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Effectiveness of accelerated solvent extraction compared to QuEChERS methods for the multiresidue analysis of pesticides in organic honey by GC-MS/MS

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Keywords

Pressurized fluid extraction, multiresidue pesticide analysis, in-line clean up, Rocket Evaporator, TSQ 8000 Triple Quadrupole GC-MS, Dionex ASE 350 Accelerated Solvent Extractor

Goal

Compare in-line ASE methods cleanup conditions for the evaluation of pesticides in honey to QuEChERS methods.

Introduction

According to the Council Directive 2001/110/EC,[1] honey is considered as a "viscous, aromatic, natural, and sweet fluid produced by *Apis mellifera* bees[2,3] from the nectar of flowers or secretions of live parts of certain plants or excretions of insects." It has been used in medicine since ancient times for treatment of burns, gastrointestinal disorders, asthma, infected wounds, and skin ulcers.[4]

World production of honey has increased in the last 20 years. In 2014, 1.5 million tons of honey were produced worldwide, with China accounting for 31% of the world total followed by Turkey, the United States, Ukraine, and the Russian Federation.

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There are two main forms of contamination of honey: cross-contamination as bees collect pollen and nectar and contamination through treatment of hives with insecticides, fungicides, and acaricides for the protection from parasites like *Varroa destructor, Acarapis woodi*, and *Paenibacillus larvae.*

Although some organizations, such as the U.S. Food and Drug Administration (FDA)[5] and the Canadian Food Inspection Agency,[6] approve the use of certain veterinary drugs, the European Union does not accept the use of antimicrobial drugs in beekeeping. The use of veterinary medicinal products in beekeeping is regulated by the European Council (EC,1804/1999). According to this regulation, the use of allopathic chemically synthesized medicinal products for preventive treatments in beekeeping is prohibited, as these fat-soluble and nonvolatile compounds can accumulate in the stored honey, where they are able to migrate from the wax comb. The Council Regulation 1804/1999 EC[7] is very restrictive regarding the production of organic honey in terms of the origin of bees, siting of the apiaries, feed, disease prevention, and veterinary treatments. In particular, it establishes that plants that can be foraged by bees, either biological or spontaneous, must be at least 3 km from any source of pollution and from any non-agricultural production sources, that can possibly lead to contamination, such as industrial areas, urban centers, or motorways.

The determination of pesticide residues in honey is a considerable analytical challenge, as honey is a mixture of more than 300 substances identified mainly as sugars and waxes, subject to variations arising from the type of plant(s) where bees collect nectar.[8] Its complexity requires a selective sample preparation because carbohydrates and other matrix substances can be co-extracted with the analytes. These unwanted co-extractables can cause buildup of nonvolatile materials on the GC injection port and the analytical column, resulting in poor analytical results and high instrument maintenance costs.

Among the available extraction techniques, accelerated solvent extraction (ASE) is characterized by shorter extraction times and reduced solvent consumption. ASE uses high temperatures combined with high pressure. The high temperature allows a higher rate of extraction due to a reduction of the viscosity and surface tension, and increases the solubility and diffusion rate into the sample. At the same time, high pressure prevents the solvents from reaching their boiling point and promotes penetration into the sample. Recent advances using ASE systems are described in several publications,[9,10] and include procedures for selective removal of interferences during sample extraction, thus combining extraction and purification into a single step.

The methods reported here are applicable for the determination of 53 pesticides, including acaricides and insecticides (both chlorinated and non-chlorinated) in organic honey and in concentrations from 1 to 100 ng/g.

Experimental

Honey Samples

This study included 45 organic honey samples. In particular, 10 orange blossom honey samples were from a German beekeeper and 35 were from Italian beekeepers of two different regions: Calabria (south Italy, 15 samples), where intensive citrus orchards are present, and Trentino-Alto Adige (north Italy, 20 samples), where intensive apple orchards are present. All samples were stored at -20°C until analysis to prevent decomposition.

Equipment

A Radwag analytical balance was used for weighing the honey samples. The extractions were carried out with a Thermo Scientific™ Dionex™ ASE™ 350 Accelerated Solvent Extractor [P/N 083114 (120 V) or 083146 (240 V)], shown in Figure 1A, equipped with 34 mL stainless steel extraction cells. The extracts were collected in 60 mL vials (Thermo Scientific, P/N 048784), treated with sodium sulfate and directly concentrated in a 2 mL autosampler glass vial (Thermo Scientific™ Chromacol™ VAGK ISP: GC 2-SVW + 9-SCK(B)-ST1) with the Thermo Scientific™ Rocket™ Evaporator [P/N 075904 (120 V) or 082766 (240 V)], shown in Figure 1B. The samples were analyzed with a Thermo Scientific™ TRACE™ 1310 Gas Chromatograph equipped Split/Splitless Injector, a fused-silica capillary column Thermo Scientific™ TraceGOLD™ TG-5SilMS (30 m × 0.25 mm × 0.25 µm), and a Thermo Scientific™ TSQ™ 8000 Triple Quadrupole GC-MS/MS.

