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## Rapid determination of residues of pesticides in honey by $\mu$ GC-ECD and GC-MS/MS: Method validation and estimation of measurement uncertainty according to document No. SANCO/12571/2013

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### ABSTRACT

A simple and straightforward method for simultaneous determination of residues of 13 pesticides in honey samples (acrinathrin, bifenthrin, bromopropylate, cyhalothrin-lambda, cypermethrin, chlorfenvinphos, chlorpyrifos, coumaphos, deltamethrin, fluralinate-tau, malathion, permethrin and tetradifon) from different pesticide classes has been developed and validated. The analytical method provides dissolution of honey in water and an extraction of pesticide residues by n-Hexane followed by clean-up on a Florisil SPE column. The extract was evaporated and taken up by a solution of an injection internal standard (I-IS), ethion, and finally analyzed by capillary gas chromatography with electron capture detection (GC- $\mu$ ECD). Identification for qualitative purpose was conducted by gas chromatography with triple quadrupole mass spectrometer (GC-MS/MS). A matrix-matched calibration curve was performed for quantitative purposes by plotting the area ratio (analyte/I-IS) against concentration using a GC- $\mu$ ECD instrument. According to document No. SANCO/12571/2013, the method was validated by testing the following parameters: linearity, matrix effect, specificity, precision, trueness (bias) and measurement uncertainty. The analytical process was validated analyzing blank honey samples spiked at levels equal to and greater than 0.010 mg/kg (limit of quantification). All parameters were satisfactorily compared with the values established by document No. SANCO/12571/2013. The analytical performance was verified by participating in eight multi-residue proficiency tests organized by BIPEA, obtaining satisfactory z-scores in all 70 determinations. Measurement uncertainty was estimated according to the top-down approaches described in Appendix C of the SANCO document using the within-laboratory reproducibility relative standard deviation combined with laboratory bias using the proficiency test data.

### ARTICLE HISTORY

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### KEYWORDS

Uncertainty estimation; top-down approach; honey; SANCO 12571/2013; pesticide residues

### Introduction

The presence of pesticide residues in food is one of the most critical problems in food safety.

Honey, because of its great use by sensitive population groups such as children and elderly, requires special attention to ensure a healthy and safe product.

Honey is regarded as a valuable natural food product: It is used as a natural sweetener and additive in bakery products consumed by many people around the world. It is being used as a natural sweetener in food manufacturing practices, cosmetics, and as a medicine in the treatment of various human infections.<sup>[1–3]</sup> The nutritional and quality aspects of honey are very important; also, safety of honey is crucial as it determines consumer acceptance, and moreover additives and preservatives are not allowed.<sup>[4–7]</sup>

However, there is recent concern regarding the presence of insecticides, acaricides, herbicides and fungicides in certain honey samples as reported by several studies.<sup>[8–13]</sup> In order to prevent economic losses, beekeepers treat their colonies with pesticides, using them inside hives, which implies a

risk of direct pollution of honey and other products thereof.<sup>[9,10]</sup>

For example, Acaricides, used to treat honeybee (*Apis mellifera* L.) colonies to control *Varroa destructor*,<sup>[14–19]</sup> is a worldwide threat to honeybee health.

Considerable attention is focused on regulating the allowable limits of pesticide residues in honey.<sup>[18–21]</sup> Consequently a new analytical method is required to quantify pesticides at low levels of concentration in honey and honeybee products. Several analytical methods with high precision and accuracy<sup>[8–13]</sup> have been reported for the measurement of pesticides to determine not only their presence but also their concentrations in honey samples. The purpose of this study is to develop a method for determining 13 pesticides (acrinathrin, bifenthrin, bromopropylate, cyhalothrin-lambda, cypermethrin, chlorfenvinphos, chlorpyrifos, coumaphos, deltamethrin, fluralinate-tau, malathion, permethrin and tetradifon) at levels equal to or greater than 0.010 mg/kg. Maximum Residue Levels (MRL), according to the European Union (EU) legislation, are reported in Table 1. In particular, the aim of this study is to estimate

**Table 1.** Pesticides MRL according to EU legislation.

Pesticide	Maximum residue level (mg/kg)	Legislation	Note
Acrinathrin	0.05*	Reg. (EC) No. 839/2008	
Bifenthrin	0.01*	Reg. (EU) No. 441/2012	
Bromopropylate	0.01*	Reg. (EU) No. 310/2011	
Cyhalothrin-lambda	0.05*	Reg. (EU) No. 834/2013	
Cypermethrin	0.05*	Reg. (EU) No. 520/2011	
Chlorfenvinphos	0.01*	Reg. (EU) No. 1138/2013	
Chlorpyrifos	0.01		Default MRL of 0.01 mg/kg according to Art. 18 (1) (b) Reg. No. 396/2005
Coumaphos	0.01	Not approved [Reg. (EC) No. 1107/2009]	Default MRL of 0.01 mg/kg according to Art. 18 (1) (b) Reg. No. 396/2005
Deltamethrin	0.03*	Reg. (EU) No. 441/2012	
Fluvalinate-tau	0.01*	Reg. (EC) No. 149/2008	According to SANCO/12427/2014 MRL will be 0.05* mg/kg in the near future
Malathion	0.02*	Reg. (EU) No. 270/2012	According to SANCO/11658/2014 MRL will be 0.05* mg/kg in the near future
Permethrin	0.01		Default MRL of 0.01 mg/kg according to Art. 18 (1) (b) Reg. No. 396/2005
Tetradifon	0.05*	Reg. (EU) No. 310/2011	

\* Concentration must be considered as an LOQ.

measurement uncertainty using validation and proficiency tests (PT) data instead of a different, more complex and time-consuming approach as recommended by Eurachem,<sup>[22]</sup> Nordtest,<sup>[23]</sup> EuroLab<sup>[24]</sup> and Codex CAC/GL 59-2006<sup>[25]</sup> guidelines. The “top-down” approach suggested by the SANCO document appears to be the most suitable and straightforward one for the evaluation of measurement uncertainty in pesticide residue testing laboratories. For this purpose, the estimation of measurement uncertainty was carried out as described in Appendix C of the SANCO document<sup>[26]</sup> by participating in eight proficiency tests organized by International Bureau For Analytical Studies (BIPEA), obtaining satisfactory z-scores in all 70 determinations.

