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VALIDATION OF THE METHOD FOR DETERMINATION OF PESTICIDE RESIDUES IN LEAFY VEGETABLES AND FRESH HERBS BY LIQUID CHROMATOGRAPHY –TRIPLE QUADRUPOLE MASS SPECTROMETRY

VJOLLCA VLADI[1]
FATOS HARIZAJ[2]
VALDETE VORPSI[2]
MAGDALENA CARA[3]

[1]Food Safety and Veterinary Institute. Tirana, Albania
[2]Faculty of Agriculture and Environment, AUT, Department of Plant Production, Tirana, Albania
[3]Faculty of Agriculture and Environment, AUT, Department of Plant Protection, Tirana, Albania

ABSTRACT

This method reports on in-house validation results and assessment of performance parameters of a complete multi-residue (32 pesticides) pesticide analysis method employing QuEChERS sample preparation kits. Sub-portions of previously homogenized samples were treated according to a standard QuEChERS method protocol (extraction and clean-up) prior to injection in the LC-MS/MS system. The separation of the analytes under examination is conducted on Phenomenex Synergy column 4u Hydro-RP 80A (150 x 2,0 mm x 4 μm) at room temperature Identification of pesticide residues was based on retention time and ion-ratio confirmation using multiple reaction monitoring (MRM) of characteristic transition ions, while quantification was calculated on matrix matched calibration and internal standardization. The method performance parameters indicate that the performance for the majority of target compounds complies with current regulatory requirements. In some cases (the set value of Correlation coefficient 0.985 wasn't met for Benomyl, Deltamethrine, Methomyl and Fluroxypir due to individual properties of compounds or strong matrix influences on the analytical results. Overall it can be concluded that the complete workflow solution offered by this method delivers the required performance for the target compounds especially regarding sensitivity, selectivity and recovery.

KEYWORDS: LC/MS/MS, Pesticide Residues, QuEChERS, Triple Quadrupole

1. INTRODUCTION

Pesticide residue analysis in food is one of the most important and challenging tasks in routine laboratory practice [8]. The European legislation, which is currently the most strict legislation (European Regulation 396/2005 and Commission Directive 2006/125/EC), sets maximum residue limits (MRL) of pesticides in different products of plant and animal origin. This presents a significant analytical challenge with respect to the low limits of quantification

(LOQ) required for some specified food matrices. A variety of GC and HPLC methods have been developed for multi-residue determination of pesticides employing a variety of sample preparation and cleanup techniques [2,3]. In recent years the QuEChERS method has become widely adopted for preparing samples of fruit and vegetables, but the continuous need for more sensitive and accurate measurements requires new developments from the instrument producers as well [1]. This method reports on in-house validation results and assessment of performance parameters of a complete multi-residue pesticide analysis method employing QuEChERS sample preparation kits and Phenomenex Synergy column 4u Hydro-RP 80A (150 x 2,0 mm x 4 µm) at room temperature.

2. Schematic of Method

Homogenization

Sample + IS

1. Weigh 10 g sample in 50 mL extraction tube and 200 µL stock IS

Extraction

2. Add 9.8 mL acetonitrile
3. Shake for 10 min, centrifuge at 3500 rpm for 5 min

Clean up

4. Transfer supernatant into a 15 mL clean-up tube
5. Centrifuge samples at 3500 rpm for 5 min
6. Transfer supernatant into a LC vial

LC-MS/MS ANALYSIS

3. Scope

The objective of this validation study was to evaluate and to validate “in house” a multi residue method for determination of pesticide residues in fruit and vegetable by LC/MS/MS determination. The method can be implemented for routine multi-residuepesticide analysis (approximately 32 pesticides)in leafy vegetables and fresh herbsmatrices.

