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Residual Determination of Multiple Pesticides in Vegetable Samples by LC-MS/MS Coupled with Modified QuEChERS-dSPE Ionic Liquid-Based DLLME Method

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Abstract: As a matter of fact, the Dietary Guidelines of the United States of America recommended the consumption of more fruits and vegetables to support the healthy condition of the body. Unfortunately, these food materials are being accumulated with pesticidal residues due to the continuous miss-management and excessive application of the chemicals during pre and post-agricultural practices, which compels multiple analysis of pesticidal residues to know their concentration levels for the betterment of food security and safety. For that matter, multi-residues of Thiamethoxam, Propamocarb, Carbaryl, Metalaxyl, Baycarb, Thiobencarb, Diazinon, and Dursban pesticides were determined in the samples of lettuce, garlic, ginger, and bell-pepper using modified QuEChERS-dSPE Ionic Liquid-based dispersive liquid-liquid microextraction (DLLME) method coupled with LC-MS/MS instrument and validated (European Union Guideline). Resultantly, the accuracy (87-127%) and precision (0–22%) were mostly within the acceptable range for the former (70-120%) and latter (≤20%). Meanwhile, the limit of detections (0.01-0.28 μg/kg) and limit of quantitations (0.03-0.93 μg/kg) were satisfactory. The concentration range (5–400 μg/kg) of calibration curves for the evaluated linearity were linear with coefficient of regressions greater than 0.99. The matrix effects for all the analyzed samples were very weak and less effective (≤ -86%). The range (1–25%) for the estimated measurement uncertainties were certifiable and acceptable (≤ 50%). Therefore, the sample preparation method prove effective as validated and useful for the multiple determination of pesticides residues in the analyzed vegetable samples, which are presumably safe for consumption against health issues.

Keywords: Pesticide residues; fruits and vegetables; QuEChERS-dSPE and DLLME cleanups; ionic liquid-based; liquid chromatography tandem mass spectrometry.

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INTRODUCTION

Fruit and vegetable foods are one of the bases that constitute healthy diets worldwide, playing vital roles nutritionally for the attainment of a healthy life (1). Moreover, fresh fruits and vegetables provide dietary fibers, carbohydrate, vitamins (particularly vitamin C), minerals (particularly electrolytes), and bioactive compounds (2-4). The bioactive compounds include phytochemicals, which possesses antioxidant, phytoestrogen activities and anti-inflammatory agents (5,6). Moreover, the dietary fibers supplied by these foods prevent gastrointestinal cancers and contribute to lowering the cholesterol level in the blood (cholesterolemia) (7). Consequently, reducing the high peril of cardiovascular diseases and the reduction of high risks of obesity (8,9).

Meanwhile, the derived nutrients and biological compounds in fruits and vegetables depend on nature, size, geographical locations they were cultivated (10). In the year 2010, it was recommended by the Dietary Guidelines of the United States of America suggested that one-half of a person's plate of food should contain fruits and vegetables (11). Unfortunately, the percentage of nutrients in fruits and vegetables has been decreasing over the years due to soil depletion of essential materials caused by intensive modern agricultural techniques (12,13), which results in consumption of more fruits and vegetables to support the healthy condition of the body (11).

Notwithstanding, the fresh vegetables and fruits of today have been accumulated with pesticide residues because of the continuous miss-management and excessive application of pesticides during pre and post-agricultural practices (14-17). For example, the triazole fungicides, carbamates, pyrethroids, and organochlorine pesticides (OCPs) are most well-known for controlling pests in vegetables and fruits (18-20). This could lead to disastrous health-related issues such as different forms of cancer and congenital disabilities (1). Based on these facts, the food quality controllers and the analytical scientists have periodically analyzed the concentration levels of pesticides residue in vegetable and fruit samples using conventional methods and instruments such as gas and liquid chromatography.

