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DETERMINATION OF SOME PESTICIDE RESIDUES IN APPLE JUICE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Velkoska-Markovska Lenche[1*], Petanovska-Ilievska Biljana[1], Markovski Aleksandar[2]

[1]Ss. Cyril and Methodius University in Skopje, Faculty of Agricultural Sciences and Food - Skopje, Republic of North Macedonia
[2]Ss. Cyril and Methodius University in Skopje, Institute of Agriculture - Skopje, Republic of North Macedonia

[*]Corresponding author: levemar@gmail.com

ABSTRACT
A new, simple and reliable reversed-phase high-performance liquid chromatography (RP-HPLC) method for determination of 2,4-D ((2,4-dichlorophenoxy)acetic acid), atrazine, malathion, fenitrothion and parathion residues in apple juices has been developed and validated. Successful separation and quantitative determination of analytes were performed on Purospher STAR RP-8e (30 x 4 mm, 3 µm) analytical column, with mobile phase consisted of acetonitrile/water (45/55, *V/V*), flow rate of 1 mL/min, constant column temperature at 25 ^{0}C and UV detection at 220 nm and 270 nm. A solid-phase extraction (SPE) was used for concentration and clean-up of analytes. Specificity, selectivity, linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ) were tested for the method validation according to European Commission guidelines for pesticide residue analytical methods, and all performance characteristics were found within acceptance criteria. Calibration curves were linear within the range less than 50 % of the maximum residue limit (MRL) values and 20 % above for all investigated pesticides (R^2 > 0.95). Under the stipulated conditions, values for relative standard deviations (RSD) of retention times and peak areas were 0.12 % - 0.28 % and 0.08 % - 8.50 %, respectively. The obtained values for recoveries ranged from 93.65 % - 118.62 %, with RSD ≤ 5.64 %. This method was successfully applied for determination of investigated pesticide residues in apple juice samples, which were taken from Macedonian markets. The run time of assay obtained from this chromatography condition was about 4 min.

Keywords: RP-HPLC, SPE, method development, validation.

INTRODUCTION
Apple is a fruit kind that grows across the world and it is one of the most consumed fruits in the world. Apples are consumed fresh, but, also they are processed into many products, and the apple juice is its most popular product. Apples and apple juice are rich in essential nutrients and phytochemicals such as phenolic compounds and flavonoids that have antioxidant activity and help against many diseases and ailments, such as cancer, asthma, cardiovascular disease, and blood pressure (Hyson, 2011; Candrawinata et al., 2015). On the other hand, in the apple production, huge amounts of pesticides are used, which can penetrate the tissues of apples and their residues cause a potential risk to human health. To protect human health, in most countries, Maximum Residue Levels (MRLs) for the pesticides in food (eg. fruit and vegetables) have been established. Among the most used pesticides in apple production are phenoxycarboxylic acids (e.g., 2,4-D), organonitrogen (e.g., atrazine) and organophosphorus (e.g., malathion, fenitrothion and parathion). The MRLs of selected pesticides in apple were set by the European Union Regulation (EC) No. 396/2005 (2005) and they were estimated at 0.05 mg/kg for 2,4-D, atrazine and parathion, 0.02 mg/kg for malathion, and 0.01 mg/kg for fenitrothion. Monitoring of pesticide residues in food samples is essential for safety purposes, therefore, development of new and improvement of existing analytical methods are crucial.
Up to now, many analytical methods for the determination of pesticides in fruits, vegetables and their juices have been published, among which the most numerous are Gas Chromatography (GC) and Liquid Chromatography (LC) equipped with different detectors, such as: Mass Spectrometry (MS) (Cunha et al., 2009), Tandem Mass Spectrometry (MS/MS) (Pang et al., 2006;), Flame Photometric Detector (FPD) (Tseng et al., 2007), Nitrogen Phosphorous Detector (NPD) (Attallah et al., 2012) etc. Although it is characterized by lower sensitivity than GC-MS and LC-MS and normally is not used for the analysis of complex samples, HPLC combined with ultraviolet (UV) and/or Diode Array Detector (DAD) is used for determination of organophosphorus and triazines in different matrices (Sanchez-Ortega et al., 2005). The