## Materials and methods

### Reagents and standards

Standard reference materials of 12 pesticides were purchased from Supelco (Milan, Italy) in solution at a concentration of 10  $\mu\text{g}/\text{mL}$  in cyclohexane. Standard reference materials of ethion, injection internal standard (I-IS), and deltamethrin were purchased from Dr. Ehrenstorfer GmbH (Ausburg, Germany) in solution at a concentration of 10  $\mu\text{g}/\text{mL}$  in cyclohexane. Honey

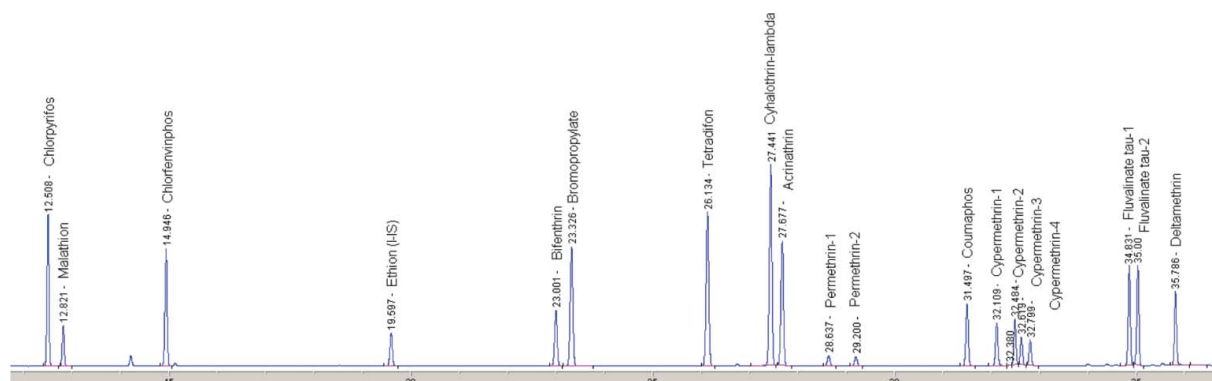
proficiency test materials were purchased from BIPEA (Paris, France).

Deionized water was obtained by Millipore Milli-Q system (Billerica, MA, USA). Isooctane RS ( $\geq 99.5\%$ ); n-Hexane RS ( $\geq 99\%$ ); diethyl ether RS ( $\geq 99.7\%$ ); sodium sulfate anhydrous ( $\geq 99\%$ ) and sodium chloride were obtained from Carlo Erba (Milan, Italy). SPE Florisil columns (500 mg/6 mL) were supplied by IST (Bologna, Italy).

Working standard solutions were prepared in isooctane from reference standard materials and stored at  $-20^\circ\text{C}$ . Matrix-matched standard solutions for calibration were prepared on the day of analysis. Honey samples from different floral origins were purchased from market (Perugia, Italy) and tested for absence of analytes of interest and possible sources of interference as described by the SANCO document by checking the absence of signal above 30% of reporting limit (RL) at pesticide retention time (RT)  $\pm 0.2$  min.

### Gas chromatography $\mu$ -ECD and mass spectrometry systems and operating conditions

Gas chromatography was carried out using an Agilent 6890N GC system equipped with a 7683 series automatic autosampler, a PTV injector and a  $\mu$ -ECD detector, all controlled by Chemstation Software Rev B.04.03 [54] (Agilent Technologies, Santa Clara, CA, USA).



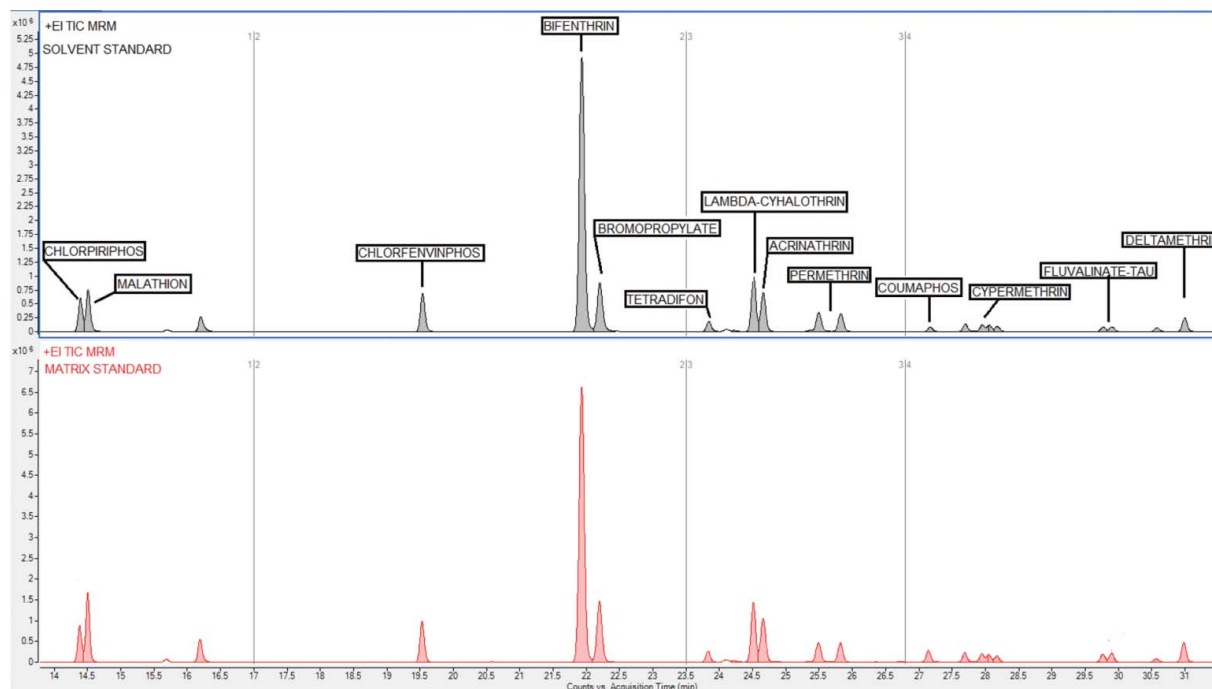
**Figure 1.** Chromatogram of matrix-matched standard solution at 0.1  $\mu\text{g}/\text{mL}$  by GC- $\mu$ ECD.

