated with an adsorbent is immersed in the wine sample and stirred so that the active substances are absorbed onto it. Desorption is done thermally or with a small amount of liquid solvent. The main advantage of SBSE over SPME is that it contains a larger amount of adsorbent, allowing 500-fold higher enrichment than SPME (Sandra et al., 2001). Although, new SPME approach, SPME arrow, has large sorbent volume, which increases sample capacity and efficiency of extraction (Lan et al., 2017). The first to commercialise SBSE was Gerstel under the name Twister. Nowadays, two types of stir bars are available: one with PDMS and one with ethylene glycol modified silicone material (EG-silicone). In our methods, dual SBSE was used, in which active substances were adsorbed on PDMS and from the same sample solution on an EG-silicone stir bar.

The determination of PPP residues can be performed by gas chromatography coupled with mass spectrometry (GC-MS) (Zambonin et al., 2004), gas chromatography coupled with tandem mass spectrometry (GC-MS/MS) (Martins et al., 2011) and/or liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) (Castro et al., 2020).

A few authors in Europe have analysed pesticide residues in wine. Vitali Čepo et al. (2018) and Pelajić et al. (2016) analysed 25 active substances in wine samples in Croatia. Ravelo-Pérez et al. (2008) analysed 11 active substances in wine samples in Spain. Čuš et al. (2010) analysed wine samples from Slovenia for 116 active substances. Many of the active substances sought in these studies were also introduced in our study.

In this paper, the multiresidual SBSE GC-MS method for determination of 50 active substances and SBSE LC-MS/MS method for determination of 27 active substances in wine are presented. The validation parameters are summarised as well as the practical application of the method on 42 commercially purchased samples of Slovenian wine from all three wine-growing regions. The contents of pesticide residues were compared with those found in the literature. Finally, a risk assessment for consumers was carried out.

MATERIALS AND METHODS

Materials

Chemicals

The certified standards were supplied by Dr. Ehrenstorfer (Augsburg, Germany) and PESTANAL (Darmstadt, Germany). The acetonitrile HPLC-grade (used for the ultrasound extraction procedure), acetone HPLC-grade (used for preparation of standards analysed by GC) and methanol HPLC-grade (used for preparation of standards analysed by LC) were supplied by J.T.Baker (Deventer, Netherlands). All other chemicals used were provided by Sigma-Aldrich (Steinheim, Germany). The water used was MilliQ deionised water. The SBSE stir bars (PDMS and EG-silicone) were supplied by Agilent Technologies (Santa Clara, USA).

Preparation of the solutions for GC-MS determination

Stock solutions of individual active substances were prepared in acetone at the concentrations of 625 μg pesticide/mL. Two mixed solutions were prepared from 50 stock solutions: the first of 46 and the second of 4 active substances, with concentrations of 5 and 1 μg/mL in acetone. The huge difference of number of pesticides in mixtures is consequence of fact that the first mixture is used for analyses of fruit, vegetables, honey and pollen as well (where also some different active substances are measured) and the second mixture is used for analyses of wine only. Besides, two mixtures were prepared since two chromatographic runs were used for wine analyses. Solutions used to determine the linearity, LOQs, measurement uncertainty and accuracy and to perform calibration during sample analysis were prepared in acetonitrile from a mixed solution of 5 μg/mL or 1 μg/mL with appropriate dilutions.

Preparation of the solutions for LC-MS/MS determination

Stock solutions of individual active substances were prepared in methanol at the concentrations of 625 μg pesticide/mL. From 27 stock solutions, one mix solution of all 27 active substances was prepared in methanol with a concentration of 3.125 μg/mL. Solutions used to determine the linearity, LOQs, measurement uncertainty and accuracy and to perform calibration during sample analysis were prepared in methanol from a mix solution of 3.125 μg/mL with appropriate dilutions.

Extraction procedure

The extraction procedure was based on literature references and our own experiments. Two SBSE stir bars with different coatings were used as our tests showed that recoveries were poor with only the first or only the second bar. Dual SBSE (two different SBSE stir bars one after another) improved the recoveries significantly (up to 50%). Sample volume was selected based on an experiment testing 10 ml and 20 ml headspace vials, filled with 10 ml and 20 ml sample volume. The recoveries for 20 ml sample volume were 10-30 % worse than those where 10 ml sample volume was chosen. The reason for this is probably that higher volumes cannot be mixed as efficiently with stir bars as low volumes. The tests showed that the highest recoveries were obtained at 3 h extraction time and extending this time by 1 h did not give significantly better results. We compared stirring speeds of 750 and 1000 rpm and there was not much difference. In the literature (Hauser et al., 2002), a steering speed of 1000 rpm was used, so it was also used in our extraction procedure. Based on the fact in the literature that salt addition improves extraction efficiency for more polar compounds (Ochiai et al., 2006), we used salt addition for the compounds analysed by LC. Acetonitrile was used as extraction solvent because it has been recommended in the literature (Hauser et al., 2002) and is used as part of QuEChERS methods in GC and LC analyses. The solvent volume was chosen as 1 ml is just enough to cover both SBSE stir bars in the 2 ml vial, allowing efficient extraction and lower detection limits (the smaller final volume means a higher final concentration). The extraction time from the SBSE stir bars to the solvent was tested for 15 and 30 min. Since 30 minutes gave slightly better results for some compounds, it was used for the final extraction procedure.