analysis of pesticide residues necessarily involves pretreatment of samples, and for this purpose some of the following techniques are used: Liquid-Liquid Extraction (LLE) (Jeannot et al., 2009), Solid Phase Extraction (SPE) (Topuz et al., 2005), Liquid-Liquid Microextraction (LLME) (Cunha et al., 2009), Solid Phase Microextraction (SPME) (Hercegová and Mőder, 2011), Matrix Solid-Phase Dispersion (MSPD) (Chua et al., 2005) and recently used, a quick, easy, cheap, effective, rugged and safe (QuEChERS) method (Zanella et al., 2013).

In a previous study HPLC method was developed for determination of selected pesticides using DAD (Velkoska-Markovska and Petanovska-Ilievska, 2013; Velkoska-Markovska et al., 2017a, 2017b). The objective of this study was to investigate the other possibilities for HPLC determination of 2,4-D, atrazine, malathion, fenitrothion and parathion residues in apple juice using different analytical column in order to achieve reducing both the duration and the cost of the chromatographic analysis.

MATERIAL AND METHODS

Instrumentation

The high-performance liquid chromatographic analysis was performed on an Agilent 1260 Infinity Rapid Resolution Liquid Chromatography (RRLC) system equipped with: vacuum degasser (G1322A), binary pump (G1312B), autosampler (G1329B), a column compartment (G1316A), UV-VIS diode array detector (G1316B) and ChemStation software. The investigations were conducted using Purospher STAR RP-8e (30 x 4 mm, 3 µm) analytical column produced by Merck (Germany). An ultrasonic bath "Elma" was used for better dissolving of the stock solutions. Solid phase extraction was performed using a vacuum manifold Visiprep (Supelco, Sigma Aldrich). The samples were vortexed with IKA Vortex Genius 3 (Germany).

Chemicals

The Pestanal analytical standards of 2,4-D (98.6 %), atrazine (98.8 % purity), malathion (97.2 % purity), fenitrothion (95.2 % purity) and parathion (98.8 % purity), as well as, HPLC-grade acetonitrile and methanol were purchased by Sigma-Aldrich (Germany). Ultrapure water was produced by TKA Smart - 2Pure 12 UV/UF water purification system (Germany). Formic acid (98 % - 100 % purity) was produced by Merck (Germany).

Preparation of Standard Solutions

Stock solutions were prepared by dissolving of the pure analytical standards of 2,4-D (25.3 mg), atrazine (11.3 mg), malathion (33.0 mg), fenitrothion (22.5 mg) and parathion (18.8 mg) in acetonitrile using a 25 mL volumetric flasks. The solutions were degassed for 15 min in an ultrasonic bath and stored in a refrigerator in the dark at 4 ^{0}C before use. Stock solutions were used for the preparation of standard mixtures with different pesticide concentrations (1.82 - 14.59 µg/mL for 2,4-D, 0.62 - 4.99 µg/mL for atrazine, 46.75 - 373.99 µg/mL for malathion, 11.18 - 89.47 µg/mL for fenitrothion and 12.851 - 102.81 µg/mL for parathion) in 10 mL volumetric flasks by dilution with the acetonitrile/water mixture (50/50, *V/V*) and for the spiking of apple juice samples.

Sample preparation

For the investigation 100 % clear apple juice samples were used. The samples were chosen from three different producers (A, B, and C), which were taken from local supermarkets in Macedonia. The sample preparation was started by filtering of apple juice samples through 0.45 µm nitrocellulose membrane filters (Millipore, Ireland). For the analytes concentration and purification of the samples, the solid-phase extraction (SPE) was performed using Supelclean ENVI-18 tubes (6 mL, 0.5 g, produced by Supelco, Sigma-Aldrich, Germany).