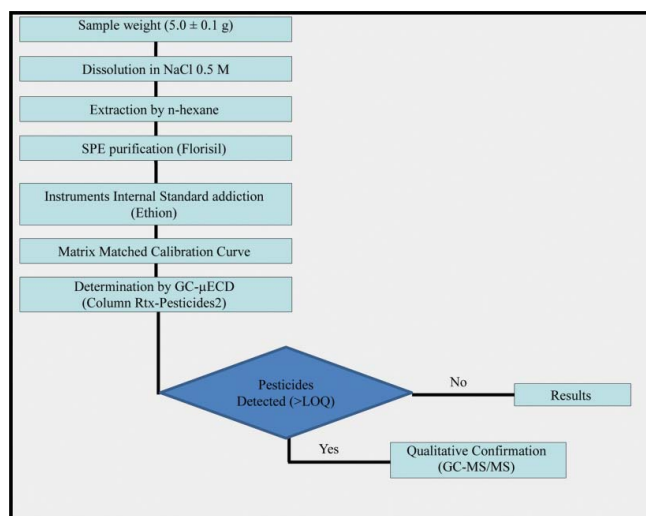
**Table 2.** GC-MS/MS parameter.

Compound name	Precursor ion (m/z)	Product ion (m/z)	Retention time (min)	Dwell time (ms)	Collision energy (V)
Chlorpyrifos	197	169	14.6	30	15
	314	258			15
Malathion	127	99	14.9		5
	158	125			5
Chlorfenvinphos	323	267	16.6		15
	267	159			10
Ethion (I-IS)	231	185	19.9		30
	231	129			10
Bifenthrin	181	165	22.1		25
	181	115			50
Bromopropylate	341	185	22.4		15
	341	155			40
Tetradifon	229	201	24.3		15
	227	164			30
Acrinathrin	208	181	24.5		10
	289	93			10
Cyhalothrin-lambda	197	141	24.5		25
	181	152			10
Permethrin	183	168	25.7–26.0		10
	183	153			15
Coumaphos	362	109	27.7		15
	362	81			40
Cypermethrin	163	127	28.1–28.4–28.5–28.6		5
	181	152			25
Fluvalinate-tau	250	200	30.2–30.3		20
	250	130			40
Deltamethrin	253	174	31.4		5
	253	93			10

Chromatographic separation (Fig. 1) was carried out using an Rtx-Pesticides2 column (30 m × 0.25 mm i.d. × 0.20 μm) supplied by Superchrom (Milan, Italy) in temperature-programmed mode as follows: 1 min at 80°C, increased to 190°C at 50°C/min, kept at 190°C for 2 min, increased to 260°C at 3°C/min, kept at 260°C for 0 min, finally increased to 300°C at 5°C/min and kept at 300°C for 6 min. Sample solution, 1 μL, was injected in splitless mode and the PTV injector temperature was programmed as

follows: 0.08 min at 100°C; increased to 300°C at 700°C/min, kept at 300°C for 20 min. Qualitative identification was conducted by an Agilent 7980A gas chromatographer equipped with a 7693 autosampler coupled with an Agilent 7000 triple quadrupole mass spectrometer with electron impact ion source (EI), all controlled by MassHunter workstation software Rev B.06.01.1312. The same Rtx-Pesticides2 column (30 m × 0.25 mm i.d. × 0.20 μm, Fig. 2) was used for qualitative confirmation of samples above reporting

**Figure 2.** Chromatogram of standard at 1.0 μg/mL by GC-MS/MS ((a) solvent; (b) matrix-matched).



**Figure 3.** Scheme of extraction purification. GC- $\mu$ ECD analysis and GC-MS/MS qualitative confirmation.

**Table 3.** Solvent calibration parameter in GC- $\mu$ ECD.

Molecule	r2	Slope (a)	Intercept (b)
Acrinathrin	0.997	2.423	0.001
Bifenthrin	0.993	1.068	0.017
Bromopropylate	0.994	2.379	0.001
Cyhalothrin-lambda	1.000	3.746	-0.035
Cypermethrin	1.000	2.769	-0.024
Chlorfenvinphos	0.994	1.962	0.002
Chlorpyrifos	0.994	2.322	-0.001
Coumaphos	1.000	1.179	0.006
Deltamethrin	0.997	2.880	-0.061
Fluvalinate-tau	1.000	3.533	-0.059
Malathion	0.990	0.604	0.017
Permethrin	0.999	0.408	0.016
Tetradifon	1.000	3.322	0.011

limit in temperature-programmed mode as follows: 0 min at 80°C, increased to 160°C at 20°C/min, kept at 190°C for 0 min, increased to 300°C at 5°C/min, kept at 300°C for 3 min. Sample solution, 2  $\mu$ L, was injected in splitless mode and the PTV injector

temperature was programmed as follows: 0.08 min at 100°C; increased to 300°C at 700°C/min, kept at 300°C for 20 min. Mass Spectrometer operates in multiple reaction monitoring (MRM) in positive ion mode. Two product ions for each pesticide were monitored, and data about mass parameters are reported in Table 2.

### Extraction and clean-up

Extraction and clean-up steps are summarized in Fig. 3. Homogenized honey, 5.0  $\pm$  0.1 g, was weighed and dissolved by 10 mL of 0.5 M NaCl solution for 5 min by Vortex, to which 10 mL of extraction solvent (n-hexane) was added. The extract was centrifuged for 10 min at 4,500 g by a Hettich (Tuttlingen, Germany) Rotina 420 R, and the supernatant extract was further purified. Purification was carried out using SPE Florisil columns, activated in oven at 105°C overnight. About 100 mg of sodium sulfate anhydrous was added on top of SPE column and conditioned with 10 mL of n-hexane. After the conditioning step, 5 mL of n-hexane extract was quantitatively loaded on the SPE column, and the column was washed with 2 mL of n-hexane. Elution of analytes from SPE column was carried out by filling the column with 10 mL of diethyl ether. The elution fraction was evaporated to dryness at 35°C under a gentle stream of nitrogen. The purified extract was finally re-suspended in 250  $\mu$ L of ethion solution at 0.5  $\mu$ g/mL.

