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Development and validation of RP-HPLC method for determination of some pesticide residues in apple samples

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ORIGINAL RESEARCH PAPER

ABSTRACT

Apples are among the most common fruits, produced on the territory of North Macedonia. Before they reach the market, it is essential to be tested for pesticide residues, which are used for protection against pests for this culture, and in order to ensure the customer safety. For this reason, a novel and simple method for simultaneous determination of captan, folpet, difenoconazole and chlorpyrifos in apple samples has been developed and validated. Acetone is used for extraction of the pesticide residues, followed by liquid-liquid (LLE) and solid–phase extraction (SPE). Separation and quantification of analytes is achieved on reversed-phase high performance liquid chromatography (RP-HPLC) with UV diode array detector (UV-DAD). The best results are obtained using analytical column LiChrospher 60 RP-select B (250 mm × 4 mm, 5 µm), with isocratic elution and acetonitrile/0.1 % acetic acid in water (70:30, *V/V*) as a mobile phase. The flow rate is 1 mL min^{-1}, and UV detection is performed at 220 and 230 nm. The linearity of the method is tested in the range of 1.50–3.60 mg kg^{-1} for captan and folpet, and 0.35 – 0.60 mg kg^{-1} for difenoconazole and chlorpyrifos. The obtained values for recovery and RSD ranged from 94.94 to 114.63 %, and 0.09–9.25 %, respectively. The validated method is successfully applied to apple samples for the determination of the investigated pesticide residues.

KEYWORDS

RP-HPLC, UV-DAD, solid-phase extraction, pesticide residues, apples, validation method

INTRODUCTION

The apple, as one of the most cultivated fruits in North Macedonia, is widely used both for consumption and in the food processing industry. In North Macedonia 61 % of the total fruit production goes to apples, making it the most common fruit. To achieve high yields, various pesticides are applied in order to repel and destroy pests. However, extensive use of pesticide can have implication on the environment and consumer health. To ensure consumer safety and regulation of foreign trade, the European Union and government agencies have established regulatives that control the maximum residual levels (MRLs). Even though North Macedonia is not a European member, its food agencies work in compliance with the same regulative norms on pesticides as the member states.

As there is a wide range of pesticides that are used for apple protection, their residue concentrations must be accurately monitored to ensure consumer safety. Among the most commonly used pesticides in apple production in North Macedonia are captan, folpet, difenoconazole and chlorpyrifos. Chemical structure of captan (IUPAC name: 2-(trichloromethylsulfanyl)-3*a*,4,7,7*a*-tetrahydroisoindole-1,3-dione) [1], folpet (IUPAC name: 2-(trichloromethylsulfanyl)isoindole-1,3-dione) [2], difenoconazole (IUPAC name: 1-[[2-[2-chloro-4-(4-chlorophenoxy)phenyl]-4-methyl-1,3-dioxolan-2-yl]methyl]-1,2,4-triazole) [3]

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and chlorpyrifos (IUPAC name: diethoxy-sulfanylidene-(3,5,6-trichloropyridin-2-yl)oxy-λ^5-phosphane) [4] are shown in Fig. 1.

Captan and folpet have similar chemical structure and properties. They belong to the group of phthalimide fungicides with a wide spectrum of action, which are used preventively, but also curatively [5]. Difenoconazole is also a fungicide with a broad spectrum of action, which is used preventively and curatively. Difenoconazole has four chiral centers, therefore it can exist in four stereoisomeric forms. Even though they are reported as sum of the signals, the chiral forms have different molecular configuration, thus influencing their stereoselective toxicity, which sometimes could not be predicted [6]. Chlorpyrifos is an organophosphate pesticide that has been used on crops, animals, and buildings, and in other settings, to kill several pests, including insects and worms. It acts on the nervous systems of insects by inhibiting the acetylcholinesterase enzyme [7]. According to the European Union (EU) Regulation (European Commission [EC]) No. 396/2005 [8], established MRLs for apple are 3.0 mg kg^{-1} for captan and folpet and 0.5 mg kg^{-1} for difenoconazole and chlorpyrifos.

For the determination of the pesticide residues in complex matrices, including apples, effective extraction techniques should be applied, followed up by matrix clean-up such as liquid-liquid extraction (LLE) and/or solid phase extraction (SPE) [9–14]. Most commonly used techniques for separation and determination of those residues are liquid chromatography (LC) and gas chromatography (GC), coupled with different detectors. Mass spectrometers are most preferred detectors for determination of pesticide residues, which can provide with unambiguous analyte identification. Even though diode array detectors (DAD) are less sensitive and preferable, they can be used as well as for pesticide residue analysis [15–20].