Spiking samples were prepared for determination of linearity, precision and recovery by fortifying 1 kg apple juice with five sets of concentrations of each analyte: 0.007, 0.025, 0.035, 0.05 and 0.06 mg/kg for atrazine and parathion; 0.0028, 0.01, 0.014, 0.02 and 0.024 mg/kg for malathion and 0.0014, 0.007, 0.005, 0.01 and 0.012 mg/kg for fenitrothion. Unspiked samples were used for blanks. The blank samples were prepared from apple juice free of tested pesticides. For each concentration level five samples (n = 5) were prepared.

Prior to use, the SPE cartridges were conditioned with 5 mL of acetonitrile, followed by 5 mL of water at a flow rate of 2 mL/min. Subsequently, 1 kg of filtered apple juice samples were passed through the cartridges at a flow rate of 8 - 10 mL/min, and then the tubes were washed with 5 mL of water. The drying process of the cartridges was carried out under a vacuum for 10 minutes. The elution of the cartridges was achieved with 2 × 2 mL of acetonitrile and the eluates were evaporated to dryness in a nitrogen evaporator. The

obtained residue was dissolved in 1 mL acetonitrile/water mixture (50/50, *V/V*) by vortexing for 1 min and filtered through 0.45 µm Iso-Disc PTFE syringe filters (Supelco, Sigma-Aldrich, Germany) just before the HPLC analysis. The injection volume of each sample was 5 µL.

RESULTS AND DISCUSSION

In order to perform a determination of 2,4-D ((2,4-dichlorophenoxy)acetic acid), atrazine, malathion, fenitrothion and parathion residues in apple juice, a reversed-phase high-performance liquid chromatography (RP-HPLC) method has been developed and validated. The investigated pesticides belong to different groups according to their chemical structures (Fig. 1): 2,4-D ((2,4-dichlorophenoxy)acetic acid, IUPAC) is a phenoxycarboxylic acid, atrazine (6-chloro-N^2-ethyl-N^4-isopropyl-1,3,5-triazine-2,4-diamine, IUPAC) belongs to triazines, malathion (diethyl (dimethoxythiophosphorylthio) succinate; *S*-1,2-bis(ethoxycarbonyl)ethyl *O*,*O*-dimethyl phosphorodithioate, IUPAC), fenitrothion (*O*,*O*-dimethyl *O*-4-nitro-*m*-tolylphosphorothioate, IUPAC) and parathion (*O*,*O*-diethyl *O*-4-nitrophenyl phosphorothioate, IUPAC) are organophosphorus pesticides (Tomlin, 1997).

The choice of the wavelength at which the chromatographic analysis was performed was made based on the UV spectra of the components of interest recorded in a solution of acetonitrile and water, with a volume ratio of 50/50 (Fig. 1). As can be seen from this figure, the analytes have absorption maxima around 220 nm. Moreover, fenitrothion shows a maximum UV absorption of about 270 nm (Fig. 1d), and the parathion of about 280 nm (Fig. 1e). As a result of this, the chromatographic analysis for their simultaneous determination was carried out at 220 nm and 270 nm.

To obtain optimum conditions for the separation of analytes with a symmetrical form of chromatographic peaks and satisfactory values for the purity index, a series of preliminary investigations have been carried out, whereby the volume ratio of acetonitrile and water to the mobile phase was changed. The optimum separation conditions of the investigated components with isocratic elution were determined using a mobile phase composed of acetonitrile and water with a volume ratio (45/55, *V/V*) (Fig. 2), flow of 1 mL/min, a constant column temperature of 25 ^{0}C and UV detection at 220 nm and 270 nm.