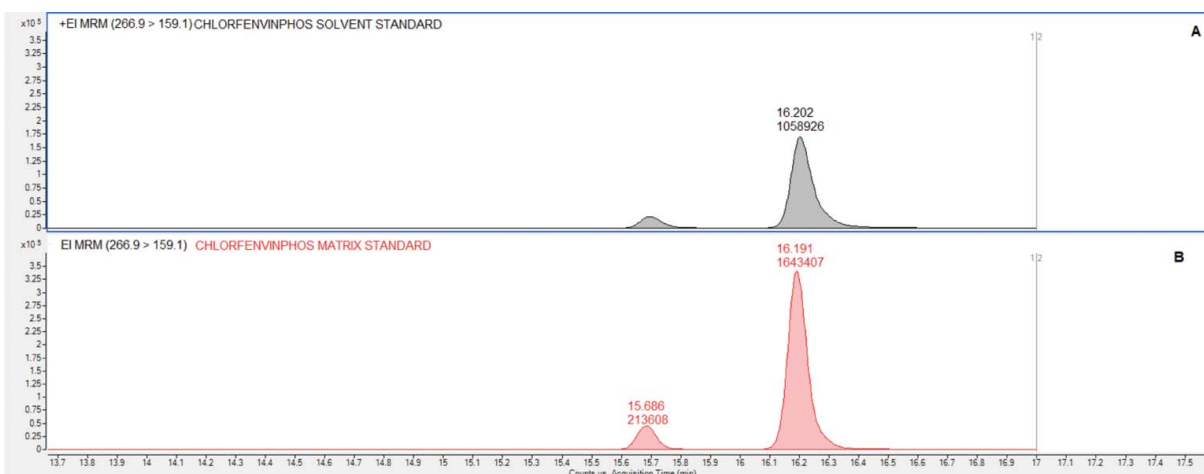
## Results and discussion

### Method validation

The performance parameters of the method in terms of repeatability, linearity, recovery, specificity and sensitivity were established by a single laboratory validation. A blank sample of honey from the market was selected for validation purposes in terms of trueness and precision, while the data from proficiency tests were used for estimation of measurement uncertainty as described in Appendix C of document No. SANCO\12571\2013.

### Linearity and matrix effect

Calibration curves have been produced for quantification. Calibration study for each analyte was in isooctane. Calibration



**Figure 4.** Chlorfenvinphos standard solution at 0.1  $\mu$ g/mL (A solvent; B matrix-matched) different peak shape and signal response.

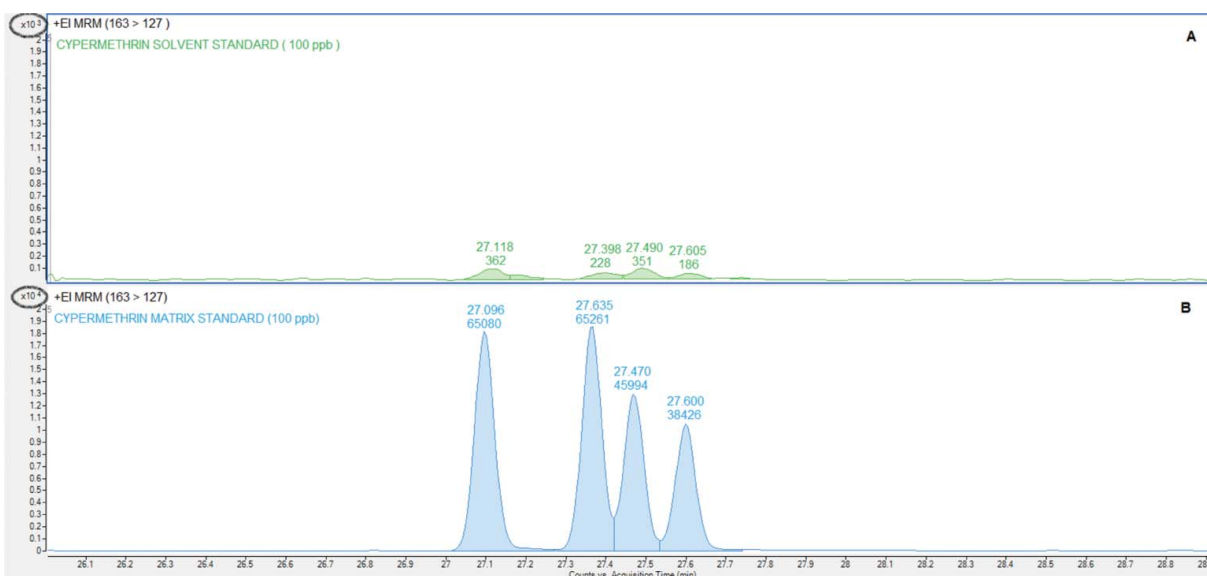


Figure 5. Cypermethrin standard solution at 0.1 µg/mL (A solvent; B matrix-matched) different peak shape and signal response.

