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OPTIMIZATION AND DEVELOPMENT OF A SPE-HPLC-DAD METHOD FOR THE DETERMINATION OF ATRAZINE, MALATHION, FENITROTHION, AND PARATHION PESTICIDE RESIDUES IN APPLE JUICE

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A precise and accurate reversed-phase high-performance liquid chromatography (RP-HPLC) method with ultraviolet–diode array detection (UV-DAD) for the simultaneous determination of atrazine, malathion, fenitrothion, and parathion residues in apple juices has been developed. For the enrichment and cleanup of compounds of interest, Supelclean ENVI-18 SPE tubes were used. Separation and quantitative determination of the analytes were performed on a LiChrospher 60 RP-select B (125 mm × 4 mm, 5 μm, Merck) analytical column, the mobile phase consisting of acetonitrile/water (55/45, *V/V*) in isocratic elution with the following set values: flow rate of 1 ml/min, constant column temperature at 25 °C, and UV detection at 220 nm and 270 nm. The gathered values of the investigated pesticides from the apple juice samples were in the range 94.2 %–117.2 %.

Keywords: atrazine; malathion; fenitrothion; parathion; pesticide residues; SPE-HPLC-DAD; apple juice

ОПТИМИЗАЦИЈА И РАЗРАБОТКА НА SPE-HPLC-DAD МЕТОД ЗА ОПРЕДЕЛУВАЊЕ НА ОСТАТОЦИ ОД АТРАЗИН, МАЛАТИОН, ФЕНИТРОТИОН И ПАРАТИОН ВО СОК ОД ЈАБОЛКО

Разработен е прецизен и точен метод со помош на реверзно-фазна високоефикасна течна хроматографија (RP-HPLC) и ултравиолетов детектор со низа диоди (UV-DAD) за истовремено определување на остатоци од атразин, малатион, фенитротион и паратион во примероци од јаболков сок. За концентрирање и прочистување на компонентите од интерес се користени колоните Supelclean ENVI-18 SPE. Раздвојувањето и квантитативното определување на аналитите е спроведено на аналитичка колона од типот LiChrospher 60 RP-select B (125 mm × 4 mm, 5 μm, Merck), под изократски режим на елуирање со мобилна фаза составена од ацетонитрил/вода (55/45, *V/V*), проток 1 ml/min, константна температура на колоната од 25 ºC и UV-детекција на 220 nm and 270 nm. Аналитичките приноси на испитуваните пестициди од примероците на јаболков сок се во интервал од 94,2 % до 117,2 %.

Клучни зборови: атразин; малатион; фенитротион; паратион; остатоци од пестициди; SPE-HPLC-DAD; сок од јаболко

1. INTRODUCTION

Pesticides are substances (natural or synthetic) or a mixture of substances intended for preventing, destroying, repelling, or mitigating any pests (insects, weeds, diseases, fungi, bacteria, etc.) in order to obtain higher yields of agricultural crops, as well as to keep them safe while in storage [1]. According to their chemical compositions, they belong to more than 100 chemi-

cal classes of compounds, of which the most famous are organochlorine (which is the banned in most of the USA and Europe), organophosphorus (e.g., malathion, fenitrothion, parathion), and organonitrogen with triazines (e.g., atrazine). Atrazine, malathion, fenitrothion, and parathion were widely used in the Republic of Macedonia until a few years ago. Because of their solubility in water, they can cause serious pollution to the environment (soil, water, and air) and health damage in humans [2]. Pesticides can also accumulate in crops (e.g., fruit and vegetables) [3], so they can be found in processed products for human consumption, such as fruit juices, which are widely consumed, especially by children [4].