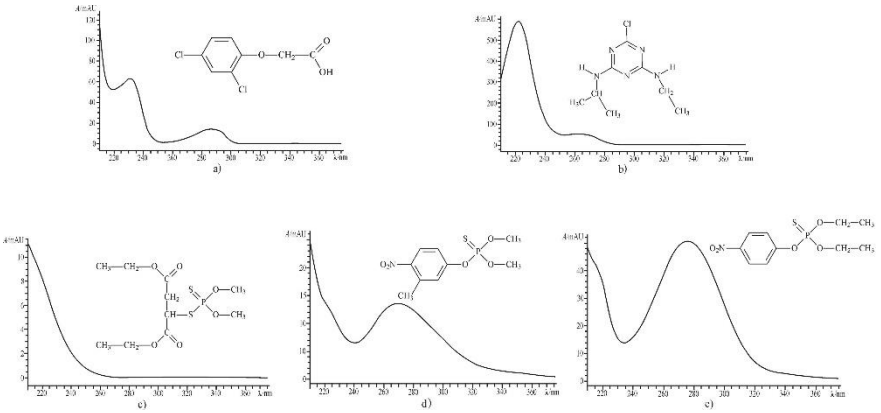

Figure 1. Chemical structures of 2,4-D (a), atrazine (b), malathion (c), fenitrothion (d) and parathion (e) and their UV spectra in acetonitrile/water (50/50, *V/V*)

Table 1 shows the obtained values for the dead time (t_0), the retention time (t_R) of the analytes, their retention factors (k'), the separation factor (α), and the resolution (R_S) at the adjacent peaks. Under the determined chromatographic operating conditions, the calculated values for the retention factor (k') were less than 20, which is the highest optimal value for this parameter, and the resolution (R_S) at the adjacent peaks was greater than 1.5, which means that under the prescribed chromatographic conditions a high separation of the investigated pesticides was achieved (Dong, 2006) in about 4 min. Compared with the previously published results (Velkoska-Markovska and Petanovska-Ilievska, 2013; Velkoska-Markovska et al., 2017b), shorter retention times for the components were obtained, thereby reducing both the duration and the cost of the analysis.

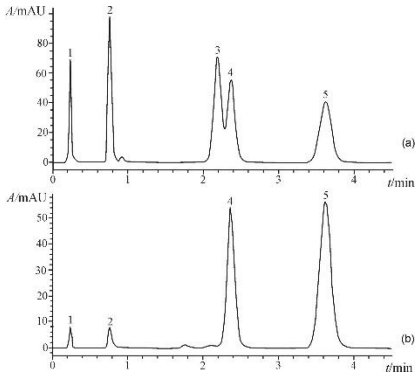

Figure 2. Chromatograms obtained from standard mixtures of 36.48 ng 2,4-D (1), 12.48 ng atrazine (2), 935.00 ng malathion (3), 223.68 ng fenitrothion (4) and 257.02 ng parathion (5) at 220 nm (a) and 270 nm (b) with developed method

Table 1. Data for dead time (t_0), retention times (t_R), retention factors (k'), separation factors (α) and resolution (Rs) for the analysed pesticides

Compound	t_R (min)	k'	α	Rs
dead time	0.19	-	-	-
2,4-D	0.23	0.21	14.05	12.21
atrazine	0.75	2.95	3.53	17.48
malathion	2.17	10.42	1.09	1.51
fenitrothion	2.34	11.32	1.57	8.57
parathion	3.57	17.79	-	-

To perform quantitative determination of the tested pesticide residues in apple juice samples using the developed method, it was necessary concentrating the components of interest, which was done by applying solid phase extraction using the Supelclean ENVI-18 column.

For the method validation, apple juice samples were prepared by fortifying with pesticides of interest, and then the samples were concentrated using solid-phase extraction. The method validation was carried out by testing specificity, selectivity, linearity, precision, recovery, limit of detection (LOD) and limit of quantification (LOQ) in accordance with the directions for analytical methods for determination of pesticide residues given by the EU Regulation and EU Guidance documents (Document N^o SANCO/12495/2011, 2011, European Commission 2010).