Figure 1. Dionex ASE 350 accelerated solvent extractor (A) and Thermo Scientific Rocket Evaporator (B).

Chemicals and reagents

Acrinathrin, bifenthrin, boscalid, bromopropylate, buprofezin, chlorfenvinphos, chlorpyrifos-methyl, chlorothalonil, cyfluthrin, cyhalothrin, cypermethrin, deltamethrin, diazinon dichlorvos, difenoconazole, dimethoate, endosulfan (α, β, and sulfate), ethion, ethoprophos, fenamiphos, fenitrothion, fenpropathrin, lindane (γ-HCH), iprodion, malathion, methamidophos, oxadixyl, permethrin, phosalone, pirimiphos-methyl, procymidon, propargite, propiconazole, pyridaben, quinoxyfen, tebuconazole, tetradifon, triadimefon, and vinclozolin were purchased from Sigma-Aldrich (St. Louis, MO, USA).

QuEChERS extraction tubes (50 mL; 4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate dihydrate tribasic, and 500 mg sodium citrate dibasic sesquihydrate), 15 mL QuEChERS clean-up tubes (1200 mg $MgSO_4$, 400 mg CUPSA/ 400 mg CE C18), acetonitrile (LC-MS grade), hexane (pesticide residue grade), Florisil® 60–100 mesh for column chromatography, 1 mL NORM-JECT® Tuberkulin syringes, and 0.2 µm nylon filters were purchased from Fisher Scientific (Schwerte, Germany).

Diatomaceous earth and ASE cellulose filters were purchased from Thermo Fisher Scientific (Waltham, MA, USA); ethylacetate (Lichrosolv for LC) was purchased from Merck (Darmstadt, Germany); Supelclean™ PSA (primary-secondary amine) was purchased from Supelco Analytical (Bellofonte, PA, USA).

The stock solution, at a concentration of 10 µg/mL, was prepared by dissolving the pesticide standards in hexane or acetonitrile for ASE extraction and QuEChERS, respectively. Stock solutions were stored at –40°C. Calibration solutions containing all the investigated compounds, in hexane or acetonitrile, were prepared daily from the stock solutions (10 µg/mL). The appropriate volume was used as a spiking solution.

Accelerated solvent extraction

A cellulose filter (Thermo Scientific, P/N 056780) was placed in the bottom of a 34 mL extraction cell (Figure 2), followed by 2 g of PSA (primary-secondary amine) or Florisil and another cellulose filter. A 5 g sample of honey was homogenized with an equal weight of Thermo Scientific™ Dionex™ ASE Prep DE (Thermo Scientific P/N 062819), and transferred into the cell. One milliliter of acetonitrile solution containing the internal standard (FBDE, 3-fluoro-2,2,4,4,6 pentabromodiphenyl ether) was added. The remaining empty volume was filled with Dionex ASE Prep DE. The extractor was programmed according to the conditions reported in Tables 1A and 1B.

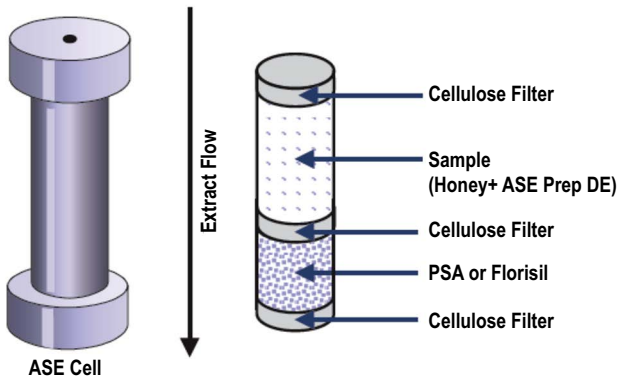

Figure 2. Extraction cell schematic.

Table 1A. Conditions for accelerated solvent extraction (acetonitrile solvent).

ASE program for acetonitrile solvent	
Adsorbent:	PSA (2 g)
Temperature:	Ambient
Pressure:	1500 psi
Static Cycles:	2
Extraction Time:	5 min
Rinse Volume:	90%
Purge Time:	90 s

Table 1B. Conditions for accelerated solvent extraction (*n*-hexane/ethyl acetate solvent).