Multiresidue methods for the analysis of the different classes of pesticides (including organophosphorus and triazines) in fruits, vegetables, and their juices have been developed using gas chromatography (GC) with mass spectrometry (MS) [5–9], nitrogen-phosphorus detection (NPD) [4, 10], flame photometric detection (FPD) [11, 12], electron capture detection (ECD) [13, 14], and liquid chromatography with tandem mass spectrometry (LC-MS/MS) [15–17]. HPLC-DAD is a chromatography technique that is also used for the determination of organophosphorus [18] and triazine [19, 20] residues in environmental and food samples.

Prior to a GC or LC analysis, samples require extraction and purification, and the most widely used technique for the determination of organophosphorus and triazine pesticide residues is based on liquid-liquid extraction (LLE) [21], solid-phase extraction (SPE) [16, 22, 23], solid-phase microextraction (SPME) [24], matrix solid-phase dispersion (MSPD) [8], etc. In the majority of studies, C-18 bonded silica and styrene-divinylbenzene copolymers sorbent are usually used for the SPE of pesticide residues [25].

However, the HPLC method for the determination of atrazine, malathion, fenitrothion, and parathion residues in apple juices using UV-DAD was not found. Although atrazine, fenitrothion, and parathion are banned for use in the EU, they are used in the United States (except parathion) and some other countries. In addition, as a result of the illegal use of these pesticides, they can be found in foodstuff. On the other hand, due to the Agreement on Technical Barriers to Trade for removing trade barriers, raw material for apple juice production contaminated with pesticides (including those examined) can be imported from a third country. In order to ensure consumers' health is protected from possible adverse effects, the content of pesticides and their residues in food (e.g., fruits and vegetables) should be controlled. Therefore, Maximum Residue Levels (MRLs) of pesticides in foodstuff in most countries have been established. The MRLs of pesticides contained in apple are laid down by the EU Regulation (EC) No. 396/2005 [26], and they are estimated to be at 0.05 mg/kg for atrazine and parathion, 0.02 mg/kg for malathion, and 0.01 mg/kg for fenitrothion. The monitoring of pesticide residues in food samples – especially fruits, vegetables, and their juices – is of particular importance for the protection of human health and the environment.

Therefore, the main objective of this work was to develop a reversed-phase HPLC-UV-DAD method for the determination of atrazine, malathion, fenitrothion, and parathion residues in apple juices using the SPE of samples on ENVI-18, filled with octadecyl (17 % C) sorbent.

2. EXPERIMENTAL

2.1. *Instrumentation*

The chromatographic analysis was performed on an Agilent 1260 Infinity Rapid Resolution Liquid Chromatography (RRLC) system equipped with a vacuum degasser (G1322A), a binary pump (G1312B), an autosampler (G1329B), a thermostatted column compartment (G1316A), a UV-VIS diode array detector (G1316B), and the ChemStation software. For the better dissolving of the stock solutions, an Elma ultrasonic bath

was used. The investigations were carried out on a LiChrospher 60 RP-select B (125 mm x 4 mm, 5 μm, Merck) analytical column. For the SPE, a Visiprep vacuum manifold (Supelco) was used; and for the vortexing of samples, an IKA Vortex Genius 3 (Germany) was used.

2.2. *Reagents and chemicals*

The Pestanal analytical standards of atrazine (98.8% purity), malathion (97.2% purity), fenitrothion (95.2% purity), parathion (98.8% purity), and HPLC-grade acetonitrile were purchased by Sigma-Aldrich (Germany). Ultrapure water was produced by TKA Smart-2Pure 12 UV/UF water purification system (Germany). The juice samples were filtered by 0.45 μm Nitrocellulose membrane filters (Millipore, Ireland), and the final extracts were filtered through 0.45 μm Iso-Disc PTFE syringe filters (Supelco), just before the application.