Illustratively, the conventional methods include liquid-liquid extraction (LLE), solid phase extraction (SPE) and liquid phase microextraction (LPME) (20). Unfortunately, most of the methods possess poor selectivity. Meanwhile, many detectors such as diode array, photodiode array and mass spectrometry instrumentally possesses poor sensitivity towards targeted analytes because most of the instruments were operated at default settings lacking optimization (21). Fortunately, the recent reports suggested the use of a modified quick, easy, cheap, effective, rugged, and safe (QuEChERS)-dispersive SPE (dSPE) coupled with LPME as dispersive liquid-liquid microextraction (DLLME) technique instrumented with an optimized liquid chromatography tandem mass spectrometry (LC-MS/MS) for analysis of multiple pesticidal residues in fruits and vegetables (15, 21). Also, the optional used of 1-hexyl-3-methylimidazolium hexafluorophosphate ($[C_6MIM][PF_6]$) ionic liquid-based in the DLLME technique increases the extraction efficiency and chromatographic properties of the analysis (22, 23).

Therefore, this study was aimed to determine the multi-pesticide residues of Thiamethoxam, Propamocarb, Carbaryl, Metalaxyl, Baycarb, Thiobencarb, Diazinon, and Dursban (Figure 1) in some selected sample of vegetables. The analyses were carried out using the modified QuEChERS-dSPE Ionic Liquid-based DLLME coupled with optimized LC-MS/MS method revealed by Lawal et al. (15) and Lawal, et al. (21). It is hoped that this study will serve as a reference guide for the future studies of pesticide residues in other vegetable samples to provide betterment of food security and safety.

MATERIALS AND METHODS

Chemicals and Reagents

The pesticidal standards (100 mg/kg) for Thiamethoxam, Propamocarb, Carbaryl, Metalaxyl, Baycarb, Thiobencarb, Diazinon, and Dursban were obtained from AccuStandard® (New Haven, USA) and were later diluted to 0.1 mg/kg (100 µg/kg) with estimated volume of methanol, respectively. Meanwhile, the LC-MS grade organic solvents were used for this research work. The solvents include methanol and ACN (Merck, Germany), acetic acid (HOAc), and formic acid were obtained from Fisher Scientific. The Millipore-filtered (deionized) water was obtained using Merck Millipore water purification system (Billerica, USA). The ProElut™ AOAC 2007.01 QuEChERS-dSPE kits for general vegetables and fruits were obtained from Dikma Technologies Inc. (Lake Forest, USA), as well as the molten salt (HPLC grade) of $[C_6MIM][PF_6]$ ionic liquid-based ($P \geq 97.0\%$) was purchased from Sigma-Aldrich, (Germany).

Apparatus and Equipments

The 2, 15, and 50 mL polypropylene centrifuge tubes by LabServ Fisher-Scientific (Kuala Lumpur, Malaysia), and 100 and 500 µL microsyringes were obtained from Agilent (Australia). The HPLC autosampler vials were purchased from Agilent Technologies (USA) and other equipments such as Dynamica refrigerated centrifuge by CNG instruments (Selangor, Malaysia), vortexer VTX-3000L by Copens Scientific (Tokyo, Japan) and glass jug blender MX-GX1581WSK (Panasonic, Malaysia) and Supelco HPLC column [Ascentis® Express C_{18} (5 cm x 2.1 mm, 2.7 µm)] (Sigma-Aldrich, USA). The others include weighing balance (Sartorius Technology Park, Germany), pH meter PB (Sartorius group, Germany) and Agilent triple quadrupole LC/MS G6490A [built in Electrosprays ESI (±) MS/MS Sensitivity and Jet stream Technology] instrument (Singapore).

Conditioning the LC-MS/MS Instrument

The setup for contributory factors of the LC-MS/MS instrument were optimized. These include; analyte injection volume (5 µL), column temperature (30 ºC), flow rate (0.1 mL/min), gas temperature (200 ºC), gas flow (14 L/min), nebulizer gas (45 psi), sheath gas temperature (400 ºC), sheath gas flow

(11 L/min), capillary voltage (3000 V) and delta$^{(+)}$ EMV (200 V). The factors were used for the determination of optimum fragmentary voltage and the four-fragmentary product ions with their respective retention time (RT) and collision energy (CE) by the Auto-tuning and Mass-Hunter instrumental optimization using 1 mg/kg multi-pesticides mixture of standard solutions (Table 1) and the total ion chromatography (TIC) were highlighted (Figure 2). Moreover, the setup was also used for the estimated gradient (elution) time for the mobile phase-B at 15% (0 - 1.6 min), 15 – 100% (1.6 – 10.4 min), and 100 – 15% (10.4 – 12 min) at the pressure of 600 bar. Moreover, the mobile phase-B (acetonitrile + 0.1% formic acids) and "A" (deionized water + 0.1% formic acid) instrumentally started from 15 and 85%, respectively, and transported through the column by a stream of nitrogen gas after the column was injected with 5 µL analyte solution.