Samples were analysed within a maximum period of 2 months after arrival at the laboratory. During this time, they were stored at -20 °C.

Extraction procedure for GC-MS analyses

10 ml of wine was transferred to a 10 mL headspace vial. PDMS stir bar was added and the vial was capped. The 10 mL vial was placed on the magnetic stirrer at 1000 rpm for 3 h. Then the PDMS stir bar was transferred to a 2 mL vial. EG-Silicone stir bar was placed in the same 10 mL headspace vial, which was resealed and placed on the magnetic stirrer for 3 h at 1000 rpm. Then EG-Silicone stir bar was transferred to a 2 mL vial that already contained PDMS stir bar. 1 mL of acetonitrile was added and the 2 mL vial was capped and placed in an ultrasonic bath for 30 min. The contents of the 2 mL vial were transferred to another 2 mL vial with 100 μL insert and analysed by

GC-MS. After use, PDMS and EG-Silicone stir bars were cleaned in 2 mL vials, filled with acetonitrile and capped in an ultrasonic bath for 15 min.

Extraction procedure for LC-MS/MS analyses

10 ml of wine was transferred to a 20 mL headspace vial and 3 g of sodium chloride (p.a.) was added. The rest of the extraction procedure was the same as for GC-MS.

Determination

Determination with GC-MS

Samples were analysed using a gas chromatograph (Agilent Technologies 7890A, Palo Alto, CA, USA) equipped with a Gerstel MPS2 multipurpose sampler (Gerstel, Mülheim an der Ruhr, Germany) and a HP-5 MS UI column (Agilent Technologies, 30 m, 0.25 mm i.d., 0.25 µm film thickness) with a constant helium flow of 1.2 mL/min and injection volume of 2 µL. For 4 active compounds (etoxazole, oxyfluorfen, piriofenone and prokvinazide), the GC oven was programmed as follows: 55 °C for 2 min, from 55 °C to 180 °C at 40 °C/min, from 180 °C to 200 °C at 10 °C/min, held at 200 °C for 20 min, from 200 °C to 280 °C at 5 °C/min, held at 280 °C for 15.875 min. For the remaining 46 active compounds, the GC oven was programmed as follows: 55 °C for 2 min, from 55 °C to 130 °C at 25 °C/min, held at 130 °C for 1 min, from 130 °C to 180 °C at 5 °C/min, held at 180 °C for 30 min, from 180 °C to 230 °C at 20 °C/min, held at 230 °C for 16 min, from 230 °C to 250 °C at 20 °C/min, held at 250 °C for 13 min, from 250 °C to 280 °C at 20 °C/min, held at 280 °C for 20 min. A mass spectrometer (Agilent Technologies 5975C, upgraded with a triple-axis detector, Palo Alto, CA, USA) was used to determine the analytes using electron ionization. The ion source temperature was 230 °C, the auxiliary temperature was 280 °C and the quadrupole temperature was 150 °C.

Retention time and ratio of selected ions in the SIM were used for qualitative determination. For each active substance, one target and two qualifier ions, shown in Table 1, were used. Calibration was performed against standards in acetonitrile.

Chromatogram of standard solution for GC-MS is shown in Figure 1 and chromatogram of sample analysed with GC-MS is shown in Figure 2.

