

# Development and validation of a method for the simultaneous determination of 20 organophosphorus pesticide residues in corn by accelerated solvent extraction and gas chromatography with nitrogen phosphorus detection

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**Abstract:** The method for simultaneous determination of 20 organophosphorus pesticide residues in corn samples has been developed and validated. For the extraction of organophosphorus pesticide residues from the samples, the accelerated solvent technique with the mixture of dichloromethane: acetone (1:1, *V/V*) was used. Clean up was done using liquid – liquid extraction with *n* – hexane, followed by solid phase extraction on primary secondary amine adsorbent, and elution with the mixture of acetone: toluene (65:35). The determination of the pesticides was carried out by gas chromatography with nitrogen phosphorus detection. Separation and quantitative determination of the analytes were performed on a fused silica capillary ZB-35 column (30 m x 0.25 mm i.d. x 0.25  $\mu$ m, Phenomenex). The recovery was investigated in blank corn samples fortified with mevinphos, diazinon, dimethoate, bromofos-methyl, chlorfenvinphos, fenamiphos, ethion and phosalone at 5 ng/g, 10 ng/g, 15 ng/g, 20 ng/g and 25 ng/g, respectively and with methacrifos, phorate, etrimfos, parathion-methyl, pirimiphos - methyl, fenitrothion, chlorpyrifos, malathion, parathion, bromofos-ethyl, phosmet and azinphos-methyl at 10 ng/g, 20 ng/g, 30 ng/g, 40 ng/g and 50 ng/g, respectively. The recovery ranged from 76.0% to 112.0%. Repeatability expressed as relative standard deviation (RSD) was less than 8.2%. Linearity expressed as correlation coefficient ( $R^2$ ) ranged from 0.9935 to 0.9996. Measurement uncertainty ( $U_x$ ) was lower than 14.2% for all tested pesticides. The limits of quantification (LOQ) were below 5 ng/g for all tested pesticides. The satisfactory Z-score results of international proficiency tests confirm good analytical performances of the developed method.

**Keywords:** Organophosphorus Pesticide Residues, Gas Chromatography, Accelerated Solvent Extraction, Solid Phase Extraction

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## 1. Introduction

Most crops are treated with pesticides on several occasions during the growing season. Pesticides enable farmers to produce some crops in areas that otherwise would not be suitable, increase their yields, preserve product quality, and extend shelf life [1]. Without pesticides, commercial crop's production would not be economically viable in many regions of the world. Insecticides and fungicides are likely to remain the major class of pesticides used for crop's protection [2, 3]. Insecticides are used to

control pests. A particular insecticide targets specific insects [2]. Fungicides are applied to control a considerable number of diseases caused by *Venturia inaequalis*, *Uncinula necator*, *Sclerotinia blight*, *Botrytis cinerea* etc [3]. At the same time, pesticides can pose risks if they are not applied according to Good Agricultural Practice (GAP). Pesticide levels tend to decline over time as the residues in crops degrade/metabolize during the growing period and following harvest if they are washed and processed before

reaching the markets. Many authors have also shown that if pesticides are applied in accordance with GAP then the Maximum Residue Levels (MRLs) will not be exceeded. [4, 5] So, levels are usually well below legal limits by the time food reaches the retail outlets.

A large number of pesticides are used on corn, which in the Republic of Macedonia is grown throughout the country both for human consumption and animal feed [6]. Conventionally grown corn requires many applications of insect-killers, weed-killers, plant growth regulators and other types of pesticides [7]. Corn is treated with organophosphorus pesticides (OPPs) in pre harvest interval, while corn grains are treated in post harvest period for prevention the infestation with insects of stored commodities. The use of OPPs as post harvest pesticides is mainly due to the lower rate of degradation under the storage conditions that exist in storehouses. The post-harvest pesticides have been attracting much attention because their residues in stored cereal grains may be hazardous to human health [8]. The presence of OPPs residues in corn is still reported [9, 10]. The monitoring of pesticide residues in corn and corn products is of a significant importance in terms of consumer safety.

To protect the health of consumers and to eliminate unsafe food, as well as to comply with the requirements of international food trade, MRLs of pesticide in food were laid down in Regulation (EC) 396/2005 [11]. For the majority of the studied pesticides, MRLs in corn have been established at the analytical limit of determination (between 10 and 50  $\mu\text{g/kg}$ ).

Due to the complex nature of cereals and the presence of lipids, fiber-carbohydrates (hemicellulose, cellulose and lignin), non fiber carbohydrates (starch, sucrose and reducing sugars) and proteins, the selection of the appropriate technique of extraction, concentration and purification of the pesticides, and its optimization is the most laborious, but very important aspect of the analysis [12]. A lot of different extraction and cleanup methods including multiple solvent extractions, supercritical fluid extraction (SFE), accelerated solvent extraction (ASE), gel permeation chromatography (GPC), solid phase extraction (SPE) were used in the determination of pesticides in cereals but there is no universal technique, which would be entirely better than the others in terms of all analytical scopes [13-20]. Many of the traditional procedures used to perform the extractions for these analyses are time consuming and solvent intensive [13, 17].

Organophosphorus pesticides are usually determined by gas chromatography (GC) with flame photometric detection (FPD) [13, 18], nitrogen-phosphorus detection (NPD) [14, 15, 21], mass spectrometry detection (MS) [16, 17, 21-25], and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) [23, 24].

