Original Article

Determination of Twenty Organophosphorus Pesticides in Wheat Samples from Different Regions of Iran

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Received: 10.01.2017 *Accepted: 15.02.2017*

ABSTRACT

Background*:* Organophosphorus pesticides are widely used in agriculture, homes, gardens, and veterinary practices. Extensive application of pesticides in agriculture often results in residues of these compounds being absorbed into the foods, including wheat. The aim of this study was to evaluate the residue levels of 20 organophosphorus pesticides in wheat samples collected from different regions of Iran.

Methods*:* This research reports a rapid, specific and sensitive multiresidue method based on the Quick Easy Cheap Effective Rugged and Safe (QuEChERS) sample preparation method and gas chromatography with mass spectrometric detection in the selected ion monitoring mode (GC–SIM–MS) to evaluate 20 organophosphorus pesticides in wheat samples.

Results*:* In the concentration range of 20-200 ng/g, the calibration curves for each analyte was linear with a determination coefficient (R^2) of 0.993 to 0.999. The limits of detection (LODs) and quantitation (LOQs) were between 2.5-6.7 and 7.5-20 ng/g, respectively. The mean recoveries obtained for three fortification levels (25, 50 and 100 ng/g, five replicates each) were 80-114% with a satisfactory precision (RSD<20%). 31.1% samples contained residues of one or more target compounds. Chlorpyrifos was the most common residue (17.8%), followed by pirimiphos-methyl (6.7%), diazinon (4.4%), chlorpyrifos-methyl (1.1%) and malathion (1.1%).

Conclusion*:* Among the detected pesticides, only diazinon and malathion are permitted pesticides for wheat production in Iran. However, their concentrations were below the maximum residue levels (MRLs) established by the Iranian National Standard Organization (INSO).

Keywords: GC-MS, Organophosphates, Pesticides, Wheat.

IJT 2017 (5): 37-44

INTRODUCTION

Pesticides are natural or synthetic chemicals used in agriculture to protect crops against destructive pests. They are also used in public health for the eradication of disease vectors and other pests. Annually, at least 4 million tons of pesticides are used for control of pests in the world. Only 1% of applied pesticides reach the target pests. Therefore, 99% of pesticides are left in the environment; finally affect living organisms [1]. Due to their widespread use, pesticides have now become a major group of environmental contaminants. They pollute the environment and remain in food chains, thereby posing health hazards to humans [2, 3]. Chemically, pesticides belong to different classes and organophosphorus pesticides constitute one of the major families, highly potent compounds used mainly as insecticides. Former restrictions on some persistent pesticides had led to the use of non-persistent alternatives such as organophosphorus compounds, carbamates and pyrethroids, which are very effective in pest control, in both agricultural and residential settings [4]. Today, organophosphates are the most widely used pesticides across the world comprising 70% of the compounds in use [5]. In 2007, nearly 35% of applied insecticides in the US comprised organophosphorus, worth about 33 million pounds each year [6].

Intensive and extensive use of these compounds is now posing a significant risk to public health because of their potential adverse effects. Exposure to organophosphates affects not only those who use them occupationally, but the

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general population exposed to low concentrations of these compounds via foodstuffs and the environment throughout their lifetime [7]. Organophosphate poisoning continues to be a major cause of morbidity and mortality in third world countries.

Chemically, organophosphates are esters of phosphoric or thiophosphoric acids. They can phosphorylate the active site of acetylcholinesterase (AChE), which hydrolyses acetylcholine (ACh) in cholinergic synapses and in neuromuscular junctions. This excessive accumulation of acetylcholine in synapses leads to activation of cholinergic receptors [8]. Acute effects of organophosphorus pesticides are well documented. They can cause dizziness, headaches, gastrointestinal distresses, bronchospasm, miosis, urination, sweating, lacrimation, bradycardia, fasciculations, muscle weakness, hypertension, liver and kidney damage, coma and ultimately death [9, 10]. However, some investigations have revealed a number of organophosphorus secondary targets not associated with the cholinergic system and may lead to immunotoxicity [11], endocrine disruption, genotoxicity, and potential carcinogenic effects such as non-Hodgkin's lymphoma [12] and some types of leukaemia [13]. Children may be more vulnerable than adults may to the effects of pesticides because of proportionally higher food and water intake relative to body weight, along with immaturity in the neurological development and detoxification pathways [14].