4. Principle

Sub-portions of previously homogenized samples were treated according to a standard QuEChERS method protocol (extraction and clean-up) prior to injection in the LC-MS/MS system. Ready to use QuEChERS kit containing both extraction and clean-up tubes and associated protocol were used for sample preparation [1]. Identification of pesticide residues was based on retention time and ion-ratio confirmation using multiple reaction monitoring (MRM) of characteristic transitionions, while quantification was calculated on matrix matched calibration and internal standardization. All method performance criteria were established according to the relevant guidelines[4,7,9].

5. Reagent List

Acetone, HPLC Grade, Acetonitrile, LC-MS Grade, Methanol LC-MS grade, Toluene, HPLC grade, Water, LC-MS grade.

6. Standard List

6.1 Pesticides

All individual pesticide compounds

Aldicarb,Benomyl, Buprofezin, Carbaryl, Carbendazim, Carbofuran,Chlorpyriphos, Deltamethrin, 2,6 Dichlorobenzamide, Dimethoate, Fluopicolide, Fluroxypir, Fenhexamid, Fenitrothion, Malaoxon, Malathion, Methacrifos, Methomyl, Methiocarb, Omethoate, Pirimicarb, Pirimicarb, desmethyl, Profenofos, Propamocarb, Pyrimethalin, Propoxur, Quinoxifen, Terbufos, Thiabendazolo, Thiocarb, Thiofanate methyl, Triadimefon. were obtained from Sigma-Aldrich and DrEhrenstorfer Standards

6.2 The Internal standard triphenylphosphate (TPP) were obtained from Sigma-Aldrich,

7. Standards and Reagent Preparation

7.1 Individual Pesticide Standard Stock Solutions

Prepared gravimetrically in ~2000 mg/L concentration by weighing 20 mg from each analyte on a five digit analytical balance and dissolving in 10 mL of appropriate solvent (acetone, toluene or acetonitrile depending on the individual compound). Concentrations of each individual standard stock solutions were calculated gravimetrically using weight of added compounds, their purity and solvents. All individual standard stocks were stored in a freezer at -20 °C [9].Validity of individual standard stock solutions was 12 months.

7.2 Intermediate Standard Stock and Working Standard Solutions

Prepared by pipetting the appropriate amount of each individual standard stock and diluting it with acetonitrile. The concentration of intermediate standard stock solutions was 5000 ng/mL. Working standards were prepared by diluting intermediate standard stock solution accordingly. Intermediate standard stock solutions were stored in a freezer at -20 °C, and the working solutions in a fridge at 4 °C [9]. Validity of intermediate stock solutions was 3 months.

7.3 Internal Standard Stock Solution

Prepared gravimetrically in ~2000 mg/L concentration by weighing 20 mg from the internal standard TPP in 10 mL acetone. Exact concentration values were determined based on the gravimetrical values weighed compound and added solvent. Internal standard stock solution were stored in a freezerat -20 °C. Validity of internal standard stock solution was 12 months.

7.4 Working Internal Standard Stock Solution

Prepared individually by pipetting the appropriate amount of each individual standard stock solution and diluting it with acetonitrile. The concentration of working internal standard stock solutions was 5000 ng/mL and was used for direct spiking of the samples [4,9]. Validity of working stock solutions was 3 months.

8. Apparatus

Precision balance (d=0.01g), Analytical balance(d=0.01mg),Vortex shaker with Variable speed control between 200 and 2500 rpm,360 Degree Vertical Multi-Function Rotator, centrifuge up to 5000 rpm,LC-MS/MSAPI 3000.

9. Consumables

LC vial kit;Finnpipette 10–100 µL, 100–1000 µL,500–5000 µL; Pipette holder; Pipette tips 0.5–250 µL,100–1000 µL, 1–5 mL; Spatula 18/10 steel; QuEChERS extraction tube 50 mL,15 mL.

10. Glassware

Volumetric flask 10 mL ;Volumetric flask 25 mL; 500 and 1000 mL bottle.

