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APPLICATION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TO THE ANALYSIS OF PESTICIDE RESIDUES IN APPLE JUICE

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Summary: The modern apple production involves the use of large amounts of pesticides that can be found in processed products such as apple juice. Harmful effects of pesticide residues on humans, especially children, are well known, hence the content of pesticide residues in fruit, vegetables and their juices should be controlled. This study presents an application of a new, relatively simple and reliable analytical method for qualitative and quantitative determination of three organophosphorus and one organonitrogen pesticide residues in apple juices. The analysis utilizes reversed-phase high-performance liquid chromatography (RP-HPLC) followed by UV diode array detection. Prior to HPLC analysis, a solid-phase extraction (SPE) was used for analytes concentration and sample clean-up. Specificity, selectivity, linearity, precision, accuracy and limit of quantification (LOQ) were examined to assess the validity of the developed method. The method had satisfactory values of multiple correlation coefficients for calibration curves ($R^2 \geq 0.95$). The precision was evaluated for the retention times and peak areas, and the estimated values for relative standard deviations (RSD) were 0.05 % - 0.18 % and 0.09 % - 0.62 %, respectively, which indicated an excellent precision of the proposed method. Under the established conditions, the recovery of analytes was 93.80 % - 119.41 %, with relative standard deviations below 0.56 %. This method was successfully applied for determination of some organophosphorus and organonitrogen pesticide residues in apple juices which were taken from Macedonian markets. The achieved values for LOQs were low enough compared to the MRLs of the investigated pesticides in apple according to the Regulation (EC) No 396/2005. Detectable residues of the examined pesticides were not found in the analyzed samples.

Key words: RP-HPLC method, solid-phase extraction, pesticide residues, apple juice

INTRODUCTION

Apple juice is the most significant product obtained from apple processing and occupies an important place in the assortment of juices in many countries; also it is characterized with many positive effects on human health (Akazone, 2004; Barth et al., 2005; Markowski et al., 2009). However, excessive use of pesticides in apple cultivation results in pesticide residues found in apples and their processed products such as apple juice. Pesticides widely used in the apple production in the Republic of Macedonia include organophosphorus and organonitrogen pesticides, some of them being malathion, fenitrothion, parathion and atrazine. Although atrazine, fenitrothion and parathion are banned for use in the EU and the R. of Macedonia, they are permitted for use in the United States (except parathion) and some other countries. Also, as a result of illegal use of these pesticides, they could be found in foodstuff. Additionally, the raw material for the apple juice production could be contaminated with pesticides (including the examined ones) and could be imported from third countries due to the Agreement on Technical Barriers to Trade for removing the trade barriers. To avoid any adverse effects on human health, the Maximum Residue Levels (MRLs) for the pesticides in food (e.g. fruit and vegetables) have been stipulated in most countries. The MRLs of selected pesticides in apple established by the EU Regulation (EC) No. 396/2005 (2005) were estimated at 0.05 mg/kg for atrazine and parathion, 0.02 mg/kg for malathion, and 0.01 mg/kg for fenitrothion.

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In order to monitor food safety, it is highly necessary to develop and employ reliable methods for determination of pesticide residues. Gas Chromatography (GC) and Liquid Chromatography (LC) are powerful techniques for determination of many pesticides (e.g. organophosphorus and organonitrogen) in fruit, vegetables and their juices using various detectors, such as: Flame Photometric Detector (FPD) (Tseng et al., 2007), Nitrogen Phosphorous Detector (NPD) (Attalah, 2012; Albero et al., 2003), Mass Spectrometry (MS) (Cunha et al., 2009; Mercer, 2005; Hu et al., 2004), Tandem Mass Spectrometry (MS/MS) (Borba da Cunha, et al., 2004), 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 (Texeira et al., 2004; Melo et al., 2005).