Since in the literature there is no known HPLC method for simultaneous determination of captan, folpet, difenoconazole and chloyrifos in apples, the objective of this paper is to present a development and validation the method for effective sample preparation, identification and quantification of above mentioned pesticide residues in apple samples using RP-HPLC method with ultraviolet diode array detection (UV-DAD).

The method is successfully applied to analyse the studied pesticide residues in apple samples produced in different regions of North Macedonia. The obtained results will contribute to the analytical methodology for the determination of pesticide residues in food using the RP-HPLC UV-DAD method, as well as insight into the presence of the investigated pesticides in apples from different regions in North Macedonia.

EXPERIMENTAL

Equipment and materials

The chromatographic analysis is carried out on Agilent 1260 Infinity Rapid Resolution Liquid Chromatography (RRLC) system equipped with: vacuum degasser (G1322A), binary pump (G1312B), autosampler (G1329B), thermostatted column compartment (G1316A), UV-VIS diode array detector (G4212B) and ChemStation version A.01.01 software. Samples and stock solutions are dissolved with aid of ultrasonic bath Elma (Schmidbauer GmbH, Germany). The following analytical columns are used for the development of the method: LiChrospher 60 RP-select B (125 mm × 4 mm, 5 µm) and LiChrospher 60 RP-select B (250 mm × 4 mm, 5 µm) produced by Merck (Germany), as well as Discovery C18 (250 mm x 4.6 mm, 5 µm) made by Supelco,

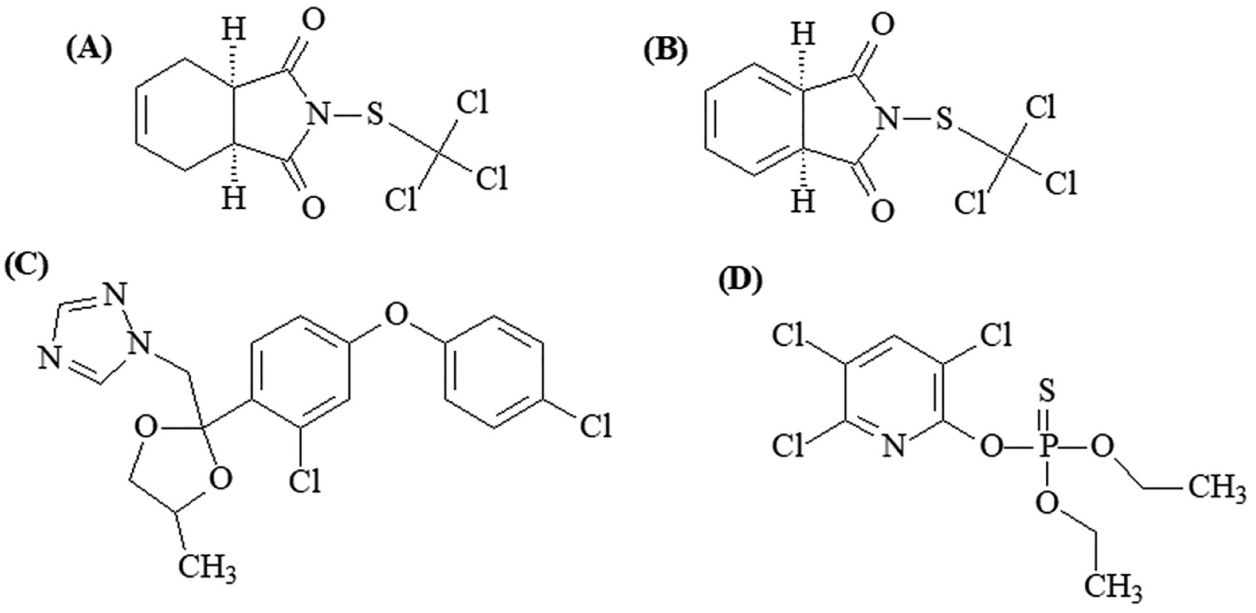

Fig. 1. Chemical structure of captan (A), folpet (B), difenoconazole (C) and chlorpyrifos (D)

Sigma Aldrich (Germany). Solid-phase extraction is performed for samples clean-up and concentrate the analytes using two SPE columns: Supelclean ENVI-18 and Supelclean ENVI carb (6 mL, 0.5 g), as well as, SPE vacuum manifold Visiprep, produced by Supleco, Sigma-Aldrich (Germany). Removal of organic solvents from samples is achieved with rotary evaporator Büchi (Switzerland). IKA Vortex Genius 3 (Germany) is used for the vortexing of samples.

Analythical standards of captan (99.5 %), folpet (99.7 %), difenoconazole (99.0 %), and chlorpyrifos (100 %), as well as HPLC-grade water, acetonitrile, etthylacetate and acetone are purchased from Sigma Aldrich (Germany). Glacial acetic acid (CH_3COOH), sodium chloride (NaCl, 99.5 %) and anhydrous sodium sulfate (Na_2SO_4, 99.0 %) are procured from Merck (Germany).

