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

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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 (R² > 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 has been maintained in Republic of Macedonia due to 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 foodstuff has 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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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 the following pesticide concentrations: 2 mg/kg for metalaxyl, 0.0100 g, 0.0242 g, and 0.0080 g of the pure analytical standards with acetonitrile 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 hundred 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 ENVIL-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]:

\[
\text{ME}(\%) = \frac{X_2 - X_1}{X_1} * 100
\]  

where X1 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 X2, 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-trihalomethylthio pesticides, and metalaxyl (methyl N-(2-methoxyacetyl)-N-(2,6-xylyl)-dt-alanine, IUPAC) is acylalanine (Figure 1) [18].

Chromatographic 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 acetamid–water (80%–40% acetamid) and acetamid–0.1% formic acid in water (80%–40% acetamid) 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)](image-url)
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 3 а), 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 ($\alpha$), and resolution ($R_s$) 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 ($\alpha$), above 1.2; and for resolution ($R_s$), 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 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)
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 1. Data for retention times ($t_R$), retention factors ($k'$), separation factors (α), and resolution ($R_s$) for the investigated pesticides |
|---|---|---|---|---|---|---|---|
| Compound | $t_R$ (min) | $k'$ | α | $R_s$ |
| 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 | – | – |

| Table 2. Statistical data for linearity of the method |
|---|---|---|---|
| Compound | Linearity range (mg/kg) | Regression equation | $R^2$ |
| Metalaxyl | 1.40–2.40 | $y = 1695.2x + 456.62$ | 0.9572 |
| Captan | 0.014–0.024 | $y = 206.48x + 163.58$ | 0.9076 |
| Folpet | 0.014–0.024 | $y = 8998.9x + 48.503$ | 0.8966 |

$\alpha = $Area, $\beta = $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 | 3591.21 ± 67.72 | 1205.29 ± 24.82 | –66.4 |
| Captan | 853.49 ± 5.90 | 512.84 ± 10.15 | –39.9 |
| Folpet | 109.87 ± 0.03 | 164.85 ± 8.30 | 50.0 |

$X_1 =$average peak area of the pesticide standard solution in solution; $X_2 =$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 = 5) |
|---|---|---|---|---|
| Compound | $t_R$ | SD | RSD (%) |
| Metalaxyl | 3.53 | 0.002 | 0.06 |
| Captan | 4.3 | 0.004 | 0.09 |
| Folpet | 9.86 | 0.22 | 2.25 |

| 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 |
| Folpet | 0.02 | 0.0251 ± 0.00049 | 104.73 | 1.93 |
| Metalaxyl | 2 | 1.8779 ± 0.040 | 93.90 | 2.16 |

| 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 | nd | – | – |
| 4 | Rose | nd | – | – |
| 5 | Red | nd | – | – |
| 6 | Red | Folpet | 0.0118 ± 0.00015 | 1.27 |
| 7 | Red | nd | – | – |
| 8 | Red | Folpet | 0.0215 ± 0.00008 | 3.72 |
| 9 | Red | nd | – | – |
| 10 | White | nd | – | – |

nd = not detected.

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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 ≤ 2.5%). 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 sampled table grape samples, and found concentrations were less than or equal to MRL according to Regulation (EC) No. 396/2005.

References

1. Regulation (EC) No. 396/2005 of the European Parliament and of the Council of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC with EEA relevance, 2005.
2. Zhou, X.; Zeng, X. H.; Wang, D. Y.; Cui, P. L.; Wang, Z. Chin. J. Anal. Chem. 2009, 37, 41.
3. Česnik, H. B.; Gregorčič, A.; Bolu, Š. V. Journal of Central European Agriculture 2009, 10, 311.
4. Alves, A. A. R.; Rodrigues, A. S.; Barros, E. B. P.; Uekane, T. M.; Bizro, H. R.; Rezende, C. M. Food Anal. Methods 2014, 7, 1834.
5. Cunha, S. C.; Fernandes, J. O.; Alves, A.; Oliveira, M. B. P. J. Chromatogr. A 2009, 1216, 119.
6. Affify, A. E. M. M. R.; Mohamed, M. A.; El-Gammal, H. A.; Attalah, E. R. J. Food Agric. Environ. 2010, 8, 602.
7. Banerjee, K.; Oulkar, D. P.; Dasgupta, S.; Patil, S. B.; Patil, S. H.; Savant, R.; Adsule, P. G. J. Chromatogr. A 2007, 1173, 98.
8. Zanella, R.; Munaretto, J. S.; Martins, M. L. Determination of pesticide multiresidues in apple, pear, and grape using modified QuEChERS and analysis by LC–QTOF MS Agrilent Technologies, Inc., 2013, 1.
9. Öreo, R. R.; Grunde, B. C.; Gandara, J. S. J. Chromatogr. A 2003, 992, 121.
10. Velkoska-Markovska, L.; Petanovska-Ilievska, B. Maced. J. Chem. Chem. Eng. 2013, 52, 299.
11. Latif, A.; Sherazi, S. T. H.; Bhanger, M. I. Pak. J. Anal. Environ. Chem. 2011, 12, 76.
12. Lian, Y. J.; Pang, G. F.; Shu, H. R.; Fan, C. L.; Liu, Y. M.; Feng, J.; Wu, Y. P.; Chang, Q. Y. J. Agric. Food Chem. 2010, 58, 9428.
13. Wang, J. F.; Luun, L.; Wang, Z. Q.; Jiang, S. R.; Fan, C. P. Chin. J. Anal. Chem. 2007, 35, 1430.
14. Zang, X.; Wang, J.; Wang, O.; Wang, M.; Ma, J.; Xi, G.; Wang. Z. Anal. Bioanal. Chem. 2008, 392, 749.
15. Nieto-García, A. J.; Romero-González, R.; Garrido Frenich, A. Food Addit. Contam. 2014, 31, 1550.
16. Satpathy, G.; Tyagi, Y. K.; Gupta, R. K. Am. J. Food Sci. Technol. 2014, 2, 53.
17. Pizzutti, I. R.; de Kok, A.; Hienstra, M.; Wickert, C.; Prestes, O. D. J. Chromatogr. A 2009, 1216, 4539.
18. Horning, C. The Pesticide Manual Incorporating the Agrochemicals Handbook 11th Ed., British Crop Protection Council, Royal Society of Chemistry Cambridge, 1997, pp. 145–146, 518–519, 610–661.
19. Drenthook, Your Guide to a Fascinating World of Chromatography Merck KGaA, Darmstadt, Germany, 2011, pp. 240–243.
20. Dong, M. W. Modern HPLC for Practicing Scientists John Wiley & Sons, Inc., Hoboken, New Jersey, 2006, p. 17–46.
21. Hollosi, L.; Mittendorf, K. Determination of Pesticides in Grapes, Baby Food and Wheat Flour by Automated Online Sample Preparation LC-MS/MS Thermo Fisher Scientific Inc., 2012, 1.
22. Fernandes, V. C.; Domingues, V. F.; Mateus, N.; Delerue-Matos, C. J. Chromatogr. Sci. 2011, 49, 715.
23. Document No SANCO/12495/2011, Method validation and quality control procedures for pesticide residues analysis in food and feed, 2011.
24. European Commission, Directorate General Health and Consumer Protection, Guidance document on pesticide residue analytical methods SANCO/825/00 rev. 8.1 2010.