11. Procedure

11.1 Sample Preparation

Blank matrix samples (Lettuce, spinach, basil, etc.) used for validation experiments were purchased in local retail stores and were homogenized, extracted and cleaned-up prior to sample preparation. Matrix extracts were used as matrix blank samples and dilution solvents for matrix-matched calibration. Ready to use QuEChERS extraction kits were used for sample preparation, and contained 4 g MgSO4, 1 gNaCl, 1 g trisodiumcitrate dehydrate and 0.5 g disodiumcitratesesquihydrate for buffered extraction of target compounds. Pre-prepared clean-up tubes contained900 mg MgSO4, 150 mg PSA. The same QuEChERS protocol was applied for all of the matrices.[1,2]

Homogenization of Matrices; Select the amount of Lettuce, spinach, basil, etc (other leafy vegetables and fresh herbs matrices) for homogenization and start homogenization at middle rotation speed(speed level 2–3) and continue to form asmooth homogenate.

Sample Extraction and Clean-up; Weigh 10 g sample into a 50 mL QuEChERS extraction tube containing 4 g MgSO4, 1 gNaCl, 1 g trisodiumcitrate dehydrate and 0.5 gdisodiumcitratesesquihydrate. Add 200 µL 5000 ng/mL internal standard to the samples. Add 10 mL ACN to all samples. Shake samples for 10 min on a vertical shaker and centrifuge with 3500 rpm for 10 min. Transfer supernatant (~7mL) into the 15 mLQuEChERS clean-up tubes containing 900 mg MgSO4, 150 mg PSA. Vortex for 1 min and centrifuge samples with 3500 rpm for 5 min. Collect supernatant and transfer 1 mL into a LC vial for instrumental analysis.[1,5]

11.2 LC-MS/MS Analysis

Instrumental conditions

The analysis by mass spectrometry is carried out with a spectrometer API 3000 triple quadrupole, equipped with Ionspray source (IS) in positive ion mode for all analytes under examination and the source temperature is set at 400 ° C. The potential applied to the source is 5000 V, the nebulizer gas is set to 8 psi, the curtain gas 10 psi and CAD gas to 5 psi. The electrical parameters that are optimized: desolvation potential (DP), the potential for transfer of ions from the source to the analyzer of the mass (EP), collision energy (CE), extraction potential of the fragments and ions from the collision cell (CXP). Fragmentations considered and the electrical parameters optimized for each analyte are shown in Table 3.

The separation of the analytes under examination (see Table 3) is conducted on Phenomenex Synergy column 4u Hydro-RP 80A (150 x 2,0 mm x 4 μm) or equivalent, at room temperature. The mobile phase used is constituted by 5 mmol ammonium formatein water [2] and 5 mmol of ammonium formate in methanol with gradient elution below

Table 1. Gradient of mobile phase for the analysis of LC-MS / MS

Time (min)	Flow (μl/min)	Phase A (%) ammonium formate 5 mmol in methanol	Phase B (%) ammonium formate 5 mmol in water
0	100	0	100
3	100	70	30
6	200	85	15
9	200	90	10
20,5	200	90	10
21	200	0	100

11.3 Calculation of Results

Internal standardization was applied for quantification of target pesticides. The relevant response factors (Rf)were defined by the equation below. Calculation of final result was performed using the following equations.

11.3.1 Equations

Calculation of the response factor:

$$R_f = \frac{A_{St} \times C_{[IS]}}{A_{[IS]} \times C_{St}}$$

R_f – the response factor

A_{St} – the area of the pesticide peak in the calibration standard

$A_{[IS]}$ – the area of the internal standard peak of the calibration standard

c_{St} – pesticide concentration of the calibration standard solution

$c_{[IS]}$ – the internal standard concentration of the calibration standard solution

