Detection and Optimization of Multiple Pesticides Residues in Honey Using Liquid Chromatography Tandem Mass Spectrometry

Abstract

This study aimed to optimize and validate a multi-residue method for identifying and quantifying pesticides in honey by using an accurate, rapid and reliable method for the simultaneous determination of 100 pesticide residues in honey by liquid chromatography coupled with tandem mass spectrometry LC–ESI (+)-MS/MS operating in multiple reaction monitoring (MRM) mode. The performance of the analytical method was validated in accordance with EU SANCO guidelines (SANTE/12682/2019). Acceptable values were obtained for the following parameters: linearity, recoveries, precision (reproducibility and repeatability) and measurement uncertainty tests (<50.0%).

A highly efficient approach for determining pesticide residues in honey with good recoveries was developed. The recovery values obtained at two fortification levels: 0.01 and 0.1 mgkg^{-1}, were calculated 73.2% and 119.7% respectively, with an RSD<17% for all the tested compounds. The estimated linearity measured at five concentration levels presented good correlation coefficients (R^2) average 0.99. All residue contaminants were detected at acceptable their MRLs. The proposed assay was successfully applied for the analysis of the studied pesticide residues in one PT sample.(write full form of PT)

Keywords: Optimization, Validation, Honey, Multiresidue, Pesticides, Liquid chromatography, Tandem mass spectrometry (MS/MS), QuEChERS, Acceptable value, Pesticide residue, Detection, Analytical method, Calibration curve, Multiple analytes,MRL.

INTRODUCTION

The analysis of pesticide residues in honey is one of the most analytical challenges task as it contains 300 substances (sugars/waxes) [1], which are affected by the type of plant from which bees collect nectar to make honey [2], in addition, the increasing consumption of honey has demanded efficient quality control for this product.

There are multiple ways to expose honeybees and other pollinators to pesticides, including direct contamination such as during spraying in flowering [3], dust deposit abraded from treated seeds during seeding [4], treatment of bee hives with acaricides in the control of *Varroa destructor* [5], contaminated water puddles [6], uptake of volatilized pesticides [7], or indirectly by collection of nectar, pollen, and guttation droplets contaminated by (systemic) pesticides of crops and even wildflowers [8]. and the pesticide intrusion into the hive by wind [9].

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BE-3001 Leuven, Belgium4Mekelle University, Department of Land Resources Management and Environmental Protection, Ethiopia POTENTIAL POLLINATORS AND FLORAL VISITORS OF INTRODUCED TROPICAL

BIOFUEL TREE SPECIES Jatropha curcas L. (Euphorbiaceae), IN SOUTHERN AFRICA African Crop Science Journal, Vol. 21, No. 2, pp. 133 - 141aks.

Consequently, residues of pesticides can be found in bee matrices like pollen pellets, bee bread, honey, wax or royal jelly [10]. Several studies have shown the common presence of pesticide residues in honey bee colonies and bee products, thus, the Monitoring of the maximum residue limits (MRLs) has become necessary in honey (Royal jelly, pollen, comb honey), it helps to assess the potential risk of this product to consumer health and gives furnish the information regarding the pesticide treatments that have been used in field crops surrounding the hives [11]. Nowadays, the most universal extraction method to analyze a wide range of pesticides is the “QuEChERS method,” which stands for quick, easy, cheap, efficient, rugged, and safe. Essentially, it is based on extraction of pesticides from sample matrix with an organic solvent (commonly acetonitrile) followed by the removal of interference using a clean-up sorbent; then, the purified extracts analyzed by the appropriate analytical technique [12].

Several studies on multi-residue determination of pesticides in honey have been reported in the literature. Multi-residue analysis of at least one hundred pesticides in honey has been achieved using LC and GC coupled to mass spectrometric (MS) or tandem mass spectrometric (MS/MS) detection [13,14,15,16,17]. The LC-MS/MS method was validated to measure 116 pesticides in honey, but 11 compounds showed recoveries at 0.010 mg/kg out of the 70–120% range [18] the total chromatographic run time was 22mins. Similarly, the method developed and evaluated the occurrence of 83 pesticides and antibiotics of different classes in honey from France using QuEChERS and LC–MS/MS in a run time of 30min [19].

The European regulation 396/2005 EC set the limit at 10 μg kg^{-1} for substances for which no MRL had been established. Since 1 September 2008 the European Commission has set new MRLs, which mostly lies in between 10 and 50 μg kg^{-1} in honey [20].

Honey, food consumption data vary between 20 g per day and per person (CVMP) (21)(EU Committee for Medicinal Products for Veterinary Use (CVMP)) [21] and 50 g per day and per person (JECFA (22). (Joint FAO/WHO Expert Committee on Food Additives (JECFA)) [22] The value of 50 g honey per day and per person represents the acute daily intake (95th percentile) for an adult of 60 kg according to EFSA [23].

The assessment of consumers' chronic exposure to the selected residues through the food consumption of honey was based on a worst-case scenario. This consisted, for each residue and based on the "maximum level of contamination" for this residue, in adding the honey contribution (via the consumption of 50 g of honey/person/day) to a theoretical maximum daily intake (TMDI) and in checking that the ADI value is not being exceeded. The contribution of honey was calculated on the basis of a residue concentration equal to the MRL. The TMDI values generally come from the EMA and take into account the residue intake via other foodstuffs (e.g., meat, milk, eggs), but sometimes via honey as well. The TMDI is, however, not always known. In that case, consumers' exposure through the consumption of honey is compared to the ADI [24]

The goal of this work is to develop and validate a multi-residue method according to European Union SANCO guidelines (SANTE/12682/2019) to identify and quantify 101 pesticide residues belonging to different chemical classes in honey using high performance liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). Also, measurement uncertainty was evaluated as well as method performance by means of participation in a proficiency test in order to evaluate the applicability on the real samples of honeys.

Materials and methods

Chemicals and Reagents

Certified analytical standards were purchased from Dr Ehrenstofer (Germany), with purity between 92.0 and 99.5%. Acetonitrile (Merk, Germany), methanol (high performance liquid chromatography [HPLC] grade, Scharlab), Formic acid (Honeywell, Germany), Ammonium formate (>99%). Ready-made QuECHERS kits were purchased from Suplco; Supel™ QuE citrate extraction (4 g $MgSO_4$, 1 g NaCl, 0.5 g NaCitrate dibasic sesquihydrate, 1 g NaCitrate tribasic dehydrate), Supel™ QuE PSA/ENVI-CARB (EN) tube 2 (150 mg Supelclean PSA, 45 mg Supelclean ENVI-Carb, 900 mg MgSO4). The solutions were prepared with Ultrapure demineralized water Milli-Q plus system (Merck-Millipore Corporations, USA).

Stock solution and Calibration curve.

Individual analytical stock solutions (1000 mg L^{-1}) of each pesticide were prepared in methanol HPLC grade, considering the purity of each pesticide standard. All solutions were stored in amber flasks at –18 ºC. Afterwards, Stock solution of 10 mg L^{-1} standard mixture of all analytes were prepared, that was diluted to 1 mg L^{-1} using methanol immediately prior to sample preparation. The calibration standards (10, 20, 50, 100 and 200 $ngml^{-1}$) were prepared from stock standard

Trueness inter-laboratory comparison.

Blank honey samples originated were obtained from a local market. Blank samples were classified by the absence of compounds of interest, with prior injection into chromatographic system. These blank samples were fortified with target analytes for the validation of the analytical method. Honey sample from the provider BIPEA, code 18-3619-0038, analyzed in the proficiency test, was maintained under refrigeration (5ºC) until analysis.

