CUSTOMER APPLICATION NOTE

Determination of pesticides and persistent organic pollutants in honey by accelerated solvent extraction and GC-MS/MS

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Introduction

Honey is a natural product that is widely used for both nutritional and medicinal purposes. It is generally considered a natural and healthy product of animal origin, free of impurities[1]. However, honeybees are subject to a number of viral, bacterial, fungal, and parasitic diseases and infestations. Insecticides, fungicides, and acaricides are used to protect colonies against infestations from hive beetle and parasites like *Varroa destructor* and *Acarapis woodi*.

Although some organizations, such as the U.S. Food and Drug Administration (FDA)[2] and the Canadian Food Inspection Agency,[3] approve the use of certain veterinary drugs, the European Union does not accept the use of antimicrobial drugs in beekeeping. In the European Union, the use of veterinary medicines in beekeeping is regulated by the European Council (EC,1804/1999)[4]. According to this regulation, the use of allopathic, chemically synthesized medicinal products for preventive treatments in beekeeping is prohibited, as these fat-soluble compounds can accumulate in the stored honey, where they are able to migrate into the wax comb.

Consumer preference for natural products and the demand for quality ingredients and clean-label products, including organic honey, is increasing. European Council Regulation 1804/1999 EC is very restrictive with regard to the production of organic honey in terms of the origin of bees, location of the apiaries, feed, disease prevention, and veterinary treatments. In particular, it establishes that the

location of the apiaries must be such that, within a radius of 3 km from the apiary site, nectar and pollen sources consist essentially of organically produced crops and/or spontaneous vegetation. Additionally, a certain distance must be maintained from non-agricultural production sources that might lead to contamination, such as industrial areas, urban centers, motorways, waste dumps and waste incinerators.

Many pollutants in the environment can also contaminate the bees themselves in addition to their pollen, honey, and other bee products. Environmental pollutants include pesticides, heavy metals, bacteria, and radioactive materials. Among the environmental contaminants, studies have documented the occurrence of organochlorines (OCs), polychlorobiphenyls (PCBs), organophosphates (OPs), and polybromodiphenylethers (PBDEs) in honey. The European Commission has set the maximum residue level values (MRLs) for feed as well for food of animal origin[5,6] for these chemicals.

In particular, organochlorine pesticides are lipophilic and very stable compounds. Therefore, they are extremely persistent in the environment because they are resistant to natural breakdown processes. Due to these characteristics, these compounds tend to bioaccumulate in the food chain.

Despite high toxicity to mammals, including humans, organophosphorous pesticides (OPs) have been increasingly used in agriculture for the control and protection of crop-eating insects. Most OPs have a short half-life in the environment, as they are degraded by microorganisms and by hydrolysis on exposure to sunlight, air, and soil. Their ability to degrade made them an attractive alternative to organochloride pesticides.

Brominated flame retardants (BFRs), especially polybrominated diphenyl ethers (PBDEs), are organobromine compounds that are applied to man-made products in order to inhibit or slow down the ignition of combustible materials in case of fire. They are commonly used in a wide range of consumer goods such as electronics, cars, furniture, and construction materials. Many brominated flame retardants are persistent, bioaccumulative, and toxic to both humans and the environment and are suspected of causing neurobehavioral effects and endocrine disruption.

In 2010, the European Food Safety Authority (EFSA) was asked by the European Commission to deliver a scientific opinion on the risks to public health related to the presence of brominated flame retardants in food[7]. In 2014 the European Commission requested the member States to monitor different classes of brominated flame retardants in food, recommending limits of quantifications (LOQs) up to 0.01 ng/g[8].

Composed of ~17% water and ~83% carbohydrate, honey is low in fat, dietary fiber, and protein. The carbohydrates present are fructose (~38%) and glucose (~31%), with remaining sugars including maltose, sucrose, and other complex carbohydrates formed from incomplete breakdown of the polysaccharides present in nectar and honeydew.

The complexity of honey requires selective sample preparation because carbohydrates and other matrix substances may 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) offers shorter extraction times and reduced solvent consumption. ASE uses high temperatures combined with high pressure. A high temperature allows a higher rate of extraction due to a reduction in 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, as described in several publications,[9-31] include procedures for selective removal of interferences during sample extraction, thus combining extraction and purification into a single step.

The method reported here is applicable for the determination of four different classes of compounds (6 PCBs, 7 PBDEs, 16 OCs, and 19 OPs) in honey. Eleven additional compounds, belonging to different classes and commonly used as agrochemicals, have also been investigated. The concentration ranges are 1 to 100 ng/g for PCBs, 0.5 to 10 ng/g for PBDEs, and 5 to 100 ng/g for OCs, OPs and all the agrochemicals.

Experimental

Sample collection

Beekeepers from three different Italian regions (Figure 1): Calabria, South Italy (14 samples); Trentino Alto Adige, North Italy (18 samples) and Lombardia, North Italy (27 samples) provided 59 organic honey samples, as summarized in Table 1. All samples were stored at -20 °C until analysis to prevent matrix decomposition.

