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Original Article

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Development a HPLC method for simultaneous determination of Azinphose methyl, Diazinon, Phosalone and Chlorpyrifos residues in fruit

Maliheh Soodi[1*], Atefeh Garshasbi[2], Soheil Eskandari[3]

[1] *Department of Toxicology, School of Medical Sciences, Tarbiat Modares University, Tehran ,Iran*

[2] *Department of Analytical Chemistry, Islamic Azad University-Shahr-e-Ray Branch, Tehran, Iran*

[3] *Food and Drug Research Center, Tehran, Iran*

Abstract

A high-performance liquid chromatography with ultraviolet detection (HPLC/UV) method which was used solid-phase extraction for cleanup, was developed for the determination of Azinphos methyl ,Phosalone, Diazinon and Chlorpyrifos residues in fruits. A full factorial experimental design was used for development of HPLC condition and evaluation of effect of three factors (pH of mobile phase, percent of acetonitril in mobile phase and flow rate) on the retention time and resolution of peaks. The samples were extracted with Hexan/Aceton(50/50) and then is loaded onto a C18 end-caped SPE cartridge for further clean up. For SPE condition optimization, several solvents (acetonitril, dichloromethane, ethyl acetate and hexane) were tested as eluent and the best recovery was found with hexane. The optimum HPLC condition was achieved with acetonitril: water (60:40) as mobile phase, flow rate 1 ml/min, on C8 column. The standard calibration curves were linear between 0.05-1μg/ml for Azinphos methyl and 0.1-2 μg/ml for others. The hexan:acetone extraction followed by the C18 end-cap SPE cleanup provided recoveries of >70% for all of pesticides and removing the greatest number of sample matrix interferences.

Keywords: Azynphos methyl, Phosalone, Diazinon, Chlorpyrifos, Organophosphate pesticide, HPLC

***Corresponding author**: Maliheh Soodi, PhD., Department of Toxicology, School of Medical Sciences, Tarbiat Modares University, Tehran, Iran.
Tel: +98 21 82884549 Fax: +98 21 82884555
E-mail address: soodi@modares.ac.ir

1. Introduction

Pesticides are chemical agents used to control and reduce pest populations. Published reports represent that the total amount of pesticides used in agriculture is increasing. Unfortunately these compounds were improperly used without attention to currency period of pesticide by farmers. As a result of the large quantities and inappropriate use of the pesticides, they are present in all areas of the natural environment and food commodity (Tadeo et al.,2008; Ecobichon 2001).

Organophosphate pesticides have been widely used as insecticide in agriculture for last decades. Because of relatively rapid decomposition of these compounds, then short half life in the environment, they were replaced organochlorine pesticide and todays extensively were used in agriculture. As a consequence, residues of these substances can be found in food, and raises significant concerns about potential risk for human health (Damalas et al.,2011) . Several Studies have shown that human exposure to these compounds can have adverse health effects like suppressing of the immune system, cancer and neurological disorder (Damalas et al.,2011; Jaga et al.,2005; Kamel et al.,2005).

Therefore, control and measurement of the pesticide residues on fruit crops is an essential measure and requires a simple and affordable method of analysis.

Chromatography-based methods are frequently applied for the determination of pesticide residues in various matrices. Gas chromatography is the conventional method for the detection and quantification of organophosphate pesticides (Hennion et al.,1997; Picó et al.,2012) . However, number of studies used high-performance liquid chromatography (HPLC) technique for analyzing the organophosphate residues in food is increasing (Picó et al.,2012; Jedrzejczuk et al., 2001; Ravelo-Pérez et al.,2006, Fernandes et al.,2011) .The major advantage of the use of HPLC method is suitability of this technique for precise analyzing of polar and thermally labile compounds. In some studies, pesticide compounds with a wide range of polarity are simultaneously measured (Fernandes et al.,2011). Derivatization is not required through this approach. Clean up procedure using SPE cartridges is compatible with HPLC and also HPLC methods are more simple and robust than GC methods (Picó et al.,2012; Jedrzejczuk et al., 2001).

Azinphose methyl, Diazinon, Phosalone and Chlorpyrifos are commonly used organophosphate pesticides in fruit gardens ,and the aim of this study was the development of a HPLC method to determine the residues of these compound in fruits.

2.Materials and Methods

Chemicals

Azinphos methyl, Phosalen, Diazinon and chlorpryphs reference standards, purity > 99%, AccuStandard® .Methanol, Acetonitrl, Hexan, Ethyl acetate, Methylen chloride, were analytical grade and were purchased from Merck.