Calculations of analyte amount in each sample (the absolute amount of pesticide extracted from the sample):

$$X_{analyte} = \frac{A_{analyte} \times X_{IS}}{A_{IS\times} \times R_f}$$

Xanalyte – the absolute amount of pesticide that was extracted from the sample

Aanalyte – the area of pesticide peak in the sample

A[IS] – the area of the internal standard peak in the sample

X[IS] – the absolute amount of internal standard added to the sample

Calculations of sample amount in each sample (the absolute amount of pesticide extracted from the sample):

$$c = \frac{X_{analyte}}{m}$$

m – the weight of sample [g]

Xanalyte – absolute analyte amount [ng]

12. Method Performance Characteristics

In-house validation of the method was carried out on allmatrices and target pesticides. European guidelines for single laboratory validation and pesticide residue analysis were used for establishing method performance criteria. All method performance parameters were compared to the relevant legislative requirements and maximum residue limit (MRLs). (EC 396/2005).For compounds containing more isoforms, only one performance criteria was established.

12.1 Selectivity

Method (MRM) selectivity was assessed based on the presence of specific ion transitions (quantifier ion and two transitions for compound confirmation) at the corresponding retention time, as well as the observed ion ratio values corresponding to those of the standards. Acceptance criteria for retention time and ionratios were set according to current quality control criteria. Matrix blank samples were also inspected for the presence of interfering peaks in close vicinity of the target retention times for which (according to SANCO guideline definitions) <30% of LOQ acceptance criteriawas applied.

12.2 Linearity, Response Factor, Matrix Effect

The calibration curves were created at six levels (matrixmatched) and injected in duplicate. Rf values for internal standardization were determined from the calibrationcurves for all matrices and internal standards [4,7,9] by calculating cumulative average response factor over the whole calibration range. The linearity of calibrationcurves was assessed in calibrationranges of 0–500 ng/g. Calibration levels were equidistantly distributed over the calibration range. Linear function was evaluated according to Mandel's fitting test and

plotting of residuals for which <20%acceptance limit was set. Correlation coefficient values were additionally established for which an artificial0.985 was set as an acceptance limit, as no legislativelimits are defined for them. The set value wasn't met for Benomyl, Deltamethrine, Methomyl and Fluroxypir based on the high LOQ values related to the calibration levels. No weighted function was applied. Matrix effects were evaluated by (Youden-) plotting of measured relative peak areas of calibration standards insolvent against the areas in the relevant matrix [6]. Nomatrix effect is observed if the difference of the slope(dif%) of the fitted line is less than 20% from the ideal(y=x) curve, while matrix effects are observed when the difference is between 20–50% (minor matrix effect) or exceeds 50% (major matrix effect).

Table 2.Concentrations of pesticides in solutions for LC-MS / MS settings.

Curva in Matrice	**Conc. Standard** (mg/kg)	**Conc. Internal Standard** (mg/kg)
ST_1-LC	0,01	0,2
ST_2-LC	0,02	0,2
ST_3-LC	0,10	0,2
ST_4-LC	0,50	0,2
ST_5-LC	1,00	0,2

12.3 Accuracy

Method trueness was assessed by recovery studies using blank matrices spiked at three concentration levels (L1,L2 and L3) and injected in six individually prepared replicates. Spiking of samples occurred prior to sample preparation. Found concentrations, recovery and relative standard deviation (% RSD) were calculated (Table 4). According to SANCO requirements recovery values are deemed acceptable if between 70–120%.

12.4 (Intermediate) Precision

Instrument injection precision was tested for both retention time and peak area for all target compounds by subsequent injections (n=6) of three concentration levels; concentration level(L1= 10 ng/g),(L2= 20 ng/g), (L3= 100 ng/g) standard solutions. Instrument injection precision for retention time was below 0.5% for all compounds for peak area without internal standard compensation indicating reliable instrument performance. Method within-day and between-day precision values were determined for each matrix at middle spiking level (L2) and expressed as %RSD over 3 days with individually prepared samples (n=6). Mean with in-

day precision values were determined as anaverage of the 3 individual days' mean precision, while between-day precision was expressed as mean of the overall precision data. According to SANCO requirements<20% was set as acceptance criteria for the target compounds and matrices. Measured values are shown in Table 4.