Preparation of standard solutions

Stock solutions are prepared by dissolving analytical standards of captan (3.6 mg), folpet (2.8 mg), difenocoazole (3.9 mg) and chlorpyrifos (3.3 mg) with acetonitrile in 10 mL volumetric flask. For better dissolution, the stock solutions are sonicated in an ultrasonic bath for 15 min and then stored in refrigerator at 4 °C before use. The stock solutions are further used for preparation of working standard solutions and a standard mixture of analytes, as well as, for fortification of apple samples. Standard mixture is prepared daily, at MRL concentrations for all analytes, by dilution with acetonitrile/water (50/50, *V/V*) in 10 mL volumetric flask.

Sample preparation (extraction procedure)

As a basis for the extraction of analytes from apple samples, the method for determination of some pesticide residues in table grape [21] and tomato samples [22] has been used with slight modification.

Approximately 1 kg of apples are chopped in small pieces and blended until homogenous sample is obtained. After that 30 g of the homogeneous mass is weighted and added to conical flask with stopper, and 100 mL of acetone is added into the flask. The mixture is sonicated on ultrasound for 60 min, making sure that the temperature does not exceed 40 °C. After extraction, the mixture is filtered through a Büchner funnel using double filter paper under vacuum. The filtrate is transferred to round-bottomed flask, using 20 mL of acetone to wash the filter paper and vacuum flask, and then concentrated using a rotary evaporator under vacuum to obtain approximately 2–5 mL aliquot. After that, the aliquot is transferred into a separatory funnel, where 100 mL 20 % NaCl solution is added and extracted twice with 40 mL ethyl acetate for each extraction. The extracts are passed through anhydrous sodium sulphate to remove residual water, and evaporated to dryness in a rotary evaporator. After that, the obtained dry residue is dissolved with 10 mL mixture of water/methanol (90/10, *V/V*) and filtered through a Büchner funnel using double filter paper under vacuum, followed by SPE. For efficiency testing, two SPE columns are used: Supelclean ENVI-18 and Supelclean ENVI carb, which are conditioned in the same way prior to use. The conditioning of SPE cartridges is performed with 3 mL of methanol, followed by 3 mL of water at a flow rate of 2 mL min^{-1}. After that, 9 mL of the sample extract is passed through the cartridges and then washed the tubes with 3 mL of water. Subsequently, the cartridges are dried for 10 min under a vacuum. The retained pesticides are eluted with 3 mL mixture of methanol and ethyl acetate (75/25, *V/V*). The eluates are evaporated to dryness under the gentle stream of nitrogen and redissolved in 1 mL methanol by vortexing for 1 min. Prior HPLC analysis samples are filtered through 0.45 µm Iso-Disc PTFE syringe filter. For this method injection volume of each sample is 20 µL.

The blank samples are prepared in the same way as apples that are not treated with tested pesticides.

For determination of linearity, LOD, LOQ, precision, and recovery, spiked samples are prepared by fortifying an apple sample with the following sets of concentrations: 1.5, 2.1, 3.0, and 3.6 mg kg^{-1} (for captan and folpet), and 0.35, 0.5, and 0.6 mg kg^{-1} (for difenoconazole and chlorpyrifos). For each concentration level, five samples ($n = 5$) are prepared.

In this research 19 different apple samples collected from different orchards, in different regions of North Macedonia are analysed.

RESULTS AND DISCUSSION

Chromatography study

A series of preliminary investigations, using three reversed-phase analytical columns, among which two with same stationary phase 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), and Discovery C-18 (250 mm × 4.6 mm, 5 µm) are carried out. Furthermore, different mixtures of acetonitrile/water (80 %–40 % acetonitrile) and acetonitrile/0.1 % acetic acid in water (80 %–40 % acetonitrile) as mobile phases are tested. In order to develop a simpler method, isocratic elution is used.

The selection of wavelength at which the chromatographic process is followed is based on the UV spectra of the analytes. As can be seen from Fig. 2, captan, folpet and difenoconazole have an absorption maximum at around 220 nm, while chlorpyrifos at around 230 nm. Therefore, the chromatographic process is monitored at 220 and 230 nm.

Successful separation of the analytes using a Discovery C18 column (250 mm × 4.6 mm, 5 µm) is obtained by isocratic elution with a mobile phase consisting of acetonitrile/water with volume ratio of 65/35 and 70/30, with the run time of 18 and 14 min, respectively. A flow rate of 1 mL min^{-1}, a column temperature of 25 °C, an injection volume of 20 µL and detection at 220 and 230 nm are used.