Figure 1. Areas of origin of the organic honey samples.

Equipment

The extractions were carried out using a Thermo Scientific™ Dionex™ ASE™ 350 Accelerated Solvent Extractor [P/N 083114 (120 V) or 083146 (240 V)], shown in Figure 2A, 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) using a Thermo Scientific™ Rocket™ Evaporator system [P/N 075904 (120 V) or 082766 (240 V)], shown in Figure 2B. The samples were analyzed using a Thermo Scientific™ TRACE™ 1310 Gas Chromatograph equipped with a PTV injector, a fused-silica capillary column (Rt-5MS Crossbond-5% diphenyl 95% dimethylpolysiloxane, 35 m × 0.25 mm × 0.25 µm, from Restek) and a Thermo Scientific™ TSQ™ 8000 Evo Triple Quadrupole GC-MS/MS.

Figure 2. Dionex ASE 350 Accelerated Solvent Extractor (A) and Genevac Rocket Evaporator (B).

Table 1. Areas of origin of the organic honey samples. (*IPM = Integrated Pest Management)

Number of Samples	Area of Origin	Botanical Source	Potential Environmental Contamination Sources
27	Lombardia (North of Italy)	Multifloral	Industrialized Area (OCPs, PBCs, PBDEs)
14	Trentino (North of Italy)	Multifloral	Intensive Apple Orchard (Pesticides utilized in IPM* plan)
18	Calabria (South of Italy)	Citrus (Monofloral)	Intensive Citrus Orchard (Pesticides utilized in IPM* plan)

Chemical and reagents

Mixtures of PCB congeners (PCB 28; PCB 52; PCB 101; PCB 138; PCB 153; PCB 180), PBDE congeners (PBDE 28; PBDE 33; PBDE 47; PBDE 99; PBDE 100; PBDE 153 and PBDE 154), PCB 209 (internal standard for PCBs) and 3-fluoro-2,2,4,4,6-pentabromodiphenyl ether (FBDE, internal standard for flame retardants) were purchased from AccuStandard® (New Haven, USA). A mixture of 19 standard OCs (α-HCH; Hexachlorobenzene; β-BHC; Lindane; Heptachlor; Aldrin; Heptachlor Epoxide; trans- Chlordane; 4,4'-DDE; Endosulphan I; 2,4'-DDT; Endrin; 4,4'-DDD; Endosulphan II; 4,4'-DDT and Endosulphan sulphate, Dieldrin, Endrin aldehyde and Methoxychlor was purchased from Restek® (Bellefonte, PA, USA). OP pesticide standards of Mevinphos, Ethopropos, Phorate, Diazinon, Disulfoton, Parathion-methyl, Fenchlorphos, Chlorpyrifos, Fenthion, Sulprofos, Coumaphos, Tetrachlorvinphos, Prothiofos, Terbufos, Azinphos-methyl, Chlorpyrifos, Penconazol, Captan, Bupiramate, Quinoxyfen, Fluazinam, Trifloxystrobin, Iprodion, Chlorantraniliprol, Spirodiclofen, Boscalid, and Pyraclostrobin were purchased from Sigma-Aldrich®, St. Louis, MO, USA. Florisil® (100–200 mesh) was purchased from Promochem® (Wesel, Germany). Hexane, isooctane, acetone, ethyl acetate [special grade for pesticide residue analysis (Pestanal®)] and 4-nonylphenol (IS for OCs and OPs) were purchased from Fluka® (Sigma- Aldrich, St. Louis, MO, USA). Working solutions were prepared by diluting the stock solution in hexane for pesticides and then stored at –40 °C. Mixed compound calibration solution, in hexane, was prepared daily from the stock solutions (10 µg/mL) and the appropriate volume was used as a spiking solution.

Extraction, concentration, and measurement

A cellulose filter (Thermo Scientific, P/N 056780) was placed in the bottom of a 34 mL extraction cell (Figure 3), followed by 5 g of activated Florisil and another cellulose filter. A 2 g sample of honey was homogenized with an equal weight of Thermo Scientific™ Dionex™ ASE™ Prep DE dispersant (Thermo Scientific, P/N 062819), sodium sulfate and transferred into the cell. One mL of isooctane solution containing the three internal standards was added. The remaining empty volume was filled with Dionex ASE Prep DE dispersant. The extractor was programmed according to the conditions reported in Table 2. 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 using the Rocket Evaporator system, dissolved in 200 µL of isooctane, and submitted to analysis by GC-MS/MS. The GC and MS conditions are summarized in Tables 3 and 4.