Sample Treatment and QuEChERS-dSPE Ionic Liquid-based DLLME Method

The 250 g for each of the purchased vegetable (Petaling Jaya, Malaysia) samples of lettuce, garlic, ginger and bell-pepper were homogenized, and refrigerated (reserved) at 4 °C. The methodological procedure occurred by transferring 20 g for each of the homogenized vegetable sample into 50 mL centrifuge tube and the content was spiked with 200 µL of 100 µg/kg multi-pesticides mixture of standard solutions. 1% HOAc in 15 mL ACN was added before covering and vortexing the tube for 1 min. A sachet of QuEChERS extraction salt was added to the tube's content, covered, shaken vigorously (1 min) and centrifuged (4000 rpm) for 2 min. 1 mL supernatant was transferred into 2 mL centrifuge tube that was occupied with a sachet of the cleanup agent. The tube was centrifuged (4000 rpm) for 5 min after vortexing it for 30 sec. Subsequently, the resulted supernatant from the d-SPE cleanup was transferred into 15 mL centrifuge tube containing 10% NaCl in 9 mL of Milli-Q-water. The tube was covered, shaken vigorously (1 min) and centrifuged (7000 rpm) for 5 min after addition of 130 µL ionic liquid-based. Then, the 100 µL [C_6MIM][PF_6] ionic liquid-based extract was diluted with 400 µL of methanol (1:5) in 2 mL HPLC auto-sampler vial and vortexed for 1 min and the solution was analyzed with LC-MS/MS instrument. Consequently, the sum of the resulted total chromatographic peak areas (TCPAs) of the multiple reaction monitoring (MRM) scans for each of the analyte was used as an index that correspond to the concentration levels of analytes quantified in the analyzed samples (24).

Similarly, results were obtained for the construction of calibration curves and validation studies for each analyte respectively using parts of the refrigerated samples, weighed equally and spiked serially with estimated volumes of analyte mixture of standard solutions to provide equivalent known concentrations.

Validation Studies of Sample Treatment Method

The sample treatment method for the analysis of multiple pesticides residues was validated to express its effectivity, desirability and certification (25). The method was validated using the European Union Guideline (26) that include; accuracy (relative recovery) and precision (relative standard deviation) were estimated using triple concentration levels (5, 100 and 300 µg/kg) and the samples were analyzed in triplicates (n=3), the limit of detections (LODs) and limit of quantitations (LOQs) were correspondingly estimated to 3 and 10 factors of signal-to-noise ratio respectively (27), the calibration curve for each analyte was used for the estimation of linearity based on the regression coefficient (R^2) at five spiked concentration levels ranging 5 – 400 µg/kg, the matrix effect (ME) was also estimated mathematically using the calibration curves (Equation 1) and ultimately, the empirical model and coverage factor (k=2) were used for the estimation of measurement uncertainties (MU) at 95% confidence level (28).

