Development and Validation of High-Performance Liquid Chromatography Method for Determination of Some Pesticide Residues in Table Grape

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Received: 19 May 2017; accepted: 19 May 2017

This study presents the development and validation of a new reversed-phase high-performance liquid chromatography (RP-HPLC) method for simultaneous determination of captan, folpet, and metalaxyl residues in table grape samples with ultraviolet–diode array detection (UV–DAD). Successful separation and quantitative determination of analytes was carried out on LiChrospher 60 RP-select B (250 × 4 mm, 5 μm) analytical column. Mixture of acetonitrile–0.1% formic acid in water (65:35, v/v) was used as a mobile phase, with flow rate of 1 mL/min, constant column temperature at 25 °C, and UV detection at 220 nm. The target residues were extracted with acetone by ultrasonication, followed by a cleanup using liquid–liquid extraction (LLE) and solid-phase extraction (SPE). The obtained values for multiple correlation coefficients ($R^2 > 0.90$), relative standard deviation (RSD) of retention times, peak areas and heights (RSD ≤ 2.25%), and recoveries ranging from 90.55% to 105.40%, with RSD of 0.02% to 5.37%, revealed that the developed method has a good linearity, precision, and accuracy for all analytes. Hence, it is suitable for routine determination of investigated fungicides in table grape samples.

Keywords: RP-HPLC, UV–DAD, captan, folpet, metalaxyl, table grape

Introduction

Viticulture is one of the leading agricultural sectors and has great economic importance for the Republic of Macedonia. Due to the favorable climate, the grapes are characterized by remarkable quality and significant export potential. In Republic of Macedonia, there is a tradition of many years of successful vines cultivation, especially the table grape sorts. The assortment of table grapes includes several classes from very early to very late varieties of table grapes. Besides many other conditions, protecting the vines from diseases is more than necessary to increase the quality grapes production. Due to these reasons, the use of fungicides is inevitable. On the other hand, because fungicides are a potential risk to human health, monitoring of pesticide residues in food especially fruits and vegetables is required.

Table grape was a part of the monitoring for pesticide residues in primary agricultural products of plant origin in Republic of Macedonia for 2013 year. Among the most commonly used fungicides to protect the vines from diseases are captan, folpet, and metalaxyl, and therefore, these fungicides have been covered by the monitoring program.

To ensure the food safety and consumers' health protection, in most countries, maximum residue levels (MRLs) of pesticides in foodstuff have been established. The MRLs of pesticides contained in table grape were set up by the European Union (EU) Regulation (European Commission [EC]) No. 396/2005 [1], and they were estimated at 0.02 mg/kg for captan and folpet, and 2 mg/kg for metalaxyl. In order to monitor food safety, it is highly necessary to develop and employ reliable methods for determination of pesticide residues.

Numerous analytical methods for determining captan, folpet, and metalaxyl residues (in combination with other pesticides) in grape and other fruit and vegetable samples have been published, among which the most widely used chromatographic techniques were gas and liquid chromatography equipped with different detectors [2–8]. High-performance liquid chromatography (HPLC) coupled with ultraviolet (UV) detector or diode array detector (DAD) [9] was, also, used for the determination of examined pesticides. Pretreatment of samples involves several extraction and purification steps utilizing the following procedures: liquid–liquid extraction (LLE) [9], solid-phase extraction (SPE) [10, 11], matrix solid-phase dispersion (MSPD) [12], micro liquid–liquid extraction (MLLE) [13], dispersive liquid–liquid microextraction (DLLME) [14], and, recently used, a quick, easy, cheap, effective, rugged, and safe (QuEChERS) method [15, 16].

However, the HPLC method for simultaneous determination of captan, folpet, and metalaxyl residues in grape using UV–DAD was not found. Hence, the objective of this paper was to develop method for the simultaneous determination of captan, folpet, and metalaxyl residues in table grape samples using rapid resolution liquid chromatography (RRLC) system coupled with UV–DAD.

Experimental

Equipment and Materials. The chromatographic analysis was performed on an Agilent 1260 Infinity RRLC system equipped with: vacuum degasser (G1322A), binary pump (G1312B), autosampler (G1329B), a thermostatted column compartment (G1316A), UV-VIS diode array detector (G1316B), and ChemStation software. For the better dissolving of the stock solutions and sample preparation, an ultrasonic bath "Elma" was used. The experiments were carried out using LiChrospher 60 RP-select B (125 mm × 4 mm, 5 μm) and LiChrospher 60 RP-select B (250 mm × 4 mm, 5 μm) analytical columns produced by Merck (Germany). Evaporation of samples was enabled with vacuum rotary evaporator Büchi (Switzerland). For the SPE, a vacuum manifold Visiprep (Supelco, Sigma-Aldrich) was employed, and for vortexing of samples, IKA Vortex Genius 3 (Germany) was used.