Unlike acute poisoning, chronic exposure of humans to small amounts of organophosphates through the air and consumption of contaminated food and water can affect a large proportion of the population. Therefore, a pesticide residue in foodstuffs continues to be the target of many studies due to the mentioned adverse effects.

Several techniques are available for the determination of these residues, but traditional pesticide analysis procedures are complicated, time-consuming and labor-intensive. In spite of the variety and complications of the matrices and low levels of pesticides in different food samples, analysis and sample preparation techniques in the area of pesticide residues have remarkably progressed [15]. In 2003, a fast and easy multiresidue technique was developed named QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method [16]. It is based on initial single-phase extraction with acetonitrile, followed by liquid–liquid partitioning by addition of anhydrous magnesium sulfate ($MgSO_4$) and sodium chloride. Removal of the water content and cleanup are achieved by $MgSO_4$ and a primary secondary amine (PSA) sorbent. This method is very flexible and serves as a template for determination of pesticide residues. Today, combination of QuEChERS method and mass spectrometry technique including LC-MS and GC-MS, have been successfully applied to determine the multiresidue levels of pesticides in various food samples [17,18].

Wheat is one of the most common staple foods in the world and especially in Iran. Most pesticide residues in wheat are due to direct application of pesticide during the development of the crop or because of post-harvest treatment of wheat. For example, the main residues detected were the storage insecticides, such as chlorpyrifos-methyl and Malathion. Owing to slow deterioration, they are employed to protect of cereal grains including wheat under storage conditions [19]. Beside the commonly used organophosphates, the presence of banned organophosphorus compounds in wheat is another significant challenge in some countries. Therefore, there is a clear need to develop quick methods for monitoring the most commonly used and forbidden organophosphorus pesticides in wheat crops.

In the present study, a validated multi-residue technique for determination of these residues in wheat, using GC-MS and the QuEChERS method is introduced. Subsequently, the validated method is applied for determination of organophosphorus residues in 90 wheat samples collected from different regions in Iran.

MATERIALS AND METHODS

Chemicals

Pesticide reference standards (purity>96.0%), triphenyl phosphate (TTP), as the internal standard and anhydrous magnesium sulfate ($MgSO_4$), were purchased from Sigma–Aldrich / Fluka / Riedel-de-Haën (Germany). Methanol (MeOH) and HPLC-grade acetonitrile (ACN) were obtained from Acros (Belgium). Ethyl acetate (EtAc), glacial acetic acid (HOAc) and sodium chloride were supplied by Merck (Darmstadt, Germany). Bondesil-primary secondary amine (PSA, 40 µm) was provided by

Interchim (France). HPLC grade water was produced by purifying demineralized water on a Milli-Q plus Ultra-Pure Water System (Millipore, Molsheim, France).

Individual stock solutions of pesticides at a concentration of 1000 µg/mL were prepared in Ethyl acetate (EtAc). A mixed intermediate standard solution at a concentration of 5µg/mL was prepared via the appropriate dilution of the stock solutions in EtAc. This solution was used as the spiking solution for the validation experiments. A stock solution of TTP in EtAc at a concentration of 20 µg/mL was used as the internal standard and an aliquot (50 µL) of this solution (20 µg/mL) was added to the spiked wheat sample as the internal standard.

Pesticide Selection

The GC-amenable investigated pesticides (Table 1) were selected based on compounds that are commonly used for crop production. The MRLs for the pesticides, including diazinon, fenitrothion, malathion and fenthion have been established by the Iranian National Standard Organization (INSO) [20]. According to the same Act, the use of some of these pesticides including phorate, methidathion, and triazophos are forbidden in Iran. The other selected compounds are the ones commonly used around the world for cereal production.

GC-MS Analysis

Gas chromatography (Model 7890 A, Agilent technologies, USA) was employed using a mass spectrometry detector (Model 5975 C, Agilent technologies, USA) equipped with split/splitless injector and an Agilent autosampler. An HP-5 19091S-436 Agilent capillary column (60 m × 0.25 mm I.D., 0.25 µm film thickness) was used along with the following oven temperature program: initial temperature 60 °C, held for 1 min, 60 °C/min ramped to 160 °C held for 3 min, 6°C/min ramped to 190 °C, 0.5 °C/min ramped to 192 °C, 2 °C/min ramped to 233 °C, 5 °C/min ramped to 280 °C, followed by 10 °C/min ramped to 300 °C and held for 4 min. Helium (99.999%) was used as the carrier gas at a constant flow rate of 1.6 mL/ min. The injection port was adjusted at 250 °C using the splitless mode. After acquiring the ion chromatogram in selected ion monitoring (SIM) mode, peaks were identified by their retention time and mass spectra. The most abundant ion that had the highest signal-to-noise ratio and showed no evidence of chromatographic interference was selected for quantification.