The main objective of this work was to develop and validate rapid, simple and reliable analytical method for the simultaneous determination of residues of twenty OPPs: azinphos-methyl; bromofos-methyl; bromofos-ethyl; chlorfenvinphos; chlorpyrifos; diazinon; dimethoate;

ethion; fenamiphos; fenitrothion; malathion; parathion; parathion-methyl; pirimiphos-methyl; phorate; phosalone; phosmet in corn including etrimfos, mevinphos and methacrifos, which have not appeared in recent reports. Extraction of the samples was performed with a low volume of organic solvent using ASE technique. *N* – hexane was used for purification of the extracts, followed by SPE on the primary secondary amine adsorbent (PSA). The OPPs in corn samples were determined by GC – NPD.

## 2. Experimental

### 2.1. Instrumentation

The sample extraction was performed in a DIONEX Accelerated Solvent extractor, ASE-100 (USA). Stainless steel extraction cells (10 mL) were used for the extraction. Helium (purity 99.999 %) was used as a purge gas.

The chromatographic analysis was performed on a Shimadzu 2010 Gas Chromatography system (GC) system equipped with nitrogen-phosphorus detector (NPD), and auto injector (AOC- 20i), and the ChromSolution software. Chromatographic separation was achieved on a fused silica ZB-35 capillary column (30 m x 0.25 mm i.d. x 0.25  $\mu\text{m}$  film thickness), supplied by Phenomenex (Torrance, USA). Operating conditions were as follows: injector port temperature, 250  $^{\circ}\text{C}$ ; injection volume, 2  $\mu\text{L}$  in splitless mode (constant pressure 110.8 kPa; total flow 10.2 mL/min); detector temperature 280  $^{\circ}\text{C}$  (make up gas - helium flow 27.5 mL/min; hydrogen flow 1.5 mL/min; air flow 145 mL/min; current 3 pA); helium as carrier gas at a flow rate of 1.2 mL/min; oven temperature programme, 90  $^{\circ}\text{C}$  (1 min), increased with the rate of 25  $^{\circ}\text{C}/\text{min}$  to 200  $^{\circ}\text{C}$ , held for 15 min, then increased to 220  $^{\circ}\text{C}$  with the rate of 10  $^{\circ}\text{C}/\text{min}$ , and held for 25 min. The total run time of the chromatographic analysis was 47.40 min. The column equilibration time was 3 min.

### 2.2. Reagents and Chemicals

All chemicals and solvents were a special grade for pesticide residue analysis. Acetone, dichloromethane (DCM), *n*-hexane, ethyl acetate, acetonitrile (ACN) and toluene were obtained from Merck (Darmstadt, Germany). Anhydrous sodium sulphate, (prepared 3 hours at 650  $^{\circ}\text{C}$ ), sodium chloride and PSA /SPE cartridges (PSA, SPE 500 mg/ 6 mL tubes) were purchased from Sigma-Aldrich/Fluka/Riedel-de-Haen (Zwijndrecht, The Netherlands). Water was deionized then distilled from glass apparatus.

The analytical standards of azinphos-methyl (97.3 %  $\pm$  2), bromofos-methyl (98.5 %  $\pm$  2), bromofos-ethyl (99.7%  $\pm$  5), chlorfenvinphos (99.0%  $\pm$  5), chlorpyrifos (98.0 %  $\pm$  5), diazinon (99.2 %  $\pm$  5), dimethoate (98.5%  $\pm$  2), ethion (98.0%  $\pm$  2), etrimfos (95.0 %  $\pm$  5), fenamiphos (95.5%  $\pm$  2), fenitrothion (95.2 %  $\pm$  5), malathion (97.2 %  $\pm$  2), mevinphos (98.0 %  $\pm$  5), methacrifos (98.5 %  $\pm$  5), parathion (99.7  $\pm$  2), parathion-methyl (99.8  $\pm$  5), pirimiphos-methyl

(99.3 %  $\pm$  5); phorate (95.8 %  $\pm$  5); phosalone (99.0  $\pm$  5) and phosmet (97.0 %  $\pm$  5) were obtained from Sigma-Aldrich/Fluka/Riedel-de-Haen (Zwijndrecht, The Netherlands). Blank corn samples were purchased from FAPAS (CSL, York, UK).

### 2.3. Preparation of the Standard Solutions

Stock solutions of individual pesticide standards were prepared in acetone at 500  $\mu$ g/mL and stored in a refrigerator at 4 °C. Standard working solution mixture (i) was prepared by transferring 5 mL of each individual stock standard solution in a 50 mL volumetric flask and diluting with methanol to a concentration of 50  $\mu$ g/mL. Standard working solution mixture (ii) was prepared by transferring 2.5 mL of working solution mixture (i) in a 25 mL volumetric flask and diluting with methanol to a concentration of 5  $\mu$ g/mL. Standard working solution mixture (iii) was prepared by transferring 5 mL of the standard working solution mixture (ii) in a 50 mL volumetric flask and diluting with methanol to a 0.5  $\mu$ g/mL. Standard working solution mixtures (ii, iii) were used for the fortification of blank samples. Standard working solution mixtures (i, ii) were used for the preparation of chromatographic standard solutions with different pesticide concentrations: 25 ng/mL – 125 ng/mL, i.e. 25 ng/mL; 50 ng/mL; 75 ng/mL; 100 ng/mL and 125 ng/mL for mevinphos, diazinon, dimethoate, bromofos-methyl, chlorfenvinphos, fenamiphos, ethion and phosalone; 50 - 250 ng/mL, i.e. 50 ng/mL; 100 ng/mL; 150 ng/mL; 200 ng/mL and 250 ng/mL for methacrifos, phorate, etrimfos, parathion-methyl, pirimiphos - methyl, fenitrothion, chlorpyrifos, malathion, parathion, bromofos-ethyl, phosmet and azinphos-methyl. Chromatographic standard solutions were prepared in a 10 mL volumetric flask class A, using ethyl acetate as a solvent.