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DOI: 10.1556/1326.2017.00330

First published online: 05 August 2017

The Pestanal analytical standards of captan (99.5% purity), folpet (99.7% purity), and metalaxyl (99.8% purity), as well as HPLC-grade acetonitrile, acetone, ethyl acetate, and water were manufactured by Sigma-Aldrich (Germany). Formic acid (98%–100% purity) and sodium sulfate decahydrate (99% purity) were produced by Merck (Germany).

Preparation of Standard Solutions. Stock solutions of captan, folpet, and metalaxyl were prepared by dissolving 0.0100 g, 0.0242 g, and 0.0080 g of the pure analytical standards with acetonitrile in a 25 mL volumetric flask. The solutions were degassed for 15 min in an ultrasonic bath and stored in a refrigerator at 4 °C. Stock solutions were used for fortification of table grape samples and for preparation of standard mixture with the following pesticide concentrations: 2 mg/kg for metalaxyl and 0.02 mg/kg for captan and folpet, in 10 mL volumetric flask by dilution with the mixture of acetonitrile–0.1% formic acid in water (65:35, v/v).

Extraction procedure. Ten different varieties (5 white, 4 red, and 1 pink) of table grape samples were taken from three vine-growing regions in Republic of Macedonia. Blank samples were prepared from table grape that was not treated with tested pesticides. For determination of linearity, precision, and recovery, spiking samples were prepared by fortifying 100 g homogenized table grape sample with three sets of concentrations: 0.014 mg/kg, 0.02 mg/kg, and 0.024 mg/kg (for captan and folpet), and 1.4 mg/kg, 2 mg/kg, and 2.4 mg/kg (for metalaxyl). Unspiked samples were used for blanks. For each concentration level, five samples ($n = 5$) were prepared.

For determination of a limit of quantification (LOQ), a 100 g homogenized table grape sample was spiked with 0.01 mg/kg of captan and folpet and with 1 mg/kg metalaxyl.

One hundered grams of homogenized sample was measured into a conical flask with stopper, and 150 mL acetone was added. The mixture was ultrasonicated for 60 min. After extraction, the mixture was filtered through a Büchner funnel using double filter paper under vacuum. Approximately 20 mL of acetone was used to wash the flask and filter residues. The extract was transferred into round-bottomed flask and concentrated using a rotary evaporator under vacuum to obtain about 5 mL of extract. After that, the extract was decanted into a separating funnel, 100 mL distilled water and 20 g NaCl were added, and extracted twice with 40 mL ethyl acetate. The extracts were dried over sodium sulfate and evaporated to dryness in a rotary evaporator.

The obtained residue was dissolved with 10 mL mixture of water and methanol (90:10, v/v) and filtered through a Büchner funnel using double filter paper under vacuum followed by SPE. The SPE procedure was carried out using Supelclean ENVI-18 tubes (6 mL, 0.5 g, produced by Supelco, Sigma-Aldrich, Germany). The conditioning of SPE cartridges was performed with 3 mL of methanol, followed by 3 mL of water at a flow rate of 2 mL/min. After that, 9 mL of the sample extract was passed through the cartridges and then washed the tubes with 3 mL of water. Subsequently, the cartridges were dried for 10 min under a vacuum. The retained pesticides were eluted with 3 mL of methanol–ethyl acetate (75:25, v/v). The eluates were evaporated to dryness under the gentle stream of nitrogen. The residues were redissolved with 1 mL of methanol by vortexing for 1 min, then filtered through 0.45 μm Iso-Disc PTFE syringe filters, and transferred into vials for HPLC analysis. The injection volume of each sample was 30 μL.

Matrix effect evaluation. The quantitative measurement of matrix effect (ME) was done by comparing the peak areas from standard solutions ($n = 3$) of the examined pesticides in solvent (acetonitrile–0.1% formic acid in water [65:35, v/v]) with the peak areas obtained from standard solutions of the same pesticides prepared in blank table grape extract, at the following concentrations: 0.02 mg/kg for captan and folpet and 2 mg/kg for metalaxyl. The ME was calculated using the following equation [17]:

$$\mathrm{ME}(\%) = (\mathrm{X}_2 - \mathrm{X}_1)/\mathrm{X}_1{}^{*}100 \qquad (1)$$

where X_1 is the average area of the pesticide standard in solvent (acetonitrile–0.1% formic acid in water [65:35, v/v]), at a specific concentration, and X_2, the average area of the pesticide standard in blank table grape extract, at the same concentration.