Furthermore, good separation of the pesticides of interest is achieved using a LiChrospher 60 RP-Select B (125 mm × 4 mm, 5 µm) column, by isocratic elution with acetonitrile/water (70/30, *V/V*) as mobile phase, a flow rate of 1 mL min^{-1},

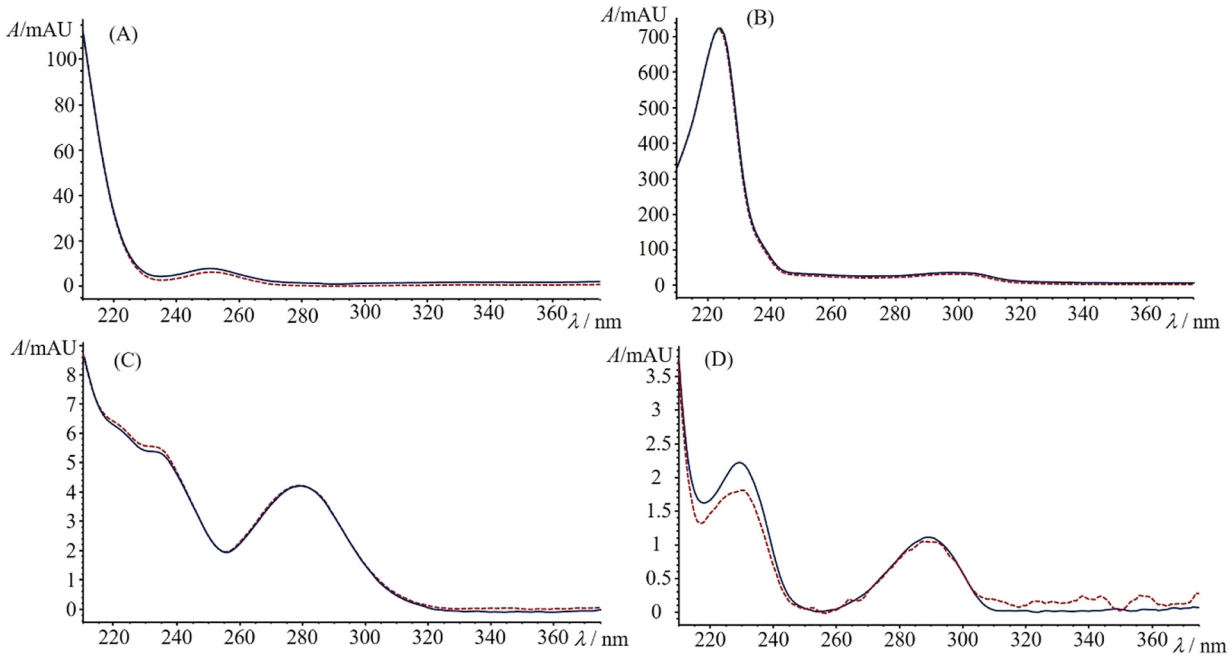

Fig. 2. The overlaid UV spectra were obtained by comparing the absorption spectra of a pure analytical standard of investigated pesticides in acetonitrile/water (50/50, *V/V*) at MRL and absorption spectra of the same analyte spiked in apple sample at the concentration equal to MRL for captan (A), folpet (B), difenoconazole (C) and chlorpyrifos (D)

a column temperature at 25 °C, an injection volume of 20 µL, and UV detection at 220 and 230 nm. Although the analytes are completely separated from the standard mixture for about 4 min, due to the complexity of the matrix and its interferents, method development is performed on the longer column to improve the selectivity of the analytes in the apple matrix.

The best chromatographic conditions are reached on the column LiChrospher 60 RP-Select B (250 mm × 4 mm, 5 µm), with isocratic elution and mobile phase consisting of acetonitrile/0.1 % CH_3COOH in water (70/30, *V/V*), a flow rate of 1 mL min^{-1}, a column temperature at 25 °C, an injection volume of 20 µL and detection at 220 and 230 nm. This is expected probably due to its higher efficiency as a result of the higher number of theoretical plates [23]. Under these established conditions, the best separation, symmetrical peak shapes and satisfying purity index of all analytes from the standard mixture are obtained (Fig. 3a). At the same time, with this column a shorter run time is reached compared to the Discovery column with the same dimensions, which is about 8.5 min.

The obtained values for column dead time (t_0), retention times (t_R) as well as retention factors (k'), separation factors (α) and resolutions (R_S) are shown in Table 1. Computed values for the retention factors for all components are below 10, which is the highest optimal value for this parameter. The separation factors are above 1.2, while the resolutions are above 2.7 which implies that the investigated peaks are fully separated under the established chromatographic conditions [24].