### 2.4. Sample Preparation

An aliquot of 5 g of grinded sample was mixed with 2.5 g of anhydrous sodium sulphate. The extraction cell was filled with the homogenized mixture, and placed in the ASE, which worked out under the conditions shown in Table 1.

Table 1. ASE operating conditions

Solvent	DCM/acetone (1:1, V/V)
Temperature	100 °C
Pressure	1500 psi
Static time	5 min
Flush volume	60 %
Purge time	140 sec
Static Cycle	1

The obtained extract (15 mL) was transferred into the separating funnel. 200 mL (10 %, W/V) sodium chloride in deionized water and 50 mL of *n*-hexane were added and vigorously shaken for 5 min and then allowed to stand for 15 min. The water portion was discarded. The hexane layer was collected through the anhydrous sodium sulphate and removed by a rotary evaporator at 40 °C. The residue was dissolved in ACN and adjusted to the volume of 1 mL with the same solvent.

Sample cleanup: 6 mL column (PSA) was conditioned with 5 mL mixture of acetone: toluene (65:35). 1 mL of the sample was transferred to a column. Pesticides were eluted from the column with 20 mL mixture of acetone: toluene (65:35). The eluted solution was concentrated to 0.5 mL by a gentle stream of nitrogen at 40 °C. The volume was adjusted to 1 mL by adding of ethyl acetate. The sample solution was used for GC-NPD determination.

The proposed method was validated in respect to the recovery, linearity, precision expressed as within day repeatability and between day reproducibility of retention time and peak area, stability, limit of detection (LOQ), limit of quantification (LOQ) and measurement uncertainty ( $U_x$ ).

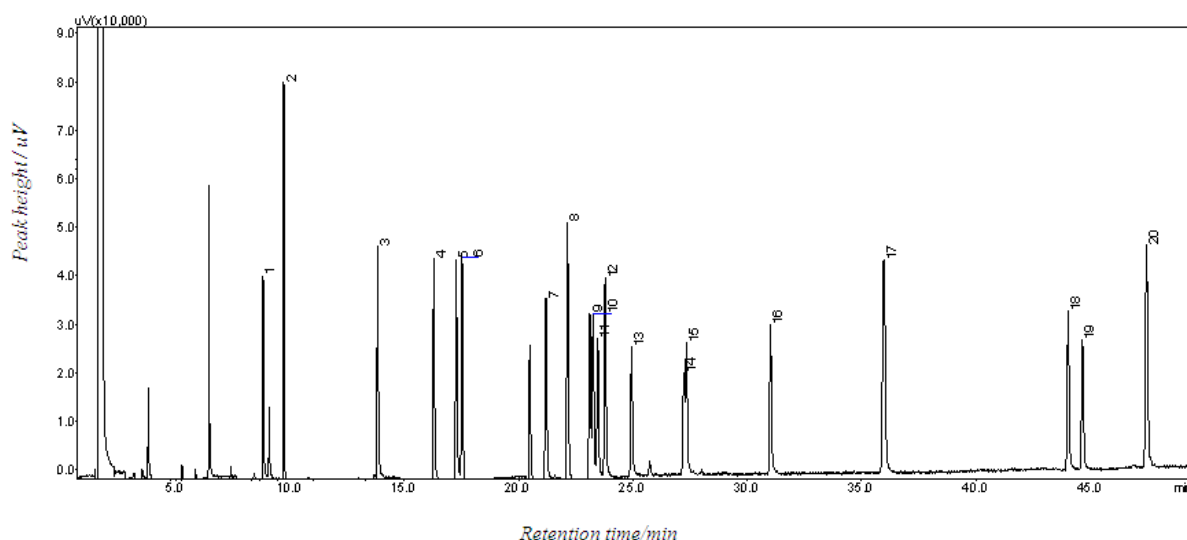


Figure 1. GC-NPD chromatogram of standard solution with OPPs in ethyl acetate at 0.1 mg/L: (1) mevinphos, (2) methacrifos, (3) phorate, (4) diazinon, (5) dimethoate, (6) etrimfos, (7) parathion-methyl, (8) pirimiphos-methyl, (9) fenitrothion, (10) chlorpyrifos, (11) malathion, (12) parathion, (13) bromophos – methyl, (14) chlorfenvinphos, (15) bromofos – ethyl, (16) fenamiphos, (17) ethion, (18) phosmet, (19) phosalone, (20) azinphos – methyl.

### 3. Results and Discussion

#### 3.1. Method Optimization

In order to obtain the best separation and get symmetrical peak shapes for all tested pesticides, a series of preliminary investigations with capillary GC columns with a different polarity of the stationary phase were tested. The optimum separation of components of interest was achieved when capillary column with an intermediate polarity (35% phenyl 65% dimethylpolysiloxane) and optimized temperature programme is used (Fig. 1).

In our research ASE technique was used for samples extraction. To obtain higher recovery for pesticides and to reduce the amount of co-extractable lipids which require extensive purification procedures as a gel permeation chromatography (GPC), we used a zeotropic mixture of DCM: acetone (1:1, *V/V*) as an extraction solvent. In the investigations of Ezzell *et al.*, high recoveries for OPPs in fortified apple puree (0.05 mg/kg), ranging from 82% for fenthion to 128% for dimethoate were obtained with ASE and azeotropic mixture of ethyl acetate: cyclohexane (1:1, *V/V*), followed by purification with GPC [20].