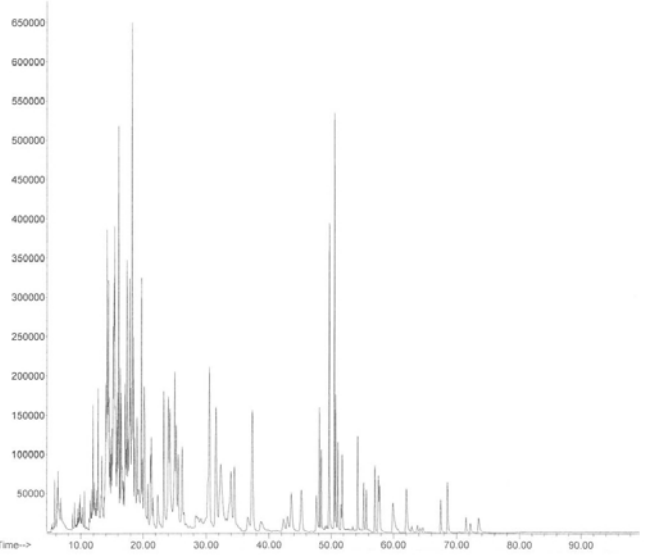

Figure 1. Chromatogram of the standard solution for GC-MS

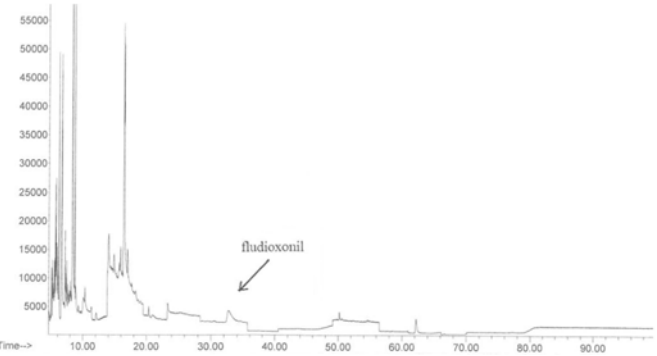

Figure 2. Chromatogram of a sample analysed with GC-MS

Determination with LC-MS/MS

The samples were analysed using a liquid chromatograph (Agilent Infinity 1290, Palo Alto, USA) on a Titan™ C18 80A column (10 cm x 2.1 mm, 1.9 µm), Supelco with the gradient of 0.1% formic acid (A) and 0.1% methanol with added formic acid (B). Each sample was injected once with injection volume of 1 µL. The flow was 0.4 mL/min and the gradient was as follows: start at 3% B and hold for 3 min, increase to 15% B in 20 min, then increase to 100% B in 2 min, hold 100% B for 5 min, decrease to 3% B in 1 min, post run 2 min at 3% B. For the determination of analytes, positive electro spray (ESI) mode and triple quadrupole mass spectrometer Agilent 6460 (Agilent Technologies Palo Alto, CA, USA) were used.

Table 1. Validation parameters and ions scanned for active substances analysed with GC-MS

Active substance	Ions scanned (m/z)[a] T, Q1, Q2	Linearity range (µg/L)	R^2	LOD[b] (µg/L)	LOQ[c] (µg/L)	Recovery (%)	RSDd (%)	U_r[e] (µg/L)	U_r[f] (%)	U_R[g] (µg/L)	U_R[h] (%)
acrinathrin	*181, 208, 289*	10-100	0.983	3	10	94.7	15.1	2.0	19.7	3.3	32.8
aldrin	263, 265, 261	10-90	0.984	3	10	88.1	11.4	1.2	12.1	2.3	23.1
bifenthrin	181, 165, 166	10-100	0.974	3	10	90.6	14.3	1.9	18.9	3.0	29.8
bromopropylate	183, 341, 185	10-100	0.989	3	10	108.5	7.6	0.8	8.0	1.9	19.1
chlorpropham	213, 127, 154	10-70	0.974	3	10	85.5	12.0	1.0	9.9	2.4	23.7
chlorpyriphos	314, 316, 197	10-90	0.977	3	10	101.2	11.9	1.3	12.8	2.8	27.8
chlorpyriphos-methyl	286, 288, 125	10-90	0.981	3	10	100.5	12.1	1.4	13.6	2.8	28.0
cyhalotrin-lambda	181, 197, 208	10-90	0.967	3	10	116.9	13.6	1.6	15.6	3.7	36.7
cypermethrin	181, 163, 165	10-100	0.984	3	10	107.9	17.0	1.6	16.5	4.2	42.5
deltamethrin	181, 251, 255	10-100	0.974	3	10	99.4	18.3	2.7	27.1	4.2	41.8
diazinon	179, 304, 199	10-80	0.980	3	10	79.2	13.0	1.1	10.8	2.4	23.7
dichlofluanid	226, 123, 167	10-80	0.987	3	10	100.0	16.0	2.0	20.4	3.7	36.8
diniconazole	268, 270, 70	10-100	0.975	3	10	94.9	12.4	1.4	14.5	2.7	27.2
diphenylamine	169, 167, 168	10-90	0.981	3	10	104.8	9.9	1.6	16.4	2.4	23.9
endosulfan-sulphate	272, 274, 387	10-100	0.988	3	10	108.6	8.2	1.0	10.5	2.0	20.5
endrin	263, 261, 265	10-100	0.989	3	10	108.5	16.8	3.3	32.7	4.2	41.6
esfenvalerate + fenvalerate	125, 167, 225	20-200	0.975	6	20	99.6	17.6	4.9	24.6	8.1	40.3
etoxazole	141, 204, 300	10-70	0.991	3	10	100.4	13.5	2.3	23.0	3.1	31.0
fenitrothion	277, 260, 109	10-70	0.977	3	10	97.2	13.1	1.5	14.6	2.9	29.4
fenthion	278, 279, 280	10-80	0.982	3	10	103.0	11.8	1.4	14.3	2.8	28.1
fludioxonil	248, 154, 127	10-90	0.961	3	10	105.9	10.0	1.7	16.7	2.4	24.3
HCH-alpha	219, 181, 183	10-100	0.970	3	10	88.2	16.1	2.0	19.6	3.3	32.7
HCH-beta + lindan	219, 181, 183	20-200	0.984	6	20	97.6	14.8	5.1	25.6	6.6	33.1
HCH-deltha	219, 181, 183	10-90	0.985	3	10	96.0	8.4	0.8	7.9	1.9	18.7
heptachlor	272, 274, 270	10-80	0.977	3	10	93.9	13.0	1.2	11.9	2.8	28.2
hexachlorobenzene	284, 286, 282	10-80	0.961	3	10	93.9	10.1	1.3	12.9	2.2	21.9