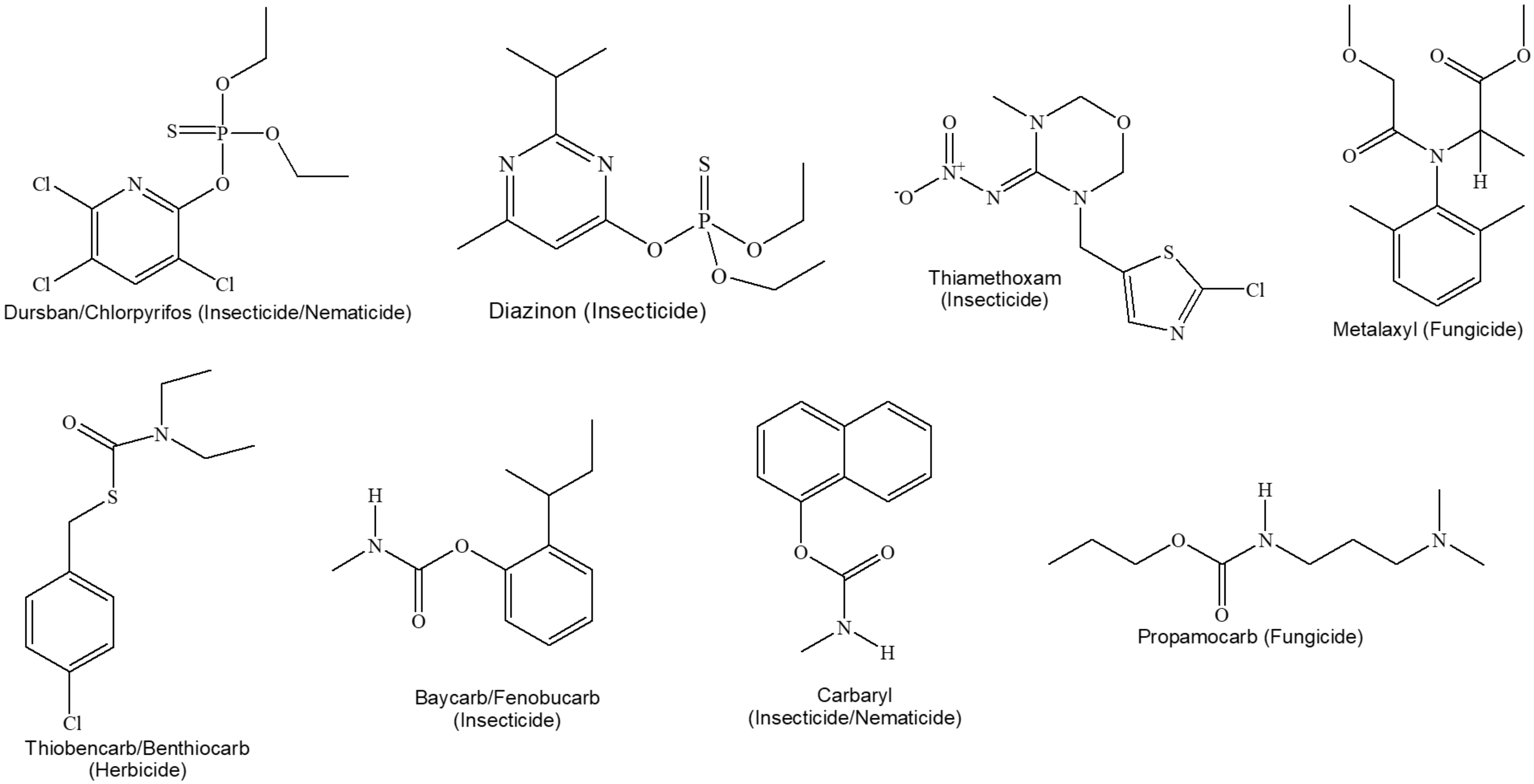
$$ME(\%)=\left[\left(\frac{\text{Slope of calibration curve for analyte in matrix}}{\text{Slope of calibration curve for analyte in ACN}}\right)-1\right]\times 100 \quad \text{(Eq. 1)}$$

RESULTS AND DISCUSSION

The modified QuEChERS-dSPE Ionic Liquid-based DLLME method was successfully validated based on the parameters that include relative recoveries (RRs), relative standard deviations (RSDs), LODs, LOQs, R^2, ME and MU. However, 98 and 99% of the RR (87 - 127%) and RSDs (3 - 22%) tabulated in Table 2 were within the recommended guideline (70 – 120%) value (26) and conforms to the report of Nantia et al. (29). The range results of 0.01-0.28 and 0.03-0.93 µg/kg for LODs and LOQs (Table 3) respectively were excellent and lower that than the least concentration of the calibration curve (5 µg/kg) and European Union maximum residue limits (EU-MRLs) recommendation (30). The R^2 obtained were linear and greater than 0.99 value as indicated in Table 4. The results were similar to the documentation of Camino-Sánchez et al. (31) and Lawal et al. (32). Table 4 also shows that the method's performance capability (matrix effect) against matrix inferences towards recovery of analytes is very strong i.e. the matrix effects for all the analyzed samples were very weak, less effective (≤ -86) as referenced by the guideline; suppression (-20%) or enhancement (20%) of analytes' recovery, which could be attributed to the excessive cleanup of matrix interferences by the modified sample preparation method. Moreover, the matrix effect results were in line with the recent reports on the analysis of fruits and vegetables (1, 15, 21). The recommended range (50%) for the measurement uncertainties (MU) supported the obtained results (Table 4) range estimated (1 – 25%). Eventually, the modified QuEChERS-dSPE Ionic Liquid-based DLLME sample treatment method coupled with the LC-MS/MS instrumentation were reliably and credibly used for quantitative analysis of the unspiked (reserved) vegetable samples and most of the analytes detected (Table 5) were lower than the LOQ and the EU-MRLs.

