P30: DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR DETERMINATION OF PESTICIDE RESIDUES IN APPLE JUICE

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Abstract – This study presents development and validation of a reversed-phase high-performance liquid chromatography (RP-HPLC) method for qualitative and quantitative determination of 2,4-D ((2,4-dichlorophenoxy)acetic acid), atrazine, malathion, fenitrothion and parathion residues in apple juices. Specificity, selectivity, linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ) were tested for the method validation and all performance characteristics were found within acceptance criteria.

Keywords: RP-HPLC, method validation, pesticide residues, apple juice

1. INTRODUCTION

Apple juice is one of the most used juices in the world with many positive effects on the human health and because of that scientists support the inclusion of apple juice in a healthy human diet [1, 2]. On the other hand, the apple production includes use of excessive amount of pesticides, which can be harmful to human health. According to USDA (US Department of Agriculture) and FDA (US Food and Drug Administration) testing data, apples are on the top of the list of fruits and vegetables with the highest levels of pesticide residues [3]. Some of the widely 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). In order to protect the human health, in most countries and internationally by Codex Alimentarius, the Maximum Residue Levels (MRLs) for the pesticides in food (eg., fruit and vegetables) have been stipulated. The MRLs of pesticides in apple were set up by European Union Regulation (EC) No. 396/2005 [4] 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. Development of new and improvement of existing analytical methods are highly necessary for monitoring of pesticide residues in food samples.

Searching the literature data shows that Gas Chromatography (GC) and Liquid Chromatography (LC) are mostly used techniques for determination of many pesticides in fruits, vegetables and their juices using various detectors, such as: Flame Photometric Detector (FPD) [5], Nitrogen Phosphorous Detector (NPD) [6], Mass Spectrometry (MS) [7, 8], etc. HPLC combined with ultraviolet (UV) and/or Diode Array Detector (DAD) is also used for determination of some pesticide residues in different matrices [9, 10]. The aim of this study was to investigate the other possibilities for determination of 2,4-D, atrazine, malathion, fenitrothion and parathion residues in apple juice using different analytical column and mobile phases in order to achieve time reducing of chromatographic analysis, thus reducing the cost of analysis.

2. EXPERIMENTAL

Equipment and Chemicals. The analysis was performed on an Agilent 1260 Infinity Rapid Resolution Liquid Chromatography (RRLC) system. The experiments were carried out using Purospher STAR RP-18e (30 x 4 mm, 3 µm) analytical column produced by Merck (Germany).

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 of 2,4-D, atrazine, malathion, fenitrothion, and parathion were prepared by separately dissolving 0.0253 g, 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 ^{0}C. Stock solutions were used for the preparation of standard mixtures with different pesticide concentrations in 10 mL volumetric flasks by dilution with the acetonitrile/water mixture (50/50, *V/V*) and for spiking of apple juice samples.

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

Extraction procedure. 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), 1 kg apple juice samples were spiked with six sets of concentrations: 0.7, 7, 25, 35, 50 and 60 µg/kg for 2,4-D, atrazine and parathion, 0.28, 2.8, 10, 14, 20 and 24 µg/kg for malathion and 0.14, 1.4, 5, 7, 10 and 12 µg/kg for fenitrothion. Unspiked samples were used for blanks. Blank samples were prepared from apple juice free of tested pesticides. For each concentration level five samples (n = 5) were prepared.

1 kg of filtered (through 0.45 µm nitrocellulose membrane filters) 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 5 µL.

3. RESULTS AND DISCUSSION

Reversed - phase high performance liquid chromatography (RP-HPLC) method was developed for simultaneous determination of selected pesticides in apple juices, using Purospher STAR RP-18e (30 x 4 mm, 3 µm) analytical column. In order to obtain better results in relation to better baseline, a better peak shape and shorter retention time several mobile phases consisted of different mixtures of acetonitrile/water (85 - 40 % acetonitrile), methanol/water (80 - 60 % methanol), as well as acetonitrile/0.1 % formic acid and methanol/0.1 % formic acid were tested. The best separation of the analytes was achieved under isocratic elution with mobile phase consisted of acetonitrile/water (47/53, *V/V*), flow rate of 1 mL/min, constant column temperature at 25 ^{0}C and UV detection at 220 nm (Figure 1a).

The developed method was applied for the determination of investigated pesticides in apple juice samples. The method validation was performed according to EU Regulation and EU Guidance documents [11, 12] and for that purpose the specificity, selectivity, linearity, precision, accuracy and limit of quantification (LOQ) were tested. Therefore, 1 kg 100 % clear apple juice samples were spiked by investigated pesticides ranged from 1.4 % of MRLs to 20 % above MRLs. For concentration and clean-up of analytes, a solid-phase extraction (SPE) was used as a necessary step prior the HPLC analysis.

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 990, which means that the chromatographic peak was not affected by any other compound. Furthermore, 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. According to EU criteria [12], additionally, to prove selectivity on Figure 1 are presented chromatograms of standards at the concentrations which are correspond to MRLs (a), matrix blank (unspiked apple juice) (b) and sample of apple juice fortified at the concentration equal to MRL for each analyte (c).