Concentration and sample clean-up

The apple represents a complex set of different compounds that are in higher concentration in relation to the presence of pesticide residues. Sample matrix usually interfere with the analytes, making it necessary to concentrate and clean-up the samples. Preliminary research has shown that not only extracting the analytes with a solvent, for example acetone, and cleaning the samples with SPE is sufficient, but liquid-to-liquid extraction should also be applied to further sample clean-up and concentrate the analytes. The extraction and clean-up procedure is also tested on two SPE columns (Supelclean ENVI 18 and Supelclean ENVI carb), and the obtained results showed that the ENVI C18 columns are more efficient. Extraction of analytes is performed by ultrasonication with acetone, purification by liquid–liquid extraction, concentration and sample clean-up using an ENVI-18 SPE column.

The explained extraction method is applied to a blank sample, as well as to an apple sample spiked with analysed pesticides at the MRL level (Fig. 3b and c). The retention time values are used for the identification of target pesticides by comparison with those of the analytical standards. The obtained results show that no matrix coeluting peaks with the same retention time as the analytes are detected on the chromatogram of the blank sample, indicating a successful sample clean-up. Moreover, from the chromatogram obtained by analysing the spiked sample, it can be observed that the peaks of the analytes are well separated and there are no coeluting peaks from the matrix. The peak purity index, which is higher than 997.5 for all analytes, is further used to confirm this claim. The obtained results revealed that the described extraction method successfully extracted and concentrated the analytes. Considering that the method is characterized by analyte selectivity, it is further validated and applied to the determination of investigated pesticide residues in apple samples.

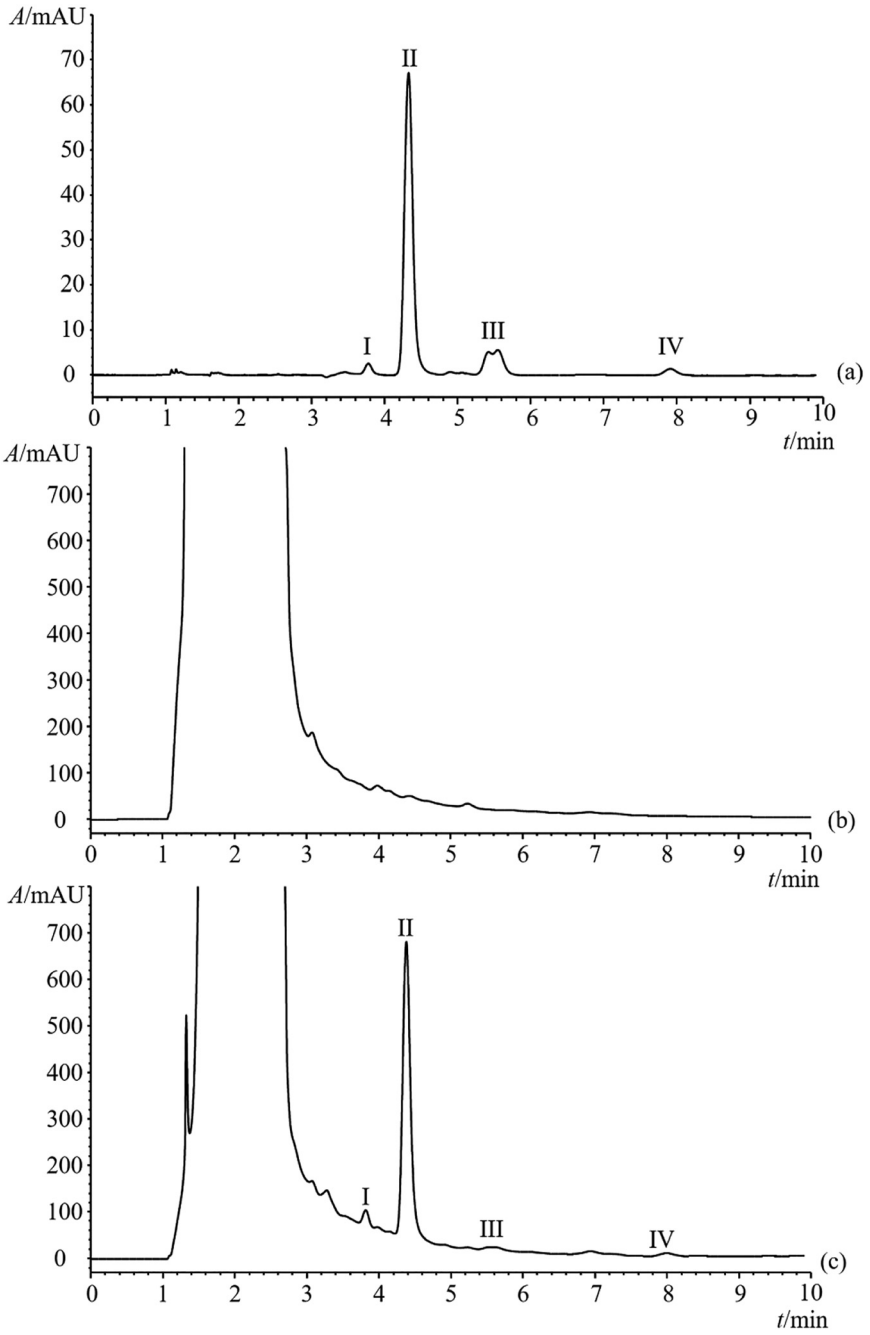

Fig. 3. Chromatograms UV–VIS from the standard mixture of captan (I), folpet (II), difenoconazole (III) and chlorpyrifos (IV) at the concentrations that correspond to MRLs (a), unspiked apple sample (b), and apple sample fortified at the concentration equal to MRL for each analyte (c) obtained with the developed method at 220 nm