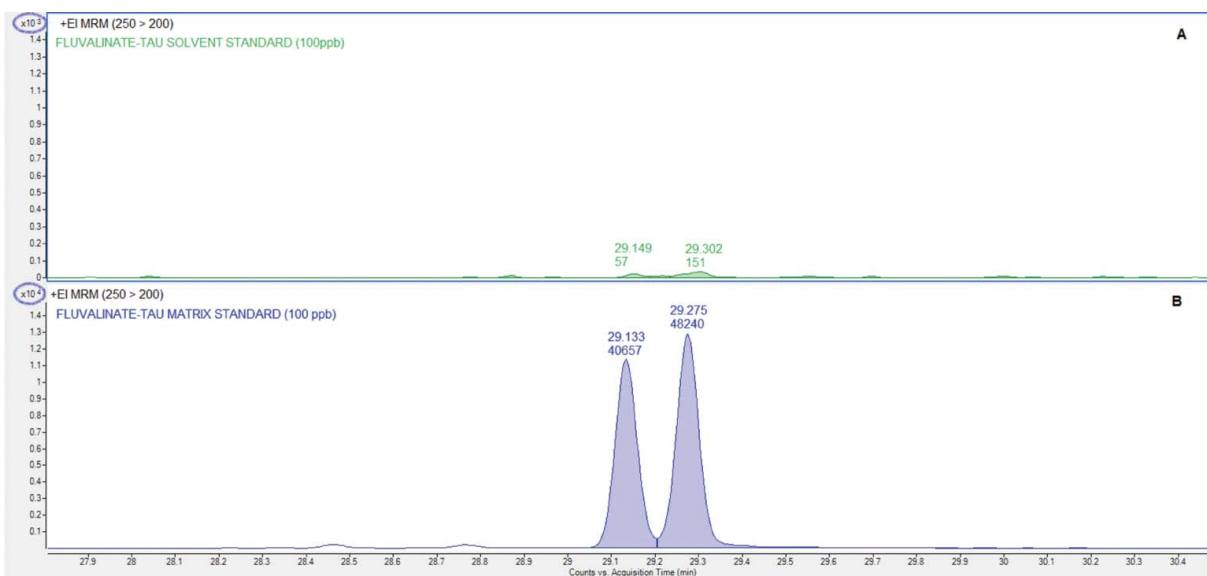


Figure 6. Fluralinate-tau standard solution at 0.1 µg/mL (A solvent; B matrix-matched) differed peak shape and signal response.

Table 4. Matrix calibration parameter in GC-µECD.

Molecule	r2	Slope (b)	Intercept (a)
Acrinathrin	0.993	2.174	0.001
Bifenthrin	1.000	1.137	0.003
Bromopropylate	1.000	2.668	-0.000
Cyhalothrin-lambda	0.991	2.960	0.018
Cypermethrin	0.999	2.320	-0.039
Chlorfenvinphos	0.999	0.649	0.013
Chlorpyrifos	1.000	2.470	0.011
Coumaphos	1.000	1.347	-0.013
Deltamethrin	0.998	3.153	-0.074
Fluralinate-tau	0.999	3.785	-0.083
Malathion	0.999	0.649	0.013
Permethrin	0.999	0.493	0.013
Tetradifon	0.998	3.283	-0.066

Table 5. Matrix calibration parameter in GC-MS/MS.

Molecule	r2	Slope (b)	Intercept (a)
Acrinathrin	0.999	1.064	-0.033
Bifenthrin	0.998	7.039	0.144
Bromopropylate	1.000	1.529	-0.003
Cyhalothrin-lambda	1.000	1.070	-0.022
Cypermethrin	0.999	0.889	-0.011
Chlorfenvinphos	0.999	0.967	0.002
Chlorpyrifos	1.000	1.041	0.003
Coumaphos	0.999	0.461	0.013
Deltamethrin	0.999	0.387	-0.013
Fluralinate-tau	0.997	0.534	-0.021
Malathion	1.000	1.958	-0.002
Permethrin	1.000	0.775	0.000
Tetradifon	1.000	0.499	0.003

**Table 6.** Trueness and precision.

Molecule	Spiking level (n = 6)			
	0.010 mg/kg		0.100 mg/kg	
	Rec (%)	RSDr	Rec (%)	RSDr
Acrinathrin	70	10.1	82	8.3
Bifenthrin	81	6.2	86	6.4
Bromopropylate	106	5.1	97	5.0
Cyhalothrin-lambda	80	8.7	85	7.3
Cypermethrin	92	8.0	89	6.6
Chlorfenvinphos	103	4.1	96	7.0
Chlorpyrifos	100	8.4	96	5.5
Coumaphos	105	3.9	93	7.2
Deltamethrin	93	2.3	83	0.8
Fluvalinate-tau	80	8.7	82	7.1
Malathion	115	4.1	101	5.6
Permethrin	87	13.6	90	6.4
Tetradifon	103	7.1	100	5.4

curves ranged from 0.1 to 2.0  $\mu\text{g/mL}$  (0.1–0.25–0.5–1.0–1.5–2.0  $\mu\text{g/mL}$ ) while ethion (I-IS) was 0.5  $\mu\text{g/mL}$ . Peak area ratio (analyte/I-IS) was plotted as a function of concentration for

each analyte, including non-experimental point (0; 0). The calibration curves were injected in both GC- $\mu\text{ECD}$  and GC-MS/MS to check instrumental linearity, which was evaluated in two different ways: correlation coefficient ( $r^2$ ) and deviation of single point from the interpolated curve on the y-axis. In this case deviation must not exceed 20%. (document No. SANCO 12571/2013, C17). In GC- $\mu\text{ECD}$ , all curves fulfilled SANCO requirements, and data are reported in Table 3.

In GC-MS/MS no satisfactory correlation was found for many analytes (Figs. 4–6) due to poor signal and peak shape (chlorfenvinphos, cypermethrin, fluvalinate-tau, etc.).<sup>[27]</sup> To enhance signal and peak shape in GC-MS/MS, matrix-matched calibration curves were made at the same concentration listed above by preparing honey samples, free from analytes, and re-suspending purified extracts by analytes solution and I-IS at suitable concentrations.

Calibration solution in matrix was injected in GC- $\mu\text{ECD}$  and GC-MS/MS and satisfactory results were obtained for both systems. Data are reported in Tables 4 and 5. Furthermore, matrix effect was evaluated comparing calibration curves in

**Table 7.** Row data for estimation of uncertainty component arising from method and laboratory bias, estimated from PT data.