By using this formula, it was possible to calculate the positive or negative matrix effect, which is an increase or decrease of the detector response.

Results and Discussion

Captan (*N*-(trichloromethylthio)cyclohex-4-ene-1,2-dicarboximide, IUPAC) and folpet (*N*-(trichloromethylthio)phthalimide, IUPAC) belong to *N*-trihalomethhylthio pesticides, and metalaxyl (methyl *N*-(2-methoxyacetyl)-*N*-(2,6-xylyl)-DL-alaninate, IUPAC) is acylalanine (Figure 1) [18].

Chromatography Study. In preliminary experiments, two reversed-phase analytical columns with same stationary phases and different length, such as LiChrospher 60 RP-select B (125 mm × 4 mm, 5 μm) and LiChrospher 60 RP-select B (250 mm × 4 mm, 5 μm), were employed. The LiChrospher 60 RP-Select B was chosen because it offers excellent separation properties for basic compounds, but also is suitable for determination of neutral and acidic substances. This sorbent prevents secondary interactions with basic substances, ensures that they are eluted as highly symmetrical peaks, delivers highly reproducible results, and secures the reliability of HPLC method [19]. Also, different mixtures of acetonitrile–water (80%–40% acetonitrile) and acetonitrile–0.1% formic acid in water (80%–40% acetonitrile) as mobile phases in isocratic elution mode were tested.

The investigations show that the better results were given on the longer column LiChrospher 60 RP-select B (250 mm × 4 mm, 5 μm), probably due to its higher efficiency as a result of the higher number of theoretical plates.

The UV spectra (Figure 2) of examined pesticides show that they have absorption maxima around 220 nm. Hence, the chromatographic analysis for their simultaneous determination was carried out at 220 nm.

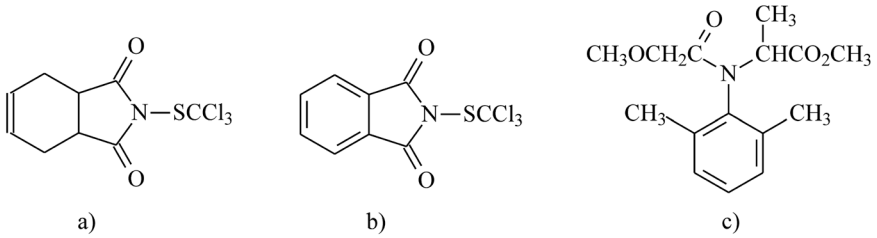

Figure 1. Chemical structures of captan (a), folpet (b), and metalaxyl (c)