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

Compound	Mobile phase	Captan	Folpet	Difenoconazole	Chlorpyrifos
t_R (min)	1.15	3.81	4.35	5.57	8.00
k'	/	2.31	2.78	3.84	5.96
α	/	/	1.20	1.38	1.55
R_S	/	2.76	5.32	7.39	/

Method validation

The validation of the method is performed in accordance with EU regulations [24, 25]. Hence, specificity, selectivity, linearity, limit of detection (LOD) and limit of quantification (LOQ), precision and recovery are tested in order to evaluate the validity of the method.

Specificity and selectivity. UV spectra of the analytical standards of the investigated pesticides (at the MRL level) are recorded using a diode array detector. To confirm the specificity of the developed method, UV–DAD is used to check the peak purity and analyte peak identity. The purity index values of all components are higher than 997.5 (whereas the maximum value is 1,000), indicating that the chromatographic peaks of analysed pesticides are not affected by any other compound. For unambiguous identification, the match factor obtained by overlaid spectra of a pure analytical standard (from spectra library) and absorption spectra of the same analyte in the apple samples is used. The obtained match factor values are 999.920 for captan, 999.999 for folpet, 999.881 for difenoconazole and 990.697 for chlorpyrifos (Fig. 2), confirming the identity of the analytes. The lowest value for the spectral matching factor is obtained for chlorpyrifos. This is probably due to the fact that the response of the signal for this component is the smallest, and its spectral characteristics is also affected by the matrix. Additionally, following the recommendation of the EU [25], to prove the selectivity of the method, on Fig. 3 presents chromatograms of standards at concentrations corresponding to the MRL (a), matrix blank (unspiked apple sample) (b) and apple sample spiked to a concentration equal to the MRL for each analyte (c).

Linearity. The linearity of the developed method is determined for all analytes separately, with triplicate injections

Table 2. Statistical data for linearity, limits of detection (LOD), limits of quantification (LOQ)

Compound	Linearity range (mg kg^{-1})	Regression equation	R^2	LOD (mg kg^{-1})	LOQ (mg kg^{-1})
Captan	1.50–3.60	$^1y = 92.74x - 81.107$	0.9460	0.45	1.50
		$^2y = 15.428x - 11.404$	0.8898		
Folpet	1.50–3.60	$^1y = 2091.8x - 1556.1$	0.9657	0.45	1.50
		$^2y = 270.04x - 166.97$	0.9225		
Difenoconazole	0.35–0.60	$^1y = 231.71x - 9.8871$	0.9957	0.11	0.35
		$^2y = 26.785x - 1.2352$	0.9834		
Chlorpyrifos	0.35–0.60	$^1y = 20.37x - 1.5125$	0.9942	0.11	0.35
		$^2y = 0.1504x + 0.2846$	0.9804		

[1]peak area, [2]peak height.

Table 3. Statistical data for intra-day precision of retention time, peak area and peak height ($n = 7$)

Compound	Parameter	$\bar{x}$	SD	RSD (%)
Captan	retention time (min)	3.83	0.0027	0.07
	peak area	185.8164	0.9804	0.53
	peak height	32.4634	0.1588	0.49
Folpet	retention time (min)	4.39	0.0033	0.07
	peak area	4724.5789	5.2671	0.11
	peak height	628.3297	1.7966	0.29
Difenoconazole	retention time (min)	5.52	0.0145	0.01
	peak area	103.8451	0.4087	0.39
	peak height	6,203	0.0500	0.43
Chlorpyrifos	retention time (min)	8.01	0.0139	0.17
	peak area	8.0514	0.4804	5.97
	peak height	0.6019	0.0165	2.74

Table 4. Results for recovery experiments ($n = 5$)