ASE program for *n*-hexane/ethyl acetate solvent (4:1, v/v)	
Adsorbent:	Florisil (2 g)
Temperature:	100°C
Pressure:	1500 psi
Static Cycles:	2
Extraction Time:	5 min
Rinse Volume:	90%
Purge Time:	90 s

The extracts were collected in 60 mL vials and treated with sodium sulfate to remove any possible water. After filtration, the organic phase was concentrated to dryness in the Rocket Evaporator, reconstituted in 1 mL of acetonitrile or hexane, and submitted for analysis by GC-MS/MS. The GC conditions are summarized in Tables 2A and 2B, and the MS conditions are summarized in Table 3.

Table 2A. Injector conditions.

Injector program (PTV, splitless mode)	
Injector Temperature:	250°C
Liner	2 mm x 2.75 mm x 120 mm, Siltek-deactivated
Injected Volume:	1 µL
Splitless Time:	1 min
Splitflow:	10 mL/min
Surge Pressure:	5 kPa
Initial Temperature:	80°C (0.05 min) 14.5°C/s to 200°C (1 min) 4.5°C/s to 320°C
Final Temperature:	320°C (12 min – cleaning phase)

Table 2B. GC conditions.

GC program	
GC Column:	TG-5SilMS (35 m x 0.25 mm x 0.25 µm)
Carrier Gas:	Helium, 99.999% purity
Flow Rate	1.0 mL/min, constant
Initial Temperature:	80°C (3 min) 10°C/min to 170°C 3°C/min to 190°C 2°C/min to 240°C 3°C/min to 280°C 10°C/min to 310°C
Final Temperature:	310°C (5 min)

Table 3. Mass spectrometer parameters.

MS parameters	
Source Temperature:	270°C
Ionization:	EI
Electron Energy:	70 eV
Emission Current:	50 µA
Q2 Gas Pressure (Argon):	1.5 mTorr
Collision Energy:	6 to 38 eV
Q1 Peak Width FWHM:	0.7 Da
Q3 Peak Width FWHM:	0.7 Da

QuEChERS extraction

QuEChERS extraction tubes (50 mL) were used for extraction. Honey (5 g) was weighed and transferred into the extraction tube, followed by 1 mL of acetonitrile solution containing the internal standard (FBDE). Acetonitrile (10 mL) was added, the tube was vigorously shaken for 10 min and centrifuged for 5 min at 5000 rpm. Supernatant (8 mL) was transferred into a 15 mL QuEChERS clean-up tube, vigorously shaken for 10 min, and centrifuged for 5 min at 5000 rpm. An aliquot of 1 mL was filtered using a 0.2 µm nylon syringe filter directly into a GC vial and submitted for analysis by GC-MS/MS.

Validation parameters

Validation was carried out following the 2015 European Union SANTE guidelines.[11] The selectivity of the method was evaluated by injecting extracted pesticide-free honey samples. The absence of signal above a signal-to-noise ratio of 3, at the retention times of the target compounds, was the parameter used to show that the method was free of interferences.

An uncontaminated honey sample was selected as control and used for the procedures of optimization and validation (QuEChERS and ASE); 5 g of the control honey was spiked by adding different volumes of the standard working solution in order to have the following concentrations: 1, 5, 10, 25, 50, and 100 ng/g, in relation to pesticide MRLs, when available, and to generate a matrix-matched calibration curve. As defined in the SANTE guidelines, the LOQ of the methods was the lowest validated spiked level meeting the requirements of recovery within the range of 70–120% and RSD ≤20%.

Finally, the extraction methods were evaluated for their repeatability, linearity, and recovery. Recoveries were calculated by comparing the concentrations of the extracted compounds with those from the matrix-matched calibration curves at three different fortification levels (10, 50, and 100 ng/g). The repeatability (evaluated as coefficient of variation, (CV %) was calculated by analyzing six replicates at a concentration of 50 ng/g.

The mass spectrometer was operated in selected reaction monitoring mode (SRM), detecting two or more transitions per analyte, which are listed together with the particular collision energies (CE) in Table 4. Identification of the compounds was carried out by comparing sample peak relative retention times with those obtained for standards under the same conditions and the MS/MS fragmentation spectra obtained for each compound.

Table 4A. Retention times, quantifications and confirmation transitions for the investigated compounds (* internal standard). CE = collision energy.