Sample pretreatment is required before carrying out chromatographic analysis, and for this purpose different procedures are used, including: Liquid-Liquid Extraction (LLE) (Jeanot et al., 2009), Solid Phase Extraction (SPE) (Koal et al., 2003; Topuz et al., 2005), Liquid-Liquid Microextraction (LLME) (Cunha et al., 2009), Solid Phase Microextraction (SPME) (Kong, 2009) 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). The aim of this study was to investigate the other possibilities for determination of atrazine, malathion, fenitrothion and parathion residues in apple juice using different analytical column and mobile phases.

MATERIAL AND METHODS

Equipment and Chemicals. The 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 thermostatted column compartment (G1316A), UV-VIS diode array detector (G1316B) and ChemStation software. For better dissolving of the stock solutions an ultrasonic bath "Elma" was used. The experiments were carried out using LiChrospher 60 RP-select B (250 mm x 4 mm, 5 µm) analytical column produced by Merck (Germany). For the SPE a vacuum manifold Visiprep (Supelco, Sigma-Aldrich) was employed, and for vortexing of samples IKA Vortex Genius 3 (Germany) was used.

The Pestanal analytical standards of 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).

Various commercial 100 % clear apple juice samples from three different producers (A, B, and C) were purchased in local supermarkets in Macedonia.

Preparation of Standard Solutions. Stock solutions of atrazine, malathion, fenitrothion, and parathion were prepared by separately dissolving 0.0113 g of atrazine, 0.0330 g of malathion, 0.0225 g of fenitrothion, and 0.0188 g of parathion, of pure analytical standards in acetonitrile in 25 mL volumetric flasks. The solutions were degassed for 15 min in an ultrasonic bath and stored in a refrigerator at 4 ^{0}C. Stock solutions were used for preparation of standard mixtures with different pesticide concentrations (4.25 – 170.25 ng/mL for atrazine, 66.74 – 2672.50 ng/mL for malathion, 49.13 – 1967.00 ng/mL for fenitrothion, and 62.77 – 2513.26 ng/mL for parathion) in 10 mL volumetric flasks by dilution with the acetonitrile/water mixture (50/50, *V/V*) and for fortification of apple juice samples.

Extraction procedure. The first step in the sample preparation for analysis was filtering of apple juice samples through 0.45 µm nitrocellulose membrane filters (Millipore, Ireland). The SPE procedure was carried out using Supelclean ENVI-18 tubes (6 mL, 0.5 g, produced by Supelco, Sigma-Aldrich, Germany).

For determination of linearity, precision, recovery and limit of quantification (LOQ), spiked samples were prepared by fortifying 1 kg apple juice with five sets of concentrations: 0.00175 mg/kg, 0.025 mg/kg, 0.035 mg/kg, 0.05 mg/kg and 0.06 mg/kg for atrazine and parathion, 0.0007 mg/kg, 0.01 mg/kg, 0.014 mg/kg, 0.02 mg/kg and 0.024 mg/kg for malathion and 0.00035 mg/kg, 0.005 mg/kg, 0.007 mg/kg, 0.01 mg/kg and 0.012 mg/kg for fenitrothion. Unspiked samples were used for blanks. Blank samples were prepared from apple juice free of the tested pesticides. For each concentration level, five samples (n = 5) were prepared.

The conditioning of SPE cartridges was performed with 5 mL of acetonitrile, followed by 5 mL of water at a flow rate of 2 mL/min. After that, 1 kg of filtered apple juice samples were passed through the cartridges at a flow rate of 10 mL/min, and then the tubes were washed with 5 mL of water. Subsequently, the cartridges were dried for 10 min under a vacuum. The retained pesticides were eluted with 2 × 2 mL of acetonitrile. The eluates were evaporated to dryness under the gentle stream of nitrogen. The residues were redissolved with 1 mL of the acetonitrile/water mixture (50/50, *V/V*) by vortexing for 1 min, then filtered through 0.45 µm Iso-Disc PTFE syringe filters (Supelco,

Sigma-Aldrich, Germany) and transferred into vials for HPLC analysis. The injection volume of each sample was 20 µL.

RESULTS AND DISCUSSION

Atrazine (6-chloro-N^2-ethyl-N^4-isopropyl-1,3,5-triazine-2,4-diamine, IUPAC) belongs to triazines, while 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 (Figure 1) (Tomlin, 1997).