To confirm the specificity of the developed method, UV-diode array detection was used to check the peak purity and analyte peak identity. The purity index for all the analytes was greater than 990 (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.

The identification of the analysed pesticides was done by comparing the retention times of analytical standards with those of the same components in the apple juice samples and by match factor values obtained by overlaid spectra of a pure analytical standard and absorption spectra of the same analyte in the apple juice samples. Additionally, in order to prove the selectivity of method and in accordance with the EU criteria (European Commission, 2010), Figure 3 presents chromatograms of a standard mixture with a concentration corresponding to MRL (a), matrix blank (apple juice free of investigated pesticides) (b) and a sample of apple juice spiked with pesticides with a concentration equal to the MRL for each analyte (c).

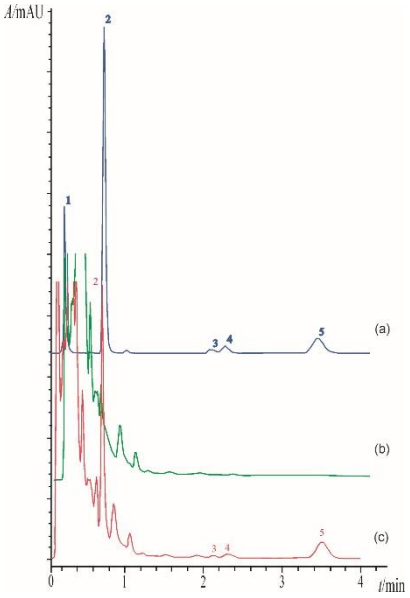

Figure 3. Chromatograms from standard mixture of 2,4-D (1), atrazine (2), malathion (3), fenitrothion (4) and parathion (5) at the concentrations which correspond to MRLs (a), matrix blank (b) and samples of apple juice fortified at the concentration equal to MRL for each analyte (c)

As can be seen from Figure 3c, using the proposed method, a qualitative and quantitative determination of the 2,4-D component cannot be performed because of its complete overlapping with peaks from the matrix, while the other components of interest: atrazine, malathion, fenitrothion and parathion can be determined by the elaborated method in apple juice samples.

The preparation of apple juice samples to determine the linearity of the method was done by adding a precisely determined quantity to each pesticide of interest at 5 concentration levels in an interval of less than 50% of the MRL to a 20% higher concentration than the MRL (Table 2), and then the samples were concentrated using SPE. For the construction of the calibration curves for each analyte, a triple injection of juice samples spiked with the tested pesticides was performed, with a volume of 5 µL. Table 2 shows the statistics on the linearity of the developed method. The values for the correlation coefficients (R^2) range from 0.9530 to 0.9975, indicating that the method is characterized by good linearity for each of the analytes.

LOD was determined as signal to noise ratio 3:1, while LOQ value was calculated as 10 times the signal height to the baseline (S/N = 10). The determined values of LOD and LOQ for the investigated compounds are given in Table 2. These results are acceptable for determining the pesticide residues, according to the EU rules (European Commission 2010).

Table 2. Statistical data for linearity of the method

Compound	Linearity range (µg/kg)	Regression equation	R^2	LOD (mg/kg)	LOQ (mg/kg)
atrazine	7.00 – 60.00	$^1y = 61335x + 269.47$ $^2y = 23073x + 118.38$	0.9865 0.9851	0.0021	0.0070
malathion	2.80 – 24.00	$^1y = 3328.1x - 0.0169$ $^2y = 536.9x + 0.1426$	0.9764 0.9975	0.00093	0.0028
fenitrothion	1.40 – 12.00	$^1y = 11798x + 10.981$ $^2y = 1538.3x + 1.2928$	0.9800 0.9847	0.00046	0.0014
parathion	7.00 – 60.00	$^1y = 22732x + 158.08$ $^2y = 2072.6x + 14.789$	0.9545 0.9530	0.0021	0.0070

