Method Validation For Determination Of Pesticides In Honey

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***Abstract*—This paper represents the use of ethyl acetate for the determination of pesticides in honey samples by LC-MS/MS detection. The method was validated for 63 pesticides in three deferent level. For method validation blank honey samples were spiked at three different levels (10, 20 and 50 µg/kg). The validation parameter included linearity, recovery, accuracy and evaluation of matrix effect. All compounds showed to have good linearity from 5 to 200 µg/kg with a R2 of 0.99, in both solvent and matrix calibration curve. The matrix effect did not pass the 20% in none of them. Most of the compound passed the SANTE criteria of the recovery between 70-120 % and RSD lower than 20%.**

Keywords—pesticides, honey , SweET, LC-MS/MS

I. INTRODUCTION

The use of pesticides has increased significantly during worldwide. A part the fact that these compounds are beneficiary for the agriculture, many of them reach other then they intended destination. If they are not used properly they can contaminate soil, water and food [1]. In Albania as well as worldwide the use of pesticides in agriculture products has been increased compere to the past. The data has shown that most of the time the farmer are not well aware of the possible contamination form the pesticides uses for their own safety and health [2] as well as for their livestock.

Honey is the substance made of nectar and sweet deposits fromplants, gathered, modified and stored in honeycombs by honeybees and as a result it has theimage of being natural, healthy and clean. However, today honey is produced in an environmentexposed to pollution by different sources of contamination. More specifically, honey caninvoluntarily be contaminated with pesticides from the environment and also from beekeepingpractice [3].

In this work, we showed the validation of a simple method using ethyl acetate[4], which is relatively fast and costeffective, for 63 different pesticides in honey using LC-MS/MS detection system.

II. MATERIAL AND METHODS

Sampling and Sample Preparation

Sample of honey has taken from honey bee farm and well homogenized prior analysis. 10 g of the homogenized honey was accurately weighed in 50 ml polypropylene tubes. The weight samples were diluted with 10 milliliter of distilled water and well homogenized by using a vortex. After homogenization in each samples were added 0.20 mg of PSA (primary secondary amine) and C18 sorbent were added. For the pesticide extraction was used 20 ml of ethyl acetate. The mixture was homogenized by 20 minutesshaking as the first extraction step. A secondextraction step was performed for another 10 minutes shaking after 10 g of sodium sulfate was added into the sample. The organic phase was separated by centrifugation and then filter. 1 ml of the filtered organic extract was transferred in 2 ml autosampler vial followed by injection into LC-MS/MS.

2.2. Method Set Up

Pesticides analysis was performed by LC-MS/MS analytical system of Agilent 1200 HPLC system (Agilent Technologies, Germany) with an automatic degasser, a binary pump and an auto sampler connected to the Agilent 6460B Triple-Quad LC/MS system with electrosprayionization interface set at positive and negative polarities. Themo BDS HYPERSIL C18 Dim.(mm) 150 x 2.1 particle size 3µ.The mobile phase used were: 10-mM ammonium formate (A) and methanol (B)following the gradient program which is shown in table I. The volume of injection was 5µL.

TABLE I. GRANDIENT PROGRAME

	Time	A	B	Flow
1	1.00 min	95.0 %	5.0 %	0.550 mL/min
2	20.00 min	5.0 %	95.0 %	0.550 mL/min
3	27.00 min	5.0 %	95.0 %	0.550 mL/min
4	27.20 min	95.0 %	5.0 %	0.550 mL/min
5	30.00 min	95.0 %	5.0 %	0.550 mL/min

Detection of the compounds was performed by Multiple Reaction Monitoring (MRM) with two mass transitions for each pesticide. MRM transitions with their fragmentor voltages (V) and collision energies (CE) are presented in Table II.

TABLE II. RETENTION TIME LC-MS/MS TRANSITIONS, FRAGMENTOR VOLTAVE AND ESI MODE.