Due to the use of elevated temperature and pressure in the extraction cell the extraction time in our experiment was approximately 15 min. The presence of cellulose filter disk in the outlet end of the extraction cell provided the clear extract, and there was no need for further filtration.

ASE is generally considered to be an exhaustive extraction technique, and often the extracts obtained from complex samples contain compounds that can interfere in the determination of the desired analytes [19, 20]. Therefore, the purification of the obtained extract was performed. In order to remove the lipids from the extract, we introduced liquid-liquid extraction with *n*-hexane in the presence of 10 % (*W/V*) NaCl in deionized water. In the study of Hirara *et al.* after multiple extractions of the sample with ACN and ethyl acetate, the lipid fraction was removed by addition of the mixture of ACN saturated with *n*-hexane and *n*-hexane saturated with ACN [17]. In a study conducted by Tang *et al.* DCM was used for purification after acetone-water extraction of cereals and kidney beans prior to GC-NPD determination of nine OPPs [13].

In our study an additional purification of the extract was performed in order to remove the residual lipid fraction. For that purposes we used SPE tubes containing PSA adsorbent. Florisil (magnesium silicate) is material mostly used in sample purification prior to GC analysis of organochlorine compounds, nitrosamines, aliphatic and aromatic compounds, etc. [19]. But, when used for purification of matrices that contain organophosphorus pesticides which have phosphorus – oxygen bond, a decomposition of the pesticide may occur, which yields a poor recovery [16]. Liu and Ma obtained a low recovery for dimethoate in corn (2.65% for 0.25 mg/kg and 9.0% for 1.0 mg/kg, respectively) using florisil for the purification of the extract [16]. Hirara *et al.* used strong anion exchange

(SAX) resin in combination with PSA for purification of the extracts obtained for fruit, vegetable and cereal samples, prior to GC-MS analysis of 186 pesticides [17]. They obtained low recovery for polar pesticides (omethoate), which was probably due to the insufficient elution with acetone: *n*-hexane (3+7) from a SAX-PSA cartridge. Mariani *et al.* found out the use of deactivated acidic alumina with the elution solvent consisted of *n*-hexane: DCM: ethyl acetate (6+3+1, *V/V*) preferable *versus* a low temperature lipid precipitation in removal of high molecular weight compounds prior to GC-NPD analysis of the OPPs in cereal extracts [14].

We use PSA also for the elimination of non-polar molecular components in the corn extracts (carotenoid pigments). This is based on its combined hydrophilic and lipophilic characteristics.

At the evaluation step of cleanup method, three different volumes (5 mL, 10 mL, 20 mL) of the solvent mixture acetone: toluene (65:35) was used for the elution of the OPPs from the PSA column. Optimization of the elution volume was done with standard mixture of OPPs in ACN at a concentration of 50 ng. The extracts were evaporated to 0.5 mL after the SPE and reconstituted in *n*-hexane: acetone (1:1). Analysis was performed by GC-NPD. The results of our study for recovery evaluation of pesticides in correlation with the volume of elution solvent acetone: toluene (65:35) presented in Table 2 show the highest recovery of all the pesticides with 20 mL of the elution mixture. The obtained recoveries are between 88.3% for chlorfenvinphos and 120.2% for etrimfos. In their study Schenck *et al.* found also high recoveries for parathion-methyl (94%) and ethion (92%), when they used 20 mL of elution solvent acetone: toluene (65:35) on PSA/carbon adsorbent in the purification of onion extracts [26].

**Table 2.** Optimization of elution solvent volume (Recovery evaluation for tested pesticides)

Pesticide	Recovery (%); n=3		
	5 mL	10 mL	20 mL
Mevinphos	45.5	89.3	95.8
Methacrifos	58.6	98.8	117.3
Phorate	34.4	75.6	88.9
Diazinon	44.5	77.6	90.3
Dimethoate	37.9	69.8	91.2
Etrimfos	55.6	79.8	120.2
Parathion-methyl	49.8	80.1	115.3
Pirimiphos-methyl	55.6	81.2	98.8
Fenitrothion	44.3	78.8	110.5
Chlorpyrifos	39.7	80.2	94.5
Malathion	44.5	87.6	110.4
Parathion	33.4	76.6	104.5
Bromofos-methyl	45.6	78.9	95.3
Chlorfenvinphos	37.5	69.8	88.3
Bromofos-ethyl	49.8	78.3	98.6
Fenamiphos	41.2	70.3	94.4
Ethion	40.4	79.8	97.3
Phosmet	35.6	67.5	89.5
Phosalone	39.8	70.7	93.2
Azinphos-methyl	44.0	76.3	98.2

### 3.2. Method Validation

#### 3.2.1. Recovery, Precision and Linearity

The recovery, precision and linearity of all the organophosphorus pesticides was determined using fortified blank corn samples which were previously tested on the presence of OPPs. In each case, 5 replicates each at 5 levels were fortified into the samples. The samples (5 g) were fortified before the extraction with 50  $\mu\text{L}$  of 0.5 ng/ $\mu\text{L}$  mixture to yield 5 ng/g (mevinphos, diazinon, dimethoate, bromofos-methyl, chlorfenvinphos, fenamiphos, ethion and phosalone); with 10  $\mu\text{L}$ , 15  $\mu\text{L}$ , 20  $\mu\text{L}$  and 25  $\mu\text{L}$  of the 5.0 ng/ $\mu\text{L}$  spiking mixture to yield 10 ng/g, 15 ng/g, 20 ng/g and 25 ng/g, respectively (mevinphos, diazinon, dimethoate, bromofos-methyl, chlorfenvinphos, fenamiphos, ethion and phosalone); and with 10  $\mu\text{L}$ , 20  $\mu\text{L}$ , 30  $\mu\text{L}$ , 40  $\mu\text{L}$  and 50  $\mu\text{L}$  of the 5.0 ng/ $\mu\text{L}$  spiking mixture to yield 10 ng/g, 20 ng/g, 30 ng/g, 40 ng/g and 50 ng/g, respectively (methacrifos, phorate, etrimfos, parathion-methyl, pirimiphos - methyl,