PT name	Pesticide	Lab result	PT-assigned value		DS PT	Qn	No. results	$\sqrt{\text{No}}$	$\frac{Q_n}{\sqrt{\text{No}}}$	
		mg/kg	(bias' <sup>2</sup> )							
October 2010 BIPEA 01/3619	Bromopropylate	0.037	0.042	0.0142	0.015	0.36	9	3.00	0.119	
	Chlorfenvinphos	0.075	0.107	0.0894	0.033	0.31	9	3.00	0.103	
	Coumaphos	0.056	0.055	0.0003	0.020	0.36	9	3.00	0.121	
	Fluvalinate-tau	0.195	0.192	0.0002	0.093	0.48	10	3.16	0.153	
	Malathion	0.22	0.192	0.0213	0.054	0.28	10	3.16	0.089	
December 2010 BIPEA 02/3619	Bromopropylate	0.166	0.182	0.0077	0.054	0.30	10	3.16	0.094	
	Chlorfenvinphos	0.151	0.174	0.0175	0.033	0.19	11	3.32	0.057	
	Coumaphos	0.124	0.139	0.0116	0.035	0.25	11	3.32	0.076	
	Fluvalinate-tau	0.022	0.045	0.2612	0.021	0.47	13	3.61	0.129	
	Malathion	0.082	0.085	0.0012	0.024	0.28	12	3.46	0.082	
April 2011 BIPEA 03/3619	Bromopropylate	0.059	0.069	0.0210	0.015	0.22	13	3.61	0.060	
	Chlorfenvinphos	0.013	0.016	0.0352	0.005	0.31	13	3.61	0.087	
	Coumaphos	0.131	0.139	0.0033	0.041	0.29	14	3.74	0.079	
	Fluvalinate-tau	0.08	0.092	0.0170	0.03	0.33	15	3.87	0.084	
	Malathion	0.026	0.029	0.0107	0.009	0.31	13	3.61	0.086	
June 2011 BIPEA 04/3619	Bromopropylate	0.164	0.156	0.0026	0.034	0.22	12	3.46	0.063	
	Chlorfenvinphos	0.158	0.142	0.0127	0.041	0.29	12	3.46	0.083	
	Coumaphos	0.098	0.081	0.0440	0.04	0.49	15	3.87	0.128	
	Fluvalinate-tau	0.179	0.135	0.1062	0.048	0.36	14	3.74	0.095	
	Malathion	0.106	0.094	0.0163	0.024	0.26	13	3.61	0.071	
October 2011 BIPEA 05/3619	Acrinathrin	0.039	0.064	0.1526	0.053	0.83	10	3.16	0.262	
	Bifenthrin	0.266	0.29	0.0068	0.156	0.54	10	3.16	0.170	
	Bromopropylate	0.083	0.076	0.0085	0.023	0.30	12	3.46	0.087	
	Chlorfenvinphos	0.145	0.127	0.0201	0.037	0.29	12	3.46	0.084	
	Chlorpyrifos	0.231	0.181	0.0763	0.084	0.46	10	3.16	0.147	
	Coumaphos	0.104	0.089	0.0284	0.048	0.54	12	3.46	0.156	
	Cyhalothrin-lambda	0.111	0.109	0.0003	0.038	0.35	10	3.16	0.110	
	Cypermethrin	0.168	0.125	0.1183	0.048	0.38	10	3.16	0.121	
	Fluvalinate-tau	0.247	0.278	0.0124	0.161	0.58	12	3.46	0.167	
	Malathion	0.061	0.057	0.0049	0.029	0.51	9	3.00	0.170	
	Permethrin	0.137	0.114	0.0407	0.044	0.39	9	3.00	0.129	
	Tetradifon	0.168	0.115	0.2124	0.061	0.53	8	2.83	0.188	
	December 2011 BIPEA 06/3619	Acrinathrin	0.135	0.186	0.0752	0.084	0.45	9	3.00	0.151
		Bifenthrin	0.076	0.113	0.1072	0.035	0.31	10	3.16	0.098
		Bromopropylate	0.042	0.041	0.0006	0.009	0.22	11	3.32	0.066
Chlorfenvinphos		0.085	0.095	0.0111	0.017	0.18	12	3.46	0.052	
Chlorpyrifos		0.042	0.044	0.0021	0.008	0.18	11	3.32	0.055	
Coumaphos		0.15	0.158	0.0026	0.044	0.28	13	3.61	0.077	
Cyhalothrin-lambda		0.035	0.051	0.0984	0.02	0.39	10	3.16	0.124	
Cypermethrin		0.086	0.085	0.0001	0.023	0.27	8	2.83	0.096	
Fluvalinate-tau		0.098	0.168	0.1736	0.08	0.48	13	3.61	0.132	
Malathion		0.106	0.092	0.0232	0.026	0.28	10	3.16	0.089	
Permethrin		0.098	0.109	0.0102	0.032	0.29	8	2.83	0.104	
Tetradifon		0.051	0.048	0.0039	0.014	0.29	9	3.00	0.097	

**Table 8.** Row data for estimation of uncertainty component arising from method and laboratory bias estimated from PT data.

