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Research Article

Application of RP-HPLC Method for the Analysis of Some Pesticide Residues in Water Samples

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Abstract

A reversed-phase high-performance liquid chromatography (RP-HPLC) method for determination of some pesticide residues in different water samples has been developed and validated. The investigated pesticides belong to a different chemical classis, such as: organonitrogen, among which triazines (e.g., atrazine), organophosphorus (e.g., malathion, fenitrothion, parathion) and phenoxycarboxylic acids (e.g., 2,4-D (2,4-dichlorophenoxy) acetic acid)), which have different chemical structures and polarities. A Purospher STAR RP-8e (30 x 4 mm, 5 µm) analytical column, mobile phase consisted of acetonitrile and water, flow rate of 1 mL/min and constant column temperature at 25 °C were used for successful separation and quantitative determination of target analytes. The UV-detection was performed at 220 nm and 270 nm. Concentration and clean-up of analytes were done by solid-phase extraction (SPE). The developed method was validated according to European Commission guidelines for pesticide residue analytical methods and specificity, selectivity, linearity, precision, accuracy, and limit of quantification (LOQ) were tested for that purpose. The obtained values for relative standard deviation of retention times and peak areas (RSD ≤ 11.26%), recoveries ranged from 95.01 - 111.20%, multiple correlation coefficients (R2 ≥ 0.99) revealed that all performance characteristics were found within acceptance criteria. The method was successfully applied for determination of target pesticide residues in different water samples.

Keywords: Method Validation; Pesticide Residues; RP-HPLC; Water Samples

Abbreviations

2,4-D: 2,4-Dichlorophenoxy Acetic Acid; LLE: Liquid-Liquid Extraction; LOQ: Limit of Quantification; MRL: Maximum Residue Level; RSD: Relative Standard Deviation; RP-HPLC: Reversed-Phase High-Performance Liquid Chromatography; SPE: Solid-Phase Extraction; UV: Ultraviolet

UV/DAD: Ultraviolet/Diode Array Detector

Introduction

Water is the most important and basic condition for the existence of life, and its pollution is a major problem nowadays. In spite of that, more than 1000 pesticides and in huge amaunt are used in the world, belonging to different groups, with different chemical structures and polarity, like organonitrogen (eg, triazines), organophosphorus, and chlorophenoxycarboxylic acids. Due to their solubility in water, pesticides can cause serious environmental pollution (soil, water and air), and adverse effects on human health, as well. Pesticides are natural or synthetic chemical compounds that are widely used in the fight against various pests (insects, weeds, diseases, fungi, bacteria, nematodes, rodents and others) in order to obtain higher yields of agricultural crops, as well as their safer storage in warehouse spaces [1]. Pollution of surface waters with pesticides is a threat to the aquatic environment, causing negative effects such as acute and chronic toxicity for aquatic organisms, accumulation in the ecosystem, loss of biodiversity, but also disruption of human health.

Among the most widely used herbicides in the world are triazines, especially atrazine. Due to their high solubility in water and their mobility, they can easily pass into underground and surface

waters [2,3]. According to Directive 2008/105/EC of the European Parliament and Council of 2008 [4], atrazine poses a significant risk to the aquatic environment and is one of the 33 priority harmful substances.

2,4-D is an herbicide from the group of chlorophenoxycarboxylic acids, which are considered non-degradable organic pollutants and represent a serious environmental problem [5], due to their relative stability and photostability in natural waters.

Organophosphorus insecticides (for example, malathion, fenitrothion and parathion) are not very toxic, due to their relatively rapid decomposition and low accumulation in living organisms [6]. However, their improper use can cause their presence in the environment, and therefore represent a cause for concern.

Monitoring of pesticide residues in water samples is of particular importance for the protection of human health and the environment, as well as for studying the mode of action and movement of pesticides within the environment. In most countries around the world, Maximum residue levels (MRLs) for pesticides in food and water have been established, in order to guarantee the safety of consumers and to regulate the presence of pesticides in the environment. The MRLs of pesticides in class I and II waters, which include drinking water, mineral waters and some surface waters, in our country and in the EU are stipulated by the Directive 98/83/EC [7] and amount to 0.1 μg/L individually for each pesticide or the total amount of all pesticides present not to exceed 0.5 μg/L.

Analytical methods are necessary for successful monitoring of pesticide residues. A large number of chromatographic methods for the determination of pesticides and their residues in different matrices can be found in the literature. Thus, for example, for the determination of residues in different water samples, the most widely used are gas [8,9] and liquid [10,11] chromatography using different detectors. Several HPLC (High Performance Liquid Chromatography) methods with ultraviolet (UV) [12] or ultraviolet/diodde array detector (UV/DAD) [13] are also known.