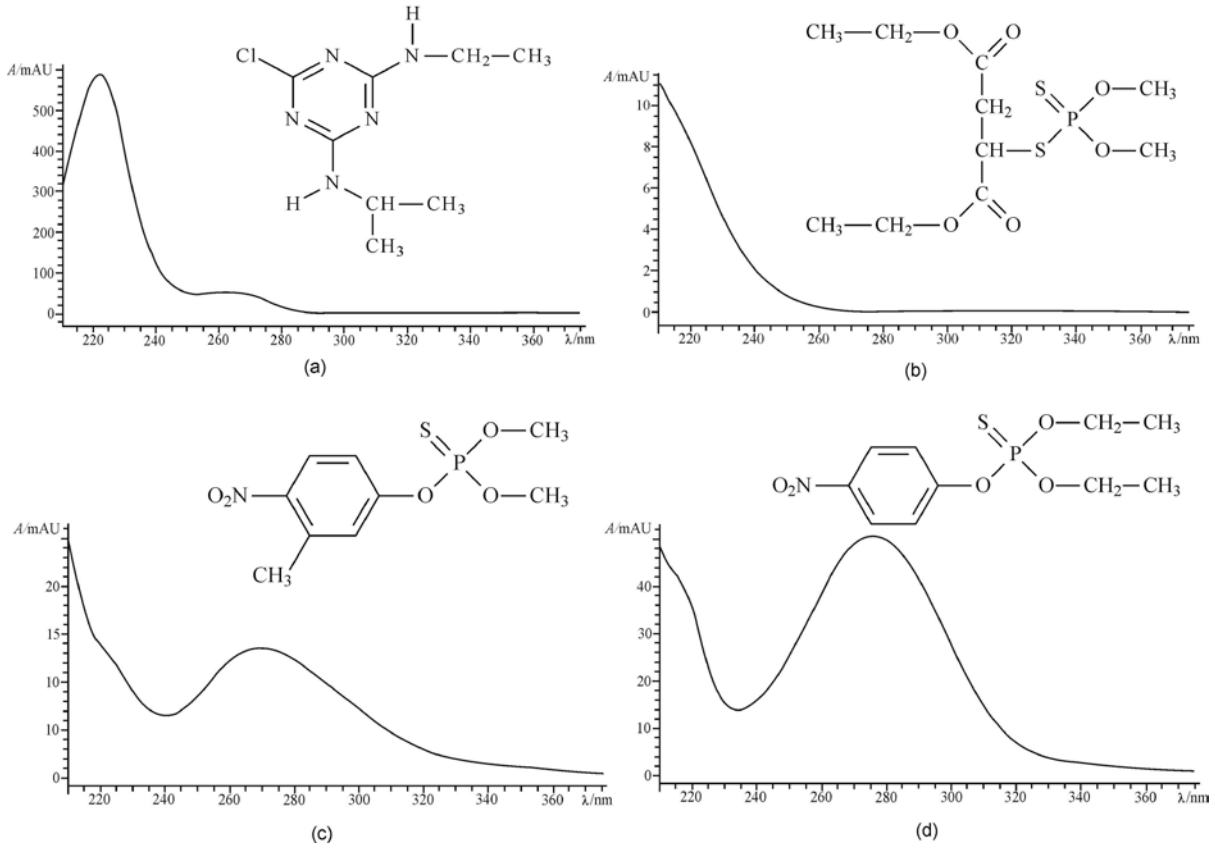

Figure 1. Chemical structures of atrazine (a), malathion (b), fenitrothion (c) and parathion (d) and their UV spectra in acetonitrile/water (50/50, V/V)

The UV spectra of the investigated pesticides in acetonitrile/water mixture (50/50, *V/V*) show that they have absorption maxima around 220 nm (Figure 1). Besides that, fenitrothion has an absorption maximum at 270 nm, while parathion has a band with higher absorption maximum at 280 nm. Hence, the chromatographic analysis for their simultaneous determination was carried out at 220 nm and 270 nm.