Compound	RT	Quantification Transition (CE)	Confirmation Transitions (CE)
Acrinathrin	25.67	181→152 (22)	208.1→180.9 (8) 289→93.1 (8)
Bifenthrin	23.72	181→165.9 (10)	165.1→163.6 (24) 181→179 (12)
Boscalid	30	112→76 (12)	139.9→76 (22) 139.9→112 (10)
Bromopropylate	23.81	340.8→185 (14)	184.9→75.5 (30) 184.9→156.9 (12)
Buprofezin	19.47	105.1→77 (18)	105.1→50.9 (32) 175→132.1 (12)
Chlorfenvinphos	17.31	266.9→159 (16)	266.9→ 203 (10) 323→266.9 (14)
Chlorfenvinphos-isomer 1	17.31	322.9→267 (12)	266.9→159 (14) 268.8→161 (12)
Chlorfenvinphos-isomer 2	17.31	266.9→159 (14)	294.9→267 (8) 323→267 (12)
Chlorothalonil	13.7	265.8→170 (24)	228.8→168 (8) 265.8→133 (36)
Chlorpyrifos-methyl	14.68	285.9→93 (20)	125→47 (12) 125→79 (6)
Cyfluthrin-isomer 1	27.67	163→65.1 (26)	163→91.1 (12) 163→127.1 (6)
Cyfluthrin-isomer 2	27.88	163→91.1 (12)	163→127 (6) 206→151.1 (18)
Cyfluthrin-isomer 3	27.98	163→91.1 (12)	163→127 (6) 226→206.1 (12)
Cyfluthrin-isomer 4	28.08	163→91.1 (12)	163→127 (6) 226→206.1 (10)
Cyhalothrin-R	25.67	197→141.1 (10)	180.9→151.9 (22) 208.1→180.9 (8)
Cyhalothrin-S	25.67	181→151.9 (22)	208.1→151.8 (28) 208.1→180.9 (8)
Cypermethrin-isomer 1	28.38	163→91.1 (12)	163→127.1 (6) 180.9→152.1 (20)
Cypermethrin-isomer 2	28.49	163→91 (12)	163→127 (6) 180.9→151.9 (18)
Cypermethrin-isomer 3	28.59	163→91 (12)	163→127 (6) 163→152.1 (12)
Cypermethrin-isomer 4	28.68	163→91 (12)	163→127.1 (6) 180.9→152.2 (20)
Deltamethrin	31.9	181→152.1 (22)	252.8→92.9 (16) 252.8→172 (8)

Table 4B. Retention times, quantifications and confirmation transitions for the investigated compounds (* internal standard). CE = collision energy.

Compound	RT	Quantification Transition (CE)	Confirmation Transitions (CE)
Diazinon	13.34	137.1→84.1 (12)	137.1→54.1 (20) 179.1→121.5 (26)
Dichlorvos	8.31	109→79 (6)	185→93 (12) 186.9→93 (12)
Difenoconazole-isomer 1	31.35	323→265 (14)	265→139 (36) 265→202.1 (16)
Difenoconazole-isomer 2	31.45	323→265 (16)	265→138.9 (36) 265→202 (18)
Dimethoate	12.7	87→42.1 (10)	93→63 (8) 125→79 (8)
Endosulfan I	18.57	240.6→205.9 (14)	194.7→125 (22) 194.7→159.4 (8)
Endosulfan II	20.54	158.9→123 (12)	194.7→159 (8) 240.6→205.8 (12)
Endosulfan sulfate	21.85	271.7→236.8 (12)	238.7→203.9 (12) 271.7→234.9 (12)
Ethion	20.64	153→97 (10)	230.9→128.9 (22)
Ethoprop (Ethoprophos)	11.64	157.9→96.9 (16)	157.9→113.9 (6) 200→158 (6)
Fenamiphos	18.64	154→139 (10)	216.9→202 (12) 303.1→195.1 (8)
Fenitrothion	14.68	127→79 (8)	277→109 (16) 277→260 (6)
Fenpropathrin	24.07	97.1→55.1 (6)	181→126.8 (28) 181→151.9 (22)
Lindane (γ-HCH)	13.27	216.89→180.91 (8)	218.89→182.91 (8)
Iprodione	23.44	314→245 (10)	315.7→247 (10) 315.7→273 (8)
Malathion	14.68	92.8→63 (8)	125→79 (8) 173.1→99 (12)
Methamidophos	8.03	141→64 (18)	141→79 (20) 141→94.8 (8)
Oxadixyl	20.58	163.1→117 (24)	131.9→117 (16) 163.1→132.1 (8)
Permethrin-isomer 1	27.25	183.1→168 (12)	163→91.1 (12) 183.1→153 (12)
Permethrin-isomer 2	27.51	183→168.1 (10)	183→153 (14) 183→165.1 (10)
Phosalone	24.97	182→111 (14)	121.1→65 (10) 182→74.8 (30)
Pirimiphos-methyl	15.41	290.1→125 (20)	290.1→233 (8) 305.→180.1 (8)

Table 4C. Retention times, quantifications and confirmation transitions for the investigated compounds (* internal standard). CE = collision energy.