CONCLUSION

The determination of the multi-pesticide residues were successfully carried out in the samples of lettuce, garlic, ginger, and bell-pepper using modified QuEChERS-dSPE Ionic Liquid-based DLLME method. The extraction method efficiently cleanup the matrix interferences toward improving the detectability, selectivity and recovery of the targeted analytes using the sensitive instrument for better determination and recovery of the targeted analytes. Consequently, the sample preparation and instrumentation techniques proved reliable and successfully used for multi-residue determination of pesticides in lettuce, garlic, ginger, and bell-pepper samples. What is more, the obtained results for their concentration levels were less than the EU-MRLs, which presumed the safe consumption of the vegetables from the sampled area.



Dursban/Chlorpyrifos (Insecticide/Nematicide)

Diazinon (Insecticide)

Thiamethoxam (Insecticide)

Metalaxyl (Fungicide)

Thiobencarb/Benthiocarb (Herbicide)

Baycarb/Fenobucarb (Insecticide)

Carbaryl (Insecticide/Nematicide)

Propamocarb (Fungicide)

Figure 1: The Structures of the analyzed residue of pesticides

Table 1: The Mass-Hunter and auto-tuned optimization for setup of the LC-MS/MS instrument

No.	Pesticides	Molecular Formula	Pesticide Type	Ionization Mode	Precursor Ion, m/z	Product Ions (m/z)	Collision Energies (eV)	RT_1;RT_2 (min)
8	Dursban	$C_9H_{11}C_{l3}NO_3PS$	Insecticide/Nematicide	$[M+H]^+$	350	97;198	34;22	11.36;11.36
7	Diazinon	$C_{12}H_{21}N_2O_3PS$	Insecticide	$[M+H]^+$	305	97;169	42;22	10.22;10.22
6	Thiamethoxam	$C_8H_{10}ClN_5O_3S$	Insecticide	$[M+H]^+$	292	132;211	26;10	2.68;2.68
5	Metalaxyl	$C_{15}H_{21}NO_4$	Fungicide	$[M+H]^+$	280	160;220	26;10	7.33;7.33
4	Thiobencarb	$C_{12}H_{16}ClNOS$	Herbicide	$[M+H]^+$	258	89;125	54;26	10.34;10.34
3	Baycarb	$C_{12}H_{17}NO_2$	Insecticide	$[M+H]^+$	208	77;95	42;10	8.34;8.34
2	Carbaryl	$C_{12}H_{11}NO_2$	Insecticide/Nematicide	$[M+H]^+$	202	127;145	30;6	7.16;7.16
1	Propamocarb	$C_9H_{20}N_2O_2$	Fungicide	$[M+H]^+$	189	74;102	26;14	1.36;1.36