N r.	Cpd Name	RT (min)	Prec Ion	Frag (V)	Quant ion /CE	Qual ion /CE	Mode
1	Amitraz	21.37	294.2	100	163.1 (12)	122 (30)	ESI+
2	Azinophos-Ethyl	17.71	346.1	73	132.2 (13)	97.1 (37)	ESI+
3	Azinophos-Methyl	15.5	318	80	160.1 (0)	132.1 (10)	ESI+
4	Azoxystrobine	16.4	404	100	372 (10)	344 (23)	ESI+
5	Boscaliti	16.65	343.3	100	307 (15)	139.9 (20)	ESI+
6	Bupiramate	18.05	317.2	150	166.1 (20)	108 (25)	ESI+
7	Buprofezin	20.22	306	100	201 (5)	116.1 (10)	ESI+
8	Carbaryl	13.6	202	100	145 (5)	127 (25)	ESI+
9	Carbendazim	9.5	192.1	110	160 (18)	132 (33)	ESI+
10	Chlorantraniliprole	15.73	484	110	453.1(16)	286.1 (8)	ESI+
11	Clofentrezine	18.95	303	80	138 (10)	102 (40)	ESI+
12	Coumaphos	18.54	363.1	140	307 (17)	227 (27)	ESI+
13	Cyprodinil	18.32	226	135	108 (25)	93 (33)	ESI+
14	Difenconazole	19.44	406	150	251 (25)	-	ESI+
15	Diflubenzuron	17.94	309	93	289.1 (1)	155.9 (5)	ESI-
16	Epoxiconazole	17.69	330.1	130	121.1 (25)	100.9 (50)	ESI+
17	Ethion	20.29	385	100	199 (5)	97 (46)	ESI+
18	Ethirimol	14.71	210.2	120	140 (20)	98 (27)	ESI+
19	Ethoprophos	17.65	243.1	100	173 (10)	359 (5)	ESI+
20	Etofenprox	22.2	394	100	177 (10)	10	ESI+
21	Febunconazole	17.97	337	150	125 (15)	70 (15)	ESI+
22	Fenamidone	16.55	312.2	145	236.1 (10)	-	ESI+
23	Fenarimol	17.55	331	150	268.1 (20)	81.1 (30)	ESI+
24	Fenpropimorph	20.4	305	150	147.3 (30)	117.2 (50	ESI+
25	Fenvalerate	21.55	437	90	167.1(9)	125.1 (45)	ESI+
26	Fenzaquine	21.49	307	120	161.1 (15)	57 (25)	ESI+
27	Fipronil	18.08	437	130	332 (12)	250 (25)	ESI-
28	Fipronol desulfinil	17.79	387	110	351 (10)	282 (35)	ESI-
29	Fipronol Sulfon	18.65	453	140	415 (13)	282 (25)	ESI-
30	Fludioxonil	16.55	266	90	185.1 (20)	158 (35)	ESI+
31	Fluopiram	17.44	397.2	83	208 (19)	173 (20)	ESI+
32	Fluquiconazole	17.29	376	100	349.1 (15)	307.1 (25)	ESI+
33	Flusilazole	18.12	316	120	247 (15)	165(25)	ESI+
34	Hexaconazole	18.83	314	130	159.1 (40)	70.1 (20)	ESI+
35	Indoxacarb	19.57	528	120	203 (40)	150 (20)	ESI+
36	Kresoxim-methyl	18.27	314	90	267 (0)	116 (10)	ESI+
37	Linuron	15.91	248.9	110	181.9 (10)	160.1 (15)	ESI+
38	Malathion	16.82	331	110	284 (5)	127 (5)	ESI+
39	Mandipropamid	16.9	412.3	110	328.2 (8)	124.9 (30)	ESI+
40	Mepanipyrin	17.21	224	120	106 (25)	77 (45)	ESI+
41	Metaflumizone_A	19.61	505.2	150	302.1 (15)	285.1 (45)	ESI-
42	Metalaxyl	15.27	280.1	100	220 (10)	-	ESI+
43	Methidathion	15.21	303	100	145 (0)	85 (15)	ESI+
44	Methiocarb	16.31	226	100	169 (5)	121 (15)	ESI+
45	Methiocarb-Sulphone	10.29	258.1	130	201.2 (0)	122.2 (15)	ESI+
46	Myclobutanil	17.18	289	120	125 (40)	70 (15)	ESI+
47	Paclobutrazol	16.77	294.2	130	125.1 (40)	70 (15)	ESI+
48	Penconazole	18.4	284	120	159 (30)	70.1 (15)	ESI+
49	Pencycuron	19.14	329.21	140	218 (5)	125.0 (25)	ESI+
50	Phosmet	15.56	318	135	160 (22)	77 (43)	ESI+
51	Primicarb	14.43	239.1	110	182.1 (10)	72.1 (20)	ESI+
52	Prochloraz	19.05	376	100	308 (5)	266 (10)	ESI+
53	Propiconazol	18.72	342.1	140	159.2 (25)	69 (20)	ESI+