12.5 Limit of Detection, Limit of Quantification

Limits of detection and quantification were estimated following the IUPAC. An artificial MRL=10 ng/g was set as target value for compounds, for which no MRL values are legislatively defined. The expectation of the method was to meet MRL values at least at LOQ level which was achieved for the all target compounds.

13. Conclusion

Full in-house validation of a complete method intended for routine pesticide residue measurements was carried out. The goal of the study was to obtain an objective and realistic overview of the analytical performance of awidely used and accepted sample preparation method combined with state of the art analytical instrumentation. The validation was made only for the components that are determined in LC/MS/MS coupled with Phenomenex Synergy column 4u Hydro-RP 80A (150 x 2,0 mm x 4 µm) or equivalent. The method performance parameters indicate that the performance for the majority of target compounds complies with current regulatory requirements (Table 4).For pesticides: Benomyl, Deltamethrine, Methomyl and Fluroxypircases method performance parameters could not be established or measured values fell outside of the targeted range due to individual properties of compounds or strong matrix influences on the analytical results. For those compounds (in the relevant matrix), individually optimized sample preparation (additional or special clean-up) and instrumental methods have to be applied. Overall it can be concluded that the complete workflow solution offered by this method delivers the required performance for the target compounds especially regarding sensitivity, selectivity and recovery.

Table 3. Ionic transitions and Indicativeelectrical parameters for LC-MS / MS analysis

		I transition			II transition			I-II transition	
Analyte	Precursor [m/z]	Product [m/z]	CE	CXP	Product [m/z]	CE	CXP	CE	CXP
Aldicarb	208.1	89.1	21	6	116.0	13	6	11	10
Benomyl	291.3	192.1	21	20	160.0	35	13	45	4
Buprofezin	306.3	201.0	19	16	116.0	25	11	32	5
Carbaryl	202.0	144.9	15	8	127.0	17	8	11	10
Carbendazim	192.0	160.1	36	19	132.1	42	13	48	5
Carbofuran	222.1	165.1	17	8	123.0	29	8	16	10
Chlorpyrifos	350.2	97.0	55	18	198.0	35	34	23	6
Deltamethrin	523.1	281.0	23	7.5	479.0	17	7	52	8
2,6-Dichlorobenzamide	190.2	173.0	27	11	145.0	41	10	48	9
Dimethoate	230.1	199.0	15	13	125.0	31	8	20	11
Fluopicolide	383.2	173.0	35	11	365.0	23	11	53	11
Fluroxypir	255.0	237.0	17	14	209.0	23	14	42	13
Fenhexamid	302.2	97.1	33	5	55.1	63	10	54	11
Fenitrothion	278.0	125.0	30	11	246.0	25	20	81	10
Iprodione	330,0	245,1	20	20	187,8	40	14	67	8
Malaoxon	315.2	127.0	19	24	99.0	35	19	24	10
Malathion	331.1	127.0	19	7	99.0	32	5	26	9
Methacrifos	258.1	209.0	19	14	125.0	37	7	12	13
Methomyl	163.1	88.0	13	7.5	106.0	15	10	49	5
Methiocarb	243,0	169.0	17	8	/	/	/	11	10
Methiocarb	226.0	/	/	/	121.0	25	8	61	100
Omethoate	214.2	183.0	17	15	154.9	23	15	36	13
Pirimicarb	239.2	71.9	32	7	182.1	23	11	85	9
Pirimicarbdesmethyl	224.9	71.9	33	7	168.2	21	16	85	11
Profenofos	373.1	302.9	27	8	345.0	19	10	25	15
Propamocarb	189.3	102.1	27	10	144.0	19	13	18	10
Propoxur	210.2	168.1	13	11	110.9	21	6	20	14
Pyrimethanil	200.0	107.0	35	10	183.0	34	15	50	12
Quinoxifen	308.1	197.0	47	13	272.0	37	7	40	11
Terbufos	289.1	102.9	13	5	232.9	9	17	30	5
Thiabendazole	201.9	175.0	36	13	131.0	46	9	56	6
Thiodicarb	355.0	88.0	25	8	106.0	24	11	46	9
Thiophanate- methyl	343.1	151.0	27	14	192.0	24	13	40	15
Triphenylphosphate	327.3	77.3	40	19	/	/	/	45	10
Triadimefon	294.2	197.2	27	10	69.0	19	13	18	10