Compound	Fortification level (mg kg^{-1})	Total analyte found (mg kg^{-1} ± SD)	Recovery (%)	RSD (%)
Captan	2.10	2.4073 ± 0.0132	114.63	0.55
	3.00	2.8483 ± 0.0080	94.94	0.28
	3.60	3.6206 ± 0.0069	100.57	0.19
Folpet	2.10	2.3350 ± 0.0032	111.19	0.14
	3.00	3.0212 ± 0.0026	100.71	0.09
	3.60	3.5177 ± 0.0067	97.71	0.19
Difenoconazole	0.35	0.3538 ± 0.0246	101.08	6.95
	0.50	0.4905 ± 0.0062	98.10	1.27
	0.60	0.5841 ± 0.0340	100.95	5.61
Chlorpyrifos	0.35	0.3544 ± 0.4673	101.27	8.19
	0.50	0.4889 ± 0.6863	97.79	8.12
	0.60	0.6067 ± 1.0034	101.11	9.25

(20 µL) of the spiked standards in the apple sample matrix at following levels: 50 % MRL, 70 % MRL, MRL and 120 % MRL for captan and folpet, and 70 % MRL, MRL and 120 % MRL for difenoconazole and chlorpyrifos (Table 2). Calibration graphs are constructed by plotting the concentration of analyte spiked in an apple sample as a function of peak area and peak height.

Table 5. The determined concentrations of pesticide residues in apple samples (n = 3)

Sample (n = 3)	Detected Pesticide	Determined concentration (mg kg^{-1} ± SD)	RSD (%)
1	nd	–	–
2	Difenoconazole	2.26 ± 0.0208	0.92
3	nd	–	–
4	Difenoconazole	1.18 ± 0.0296	2.50
5	Difenoconazole	1.37 ± 0.0509	3.72
6	Difenoconazole	0.93 ± 0.0229	2.47
7	Difenoconazole	0.17 ± 0.0084	4.98
8	Difenoconazole	0.17 ± 0.0155	9.08
9	Difenoconazole	0.21 ± 0.0028	1.29
10	nd	–	–
11	Difenoconazole	0.25 ± 0.0125	4.94
12	Difenoconazole	0.44 ± 0.0261	5.88
13	Chlorpyrifos	0.43 ± 0.0140	3.23
14–19	nd	–	–

nd = not detected.

According to the obtained chromatograms (Fig. 3) from the mixture of analytical standards and spiked sample, difenoconazole is consisted of two peaks (isomers) that are not fully separated. According to SANCO/825/00 rev. 8.1 2010 [25] and SANCO/12495/2011 [26], pesticides that are mixture of isomeric forms can be determined and validated by using either the sum of peak areas, the sum of peak heights, or measurement of a single component, whichever is the most accurate. Hence, for this validation the sum of peak area has been used for the determination of difenoconazole.

The calibration curves are linear over the concentration range of 1.50–3.60 mg kg^{-1} for captan and folpet, and 0.35 – 0.60 mg kg^{-1} for difenoconazole and chlorpyrifos. The obtained results for multiple correlation coefficients ($R^2 \geq 0.90$) indicated, preferably the use of peak area as a variable. The method has a satisfactory linearity for all analytes (Table 2).

Limit of detection and limit of quantification. Limit of detection (LOD) and limit of quantification (LOQ) are two crucial characteristics in method validation, which allow to estimate the sensitivity of the method [27]. The limit of detection (LOD) is defined as the amount of analyte for which the signal-to-noise ratio (S/N) is 3, whereas the limit of quantification (LOQ) is defined as the amount of analyte for which S/N = 10. The LOD and LOQ for each compound