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

2.3. *Preparation of standard solutions*

Stock solutions of atrazine, malathion, fenitrothion, and parathion were prepared by separately dissolving 0.0113 g, 0.0330 g, 0.0225 g, and 0.0188 g, respectively, of the 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 °C. Stock solutions were used for the fortification of apple juice samples and for the preparation of standard mixtures with different pesticide concentrations (1.42 ng/ml – 170.25 ng/ml for atrazine, 22.23 ng/ml – 2672.50 ng/ml for malathion, 16.36 ng/ml – 1967.00 ng/ml for fenitrothion, and 20.90 ng/ml – 2513.26 ng/ml for parathion) in 10 ml volumetric flasks by dilution with the acetonitrile/water mixture (50/50, *V/V*).

2.4. *Solid-phase extraction* (*SPE*)

The SPE procedure was accomplished using Supelclean ENVI-18 SPE tubes, 6 ml, 0.5 g (Supelco). Before the analysis, apple juice samples were filtered through 0.45 μm nitrocellulose membrane filters. Spiking samples were prepared for the determination of linearity, precision, and recovery by fortifying 1 kg apple juice with different concentration levels of each analyte (Tables 2, 3). Unspiked samples were used for blanks. The conditioning of SPE cartridges were 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 8–10 ml/min, and then the tubes were washed with 5 ml of water. Subsequently, the cartridges were dried for 20 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 and transferred into vials for HPLC analysis. The injection volume of each sample was 20 μl.

The proposed method was validated in terms of specificity, selectivity, linearity, LOD, LOQ, precision expressed as repeatability of retention time and peak area, and recovery.

3. RESULTS AND DISCUSSION

The investigated pesticides belong to different groups according to their chemical structures: atrazine (6-chloro-*N2*-ethyl-*N4*-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) [27].

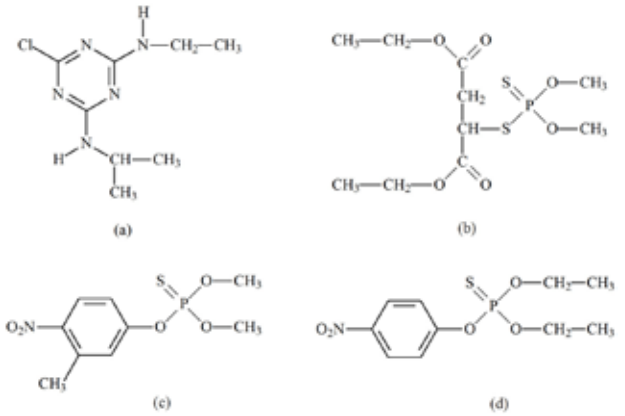

Fig. 1. Chemical structures of atrazine (a), malathion (b), fenitrothion (c), and parathion (d)