54	Pyraclostrobin	18.89	388	100	194.1(5)	163 (20)	ESI+
55	Pyridaben	20.37	322.2	100	227.1(10)	96 (10)	ESI+
56	Quinoxyfen	20.48	308	120	272 (30)	197 (35)	ESI+
57	Tebuconazole	18.45	308.2	140	124.9 (35)	70.1 (20)	ESI+
58	Tebufenozide	18.17	353.1	80	297.1(8)	133 (20)	ESI+
59	Tetraconazole	17.73	372	100	159.1 (25)	70.2 (20)	ESI+
60	Thiabendazole	11.12	202	100	175 (24)	131 (35)	ESI+
61	Thiacloprid	10.61	253	90	126 (20)	99 (50)	ESI+
62	Triadimenol	16.76	296	80	70 (10)	43 (35)	ESI+
63	Triticonazole	17.57	318.2	110	125.2 (40)	70.1 (15)	ESI+

III. RESULTS AND DISCUSSION

Calibration Curves and Linearity

Calibration curves of 6 different concentrations were plotted by using matrix matched calibration curve. Concentration range varies from 5-200 µg/kg (using 5, 10, 20, 50, 100 and 200 µg/kg as calibration points). The correlation coefficient (R2) of the calibration curves of all pesticides was ≥0.99.The lowest calibrated level (5 µg/kg) is corresponding the limit of detection (LOD).

TABLE III. CALIBRATION WEIGHT, R2 VALUES AND CALIBRATION FORMULAS OF CALIBRATION CURVE IN MATRIX.

Nr.	**Cpd. Name**	**CF Weight**	**CF R2**	**CF Formula**
1	Amitraz	1/x	1.00	y = 3925.432970 * x - 3479.189550
2	Azinophos-Ethyl	1/x	1.00	y = 227.120222 * x - 29.929356
3	Azinophos-Methyl	1/x	0.99	y = 921.017769 * x - 1614.907524
4	Azoxystrobine	1/x	0.99	y = 11986.041882 * x - 18321.169620
5	Boscaliti	1/x	0.99	y = 287.706296 * x - 454.181537
6	Bupiramate	1/x	0.99	y = 3207.049127 * x - 5339.291906
7	Buprofezin	None	0.99	y = 7238.068408 * x - 17390.578834
8	Carbaryl	1/x	0.99	y = 3324.118611 * x - 5209.865719
9	Carbendazim	None	1.00	y = 7698.928313 * x - 9176.023310
10	Chlorantraniliprole	1/x	0.99	y = 681.444303 * x - 916.052520
11	Clofentrezine	1/x	0.99	y = 267.679288 * x - 381.550935
12	Coumaphos	1/x	1.00	y = 1216.399620 * x + 1153.730277
13	Cyprodinil	1/x	0.99	y = 1481.906493 * x - 2227.902108
14	Difenconazole	1/x	0.99	y = 1001.275982 * x - 1826.995321
15	Diflubenzuron	1/x	0.99	y = 416.326481 * x + 16.988936
16	Epoxiconazole	1/x	0.99	y = 1428.431848 * x - 2212.036760
17	Ethion	None	0.99	y = 2136.706635 * x - 4882.152482
18	Ethirimol	1/x	0.99	y = 3083.246487 * x - 5226.246593
19	Ethoprophos	None	0.99	y = 1831.471263 * x - 4007.821222
20	Etofenprox	1/x	0.99	y = 1013.861453 * x - 1105.107523
21	Febunconazole	1/x	0.99	y = 605.491338 * x - 1100.807523
22	Fenamidone	1/x	0.99	y = 1189.302089 * x - 1895.439594
23	Fenarimol	None	0.99	y = 76.288728 * x - 218.069703
24	Fenpropimorph	None	0.99	y = 377.555795 * x - 1135.502076
25	Fenvalerate	None	0.99	y = 7.161244 * x - 9.798213
26	Fenzaquine	1/x	0.99	y = 6115.139450 * x - 4696.633730
27	Fipronil	1/x	0.99	y = 2001.631008 * x - 2780.456051
28	Fipronol desulfinil	None	1.00	y = 11020.093444 * x - 8407.639202
29	Fipronol Sulfon	None	1.00	y = 6159.549343 * x - 3464.896047
30	Fludioxonil	1/x	0.99	y = 64.705483 * x - 58.707072
31	Fluopiram	1/x	0.99	y = 3422.396525 * x - 5173.598216
32	Fluquiconazole	1/x	0.99	y = 82.992716 * x - 57.295303
33	Flusilazole	1/x	0.99	y = 933.397852 * x - 1319.947013
34	Hexaconazole	1/x	0.99	y = 550.559310 * x - 764.540654