Continued

Active substance	Ions scanned (m/z)[a] T, Q1, Q2	Linearity range (µg/L)	R^2	LOD[b] (µg/L)	LOQ[c] (µg/L)	Recovery (%)	RSDd (%)	U_r[e] (µg/L)	U_r[f] (%)	U_R[g] (µg/L)	U_R[h] (%)
indoxacarb	218, 264, 527	10-100	0.968	3	10	107.7	15.7	1.5	14.5	3.9	39.2
kresoxim-methyl	116, 206, 131	10-100	0.988	3	10	93.8	13.3	1.3	12.7	2.9	28.9
metrafenone	393, 408, 379	10-100	0.974	3	10	108.4	9.2	0.7	7.3	2.3	23.0
oxyfluorfen	252, 361, 300	10-90	0.996	3	10	119.5	12.9	1.9	19.0	3.5	35.0
parathion	291, 292, 235	10-70	0.976	3	10	109.2	15.4	1.5	14.7	3.9	38.9
parathion-methyl	263, 109, 125	10-70	0.985	3	10	92.7	13.9	1.5	15.3	3.0	29.7
permethrin	183, 163, 165	10-100	0.975	3	10	113.3	14.1	1.7	17.5	3.7	36.8
phorate	231, 260, 97	10-80	0.962	3	10	99.2	13.7	2.3	22.6	3.1	31.1
phosalone	182, 367, 121	10-70	0.966	3	10	109.9	16.3	1.8	17.6	4.1	41.3
pirimiphos-methyl	290, 305, 276	10-100	0.981	3	10	73.4	11.3	1.1	11.1	1.9	19.1
profenofos	208, 139, 339	10-70	0.970	3	10	103.3	16.5	1.0	9.9	4.0	39.6
proquinazid	288, 372, 245	10-90	0.991	3	10	115.7	12.2	2.2	22.0	3.2	32.0
pyriofenone	350, 334, 209	10-80	0.994	3	10	90.9	6.9	1.4	14.0	1.5	15.0
quinalphos	146, 298, 157	10-100	0.981	3	10	89.1	12.1	1.1	11.4	2.5	25.0
quinoxyfen	237, 272, 307	10-100	0.983	3	10	104.4	12.8	0.9	8.8	3.1	31.0
tetraconazole	336, 338, 337	10-80	0.964	3	10	81.5	14.0	1.1	11.1	2.6	26.3
tetradifon	159, 229, 356	10-100	0.988	3	10	107.6	10.1	1.2	12.0	2.5	25.0
tolclofos-methyl	265, 267, 250	10-90	0.982	3	10	98.7	11.7	1.4	14.1	2.7	26.6
tolylfluanid	238, 137, 240	10-100	0.973	3	10	103.2	17.3	1.1	11.2	4.1	41.5
triazophos	161, 162, 285	10-100	0.966	3	10	88.7	18.0	1.1	11.3	3.7	37.0
trifloxystrobin	116, 222, 186	10-100	0.977	3	10	104.5	7.8	0.9	8.8	1.9	18.8
vinclozolin	285, 124, 187	10-100	0.985	3	10	83.4	14.7	1.1	10.8	2.8	28.3

[a] T=target ion, Q=qualifier ion
[b] LOD=limit of detection
[c] LOQ=limit of quantification
[d] RSD was obtained during recovery analyses
[e,f] U_r=uncertainty of repeatability
[g,h] U_R=uncertainty of reproducibility

Table 2 Validation parameters and MRM transitions for active substances analysed with LC-MS/MS

Active substance	MRM transitions (m/z)	Linearity range (µg/L)	R^2	LOD[a] (µg/L)	LOQ[b] (µg/L)	Recovery (%)	RSD[c] (%)	U_r[d] (µg/L)	U_r[e] (%)	U_R[f] (µg/L)	U_R[g] (%)
beflubutamid	356 → 162 356 → 91	2-50	0.999	0.6	2	78.4	4.9	0.2	10.0	0.2	10.0
cinerin I	317 → 149 317 → 107	5-50	0.996	1.5	5	79.4	8.8	0.4	8.0	0.8	16.0
cinerin II	361 → 149 361 → 107										
clofentezine	303 → 138 303 → 102	2-100	0.999	0.6	2	82.3	8.9	0.3	15.0	0.5	25.0
cyazofamid	325 → 261 325 → 108	2-100	0.998	0.6	2	73.9	5.9	0.2	10.0	0.3	15.0
cyflufenamid	413 → 295 413 → 241	2-100	0.999	0.6	2	83.8	7.0	0.3	15.0	0.3	15.0
desmedipham	301 →182 301 → 136	2-50	0.996	0.6	2	75.4	11.7	0.8	40.0	0.9	45.0
difenoconazole	406 → 337 406 → 251	2-100	0.999	0.6	2	75.9	13.2	0.2	10.0	0.5	25.0
diflufenican	395 → 266 395 → 238	2-100	0.999	0.6	2	80.8	6.0	0.2	10.0	0.3	15.0
dimethomorph	388 → 301 388 → 165	2-100	0.999	0.6	2	73.1	6.9	0.2	10.0	0.9	45.0
fenezaquin	307 → 161 331 → 81	2-100	0.997	0.6	2	75.6	5.8	0.2	10.0	0.3	15.0
fenoxaprop-P-ethyl	364 → 290 364 → 288	2-100	0.999	0.6	2	71.3	7.2	0.2	10.0	0.3	15.0
fenoxycarb	302 → 116 302 → 88	2-100	0.998	0.6	2	77.2	6.4	0.2	10.0	0.3	15.0
fipronil	454 → 437 454 → 368	2-100	0.993	0.6	2	70.5	7.4	0.1	5.0	0.2	10.0
fluazifop butyl	384 → 328 384 → 282	2-100	0.998	0.6	2	79.0	6.6	0.2	10.0	0.3	15.0
flufenacet	364 → 194 364 → 152	2-100	0.998	0.6	2	71.1	7.2	0.2	10.0	0.2	10.0
fluorochloridone	312 → 292 312 → 145	2-100	0.999	0.6	2	77.5	9.7	0.3	15.0	0.4	20.0
hexythiazox	353 → 228 353 → 168	2-100	0.999	0.6	2	78.5	6.0	0.2	10.0	0.3	15.0
pendimethalin	282 → 212 282 → 194	2-100	0.999	0.6	2	92.6	6.0	0.2	10.0	1.0	50.0