fenitrothion, chlorpyrifos, malathion, parathion, bromofos-ethyl, phosmet and azinphos-methyl). Gas tight glass syringes (10  $\mu\text{L}$ , 50  $\mu\text{L}$  and 100  $\mu\text{L}$ ) were used for the addition of spiking mixtures.

According to a guidance document on pesticide residue analytical methods, the precision of the proposed method should be presented as a relative standard deviation (RSD) of the recovery at each fortification level. The linearity of the method was expressed as correlation coefficient ( $R^2$ ), obtained from regression analysis of the fortified samples [27].

The recovery, precision and linearity of the tested method are presented in Table 3.

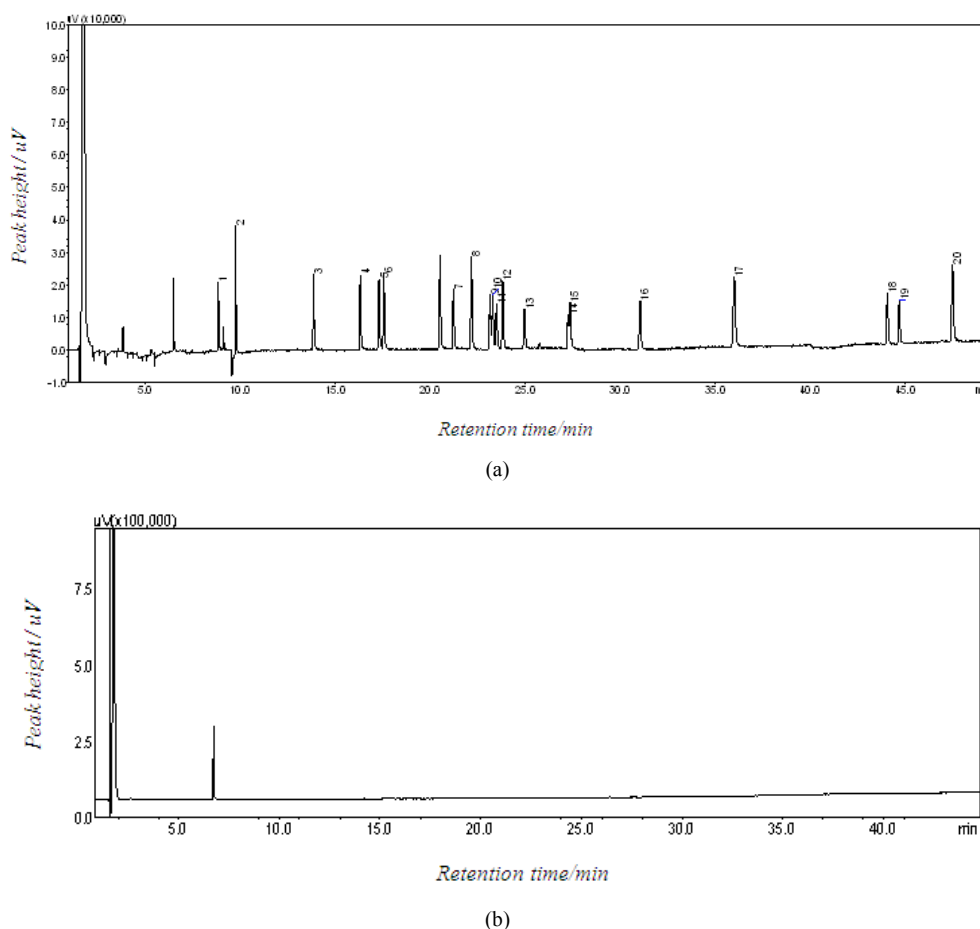
The obtained values for recovery and RSD are between 76.0% and 112.0% and 1.1% – 8.2%, respectively. According to the EU criteria the mean recovery at each fortification level should be in the range of 70% - 120% with  $\text{RSD} \leq 20\%$  [27].