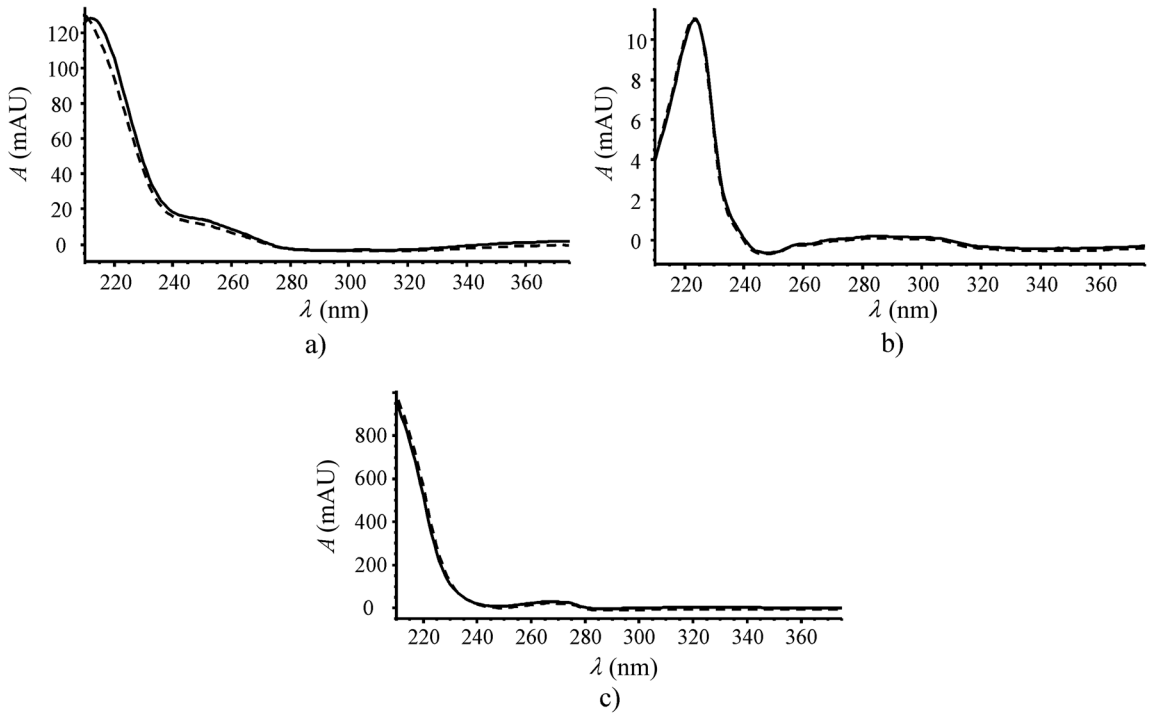

Figure 2. The overlaid UV spectra obtained by comparing the absorption spectra of a pure analytical standard of investigated pesticide and absorption spectra of the same analyte in the grape table sample for captan (a), folpet (b), and metalaxyl (c)

The best separation of the analytes with symmetrical peak shapes and satisfy purity indexes was achieved under isocratic elution with mobile phase consisted of acetonitrile–0.1% formic acid in water (65:35, v/v) (Figure 3a), flow rate of 1 mL/min, constant column temperature at 25 °C, and UV detection at 220 nm.

The obtained values for column dead time, retention times of components (t_R), the calculated values for retention factors (k'), separation factors (α), and resolution (Rs) are given in Table 1. As can be seen from this table, computed values for retention factors (k') were below 20, which is the highest optimal value for this parameter; for separation factors (α), above 1.2; and for resolution (Rs), above 7, which implies that, under the stipulated chromatographic conditions, high separation of the investigated pesticides was reached [20].

For quite some time, ultrasonication was the applied procedure for the extraction of many substances, among which are the pesticides [21]. The most commonly used solvent for extraction of pesticide residues was acetone, due to several advantages including high volatility and effectiveness and low toxicity and cost. Also, acetone is completely miscible with water, thus allowing a good penetration in the aqueous part of the sample [22]. Therefore, the target residues firstly were extracted with acetone by ultrasonication, followed by a cleanup using LLE and SPE before the analysis.

Method validation. Specificity, selectivity, linearity, matrix effect, precision expressed as repeatability of retention time, peak area and peak height, and recovery were examined to assess the validity of the developed method in accordance with EU regulations and EU documents [23, 24].

Specificity and selectivity. To confirm the specificity of the developed method, UV–DAD was used to check the peak purity and analyte peak identity. The purity index for all analytes was greater than 999 (the maximum value for the peak purity index [PPI] should be 1000), which means that the chromatographic peak was not affected by any other compound. In addition, identification of the analytes was done using the values for the retention time and match factor obtained by overlaid spectra of a pure analytical standard (from spectra library) and absorption