Continued

Active substance	MRM transitions (m/z)	Linearity range (µg/L)	R^2	LOD[a] (µg/L)	LOQ[b] (µg/L)	Recovery (%)	RSD[c] (%)	U_r[d] (µg/L)	U_r[e] (%)	U_R[f] (µg/L)	U_R[g] (%)
phoxim	299 → 129 299 → 77	2-100	0.998	0.6	2	85.0	5.9	0.2	10.0	0.3	15.0
piraclostrobin	388 → 194 388 → 163	2-100	0.996	0.6	2	78.6	4.9	0.2	10.0	0.3	15.0
propaquizafop	444 → 299 444 → 100	2-100	0.999	0.6	2	80.5	8.1	0.3	15.0	0.4	20.0
prosulfocarb	252 → 128 252 → 91	2-100	0.999	0.6	2	85.2	5.7	0.2	10.0	0.3	15.0
pyraflufen ethyl	413 → 339 413 → 289	2-100	0.999	0.6	2	87.5	6.6	0.3	15.0	0.5	25.0
pyrazophos	374 → 222 374 → 70	2-100	0.998	0.6	2	83.6	7.5	0.3	15.0	0.3	15.0
spirodiclofen	411 → 313 411 → 71	2-100	0.997	0.6	2	70.4	4.7	0.2	10.0	0.2	10.0
tebufenpyrad	334 → 145 334 → 117	2-100	0.999	0.6	2	84.3	5.7	0.2	10.0	0.3	15.0
teflubenzuron	381 → 158 381 → 141	2-100	0.998	0.6	2	86.3	15.1	0.3	15.0	0.6	30.0

[a] LOD = limit of detection
[b] LOQ = limit of quantification
[c] RSD was obtained during recovery analyses
[d,e] U_r = uncertainty of repeatability
[f,g] U_R = uncertainty of reproducibility

The source temperature was 250 °C, gas flow 6 L/min, sheath gas flow 10 L/min, sheath gas temperature 375 °C and nebuliser pressure 35 psi. Quadropole temperatures were 100 °C. For each compound, two transitions were monitored, and therefore the fragmentor and collision cell parameters were optimised. The data on possible MRM transitions were found in the EURL Pesticides Data pool accessible on the internet at https://www.eurl-pesticides-datapool.eu/. The choice of relevant MRM transitions and optimisation of fragmentor and collision cell voltages was performed using Agilent Optimizer software and standard solutions of active substances in methanol (1000 µg/L).

For quantitative determination, retention time and Multi Reaction Monitoring (MRM), peak area ratios were used. MRM transitions are presented in Table 2. The calibration was performed against standards in acetonitrile.

Chromatogram of standard solution for LC-MS/MS is shown in Figure 3 and chromatogram of sample analysed with LC-MS/MS is shown in Figure 4.

Validation of methods

LOQ and linearity

Linearity was verified with the standards in acetonitrile (three replicates for one concentration level, four to ten concentration levels for the calibration curve). Number of levels was reduced from 10 to 4 when curve was no longer linear but curved instead. Linearity and range were determined by linear regression, using the F test to conclude whether the linear model is fit and stays linear over the whole range.

LOQs were estimated from the chromatograms of the standards in acetonitrile. LOQs were chosen with a minimum of S/N = 10.

Precision

The Malvazija white wine was store-bought and analysed to prove that it did not contain target active substances (blank wine). To determine precision (ISO 5725), i.e. repeatability and reproducibility, extracts of fortified blank wine were analysed at LOQ. Within a period of 10 days, two parallel extracts were prepared