Table 1. Summary of molecular weights, diagnostic, quantitative ions and retention times for the studied pesticides.

No.	Compound	Molecular Weight (g/mol)	Diagnostic Ions	Quantitative Ions	Retention Time (min)
1	Phorate	260.38	121,97,170	75	12.85
2	Thiometon	246.35	125, 89,93	88	13.47
3	Dimethoate	229.26	93	87	13.96
4	Diazinon	304.35	137, 199	304	16.08
5	Disulfoton	274.4	89	88	16.45
6	Chlorpyrifos-methyl	322.5	289	286	19.36
7	Fenitrothion	277.23	277,109	260	21.32
8	Pirimiphos-methyl	305.33	276,305	290	21.43
9	Malathion	330.35	125,127	173	22.08
10	Fenthion	278.33	125	278	22.74
11	Chlorpyrifos	350.59	199	197	22.88
12	Methidathion	302.3	85	145	27.33
13	Fenamiphos	303.35	154	303	29.18
14	Profenphos	373.63	208	139	29.88
15	Ethion	384.48	97, 153	231	34.07
16	Triazophos	313.31	162,172	161	35.22
17	Edifenphos	310.37	173, 310	109	36.01
18	Triphenylphosphate*	326.28	325	326	38.09
19	Phosmet	317.32	161	160	39.88
20	Azinphos-methyl	317.32	132	160	42.15
21	Phosalone	367.81	121	182	42.15

* Internal standard

Sample Preparation

Ninety wheat samples were collected from different regions in Iran. One-hundred gram portions of each sample were ground with 100 g of dry ice. Extraction was performed by QuEChERS method [16]. Then, 5 g of each homogenized sample was accurately weighed and placed in a 50 mL centrifuge tube. Appropriate concentrations of the mixed working standard solution (for spiking) and internal standard were added to the tube and 10 mL of ACN was added. The mixture was vortex mixed for 2.0 min, followed by the addition of a mixture of 2 g anhydrous $MgSO_4$ and 1.5 g sodium chloride and subjected to vortex mixing for 2.0 min again. The mixture was centrifuged (Hettich, universal 320r from Germany) for 5 min at 5433×g, and 5 mL of the supernatant was then transferred into an appropriate tube placed in a nitrogen evaporator and dehydrated at 40 °C until dryness. The residue was reconstituted in 0.5 mL ACN. The mixture was vortex mixed for 2.0 min followed by sonication for 4.0 min. Then the solution was transferred to a tube containing 60 mg anhydrous MgSO4 and 20 mg PSA. The mixture was vortex mixed vigorously for 2 min and centrifuged for 5 min at 5433×g. Finally, a 0.5 mL aliquot of the cleaned extract was transferred into a screw cap vial and 1.0 µL of the solution was injected into the GC-MS.

Method Validation

The validation study was performed based on the European SANCO guidelines [21]. The method was tested to assess for linearity, recovery, precision, limits of detection (LOD) and quantitation (LOQ). This involved performing recovery experiments with spiked blank samples to estimate the accuracy of the method. A minimum of five replicates was mandatory (to check for precision) at both the reporting limit (to verify the sensitivity of the method), and at least another higher level. Linearity was studied using spiked calibrations by analyzing six concentration levels in triplicates, between 20 and 200 ng/g. For determination of the mean recoveries and precision (repeatability) expressed as the coefficient of variation (in percent), five spiked blank wheat samples at concentration levels of 25, 50 and 100 ng/g were prepared and treated according to the procedure described in the sample preparation. The recoveries were calculated using spiked calibration. The LOD and LOQ levels were calculated to evaluate pesticide concentrations resulting in a signal to noise ratio of 3 and 10, respectively.

Quantitation of Pesticide Residues

The amounts of pesticide residues in the real samples were calculated by interpolation of the relative peak areas for each pesticide to internal standard peak area in the sample on the matrix-matched calibration curve. In order to compensate for losses during sample processing and instrumental analysis, internal standard (TPP) was employed. Excel software was used for statistical calculations.