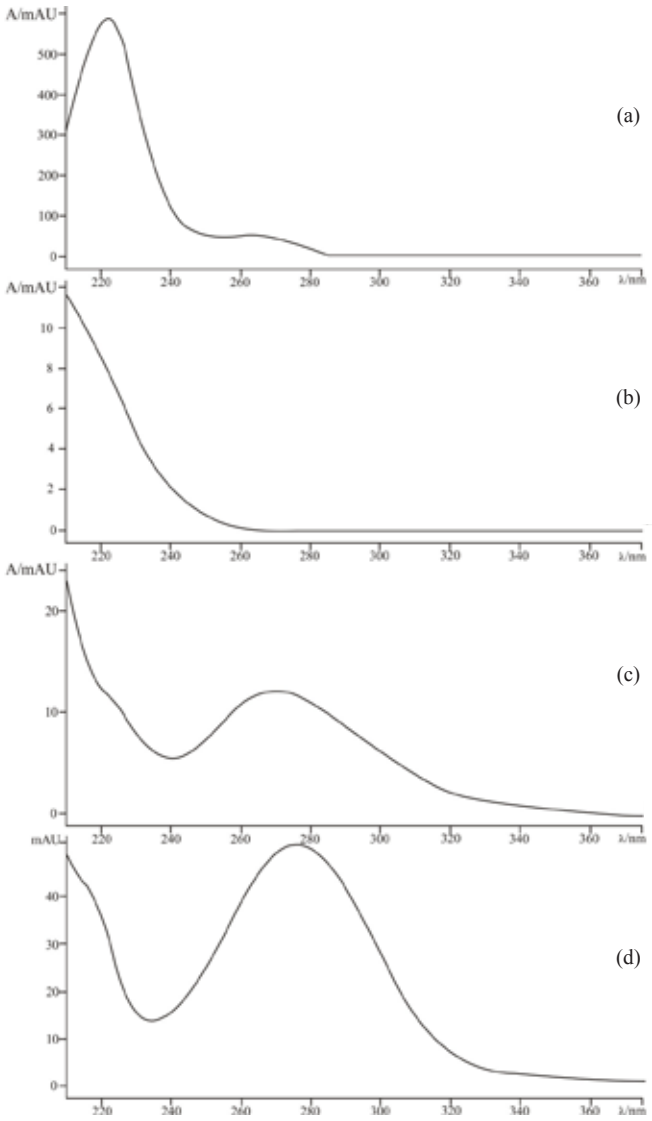

Fig. 2. The UV spectra of pure analytical standards of atrazine (a), malathion (b), fenitrothion (c), and parathion (d) in acetonitrile/water (50/50, *V/V*)

Having in mind the UV spectra of these components in acetonitrle/water mixture (50/50, *V/V*) (Figure 2.), an HPLC analysis for their simultaneous determination was carried out at 220 nm. However, fenitrothion and parathion have a band with a higher maximum absorption − *i.e.* at 270 nm for fenitrothion and 280 nm for parathion. Therefore the chromatographic determination was also performed at 270 nm. In addition, to confirm the specificity of the developed method, UV-diode array detection was used to check the peak purity and analyte peak identity [28]. The purity index for all analytes is greater than 996 (the maximum value for the peak purity index should be 1000), which means that the chromatographic peak is not affected by any other compound.

In order to obtain the best separation, get symmetrical peak shapes, and satisfy the purity indexes of the analytes from the standard mixture of pesticides, a series of preliminary investigations with different volume ratios of acetonitrile (85–50 %) and water in the mobile phase were implemented. The optimum separation of components of interest was achieved with the mobile phase consisting of acetonitrile/water (55/45, *V/V*) (Figure 3) in isocratic elution with the following set values: flow rate of 1 ml/min, constant column temperature at 25 ºC, and UV detection at 220 nm and 270 nm. Under the stated chromatographic conditions, the obtained values for column dead time, retention times of the components (t_R), the calculated values for retention factors (*k'*), separation factors (α), and resolution (*Rs*) are given in Table 1. The computed values for retention factors (*k'*) are below 10, which is the highest optimal value for this parameter. For separation factors (α), values are above 1, and for resolution (*Rs*) are above 1.5, which imply that under the stipulated chromatographic conditions, a high separation of the investigated pesticides was reached.

In the first approach, the limit of detection (LOD) and the limit of quantification (LOQ) for each compound were determined by the

T a b l e 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*
atrazine	2.51	3.18	1.92	14.40
malathion	4.27	6.12	1.07	1.69
fenitrothion	4.53	6.55	1.37	7.80
parathion	5.97	8.95	-	-

