Original Article

Simultaneous Analysis of Seven Non-authorized Pesticides Residue in Cucumber Using Spiked Calibration Curve by GC/ECD

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ABSTRACT

Background: Lack of farmers' knowledge, illegal production and import, and economical situation are the main reasons for non-authorized usage. In this study, one of the major center of cucumber cultivation at south west of Iran, Darreh shahr City in Ilam Province were studied for non-authorized pesticides usage, which health authorities and national standard organization do not recommend.

Methods: A reliable, rapid and accurate method based on spiked calibration curves and modified QuEChERS sample preparation was developed for determination of 7 non-authorized pesticides. During 2014 fifty cucumber samples were collected and analyzed by gas chromatography Electron Capture Detector (GC/ECD). For overcoming matrix effects, calibration curve was prepared by spiked samples.

Results: Method was validated with related parameters. The recovery of pesticides at five concentration levels (n=3) was in the range of 81.02-91.66%. The method was proved repeatable with RSD lower than 20%. The limits of quantification for all pesticides were 10 ng/g.

Conclusion: There are some pesticides which are legally used with identified MRL in cucumber, but for better protection farmers used another pesticides which are not permitted by health authorities, consequently are harmful for consumers. Related fields inspections showed that some specific pesticides were applied for this purpose, such as α-endosulfan, β-endosulfan, endosulfan sulfate, α-HCH, β-HCH, γ-HCH and phosalone that was found in three cucumber samples (6%).

Keywords: Cucumber, Electron Capture Detector, Gas Chromatography, Mass Spectrometry, Pesticide Residues.

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INTRODUCTION

Nowadays, according to pesticides residue in agricultural products food safety and health is very important. Pesticides are widely used to protect plants against pests and they are essential in agricultural systems pesticide residues in crops have irreparable consequences on the consumer these effects classified as acute and chronic effects [1, 2].

Acute effects appear as eye and skin irritation, nausea and acute toxicity. However, the more important is the chronic toxicity effects that arise as cancers, genetic disorders, hormonal disorders, infertility, decrease of IQ and creating allergies. For example, Alzheimer's risk associated with exposure to be in highest rate organophosphorus and paraquates are in the next order [3-6].

There are different pesticides that widely used to protect crops. Thus, analysis of pesticides residue in farming crops and comparing with the national and international implemented standards

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with criteria such as Maximum Residue Level (MRL) is necessary for supervision and control of products. MRL for pesticides describe the maximum concentration of the residues legally allowed in foods and agricultural products.

Cucumber is one of the agricultural products commonly consumed in Iran in fruit dishes, as vegetables and in traditional foods especially in summer. It is cultivated in many areas with different climates. Darreh shahr City in Ilam Province in south west of Iran is one the main area in cucumber production cultivated twice a year. Because of climate conditions on the region, most farmers use pesticides for control pests in cucumber cultivation. National standard organization issued list of pesticides authorized for each identified products, but sometimes farmers use some pesticides that are in official list but not recommended for cucumber. Seven non-authorized pesticides are applied in cucumber cultivation by farmers including α-Endosulfan, β-Endosulfan, Endosulfan sulfate, α-HCH, β-HCH, γ-HCH and Phosalone that their properties are described in Table 1.

Table 1. Physicochemical properties of the non-authorized selected pesticides.

Compound	Structure	M.F	M.W	M.P.
α–HCH	Organochlorine	$C_6H_6Cl_6$	290.83	156-161 °C
β–HCH	Organochlorine	$C_6H_6Cl_6$	290.83	≥300 °C
γ–HCH	Organochlorine	$C_6H_6Cl_6$	290.83	323 °C
α–Endosulfan	Organochlorine	$C_9H_6CL_6O_3S$	406.93	106 °C
β–Endosulfan	Organochlorine	$C_9H_6CL_6O_3S$	406.93	207-209 °C
Endosulfan Sulfate	Organochlorine	$C_9H_6CL_6O_3S$	422.92	70-100 °C
Phosalone	Organophosphorus	$C_{12}H_{15}CINO_4PS_2$	307.81	46° C

Long-term monitoring of pesticide residue in vegetables for food safety purpose and assessment of dietary exposure is necessary for most developed communities. Validated method should be applied to monitor most chemical groups of pesticides and the reliable results used for modifications on pesticides usage, changing for their MRLs and dietary exposure studies.

In this study a reliable, rapid and accurate method based on spiked calibration curves and modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged and safe) sample preparation was developed for determination of 7 non-authorized pesticide residues in cucumber collected from Darreh Shahr City of Ilam, Iran, by gas chromatography with electron capture detector (GC/ECD) during 2016.