RESULTS

GC-MS Determination

For analysis of the studied pesticides, the SIM mode was applied. Quantitation and confirmation of pesticides were performed based on the use of: one quantitative ion, at least one diagnostic (or qualifier) ion, and retention times. Table 1 summarizes molar weights, retention time, and SIM parameters obtained for the studied pesticides.

Method Validation

As shown in table 2, the investigated method was validated by determining LODs and LOQs as well as the recovery and accuracy of compounds at different levels of fortification. Method validation indicated that the calibration curves for each analyte was linear in the concentration range of 20-200 ng/g with a determination coefficient (R^2) ranging between 0.993 and 0.999. The LODs and LOQs were between 2.5-6.7 ng/g and 7.5-20 ng/g, respectively. The mean recoveries obtained for three fortification levels (25, 50 and 100 ng/g of five replicates each) were 80-114% with satisfactory precision (RSD<20%), meeting the EU guidelines for method performance criteria [21].

Analysis of Real Samples

The validated method was applied for the analysis of 90 wheat samples collected from different regions in Iran. As shown in Table 3, five pesticides including, chlorpyrifos, pirimiphos-methyl, diazinon malathion and chlorpyrifos-methyl were detected in 28 (31.1%) of wheat samples. Among the detected pesticides,

chlorpyrifos was the most common (17.8%), followed by pirimiphos-methyl (6.7%), diazinon (4.4%), chlorpyrifos-methyl (1.1%) and Malathion (in 1.1% of the samples).

Table 2. Mean recoveries (%), relative standard deviations (RSD, %), LOQs, LODs (ng/g) and determination coefficients (R^2) obtained for studied pesticides in wheat samples, spiked at 25, 50 and 100 ng/g levels (n=5).

NO.	Compounds	25 ng/g		50 ng/g		100 ng/g		LOQ[a]	LOD[b]	R^2
		Mean	RSD	Mean	RSD	Mean	RSD			
1	Phorate	92	10	94	8	93	12	15.5	5.2	0.999
2	Thiometon	89	7	86	11	91	3	20.0	6.7	0.995
3	Dimethoate	98	9	97	9	80	7	16.5	5.5	0.999
4	Diazinon	90	15	95	12	100	9	16.0	5.3	0.993
5	Disulfoton	95	6	95	4	85	9	11.5	3.8	0.998
6	Chlorpyrifos-methyl	111	2	84	6	86	2	19.0	6.3	0.999
7	Fenitrothion	108	9	105	7	95	3	9.0	3.0	0.999
8	Pirimiphos-methyl	89	1	102	6	88	6	7.5	2.5	0.997
9	Malathion	98	4	81	7	100	2	13.5	4.5	0.999
10	Fenthion	85	12	93	16	103	9	19.5	6.5	0.998
11	Chlorpyrifos	106	13	93	11	97	6	13.0	4.3	0.995
12	Methidathion	88	11	87	8	109	6	14.0	4.7	0.994
13	Fenamiphos	109	14	88	16	97	9	17.0	5.7	0.999
14	Profenphos	101	5	90	11	110	9	18.5	6.2	0.999
15	Ethion	101	9	89	5	113	9	17.0	5.7	0.999
16	Triazophos	97	8	110	9	83	13	15.5	5.2	0.993
17	Edifenphos	100	9	111	7	114	6	19.5	6.5	0.997
18	Phosmet	107	17	99	10	112	2	18.5	6.2	0.999
19	Azinphos-methyl	85	3	99	7	94	1	19.0	6.3	0.999
20	Phosalone	108	5	102	2	86	7	15.0	5.0	0.999

a. Limit of Quantitation
b. Limit of Detection

Table 3. Pesticide residues determined in real wheat samples from different parts of Iran.

No.	Pesticides	No. of positive samples	LOD[a] (ng/g)	LOQ[b] (ng/g)	Min level (ng/g)	Max level (ng/g)	INSO[c] MRLs[d] (ng/g)
1	Chlorpyrifos[e]	16(17.8%)	4.3	13	29	50	-
2	Pirimiphos-methyl[e]	6(6.7%)	2.5	7.5	21	40	-
3	Diazinon	4(4.4%)	5.3	16	34	46	50
4	Chlorpyrifos-methyl[e]	1(1.1%)	6.3	19	-	53	-
5	Malathion	1(1.1%)	4.5	13.5	-	40	500

a. Limit of Detection
b. Limit of Quantitation
c. Iranian National Standard Organization
d. Maximum residue levels
e. Prohibited pesticides for wheat production in Iran

DISCUSSION

Organophosphorus pesticides are very toxic and are most often involved in acute poisoning by inhibiting acetylcholinesterase [22]. However, chronic toxicity of organophosphates including immunotoxicity, endocrine disruption, genotoxicity and carcinogenic effects are not associated with the cholinergic system. Human exposure to organophosphorus pesticides occurs through the three main ways: daily intake of different foods, drinks and air [23]. The toxicological effects of pesticide residues depend on three factors: the residue content of the food, the quantity of the contaminated food consumed and the duration of the time over which the consumption occurs [24]. Therefore, detection and

determination of their residues in different foods, including wheat is very important.

