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APPLICATION OF RAPID RESOLUTION LIQUID CHROMATOGRAPHY TO THE ANALYSIS OF SOME PESTICIDE RESIDUES IN APPLE JUICE

SUMMARY

This paper presents the application of a new, precise, accurate and reliable rapid resolution liquid chromatography (RRLC) method with ultraviolet-diode array detection (UV-DAD) for the determination of some organonitrogen and organophosphorus pesticide residues in apple juice samples. The successful separation and quantitative determination of analytes were achieved using a Poroshell EC 120-C18 (50 mm x 3 mm; 2.7 μm) analytical column maintained at 25 ^{0}C and the detection was monitored at 220 nm and 270 nm. The mixture of acetonitrile/water (50/50, *V/V*) was used as a mobile phase, with flow rate of 1 mL/min. Specificity, selectivity, linearity, precision, accuracy and limit of quantification (LOQ) were examined to assess the validity of the developed method according to European Commission guidelines for pesticide residue analytical methods and all the performance characteristics were found within acceptance criteria. The obtained values for multiple correlation coefficients (R^2) were > 0.96, relative standard deviation (RSD) of retention times and peak areas were ≤ 1.15 %, and recoveries ranged from 93.98 % - 118.60 %, with RSD ≤ 1.77 %. The proposed method was successfully applied for the determination of investigated pesticides in apple juice samples. The detectable residues of examined pesticides were not found in the analysed samples.

Keywords: RRLC method, UV-DAD, pesticide residues, apple juice

INTRODUCTION

Numerous research studies suggest that apples may provide health benefits linked to lowered risk for many chronic and age-related diseases and the nutrients in whole apples are “passed along” when the fruit is processed into apple juice. Apple juice is rich in phytonutrients, which have powerful antioxidant effects and therefore many scientists suggest its inclusion in a healthy human diet (Barth *et*

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al. 2005, Boyer and liu 2004, Candrawinata *et al*. 2012, Crozier *et al*. 2009, Markowski *et al*. 2009, Lee *et al*. 2003).

On the other hand, this fruit is highly susceptible to many insects, pests and diseases, so in order to protect apples from pests, to increase yields and to preserve quality, excessive amounts of pesticides, both pre- and post-harvest are applied in apple production. Apples are on the top of the list of fruits and vegetables with the highest levels of pesticide residues, based on USDA (US Department of Agriculture) and FDA (US Food and Drug Administration) testing data (Grace Communication Foundation, 2017). It is well-known that the exposure to pesticides is dangerous to humans. In other words, the pesticides have been linked to a number of health problems, including neurologic and endocrine (hormone) system disorders, birth defects, cancer, and other diseases. To ensure the food safety and consumers' health protection in most countries Maximum residue levels (MRLs) of pesticides in foodstuff have been established. There is a wide range of pesticides which are used in apple production, some of them being organonitrogen (e.g., atrazine) and organophosphorus (e.g., malathion, fenitrothion and parathion) pesticides. The MRLs of pesticides in apples were set up by European Union Regulation (EC) No. 396/2005 (2005) and they were estimated at: 0.05 mg/kg for atrazine and parathion, 0.02 mg/kg for malathion, and 0.01 mg/kg for fenitrothion. In order to monitor food safety, it is highly necessary to develop and employ reliable methods for the determination of pesticide residues.