The supervisory authorities have analyzed pesticides residue in various vegetable such as cucumber in around the world [7-11]. The present study is the first attempt for simultaneous determination 7 non-authorized pesticides in cucumber grown Darreh Shahr, according to use of these pesticides continuously in this city.

This study aimed to determine the residues of non-authorized pesticides in samples of cucumber and to assess the relative safety of Darreh Shahr, cucumbers based on standard pesticide residues limits.

MATERIALS AND METHODS

Chemicals

The organic solvents were of liquid chromatography grade and were provided from Merck (Darmstadt, Germany). The Primary Secondary Amine (PSA) and Graphite Carbon Black (GCB) were obtained from Supelco (Bellefonte, USA). Anhydrous $MgSO_4$ was provided from SIGMA Aldrich CO. (Japan). Sodium chloride (NaCl) was obtained from Merck (Darmstadt, Germany); all pesticide reference standards were provided from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

GC/ECD

Nitrogen was used as carrier gas at a constant flow of 1 ml min^{-1}. Injection port was adjusted at 260 °C. Briefly, the oven temperature program for separating compounds initially programmed was 75 °C and remained for 3 min; after, it increased to 120 °C at 25 °C/min ramp rate and finally, increased to 300 °C at 5 °C/min ramp, holding at 300 °C for 20 min.

Calibration

Initially ethyl acetate solution of each standard pesticide with concentration of 1 mg/mL and ethyl acetate solution of internal standard pentachloronitrobenzene (PCNB) with concentration of 1 mg/mL were provided.

Mixed standard solutions of 7 pesticides with concentration of 10 µg/ml were produced in ethyl acetate. Spiked calibration standards at 6 levels of 10, 25, 50, 100, 200, and 300 ng/gr triplicate were prepared using to add of 10 µL, 25 µL, 50 µL, 100 µL, 200 µL, 300 µL, of the standard mixture of pesticides to 10 gr of blank cucumber samples in each case and also was add 5 µL of the internal standard to the studied samples.

Triphenylmethane (TPM) is used as an internal standard for confirmed results by GC/MS. Then, the samples were treated as described in next section.

Sample Preparation

Firstly, 10 mL acetonitrile was added to 10 gr milled and homogenized cucumber sample in a 50 mL falcon tube high-speed shaker was applied for mixing for 1 min.

Then, one gr NaCl was added into falcon tube and mixed for 1 min by shaker. The samples were centrifuged at 4500 rpm, for 10 min in -5 °C. Upper layer was transferred to the 15 mL falcon tube containing 2-gr magnesium sulfate and 0.3 gr PSA and 0.1 gr GCB. The mixture was mixed by vertex for 2 min and then centrifuged at 4500 rpm, for 10 min in -5 °C. Totally, 4 ml of the material was next conducted to a vial and evaporated to dryness under a gentle flow of nitrogen gas. The residue was reconstituted by toluene until 1 ml and ultimately, the vials shakes for mixing for 3 min and 2 µl of the solution was injected into GC/ECD.

Recovery Studies

To determine the recovery and precision of the procedure, 10 gr of blank cucumber were spiked using a standard mixture of 7 pesticides with the concentration of 10 µg/mL at concentration levels of 15, 45, 75, 150, 250 and 450 ng/g respectively and in presence of 5 µl of the internal standard and then, the samples were analyzed. This work was repeated on three different days, and then average recovery and relative standard deviation (RSD) were calculated using the calibration curves obtained from spiked samples.

Quantitation

Mixed standard solutions of 7 pesticides with concentration of 10 µg/ml were produced in ethyl acetate and then, the mixed standard solution was injected into gas chromatograph. Table 2 presents the retention time of pesticides and internal standard used in this analysis.

The chromatogram from the simultaneous analysis of pesticides and internal standard (PCNB) by GC/ECD is present in Figure 1. The standard curves were designed using obtained values from division area under the curve of the pesticides in spike samples to the area under curve of the PCNB as internal standard.

Table 2. The retention time, selected pesticides and internal standard (PCNB).

No	Compound	Retention Time (min)
1	α–HCH	21.177
2	β–HCH	22.348
3	γ–HCH	22.554
4	α–Endosulfan	29.853
5	β–Endosulfan	32.102
6	Endosulfan Sulfate	33.698
7	Phosalone	36.95
8	PCNB	22.75