LiChrospher 60 RP-select B stationary phase 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 (ChromBook, 2011). Compared to the previous study (Velkoska-Markovska and Petanovska-Ilievska, 2013), in this investigation a longer column LiChrospher 60 RP-select B (250 mm x 4 mm, 5 µm) was chosen, which is characterised with higher efficiency as a result of the higher number of theoretical plates. The experiment also included a series of preliminary examinations with different mixtures of acetonitrile/water (80 - 40 % acetonitrile), methanol/water (80 – 65 % methanol), a mixture of equal volumes of

acetonitrile and methanol/water (70 – 60 % mixture of equal volumes of acetonitrile and methanol), as well as methanol/0.1 % formic acid as mobile phases in isocratic elution mode.

The conducted experiments showed that better results in terms of better baseline, a better peak shape and shorter retention time were obtained with a mobile phase composed of acetonitrile/water. The best separation of the analytes with symmetrical peak shapes and satisfactory purity indexes was achieved under isocratic elution with mobile phase consisting of acetonitrile/water (60/40, *V/V*), flow rate of 1 mL/min, constant column temperature at 25 ^{o}C and UV detection at 220 nm and 270 nm (Figure 2).

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, the computed values for retention factors (*k'*) were below 10, for separation factors (α) were above 1 and for resolution (*Rs*) were above 2, which implies that under the stipulated chromatographic conditions high separation of the investigated pesticides was reached (Dong, 2006).

The development and validation of an analytical method for simultaneous determination of the examined pesticides in apple juices was accomplished according to EU Regulation and EU Guidance documents (Document N^{o} SANCO/12495/2011, 2011; European Commission, 2010). Therefore, specificity, selectivity, linearity, precision expressed as repeatability of retention time and peak area, recovery and limit of quantification (LOQ) for all analytes were checked. For this purpose, 1 kg 100 % clear apple juice samples were fortified by the investigated pesticides ranging from 3.5 % of MRLs to 20 % above MRLs. Prior the HPLC analysis, SPE was conducted as a necessary step for analytes concentration and sample clean-up. Among the most commonly used sorbents for SPE of pesticide residues is C-18 (Pico, 2004), so ENVI-18 tubes were used in order to achieve this purpose.