35	Indoxacarb	1/x	0.99	y = 109.711636 * x - 13.084500
36	Kresoxim-methyl	None	0.99	y = 700.189445 * x - 1592.559793
37	Linuron	None	0.99	y = 29.154845 * x + 62.754474
38	Malathion	1/x	0.99	y = 2462.687513 * x - 4210.434022
39	Mandipropamid	1/x	0.99	y = 107.188292 * x - 58.429800
40	Mepanipyrin	1/x	0.99	y = 1949.208736 * x - 3071.136083
41	Metaflumizone_A	1/x	0.99	y = 1281.323629 * x - 1928.084453
42	Metalaxyl	1/x	0.99	y = 338.675214 * x - 91.938048
43	Methidathion	1/x	0.99	y = 1954.376008 * x - 3091.002452
44	Methiocarb	1/x	0.99	y = 3566.894243 * x - 5424.704330
45	Methiocarb-Sulphone	1/x	0.99	y = 168.306655 * x - 232.404110
46	Myclobutanil	1/x	0.99	y = 775.865216 * x - 1019.725373
47	Paclobutrazol	1/x	0.99	y = 1656.280328 * x - 2276.221347
48	Penconazole	1/x	0.99	y = 1324.807288 * x - 1538.257694
49	Pencycuron	1/x	0.99	y = 339.352933 * x - 305.995487
50	Phosmet	None	0.99	y = 28.271880 * x - 114.973685
51	Primicarb	None	1.00	y = 14712.070106 * x - 54258.981409
52	Prochloraz	1/x	0.99	y = 107.679339 * x - 68.566912
53	Propiconazol	1/x	0.99	y = 791.274055 * x - 909.351547
54	Pyraclostrobin	1/x	0.99	y = 3885.615936 * x - 5635.817691
55	Pyridaben	1/x	0.99	y = 7467.848775 * x - 11461.127197
56	Quinoxyfen	1/x	0.99	y = 503.217577 * x - 711.571170
57	Tebuconazole	1/x	0.99	y = 1555.420127 * x - 1812.516124
58	Tebufenozide	1/x	0.99	y = 281.545533 * x - 421.717974
59	Tetraconazole	1/x	0.99	y = 309.648251 * x - 401.284151
60	Thiabendazole	1/x	0.99	y = 203.351918 * x - 263.440544
61	Thiacloprid	1/x	0.99	y = 162.441346 * x - 67.979064
62	Triadimenol	1/x	0.99	y = 233.071926 * x - 267.331958
63	Triticonazole	None	0.99	y = 74.779013 * x - 15.099546

Matrix Effects

Matrix effect was Calculated based on slopes of calibration curves following the above formula:

$$\%ME = \frac{\text{Slope Of MMC Curve}}{\text{Slope SMC Curve}} * 100$$

From the percentage of matrix effect is given in table III.