2.4 Extraction Protocol

QuEChERS approach for the extraction of pesticide residues in honey

The blank honey samples (Pesticide free) were acquired from the consumer market used for validation experiments. The samples were stored at ambient temperature (-20ºC) until analysis. Based on the QuECheRS method reported by Anastassiades and Coworkers [12], the following sample preparation steps were conducted:

1) Weigh 2 g of honey into 50 mL PTFE centrifuge tubes and spiked with appropriate amounts of pesticides in working solutions. Next 10.0 mL of demineralized water was added, shook well and vortexed for 30 s and left for 30 minutes.
2) Added 10 mL of acidified acetonitrile and shook/ vortexed properly for 4-5min for proper interaction of analytes and solvent
3) Added Supel™ QuE citrate extraction. Immediately Shook/vortexed well the mixture and then, centrifuge for 5 min at 3000 rpm.
4) Transferred 6 mL of supernatant into a single-use polypropylene centrifuge tube containing 150 mg Supelclean PSA, 45 mg Supelclean ENVI-Carb, 900 mg MgSO4 vortex the mix and centrifuge it for 5 min at 5000 rpm.
5) One ml of the supernatant solution was evaporated to complete dryness using vacuum concentrator at 40ºC. Finally, the dried residues of pesticide were reconstituted with injection solution and filtered using a PTFE syringe filter 0.22µm into the autosampler vials for LC–MS/MS analysis
6) The procedural internal standard (P-IS) was Triphenyl phosphate (TPP)

Liquid chromatography–mass spectrometry and data analysis

LC-MS/MS (liquid chromatography tandem mass spectrometric detection) analysis was performed using an Agilent 6460 Triple Quadrupole LC/MS system. Chromatographic separations were carried out on a Zorbax SB-C18 Rapid Resolution HT column (4.6 × 150 mm, 1.8 µm) at a 60 ◦C column temperature. The mobile phases were water containing 5 mM ammonium formate and 0.01% formic acid (phase A) and methanol containing 5 mM ammonium formate and 0.01% formic acid (phase B), with gradient elution at a flow rate of 0.5 mL/min. The total chromatographic run time was 22 min. The injection volume was 5 µL. For mass spectrometric analysis, an electrospray ionization (ESI) source was used in both negative

(ESI-) and positive (ESI+) modes. Before the calibration and quantification of pesticides it was necessary to set an acquisition method, setting chromatographic conditions, precursor and product ion so called monitoring mode of ion transfer (MRM), fragmentation energy (Frag.) and energy of collision cell (CE) were all determined. All pesticides were detected in the multiple reaction monitoring modes (MRM). Targeted analytes were identified by monitoring two transition ions where possible, for each analyte as recommended by SANCO guidelines for LC-MS/MS analysis. The most dominant transition ion was used for quantification whereas the second most intense ion as a qualifier for confirmation purposes. The MRM transitions including the precursor and product ions, fragmentor, collision energy, and retention time for the target pesticides are summarized in Table1. The analysis was run according to all requirements for identifying analytes by MS/MS established by European Union SANTE/12682/2019 [25].

Table 1 Chromatographic parameters and MS/MS (tandem mass spectrometric) detection for compounds analyzed by LC-MS/MS (liquid chromatography tandem mass spectrometric detection).

Target Pesticide	Precursor ion (m/z)	Retention Time	Quantification		Confirmation		Frag (V)	Polarity
			Product ions (m/z)	CE (V)	Product ions (m/z)	CE (V)		
Acephate	184	1.87	143	8	49.1	20	70	ESI+
Acetamiprid	223.1	3.64	126	27	90	45	80	ESI+
Aldicarb	116	2.3	89.1	4	70.1	4	70	ESI+
Ametryn	228.1	6.01	186.1	16	71.2	28	130	ESI+
Aminocarb	209.1	2.28	152.2	12	137.2	24	105	ESI+
Atrazine	216.1	5.66	174.1	16	68	40	125	ESI+
Azaconazole	300	5.97	231	20	159	28	130	ESI+
Azinphos-methyl	318	6.6	261	8	132.1	8	60	ESI+
Azoxystrobin	404.1	7.3	372.1	8	329.1	32	110	ESI+
Benalaxyl	326.2	9.6	148.1	27	91.1	48	90	ESI+
Bitertanol	338.2	10	269.2	5	70	4	70	ESI+
Boscalid	343	7.5	307.1	16	271.2	32	145	ESI+
Bromuconazole	378	9.7	159	32	70	35	115	ESI+
Cadusafos	271.1	10.4	159	8	130.9	20	90	ESI+
Carbaryl	202.1	5.04	145.1	4	127.1	28	65	ESI+
Carbendazim	192.1	2.96	160.1	16	132.1	32	105	ESI+
Carbofuran	222.1	4.84	165.1	20	123.1	30	80	ESI+
Carboxin	236.1	5.03	143.1	12	43.2	48	105	ESI+
Chloridazon	222	3.68	146.1	28	77	36	130	ESI+
Chloroxuron	291.1	8	72	20	46.1	20	130	ESI+
Cyanazine	241.1	4.54	214.1	18	68	40	120	ESI+
Cycluron	199.2	6.01	89.1	10	72	20	120	ESI+
Diazinon	305.1	9.6	169.1	32	97	40	105	ESI+
Dicrotophos	238.09	3.29	112.1	8	72.1	28	75	ESI+
Diethofencarb	268.2	7.1	226.1	12	124	30	70	ESI+
Dimethenamide	276.1	7.4	244.1	10	168.1	20	125	ESI+
Dimethirimol	210.16	4.35	140	20	98	25	120	ESI+
Dimethomorph(E)	388.1	8	301.1	20	165.1	32	145	ESI+
Dimethomorph(Z)	388.1	7.05	301.1	20	165.1	32	145	ESI+

Dimoxystrobin	327.2	9.2	205.1	12	116	20	115	ESI+
Diniconazole	326.1	10.1	159	28	70	28	110	ESI+
Diuron	233	5.26	72.1	20	46.1	16	110	ESI+
DMST	215.1	4.87	106.1	10	77	48	90	ESI+
Ethiofencarb	226.09	5.26	107	12	77	48	70	ESI+
Ethirimol	210.16	4.35	140.1	20	98.1	28	145	ESI+
Etrimfos	293.1	9.4	265	26	125	28	120	ESI+
Fenamidone	312	7.5	92.2	28	65.1	56	100	ESI+
Fenamiphos	304.1	9	217.1	20	202	36	120	ESI+
Fenpiclonil	237	6.23	202	16	140.1	40	90	ESI+
Fenpropimorph	304.3	6.69	147.1	30	117.1	64	165	ESI+
Fluometuron	233.1	5.55	72	16	46.1	16	105	ESI+
Flusilazol	316.1	9	247.1	12	165	24	120	ESI+
Fuberidazole	185.1	3.51	157.1	20	156	32	145	ESI+
Furathiocarb	383.2	11.4	195	16	167	24	110	ESI+
Heptenophos	251.02	5.97	127.02	15	125	25	80	ESI+
Hexaconazole	314.1	9.8	159	30	70.1	20	95	ESI+
Hexazinone	253.2	4.85	171.1	20	85.1	40	120	ESI+
Imazalil	297.1	5.43	159	20	69	16	115	ESI+
Imidacloprid	256	3.34	208.9	18	175	18	80	ESI+
Ipconazole	334.1	10.9	125	20	70	44	115	ESI+
Iprobenfos	289.1	9.2	205	10	91.1	20	120	ESI+
Iprovalicarb	321.2	8.4	119	16	91.1	56	80	ESI+

Table 1. (continued

Target Pesticide	Precursor ion (m/z)	Retention Time	Quantification		Confirmation		Frag (V)	Polarity
			Product ions (m/z)	CE (V)	Product ions (m/z)	CE (V)		
Isoproturon	207.15	6.1	72.1	10	46.1	20	120	ESI+
Kresoxim methyl	314.1	9.2	267	0	222.1	10	85	ESI+
Malaoxon	315	4.93	127.1	20	99.2	4	85	ESI+
Malathion	331	7.6	126.9	5	99	10	80	ESI+
Mandipropamid	412.13	7.6	356.1	4	328.1	8	110	ESI+
Mefenpyr-Diethyl	373.07	9.8	327.03	15	186	35	80	ESI+
Mepronil	270.1	7.7	119	20	91.1	40	130	ESI+
Metalaxyl	280.2	5.96	148.2	4	220.0	10	95	ESI+
Metamitron	203.1	3.57	175.1	12	104.1	20	100	ESI+
Metazachlor	278.1	5.79	210.1	4	134.2	15	70	ESI+
Metconazole	320.1	9.9	125	48	70.1	24	130	ESI+
Methoxyfenozide	369.2	8	313.1	0	149	10	85	ESI+
Nitenpyram	271.1	2.66	90	48	56.1	36	95	ESI+
Ofurace	282	4.87	254	8	236	12	90	ESI+
Oxadixyl	279.1	4.42	219.2	5	117	60	70	ESI+
Paclobutrazol	294.1	7.6	125.2	36	70.1	16	115	ESI+
Paraoxon-methyl	294.1	8.19	125.2	36	70.1	16	115	ESI+
Penconazole	284.1	9.3	159	30	70.1	15	70	ESI+
Pencycuron	329.1	10.4	125.1	24	89.1	60	120	ESI+
Phosphamidon	300	4.49	127.1	16	72.1	28	110	ESI+
Picoxystrobin	368.1	9.1	205.2	4	145	20	70	ESI+
Pirimicarb	239.15	4.67	182.1	12	72.1	20	100	ESI+
Prochloraz	376	9.7	308	4	70.1	24	70	ESI+
Promecarb	208.1	7.5	151	0	109.1	10	80	ESI+
Prometon	226.2	5.46	184.1	16	142.1	24	120	ESI+
Prometryn	242.1	7.5	200.1	20	158	28	120	ESI+
Propachlor	376	6.2	308	4	70.1	24	70	ESI+
Propazine	230.1	6.72	146	22	79	44	100	ESI+
Propiconazole	342.1	9.5	159	32	69.1	16	115	ESI+
Propoxur	210.11	4.78	168.1	24	111.1	8	55	ESI+
Propyzamide	256	7.6	190	10	145	36	105	ESI+