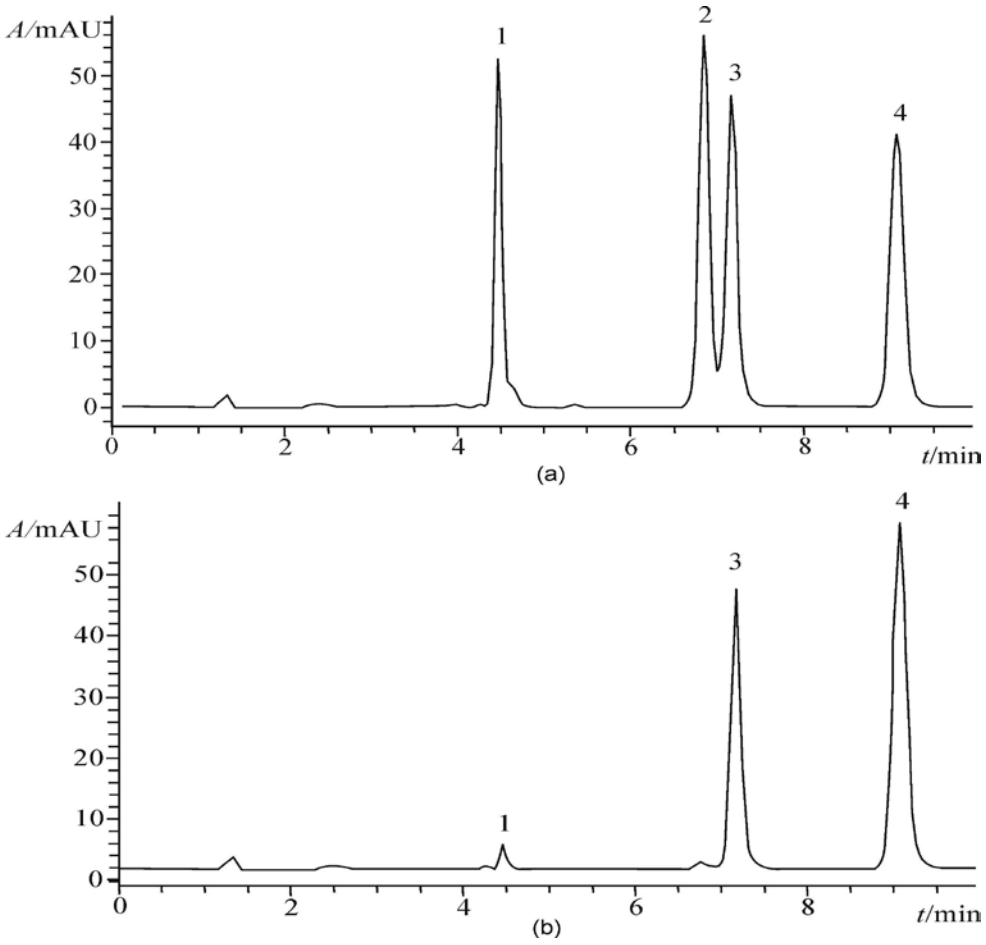

Figure 2. Chromatograms obtained from standard mixtures of atrazine (1), malathion (2), fenitrothion (3) and parathion (4) at 220 nm (a) and 270 nm (b) with developed method

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	1.09	-	-	-
atrazine	4.45	3.08	1.71	19.02
malathion	6.82	5.26	1.06	2.18
fenitrothion	7.14	5.55	1.31	11.54
parathion	9.05	7.30	-	-

Specificity and selectivity. 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 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. Additionally, 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 and absorption spectra of the same analyte in the apple juice samples. Furthermore, as recommended by European Commission (2010), to prove selectivity of the method, Figure 3 presents chromatograms of a standard mixture of target pesticides at the concentrations which corresponded to MRLs (a), matrix blank (unspiked apple juice sample, which was apple juice free of the investigated pesticides) (b), and a sample of apple juice fortified at the concentration equal to MRL for each analyte (c). As can be seen from Figure 3, the selected pesticide residues were separated from each other and from the matrix, hence they can be determined by the developed method.

Linearity. The linearity of the developed method was determined for all compounds separately, by construction of calibration curves at 5 concentration levels, with triplicate injections (20 μL) of the spiked standards in the apple juice sample matrix in the range of 0.00175 – 0.06 mg/kg for atrazine and parathion, 0.0007 – 0.024 mg/kg for malathion and 0.00035 – 0.012 mg/kg for fenitrothion. For these concentration ranges and using data for the peak areas and peak heights, curves were constructed and the correlation coefficients (R^2) were calculated (Table 2). The curves followed Lambert-Beer's law and the calculated results for multiple correlation coefficients ($R^2 \geq 0.95$) suggested that the method has a satisfactory linearity for all analytes (Table 2).

Table 2. Statistical data for linearity of the method

Compound	Linearity range (μg/kg)	Regression equation	R^2
atrazine	1.75 - 60.00	$^1y = 262152x + 643.87$ $^2y = 38104x + 116.32$	0.9880 0.9850
malathion	0.7 - 24.00	$^1y = 15196x + 16.885$ $^2y = 1594x + 1.807$	0.9904 0.9917
fenitrothion	0.35 - 12.00	$^1y = 41804x + 28.4$ $^2y = 4615.6x + 2.5208$	0.9829 0.9877
parathion	1.75 - 60.00	$^1y = 95990x + 452.05$ $^2y = 8433.6x + 41.319$	0.9596 0.9579

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

Limit of quantification. The signal-to-noise ratio (S/N) at the lowest concentration level for each compound was found to be ≥ 10 for all examined pesticides. Therefore, the LOQ was estimated to be 0.00175 mg/kg of atrazine and parathion, 0.0007 mg/kg of malathion and 0.00035 mg/kg of fenitrothion in this study. These results showed that the obtained values for LOQs were low enough compared to the MRLs of the selected pesticides in the apples (Regulation (EC) No 396/2005, 2005) and they are acceptable for determining the pesticide residues, according to the European Commission (2010) rules.