Although chromatographic methods are very powerful techniques for the analysis of pesticides in environmental (water, soil, air) and food samples, pre-preparation of the sample, such as extraction or concentration of the sample before their chromatographic determination, is usually required. For this purpose, the most used and most suitable are liquid-liquid extraction (LLE) and solid-phase extraction (SPE) [9,14,15].

In our previous research, we have developed and validated several HPLC methods for the determination of atrazine, 2,4-D, fenitrothion, malathion and parathion in various water samples [16-18]. However, there is always a need for the development of new analytical methods, as well as the improvement of existing methods. Therefore, the purpose of this paper was to investigate the possibility of developing a new, precise and accurate reversed-phase high-performance liquid chromatography (RP-HPLC) method for the determination of residues of atrazine, 2,4-D, fenitrothion, malathion and parathion in water samples. The method has been validated according to European Commission guidelines for pesticide residue analytical methods [19] and successfully applied for the determination of selected pesticide residues in various water samples, such as bottled water, tap water, and water from the Vardar River, the largest river in the Republic of North Macedonia.

Materials and Methods

Equipment

The chromatographic analyses were performed using 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. An analytical column Purospher STAR RP-8e (30 x 4 mm, 5 μm), produced by Merck was utilized for the separation and determination of investigated pesticide residues. In order to better dissolving of the stock solutions an ultrasonic bath "Elma" was used. A SPE was carried out using a vacuum manifold Visiprep (Supelco). For vortexing of samples was used IKA Vortex Genius 3 (Germany).

Chemicals and reagents

Pestanal analytical standards of atrazine (98.8%), 2,4-D (98.6%), fenitrothion (95.2%), malathion (97.2%), and parathion (98.8%) manufactured by Sigma Aldrich (Germany) were used for HPLC analyses. For the preparation of the mobile phase, HPLC grade acetonitrile (CH_3CN) produced by Sigma Aldrich (Germany) was used, as well as ultrapure water obtained from a water purification system (TKA Smart2 Pure 12 UV/UF, Germany).

The water samples for the analysis of the targeted pesticide residues were taken from drinking tap water (A), bottled water, purchased from markets in Skopje (B) and water from the Vardar River (near the village of Dolno Lisiche) (C).

Preparation of stock solutions

The stock solutions were prepared by dissolving an appropriate mass of the analytical standard of the investigated pesticides with acetonitrile. Namely, a mass of 0.0113 g of atrazine; 0.0253 g of 2,4-D; 0.0225 g of fenitrothion; 0.0330 g of malathion; and 0.0188 g of parathion were measured in separate flasks of 25 mL. Then the flasks were filled with acetonitrile and placed in an ultrasonic bath for 15 min in order to achieve complete dissolution of the analytical standards. Then, after cooling the flasks, they were made up to the mark with acetonitrile. The stock solutions were kept in a refrigerator at a temperature of 4 °C.

The stock solutions were used to enrich the water samples to test the validation of the method for the quantitative determination of residues of the analysed pesticides in the water samples.

Preparation of water samples for pesticide residue analysis

The samples from the Vardar River were taken in brown glass bottles of 2.5 L. Immediately after arriving at the laboratory, the samples were filtered through a 0.45 μm nitrocellulose membrane filter.

The calibration curves for determining the linearity of the proposed RP-HPLC method were obtained by triple injection of samples of distilled water enriched with the investigated pesticides, in 3 concentration levels (0.1 μg/L; 0.2 μg/L and 0.5 μg/L for each analysed pesticide) after performing solid-phase extraction through Supelclean ENVI-18 columns. A volume of 5 μL of each solution was injected.

The recovery of the method was determined by adding an exactly determined concentration (in three concentration levels) of each analysed pesticide to 1 L of distilled water, namely: 0.1 μg/L; 0.2 μg/L and 0.5 μg/L. Samples of distilled water to which no pesticides have been added were used as blanks. For each concentration level, 4 samples were prepared (n = 4). Furthermore, the samples were subjected to solid-phase extraction and HPLC analysis, and 5 μL was injected from each sample.