Pyraflufen-ethyl	413	9.6	339	25	261	40	120	ESI+
Pyrifenox	295	8	93	20	66.1	60	100	ESI+
Quinalphos	299	9	163	20	97	32	90	ESI+
Secbumeton	226.2	5.42	142	25	67.9	50	100	ESI+
Simazine	202.1	4.78	132	22	68	40	120	ESI+
Spiroxamine	298.28	7.3	144.1	16	100.1	32	125	ESI+
Tebutam	234.2	8.6	91.1	36	65	40	120	ESI+
Tebuthiuron	229.1	4.97	172.1	12	116	24	105	ESI+
Terbumeton	226.2	5.58	170.1	26	69	40	120	ESI+
Terbutryn	242.1	7.5	186.1	16	68.1	48	110	ESI+
Tetrachlorvinphos	364.9	9.1	203.9	40	127	16	120	ESI+
Thiacloprid	253	3.92	126	16	90	40	100	ESI+
Thiamethoxam	292.03	2.86	181.1	20	132	24	85	ESI+
Thiodicarb	355.06	5.34	163.3	0	88.1	8	85	ESI+
Triphenyl Phosphate	327.08	9.9	215	30	77.04	35	160	ESI+
Triazophos	314.1	8.2	162.1	16	119.1	36	110	ESI+
Tricyclazole	190	4.14	163.1	24	136.2	28	130	ESI+
Zoxamide	336	9.5	187	16	159	44	120	ESI+

CE = Collision energy.
Frag = Fragmentor

Method Validation

The methods of extraction, clean-up, detection and quantification of 100 pesticides (or their metabolites) from the honey matrix were optimized and validated in compliance with the document SANTE/12682/2019 [25] entitled “Guidance document on analytical quality control and method validation procedures for pesticide residue analysis in food and feed”, which was issued by the European Commission Directorate General for Health and Food Safety, and became effective on January 1, 2020 by evaluating the following performance parameters: linearity, accuracy (expressed as recovery percentage), intermediate precision (relative standard deviation <20.0%).

Linearity: The quantification of pesticide was based on a five-point matrix-matched calibration graph. The LC-MS/MS system’s linearity was evaluated by assessing the signal responses of the calibration standards. The regression equations with slope, y-intercept, and coefficient of correlation (r 2) were evaluated for all tested compounds.

Accuracy and Precision: the reliability of the method was evaluated by estimating the accuracy (expressed as recovery percentage), precision (% relative standard deviation): The main goal of the recovery experiments is to determine the method accuracy, via comparison of the real concentration of each pesticide measured by performing the complete procedure with the known pesticide concentration initially added to the matrix. The method precision is expressed as the repeatability (RSD%) of the recovery determinations at the two different spiking levels (0.01and 0.1 mgkg^{-1}). Five spiked samples were analyzed at each level and also the blank union matrix analysis was performed two times. This blank extract was also used for preparation of standard solutions in matrix. Calibration curves were obtained for the levels of concentration 10, 20, 50, 100 and 200 µg L^{-1} in methanol and in the matrix blank extract, corresponding to a range of 10 to 200 µg L^{-1} in the sample (method factor of 2).

Method recovery studies were performed at two spiking concentration levels (10 µg L^{-1} and 100 µg L^{-1}), based on the QuECheRS method reported by Anastassiades *et al.*, 2002 [12]. The blank honey samples (Pesticide free) were acquired from the consumer market used for validation experiments. The samples were stored at ambient temperature (-20ºC) until analysis. The following steps were conducted:

1) Weigh 2 g of honey into 50 mL PTFE centrifuge tubes and added 10.0 mL of demineralized water shook well and vortexed for 30 s and left for 30 minutes.
2) Added 10 mL of acidified acetonitrile and shook/ vortexed properly for 4-5min for proper interaction of analytes and solvent.
3) Added Supel™ QuE citrate extraction. Immediately Shook/vortexed well the mixture and then, centrifuge for 5 min at 3000 rpm.
4) Transferred 6 mL of supernatant into a single-use polypropylene centrifuge tube containing 150 mg Supelclean PSA, 45 mg Supelclean ENVI-Carb, 900 mg MgSO4 vortex the mix and centrifuge it for 5 min at 5000 rpm.
5) One ml of the supernatant solution was evaporated to complete dryness using vacuum concentrator at 40ºC. Finally, the residues of pesticide were re-dissolved in injection solution and filtered using a PTFE syringe filter 0.22µm into the auto-sampler vials for LC–MS/MS analysis. The extract was analyzed by LC-MS/MS making 5 replicates for each concentration. The average percentage of recovery and the relative standard deviation (RSD, repeatability) were evaluated.

RESULTS AND DISCUSION

To ensure the safety and quality control of honey, it is necessary to monitor chemical pollution in honey in order to be sure ensure that the natural product does not contain any toxic residues at levels that would be harmful to consumers. The multi-residue QuEChERS method in combination with high-performance liquid chromatography with tandem mass spectrometry (LC–MS/MS) was used described for honey [26, 27]. This analytical method was sufficiently validated for 101 pesticide standards at the default MRL values LOQ of (0.01 mgkg^{-1} and at 0.1 mgKg^{-1})Which provided great recoveries with the best reproducibility across multiple analytes during method development. Thus, a sufficiently validated analytical method is available that can be used for MRL enforcement.

Extraction method

The QuEChERS extraction method was chosen for the analysis of pesticides in honey based on available literature data demonstrating its applicable method, since it offers good selectivity, performance and sensitivity for extraction of pesticides from honey [28, 29, 30, 31, 32,33, 34.

35]. After investigating different conditions regarding sample weight, amount of water for sample dilution, type of extraction solvent and clean-up salts which eliminate possible LC-MS/MS interferences, based on data from recovery the final technique as mentioned above which has the advantages of being fast, easy, economical, effective, robust and secure, can be applied in any laboratory, due to the simplification of the steps

Method Validation.

According to the European Union SANTE/12682/2019 guidelines (SANTE, 2019), the precursor (parent) ion and the two transitions (quantification and identification ions) should be present with a signal-to-noise (S/N) ratio greater than 3 (in the lowest calibration level this ratio should be higher than 6); and the ratio of the quantification/confirmation transitions in the sample and the previously injected standard should not differ by more than ±30%. Therefore, two transitions were selected for each compound (Table 1) and these criteria were evaluated. Figure 1 shows The total ion chromatograms (TIC) of QuEChERS extracts spiked at 0.1 mg/kg with 101 pesticide standard described in Table 1 are depicted in Figure 1. All chromatograms are on the same scale and have been enlarged along the Y axis to show the inherent complexity of each extract. The absence of signal above a signal-to-noise ratio of 3 at the retention times of the target compounds showed that the method was free of interferences.

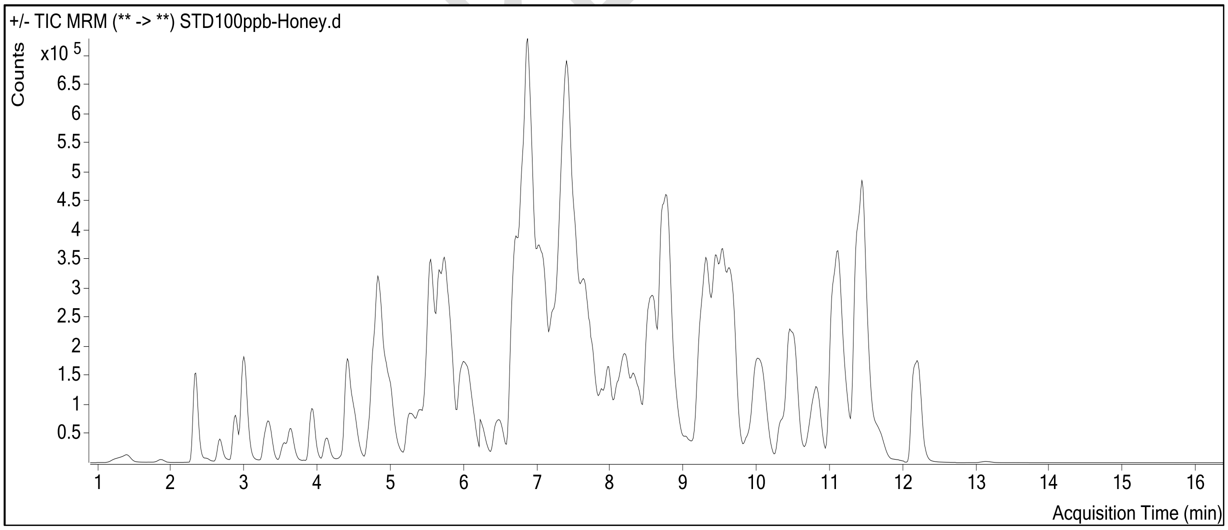


Fig. 1 Total ion chromatograms (TIC) and MRM chromatograms of 101 pesticides extracted from a spiked honey sample at 0.1mgKg^{-1} level obtained by LC-MS/MS (ESI positive mode)

Based on table 1 the performance of liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) has shown great success in multi-residue pesticide analysis in complex food matrices such as honey This technique provides information regarding the characteristic ion of each analyte as well as two or more transitions of these ions, useful to quantify and confirm the analytes at concentrations consistent with maximum residue levels (MRLs) established.