In the present investigation, the residue levels of different organophosphorus pesticides were studied in wheat samples. According to the Iranian regulations, the studied organophosphorus can be divided into three groups: 1) forbidden organophosphorus, 2) permitted and, 3) prohibited organophosphorus for wheat production in Iran. Group 1, including, phorate, methidathion, and triazophos are forbidden for crop production, including wheat in Iran. These pesticides severely affected human health and, their chronic toxicity has been documented. For example, phorate has been demonstrated to induce genotoxicity by causing cytogenetic changes [25] and leading to prostate cancer [26]. These chemicals can be smuggled into Iran and illegally used. Therefore, it is necessary to detect of banned pesticides in wheat. The results showed that none of the detected pesticide was forbidden.

Group 2, including diazinon, fenitrothion, malathion and fenthion are permitted for wheat production in Iran and the MRLs for them have been established by the Iranian National Standard Organization. As shown in table 3, among the permitted pesticides, only diazinon and malathion found in positive wheat samples and their amounts were below the INSO-specified MRLs.

The other pesticides in Table 1 belong to group 3. These chemicals are used in other crop production, like apple, cucumber, rice etc., but are prohibited for wheat production, and MRLs have not been established for them by INSO. It is important to observe that none of the detected pesticides including, chlorpyriphos, chlorpyriphos-methyl and pirimiphos-methyl are permitted for wheat production in Iran. Hence, their occurrence in wheat samples is a major concern.

Chlorpyrifos (O,O-diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate) was the most commonly detected residue in our real wheat samples. It is an effective organophosphorus pesticide used across the world in agricultural and domestic pest control. Toxicity mechanisms of chlorpyrifos in mammals occur by inhibiting AChE, oxidative stress damage, and endocrine disrupting effects. Inhibition of AChE causes the neurons become over-stimulated leading to nausea, hypersecretion, psychiatric disorders, muscle incoordination, disturbance of motor function, respiratory failure, convulsions, and death [27]. Chlorpyrifos causes oxidative stress in animals. Oxidative stress and AChE inhibition, lead to developmental neurotoxicity in humans and animals [28]. Furthermore, chlorpyrifos causes damage to the dopaminergic neurons via oxidative stress and this mechanism may lead to neurodegenerative disease such as Parkinson's disease [29]. Chlorpyrifos is a potent inhibitor of the liver CYP450-dependent monooxygenase system and, chronic exposure can cause liver damage and disturbance of metabolic function in the liver [30]. Chronically, chlorpyrifos causes structural and functional alterations of the kidney that may lead to renal failure [31]. In addition, chlorpyrifos is associated with lung and rectal cancers in humans [32]. Because of severe chronic toxicity of detected pesticides and high per capita consumption of bread in Iran, it is very important to develop modern methods for analysis of the organophosphorus residues in wheat.

Additionally, some wheat samples contained more than one residue; the reason being that wheat cultivated under some conditions is highly sensitive to pests and requires successive applications of different pesticide treatments. Therefore, studies that are more detailed are necessary with respect to the Iranian food consumption patterns and the residue contents of other foodstuffs to determine the total intake of organophosphorus residues and their toxicological effects.

CONCLUSION

An accurate, precise, sensitive and selective method was used for detection, quantification and confirmation of 20 organophosphate residues in wheat using QuEChERS sample preparation procedure and GC-MS. Five pesticides were found in 28 (31.1%) samples. Chlorpyrifos was the most common detected residue, followed by pirimiphos-methyl, diazinon, chlorpyrifos-methyl and Malathion. Among the detected pesticides, diazinon and malathion are permitted pesticides for wheat production in Iran. However, their concentrations were below the MRLs established by the Iranian National Standard Organization.

ACKNOWLEDGMENTS

The authors are very grateful to Mohammad Hossein Shojaei, Head of Faroogh Life Sciences Research Laboratory.

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