CE (collision energy)

CXP (collision cell exit potential)

DP (declustering potential)

EP (entrance potential)

Table 4. Linearity, Precision and recovery for all analytes.

				Fortification level 10 (µg/kg)			Fortification level 20 (µg/kg)			Fortification level 100 (µg/kg)	
	Analyte			**LOQ** (µg/kg)			**2 LOQ** (µg/kg)			**10 LOQ** (µg/kg)	
No		***Calibration range [ng/g]***	***r^2***	***mean recovery %***	***RSD%***		***mean recovery %***	***RSD%***		***mean recovery %***	***RSD %***
1	Aldicarb	5-1000	0.99275	82	12		80	8		80	7
2	Benomyl	5-1000	0.97432	77	10		79	2		86	5
3	Buprofezin	5-1000	0.99517	116	5		117	5		117	6
4	Carbaryl	5-1000	0.99179	82	9		76	4		78	4
5	Carbendazim	5-1000	0.98416	80	12		73	4		66	6
6	Carbofuran	5-1000	0.99704	90	8		84	4		78	4
7	Chlorpyriphos	5-1000	0.99535	117	15		126	5		134	4
8	Deltamethrin	5-1000	0.96331	81	14		90	12		95	19
9	2,6Dichlorobenzamide	5-1000	0.99128	71	15		77	6		72	3
10	Dimethoate	5-1000	0.98750	79	8		75	2		69	6
11	Fluopicolide	5-1000	0.99494	92	18		93	12		105	6
12	Fluroxypir	5-1000	0.97432	77	10		89	12		86	15
13	Fenhexamid	5-1000	0.99147	63	12		74	13		87	19
14	Fenitrothion	5-1000	0.99147	72	13		104	16		103	6
15	Malaoxon	5-1000	0.99389	85	9		78	3		80	7
16	Malathion	5-1000	0.99730	109	5		105	3		106	6
17	Methacrifos	5-1000	0.99140	104	9		95	4		101	4
18	Methomyl	5-1000	0.97482	70	9		78	2		88	4
19	Methiocarb	5-1000	0.99721	102	9		94	7		91	3
20	Omethoate	5-1000	0.97504	64	11		79	3		81	7
21	Pirimicarb	5-1000	0.98502	89	12		76	6		73	5
22	Pirimicarbdesmethyl	5-1000	0.98114	105	21		85	14		93	15
23	Profenofos	5-1000	0.99421	112	7		109	4		111	5
24	Propamocarb	5-1000	0.99203	72	16		84	8		89	5
25	Pyrimethalin	5-1000	0.98938	87	9		84	4		83	4
26	Propoxur	5-1000	0.99619	89	7		86	5		80	5
27	Quinoxifen	5-1000	0.98337	180	36		150	18		137	27
28	Terbufos	5-1000	0.99395	115	9		113	4		115	5
29	Thiabendazolo	5-1000	0.99056	73	10		73	4		88	7
30	Thiocarb	5-1000	0.98620	84	11		79	5		72	6
31	Thiofanate methyl	5-1000	0.99390	78	10		73	6		71	7
32	Triadimefon	5-1000	0.99966	108	7		97	6		95	4

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