Instrumentation

The chromatography was performed on a Thermo Scientific Dionex UltiMate 3000 HPLC system, which comprised online degasser, autosampler, column oven, and UV detector. This system was controlled by Cromoleon software 6.8. An RP column (Prontosil 120,C8 ACE-EPS, 5μm, 25 × 0.46 cm) was used.

Preparation of Standard solutions

Individual stock standards solutions of each compound were prepared by weighing the powder and dissolution in 10 mL of Acetonitril to prepare 1000 μg/ml stock solution then this stock solution was diluted by acetonitril to prepare 100 μg/ml working standard . Next,

pesticide mixture solutions were prepared by adequate dilution of the corresponding stock solution with acetonitril. The concentration of standard solutions were 0.1, 0.2, 0.4, 0.8, 1.6 and 2 μg/ml for Diazinon , Chlorpryphos and Phosalen, and 0.05, 0.1, 0.2, 0.4, 0.8 and 1 μg/ml for Azinphos methyl.

Extraction

The chopped fresh fruits were blended in a blender and 20 gr of well blended fruits were weighed in two 250 mL glass bottles fitted with screw caps. One of these weighed samples was used as blank sample and another one was used as spiked sample. The spiked sample was prepared by adding proper amount of stock solution of pesticides, in which final concentrations of 0.25 μg/ml for Azinphos methyl and 0.5 μg/ml for Diazinon , Chlorpryphos and Phosalen were achieved. The spiked samples were left standing for 1 h before beginning the extraction step. Two samples were extracted as described by Talebi([Talebi, 2006). Briefly, Solvent (acetone:hexane, 50:50 v/v) in the amount of 150 mL was added and agitated for 20 min using a shaker. The sample was filtered, and the resultant liquid was transferred into a separatory funnel containing 50 mL of sodium chloride solution (20 g/l).The solution was shaken vigorously and left on the lab bench for layers to separate. The upper layer was separated, and the lower layer was re-extracted with 25 mL of hexane. The combined hexane extract evaporated to dryness on a vacuum rotary evaporator, The obtained residue was re-dissolved in 5 mL of hexan and 1 ml of this extract was loaded on SPE cartridge for further clean up.

Clean up

The C18 SPE cartridge (CHROMBOND®, end-capped) was used for clean up the extracts. The SPE sorbent was conditioned with 3 ml Methanol followed by 3 ml water and then loaded with1 ml of extract. Different solvents including Hexan, Acetonitrl, Dichloro methan and Ethyl acetate) were tried as eluent to obtain the optimum recovery for elution .Finally, the eluate was saved and evaporated to dryness under a gentle nitrogen stream then residue was re-dissolved in 2 ml mobile phase and injected to the HPLC.

HPLC method development

The mixed standard solution of pesticides was used for the optimization of HPLC conditions. The eluate was monitored by UV absorbance at 235 nm. The preliminary experiment conducted with C18 column as stationary phase and water/acetonitril mixture with varying percent as mobile phase .But these conditions were not resulted in good separation of pesticide peaks, even with changing percent of acetonitrl in mobile phase and flow rate (figure 1), therefore the experiments were conducted by C8 column.

Experimental design method was used for estimation of optimum HPLC condition. a

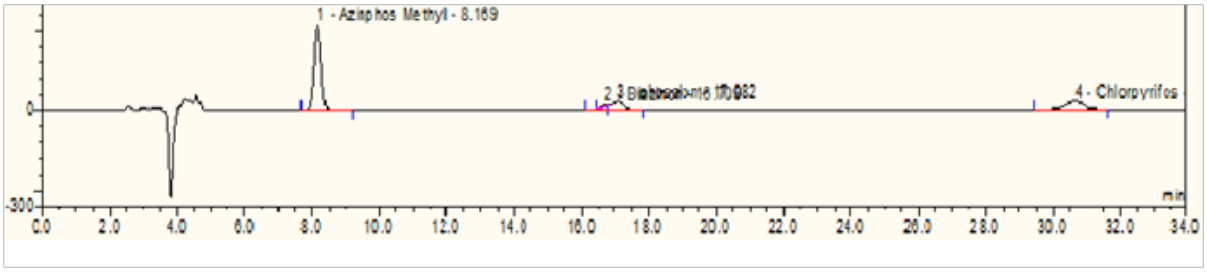

Figure 1. Chromatogram of the mixed standard solution of pesticides on C18 column

23 full factorial design was proposed for assessing effect of three major factors (pH of mobile phase , percent of acetonitril in mobile phase and flow rate) on the retention times and resolution of pesticide peaks , and for estimating the best value for these factors in which the pesticide were eluted in minimum retention time and optimum peak resolution. In this method each factor was tested in two levels , 2.5 and 7 for pH, 50 and 70 for percent of acetonitril in mobile phase and 0.7 and 1.3 for flow rate, and each condition was repeated three times, then the total 24 experiments were performed (Table 1). Design Expert® 6.01 software (Stat- Ease Inc.) was used for planning and interpreting of this design.