Solid-phase extraction

Solid-phase extraction was performed using Supelclean ENVI-18 columns (Supelco), with a volume of 6 mL and adsorbent mass of 0.5g. The solid-phase extraction procedure consists of the following steps: Conditioning the columns (by passing 5 mL of acetonitrile and then 5 mL of water at a flow rate of 2 mL/min); Passing the sample (1 L of water, prefiltered through a nitrocellulose membrane filter with a pore size of 0.45 μm was passed through the column at a flow rate of 8-10 mL/min); Column washing (by passing 5 mL of distilled water, the column was washed from any residual amount of the sample, as well as washing of the interfering substances); Column drying (under vacuum for 20 min) and Elution of the components of interest (with two portions of 2 mL of acetonitrile). After that, the eluates were evaporated to dryness using nitrogen at a temperature of 40 °C, and then the dry residue was dissolved with 1 mL of a mixture of acetonitrile and water (50/50, *V/V*) using a Vortex for 1 min. Before performing the HPLC analysis, the final extract was filtered through an Iso-Disc PTFE syringe filter with a pore size of 0.45 μm and transferred into appropriate vials for analysis. A volume of 5 μL was injected from each sample.

Results and Discussion

Purospher STAR RP-8e was chosen as the stationary phase for the chromatographic separation and quantitative determination of the analytes. These columns are constructed of exceptionally pure silica gel, free from the presence of metal ions, and enable excellent separation of acidic, basic and complex compounds, with excellent peak symmetry, without the formation of chromatographic tails. This stationary phase is characterized by high reproducibility of results, excellent efficiency and pro-long working life. As a result of the lower hydrophobicity of C-8 radicals relative to C-18, analytes will elute faster on Purospher STAR RP-8 endcapped compared to Purospher STAR RP-18 endcapped [20], which was also confirmed by our previous research [18].

In a previous study [21], the optimal conditions for the separation of the investigated components were determined on the Purospher STAR RP-8e analytical column (30 mm x 4 mm; 3 μm) and was used isocratic elution using a mobile phase composed of acetonitrile and water (45/55, *V/V*), flow rate of 1 mL/min, constant column temperature of 25 °C and UV detection at 220 nm and 270 nm. Under these chromatographic conditions, a good separation of all analysed pesticides was achieved, with well-formed chromatographic peaks in a time shorter than 5 min. Compared to the longer LiChrospher 60 RP-select B columns with a length of 125 mm [16] and 250 mm [17], as well as to the Purospher C-18 column [18], shorter retention times were obtained for the components, thereby reducing the duration of the analysis, as well as the consumption of organic solvent. This method has been applied to the determination of atrazine, malathion, fenitrothion and parathion in apple juice samples [21]. In this paper, the possibility of applying this method for the determination of 2,4-D, atrazine, malathion, fenitrothion and parathion in different water samples was investigated. For this purpose, it was necessary to first concentrate the analytes and purify the matrix from coeluting components, which

was achieved by applying solid-phase extraction (SPE). The method was validated according to European Commission guidelines for pesticide residue analytical methods [19] and specificity, selectivity, linearity, precision, recovery, and limit of quantification (LOQ) were tested for that purpose.

Identification of the analytes was performed by comparing the retention times of the analytical standards with those of the same components of the water sample spiked with the analytes and using match factor values obtained by overlapping the UV spectra of the pure analytical standard and the absorption spectrum of the same analyte present in the water samples. Additionally, to prove the selectivity of the method, in Figure 1 are shown chromatograms of the standard mixture with a concentration corresponding to the MRL (a), a blank sample (distilled water) (b) and a sample of distilled water enriched with pesticides with a concentration equal to the MRL for each analyte (c). In the blank chromatogram (Figure 1b), a peak (X) appears at the beginning with approximately the same retention time as the 2,4-D component, but with much lower intensity and a completely different spectrum, thus proving that it is not originates from the test substance 2,4-D.

The linearity of the method was tested by constructing calibration curves, which were obtained by triplicate injection of distilled water samples enriched with the tested pesticides in 3 concentration levels (0.1 µg/L; 0.2 µg/L and 0.5 µg/L for each analysed pesticide). The tested pesticides were concentrated using solid-phase extraction, and then analysed using the proposed method. Table 1 shows the statistical data for determining the linearity of method applied to the determination of 2,4-D, atrazine, malathion, fenitrothion and parathion residues in water samples using the Purospher STAR RP-8e analytical column (30 mm x 4 mm; 3 µm).