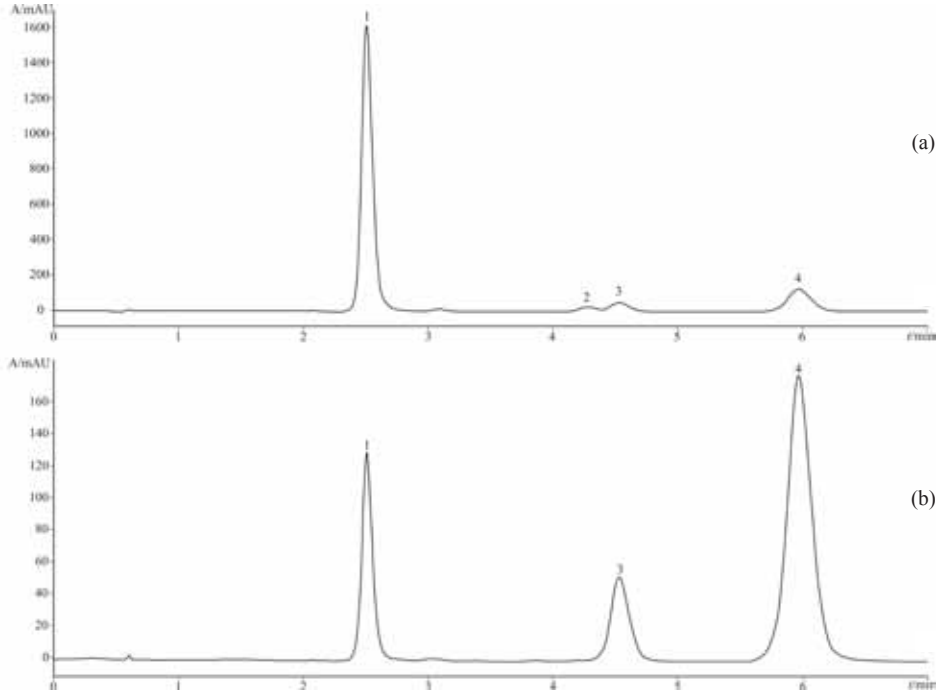

Fig. 3. Chromatograms obtained from standard mixtures of 1 μg atrazine (1), 0.4 μg malathion (2), 0.2 μg fenitrothion (3), and 1 μg parathion (4) at 220 nm (a) and 270 nm (b) on LiChrospher 60 RP-select B column with acetonitrile/water (55/45, *V/V*) as the mobile phase

construction of calibration curves in the low concentration region (1.42 ng/ml – 170.25 ng/ml for atrazine, 22.23 ng/ml – 2672.5 ng/ml for malathion, 16.36 ng/ml – 1967.0 ng/ml for fenitrothion, and 20.90 ng/ml – 2513.26 ng/ml for parathion) using standard mixtures of pesticides directly injected into the HPLC. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated according to the formulas LOD = 3.3·SD/slope and LOQ = 10·SD/slope [29]. The computed values for LOD (0.19 mg/l for atrazine, 3.04 mg/l for malathion, 2.23 mg/l for fenitrothion, and 2.85 mg/l for parathion) and LOQ (0.58 mg/l, 9.21 mg/l, 6.75 mg/l, and 8.63 mg/l for atrazine, malathion, fenitrothion, and parathion, respectively) are higher than MRLs, so the concentrations of analytes in juice samples are necessary. SPE has been successfully used for analyte enrichment or sample cleanup before the HPLC analysis [25].