In the literature there are a lot of papers describing numerous techniques and analytical methods for the determination of many pesticide residues (e.g. organophosphorus and organonitrogen) in fruits, vegetables and their juices, among which the most widely used are Gas Chromatography (GC) equipped with different detectors, such as: Mass Spectrometry (MS) (Mercer, 2005, Chua *et al*. 2005, Cunha *et al*. 2009, Wang *et al*. 2006, Hu *et al*. 2004), Flame Photometric Detector (FPD) (Tseng *et al*. 2007), Nitrogen Phosphorous Detector (NPD) (Albero *et al*. 2003, Attallah *et al*. 2012), and Liquid Chromatography (LC) with Tandem Mass Spectrometry (MS/MS) (Perret *et al*. 2002, Pang *et al*. 2006, Borba da Cunha *et al*. 2004), Fluorescent detector (FD) (Fillion *et al*. 2000), etc. Although it is characterized by lower sensitivity than GC-MS and LC-MS and is not normally used for the analysis of complex samples, HPLC combined with ultraviolet (UV) and/or Diode Array Detector (DAD) is used for the determination of organophosphorus and triazines in different matrices (Rodriguez-Cuesta *et al*. 2005, Sanchez-Ortega *et al*. 2005, Baranowska *et al*. 2005, Texeira *et al*. 2004, Melo *et al*. 2005). The pretreatment of samples involves several extraction and purification steps utilizing the following procedures: Liquid-Liquid Extraction (LLE) (Jeannot *et al*. 2009), Solid Phase Extraction (SPE) (Topuz *et al*. 2005, Koal *et al*. 2003), Liquid-Liquid Microextraction (LLME) (Cunha *et al*. 2009), Solid Phase Microextraction (SPME) (Kong, 2009, 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 the determination of selected pesticides using DAD (Velkoska-Markovska and Petanovska-Ilievska

2013). To date, no RRLC method for the determination of target pesticide residues in apple juice has been developed. Hence, the objective of this paper was to develop and validate a new RRLC method that has been applied to the simultaneous determination of atrazine, malathion, fenitrothion and parathion residues in apple juice samples using UV-DAD.

MATERIAL AND METHODS

Equipment and Materials

The 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. For the better dissolving of the stock solutions an ultrasonic bath "Elma" was used. The experiments were carried out using Poroshell EC 120-C18 (50 mm x 3 mm; 2.7 μm) analytical column produced by Agilent Technologies (USA). A vacuum manifold Visiprep (Supelco, Sigma Aldrich) was used for solid phase extraction, while the samples were vortexed with IKA Vortex Genius 3 (Germany). The SPE procedure was performed on Supelclean ENVI-18 tubes, 6 mL, 0.5 g (Supelco, Sigma Aldrich, Germany).

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 were prepared by dissolving adequate mass of the pure analytical standards of 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 $^{\circ}$C before use. Stock solutions were used for the 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 the spiking of apple juice samples.

Sample preparation

The preparation of the samples for analysis was performed in a few steps. The first step in sample preparation procedures for RRLC determination was filtering of apple juice samples through 0.45 μm nitrocellulose membrane filters (Millipore, Ireland). The solid-phase extraction (SPE) was employed for the concentration and purification of the samples. The SPE procedure was carried out

using Supelclean ENVI-18 tubes (6 mL, 0.5 g, produced by Supelco, Sigma-Aldrich, Germany).

For the determination of linearity, precision, recovery and limit of quantification (LOQ), spiking samples were prepared by fortifying 1 kg apple juice with six sets of concentrations: 0.0007, 0.007, 0.025, 0.035, 0.05 and 0.06 mg/kg for atrazine and parathion, 0.00028, 0.0028, 0.01, 0.014, 0.02 and 0.024 mg/kg for malathion and 0.00014, 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 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 RRLC analysis. The injection volume of each sample was 5 µL.

RESULTS AND DISCUSSION

Rapid resolution liquid chromatography (RRLC) method was developed for the simultaneous determination of atrazine, malathion, fenitrothion and parathion residues in apple juice samples. According to their chemical structures (Figure 1) the target pesticides belong to different groups: atrazine (6-chloro-N^2-ethyl-N^4-isopropyl-1,3,5-triazine-2,4-diamine, IUPAC) is organonitrogen, and 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 identification of these pesticides was accomplished using UV-DAD.

From the UV spectra of investigated pesticides in acetonitrile/water mixture (50/50, *V/V*) (Figure 1) it can be seen that they have absorption maxima around 220 nm. Also, it is evident that fenitrothion has an absorption maximum at 270 nm, while parathion has a band with higher absorption maximum at 280 nm. Therefore, the chromatographic analysis for their simultaneous determination was carried out at 220 nm and 270 nm.