The obtained results (Table 1) show that the proposed method was linear for all components of interest (R^2 > 0.99). For the components 2,4-D, atrazine and malathion the linearity was better when the peak height was used as the dependent variable, while for fenitrothion and parathion the linearity of method was better when the peak area was used as the dependent variable. For these reasons, when calculating the recovery of 2,4-D, atrazine, and malathion, the peak height was used, and for fenitrothion and parathion, the peak area was used (Table 1).

Determination of the limit of quantification (LOQ) was made on the basis of signal-to-noise ratio (S/N), which was found to be ≥ 10 for all investigated pesticides at the lowest concentration level

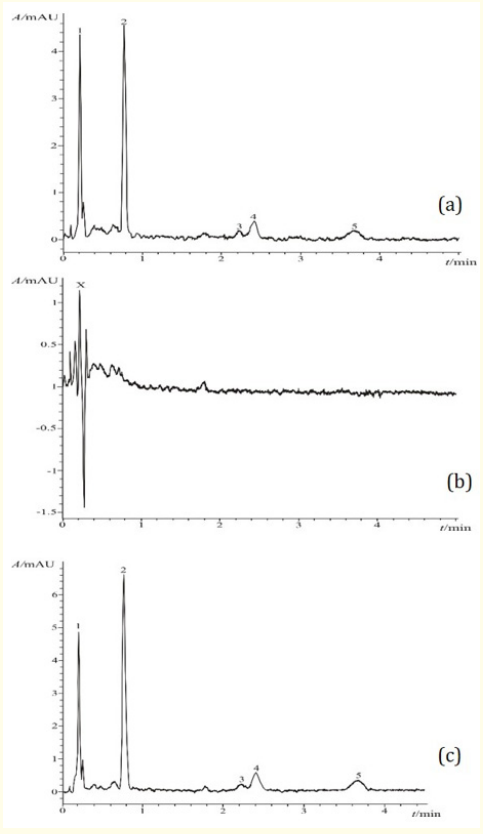

Figure 1: Chromatograms obtained from a standard mixture of 2,4-D (1), atrazine (2), malathion (3), fenitrothion (4) and parathion (5) with a concentration corresponding to the MRL after performing SPE (a), a blank sample (distilled water) (b) and a sample of distilled water enriched with pesticides with a concentration equal to the MRL (c) at 220 nm obtained by the proposed method.

Compound	Linearity range(µg/L)	Regression equation	R^2
2,4-D	0.1 - 0.5	$^1y = 36.083x + 27.867$ $^2y = 16.461x + 11.933$	0.9968 0.9972
atrazine	0.1 - 0.5	$^1y = 80.463x + 17.722$ $^2y = 31.844x + 6.1287$	0.9900 0.9998
malathion	0.1 - 0.5	$^1y = 6.1847x - 0.1218$ $^2y = 0.9062x + 0.0253$	0.9985 0.9995
fenitrothion	0.1 - 0.5	$^1y = 14.93x + 0.8219$ $^2y = 2.0862x + 0.1378$	0.9994 0.9988
parathion	0.1 - 0.5	$^1y = 18.155x + 0.2712$ $^2y = 1.5783x + 0.0793$	0.9996 0.9995
^1y - peak area; ^2y - peak height			

Table 1: Statistical data for the method linearity.

Compound	t_R (min) ± SD	RSD (%)	peak area ± SD	RSD (%)
2,4-D	0.22 ± 0.005	0.23	31.02 ± 0.33	1.06
atrazine	0.76 ± 0.005	0.06	24.34 ± 0.05	0.22
malathion	2.21 ± 0.008	0.36	0.47 ± 0.04	9.57
fenitrothion	2.41 ± 0.005	0.20	2.44 ± 0.11	4.65
parathion	3.67 ± 0.009	0.24	2.04 ± 0.23	11.26

Table 2: Statistical data for the method precision (n = 5).

for each compound (0.1 µg/L). According to the rules of European Commission guidance document on pesticide residue analytical methods [19], these obtained values for LOQs were acceptable for determining the pesticide residues in water samples.

By statistical processing of the experimental results obtained from five consecutive injections (5 µL) of a sample of distilled water enriched with the investigated pesticides at the level of the MRL (0.1 µg/L), the precision of the method was determined, expressed as the repeatability of the obtained results for retention time and area under the chromatographic peak for each analyte (Table 2). The RSD values for the retention times range from 0.06% to 0.36%, and for the area under the chromatographic peaks of the analytes, values from 0.22% to 11.26% were obtained. From the RSD values obtained, it can be concluded that the method has good intraday precision for the quantitative determination of residues of the analysed pesticides in water samples.