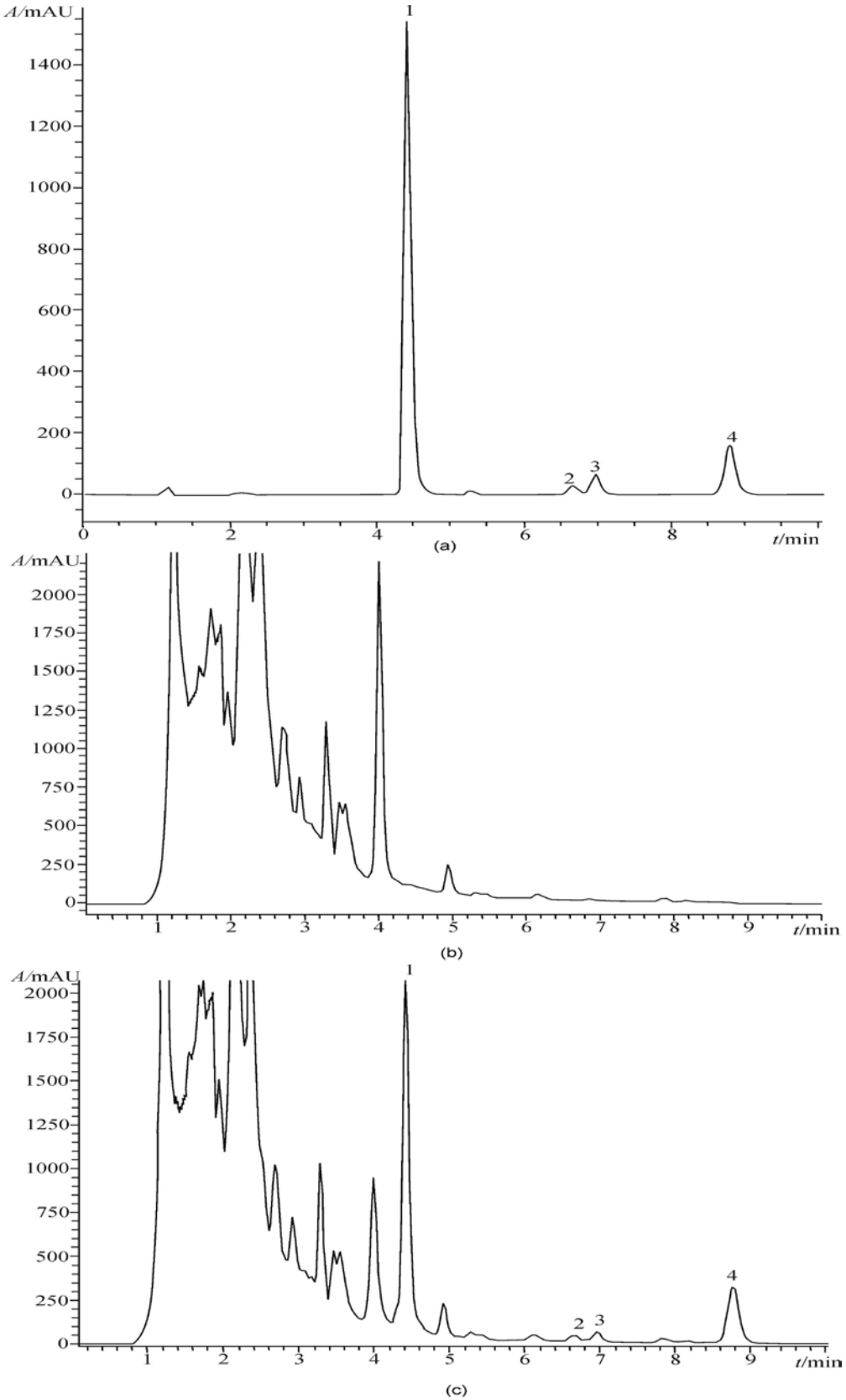

Figure 3. Chromatograms from standard mixture of atrazine (1), malathion (2), fenitrothion (3) and parathion (4) at the concentrations which corresponded to MRLs (a), matrix blank (b) and sample of apple juice fortified at the concentration equal to MRL for each analyte (c)