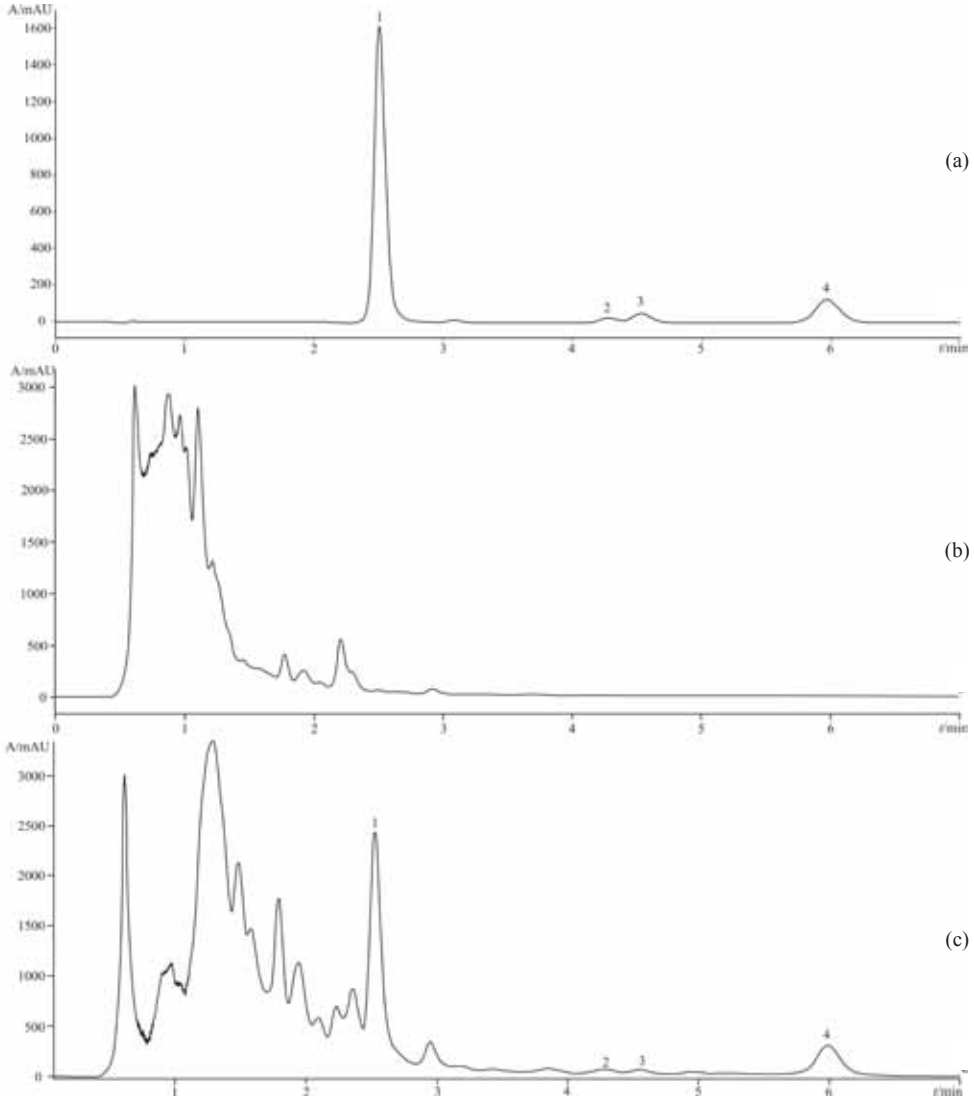

Fig. 4. Chromatograms obtained from standard mixtures of atrazine (1), malathion (2), fenitrothion (3), and parathion (4) at the corresponding concentrations to MRLs (a), unspiked apple juice (b), and sample of apple juice fortified at the MRL for each analyte (c) at 220 nm on LiChrospher 60 RP-select B column, with acetonitrile/water (55/45, *V/V*) as the mobile phase

The developed method based on solid-phase extraction was applied for the determination of investigated pesticide residues from apple juice samples. For the analytes' identities, the values of the retention time and match factor obtained by the overlaid spectra of a pure analytical standard and the absorption spectra of the same analyte in the apple juice samples were used. The chromatograms of standards at the MRLs (a), matrix blank (unspiked apple juice) (b), and samples of apple juice fortified for each analyte at the MRLs (c) are presented in Figure 4 [30].

The linearity of all investigated pesticides was determined with triplicate injections (20 μl each) of the spiked standards in the sample matrix at 4 concentration levels (the range being within 50 % less than MRLs and 20 % above, Table 2). The obtained results for multiple correlation coefficients (R^2), ranging from 0.9675 to 1, indicated that the method has a good linearity for all analytes (Table 2).