Compound	RT	Quantification Transition (CE)	Confirmation Transitions (CE)
Procymidone	26.02	181→151.9 (22)	208.1→151.8 (28) 289→93.1 (8)
Propargite	22.52	132.1→77.1 (26)	135.1→107.1 (12) 150.1→135.1 (8)
Propiconazole-isomer 1	21.73	172.9→145 (16)	172.9→74 (38) 172.9→109 (26)
Propiconazole-isomer 2	21.95	172.9→145 (14)	172.9→74 (38) 172.9→109 (24)
Pyridaben	27.56	147.1→117.1 (20)	147.1→119.1 (8) 147.1→132.1 (12)
Quinoxyfen	21.81	237→208 (26)	271.8→237.1 (12) 307→237 (18)
Tebuconazole	22.49	250v125 (20)	125→89 (16) 125→99 (16)
Tetradifon	24.77	159→131 (10)	159→74.8 (32) 159→111 (20)
Triadimefon	16.32	208→180.8 (8)	208→111 (20) 208→126.7 (12)
Vinclozolin	14.25	241.1→58.1 (12)	241.1→184.1 (10) 286.9→214 (15)
FBDE*	38.01	583.6→423.8 (10)	421.8→314.8 (30) 423.7→314.9 (30)

Results and discussion

Several methods have been proposed for the extraction of pesticides from honey samples, including solid phase extraction (SPE),[12,13] solid-phase microextraction (SPME),[14] pressurized liquid extraction (PLE),[12] solvent extraction (SE),[15] matrix solid-phase dispersion (MSPD),[16] liquid-liquid extraction and low temperature purification (LLE-LTP),[17] and QuEChERS.[12,18]

Due to the complexity of the honey matrix, which can contain a great number of sugars and other substances, such as phenolic compounds, these interferences, mainly waxes and pigments, should be removed before the chromatographic analysis to improve the limit of quantification of the method. An inappropriate cleanup can lead to adverse effects related to the quality of the generated results and to contamination of the GC system. The effectiveness of pressurized fluid extraction in combination with an in-line cleanup step for the determination of pesticides and persistent organic pollutants in honey has been previously demonstrated by the same group.[9,10] The scope of the work has now been extended in order to compare the performance of the pressurized fluid extraction with QuEChERS, both in terms of extraction solvent (acetonitrile) and interference retainer (PSA).

The linearity of the three investigated methods was very good, with coefficients of determination (r^2) > 0.98 for most of the compounds detected in both solvent and matrix. In addition, evaluating the average coefficients of determination and LOQs obtained in the matrix, the in-line ASE method using PSA as an interference retainer showed the best results compared with QuEChERS (0.9916, 7 ng/g for ASE and 0.9480, 22 ng/g for QuEChERS). Recoveries of the investigated compounds were measured at three different levels (10, 50, and 100 ng/g, Table 5). Matrix-matched standards coupled with internal standard calibration were used to compensate for potential matrix effects and to avoid any under/overestimation during quantification.

The recoveries calculated at the three concentration levels and reported in three different groups (< 70%, 70–120%, and > 120%) are presented in Figure 3. In general, the extraction and cleanup using both QuEChERS and ASE with acetonitrile and PSA yielded recoveries within the acceptable SANTE range of 70–120%. In contrast, when the pressurized fluid extraction was performed with *n*-Hexane/ethyl acetate and Florisil as adsorbent, several compounds showed recoveries below the lower limit of 70%. The repeatability, expressed as coefficient of variation (CV %), of the three investigated methods is reported in Figure 4. Most of the compounds showed a CV < 20% (blue color). The similarity in the CV between the QuEChERS and the pressurized fluid extraction with acetonitrile and PSA is remarkable.

Table 5A. Mean recovery (%) of selected pesticides in organic honey at different spiked levels. ASE*: extraction with *n*-hexane/ethyl acetate and Florisil; ASE **: extraction with acetonitrile and PSA.