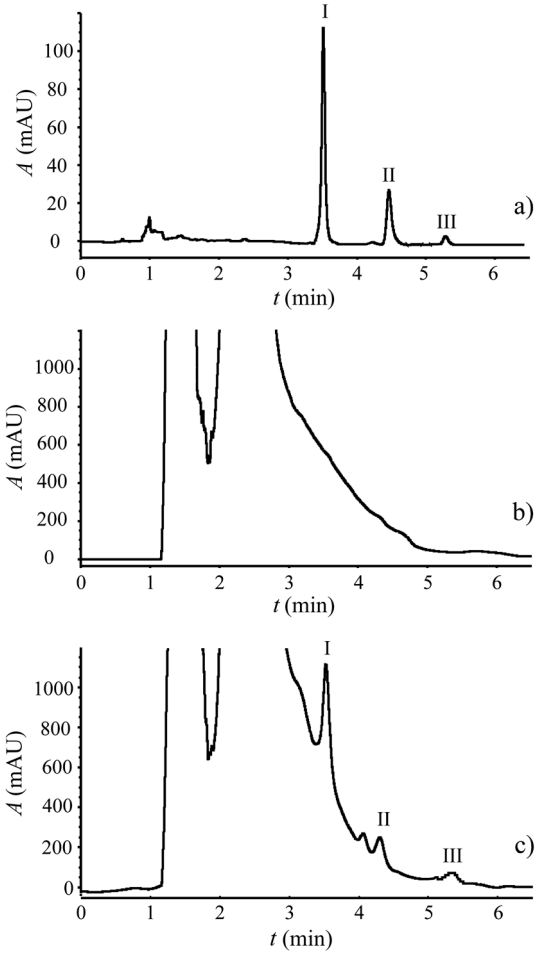

Figure 3. Chromatograms from standard mixture of metalaxyl (I), captan (II), and folpet (III) at the concentrations that correspond to MRLs (a), unspiked table grape sample (b), and sample of table grape fortified at the concentration equal to MRL for each analyte (c) obtained with the developed method

Table 1. Data for retention times (t_R), retention factors (k'), separation factors (α), and resolution (Rs) for the investigated pesticides

Compound	t_R (min)	k'	α	Rs
Dead time	0.74	–	–	–
Metalaxyl	3.53	3.77	1.28	7.02
Captan	4.30	4.81	1.27	7.61
Folpet	5.27	6.12	–	–

spectra of the same analyte in the grape samples. The obtained values for match factors (997.857 for captan, 999.923 for folpet, and 999.983 for metalaxyl) confirmed the identity of the analytes. Additionally, on the recommendation of EU [24], to prove selectivity of the method, on Figure 3 are presented chromatograms of standards at the concentrations that correspond to MRLs (a), matrix blank (unspiked grape sample) (b), and sample of table grape fortified at the concentration equal to MRL for each analyte (c).

Linearity. The linearity of the developed method was determined for all compounds separately, with triplicate injections (30 μL) of the spiked standards in the table grape sample matrix in the range from 30% less than MRLs to 20% above (Table 2). The obtained results for multiple correlation coefficients ($R^2 \geq 0.90$) suggested that the method has a satisfactory linearity for all analytes (Table 2).

Matrix effect. The quantitative determination of matrix effect was done using the Eq. (1). Matrix effect represents the noticed effect of an increase (enhancement) or decrease in detector response (a positive or negative matrix effect, respectively) of a pesticide present in a matrix extract compared with the same pesticide present in just solvent [17]. The calculated matrix effect for investigated pesticides exceeded 39% (Table 3) and indicated a significant matrix effect. Captan and metalaxyl showed a significant negative matrix effect, while significant positive matrix effect was noticed for folpet. When matrix effects are significant (i.e., >20%), calibration should be generated using standards prepared in blank matrix extracts (matrix matched standards) [23, 24]. For these reasons, the calibration was conducted this way.

Limit of quantification. The LOQ for each compound was determined by spiking a table grape sample with 0.01 mg/kg of captan and folpet and with 1 mg/kg metalaxyl, the concentrations of which correspond to 50% of MRL for each compound.

The signal-to-noise ratio (S/N) at the concentration level corresponding to 50% of MRL for each compound (0.01 mg/kg for captan and folpet and 1 mg/kg for metalaxyl) was found to be ≥10 for all examined pesticides. Therefore, the LOQ was estimated to be ≤0.01 mg/kg for captan and folpet and ≤1 mg/kg for metalaxyl in this study. These results are acceptable for determining the pesticide residues, according to the EU rules [24].

Precision. The precision was expressed as repeatability of obtained results from eight successive injections (30 μL) of the spiked table grape samples at MRLs for each of the analytes (Table 4). The computed values of RSD for retention time, peak area, and peak height indicated an excellent precision of the proposed method.

Table 2. Statistical data for linearity of the method

Compound	Linearity range (mg/kg)	Regression equation	R^2
Metalaxyl	1.40–2.40	[a] $y = 1669.2x + 456.62$	0.9572
		[b] $y = 206.48x + 163.58$	0.9076
Captan	0.014–0.024	[a] $y = 93519x - 830.49$	0.9032
		[b] $y = 8998{,}9x - 48{,}503$	0.8896
Folpet	0.014–0.024	[a] $y = 11199x - 108.44$	0.9925
		[b] $y = 1048.4x - 10.116$	0.9829

[a]Area.
[b]Height.

Table 3. Average matrix effect (%) for investigated pesticides ($n = 3$)

Compound	Concentration (mg/kg)	$X_1 \pm SD$	$X_2 \pm SD$	Matrix effect (%)
Metalaxyl	2	3591.21 ± 67.72	1205.29 ± 24.82	−66.4
Captan	0.02	853.49 ± 5.90	512.84 ± 10.15	−39.9
Folpet	0.02	109.87 ± 0.03	164.85 ± 8.30	50.0

X_1 = average peak area of the pesticide standard solution in solvent; X_2 = average peak area of the pesticide standard solution in table grape extract.