The successful separation and quantification of examined pesticides were carried out using a reverse phase Poroshell EC 120-C18 (50 mm x 3 mm; 2.7 µm) analytical column. A series of preliminary examinations with different mixtures of acetonitrile/water (80 - 40% acetonitrile), methanol/water (80 – 65%

methanol), as well as acetonitrile/0.1% formic acid and methanol/0.1 % formic acid as mobile phases in isocratic elution mode were used. The investigations 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 satisfying purity indexes was achieved under isocratic elution with acetonitrile/water (50/50, *V/V*) as a mobile phase, flow rate of 1 mL/min, constant column temperature at 25 °C and UV detection at 220 nm and 270 nm (Figure 2).

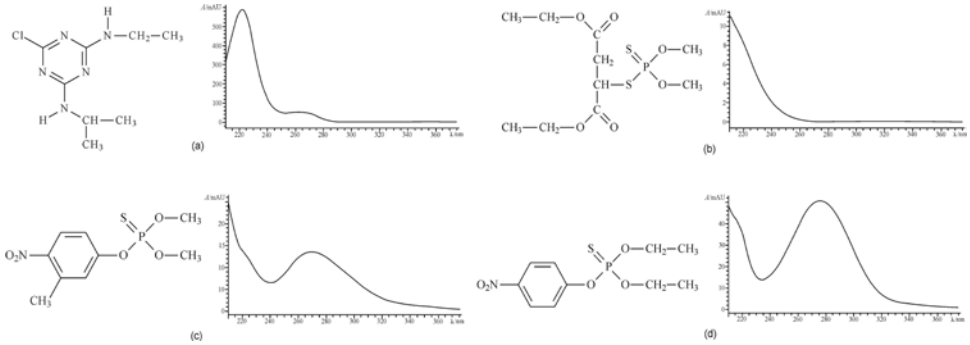

Figure 1. Chemical structures of atrazine (a), malathion (b), fenitrothion (c) and parathion (d) and their UV spectra in acetonitrile/water (50/50, *V/V*)

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 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. The values of retention factor below 20 and for resolution above 1.5 indicated that the separation of analytes under used chromatographic conditions was successful (Dong, 2006). Compared with the results of the previous study (Velkoska-Markovska and Petanovska-Ilievska 2013), conducted on the LiChrospher 60 RP-select B (125 mm x 4 mm, 5 µm) column, shorter retention times for components were obtained, which means less time for chromatographic analysis (3.5 min). In other words, this analysis requires a small volume (< 2 mL) of the organic solvent (acetonitrile), thereby reducing the cost of the analysis.

The developed method was applied for the determination of selected pesticide residues in apple juice samples. Prior to the quantitative determination of the content of tested pesticide residues in apple juice samples it is necessary to perform the enrichment and clean-up of analytes. The sample preparation is a crucial step in the analysis, which has great impact on the reliability and accuracy

of the result. For achieving that goal, a solid-phase extraction was conducted using the Supelclean ENVI-18 tubes. These SPE columns were chosen because C-18 is the most commonly used sorbent for SPE of pesticide residues in various samples (Pico *et al*. 2004). The method validation was performed in accordance with EU Regulation and EU Guidance documents (Document N^{o} SANCO/12495/2011 2011, European Commission 2010) and for that purpose 1 kg 100% clear apple juice samples were fortified by investigated pesticides ranged from 1.4% of MRLs to 20 % above MRLs.