PT name	Pesticide	Lab result	PT assigned value mg/kg	(bias <sub>i</sub> ) <sup>2</sup>	DS PT	Q <sub>n</sub>	No. results	√No	$\frac{Q_n}{\sqrt{N_o}}$	
April 2012 BIPEA 07/3619	Acrinathrin	0.084	0.081	0.0014	0.02	0.25	9	3.00	0.082	
	Bifenthrin	0.015	0.019	0.0443	0.007	0.37	7	2.65	0.139	
	Bromopropylate	0.083	0.092	0.0096	0.017	0.18	10	3.16	0.058	
	Chlorfenvinphos	0.138	0.147	0.0037	0.041	0.28	10	3.16	0.088	
	Chlorpyrifos	0.144	0.13	0.0116	0.018	0.14	11	3.32	0.042	
	Coumaphos	0.025	0.027	0.0055	0.007	0.26	11	3.32	0.078	
	Cyhalothrin-lambda	0.157	0.178	0.0139	0.042	0.24	8	2.83	0.083	
	Cypermethrin	0.148	0.135	0.0093	0.031	0.23	8	2.83	0.081	
	Deltamethrin	0.094	0.075	0.0114	0.016	0.21	7	2.65	0.081	
	Fluvalinate-tau	0.014	0.015	0.0044	0.004	0.27	11	3.32	0.080	
	Malathion	0.108	0.088	0.0517	0.023	0.26	10	3.16	0.083	
	Permethrin	0.016	0.02	0.0400	0.006	0.30	8	2.83	0.106	
	Tetradifon	0.171	0.168	0.0003	0.039	0.23	9	3.00	0.077	
	June 2012 BIPEA 08/3619	Acrinathrin	0.136	0.135	0.0001	0.056	0.41	10	3.16	0.131
		Bifenthrin	0.105	0.132	0.0418	0.033	0.25	9	3.00	0.083
		Bromopropylate	0.123	0.164	0.0625	0.033	0.20	11	3.32	0.061
Chlorfenvinphos		0.015	0.018	0.0278	0.003	0.17	11	3.32	0.050	
Chlorpyrifos		0.162	0.181	0.0110	0.038	0.21	12	3.46	0.061	
Coumaphos		0.196	0.168	0.0278	0.041	0.24	12	3.46	0.070	
Cyhalothrin-lambda		0.043	0.053	0.0356	0.015	0.28	10	3.16	0.089	
Cypermethrin		0.017	0.016	0.0039	0.004	0.25	7	2.65	0.094	
Deltamethrin		0.18	0.16	0.0156	0.04	0.25	9	3.00	0.083	
Fluvalinate-tau		0.067	0.079	0.0231	0.021	0.27	12	3.46	0.077	
Malathion		0.172	0.17	0.0001	0.031	0.18	11	3.32	0.055	
Permethrin		0.178	0.196	0.0084	0.054	0.28	9	3.00	0.092	
Tetradifon		0.052	0.05	0.0016	0.009	0.18	10	3.16	0.057	
No. of results (m)				$\sum (bias_i)^2$	2.40308	70		$\sum \frac{Q_n}{\sqrt{N_o}}$	6.8739	70

**Table 9.** Raw data for estimation of uncertainty component arising from in-house validation.

Pesticide	Spiking level	[c] (mg/kg)	Recovery	Spiking level	[c] (mg/kg)	Recovery
Malathion	~ 0.010 (mg/kg)	0.011	1.14	~ 0.100 (mg/kg)	0.106	1.06
Chlorfenvinphos		0.010	1.00		0.093	0.93
Bromopropylate		0.011	1.10		0.097	0.97
Coumaphos		0.010	1.06		0.092	0.92
Fluvalinate-tau		0.008	0.86		0.088	0.89
Malathion		0.012	1.17		0.092	0.92
Chlorfenvinphos		0.010	1.02		0.085	0.85
Bromopropylate		0.011	1.13		0.089	0.89
Coumaphos		0.011	1.06		0.081	0.81
Fluvalinate-tau		0.009	0.88		0.074	0.74
Malathion		0.012	1.23		0.099	1.00
Chlorfenvinphos		0.010	1.06		0.096	0.97
Bromopropylate		0.010	1.09		0.097	0.97
Coumaphos		0.010	1.10		0.092	0.93
Fluvalinate-tau		0.008	0.85		0.084	0.85
Malathion		0.011	1.14		0.107	1.07
Chlorfenvinphos		0.010	1.04		0.104	1.04
Bromopropylate		0.009	0.98		0.104	1.04
Coumaphos		0.010	1.06		0.100	1.00
Fluvalinate-tau		0.007	0.75		0.085	0.86
Malathion		0.011	1.10		0.100	1.00
Chlorfenvinphos		0.010	1.00		0.097	0.97
Bromopropylate		0.010	1.03		0.096	0.96
Coumaphos		0.010	0.99		0.093	0.93
Fluvalinate-tau		0.008	0.76		0.076	0.77
Malathion		0.011	1.12		0.103	1.04
Chlorfenvinphos		0.011	1.11		0.100	1.01
Bromopropylate		0.011	1.05		0.100	1.01
Coumaphos		0.010	1.01		0.098	0.99
Fluvalinate-tau		0.007	0.71		0.079	0.80
Chlorpyrifos		0.010	1.05		0.099	0.99
Bifenthrin		0.008	0.83		0.093	0.93
Tetradifon		0.011	1.10		0.104	1.04
Cyhalothrin-lambda		0.009	0.90		0.096	0.93
Acrinathrin		0.008	0.77		0.092	0.92
Permethrin		0.010	1.06		0.097	0.97
Cypermethrin		0.010	1.00		0.095	0.95
Deltamethrin		0.009	0.93		0.084	0.84

solvent with matrix-matched calibration curves by GC- $\mu$ ECD. The slopes of the two curves were compared using t-test to evaluate their differences. A significant alpha-level of 0.05 (degrees of freedom,  $n = 12$ , critical; t-value for  $n - 4$  degrees of freedom = 2.306) was chosen. According to Soliani,<sup>[28]</sup> experimental t-values for each analyte were calculated by Eq. (1):

$$t_{(n-4)} = \frac{(b_1 - b_2)}{es(b_1 - b_2)}, \quad (1)$$

where  $(n - 4)$  is the total degrees of freedom;  $b_1$  and  $b_2$  are the slopes of the two curves and  $es(b_1 - b_2)$  is the standard error of the difference between the two slopes. According to Eq. (1), no significant matrix effect was evidenced for any analyte, except for chlorfenvinphos, because experimental t-value was lower than reference t-value (2.306). In order to prevent bad performances in GC-MS/MS and to overcome matrix effect for chlorfenvinphos, matrix-matched calibration was chosen for quantitative purpose.

### Specificity

In conformity with the SANCO validation protocol (Appendix A), specificity was evaluated by detector response at the retention time of analyte  $\pm 0.2$  min by verifying absence of signals above 30% of the reporting level (0.01 mg/kg). In order to evaluate this effect, 20 blank honey samples of different floral origins were analyzed by both instruments, and no signal above the limit mentioned above was found, ensuring specificity of the method.

### Trueness, precision, repeatability and practical limit of quantification (LOQ)

To investigate the precision and trueness of the proposed GC- $\mu$ ECD method, six independent replicate analyses of honey spiked with all 13 pesticides at two different levels (0.010 and 0.100 mg/kg) were performed. Concentrations and recoveries were calculated from the matrix-matched calibration curves. Blank analyses were performed to determine possible interference from the sample. Trueness (as recovery %) and precision in repeatability conditions (as RSD<sub>r</sub>) were calculated for each batch and the results were compared with the values stated by document No. SANCO 12571/2013. Results are reported in Table 6.