No., identified pesticide analyte on the TIC chart

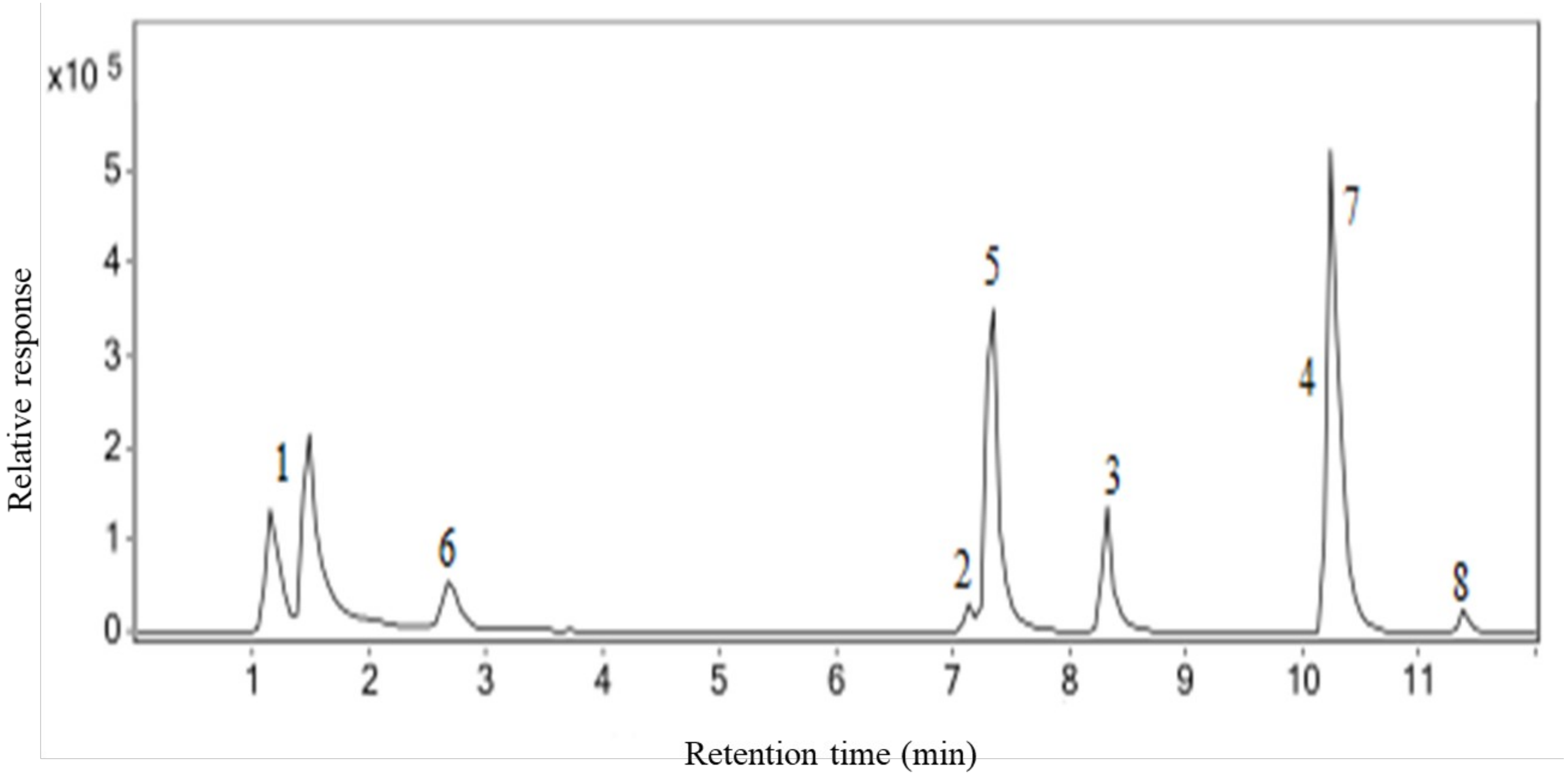


Figure 2: The TIC chart of the multi-pesticide analytes.

Table 2: The accuracies, precisions at three concentration levels for the analyzed samples.

Pesticides		**Lettuce**		**Garlic**		**Ginger**		**Bell-pepper**	
	Spike (μg/kg)	RR (%)	RSD (%)	RR (%)	RSD (%)	RR (%)	RSD (%)	RR (%)	RSD (%)
Durban	5	99	6	111	6	106	2	108	6
	100	99	6	100	5	100	0	96	5
	300	99	4	99	10	100	13	101	7
Diazinon	5	100	4	103	5	99	2	105	4
	100	101	4	97	7	101	4	99	3
	300	100	6	97	4	101	4	99	4
Thiamethoxam	5	111	7	103	7	100	2	91	4
	100	102	4	99	2	104	1	99	3
	300	99	5	99	8	96	1	100	5
Metalaxyl	5	91	6	102	3	87	0	101	10
	100	99	7	98	12	100	11	99	12
	300	100	22	100	11	101	8	100	16
Thiobencarb	5	88	11	100	7	102	5	91	3
	100	100	4	102	8	101	3	101	4
	300	101	5	98	1	101	3	98	3
Baycarb	5	112	4	106	2	124	0	102	2
	100	99	5	101	3	99	0	100	3
	300	100	5	97	2	99	1	100	4
Carbaryl	5	95	6	101	6	94	1	98	3
	100	101	6	99	4	99	4	100	2
	300	100	4	99	8	99	15	101	11
Propamocarb	5	106	6	106	5	127	3	92	5
	100	101	3	99	4	101	1	103	5
	300	100	4	100	2	99	17	100	3
Ranges	5-300	88-112	3-22	97-111	1-12	87-127	0-17	91-108	2-16

Table 3: The pesticides detection and quantitation limits for the analyzed samples.