Linearity of Calibration Curve.

To determine whether a detection method holds a linear response between amount of analyte and acquired response from LC-MS/MS. According to the validation guidelines, the linearity of the method is considered satisfying (0.95- 1.00) for all investigated pesticides. The target Pesticides showed linearity in MRM mode. Linear spiked calibration curves for all the interest pesticides were obtained with correlation coefficient ($r2$) with average 0.99 (Table 2), calibration curves for some selected representative pesticides are shown in Figure 2, 3, 4, 5.

Recovery and Precision (Repeatability)

Recovery percentage experiments were performed at two spiking levels 10 µg L^{-1} and 100 µg L^{-1} of target pesticides (n = 5 replicates per level). Table 2 shows the relative recoveries, precision (repeatability), measured as % RSD, relative standard deviation, relative percentage error and uncertainty measurement (%). The percent recovery of pesticides is in the range of 71.5 (Iprovalicarb) to 119.8% (Paraoxon-methyl) at 0.01mgkg^{-1} and 73.21 (Quinalphos) to 119.79% (Tebuthiuron) at 100 µg L^{-1} fortification level.

Table 2: Validation results of 101 compounds, average recoveries (%), relative standard deviations (%), relative percentage error and uncertainty measurement (%) of pesticides obtained by LC-MS/MS analysis of honey samples at 2 spiking levels (n=5).

Target Pesticide	Coefficient of Variation (r^2)	Recovery and %RSD						Uncertainty measurement	
		10 µg L^{-1}			100 µg L^{-1}				
		Rec	RSD	RE	Rec	RSD	RE	10 µg L^{-1}	100 µg L^{-1}
Acephate	0.989982	97.04	10.73	-2.96	95.00	5.70	-5.00	21.45	11.39
Acetamiprid	0.999704	114.36	8.25	14.36	106.41	7.05	6.41	16.51	14.10
Aldicarb	0.991753	116.71	5.97	16.71	102.13	15.98	2.13	11.95	31.96
Ametryn	0.992602	97.78	9.60	-2.22	100.07	4.73	0.07	19.21	9.47
Aminocarb	0.996648	106.80	6.26	6.80	104.18	6.42	4.18	12.53	12.83
Atrazine	0.999697	113.47	12.06	13.47	109.80	4.21	9.80	24.12	8.41
Azaconazole	0.999109	116.35	6.16	16.35	116.44	3.05	16.44	12.32	6.10
Azinphos-methyl	0.994295	93.12	14.55	-6.88	89.55	6.23	-10.45	29.11	12.47
Azoxystrobin	0.999064	108.36	7.78	8.36	107.30	3.07	7.30	15.57	6.15
Benalaxyl	0.998954	98.30	7.20	-1.70	99.51	2.88	-0.49	14.40	5.75

Bitertanol	0.997989	91.50	8.05	-8.50	90.97	2.92	-9.03	16.11	5.84
Boscalid	0.999649	115.06	4.65	15.06	96.61	1.27	-3.39	9.30	2.55
Bromuconazole	0.994796	83.25	7.51	-16.75	79.24	3.01	-20.76	15.01	6.02

Table 2. (continued)

Target Pesticide	Coefficient of Variation (r^2)	Recovery and %RSD						Uncertainty measurement	
		10 µg L^{-1}			100 µg L^{-1}				
		Rec	RSD	RE	Rec	RSD	RE	10 µg L^{-1}	100 µg L^{-1}
Cadusafos	0.995857	87.57	6.21	-12.43	84.05	8.58	-15.95	12.43	17.17
Carbaryl	0.954257	88.85	9.93	-11.15	104.84	9.25	4.84	19.86	18.50
Carbendazim	0.999451	88.33	11.71	-11.67	80.29	10.45	-19.71	23.42	20.90
Carbofuran	0.997578	117.02	1.51	17.02	113.75	3.79	13.75	3.01	7.57
Carboxin	0.998616	115.37	8.89	15.37	115.91	5.02	15.91	17.78	10.04
Chloridazon	0.99928	116.24	6.10	16.24	103.42	6.03	3.42	12.21	12.05
Chloroxuron	0.999286	108.09	11.14	8.09	106.79	6.76	6.79	22.29	13.52
Cyanazine	0.999829	103.64	7.85	3.64	104.44	7.73	4.44	15.69	15.46
Cycluron	0.99939	116.89	11.44	16.89	111.44	5.92	11.44	22.87	11.84
Diazinon	0.993343	96.44	11.90	-3.56	86.12	6.39	-13.88	23.79	12.78
Dicrotophos	0.995438	106.88	9.81	6.88	102.03	3.84	2.03	19.63	7.67
Diethofencarb	0.99947	107.20	5.68	7.20	93.60	6.56	-6.40	11.36	13.12
Dimethenamide	0.99937	105.67	8.62	5.67	103.26	2.97	3.26	17.23	5.93
Dimethirimol	0.99592	99.61	11.42	-0.39	102.64	6.57	2.64	22.83	13.15
Dimethomorph(E)	0.999383	113.56	8.61	13.56	108.51	4.10	8.51	17.23	8.21
Dimethomorph(Z)	0.999383	113.56	8.61	13.56	108.51	4.10	8.51	17.23	8.21
Dimoxystrobin	0.999461	98.40	7.68	-1.60	97.75	2.35	-2.25	15.35	4.69
Diniconazole	0.998801	79.77	11.41	-20.23	74.82	7.25	-25.18	22.83	14.50
Diuron	0.999909	116.11	13.32	16.11	113.76	3.58	13.76	26.64	7.17
DMST	0.994969	111.18	8.70	11.18	113.92	8.74	13.92	17.41	17.47
Ethiofencarb	0.991423	100.54	9.07	0.54	106.22	5.60	6.22	18.15	11.20
Ethirimol	0.986501	85.04	10.49	-14.96	93.98	4.11	-6.02	20.99	8.22
Etrimfos	0.997098	100.12	8.51	0.12	84.84	9.87	-15.16	17.02	19.73
Fenamidone	0.999045	109.33	8.27	9.33	106.45	4.09	6.45	16.55	8.18
Fenamiphos	0.999721	91.47	12.22	-8.53	88.84	3.89	-11.16	24.44	7.79
Fenpiclonil	0.988895	114.85	7.95	14.85	117.16	10.37	17.16	15.91	20.75
Fenpropimorph	0.998394	102.36	6.90	2.36	101.66	1.61	1.66	13.79	3.22
Fluometuron	0.998932	113.43	14.35	13.43	110.27	2.32	10.27	28.70	4.65
Flusilazol	0.999819	92.13	13.37	-7.87	85.61	3.72	-14.39	26.75	7.44
Fuberidazole	0.997955	91.91	9.22	-8.09	85.05	6.66	-14.95	18.44	13.33
Furathiocarb	0.994415	71.51	6.56	-28.49	73.62	3.25	-26.38	13.13	6.50
Heptenophos	0.999879	104.22	9.88	4.22	88.63	7.25	-11.37	19.77	14.49
Hexaconazole	0.997095	116.99	3.05	16.99	109.71	3.02	9.71	6.10	6.04
Hexazinone	0.999591	116.55	7.13	16.55	115.03	2.10	15.03	14.26	4.19
Imazalil	0.997363	104.80	5.64	4.80	96.61	7.09	-3.39	11.29	14.19
Imidacloprid	0.998175	113.72	9.03	13.72	103.81	6.09	3.81	18.06	12.19
Ipconazole	0.996188	84.47	6.78	-15.53	77.90	2.96	-22.10	13.56	5.92
Iprobenfos	0.999498	109.91	10.62	9.91	110.01	4.97	10.01	21.24	9.93
Iprovalicarb	0.996473	75.53	6.65	-24.47	77.23	7.24	-22.77	13.31	14.48
Isoproturon	0.999309	115.04	10.32	15.04	112.72	5.96	12.72	20.65	11.93
Kresoxim methyl	0.99844	113.93	14.68	13.93	103.33	4.86	3.33	29.36	9.73
Malaoxon	0.871943	101.00	14.52	1.00	112.44	12.61	12.44	29.03	25.23
Malathion	0.949669	111.99	8.50	11.99	116.46	9.13	16.46	16.99	18.26
Mandipropamid	0.999268	102.42	7.96	2.42	100.03	3.03	0.03	15.91	6.05
Mefenpyr-Diethyl	0.931729	98.56	12.81	-1.44	99.62	9.06	-0.38	25.62	18.11
Mepronil	0.998193	116.04	7.18	16.04	109.49	3.38	9.49	14.37	6.76