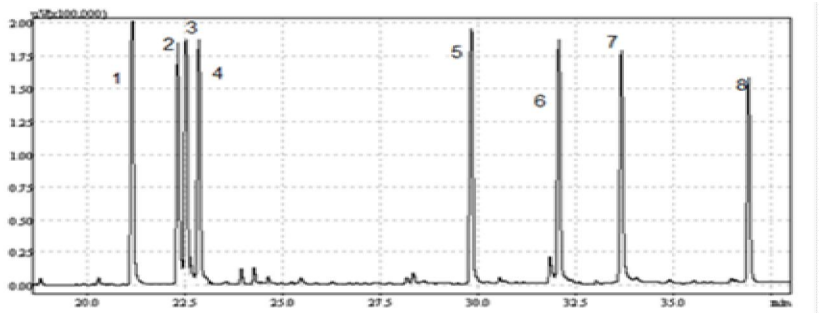

Figure 1. The representative chromatogram obtained by GC/ECD for the 7 pesticides and the internal standard. 1– α–HXH, 2– β–HXH, 3– γ–HXH, 4– ΠΧΝΒ, 5– α–Ενδοσυλφαν, 6– β–Ενδοσυλφαν, 7- Endosulfan Sulfat, 8- Phosalon.

RESULTS

Method Validation

Linearity of the Calibration Curves

The calibration curves of pesticides were linear in the range of 10-300 ng/g (Table 3). The correlation factors for all the pesticides were obtained higher than 0.994.

Limits of Detection and Limits of Quantification (LOD and LOQ)

Limits of detection and limits of quantification of the present method were calculated by considering value 3 and 10 times that of background noise in spiked cucumber samples.LOD and LOQ for all pesticides were ≤3 ng/g and ≤10 ng/g, respectively.

Recovery

The recovery of pesticides at 5 concentration levels (n=3) was in range of 81.02-91.66%. The technique was proved repeatable with RSD lower than 20%. Both the recoveries and repeatability are in accordance with the criteria set by European guidelines SANCO (2003) (Table 4).

Real Samples

Fifty samples of cucumber were collected from Darreh Shahr, and analyzed. For evaluation of analysis, two quality control (QC) sample at 15 and 100 ng/g levels were carried out in each working round.

Three samples was contaminated with Endosulfan sulfate ,α-Endosulfan and , γ-HCH and β-HCH that one of three contaminated samples have contamination more than MRL established by the Iranian national standards (ISIRI, 2009) (Table5) [12-14]. The results have been confirmed by GC/MS (Table 6). Figure 2 shows a representative chromatogram of cucumber sample contaminated by α-Endosulfan and also presents chromatogram of cucumber sample without any contamination in Figure3.

Table 3. The Calibration data (equation and regression coefficient) of the target pesticides in spiked cucumber calibration curves.

Compound	Equation	Regression Coefficient
α-HCH	y=0.0076x-0.0409	0.9996
	y=0.0062x+0.108	0.9939
β–HCH	y= 0.0063x-0.0461	0.9949
	y= 0.0054 +0.0983	0.9876
γ–HCH	y=0.0076x-0.0232	0.9991
	y=0.0065x+0.0793	0.9996
α–Endosulfan	y=0.0101x-0.0626	0.9990
	y=0.0078x+0.2843	0.9926
β–Endosulfan	y=0.0079x-0.0058	0.9970
	y=0.0072x+0.1631	0.9982
Endosulfan sulfat	y=0.0076x-0.0019	0.9989
	y=0.0072x+0.1163	0.9943
Phosalone	y=0.0048x-0.0267	0.9995
	y=0.0058+0.1697	0.9988

Table 4. The mean recoveries (%) and average RSD % in pesticides obtained by GC/ECD analysis of cucumber samples at 5 spiking levels (n= 3).

Compound	Mean recovery (%) (n=3)					Total Average Recovery(%)(n=15)	Average RSD (%)
	450 ng/g	250 ng/g	150 ng/g	75 ng/g	15 ng/g		
α–HCH	77.37	76.66	84.96	95.99	113.42	89.60	8.08
β–HCH	72.95	78.58	102.16	97.01	107.64	91.66	7.12
γ–HCH	79.93	76.18	84.57	82.84	81.62	81.02	8.96
α–Endosulfan	78.89	79.07	84.31	88.58	83.18	82.80	7.64
β–Endosulfan	77.20	82.19	97.37	90.09	85.09	86.38	8.71
Endosulfan sulfate	77.79	87.37	89.76	81.68	81.14	83.54	6.94
Phosalone	73.16	82.53	88.81	90.97	88.02	84.69	5.91

Table 5. The pesticides residue and their concentrations in the cucumber samples by GC/ECD

Sample	Pesticide	Concentration (mg/kg)	MRL by ISIRI (mg/kg)
Sample A_7	β-HCH	0.0380	0.05
Sample A_7	γ -HCH	0.0063	0.05
Sample A_{16}	α-Endosulfan	0.0664	0.05
Sample A_{47}	Endosulfan sulfate	0.0063	0.05
Sample A_{47}	γ-HCH	0.0091	0.05

Table 6. The pesticides residue and their concentrations in the cucumber samples by GC/MS