Table 1. Assignment of factors and levels of the factorial design

Experiments	Factors		
	pH	Flow (ml/min)	ACN (%)
1	2.5	0.7	50
2	7.2	0.7	50
3	2.5	1.3	50
4	7.2	1.3	50
5	2.5	0.7	70
6	7.2	0.7	70
7	2.5	1.3	70
8	7.2	1.3	70

3. Results and discussion

According to model that software was prepared between factors and responses , the pH factor did not any significant effect on responses and the retention time of peaks and peak resolution were not affected by changing of pH of mobile phase (p value > 0.05 with ANOVA analysis), but two other factors significantly affect the responses.(Table 2). The retention time of

Table 2. Results of experiments

	Retention time(s)				Resolution		
Experiments	Azinphos methyl	Diazinon	Phosalon	Clorpiryfos	R1	R2	R3
1	14.9 ± 1.4	22.6 ± 2.2	37.8 ± 1.7	55.8 ± 2.8	2.235 ± 0.75	16.5 ± 1.2	12.53 ± 1.6
2	15.7 ± 1.8	25.6 ± 2.8	41.1 ± 2	60.6 ± 3.1	13.46 ± 0.53	14.33 ± 1.5	12.2 ± 1.75
3	8 ± 1.2	12.3 ± 1.8	20.8 ± 1.9	30.5 ± 1.9	10.185 ±1.01	14.205 ± 0.9	11.455 ± 1.4
4	8.3 ± 0.9	13.7 ± 1.3	22.1 ± 1.5	32.5 ± 2.1	12.1 ± 0.72	13.59 ± 0.8	12.085 ± 1.21
5	6.6 ± 0.4	8.4 ± 0.9	9.7 ± 0.5	12.3 ± 0.8	4.85 ± 0.32	3.325 ± 0.6	5.64 ± 0.59
6	6.7 ± 0.7	8.7 ± 0.5	9.9 ± 0.8	12.6 ± 0.6	5.235 ± 0.42	2.98 ± 0.62	5.865 ± 0.41
7	3.5 ± 0.4	4.5 ± 0.7	5.2 ± 0.6	6.6 ± 0.7	4.175 ± 0.45	2.86 ± 0.4	5.315 ± 0.48
8	3.6 ± 0.6	4.6 ± 0.4	5.3 ± 0.3	6.7 ± 0.5	4.59 ± 0.34	2.54 ± 0.21	5.115 ± 0.37

Each result represents Mean ± SD of three replicate for each condition
R1: resolution of Azinphos methyl and Diazinon peaks
R2: resolution of Diazinon and Phosalon peaks
R3: resolution of Phosalon and Clorpiryfos peaks

peaks and peak resolution were significantly varied by changing the percent of acetonitril in mobile phase and flow rate (p value < 0.05 with ANOVA analysis). More increasing in the percent of acetonitril in mobile phase and flow rate, more decreasing in the retention time of peaks and peak resolutions. The optimum HPLC condition included best separation of peaks with acceptable peak resolution and the least retention time for the last peak, which gives reasonable run time, was searched by the software. The estimated HPLC condition was mobile phase included acetonitril / water 60/30 and flow rate 1ml/min, this condition was chosen for the rest of experiments. The chromatogram of mixed standard solution of pesticides in the optimum HPLC condition is represented in figure 2.

The use of experimental design for development of HPLC condition is the best way, because it reduced the number of experiments that must be performed to find the best condition

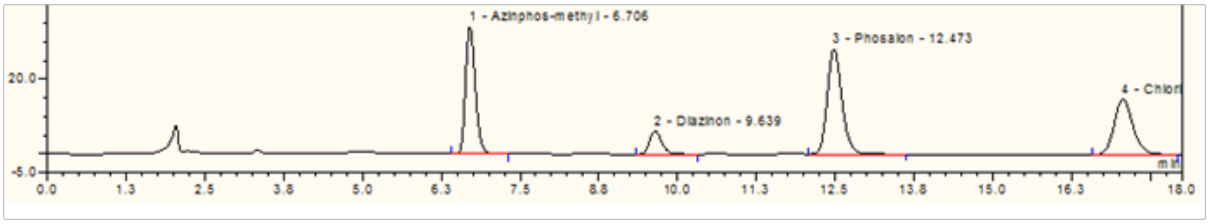

Figure 2. Chromatogram of the mixed standard solution of pesticides in optimum HPLC condition