Precision. Furthermore, the precision was expressed as repeatability of the obtained results from five successive injections (20 µL) of the spiked apple juice samples at MRLs for each of the analytes. The computed values of relative standard deviation (RSD) for retention time were in the interval from 0.05 to 0.18 %, and for peak area in the range of 0.09 – 0.62 %, which indicated an excellent precision of the proposed method (Table 3).

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	4.42 ± 0.0008	0.18	13402 ± 11.78	0.09
malathion	6.65 ± 0.003	0.05	307.01 ± 0.68	0.22
fenitrothion	6.95 ± 0.003	0.05	428.28 ± 2.67	0.62
parathion	8.76 ± 0.005	0.06	4954.08 ± 9.29	0.19

Accuracy. The accuracy of the method was determined by recovery studies in apple juice samples (pesticides free) spiked with the investigated pesticides at three concentration levels (Table 4). The obtained values for recovery and for relative standard deviation were within the following ranges 93.80 – 119.41 % and 0.06 – 2.42 %, 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 the European Commission (2010) criteria. Consequently, it can be concluded that the proposed method is convenient for determination of the target pesticide residues in apple juice.

The developed RP-HPLC method was successfully applied to determine the target pesticide residues in apple juice samples under the defined experimental conditions. Samples from three different producers marked as: A, B and C were purchased from Macedonian market. Samples were concentrated and cleaned-up using SPE prior to HPLC analysis. Each analysis was repeated five times.

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

Compound	Fortification level (mg/kg)	Total analyte found (mg/kg ± SD)	Recovery (%)	RSD (%)
atrazine	0.035	0.039 ± 0.00002	110.77	0.06
	0.050	0.049 ± 0.00003	97.30	0.07
	0.060	0.058 ± 0.0003	96.9	0.56
malathion	0.014	0.015 ± 0.00004	110.00	0.28
	0.020	0.019 ± 0.00001	95.35	0.07
	0.024	0.024 ± 0.0006	98.54	2.42
fenitrothion	0.007	0.008 ± 0.00002	110.44	0.26
	0.010	0.010 ± 0.00001	97.10	0.11
	0.012	0.012 ± 0.00002	97.31	0.15
parathion	0.035	0.042 ± 0.00006	119.41	0.5
	0.050	0.047 ± 0.0001	93.80	0.21
	0.060	0.057 ± 0.0002	95.61	0.39

The typical chromatograms of apple juice samples are presented in Figure 4. In the chromatogram shown in Figure 4b there are two peaks (X_1 and X_2) near the expected peak of atrazine with the difference in retention time of ± 0.3 min (one before and one after the expected peak of atrazine). By comparing the UV spectra of these unknown components with those of the standard atrazine, it was found that they were completely different substances.