**Table 3.** Statistical data for mean recovery, precision data and linearity of the method

Pesticide	Fortification level (mg/kg)	Pesticide found - mean value (mg/kg $\pm$ SD)	Recovery (%), n=5	RSD (%)	Regression equation
Mevinphos	0.005	0.0045 $\pm$ 0.0003	90.0	6.6	$y = 1.004x - 0.0006$ $R^2 = 0.9991$
	0.010	0.0092 $\pm$ 0.0005	92.0	5.4	
	0.015	0.0143 $\pm$ 0.0007	95.3	4.9	
	0.020	0.0198 $\pm$ 0.0008	99.0	4.0	
	0.025	0.0243 $\pm$ 0.0009	97.2	3.7	
Methacrifos	0.010	0.0110 $\pm$ 0.0002	110.0	1.8	$y = 0.945x + 0.0004$ $R^2 = 0.9961$
	0.020	0.0185 $\pm$ 0.0005	92.5	2.7	
	0.030	0.0275 $\pm$ 0.0006	91.7	2.2	
	0.040	0.0380 $\pm$ 0.0007	95.0	1.8	
	0.050	0.0485 $\pm$ 0.0008	97.0	1.6	
Phorate	0.010	0.0082 $\pm$ 0.0002	82.0	2.4	$y = 0.911x - 0.0004$ $R^2 = 0.9969$
	0.020	0.0175 $\pm$ 0.0006	87.5	3.4	
	0.030	0.0281 $\pm$ 0.0005	93.4	1.8	
	0.040	0.0364 $\pm$ 0.0004	91.0	1.1	
	0.050	0.0443 $\pm$ 0.0006	88.6	1.3	
Diazinon	0.005	0.0038 $\pm$ 0.0003	76.0	7.9	$y = 0.968x - 0.0011$ $R^2 = 0.9954$
	0.010	0.0083 $\pm$ 0.0003	83.0	3.6	
	0.015	0.0140 $\pm$ 0.0005	80.7	3.6	
	0.020	0.0175 $\pm$ 0.0006	87.5	3.4	
	0.025	0.0234 $\pm$ 0.0007	93.6	3.0	
Dimethoate	0.005	0.0041 $\pm$ 0.0003	82.0	7.3	$y = 0.992x - 0.0012$ $R^2 = 0.9964$
	0.010	0.0087 $\pm$ 0.0005	87.0	5.7	
	0.015	0.0129 $\pm$ 0.0007	86.0	5.4	
	0.020	0.0185 $\pm$ 0.0006	92.5	3.2	
	0.025	0.0240 $\pm$ 0.0008	96.0	3.3	
Etrimfos	0.010	0.0112 $\pm$ 0.0005	112.0	4.5	$y = 0.911x + 0.0016$ $R^2 = 0.9994$
	0.020	0.0195 $\pm$ 0.0006	97.5	3.1	
	0.030	0.0287 $\pm$ 0.0008	95.7	2.8	
	0.040	0.0380 $\pm$ 0.0005	95.0	1.3	
	0.050	0.0475 $\pm$ 0.0007	95.0	1.5	
Parathion-methyl	0.010	0.0102 $\pm$ 0.0006	102.0	5.9	$y = 0.983x - 0.0005$ $R^2 = 0.9966$
	0.020	0.0187 $\pm$ 0.0008	93.5	4.3	
	0.030	0.0277 $\pm$ 0.0007	92.3	2.5	
	0.040	0.0396 $\pm$ 0.0008	99.0	2.0	
	0.050	0.0489 $\pm$ 0.0006	97.8	1.3	
Pirimiphos-methyl	0.010	0.0095 $\pm$ 0.0005	95.0	5.3	$y = 1.015x - 0.0018$ $R^2 = 0.9935$
	0.020	0.0180 $\pm$ 0.0007	90.0	3.9	
	0.030	0.0276 $\pm$ 0.0006	92.0	2.2	