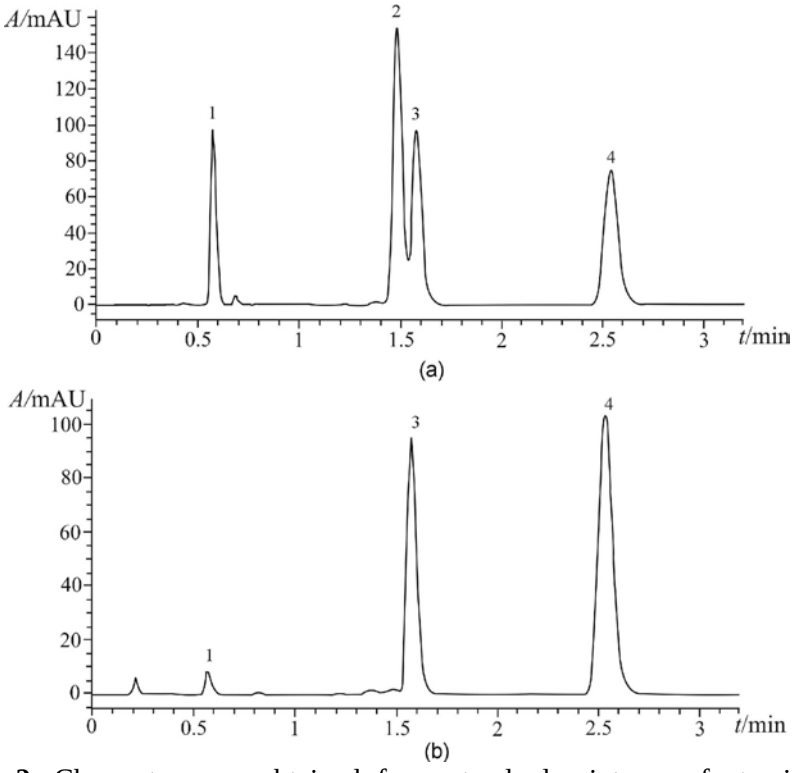

Figure 2. Chromatograms obtained from standard mixtures of atrazine (1), malathion (2), fenitrothion (3) and parathion (4) at 220 nm (a) and 270 nm (b) with developed method

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

Compound	**t_R (min)**	***k'***	***α***	***Rs***
dead time	0.19	-	-	-
atrazine	0.56	1.95	3.43	21.38
malathion	1.46	6.68	1.07	1.69
fenitrothion	1.55	7.16	1.69	14.25
parathion	2.49	12.10	-	-

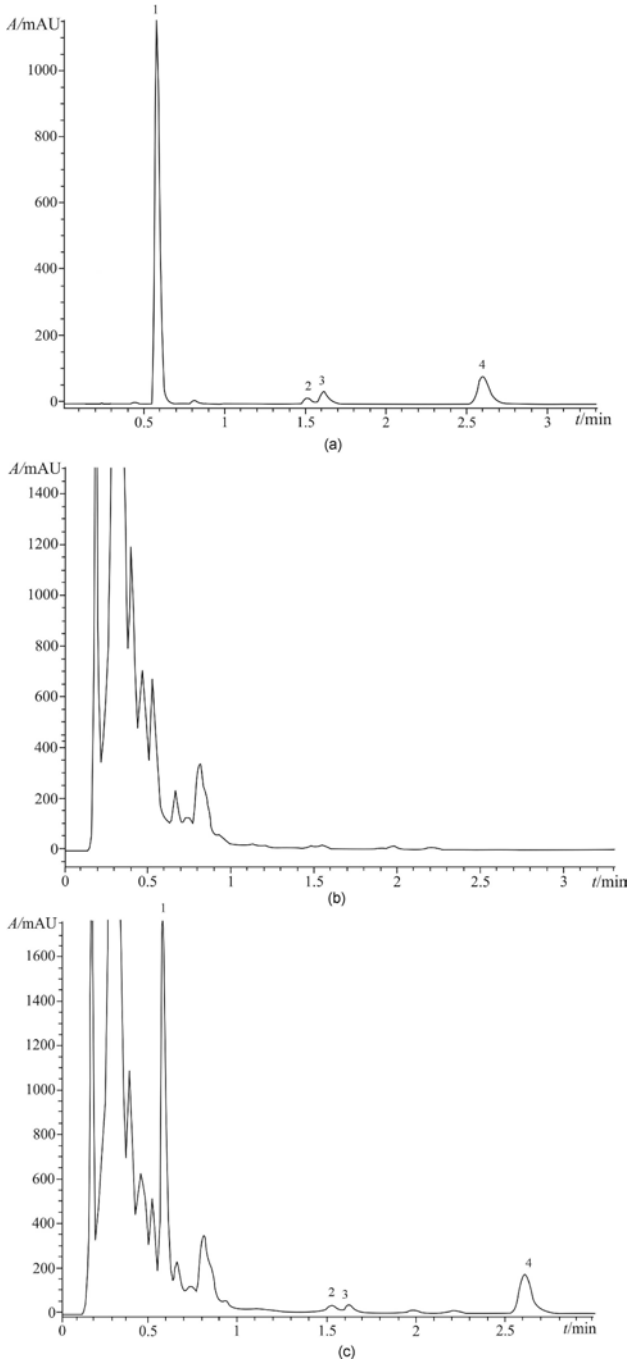

Figure 3. Chromatograms from standard mixture of atrazine (1), malathion (2), fenitrothion (3) and parathion (4) at the concentrations which correspond to MRLs (a), matrix blank (b) and samples of apple juice fortified at the concentration equal to MRL for each analyte (c).