To determine the method recovery in 1 L of distilled water, a precisely determined concentration of each analysed pesticide was added, namely: 0.1 µg/L; 0.2 µg/L and 0.5 µg/L, and then solid-phase extraction was carried out. The quantitative determination of the analytes was performed using the proposed method. The calculated values for the recovery (95.01% - 111.20%) and the relative standard deviation (Table 3) confirm that the method was precise (RSD ≤ 13.36%) and can be used for accurate determination of 2,4-D, atrazine, malathion, fenitrothion and parathion residues in water samples (according to EU criteria [19]).

Figure 2 presents typical chromatograms of the analyzed samples of tap water for drinking (a), bottled water bought in a local market (b) and a sample taken from the Vardar River (c). In the chromatograms of the examined water samples shown in Figure 2, a peak (X1) with a similar retention time as 2,4-D was observed at the beginning, but the analysis of their UV spectra showed that these samples did not contain this component in a concentration corresponding to the MRL or higher. At about 0.7 min (Figure 2b and 2c) a peak (X2) can be observed around the time of the appearance of atrazine, but it has been determined that this substance was not involved. Around 2.2 min, a peak (X3) was observed in the sample from the Vardar River (Figure 2c), but it was not originate from malathion, nor from fenitrothion. Also, a peak at 3.7 min (X4) was observed on all three chromatograms, which was not originate from parathion.

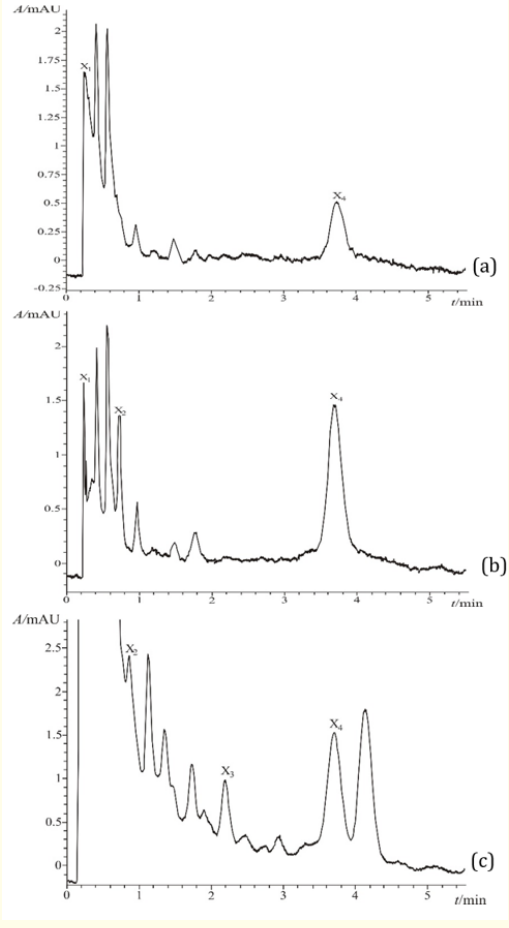

Figure 2: Typical chromatograms of water samples obtained from tap water (a), bottled water purchased from a local market (b) and water from the Vardar River (c) at 220 nm obtained by the proposed method.

As can be seen from the obtained chromatograms (Figure 2), no residues of the tested pesticides were found in the analysed samples in a concentration corresponding to the MRL or higher.

Conclusion

A new possibility of successful determination of atrazine, 2,4-D, fenitrothion, malathion and parathion residues in tap water, bottled

water and water from Vardar River has been described. The best chromatographic conditions were obtained using Purospher STAR RP-8e analytical column (30 mm x 4 mm; 3 µm), isocratic elution using a mobile phase consisted of acetonitrile and water (45/55, V/V), flow rate of 1 mL/min, constant column temperature of 25 °C and UV detection at 220 nm and 270 nm. Using the Purospher STAR RP-8e (30 mm x 4 mm; 3 µm) analytical column resulted in shorter retention times for the components compared to the same length Purospher C-18 column (30 mm x 4 mm; 3 µm) and the longer LiChrospher 60 RP-select B columns (125 mm and 250 mm length). Smaller values for retention times for the components mean that the duration of the analysis was reduced, as well as the consumption of organic solvent.

The developed reversed-phase high-performance liquid chromatography (RP-HPLC) method was validated according to European Commission guidelines for pesticide residue analytical methods, and all performance characteristics were found within acceptance criteria. Consequently, it was concluded that the method has excellent linearity, precision and accuracy and it was suitable for the determination of selected pesticide residues in different water samples.

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