for analysis and it identifies the main effects with interaction of all factors on responses (Anderson et al.,2007; Ferreira et al.,2007; Brynn Hibbert 2012). Because it is time saving methods, several studies were applied this method for predicting the effect of factors on the responses (Zecević et al., 2004; Song et al.,2008; Khamanga and Walker ,2011). In this study our aim was to find the main effect of factors and their interactions, and just was to estimate the proper condition for the separation of pesticide peaks in acceptable run time , we used the 23 full factorial design, and only with performing 24 runs, reached to our aim and found the optimum choromathographic condition.

Method validation

For testing linearity triplicate analysis of mixed standard solution of pesticides was performed at concentration ranges from 0.1-2 μg/ml for Diazinon , chlorpryphos and Phosalen, and 0.05-1 μg/ml for Azinphos methyl. Peak areas of Diazinon , chlorpryphos and Azinphos methyl were plotted against the concentration of them. Peak heights of Phosalon were plotted against the concentration of it. Correlation coefficient more than 0.99 were found for all pesticides, indicating a good linear relationship for all pesticide in concentration ranges studied.

The limit of detection (LOD) and limit of

Table 3. LOD and LOQ for pesticides

Pesticides	LOD (µg/ml)	LOQ (µg/ml)
Azinphos methyl	0.015	0.05
Diazinon	0.035	0.11
Phosalon	0.046	0.14
Chlorpyrifos	0.044	0.13

quantification (LOQ) were determined by following folmula: LOD= 3.3SD/m , LOQ= 10SD/m where SD is residual standard deviation of regression line and m is the slope of regression line (Sivakumar et al.,2007). The resultant LOD and LOQ for each pesticide represent in table 3.

The intraday precision of the method was determined by injecting the mixed standard solutions of pesticides three times in each concentration and for interday precision determination, analysis of mixed standard solutions of pesticides, in each concentration, were performed for four consecutive days. Table 4 represents RSD% value for each concentration. The sample was spiked

Table 4. The intraday and between day precision

Pesticides	Concentrations (µg/ml)	Intraday RSD (%)	Between days RSD (%)
Azinphos	0.05	1.7	5.5
methyl	0.2	0.3	5.6
	0.5	0.14	3.7
	1	0.06	2.9
Diazinon	0.1	1.16	7.1
	0.4	0.86	4.9
	1	0.46	4.9
	2	0.23	3.8
Phosalon	0.1	0.72	5.6
	0.4	0.62	3
	1	0.25	2.9
	2	0.48	2.7
Chlorpyrifos	0.1	1.95	4.6
	0.4	2.5	2.5
	1	0.3	0.34
	2	0.38	0.46

at 0.5 μg/g for Azinphos methyl and 1 μg/g for three others pesticide, with stock solution of each pesticide, for assessing the recovery of method. After extraction of sample with hexan/acetone as described above, 1 ml of extract was loaded on the SPE cartridge and the pesticide were eluted by different solvent to obtain the best recovery .As shows on table 5, elution with dichloromethane, acetonitril and etylacetate were not given proper recovery for all pesticide but elution with hexan was resulted in the best recovery for all pesticide above 70%.

All experiments were done on apple samples. The choromathogram of spiked sample is showed in figure 3.

Table 5. Recovery of pesticides in different conditions for SPE clean up

Eluent	Recovery (%)			
	Azinphos methyl	Diazinon	Phosalon	Chlorpyrifos
Dichloromethane	2 ± 1.4	5 ± 2.1	10 ± 4.3	15 ± 3.7
Acetonitril	18 ± 4.6	43 ± 6.7	4 ± 3.6	90 ± 5.5
Etylacetate	25 ± 5.9	30 ± 4.6	14 ± 3.2	3 ± 1.3
Hexane	84.2 ± 7.6	83.8 ± 5.5	93.46 ± 5.4	83.24 ± 6.6

Each value represents Mean ± SD for three replicate.