Specificity, selectivity, linearity, precision expressed as repeatability of retention time and peak area, recovery and limit of quantification (LOQ) for all analytes were tested for the method validation.

The chromatograms of the standard mixture of investigated pesticides at the concentrations which correspond to MRLs (a), matrix blank (unspiked apple juice sample, which was apple juice free of investigated pesticides) (b) and sample of apple juice fortified at the concentration equal to MRL for each analyte (c) are presented in Figure 3. The 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.

Table 2. Statistical data for linearity of the method

Compound	Linearity range (μg/kg)	Regression equation	R^2
atrazine	0.70 - 60.00	$^1y = 59103x + 112.16$ $^2y = 30711x + 78.109$	0.9924 0.9894
malathion	0.28 - 24.00	$^1y = 6336.7x + 8.4782$ $^2y = 1478.2x + 2.1872$	0.9690 0.9737
fenitrothion	0.14 - 12.00	$^1y = 10302x + 2.7927$ $^2y = 3008x + 1.1414$	0.9951 0.9920
parathion	0.70 - 60.00	$^1y = 24531x + 88.11$ $^2y = 4766.7x + 16.607$	0.9688 0.9698

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

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	0.58 ± 0.0004	0.07	2998.84 ± 3.35	0.11
malathion	1.53 ± 0.001	0.09	127.33 ± 0.61	0.48
fenitrothion	1.63 ± 0.002	0.10	101.52 ± 1.16	1.15
parathion	2.61 ± 0.004	0.17	1240.44 ± 2.54	0.20

The linearity of the developed method was determined for all compounds separately, by construction of calibration curves at 6 concentration levels, with triplicate injections (5 μL) of the spiked standards in the apple juice sample matrix in the range of: 0.0007 - 0.06 mg/kg for atrazine and parathion, 0.00028 - 0.024 mg/kg for malathion and 0.00014 - 0.012 mg/kg for fenitrothion. For these concentration ranges and using the data for the peak areas and peak heights the 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 ($R^2 \geq 0.96$) suggested that the method has a satisfactory linearity for all analytes (Table 2).

Furthermore, the precision was expressed as repeatability of the obtained results from five successive injections (5 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 0.07 to 0.17 %, and for the peak area in the range of 0.11 – 1.15%, indicated an excellent precision of the proposed method (Table 3).

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	108.90	0.18
	0.050	0.049 ± 0.00006	97.66	0.12
	0.060	0.058 ± 0.00003	97.27	0.06
malathion	0.014	0.016 ± 0.0001	114.59	0.64
	0.020	0.019 ± 0.00009	95.65	0.49
	0.024	0.023 ± 0.00008	95.30	0.37
fenitrothion	0.007	0.007 ± 0.0001	107.44	1.21
	0.010	0.010 ± 0.0001	95.62	1.21
	0.012	0.012 ± 0.0002	98.89	1.77
parathion	0.035	0.041 ± 0.00005	118.60	0.12
	0.050	0.047 ± 0.0001	93.98	0.23
	0.060	0.057 ± 0.00005	95.34	0.10

The accuracy of the method was determined by the recovery studies of 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 93.98 – 118.60% and 0.06 – 1.77%, respectively. The mean recovery at each fortification level in the range of 70 - 120% and relative standard deviation (RSD) ≤ 20% per level are acceptable according to EU criteria (European Commission, 2010). Consequently, it can be concluded that the proposed method is convenient for the determination of the target pesticide residues in apple juice.