Compound	Recovery %								
	QuEChERS			ASE*			ASE**		
	Fortification Level (ng/g)			Fortification Level (ng/g)			Fortification Level (ng/g)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High
	10	50	100	10	50	100	10	50	100
Acrinathrin	47	61	96	64	138	113	84	119	119
Bifenthrin	88	88	79	74	76	80	100	119	79
Boscalid	66	80	87	82	140	n.d.	114	90	101
Bromopropylate	76	76	83	111	94	83	104	116	93
Buprofezin	72	83	84	61	75	72	91	102	98
Chlorfenvinphos	68	80	98	n.d.	9	13	112	107	116
Chlorfenvinphos-isomer 1	80	84	88	18	13	12	106	113	93
Chlorfenvinphos-isomer 2	80	84	88	18	13	12	112	107	116
Chlorothalonil	67	95	102	54	86	45	95	115	110
Chlorpyrifos-methyl	45	68	80	58	68	77	115	117	114
Cyfluthrin-isomer 1	58	58	71	72	98	103	62	95	105
Cyfluthrin-isomer 2	91	81	97	46	127	114	89	129	112
Cyfluthrin-isomer 3	64	72	92	21	99	88	65	98	89
Cyfluthrin-isomer 4	71	67	84	68	102	101	71	103	100
Cyhalothrin-R	88	84	98	91	113	104	92	104	93
Cyhalothrin-S	10	27	42	77	130	114	104	113	78
Cypermethrin-isomer 1	29	68	85	53	113	92	36	110	91
Cypermethrin-isomer 2	100	73	81	57	99	93	103	99	95
Cypermethrin-isomer 3	120	73	78	56	97	101	115	96	103
Cypermethrin-isomer 4	105	71	78	36	104	n.d.	102	108	65
Deltamethrin	86	68	89	78	106	94	101	102	119
Diazinon	87	78	82	73	71	75	109	116	113
Dichlorvos	35	51	68	9	3	n.d.	69	103	100
Difenoconazole-isomer 1	24	68	87	100	61	n.d.	126	100	121
Difenoconazole-isomer 2	127	75	82	101	89	82	130	94	106
Dimethoate	72	76	86	n.d.	n.d.	49	147	91	97
Endosulfan I	71	93	95	98	58	77	80	117	96

Table 5B. Mean recovery (%) of selected pesticides in organic honey at different spiked levels. ASE*: extraction with *n*-hexane/ethyl acetate and Florisil; ASE **: extraction with acetonitrile and PSA.

Compound	Recovery %								
	QuEChERS			ASE*			ASE**		
	Fortification Level (ng/g)			Fortification Level (ng/g)			Fortification Level (ng/g)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High
	10	50	100	10	50	100	10	50	100
Endosulfan II	75	78	82	91	83	72	85	131	110
Endosulfan sulfate	63	74	82	95	137	134	87	119	46
Ethion	85	85	91	78	90	91	98	109	118
Ethoprop (Ethoprophos)	71	75	83	53	55	63	98	117	93
Fenamiphos	64	65	97	38	23	24	81	113	108
Fenitrothion	61	88	102	82	154	132	97	118	120
Fenpropathrin	104	82	88	147	118	94	104	100	82
Lindane (γ-HCH)	70	79	87	74	82	77	104	119	98
Iprodione	121	83	92	30	159	99	120	119	142
Malathion	124	74	82	84	145	120	106	119	103
Methamidophos	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Oxadixyl	75	90	101	n.d.	n.d.	n.d.	78	84	113
Permethrin-isomer 1	83	80	86	81	81	80	105	122	118
Permethrin-isomer 2	79	83	89	74	79	80	95	109	87
Phosalone	58	69	82	93	150	129	107	104	98
Pirimiphos-methyl	76	77	84	71	81	81	112	121	109
Procymidone	80	97	83	87	124	105	102	108	101
Propargite	138	77	80	72	96	89	94	101	107
Propiconazole-isomer 1	106	90	89	80	90	85	99	108	92
Propiconazole-isomer 2	106	90	89	78	89	85	101	98	92
Pyridaben	74	80	85	82	82	83	101	114	93
Quinoxyfen	87	80	82	81	78	78	100	114	89
Tebuconazole	79	89	97	65	76	76	105	104	86
Tetradifon	60	88	88	73	89	96	94	114	116
Triadimefon	88	85	88	104	135	128	104	105	76
Vinclozolin	65	79	89	85	90	92	72	95	93