Metalaxyl	0.997694	111.83	8.57	11.83	107.80	3.45	7.80	17.13	6.90
Metamitron	0.998433	117.55	13.59	17.55	104.41	2.18	4.41	27.18	4.37
Metazachlor	0.999368	110.50	9.91	10.50	109.86	5.55	9.86	19.82	11.09

Table 2. (continued)

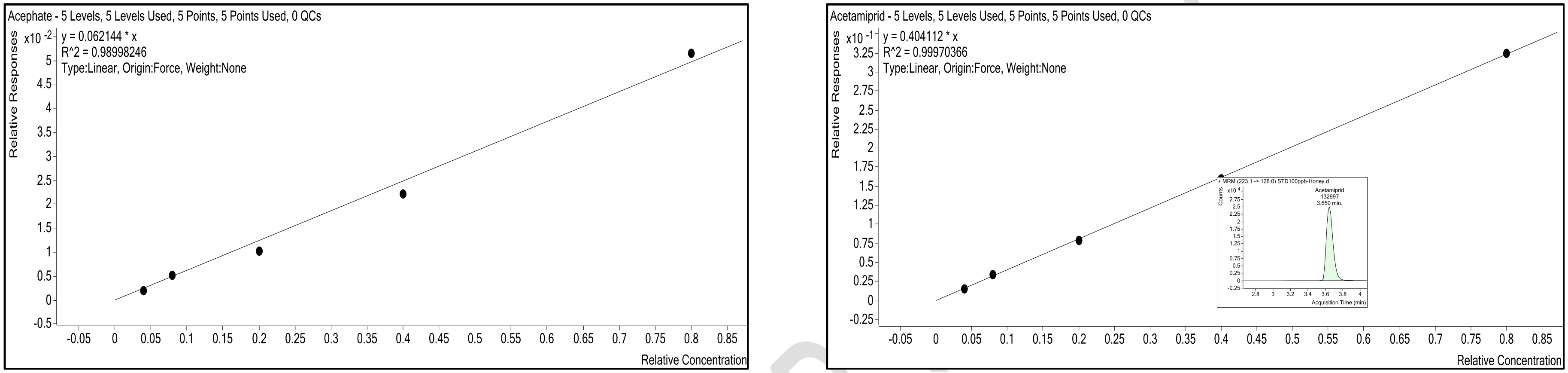
Target Pesticide	Coefficient of Variation (r^2)	Recovery and %RSD						Uncertainty measurement	
		10 µg L^{-1}			100 µg L^{-1}				
		Rec	RSD	RE	Rec	RSD	RE	10 µg L^{-1}	100 µg L^{-1}
Metconazole	0.999614	97.63	9.53	-2.37	95.79	1.46	-4.21	19.06	2.93
Methoxyfenozide	0.998577	112.05	11.26	12.05	109.99	4.46	9.99	22.53	8.93
Nitenpyram	0.995237	98.95	8.95	-1.05	93.88	6.60	-6.12	17.91	13.19
Ofurace	0.997924	117.30	9.53	17.30	114.75	3.95	14.75	19.07	7.90
Oxadixyl	0.999726	117.81	2.73	17.81	112.07	3.19	12.07	5.45	6.38
Paclobutrazol	0.999431	119.85	5.15	19.85	117.05	3.97	17.05	10.29	7.94
Paraoxon-methyl	0.999431	119.85	5.15	19.85	117.05	3.97	17.05	10.29	7.94
Penconazole	0.998213	95.12	16.79	-4.88	110.21	4.01	10.21	33.58	8.02
Pencycuron	0.995917	88.64	6.26	-11.36	86.26	5.33	-13.74	12.51	10.67
Phosphamidon	0.998388	105.12	8.64	5.12	103.00	6.16	3.00	17.28	12.31
Picoxystrobin	0.997949	92.31	8.04	-7.69	95.51	4.98	-4.49	16.08	9.97
Pirimicarb	0.997515	94.17	8.33	-5.83	89.27	6.08	-10.73	16.65	12.15
Prochloraz	0.993871	81.95	9.30	-18.05	79.15	3.61	-20.85	18.59	7.22
Promecarb	0.998289	108.89	5.95	8.89	101.96	5.68	1.96	11.91	11.36
Prometon	0.996548	108.49	7.29	8.49	107.74	4.11	7.74	14.58	8.23
Prometryn	0.995668	104.80	11.30	4.80	103.21	6.50	3.21	22.60	13.00
Propachlor	0.998307	97.60	6.67	-2.40	85.54	5.24	-14.46	13.34	10.48
Propazine	0.998991	115.11	3.71	15.11	109.65	2.28	9.65	7.43	4.56
Propiconazole	0.999791	109.42	11.77	9.42	97.98	3.02	-2.02	23.54	6.04
Propoxur	0.998656	118.11	8.26	18.11	116.38	8.13	16.38	16.52	16.26
Propyzamide	0.999542	104.22	17.02	4.22	102.97	3.87	2.97	34.03	7.74
Pyraflufen-ethyl	0.853591	105.47	4.93	5.47	95.12	13.55	-4.88	9.87	27.11
Pyrifenox	0.997701	119.65	11.17	19.65	105.59	4.22	5.59	22.35	8.44
Quinalphos	0.999617	95.64	5.98	-4.36	73.21	4.21	-26.79	11.97	8.42
Secbumeton	0.97987	105.84	8.75	5.84	108.13	2.35	8.13	17.51	4.70
Simazine	0.998182	113.34	11.39	13.34	101.90	3.30	1.90	22.78	6.59
Spiroxamine	0.997565	96.09	5.40	-3.91	108.70	17.47	8.70	10.80	34.94
Tebutam	0.995862	86.90	9.14	-13.10	82.29	4.52	-17.71	18.29	9.04
Tebuthiuron	0.997582	119.50	5.70	19.50	119.79	5.59	19.79	11.40	11.18
Terbumeton	0.97987	105.84	8.75	5.84	108.13	2.35	8.13	17.51	4.70
Terbutryn	0.998449	117.63	7.18	17.63	107.06	5.02	7.06	14.37	10.05
Tetrachlorvinphos	0.99961	97.10	6.48	-2.90	89.12	1.76	-10.88	12.96	3.53
Thiacloprid	0.999259	107.08	9.91	7.08	106.37	6.74	6.37	19.82	13.49
Thiamethoxam	0.998303	104.13	12.14	4.13	96.83	6.60	-3.17	24.29	13.19
Thiodicarb	0.881298	96.10	8.77	-3.90	104.98	12.91	4.98	17.55	25.81
Triazophos	0.999636	102.59	10.00	2.59	93.83	4.69	-6.17	20.00	9.38
Tricyclazole	0.999504	91.22	13.25	-8.78	87.96	6.92	-12.04	26.49	13.85
Zoxamide	0.99637	79.27	9.59	-20.73	77.57	3.19	-22.43	19.18	6.37

Rec: Relative recoveries
RSD: Relative Standard Deviation Percent
RE: Relative Error

All target pesticides had good recoveries, these are in agreement with the recommended criteria in (SANTE/12682/2019) document, which recommend general recovery limits of 70–120%

within laboratory repeatability ≤20% [25]. Therefore, the method could be considered sufficiently accurate and precise for the purpose.

Figure 2. Calibration curves for four selected representative pesticides from a spiked honey sample at 0.1mgKg^{-1} level obtained by LC-MS/MS