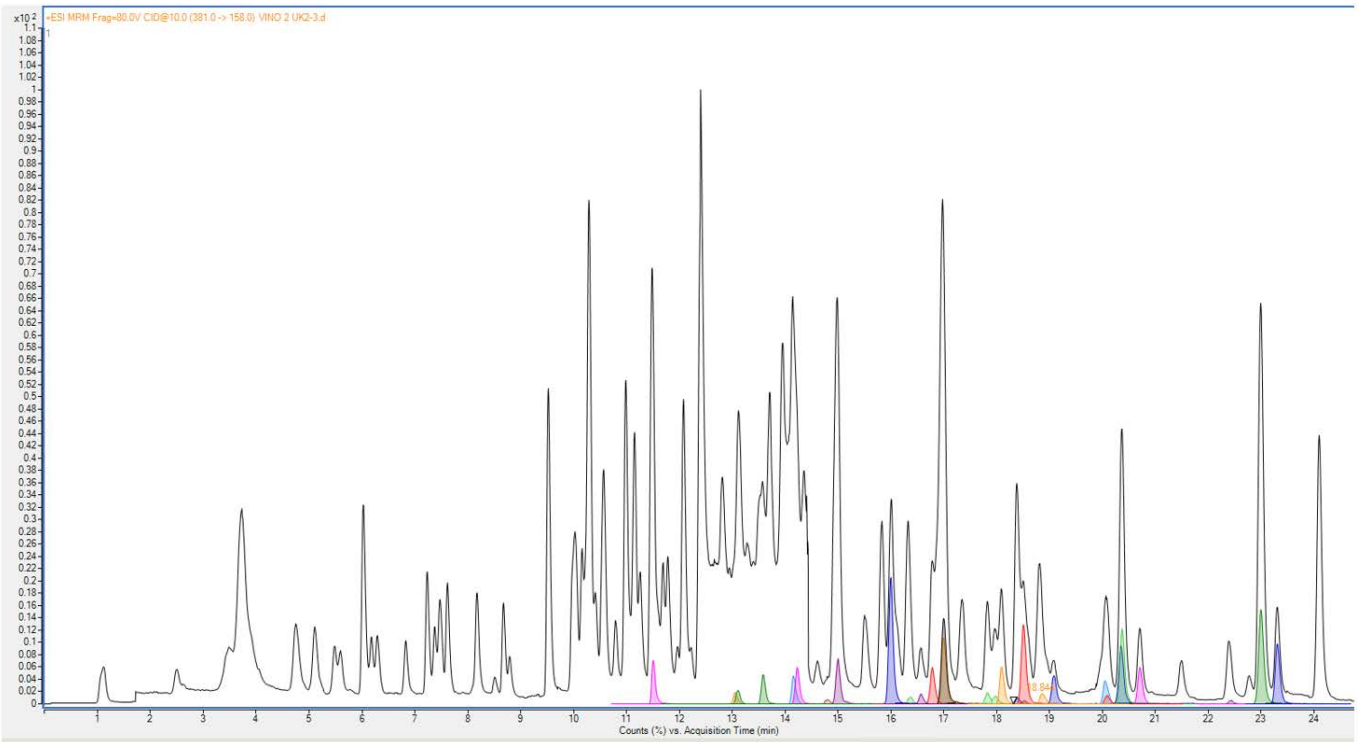

Figure 3. Chromatogram of the standard solution for LC-MS/MS

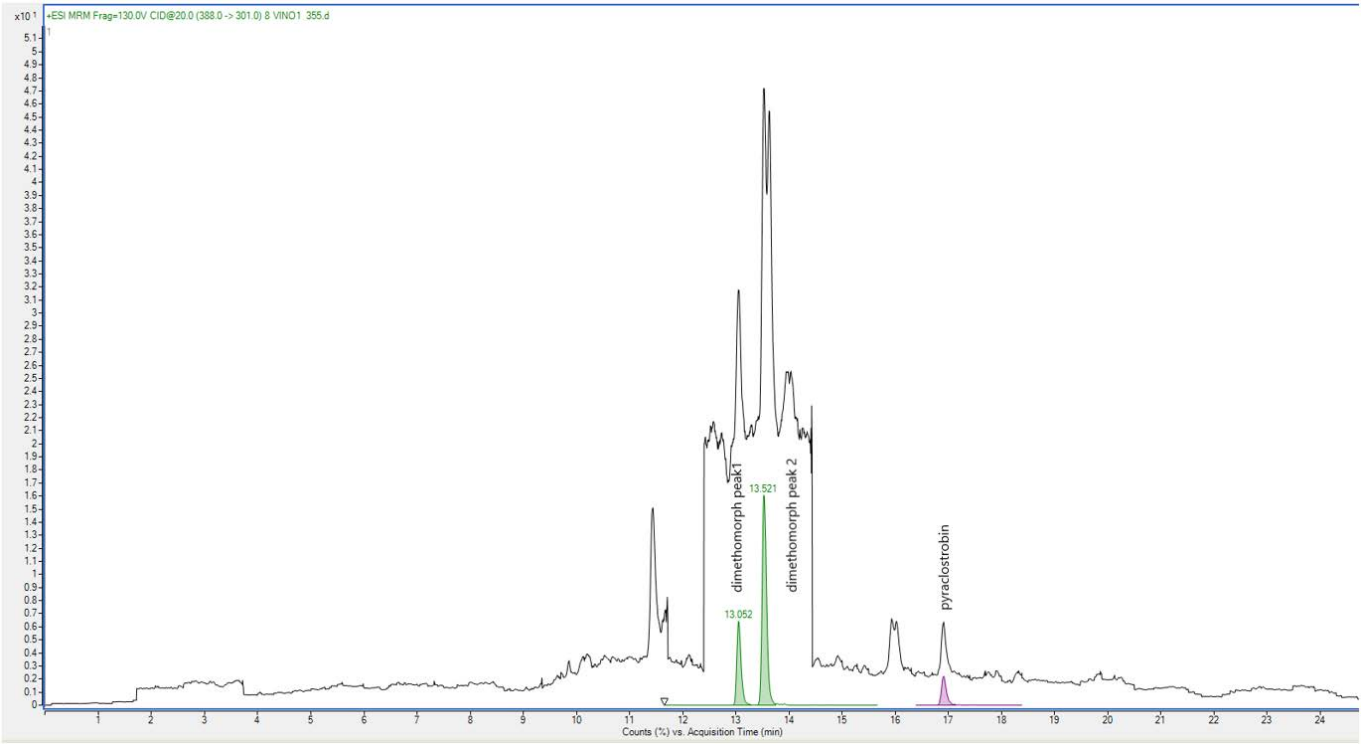

Figure 4. Chromatogram of a sample analysed with LC-MS/MS

each day for each concentration level. Then, both the standard deviation of repeatability of the level and the standard deviation of reproducibility of the level were calculated.