Table 4. Statistical data for intra-day precision of retention time, peak area, and peak height ($n = 8$)

Compound		$\bar{x}$	SD	RSD (%)
Metalaxyl	Retention time (min)	3.53	0.002	0.06
	Peak area	3481.87	47.37	1.36
	Peak height	524.55	8.22	1.57
Captan	Retention time (min)	4.3	0.004	0.09
	Peak area	843.23	7.86	0.93
	Peak height	111.38	0.64	0.57
Folpet	Retention time (min)	5.27	0.007	0.13
	Peak area	108.06	2.32	2.15
	Peak height	9.86	0.22	2.25

Accuracy. The accuracy of the method was determined by recovery studies in table grape samples (pesticides free) spiked with the investigated pesticides at three concentation levels (Table 5). The obtained values for recovery and for relative standard deviation were within the following ranges: 90.55%–105.40% and 0.02%–5.37%, respectively. The mean recovery at each fortification level in the range of 70%–120% and relative standard deviation (RSD) ≤20% per level are acceptable according to EU criteria [24]. Consequently, it can be concluded that the proposed method is convenient to determination of the target pesticide residues in table grape.

The investigations show that only folpet residues were present in table grape samples. Residues of folpet were found in all samples of red grapes, but they were also in some white grape samples. As can be seen from Table 6, in four samples, including rose grape samples, measurable quantities of the target fungicide residues were not detected. The determined concentration of folpet was below the MRL in three samples, close below MRL in one sample, and, in two samples, was equal to MRL.

Table 5. Results from recovery experiments ($n = 5$)

Compound	Fortification level (mg/kg)	Total analyte found (mg/kg ± SD)	Recovery (%)	RSD (%)
Captan	0.014	0.0148 ± 0.00068	105.40	4.63
	0.02	0.0181 ± 0.00015	90.55	0.85
	0.024	0.0251 ± 0.00049	104.73	1.93
Folpet	0.014	0.0142 ± 0.00012	101.43	0.83
	0.02	0.0195 ± 0.000005	97.48	0.02
	0.024	0.0243 ± 0.00118	101.25	4.87
Metalaxyl	1.40	1.4489 ± 0.0273	103.49	1.89
	2	1.8779 ± 0.040	93.90	2.16
	2.40	2.4733 ± 0.1328	103.05	5.37

Table 6. The determined concentration of pesticide residues in table grape samples

Sample ($n = 3$)	Type of grape	Detected pesticide	Determined concentration (mg/kg ± SD)	RSD (%)
1	White	nd	–	–
2	Red	Folpet	0.0108 ± 0.0003	2.79
3	White	Folpet	0.0123 ± 0.000058	0.47
4	Rose	nd	–	–
5	White	nd	–	–
6	Red	Folpet	0.0118 ± 0.00015	1.27
7	Red	Folpet	0.0188 ± 0.000073	0.39
8	Red	Folpet	0.0215 ± 0.0008	3.72
9	Red	Folpet	0.0196 ± 0.00038	1.94
10	White	nd	–	–

nd = not detected.

Conclusions

A new, precise, accurate, and reliable method for simultaneous determination of metalaxyl, captan, and folpet residues in table grape samples using RP-HPLC and UV–DAD has been developed and validated. Successful separation and quantification were achieved using isocratic elution with mobile phase consisted of acetonitrile–0.1% formic acid in water (65:35, v/v), flow rate of 1 mL/min, constant column temperature at 25 °C, and UV detection at 220 nm. The run time of analysis under the stipulated chromatographic conditions was about 6 min. The results from the method validation revealed that the proposed method has a satisfactory linearity ($R^2 > 0.90$) and excellent precision of retention times, peak areas, and heights (RSD ≤ 2.25%). The obtained values for recoveries ranging from 90.55% to 105.40%, with RSD of 0.02%–5.37%, revealed that the proposed method is convenient for routine determination of investigated fungicides in table grape samples.

This method was successfully applied to determine the captan, folpet, and metalaxyl residues in table grape samples from ten different varieties (5 white, 4 red, and 1 pink) taken from three vine-growing regions in Republic of Macedonia. The obtained results show that folpet was frequently detected fungicide in the analyzed table grape samples, and found concentrations were less or equal to MRL according to Regulation (EC) No. 396/2005.

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