Target Compound: **Acephate**

Target Compound: **Acetamiprid**

Target Compound: **Aminocarb**

Target Compound: **Atrazine**

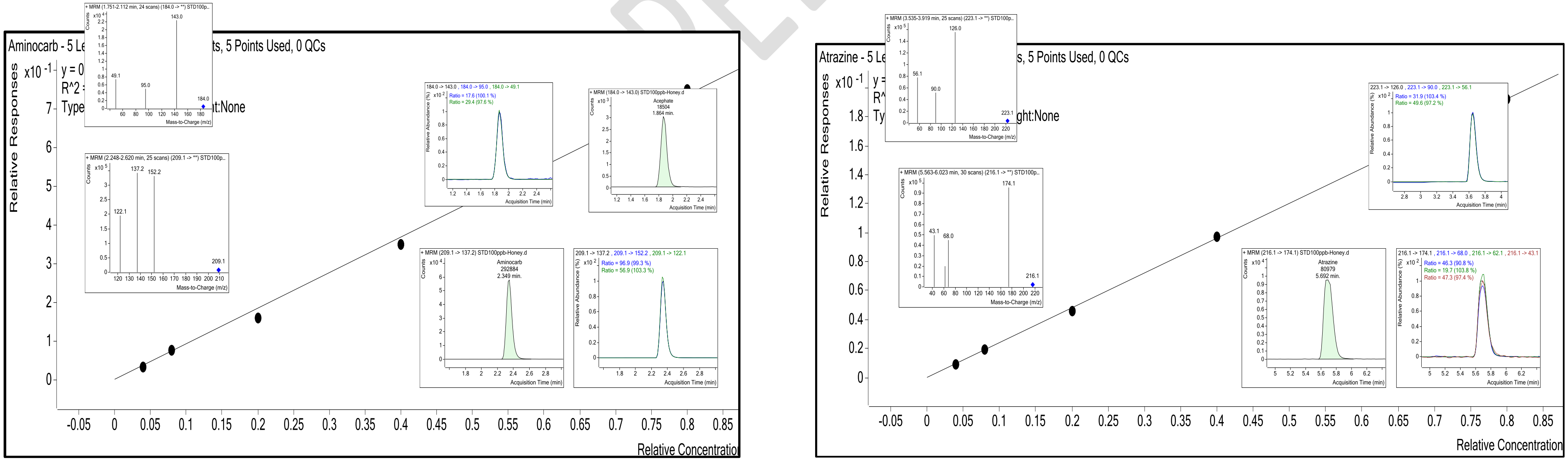
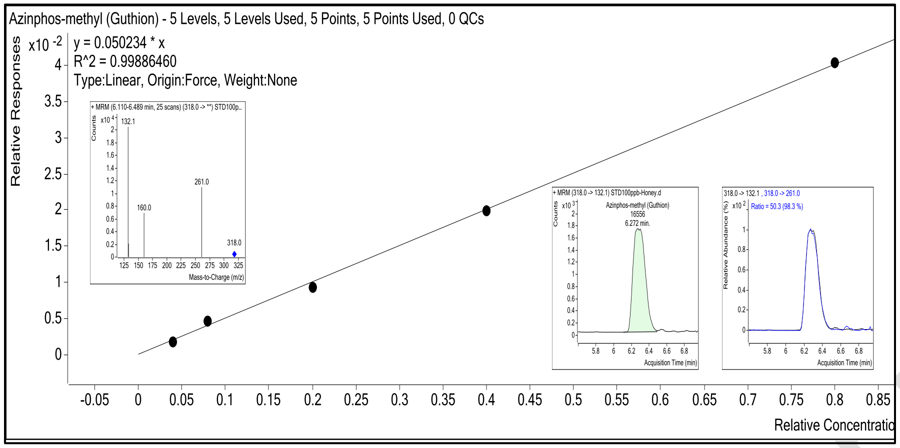
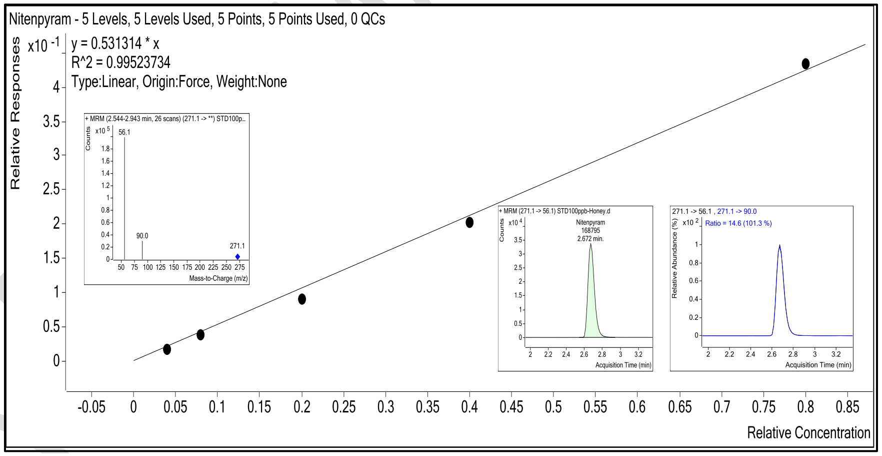


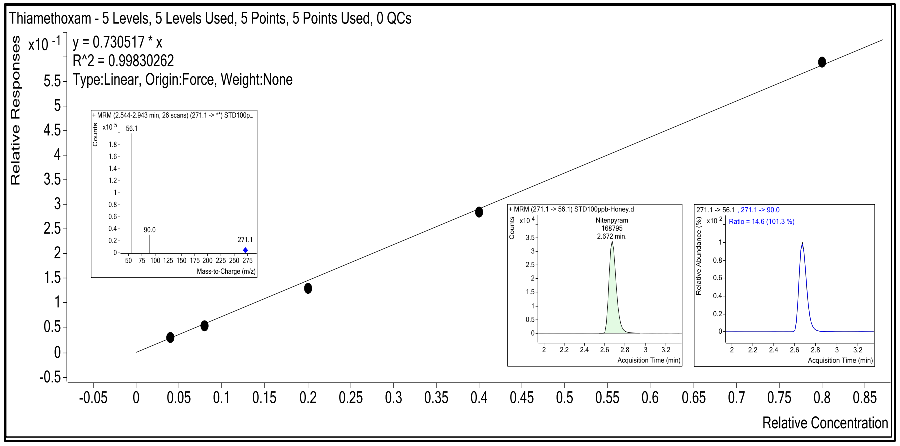
Figure 3. Calibration curves for four selected representative pesticides from a spiked honey sample at 0.1mgKg^{-1} level obtained by LC-MS/MS



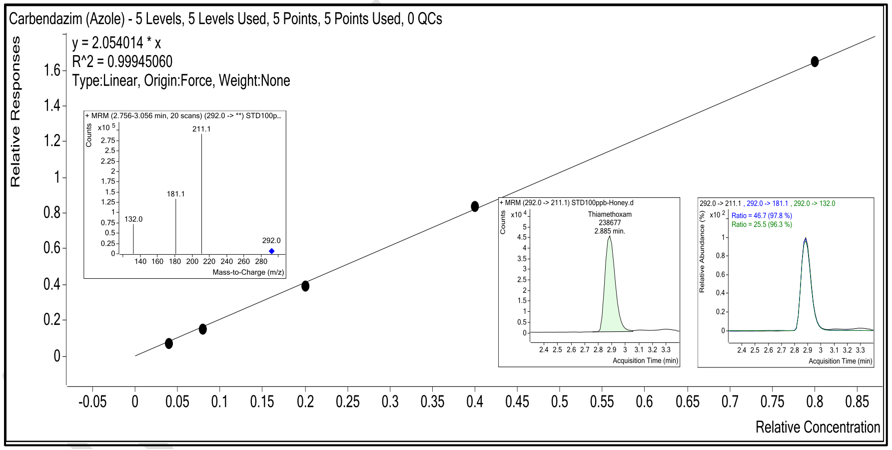
Target Compound: **Azinphos-methyl**



Target Compound: **Nitenpyram**



Target Compound: **Thiamethoxam**

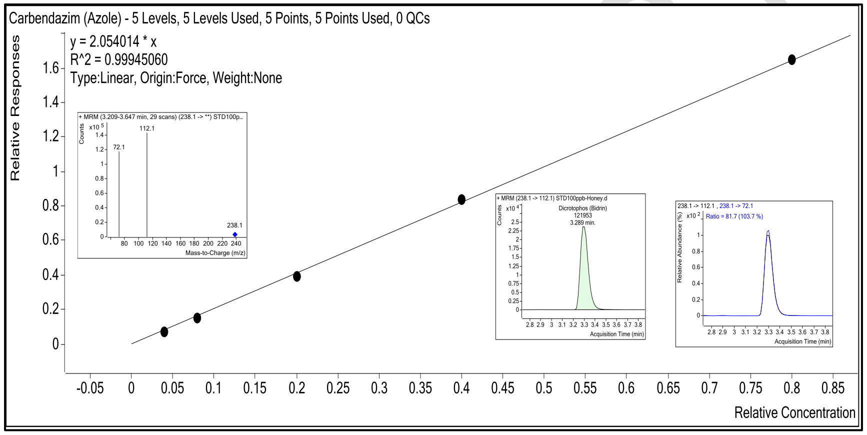


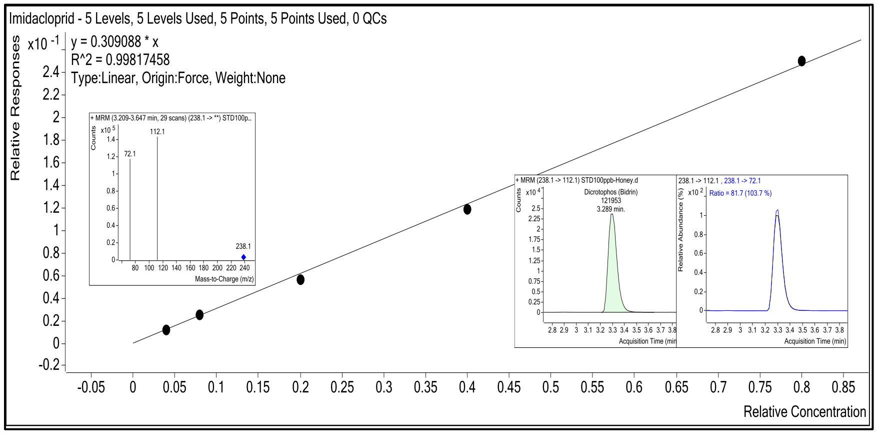
Target Compound: **Carbendazim**

Figure 4. Calibration curves for four selected representative pesticides from a spiked honey sample at 0.1mgKg^{-1} level obtained by LC-MS/MS