Uncertainty of repeatability and uncertainty of reproducibility

The uncertainty of repeatability and the uncertainty of reproducibility were calculated by multiplying the standard deviation of repeatability and the standard deviation of reproducibility by Student's t factor, for nine degrees of freedom and a 95% confidence level ($t_{95;9}$ = 2.262).

$$U_r = t_{95}; 9 \times s_r; U_R = t_{95}; 9 \times s_R$$

The measurement uncertainty for PPP residues should be 50%, as proposed in SANTE/11813/2017. During validation, analysts must demonstrate that their measurement uncertainty is below or equal to the proposed measurement uncertainty.

Accuracy

Accuracy was verified by checking the recoveries. The average of the recoveries from the tests for precision (10 days, 2 parallel samples per day) was calculated. According to the requirements for method validation procedures (SANTE/11813/2017), acceptable mean recoveries are those in the range of 70% to 120%, with an associated repeatability of RSD ≤ 20%.

According to the guidelines for single-laboratory validation (Alder et al., 2000), acceptable mean recoveries at a level of >1 µg/L ≤ 10 µg/L are those in the range of 60% to 120%, with an associated repeatability RSD ≤ 30%.

Consumer risk assessment

Long-term and short-term exposure was calculated using the United Kingdom model, which, unlike EFSA PRIMo model, includes consumer wine consumption. For chronic exposure, Supervised Trial Median Residue (STMR) was calculated and used as the input value. Chronic consumer exposure was expressed as a % of the Acceptable daily intake (ADI). The acceptable limit for long-term exposure is 100% of the ADI. For acute exposure, the highest residue (HR) was used as the input value. Acute consumer exposure was expressed as % of

Acute Reference Dose (ARfD). The acceptable limit for short-term exposure is 100% of the ARfD.

Sampling

A total of 42 wine samples (21 white and 21 red) were purchased from a shop in Slovenia in February 2021. The samples were collected from all 3 wine-growing regions in Slovenia. The distribution of the samples is shown in Table 3.

Table 3. Number of wine samples from different wine-growing regions in Slovenia in 2021 from one shop

Wine-growing region	Number of white wine samples	Number of red wine samples	Total
Posavje	4	6	10
Podravje	6	7	13
Primorska	11	8	19
Total	21	21	42

RESULTS AND DISCUSSION

Validation of methods

LOQ and linearity of GC-MS method

The linear model is valid for all active substances shown in Table 1. Linearity was demonstrated in the range from 10 μg/L to 70 μg/L for 7 active substances, in the range from 10 μg/L to 80 μg/L for 8 active substances, in the range from 10 μg/L to 90 μg/L for 10 active substances, in the range from 10 μg/L to 100 μg/L for 21 active substances and in the range from 20 μg/L to 200 μg/L for 4 active substances. R^2 ranged from 0.961 to 0.996. LOQs for all active substances were 10 μg/L the exception was active substances whose peaks were not resolved and were presented as a sum (esfenvalerate + fenvalerate and HCH-beta + lindan). The results are presented in Table 1.

LOQ and linearity of LC-MS/MS method

The linear model is valid for all active substances shown in Table 2. Linearity was demonstrated in the range from 2 μg/L to 50 μg/L for 2 active substances, in the range from 2 μg/L to 100 μg/L for 24 active substances and in the range from 5 μg/L to 50 μg/L for 1 active substance. R^2 ranged from 0.993 to 0.999. LOQs for all active substances were 2 μg/L the; exception was cinerin (sum of isomers cinerin I and cinerin II), where LOQ was 5 μg/L.

Accuracy of GC-MS method

The results for the recoveries are shown in Table 1. The recoveries at LOQs for active substances scanned by GC-MS ranged from 73.4% to 119.5%, with RSDs ranging from 6.9% to 18.3%.

Accuracy of LC-MS/MS method

The results for the recoveries are shown in Table 2. The recoveries at LOQs for active substances scanned by LC-MS/MS ranged from 70.4% to 92.6%, with RSDs ranging from 4.7% to 15.1%.

All recoveries and RSDs for GC-MS and LC-MS/MS determination are within the required ranges from the literature (Alder et al., 2000, SANTE/11813/2017).

Uncertainty of repeatability and uncertainty of reproducibility of GC-MS method

The uncertainty of repeatability and uncertainty of reproducibility were determined at contents corresponding to LOQs. The results are presented in Table 1. The uncertainty of repeatability ranged from 0.7 μg/L to 5.1 μg/L, which is 7.3% to 32.7% of LOQ, and the uncertainty of reproducibility ranged from 1.5 μg/L to 8.1 μg/L, which is 15.0% to 42.5% of LOQ.