Sample	Pesticide	Concentration (mg/kg)	MRL by ISIRI (mg/kg)
Sample A_7	γ-HCH	< LOQ	0.05
Sample A_7	γ -HCH	< LOQ	0.05
Sample A_{16}	α-Endosulfan	0.0664	0.05
Sample A_{47}	Endosulfan sulfate	< LOQ	0.05
Sample A_{47}	γ-HCH	< LOQ	0.05

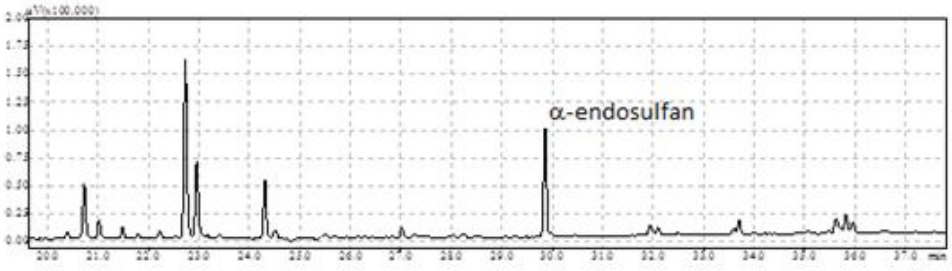

Figure 2. Chromatogram of cucumber sample contaminated by α –endosulfan

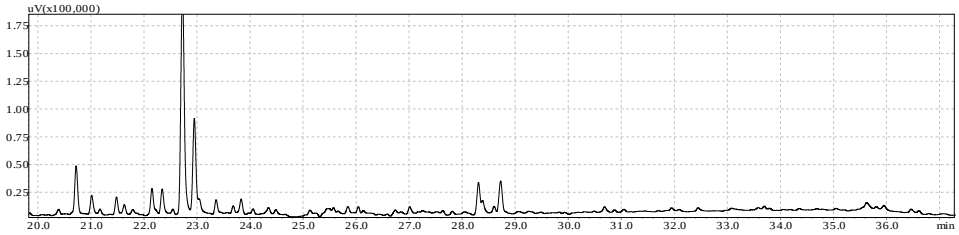

Figure 3. Chromatogram of cucumber sample without any contamination.

DISCUSSION

Multi residue analysis of pesticides in food samples is one of the most important procedures in food control authorities [15, 16]. There are references methods for simultaneous analysis, but the key point is how to match the food matrix with the selected method and with interested pesticides. From food control aspects, there are many national and international standards and rules to limit use of pesticides, also the illegal use should be considered [17-22].

The present study is a reliable, rapid and accurate method based on spiked calibration curves and modified QuEChERS sample preparation was developed for determination of 7 non-authorized pesticide residues in 50 cucumber sample collected from Darreh Shahr, by gas chromatography Electron Capture Detector (GC/ECD).

Spiked calibration curve is a new approach in food analysis which matrix is complex and some interference may be occurred in extraction and analysis procedure. In this approach, standard solutions were directly spiked on real sample matrix, pesticides were extracted form matrices and calibration curve is drawn with acquired data.

There is no data bank on pesticide residues in cucumber grown Drreh Shahr. The place is one of the important agricultural areas in cucumber production cultivated twice per year and

distributed overall country. Pesticides was selected based on list of the Iranian Standards Institute (ISIRI 2009) which do not exist in the list and were applied illegally or lack of knowledge of farmers. Controls of pesticide residue in cucumber are routinely done by authorized organizations and in identified laboratories based on the standards lists and their MRLs.

In one study, a novel strategy was applied for optimization and validation of method using GC-MS for simultaneous analysis of pesticides in tea samples in order to improvement of recovery. Fortified, extracted, and cleaned-up tea samples instead of calibration standards for quantitation were applied, which substantially reduced adverse matrix-related effects and negative recovery affected by graphite carbon black (GCB) on pesticide analysis [23].

The amount of organochlorine pesticides (DDT, γ–HXH, methoxychlor, aldrin, dieldrin and pyrethroid) was investigated in fruits and vegetables such as cucumber by gas chromatography and Electron Capture detector (ECD).

In this survey, residual pesticides were found in 19% of the samples gave resulted with levels of pesticide residues above the maximum residue limit

Besides, residues of pesticides in cucumber produced from different farming systems were examined by gas chromatography with pulsed-flame photometric detection (GC/PFPD) and then the results has been confirmed by GC/MS. Greenhouse cucumber had contamination rates higher than in various cucumbers (1.016 mg/kg).

CONCLUSION

A simple and rapid method was developed to determine banned pesticide residues in cucumber as main vegetable in Iranian food basket. According to the results and compared to MRL, consumption of cucumber based on Iranian food basket is safe but routinely monitoring of pesticides residue in all selected population of country is essential requirement.

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