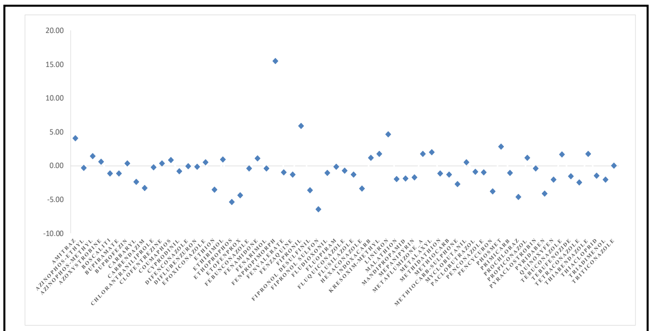

Fig. 1. *Matrix effect plot for all the pesticides*

None from the 63 compounds represented in this study shows to havematrix effect lower than 20 %, this can be due to the dilution of sample before analysis [5].

Precision in Spiked Matrix Samples

Method performance was evaluated by checking two parameters the accuracy and precision according to SANTE guidance 2017[6].

In order to study the recovery, prior extraction the honey samples were spikes in three different levels (10, 20 and 50 µg/kg). Number of replicates used were six for each spiking level, the recovery and RSD was calculated for each level. The analytical performance parameters for all the pesticides tested in this study using LC-MS/MS system are given intable III.

In the figure 2 are giving the recovery measurements versus RSD in percentage, for each recovery level.

In the first spiking level of 10 µg/kg 59out of 63 - pesticides have meet the recovery criteria between 70-120 % and the RSD <20%. Although 3 compounds (fenvalerate, linuron, triticonazol) have the recovery between 70 and 120 % their RSD was very high, which is mean that these three compounds did not meet the SANTE criteria for recovery and RSD. On the other hand, Amitraz had a recovery of 34 % but it RSD was 3.9, which according SANTE guidance is accepted. Ethoprophos did not pass this validation level because it has a low recovery as well as a high RSD.