Pesticide	Fortification level (mg/kg)	Pesticide found - mean value (mg/kg $\pm$ SD)	Recovery (%), n=5	RSD (%)	Regression equation
Fenitrothion	0.040	0.0375 $\pm$ 0.008	93.7	2.1	$y = 1.001x + 0.0001$ $R^2 = 0.9987$
	0.050	0.0505 $\pm$ 0.009	101.0	1.8	
	0.010	0.0102 $\pm$ 0.006	102.0	5.9	
	0.020	0.0195 $\pm$ 0.009	97.5	4.6	
	0.030	0.0310 $\pm$ 0.008	103.3	2.6	
	0.040	0.0404 $\pm$ 0.006	101.0	1.5	
	0.050	0.0498 $\pm$ 0.007	99.6	1.4	
Chlorpyrifos	0.010	0.0089 $\pm$ 0.006	89.0	6.7	$y = 0.988x - 0.001$ $R^2 = 0.9995$
	0.020	0.0190 $\pm$ 0.004	95.0	2.1	
	0.030	0.0280 $\pm$ 0.007	93.3	2.5	
	0.040	0.0386 $\pm$ 0.005	96.5	1.3	
	0.050	0.0485 $\pm$ 0.009	97.0	1.8	
	0.010	0.0104 $\pm$ 0.005	104.0	4.8	
	0.020	0.0184 $\pm$ 0.004	92.0	2.2	
Malathion	0.030	0.0305 $\pm$ 0.009	101.7	2.9	$y = 0.958x + 0.0004$ $R^2 = 0.9957$
	0.040	0.0380 $\pm$ 0.007	95.0	1.8	
	0.050	0.0485 $\pm$ 0.006	97.0	1.2	
	0.010	0.0095 $\pm$ 0.007	95.0	7.4	
Parathion	0.020	0.0190 $\pm$ 0.008	94.9	4.2	$y = 0.986x - 0.001$ $R^2 = 0.9945$
	0.030	0.0265 $\pm$ 0.009	88.3	3.4	
	0.040	0.0390 $\pm$ 0.007	97.5	1.8	
	0.050	0.0488 $\pm$ 0.010	97.6	2.0	
Bromofos-methyl	0.005	0.0044 $\pm$ 0.005	88.0	6.8	$y = 0.978x - 0.0009$ $R^2 = 0.997$
	0.010	0.0085 $\pm$ 0.007	85.0	8.2	
	0.015	0.0138 $\pm$ 0.007	92.0	5.1	
	0.020	0.0182 $\pm$ 0.006	92.1	3.0	
Chlorfenvinphos	0.025	0.0240 $\pm$ 0.008	96.0	3.3	$y = 0.934x - 0.0007$ $R^2 = 0.9947$
	0.005	0.0043 $\pm$ 0.003	86.0	3.5	
	0.010	0.0080 $\pm$ 0.005	80.0	6.2	
	0.015	0.0134 $\pm$ 0.004	89.3	4.5	
	0.020	0.0187 $\pm$ 0.005	93.5	5.3	
Bromofos-ethyl	0.025	0.0223 $\pm$ 0.006	89.2	6.7	$y = 0.903x + 0.0007$ $R^2 = 0.9973$
	0.010	0.0095 $\pm$ 0.0004	95.0	4.2	
	0.020	0.0187 $\pm$ 0.0007	93.5	3.7	
	0.030	0.0278 $\pm$ 0.0006	92.6	2.1	
	0.040	0.0380 $\pm$ 0.0005	95.0	1.3	
Fenamiphos	0.050	0.0450 $\pm$ 0.0008	90.0	1.8	$y = 0.996x - 0.0005$ $R^2 = 0.9983$
	0.005	0.0048 $\pm$ 0.0003	96.0	6.2	
	0.010	0.0089 $\pm$ 0.0005	89.0	5.6	
	0.015	0.0145 $\pm$ 0.0005	96.6	3.4	
	0.020	0.0195 $\pm$ 0.0007	97.5	3.6	
Ethion	0.025	0.0244 $\pm$ 0.0007	97.6	2.9	$y = 0.932x - 6E-0.5$ $R^2 = 0.9996$
	0.005	0.0047 $\pm$ 0.0003	94.0	6.0	
	0.010	0.0093 $\pm$ 0.0004	93.0	4.0	
	0.015	0.0140 $\pm$ 0.0007	91.3	4.7	
	0.020	0.0185 $\pm$ 0.0005	92.5	2.5	
Phosmet	0.025	0.0234 $\pm$ 0.0008	93.6	3.2	$y = 1.015x - 0.0017$ $R^2 = 0.9995$
	0.010	0.0085 $\pm$ 0.0004	85.0	4.7	
	0.020	0.0182 $\pm$ 0.0006	91.0	6.6	
	0.030	0.0290 $\pm$ 0.0006	96.7	6.2	
	0.040	0.0393 $\pm$ 0.0008	98.2	5.1	
Phosalone	0.050	0.0487 $\pm$ 0.0004	97.4	4.1	$y = 1.062x - 0.0014$ $R^2 = 0.9943$
	0.005	0.0044 $\pm$ 0.0003	88.0	6.8	
	0.010	0.0089 $\pm$ 0.0005	89.0	5.6	
	0.015	0.0144 $\pm$ 0.0006	96.0	4.2	
	0.020	0.0190 $\pm$ 0.0004	95.0	2.1	
Azinphos-methyl	0.025	0.0259 $\pm$ 0.0007	103.6	2.7	$y = 0.972x + 0.0013$ $R^2 = 0.9969$
	0.010	0.0092 $\pm$ 0.0003	92.0	3.3	
	0.020	0.0180 $\pm$ 0.0005	90.0	2.8	
	0.030	0.0265 $\pm$ 0.0004	88.3	1.5	
	0.040	0.0380 $\pm$ 0.0006	95.0	1.6	
	0.050	0.0478 $\pm$ 0.0005	95.6	1.1	



**Figure 2.** GC-NPD chromatograms: (a) fortified blank sample with OPPs at 10 ng/g (0.05 mg/L): (1) mevinphos, (2) methacrifos, (3) phorate, (4) diazinon, (5) dimethoate, (6) etrimfos, (7) parathion-methyl, (8) pirimiphos-methyl, (9) fenitrothion, (10) chlorpyrifos, (11) malathion, (12) parathion, (13) bromofos – methyl, (14) chlorfenvinphos, (15) bromofos – ethyl, (16) fenamiphos, (17) ethion, (18) phosmet, (19) phosalone, (20) azinphos – methyl; (b) blank corn extract

**Table 4.** Statistical data for within day repeatability and between day reproducibility

Pesticide	$t_R/min$	Within day Repeatability (RSD, %; n=10)		Between day Reproducibility (RSD, %; n=25)	
Mevinphos	8.834	0.102	3.17	0.155	4.11
Methacrifos	9.741	0.113	0.91	0.172	1.23
Phorate	13.847	0.130	1.40	0.169	1.78
Diazinon	16.299	0.092	2.73	0.118	3.34
Dimethoate	17.281	0.121	3.61	0.144	4.35
Etrimfos	17.540	0.103	3.23	0.132	4.56
Parathion-methyl	21.208	0.071	3.00	0.098	4.46
Pirimiphos-methyl	22.146	0.063	3.29	0.089	3.90
Fenitrothion	23.121	0.052	2.79	0.077	3.65
Chlorpyrifos	23.267	0.064	2.85	0.080	3.90
Malathion	23.467	0.081	2.21	0.102	4.05
Parathion	23.796	0.046	3.03	0.066	4.90
Bromofos-methyl	24.956	0.052	2.35	0.068	4.40
Chlorfenvinphos	27.232	0.055	3.19	0.072	4.56
Bromofos-ethyl	27.346	0.051	1.71	0.089	3.98
Fenamiphos	31.029	0.051	2.18	0.097	4.87
Ethion	35.978	0.025	2.37	0.046	3.98
Phosmet	44.039	0.027	1.97	0.051	3.30
Phosalone	44.661	0.031	2.10	0.058	4.01
Azinphos-methyl	47.462	0.019	2.69	0.045	4.30

From the obtained results, it can be noticed that the proposed method is accurate and precise enough for the determination of azinphos-methyl, bromofos-methyl, bromofos-ethyl, chlorfenvinphos, chlorpyrifos, diazinon, dimethoate, ethion, etrimfos, fenamiphos, fenitrothion, malathion, mevinphos, methacrifos, parathion, parathion-methyl, pirimiphos-methyl, phosalone, phorate and phosmet in corn. The obtained values for multiple correlation coefficients, ranged from 0.9935 to 0.9996, indicated that the method has a good linearity for all the pesticides.