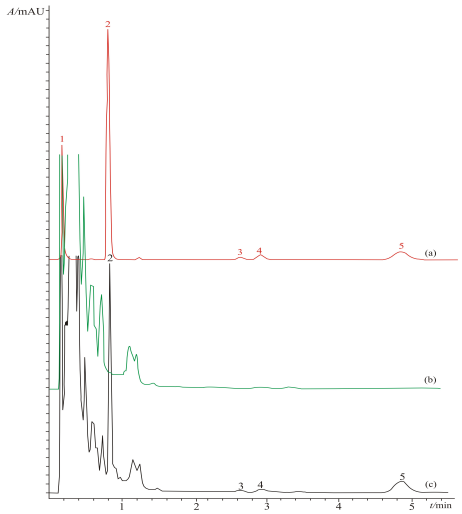

Figure 1. Chromatograms from standard mixture of 2,4-D (1), atrazine (2), malathion (3), fenitrothion (4) and parathion (5) at the concentrations which were correspond to MRLs (a), matrix blank (b) and sample of apple juice fortified at the concentration equal to MRL for each analyte (c)

As can be seen from the Figure 1, the component 2,4-D cannot be determined, because its chromatographic peak was overlapped with peaks originating from the matrix which were eluted at the beginning of the chromatographic process. The other investigated components were separated from each other and from the matrix, hence, they can be determined by developed method.

The linearity of the method was determined 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 given in Table 1. The calculated values for multiple correlation coefficients (R^2 ≥ 0.96) suggested that the method has a satisfactory linearity for all analytes (Table 1).

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 0.0007 mg/kg of atrazine and parathion, 0.00028 mg/kg of malathion and 0.00014 mg/kg of fenitrothion in this study. These results showed that the obtained values for LOQs were low enough compared to the MRLs of selected pesticides in apple [4] and they are acceptable for determining the pesticide residues, according to the European Commission rules [12]. Also, these values for LOQ were lower compared with those of the previous study [10].

Table 1. Statistical data for linearity of the method

Compound	Linearity range (µg/kg)	Regression equation	R^2
atrazine	0.70 - 60.00	^{1}y = 63169x + 81.612 ^{2}y = 23710x + 53.286	0.9978 0.9907
malathion	0.28 - 24.00	^{1}y = 3896.4x + 3.2084 ^{2}y = 501.57x + 0.366	0.9878 0.9867
fenitrothion	0.14 - 12.00	^{1}y = 11119x + 4.1624 ^{2}y = 1331.7x + 0.5522	0.9905 0.9896
parathion	0.70 - 60.00	^{1}y = 24540x + 90.046 ^{2}y = 1746.4x + 6.2502	0.9644 0.9661

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

The precision was expressed as repeatability of 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 and peak area indicated an excellent precision of the proposed method (Table 2).

Table 2. 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	0.83 ± 0.0006	0.08	3187.69 ± 4.27	0.13
malathion	2.62 ± 0.003	0.12	78.22 ± 0.37	0.47
fenitrothion	2.91 ± 0.003	0.12	112.06 ± 1.24	1.18
parathion	4.85 ± 0.01	0.21	1245.79 ± 4.81	0.39

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 3). The obtained values for recovery and for relative standard deviation are acceptable according to EU criteria [12]. Consequently, it can be concluded that the proposed method is convenient for determination of the target pesticide residues in apple juice.

The investigations show that residue of selected pesticides in concentrations which were correspond to MRLs or higher were not detected in none of the tested apple juice samples. The run time of analysis under the described chromatographic conditions was about 6 min.

Table 3. Results from recovery experiments (*n* = 5)

Compound	Fortifi-cation level (mg/kg)	Total analyte found (mg/kg ± SD)	Recovery (%)	RSD (%)
atrazine	0.035	0.036 ± 0.00004	104.21	0.12
	0.050	0.049 ± 0.00007	98.32	0.15
	0.060	0.059 ± 0.001	97.91	1.68
malathion	0.014	0.016 ± 0.0001	114.25	0.77
	0.020	0.019 ± 0.00005	96.01	0.25
	0.024	0.023 ± 0.0002	95.89	0.84
fenitrothion	0.007	0.008 ± 0.00004	110.71	0.54
	0.010	0.010 ± 0.00002	96.97	0.25
	0.012	0.012 ± 0.00005	96.65	0.41
parathion	0.035	0.042 ± 0.0001	119.92	0.26
	0.050	0.047 ± 0.0002	94.07	0.37
	0.060	0.057 ± 0.0001	94.52	0.22

4. CONCLUSIONS

This paper presents a new, simple and reliable reversed - phase high performance liquid chromatography (RP-HPLC) method for simultaneous determination of atrazine, malathion, fenitrothion and parathion in apple juices using ultraviolet - diode array detection (UV-DAD). The developed method has been validated according to 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. Compared with the results of the previous study, lower values for LOQ and shorter retention times for components were obtained, which means less time for chromatographic analysis (6 min).

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