TABLE IV. AVERAGE RECOVERIES (N = 6), AND RSD FOR SAMPLES FORTIFIED AT 10, 20, 50 μG/KG

		Spiking level		Spiking level		Spiking level		
		0.01	mg/kg	0.02	mg/kg	0.05	mg/kg	
Nr.	Cpd. Name	Recovery%	RSDr %	Recovery%	RSDr %	Recovery %	RSDr %	
1	Amitraz	39.1	3.9	47.9	7.48	47.9	7.5	4.04
2	Azinophos-Ethyl	79.4	11.7	82.5	5.0	85.6	6.4	-0.39
3	Azinophos-Methyl	99.2	2.7	92.2	3.0	87.8	3.3	1.38
4	Azoxystrobine	91.4	2.9	89.8	2.9	89.6	3.7	0.52
5	Boscaliti	100.2	3.4	94.8	7.9	86.7	3.3	-1.24
6	Bupiramate	86.8	3.5	85.4	3.4	81.5	5.4	-1.21
7	Buprofezin	90.7	3.7	84.6	5.5	75.8	5.8	0.27
8	Carbaryl	89.6	2.3	90.3	3.3	90.4	2.6	-2.46
9	Carbendazim	91.8	4.1	90.0	0.7	88.6	1.5	-3.32
10	Chlorantraniliprole	94.9	2.6	90.6	5.1	89.7	3.8	-0.32
11	Clofentrezine	86.3	11.8	87.0	5.6	79.1	5.7	0.32
12	Coumaphos	92.1	5.6	81.8	9.3	73.7	5.0	0.80
13	Cyprodinil	91.4	4.4	86.9	4.7	84.6	6.1	-0.91
14	Difenconazole	93.5	5.0	87.0	3.2	79.4	5.1	-0.11
15	Diflubenzuron	84.0	13.8	89.6	7.3	93.0	3.3	-0.19
16	Epoxiconazole	100.7	2.8	95.3	4.6	88	2.93	0.46
17	Ethion	86.5	2.9	83.3	7.5	107.1	6.4	-3.59
18	Ethirimol	93.6	1.3	91.9	3.0	89.2	1.8	0.85
19	Ethoprophos	39.4	21.5	87.8	7.8	115.6	7.4	-5.42
20	Etofenprox	91.1	8.7	87.8	7.8	65.9	6.1	-4.43
21	Febunconazole	95.7	5.8	91.7	6.2	87.3	4.2	-0.43
22	Fenamidone	93.4	3.9	88.3	6.9	87.2	4.3	1.04
23	Fenarimol	101.7	11.5	104.5	9.8	91.7	10.4	-0.48
24	Fenpropimorph	77.3	12.8	77.8	11.1	67.5	5.6	15.50
25	Fenvalerate	76.1	43.7	77.6	28.5	84.0	17.5	-0.99
26	Fenzaquine	81.8	7.1	81.7	5.2	76.9	6.7	-1.40
27	Fipronil	88.2	3.0	85.6	2.7	79.5	4.4	5.89
28	Fipronol desulfinil	78.3	6.4	78.2	8.4	74.1	3.8	-3.66
29	Fipronol Sulfon	73.6	7.0	75.4	7.8	70.0	5.3	-6.51
30	Fludioxonil	85.1	13.8	89.9	7.7	84.3	7.8	-1.11
31	Fluopiram	90.7	2.5	87.9	2.8	85.2	3.1	-0.16
32	Fluquiconazole	88.4	19.9	88.5	8.9	89.4	6.9	-0.79
33	Flusilazole	93.4	8.4	92.6	5.9	85.2	4.6	-1.39
34	Hexaconazole	94.9	10.2	90.0	3.6	87.3	6.3	-3.45
35	Indoxacarb	82.0	10.9	79.7	8.2	76.5	11.6	1.08
36	Kresoxim-methyl	92.1	3.8	85.6	2.3	82.9	6.2	1.69
37	Linuron	105.7	52.4	69.1	10.8	82.3	19.0	4.60
38	Malathion	75.5	7.8	83.0	3.1	84.2	2.4	-1.99
39	Mandipropamid	88.9	4.9	89.2	8.7	86.0	5.8	-1.92
40	Mepanipyrin	93.0	3.4	90.3	2.3	87.1	3.1	-1.81
41	Metaflumizone_A	89.0	3.8	82.9	6.9	74.5	7.8	1.67
42	Metalaxyl	86.8	5.5	94.9	2.0	91.6	2.6	1.96
43	Methidathion	87.5	3.1	89.6	2.5	88.4	2.7	-1.17
44	Methiocarb	83.4	3.7	85.8	2.9	87.0	3.6	-1.32
45	Methiocarb-Sulphone	77.1	4.4	82.6	3.9	82.9	4.4	-2.79
46	Myclobutanil	93.9	7.4	91.4	2.5	92.2	3.6	0.48
47	Paclobutrazol	89.6	5.6	90.6	3.2	89.4	4.3	-0.96
48	Penconazole	93.9	4.6	92.9	3.0	86.4	3.6	-1.05
49	Pencycuron	92.8	11.3	87.7	12.4	77.4	4.4	-3.87
50	Phosmet	109	16.04	89.4	8.5	82.1	7.3	2.76
51	Primicarb	99.3	2.4	96.2	1.5	89.4	2.2	-1.13
52	Prochloraz	110.6	17.7	87.2	14.2	89.7	8.6	-4.64
53	Propiconazol	94.4	5.5	90.4	7.1	88.5	4.2	1.13
54	Pyraclostrobin	86.8	4.6	83.0	5.5	76.8	5.9	-0.44
55	Pyridaben	76.2	4.7	75.0	6.6	70.0	4.98	-4.14