GC-NPD chromatograms of fortified blank corn samples with OPPs at 10 ng/g (a), as well as blank corn extract (b), are shown in Fig. 2.

### 3.2.2. Repeatability and Reproducibility

The within day repeatability of our method was determined by performing the analysis of 10 blank samples fortified with OPPs at 20 ng/g. After the extraction and purification the obtained extracts were analyzed by GC-NPD within the same day under the chromatographic conditions described in the section 2.1. The between day reproducibility of the method was determined by performing

the analysis of the extracts obtained from 5 fortified blank sample (20 ng/g of OPPs). After the extraction and purification the extracts were analyzed within 5 different days, under the same chromatographic conditions (section 2.1). The calculated RSD values for the retention time ( $t_R$ ), the peak areas, within day repeatability and between day reproducibility are shown in Table 4.

The calculated RSD values for within day repeatability for the  $t_R$  ranged from 0.019% to 0.13%, whereas for peak areas RSD values ranged from 0.91% to 3.61%, indicating good precision of the  $t_R$  within the same day. The calculated RSD values for between day reproducibility for the  $t_R$  ranged from 0.045% to 0.172%, whereas for peak areas RSD values ranged from 1.23% to 4.90%, indicating good precision of the  $t_R$  within different days.

### 3.2.3. Stability

Stock standard solutions and working standard solutions were found to be stable for at least 3 months, respectively, when stored at 4 °C. Moreover, the stability of a fortified blank sample at a concentration of 20 ng/g kept in the auto injector for 24 hours was assayed, and differences of < 3.5 % were obtained.

### 3.2.4. Limit of Detection, Limit of Quantification, Measurement Uncertainty

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated according to the formulas  $LOD = 3.3 \cdot SD/slope$  and  $LOQ = 10 \cdot SD/slope$  [28]. The computed values for all the tested pesticides were found to be well below the MRLs (Table 5).

**Table 5.** Statistical data for LOD, LOQ,  $U_x$  and MRLs for pesticide residues in corn

Pesticide	LOD (mg/kg)	LOQ (mg/kg)	$U_x$ (%)	MRLs (mg/kg)
Mevinphos	0.0015	0.0045	12.5	0.01
Methacrifos	0.0010	0.0030	14.2	0.05
Phorate	0.0014	0.0042	11.3	0.05
Diazinon	0.0013	0.0039	12.9	0.01
Dimethoate	0.0011	0.0033	10.8	0.02
Etrifos	0.0010	0.0030	9.6	5
Parathion-methyl	0.0012	0.0036	11.6	0.02
Pirimiphos-methyl	0.0009	0.0027	10.5	5
Fenitrothion	0.0012	0.0036	8.7	0.05
Chlorpyrifos	0.0015	0.0045	9.5	0.05
Malathion	0.0013	0.0039	11.2	8
Parathion	0.0011	0.0033	9.6	0.05
Bromofos-methyl	0.0012	0.0036	11.7	-
Chlorfenvinphos	0.0011	0.0033	13.4	0.02
Bromofos-ethyl	0.0012	0.0036	11.2	0.05
Fenamiphos	0.0011	0.0033	10.4	0.02
Ethion	0.0008	0.0024	10.6	0.01
Phosmet	0.0014	0.0042	12.3	0.05
Phosalone	0.0011	0.0033	10.9	0.01
Azinphos-methyl	0.0013	0.0039	13.7	0.05

The values for measurement uncertainty ( $U_x$ ) were calculated according to a Eurachem/CITAC Guide [29]. The obtained values showed in Table 5, ranged from 8.7% to 14.2% and were in accordance with the recommended EU criteria [27].

## 4. Conclusions

A reliable, accurate and precise GC-NPD method, after extraction with ASE and the combination of liquid-liquid and SPE/PSA purification, was developed, optimized and validated for the simultaneous determination of azinphos-methyl, bromofos-methyl, bromofos-ethyl, chlorfenvinphos, chlorpyrifos, diazinon, dimethoate, ethion, etrimfos, fenamiphos, fenitrothion, malathion, mevinphos, methacrifos, parathion, parathion-methyl, pirimiphos-methyl, phosalone, phorate and phosmet in corn samples. With the proposed extraction procedure, the extraction and filtration can be performed in a single step requiring a low volume of organic solvent. The proposed purification procedure, reduce the amount of co extractable lipids and disables the appearance of the background peaks in the chromatogram, above signal to noise ratio of 3, at the retention times of targeted pesticides. NPD was chosen for this analysis due to its selectivity for the compounds of interest. Validation parameters obtained for determination of OPPs in corn demonstrate that the developed analytical method meets the method performance acceptability criteria (mean recovery in the range 76% -112.0%; precision with  $RSD < 8.2\%$ ;  $LOQ < MRL$ ; measurement uncertainty  $< 14.2\%$ ). The results of validation and verification of the method in the international proficiency tests (FAPAS 0951) showed it's usefulness for routine analysis. Namely, the satisfactory Z scores were obtained for all present organophosphorus pesticides as:  $Z=0.0$  for etrimfos,  $Z=-0.2$  for fenitrothion,  $Z=0.1$  for malathion and  $Z=2.0$  for pirimiphos-methyl with not false positive Z scores for any of the designated pesticides.

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