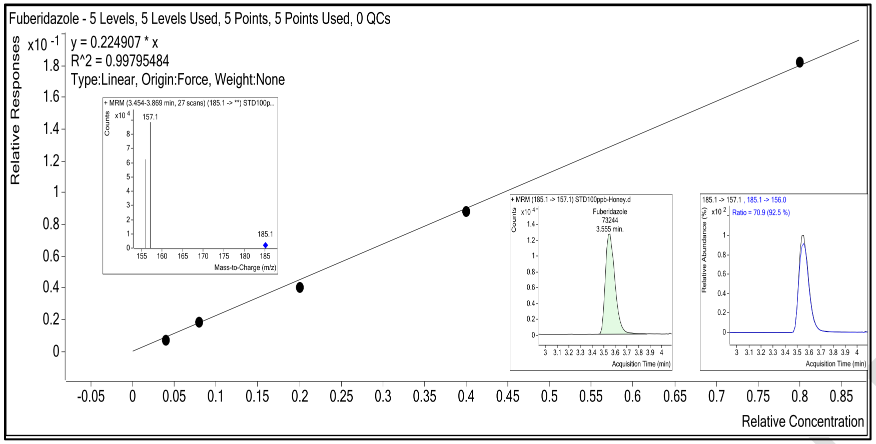
Target Compound: **Dicrotophos**

Target Compound: **Imidacloprid**





Target Compound: **Fuberidazole**



Target Compound: **Metamitron**

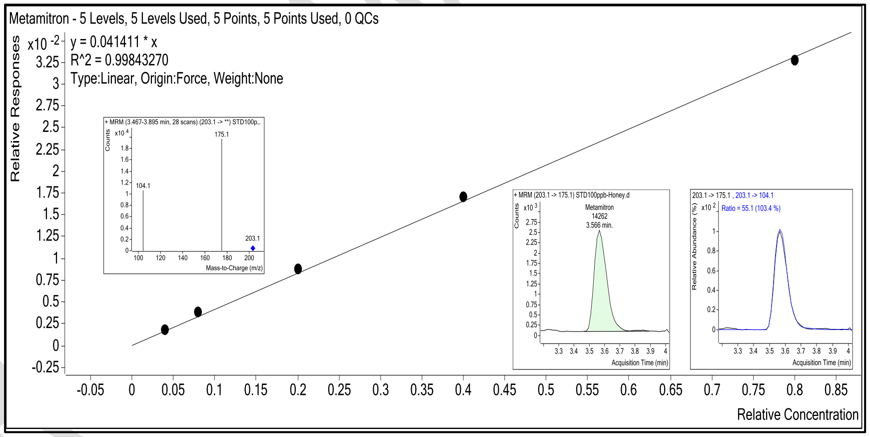
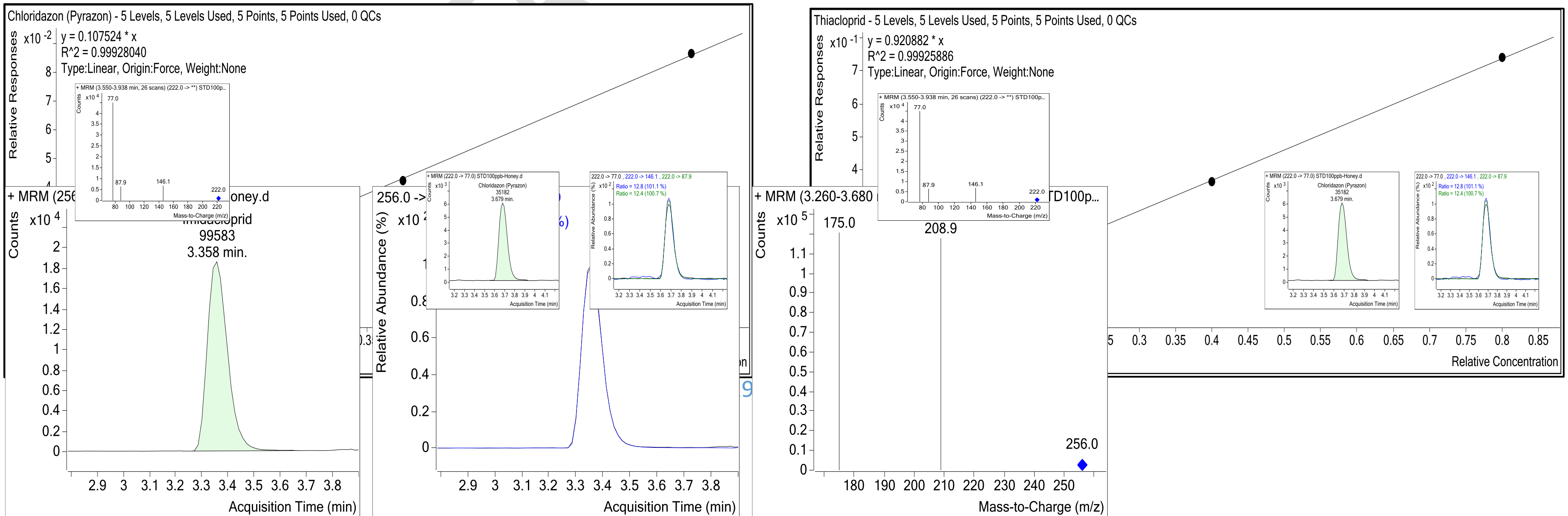


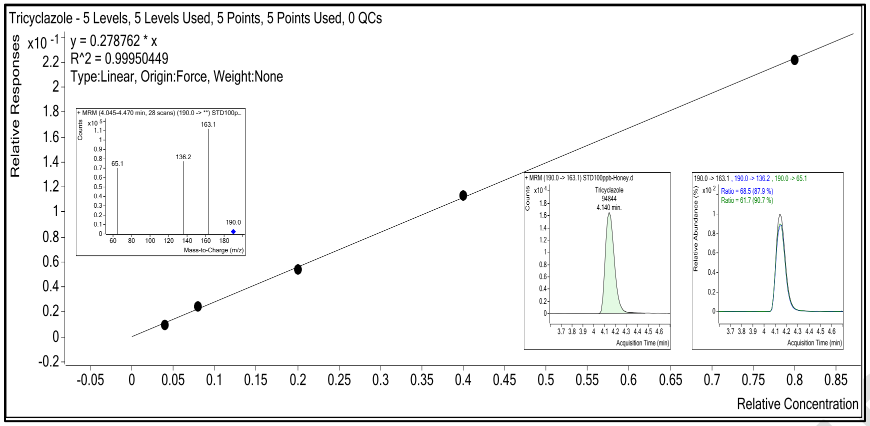
Figure 5. Calibration curves for four selected representative pesticides from a spiked honey sample at 0.1mgKg^{-1} level obtained by LC-MS/MS

Target Compound: **Chloridazon**

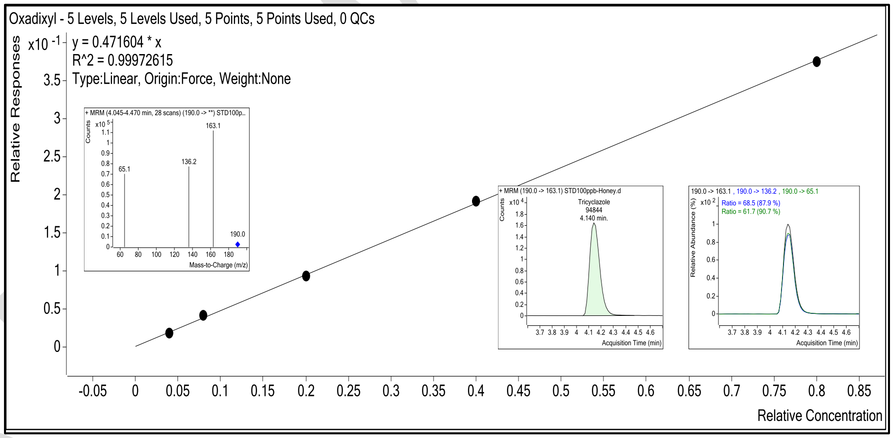
Target Compound: **Thiacloprid**



Target Compound: **Tricyclazole**



Target Compound: **Oxadixyl**



Accuracy

Accuracy measurements are usually expressed in terms of two components: “Trueness” and “Precision”.