Uncertainty of repeatability and uncertainty of reproducibility of LC-MS/MS method

The uncertainty of repeatability and uncertainty of reproducibility were determined at contents corresponding to LOQs. The results are presented in Table 2. The uncertainty of repeatability ranged from 0.1 μg/L to 0.8 μg/L, which is 5.0% to 40.0% of LOQ, and the uncertainty of reproducibility ranged from 0.2 μg/L to 1 μg/L, which is 10.0% to 50.0% of LOQ.

Measurement uncertainty for GC-MS and LC-MS/MS determination are acceptable according to SANTE/11813/2017.

Survey of pesticide residues in wine samples

The Ministry of Agriculture, Forestry and Food of Republic Slovenia reported that 582 PPPs, containing 239 active substances, were authorised for use on various agricultural products in Slovenia in 2020. The Statistical Office reported that in 2019, 1,000 tonnes of active substances were sold in Slovenia, where we have 476,000 hectares of agricultural land. In the last 28 years, a decreasing trend in the amount of PPPs sold in Slovenia by more than 50% has been observed. Fungicides account for more than two thirds of all PPPs used in Slovenia.

Of the 42 wine samples analysed, no pesticide residues were found in 21.4% (9 samples). 57.1% (24 samples) contained 1 active substance, 19.1% (8 samples) contained 2 active substances and 2.4% (1 sample) contained 3 active substances. Since no maximum residue levels (MRLs) are set for wine in the European Union, no comparison with MRLs is possible. However, the highest residue observed was 10 μg/L and 10 μg/kg is normally the lowest MRL for fruits and vegetables. Three active substances were found: dimethomorph in the range of <2 to 7 μg/L, fludioxonil in the range of <10 to 10 μg/L and pyraclostrobin at level <2 μg/L. Dimethomorph was found most frequently in 78.6% of the samples, followed by fludioxonil in 19.0% of the samples and pyraclostrobin in 4.8% of the samples. In Slovenia, dimethomorph, fludioxonil and pyraclostrobin are authorised fungicides for use on wine grapes, therefore their use was in compliance with the regulatory requirements.

A consumer risk assessment was performed using the United Kingdom model. This model was used because Slovenia did not create its own model. The same model is used in the process of registration of PPPs in Slovenia. For chronic exposure, the input values were STMRs (3 μg/L for dimethomorph, 10 μg/L for fludioxonil and 2 μg/L for pyraclostrobin) and ADIs (0.05 mg/kg bw/d for dimethomorph, 0.37 mg/kg bw/d for fludioxonil and 0.03 mg/kg bw/d for pyraclostrobin). Chronic exposure to dimethomorph, fludioxonil and pyraclostrobin represented < 1% of ADI for all groups (adults, children, vegetarians, elderly). For acute exposure, the input values were HRs (7 μg/L for dimethomorph and 2 μg/L for pyraclostrobin) and ARfDs (0.6 mg/kg bw for dimethomorph and 0.03 mg/kg bw for pyraclostrobin). The acute exposure for dimethomorph and pyraclostrobin represented < 1% of ARfD for all groups (adults, children, vegetarians, elderly). Since no Acute Reference Dose was established for fludioxonil, no acute exposure was calculated for this active substance. Based on these calculations, it was concluded that the wine samples analysed do not represent a cause for concern for consumers.

Our results were compared with the results from other scientific papers. Tworesearches were conducted in Croatia: one in 2016 (Pelajić et al., 2016) and the second in 2018 (Vitali Čepo et al., 2018). In the 2016 study, fludioxonil was found in a concentration range of 0.52-1.4 μg/L, and in the 2018 study in an average concentration 3 μg/L, which is lower than in our current study. Among active substances searched in the present investigation, kresoxim-methyl was also found in the range of 0.01-0.18 μg/L and spirodiclofen in the range of 0.02-0.34 μg/L in the 2016 Croatian investigation. Our detection limit for kresoxim-methyl was 3 μg/L and for spirodiclofen 0.6 μg/L, which is higher than the maximum concentration found in Croatia.

In our earlier investigations, dimethomorph and fludioxonil were also found. Dimethomorph was found in Teran PTP wine in the concentration range of 10-100 μg/L (Baša Česnik et al., 2015) and in Malvazija wine in the concentration range of 20-70 μg/L (Baša Česnik et al., 2016), which is higher than the maximum concentration from the present study. Fludioxonil was found in Teran PTP wine in the concentration range of 10-90 μg/L (Baša Česnik et al., 2015) and in Malvazija wine in the concentration of 20 μg/L (Baša Česnik et al., 2016), which is equal or higher than the maximum concentration from the present study. Pyraclostrobin was not found in previous studies, only in the present study.

CONCLUSIONS

In our research, SBSE GC-MS and LC-MS/MS methods were introduced and validated for the determination of pesticide residues in wine. The GC-MS method was

found to be suitable for measuring contents of 50 active substances and the LC-MS/MS method for 27 active substances.

The methods were used to analyse 42 wine samples from all 3 wine-growing regions of Slovenia. A total of 77 active substances were searched for, but only the fungicides dimethomorph, fludioxonil and pyraclostrobin were found in 78.6% of these samples. The active substances sought were not detected in 21.4% of analysed samples. A risk assessment concluded that the Slovenian wine samples do not give rise to concern for consumers.

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