^1y = peak area, ^2y = peak height

To determine the precision of the method, expressed as repeatability of the results obtained for the retention time and the peak area for each analyte, five consecutive injections (5 µL) of apple juice samples spiked with the examined pesticides at a level corresponding to MRL were done. The obtained results are shown in Table

3. The calculated values for RSD of retention times are in the range of 0.12 % to 0.28 %, while for the peak areas of the analytes are in the range 0.08 % - 8.50 %, from which, it can be concluded that the method is characterized by excellent precision in relation to the retention time and the peak area for each pesticide of interest.

Table 3. Statistical data for Intra-day precision of retention time and peak area (n = 5)

Compound	t_R (min) ± SD	RSD (%)	peak area ± SD	RSD (%)
atrazine	0.76 ± 0.0009	0.12	3246.11 ± 2.71	0.08
malathion	2.21 ± 0.006	0.27	65.21 ± 5.54	8.50
fenitrothion	2.40 ± 0.007	0.28	123.48 ± 0.28	0.23
parathion	3.65 ± 0.01	0.28	1223.55 ± 6.13	0.50

Furthermore, the accuracy of the method was determined by the recovery studies of apple juice samples fortified with the target pesticides at three concentration levels (Table 4). The obtained values for the recovery (93.65 % - 118.62 %) and the relative standard deviation (0.07 % - 5.64 %) for each concentration level are given in Table 4. The calculated values were within the allowed deviations for these parameters in accordance with the guidelines for analytical methods for the determination of pesticide residues (European Commission, 2010). These values indicated that the elaborated method is suitable for the determination of atrazine, malathion, fenitrothion and parathion residues in apple juice.

Table 4. Results from recovery experiments (n = 5)

Compound	Fortification level (mg/kg)	Total analyte found (mg/kg ± SD)	Recovery (%)	RSD (%)
atrazine	0.035	0.038 ± 0.00007	109.33	0.18
	0.050	0.048 ± 0.00004	97.03	0.08
	0.060	0.059 ± 0.00007	98.03	0.12
malathion	0.014	0.013 ± 0.00003	94.66	0.23
	0.020	0.019 ± 0.001	97.10	5.64
	0.024	0.024 ± 0.00002	101.39	0.07
fenitrothion	0.007	0.008 ± 0.00001	110.05	0.19
	0.010	0.010 ± 0.00002	96.09	0.20
	0.012	0.012 ± 0.00004	98.24	0.33
parathion	0.035	0.041 ± 0.0003	118.62	0.8
	0.050	0.047 ± 0.0003	93.65	0.61
	0.060	0.058 ± 0.0005	96.20	0.90

The developed reversed-phase high-performance liquid chromatography method was successfully applied for the determination of the selected pesticide residues in apple juice samples. In the examined samples purchased in local markets, no residues of the investigated pesticides in a concentration corresponding to the MRL or higher were found.

CONCLUSIONS

Using a Purospher STAR RP-8e (30 mm x 4 mm; 3 µm) analytical column, a new, simple, precise and accurate reversed-phase high-performance liquid chromatography (RP-HPLC) method has been developed

and validated, which has been successfully applied to determine residues of atrazine, malathion, fenitrothion and parathion in apple juice. The best separation of analytes was achieved using a mobile phase consisting of acetonitrile/water (45/55, *V/V*), flow rate of 1 mL/min, column constant temperature of 25 ^{0}C and UV detection at 220 nm and 270 nm. By applying this method, component 2,4-D cannot be determined in apple juice samples, because the chromatographic peaks originated from the matrix overlapped the peak of this component. The method validation was accomplished according to the EU Regulation and EU Guidance document, and all performance characteristics were found within acceptance criteria. This investigation shown that in the analysed apple juice samples, no residues of the targeted pesticides were found. The run time of analysis was about 4 min.

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