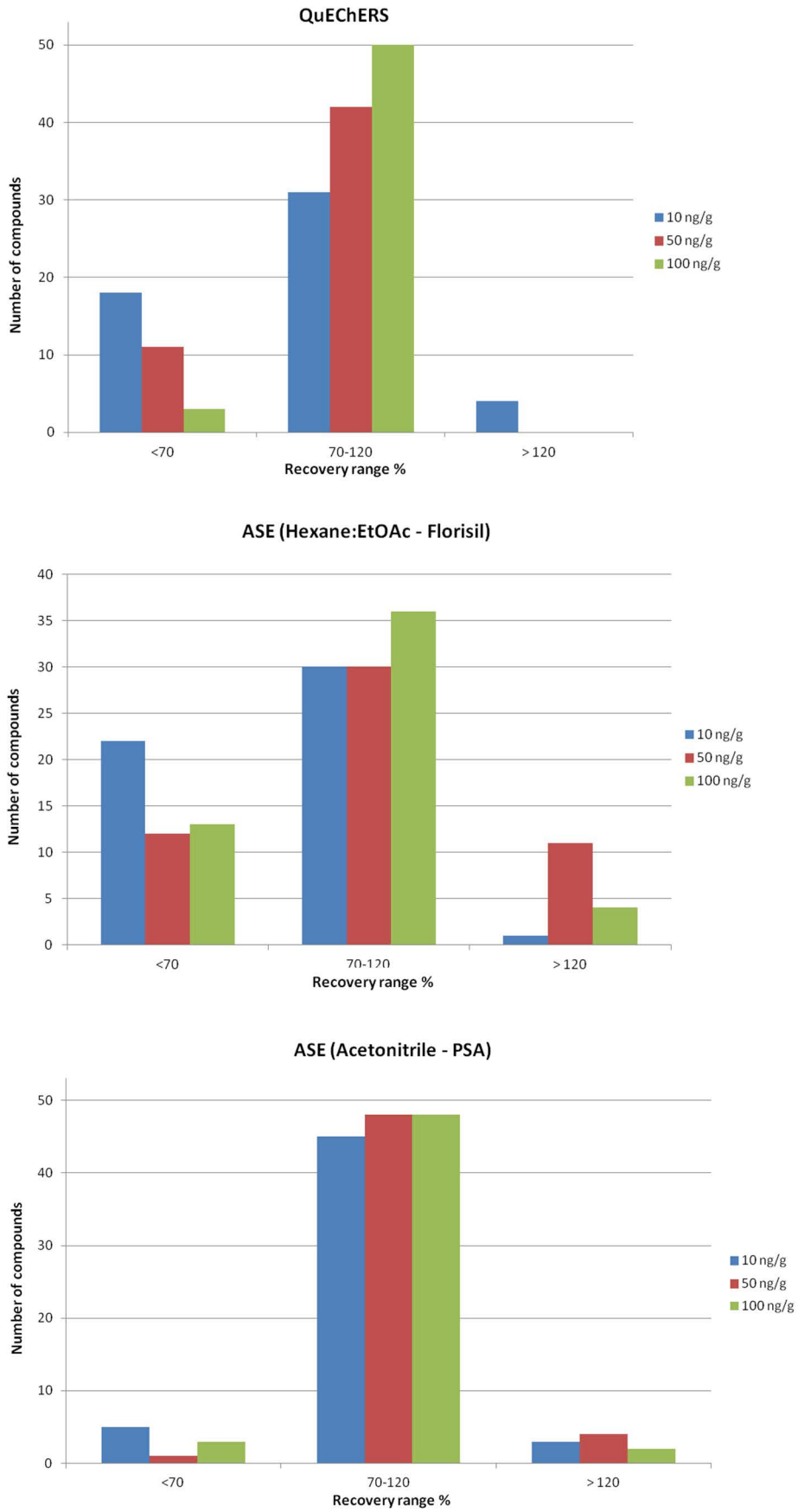

Figure 3. Recovery rates at three concentration levels. The data are reported in three different groups (< 70%, 70–120%, and > 120%).

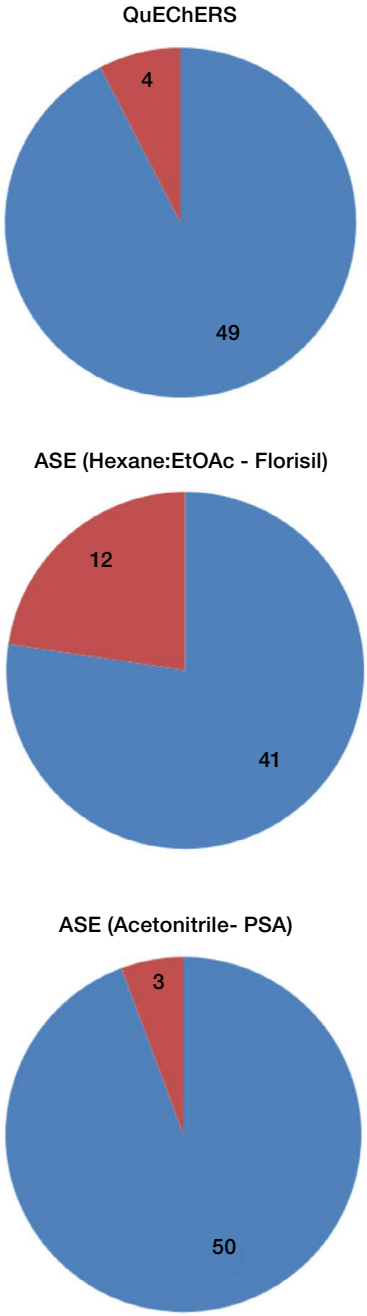

Figure 4. Repeatability (expressed as coefficient of variation – CV%) for the investigated methods. CV < 20% are in blue, CV > 20% are in red.