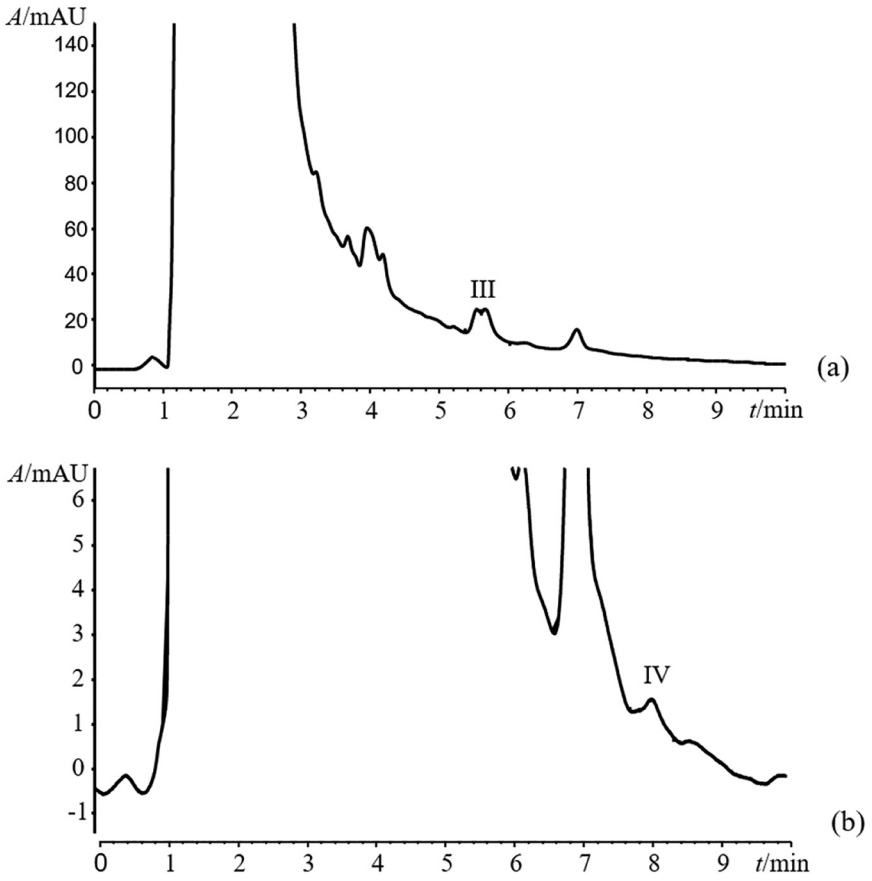

Fig. 4. Chromatograms UV–VIS from apple samples in which difenoconazole (0.93 mg kg^{-1}) at 220 nm (a) and chlorpyrifos (0.43 mg kg^{-1}) at 230 nm (b) are determined

are listed in Table 2. According to the S/N, the established limit of quantification (LOQ) for captan and folpet is 1.50 mg kg^{-1}, while for difenoconazole and chlorpyrifos is 0.35 mg kg^{-1}. The values of LOQ are lower than MRLs set by the European Union for analysed pesticides [8]. According to the obtained results, the developed method allows to identify and quantify the pesticides in the tested concentration range (Table 2).

Precision. The precision is expressed as repeatability of obtained results from seven injections with the volume of 20 µL of the spiked apple samples at MRL for each of the analytes, under the same experimental conditions, during the same day (intra-day precision) (Table 3). The computed values of RSD for retention time, peak area, and peak height, ranged from 0.01 to 5.97 %, indicating an excellent precision of the proposed method.

Accuracy. The accuracy of the method is determined by recovery studies in apple samples (pesticides free) spiked with the analysed pesticides at three concentation levels (70 % MRL, MRL and 120 % MRL) (Table 4). According to SANCO/825/00 rev. 8.1 [25], the mean recovery at each fortification level in the range of 70 %–120 % and relative standard deviation (RSD) $\leq$ 20 % per level are acceptable. The obtained values for relative standard deviation (RSD) are within the following ranges: 94.94 – 114.63 % and 0.09 – 9.25 %, respectively, which complies with the established criteria. Consequently, it can be concluded that the proposed method is characterized by satisfactory accuracy and, hence, it is suitable for the determination of the tested pesticide residues in apple samples.

Apple samples

The developed and validated method is applied to the monitoring of captan, folpet, difenoconazole and chlorpyrifos residues in apple samples. For that purpose, 19 apple samples are randomly collected from orchards in different regions of North Macedonia. The investigations showed that difenoconazole residues are most often found in the analysed apple samples. Specifically, in nine samples difenoconazole is detected, of which four samples showed concentrations lower than the established MRL (0.50 mg kg^{-1}) and in five of the tested samples difenoconazole exceeded the MRL. Chlorpyrifos is detected in one sample with concertation of 0.43 mg kg^{-1}, which is lower than its' MRL of 0.50 mg kg^{-1}. As can be seen, from Table 5, captan and folpet are no detected in none of the analysed samples. In Fig. 4 are presented typical chromatograms from apple samples in which difenoconazole and chlorpyrifos are detected and quantified.

CONCLUSIONS

The development of simple and reliable analytical methods to monitor pesticide residues in fruits is of significant importance. In this study, a selective, accurate and suitable RP-HPLC and UV–DAD method for simultaneous determination of the most commonly used pesticides for apple protection in North Macedonia has been described. Successful separation and quantification is achieved in 8.5 min, using isocratic elution with mobile phase consisting of acetonitrile/0.1 % acetic acid in water (70/30, *V/V*), flow rate of 1 mL min^{-1}, constant column temperature at 25 °C, and UV detection at 220 and 230 nm. Before identification and quantification of analysed samples, the extraction and sample clean-up are performed. Furthermore, the developed method is validated according to the EU Regulation and EU Guidance document and the obtained results revealed that the proposed method has a satisfactory linearity, sensitivity (LOD and LOQ), precision and accuracy for all analytes and it is convenient for routine determination of investigated pesticides in apple samples.

Moreover, the method is applied for determination of captan, folpet, difenoconazole and chlorpyrifos residues in apple samples taken from different regions in North Macedonia. The obtained results show that difenoconazole is frequently detected fungicide in the analysed apple samples, and its concentration in some samples exceeded the MRL according to EU Regulation [8].

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