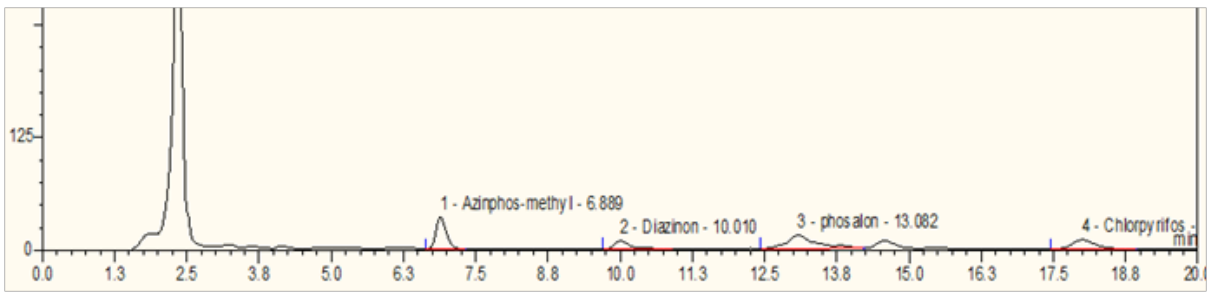

Figure 3. Chromatogram of spiked sample

4. Conclusion

In this study we could successfully apply the experimental design method for the development of HPLC condition and only with 24 experiment, we could find the proper condition for separation of pesticide peaks. This method is a simple and time saving method for estimating the appropriate chromatographic condition, and then we introduced a simple clean up stage with C18 cartridge for preparation of samples for HPLC analysis. Elution of loaded samples on C18 cartridge with hexan gave the acceptable recovery for all pesticides. Extraction of samples with hexan/aetonitril ,then further clean up on C18 cartridge and elution with hexan could remove the intractable material from the sample and the resulted chromatogram is clean enough for tracing the pesticide and quantitatively analysis of them. We used a simple HPLC condition with UV detection that could be found about almost of analytical laboratories.

5. References

Anderson MJ, Whitcomb PJ. DOE Simplified Practical Tools for Effective Experimentation. NY: Productivity Press, 2007:2-35.

Hibbert DB. Experimental design in chromatography: A tutorial review. J Chromatogr B 2012: in press.

Damalas CA, Eleftherohorinos IG. Pesticide exposure, safety issues, and risk assessment indicators. Int J Environ Res Public Health 2011; 8:1402-1419.

Ecobichon DJ. Pesticide use in developing countries. Toxicology 2001; 160:27-33.

Fernandes VC, Domingues VF, Mateus N, Delerue-Matos C. Determination of Pesticides in Fruit and Fruit Juices by Chromatographic Methods An Overview. J Chromatogr Sci. 2011; 49:715-30

Ferreira SLC, Bruns RE, da Silva EGP, dos Santos WNL, Quintella CM, David JM et al. Statistical designs and response surface techniques for the optimization of chromatographic systems. J Chromatogr A. 2007; 1158: 2–14.

Hennion MC, Barcelo D. Trace Determination of Pesticides and their Degradation Products in Water, Amesterdam: Elsevier, 1997:157-254

Jaga K, Dharmani C. The epidemiology of pesticide exposure and cancer: A review. Rev Environ Health. 2005; 20:15-38.

Jedrzejczuk A, Góralczyk K, Czaja K, Struciński P, Ludwicki JK. High performance liquid chromatography: application in pesticide residue analysis. Rocz Panstw Zakl Hig. 2001; 52:127-137.

Kamel F, Hoppin JA. Association of pesticide exposure with neurologic dysfunction and disease. Environ Health Perspect. 2004; 112:950-8.

Khamanga SM, Walker RB. The use of experimental design in the development of an HPLC–ECD method for the analysis of captopril. Talanta 2011; 83:1037-1049.

Picó Y, Font G, Carlos Moltó J. Food analysis by HPLC. Nollet LML, Toldra F ed. London: CRC Press, 2012:717-759.

Ravelo-Pérez LM, Hernández-Borges J, Rodríguez-Delgado MA. Pesticides analysis by liquid chromatography and capillary electrophoresis. J Sep Sci. 2006; 29:2557-77.

Sivakumar T, Manavalan R, Valliappan K. Development and validation of a reversed-phase HPLC method for simultaneous determination of Domperidone and Pantoprazole in pharmaceutical dosage. Acta Chromatogr. 2007; 18:130-142.

Song JZ, Qiao CF, Li SL, Han QB, Xu HX. Purity determination of yunaconitine reference standard using HPLC with experimental design and response surface optimization J Sep Sci. 2008; 31:3809-3816.

Tadeo JL, Sánchez-Brunete C, González L. Analysis of Pesticides in Food and Environmental Samples, London:CRC Press, 2008;60-80.

Talebi K. Dissipation of Phosalone and Diazinon in Fresh and Dried Alfalfa. J Environ Sci Health B. 2006; 41:595–603.

Zecević, M, Minić D, Zivanović Lj, Ivanović I. Application of experimental design to the development of an HPLC method for the analysis of ochratoxin A in Triticum aestivum grain. Pharmazie 2004; 59:175-7.