The method was evaluated for its repeatability, linearity, recovery, limit of detection (LOD), and limit of quantification (LOQ). The LODs and LOQs were calculated from the calibration curve in the concentration range corresponding to the lower concentration levels according to MRL for each pesticide when the standard was available. LOD was calculated using the equation LOD = 3.3 SD0/slope, where SD0 is the residual standard deviation. The limit of quantification was calculated as LOQ = 3 LOD. Recovery of the analytes studied was carried out at a fortification level of 50 ng/g while the method repeatability (expressed as coefficient of variation, CV%) was evaluated analyzing six replicates each by adding known quantities of POPs standard solution (10 ng/g) to 2 g of honey.

Among the 53 investigated pesticides, boscalid, diazinon, and chlorpyrifos-methyl were found in all the honey samples. All these compounds are used in apple and citrus orchards.[19,20] Diazinon and boscalid were found in all samples from Trentino-Alto Adige at concentrations ranging from 1.13 to 1.15 ng/g, while in samples from Calabria they were detected with a prevalence of 64% and a maximum concentration of 1.14 ng/g. Intensively cultivated apple and citrus plantations are subject to an extensive use of pesticides to control most agricultural pests, even if the integrated pests management (IPM) system is applied during the growing season, leading to a contamination of bee products.

Chlorpyrifos-methyl has also been detected at high concentrations in honey samples from Germany, Figure 5. This is of no surprise as chlorpyrifos, an organophosphate insecticide, acaricide, and miticide used primarily to control foliage and soil-borne insect pests on a variety of food and feed crops, is one of the most used compounds worldwide.[20] Remarkably, no MRLs are provided for this compound.

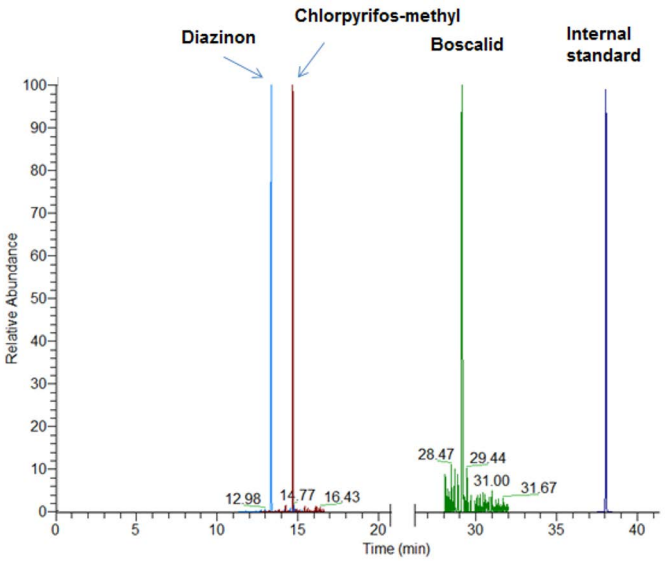

Figure 5. GC-MS/MS chromatogram of a naturally contaminated organic honey sample.

Table 6. Pesticide concentrations (in ng/g) and detection frequency in organic honey samples.

Pesticide	Organic Honey Samples - Germany					
	Percentile					Detection Frequency
	Min	25th	50th	75th	Max	(n= 10)
Boscalid	n.d.	10.04	10.15	10.42	10.67	86%
Diazinon	n.d.	n.d.	1.13	1.13	1.14	64%
Chlorpyrifos-methyl	n.d.	n.d.	n.d.	8.67	389.50	29%
	Organic Honey Samples – South Italy (Calabria)					
Boscalid	n.d.	n.d.	3.12	3.18	6.68	74%
Diazinon	n.d.	n.d.	n.d.	2.54	5.44	38%
	Organic Honey Samples – North Italy (Trentino					
Boscalid	1.13	1.13	1.14	1.14	1.15	100%
Diazinon	n.d.	n.d.	n.d.	n.d.	n.d.	17%

Conclusion

Two in-line ASE methods using Florisil and PSA as an interference retainer were developed and compared with QuEChERS. In particular, the extraction with acetonitrile with PSA as interferences retainer showed excellent performance in terms of recovery, linearity, and repeatability for all the 53 investigated pesticides according to the 2015 SANTE guidelines. ASE with in-line cleanup is cost-effective and minimizes waste generation compared with traditional methods. The combination of extraction and cleanup in a single step drastically reduces the time required for the analysis. This study is the first to compare in-line ASE methods cleanup conditions to QuEChERS for the evaluation of pesticides in honey.

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