The developed RRLC method was successfully applied for the determination of the investigated pesticide residues in apple juice samples under the defined experimental conditions. The typical chromatograms of apple juice samples are presented in Figure 4. The samples from three different producers marked as: A, B and C were purchased from Macedonian market. The samples were concentrated and the clean-up using SPE prior to RRLC analysis. Each analysis was repeated five times.

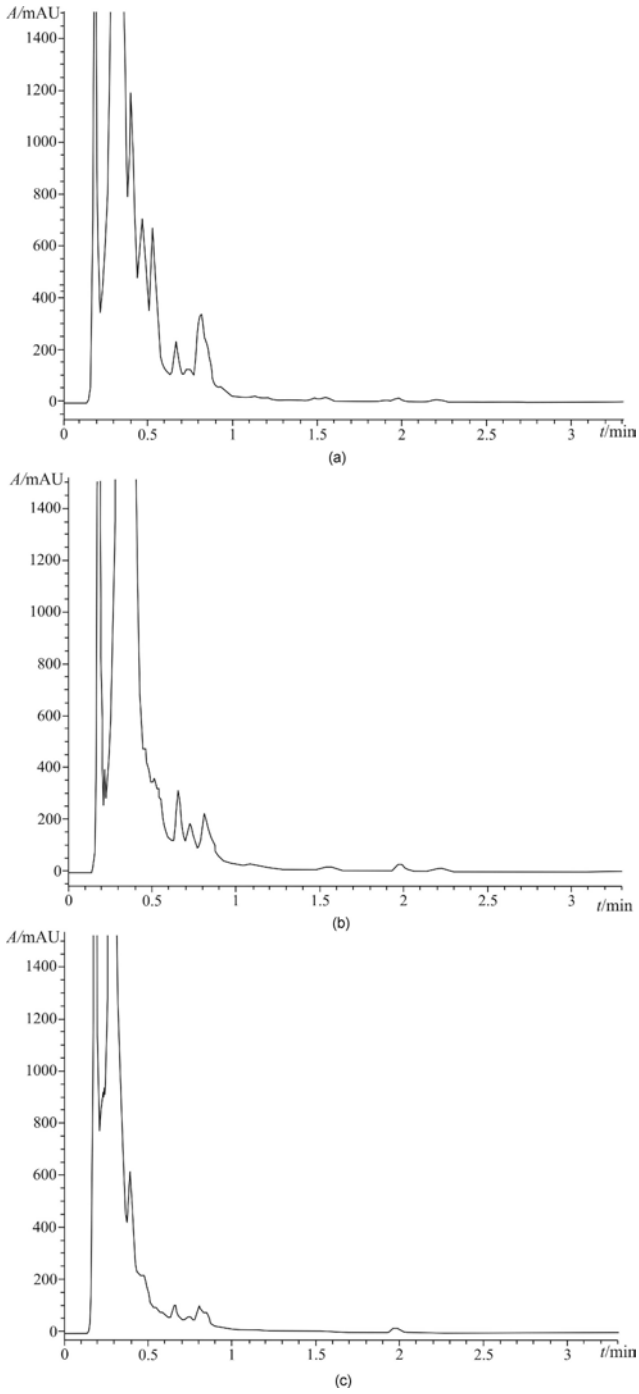

Figure 4. Typical chromatograms of apple juice samples A (a), B (b) and C (c) at 220 nm

The investigations show that residue of analysed pesticides in concentrations which correspond to MRLs or higher were detected in none of the tested apple juice samples.

CONCLUSIONS

This study describes a new, simple, fast and low-cost rapid resolution liquid chromatography method with ultraviolet - diode array detection that has been successfully applied to the simultaneous determination of atrazine, malathion, fenitrothion and parathion residues in apple juice samples. The best separation of the analytes with symmetrical peak shapes and satisfactory purity indexes was achieved under isocratic elution with acetonitrile/water (50/50, *V/V*) as a mobile phase, flow rate of 1 mL/min, constant column temperature at 25 °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 and the obtained results revealed that the proposed method has a satisfactory linearity, precision and accuracy for all analytes. The obtained results indicated that the analysed samples did not contain detectable residues of analysed pesticides. The run time of analysis was about 3.5 min.

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