56	Quinoxyfen	81.8	8.4	79.9	4.9	72.5	7.0	-2.08
57	Tebuconazole	97.6	3.0	95.5	3.6	87.9	5.9	1.65
58	Tebufenozide	97.1	8.8	88.3	8.3	87.5	13.6	-1.62
59	Tetraconazole	91.5	10.5	91.3	8.0	83.6	7.1	-2.55
60	Thiabendazole	102.1	2.0	98.3	4.1	93.9	2.7	1.67
61	Thiacloprid	97.9	2.5	98.4	3.5	93.0	2.8	-1.52
62	Triadimenol	89.0	4.2	92.3	6.3	91.1	4.6	-2.14
63	Triticonazole	82.5	23.3	92.7	15.0	94.8	13.5	-0.01

The second level of validation was 20 µg/kg. In this level only one compound, fenvalerate and linuron, did not met the SANTE [6], and amitraze still has the same tendency of low recovery and low RSD.

All the compound did pass the criteria of having a recovery between 70-120 % and RSD lower than 20 %. Amitraz still have the same tendency even in this spiking level to have low recovery and low RSD.

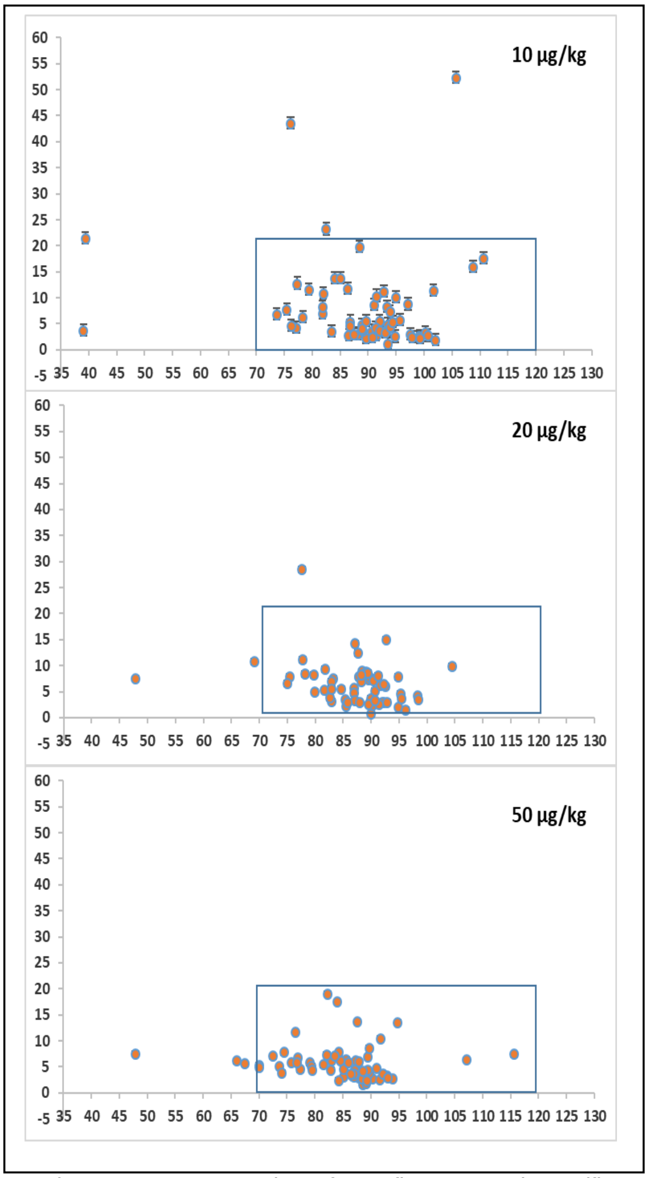

Fig. 2. *Example of a figure caption. (figure caption)*

IV. CONCLUSION

In this study we presentsa reliable and cost effective multi residue method for the determination of different pesticides in honey, which can be either used in apicultureor in the surrounding agriculture in the context food safety study. Theprocedure includes honey dilution with distillate water and the ethyl acetateextraction followed by analytical measurement in LC-MS/MS without further clean up procedure. The method that we validated complies with the validation requirements of the SANTE document.

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