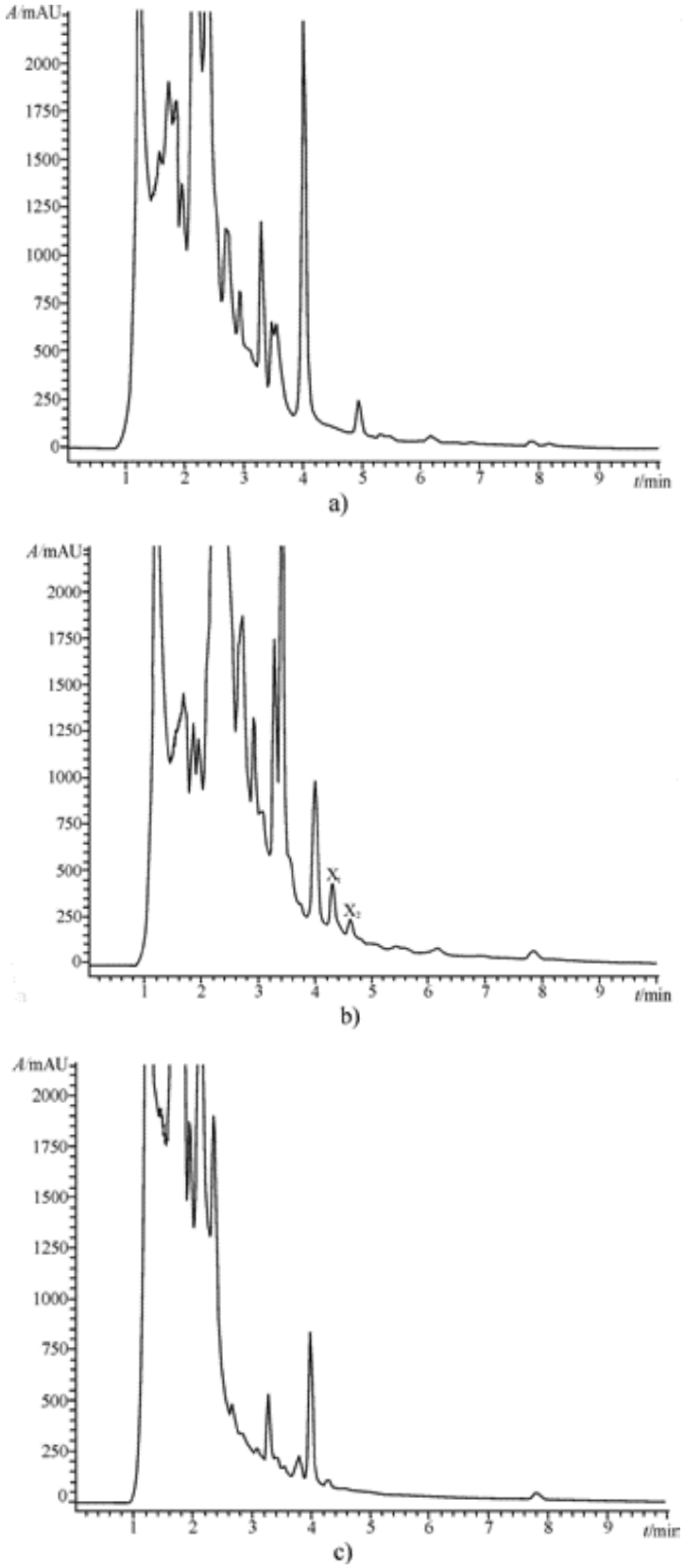

Figure 4. Typical chromatograms of apple juice samples A (a), B (b), and C (c) at 220 nm

The investigations show that residues of the analysed pesticides in the concentrations which corresponded to MRLs or higher were not detected in any of the tested apple juice samples.

CONCLUSIONS

This study describes a new possibility of successful determination of atrazine, malathion, fenitrothion and parathion residues in apple juice samples using reversed-phase high-performance liquid chromatography and ultraviolet diode array detection. Successful separation of the analytes was achieved under isocratic elution with mobile phase consisting of acetonitrile/water (60/40, *V/V*), flow rate of 1 mL/min, constant column temperature at 25 ^{0}C and UV detection at 220 nm and 270 nm. The developed method has been validated according to the EU Regulation and EU Guidance document. Solid-phase extraction was used for sample concentration and clean-up.

The results from the method validation revealed that the proposed method has a satisfactory linearity, precision and accuracy for all analytes, so it is convenient for routine determination of investigated pesticides in apple juice samples. The obtained values for LOQs were 0.00175 mg/kg for atrazine and parathion, 0.0007 mg/kg for malathion and 0.00035 mg/kg for fenitrothion, so they are acceptable for determining pesticide residues according to the Regulation (EC) No 396/2005. The analysis shows that residues of the investigated pesticides in the concentrations which corresponded to MRLs or higher were not detected in any of the tested apple juice samples. The run time of the analysis under the stipulated chromatographic conditions was about 10 min.

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