After the SPE procedure, the limits of detection (LOD) were determined by considering a value 3.3 times the background noise obtained for

the triplicate injection of blank samples (LOD = average (blank) + 3.3·SD (blank)), whereas the limits of quantification (LOQ) were determined by considering a value 10 times the background noise (LOQ = average (blank) + 10·SD (blank)) (Table 2) [31].

The precision was expressed as a repeatability of the obtained results for retention times and peak areas. For that purpose, 20 μl of the spiked apple juice samples at MRLs were injected (Table 2). The calculated values of RSD for retention times ranged from 0.16 to 0.23 %, and for areas ranged from 0.62 to 7.78 %, indicating that the precision of the method for the quantification of the investigated pesticide residues in apple juice was satisfactory.

T a b l e 2

Statistical data for linearity, limits of detection (LOD), limits of quantification (LOQ), and repeatability

Compound	t_R (min)	Linearity range (mg/kg)	Regression equation	R^2	LOD (μg/kg)	LOQ (μg/kg)	Repeatability (RSD/%)	
							t_R	Peak area
Atrazine	2.52	0.00175–0.0600	$^1y = 253126x - 17.976$ $^2y = 41046x + 78.826$	0.9998 0.9913	0.30	0.90	0.16	6.86
Malathion	4.27	0.0007–0.024	$^1y = 20050x + 0.5192$ $^2y = 1808.3x + 0.1851$	1 0.9989	1.00	3.00	0.23	0.62
Fenitrothion	4.54	0.00035–0.012	$^1y = 39992x + 14.311$ $^2y = 4180.3x + 1.7314$	0.9909 0.9874	0.07	0.21	0.18	3.31
Parathion	5.99	0.00175–0.0600	$^1y = 101252x + 329.23$ $^2y = 7251.4x + 23.957$	0.9675 0.9682	0.05	0.15	0.20	7.78

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

For the determination of the recovery, 1 kg apple juice samples were fortified at three concentration levels: 0.035 mg/kg, 0.05 mg/kg, and 0.06 mg/kg for atrazine and parathion; 0.014 mg/kg, 0.02 mg/kg, and 0.024 mg/kg for malathion; and 0.007 mg/kg, 0.01 mg/kg, and 0.012 mg/kg for fenitrothion before the solid-phase extraction. For each fortification level, five samples ($n = 5$) were prepared. The injection volume of each sample was 20 μl.

According to a guidance document on pesticide residue analytical methods [30], the precision of a proposed method should be presented as a relative standard deviation (RSD) of the recovery at each fortification level.

The recovery and precision of the tested method are presented in Table 3. The obtained values for recovery and relative standard deviation are between 94.2 % and 117.2 % and 0.1–8.7 %, respectively. According to EU criteria [30], the mean recovery at each fortification level should be in the range of 70% – 120 %, with relative standard deviation (RSD) ≤ 20 % per level. Consequently, it can be noticed that the proposed method is accurate and precise enough for the determination of atrazine, malathion, fenitrothion, and parathion residues in apple juices.

The typical chromatograms of apple juice samples (three) taken from Macedonian markets are presented in Figure 5. Investigated pesticide residues at MRL levels or higher were not found in the examined apple juice samples.

T a b l e 3

Mean recovery and precision data for investigated pesticides (n = 5)

Compound	Fortification level (mg/kg)	Total analyte found (mg/kg) (±SD)	Recovery (%)	RSD (%)
Atrazine	0.035	0.037 ± 0.003	106.5	8.7
	0.050	0.051 ± 0.003	102.4	6.8
	0.060	0.060 ± 0.001	99.8	1.3
Malathion	0.014	0.014 ± 0.0001	100.4	0.7
	0.020	0.021 ± 0.0005	104.7	2.2
	0.024	0.024 ± 0.0002	99.2	0.8
Fenitrothion	0.007	0.008 ± 0.00004	111.1	0.5
	0.010	0.010 ± 0.0003	100.2	3.4
	0.012	0.011 ± 0.00001	96.3	0.1
Parathion	0.035	0.041 ± 0.0002	117.2	0.5
	0.050	0.051 ± 0.004	101.7	8.3
	0.060	0.056 ± 0.0003	94.2	0.5