**Table 10.** Row data for estimation of uncertainty component arising from in-house validation.

Pesticide	Spiking level	[c] (mg/kg)	Recovery	Spiking level	[c] (mg/kg)	Recovery
Chlorpyrifos	~ 0.010 (mg/kg)	0.011	1.11	~ 0.100 (mg/kg)	0.087	0.87
Bifenthrin		0.009	0.86		0.079	0.79
Tetradifon		0.011	1.10		0.091	0.91
Cyhalothrin-lambda		0.009	0.85		0.079	0.77
Acrinathrin		0.007	0.74		0.074	0.74
Permethrin		0.009	0.95		0.083	0.83
Cypermethrin		0.010	1.00		0.082	0.82
Deltamethrin		0.009	0.92		0.083	0.83
Chlorpyrifos		0.009	0.93		0.094	0.94
Bifenthrin		0.008	0.85		0.088	0.89
Tetradifon		0.010	1.04		0.098	0.98
Cyhalothrin-lambda		0.008	0.82		0.088	0.86
Acrinathrin		0.008	0.76		0.084	0.85
Permethrin		0.009	0.87		0.091	0.92
Cypermethrin		0.009	0.94		0.090	0.90
Deltamethrin		0.009	0.94		0.083	0.82
Chlorpyrifos		0.009	0.88		0.101	1.01
Bifenthrin		0.008	0.78		0.090	0.90
Tetradifon		0.009	0.90		0.105	1.05
Cyhalothrin-lambda		0.007	0.73		0.092	0.90
Acrinathrin		0.007	0.68		0.086	0.86
Permethrin		0.008	0.77		0.095	0.95
Cypermethrin		0.008	0.83		0.094	0.94
Deltamethrin		0.009	0.89		0.083	0.84
Chlorpyrifos		0.010	1.00		0.093	0.93
Bifenthrin		0.008	0.77		0.080	0.80
Tetradifon		0.010	0.99		0.097	0.97
Cyhalothrin-lambda		0.008	0.76		0.082	0.80
Acrinathrin		0.007	0.65		0.076	0.76
Permethrin		0.008	0.82		0.084	0.84
Cypermethrin		0.009	0.87		0.082	0.82
Deltamethrin		0.009	0.94		0.083	0.83
Chlorpyrifos		0.010	1.04		0.098	0.99
Bifenthrin		0.007	0.74		0.084	0.85
Tetradifon		0.011	1.06		0.102	1.03
Cyhalothrin-lambda		0.008	0.75		0.086	0.85
Acrinathrin		0.006	0.59		0.079	0.80
Permethrin		0.007	0.74		0.087	0.88
Cypermethrin		0.009	0.86		0.088	0.89

As shown in Table 6, all values fulfilled the requirements of Table 1 of Document No. SANCO 12571/2013. According to this, the LOQ for all pesticides was fixed at 0.01 mg/kg, which represents the smallest amount of analyte that can be reliably detected or differentiated from the background for a particular matrix (by a specific method). The LOQ proposed fulfills the requirement of European legislation because it is equal to or lower than the MRL reported in Table 1.

### Estimation of measurement uncertainty

Measurement uncertainty was estimated according to the top-down approaches described in Appendix C of the SANCO document using the within-laboratory reproducibility relative standard deviation combined with the laboratory bias using proficiency test data applying Eq. (2):

$$u' = \sqrt{u'(RSD_{WR})^2 + u'(bias)^2} = 0.2407, \quad (2)$$

where:

$u'$  is the combined standard uncertainty,

$u'(RSD_{WR})$  is the within-laboratory reproducibility,

$u'(bias)$  is the uncertainty component arising from the method and laboratory bias estimated from the proficiency test data.

The expanded measurement uncertainty ( $U'$ ), calculated by multiplying the combined standard uncertainty ( $u'$ ) obtained in Eq. (2) by a coverage factor  $k = 2$ , was below 50%, and therefore, according to SANCO, a default value of 50% for expanded uncertainty can be used by regulatory authorities in cases of enforcement decisions (MRL exceedance) as reported in Eq. (3),

$$U' = k \times u' = 2 \times 0.2407 = 0.48. \quad (3)$$

Raw data for evaluation of each component of Eq. (2), used to calculate measurement uncertainty, are reported in Tables 7–10 and Eqs. (4)–(7):

$$u'(bias) = \sqrt{RMS'_{bias^2} + u'(c_{ref})^2} = 0.2097, \quad (4)$$

$$RMS'_{bias} = \sqrt{\sum \frac{(bias_i)^2}{m}} = 0.1853, \quad (5)$$

$$\frac{u'(c_{ref}) = \sum_i \frac{Q_n}{\sqrt{N_o}}}{m = 0.0982}, \quad (6)$$

$$RSD_{WR} = 0.1181. \quad (7)$$

### Conclusions

A rapid method has been developed and optimized for simultaneous determination of different pesticides from honey samples. A matrix-matched standard calibration is required to ensure effective qualitative and quantitative analysis of pesticides in purified extracts of honey.

Measurement uncertainty estimation is a requirement of ISO/IEC 17025 for routine analysis. The recommendation to use the

expanded uncertainty of 50% as described by the SANCO Document is a useful approach to standardize this parameter throughout the EU and to homogenize the assessment of conformity of food processed independently by each laboratory. The introduction of standardized approaches on how to calculate the uncertainty itself is very useful to obtain comparable results from different laboratories and facilitate the method of calculation. Our results showed an expanded uncertainty of about 48%, which is compliant to SANCO Document.

The method developed has been routinely tested by participation in at least one proficiency test each year in last five years, achieving acceptable z-score ( $z\text{-score} < |2|$ ) for all pesticides. Moreover it was used for about four years in the official control of over 100 honey samples of different botanical and geographical origins (EU and extra EU) and none of them was found non-compliant.

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