	Lettuce		**Garlic**		**Ginger**		**Bell-pepper**	
	LOD (µg/kg)	LOQ (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)
Dursban	0.05	0.17	0.08	0.28	0.01	0.03	0.11	0.37
Diazinon	0.03	0.12	0.20	0.66	0.10	0.33	0.08	0.27
Thiamethoxam	0.09	0.29	0.05	0.18	0.28	0.93	0.05	0.18
Metalaxyl	0.05	0.17	0.08	0.26	0.08	0.27	0.04	0.13
Thiobencarb	0.05	0.17	0.11	0.36	0.07	0.23	0.10	0.33
Baycarb	0.02	0.07	0.16	0.53	0.06	0.21	0.03	0.09
Carbaryl	0.05	0.15	0.07	0.22	0.10	0.32	0.04	0.12
Propamocarb	0.06	0.19	0.02	0.07	0.10	0.33	0.07	0.23
Ranges	0.02-0.09	0.07-0.29	0.02-0.20	0.07-0.66	0.01-0.28	0.03-0.93	0.03-0.11	0.09-0.37

Table 4: The linearity of regression coefficient, matrix effects and measurement uncertainties for the analyzed samples.

Pesticides	**Lettuce**			**Garlic**			**Ginger**			**Bell-pepper**		
	R^2	ME (%)	MU (%)	R^2	ME (%)	MU (%)	R^2	ME (%)	MU (%)	R^2	ME (%)	MU (%)
Dursban	0.9998	-91	11	0.9996	-98	14	0.9999	-91	10	0.9994	-86	12
Diazinon	0.9999	-97	9	0.9986	-98	11	0.9996	-100	7	0.9996	-96	7
Thiamethoxam	0.9996	-100	11	0.9998	-100	11	0.9973	-100	3	0.9999	-100	8
Metalaxyl	0.9999	-99	23	0.9997	-99	17	0.9998	-100	13	0.9998	-100	25
Thiobencarb	0.9999	-96	13	0.9995	-98	11	0.9997	-98	7	0.9996	-93	7
Baycarb	0.9998	-98	9	0.9990	-96	5	0.9996	-97	1	0.9999	-88	6
Carbaryl	0.9999	-100	11	0.9997	-100	12	0.9996	-100	13	0.9999	-100	11
Propamocarb	0.9998	-100	9	0.9999	-100	7	0.9994	-100	14	0.9998	-100	9
Ranges	> 0.999	≤ -91	≤ 23	> 0.99	≤ -96	≤ 17	> 0.99	≤ -91	≤ 14	> 0.999	≤ -86	≤ 25

Table 5: The pesticides residues in the analyzed samples.

	Lettuce		**Garlic**		**Ginger**		**Bell-pepper**	
	RC (μg/kg)	EU-MRL (μg/kg)	RC (μg/kg)	EU-MRL (μg/kg)	RC (μg/kg)	EU-MRL (μg/kg)	RC (μg/kg)	EU-MRL (μg/kg)
Dursban	< LOQ	10	< LOQ	200	95.99±3.1	3000	< LOQ	10
Diazinon	< LOQ	50	< LOQ	20	7.34±2.4	10	< LOQ	50
Thiamethoxam	9.03±1.2	20	< LOQ	10	178.30±7	300	393±4.1	700
Metalaxyl	< LOQ	1000	< LOQ	500	13.41±1.0	100	< LOQ	50
Thiobencarb	< LOQ	100	8.50±2.5	10	9.41±1.0	10	< LOQ	10
Baycarb	< LOQ	10	< LOQ	10	< LOQ	10	< LOQ	10
Carbaryl	7.23±1.2	10	12.06±1.1	20	6.83±2.1	10	8.29±2.5	10
Propamocarb	4.15±0.5	700	< LOQ	2000	45.06±0.5	50	< LOQ	3000

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CONFLICT OF INTEREST

The authors of this research agreed with no conflicts of interest.

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