Trueness Inter-Laboratory Comparison (Proficiency tests)

The method trueness was confirmed by participation in inter laboratory comparison with BIPEA. To analyze the sample, a matrix-matched calibration curve was prepared with a blank extract. Table 3 shows z-scores values for the identified analytes (from -1.91 to 1.9), demonstrating the suitability of the method for the purpose, concerning the acceptable limit of ±2.0. The identified analytes were Acetamiprid, Boscalid, Carbaryl, Carbendazim, Chlorfenvinphos, Imidaclopride, Malathion, Metalaxyl, Thiacloprid and Thiamethoxam.

Table 3. Interlaboratory comparison between Egyptian and Finnish laboratories

Pesticide	PT assigned value	BIPEA assigned value	z-score*
Acetamiprid	0.045	0.044	0.09
Boscalid	0.04	0.035	0.5
Carbaryl	0.064	0.043	1.9
Carbendazim	0.023	0.045	-1.91
Chlorfenvinphos	0.04	0.031	1.5
Imidaclopride	0.071	0.072	-0.05
Malathion	0.087	0.069	1.2
Metalaxyl	0.032	0.024	1.3
Thiacloprid	0.066	0.064	0.12
Thiamethoxam	0.049	0.044	0.45

* Accepted z-score: $-2 < z < 2$.

Precision – repeatability

The mean repeatability (Inter-day precision) expressed as % relative standard deviation (RSDr), obtained in one day with freshly prepared organic blank sample spiked with 10 and 100 μgL^{-1} at five replicates for each level. Table 2 showed the precision of the target pesticides. Repeatability-precision was excellent, showing less than 15% RSD for over 98% of the pesticides and met are in accordance with those reported by European Union SANTE/12682/2019 for precision.which support the results obtained in the present study.

Method uncertainty

When the uncertainty of the result is reported, the combined standard uncertainty is multiplied with a so-called coverage factor, yielding an expanded uncertainty. A factor $k = 2$ was used because of the resemblance of the expanded uncertainty to a 95% confidence interval. The document no. SANTE/12682/2019 recommended a default expanded uncertainty of 50% to be used by regulatory authorities in cases of enforcement decisions (MRL exceedances) [25].

Our results showed a relative uncertainty (%) ranging from 3.01 (Carbofuran) to 34.03% (Propyzamide) at levels of 10 μgL^{-1}.

The expanded uncertainty, expressed as percentage (MU%, Table 2), for each pesticide was determined in each fortification level. As can be seen in Table 2, the MU calculated for each pesticide showed values below 50%. The mean expanded measurement uncertainty (U) for all levels was 14.36%. These results were in accordance with the acceptable criteria established in SANTE/12682/2019 document. Thus, the measurement uncertainty for these pesticides expressed as expanded uncertainty and in terms of relative standard deviation (at 95 % confidence level) will be within the EU accepted range of 50 % (table 2). The recovery of most pesticides was in the range from 70 % to 120 %, the reproducibility expressed as relative standard deviation was less than 20%. The validated method which uses the LC-MS/MS provides appropriate linearity, a very high sensitivity, good repeatability and can be applied with the high reliability to the analysis of pesticide residues in trace levels.

The maximum residue levels (MRLs)

Based on European Union regulations, maximum residue levels (MRLs) of pesticides in honey are have MRLs are in the range of 0.01–0.05 $mgkg^{-1}$ Table 4, but there are exceptions, for example, Fipronil MRL = 0.005 $mgkg^{-1}$. Therefore, the determination of pesticide residues in honey is a challenge, especially because of the low concentration of analytes and large amounts of interfering substances which can be co-extracted with analytes. and in most cases, adversely affect analysis results [36, 37]. Table 4 shown MRL detected in honey according to EUPD and Substance authorized in the EU (as a plant protection product)

Table 4: Tested pesticides authorized reported in honey as a plant protection product and its MRL according to EUPD

Pesticides	[a] Substance authorized in the EU (as a plant protection product)	EUPD MRLs (mg/Kg)[b]
Acephate	yes	0.02
Acetamiprid	yes	0.05
Aldicarb	no	0.01 (=default MRL)
Ametryn	no, because not listed	
Aminocarb	no, because not listed	
Atrazine	yes	0.05
Azaconazole	no, because not listed	
Azinphos-methyl	yes	0.05

Table 4 (continued)

Pesticides	[a] Substance authorized in the EU (as a plant protection product)	EUPD MRLs (mg/Kg)[b]
Azoxystrobin	yes	0.05
Benalaxyl	yes	0.05
Bitertanol	yes	0.05
Boscalid	yes	0.15
Bromuconazole	yes	0.05
Cadusafos	no	0.01 (=default MRL)
Carbaryl	yes	0.05
Carbendazim	yes	1
Carbofuran	yes	0.05
Carboxin	yes	0.05
Chloridazon	yes	0.1
Chloroxuron	yes	0.05
Cyanazine	no, because not listed	
Cycluron	no, because not listed	
Diazinon	no	0.01 (=default MRL)
Dicrotophos	no, because not listed	
Diethofencarb	yes	0.05
Dimethenamide	yes	0.05
Dimethirimol	no, because not listed	
Dimethomorph(E)	yes	0.05
Dimethomorph(Z)	yes	0.05
Dimoxystrobin	yes	0.05
Diniconazole	yes	0.05
Diuron	yes	0.05
DMST	no, because not listed	
Ethiofencarb	no, because not listed	
Ethirimol	yes	0.05
Etrimfos	no, because not listed	
Fenamidone	yes	0.05
Fenamiphos	no	0.01 (=default MRL)
Fenpiclonil	no, because not listed	
Fenpropimorph	yes	0.05
Fluometuron	yes	0.05
Flusilazol	yes	0.05
Fuberidazole	yes	0.05
Furathiocarb	no, because not listed	
Heptenophos	no, because not listed	
Hexaconazole	no, because not listed	
Hexazinone	no, because not listed	
Imazalil	yes	0.05
Imidacloprid	yes	0.05
Ipconazole	yes	0.05
Iprobenfos	no, because not listed	
Iprovalicarb	yes	0.05
Isoproturon	yes	0.05
Kresoxim methyl	yes	0.05
Malaoxon	yes	0.05
Malathion	yes	0.05
Mandipropamid	yes	0.05
Mefenpyr-Diethyl	no, because not listed	
Mepronil	no, because not listed	
Metalaxyl	yes	0.05
Metamitron	yes	0.05
Metazachlor	yes	0.05
Metconazole	yes	0.05

Table 4 (continued)

Pesticides	[a] Substance authorized in the EU (as a plant protection product)	EUPD MRLs (mg/Kg)[b]
Methoxyfenozide	yes	0.05
Nitenpyram	no, because not listed	
Ofurace	no, because not listed	
Oxadixyl	no	0.01 (=default MRL)
Paclobutrazol	yes	0.05
Paraoxon-methyl	no	0.01 (=default MRL)
Penconazole	yes	0.05
Pencycuron	yes	0.05
Phosphamidon	yes	0.05
Picoxystrobin	yes	0.05
Pirimicarb	yes	0.05
Prochloraz	yes	0.15
Promecarb	no, because not listed	
Prometon	no, because not listed	
Prometryn	no, because not listed	
Propachlor	yes	0.02
Propazine	no, because not listed	
Propiconazole	yes	0.05
Propoxur	no, because not listed	
Propyzamide	yes	0.05
Pyraflufen-ethyl	yes	0.05
Pyrifenox	no, because not listed	
Quinalphos	yes	0.05
Secbumeton	no, because not listed	
Simazine	no	0.01 (=default MRL)
Spiroxamine	yes	0.05
Tebutam	no, because not listed	
Tebuthiuron	no, because not listed	
Terbumeton	no, because not listed	
Terbutryn	no, because not listed	
Tetrachlorvinphos	no, because not listed	
Thiaclo9prid	no, because not listed	
Thiamethoxam	yes	0.05
Thiodicarb	yes	0.2
Triazophos	yes	0.05
Tricyclazole	yes	0.05
Zoxamide	yes	0.05

[a]MRL= Maximum residue limits; [b]EUPD EU Pesticides Database (http://ec. europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=homepage&language=EN)

4. CONCLUSIONS

The validated method has been proved to be successful as a real quantitative, multi-residue method for 101 pesticide residues analysis in honey, which is known to be a difficult matrix. It can be recommended for routine application in monitoring studies or surveys. Very good analytical results were obtained, including recovery, precision, limit of quantification, and uncertainty. Mean recovery values were within the range of 70–120% satisfied the European Community recommendations for pesticide residues in SANTE/12682/2019 document. In conclusion, the present procedure proved to be a useful tool for simultaneous determination of pesticides residues in honey, moreover our study is recommended that regulatory agencies conduct surveillance programs at the national level to protect consumer health to assess pesticide residues in honey to avoid unnecessary consumer exposure to these toxic compounds.

5. COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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