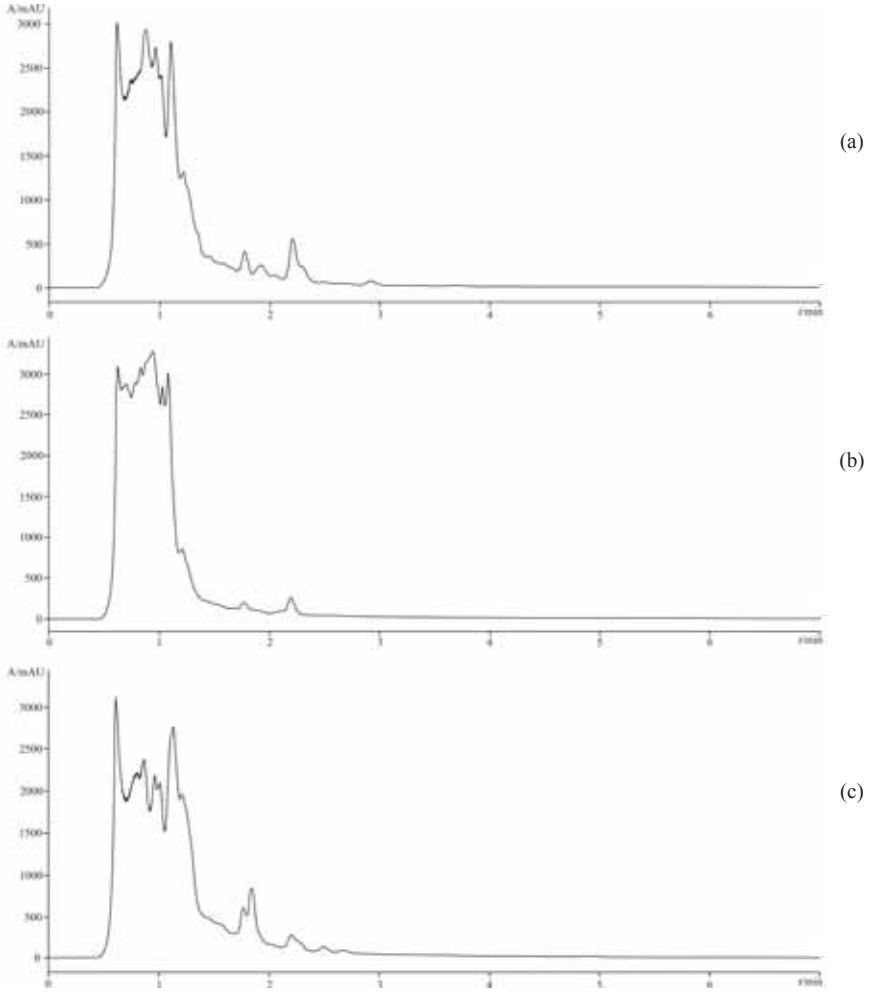

Fig. 5. Typical chromatograms of apple juice samples A (a), B (b), and C (c) at 220 nm on LiChrospher 60 RP-select B column, with acetonitrile/water (55/45, *V/V*) as the mobile phase

CONCLUSION

A precise and accurate RP-HPLC method with UV-DAD, after the solid-phase extraction (SPE) procedure, was developed and optimized for the determination of atrazine, malathion, fenitrothion, and parathion residues in apple juice samples. The proposed method showed good values of multiple correlation coefficients for calibration equations of all pesticides, satisfactory repeatability of retention times and peak areas, and acceptable values for recovery. The run time of analysis under the stipulated chromatographic conditions was about 7 min. The developed method is suitable for the routine determination of investigated pesticides in apple juice samples.

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