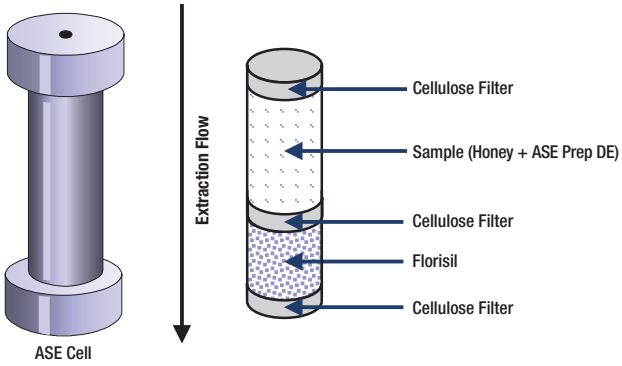

Figure 3. Extraction cell schematic.

Table 2. Conditions for accelerated solvent extraction.

ASE Program	
Solvent	*n*-Hexane/Ethyl Acetate (4:1, v/v)
Temperature	80 °C
Pressure	1500 psi
Static Cycles	3
Extraction Time	3 min
Rinse Volume	90%
Purge Time	90 s
Total Extraction Time (per sample)	~15 min
Total Solvent Volume (per Sample)	~50 mL

Table 3. GC and injector conditions.

Injector Program (PTV, splitless mode)	
Injector Temperature	250 °C
Liner	2 mm × 2.75 mm × 120 mm, Siltek-deactivated
Injected Volume	1 µL
Splitless Time	0.5 min
Split Flow	20 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)
GC Program	
GC Column	Rt-5MS (35 m × 0.25 mm × 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 4. MS conditions.

MS Program	
Source Temperature	270 °C
Ionization	EI
Electron Energy	70 eV
Emission Current	50 µA
Q2 Gas Pressure (Argon)	1.5 mTorr
Collision Energy	10 to 30 eV
Q1 Peak Width FWHM	0.7 Da
Q3 Peak Width FWHM	0.7 Da
Total Solvent Volume per Sample	~40 mL

An uncontaminated honey sample used as a control was selected for the optimization of all procedures. For honey fortification, 2 g of the control sample was spiked by adding an appropriate volume of the standard working solution to cover the concentration range from 1 to 100 ng/g for PCBs, from 0.5 to 10 ng/g for PBDEs, and from 5 to 100 ng/g for OCs and OPs and also in relation to pesticide MRLs, when available, in order to realize the matrix-matched calibration curves.

The triple quadrupole mass spectrometer was operated in selected reaction monitoring (SRM) mode, detecting two to three transitions per analyte. These transitions are listed together with the particular collision energies in Tables 5–9. Identification of POPs 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. Thermo Scientific™ Xcalibur™ software and Thermo Scientific™ TraceFinder™ software were used for data analysis and reporting.

The method was evaluated for its repeatability, linearity, recovery, limit of detection and quantification. The limits of detection (LOD) and quantification (LOQ) were calculated from the calibration curve in the concentration range corresponding to the lower concentration levels according to MRL for each pesticide when available. LOD was calculated using the equation LOD = 3.3 SD_0/slope, where SD_0 is the residual standard deviation. The limit of quantification was calculated as LOQ = 3 LOD. Recovery of the analytes studied were carried out at a fortification level of 10 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.

Table 5. SRM transitions for PCBs ([i]internal standard).

PCB #	Compound Name	Molecular Formula	Retention Time	Nominal Mass	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Collision Energy (eV)
28	2,4,4'-Trichlorobiphenyl	$C_{12}H_7Cl_3$	18.76	258	256	**186***	20
					258	186	25
52	2,2',5,5'-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$	20.25	292	292	**222***	25
					292	257	10
101	2,2',4,5,5'-Pentachlorobiphenyl	$C_{12}H_5Cl_5$	24.46	326	324	254	25
					326	**256***	25
					328	256	25
138	2,2',3,4,4',5'-Hexachlorobiphenyl	$C_{12}H_4Cl_6$	28.99	361	360	**290***	25
					360	325	10
153	2,2',4,4',5,5'-Hexachlorobiphenyl	$C_{12}H_4Cl_6$	30.25	361	360	**290***	20
					360	325	30
180	2,2',3,4,4',5,5'-Heptachlorobiphenyl	$C_{12}H_3Cl_7$	34.06	395	394	**324***	25
					394	359	10
					396	324	25
209[i]	Decachlorobiphenyl	$C_{12}Cl_{10}$	40.06	499	498	426	26
					498	**428***	22
					500	428	24

Table 6. SRM transitions for PBDEs ([1]FBDE, internal standard).

PBDE #	Compound Name	Molecular Formula	Retention Time	Nominal Mass	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Collision Energy (eV)
28	2,4,4'-Tribromodiphenyl Ether	$C_{12}H_7Br_3O$	27.95	407	246	139	10
					248	**139***	10
					408	248	10
33	2',3,4-Tribromodiphenyl Ether	$C_{12}H_7Br_3O$	28.05	407	246	139	30
					248	**139***	30
					406	246	10
47	2,2',4,4'-Tetrabromodiphenyl Ether	$C_{12}H_6Br_4O$	34.34	486	326	217	30
					328	219	30
					484	**326***	30
99	2,2',4,4',5-Pentabromodiphenyl Ether	$C_{12}H_5Br_5O$	38.17	565	410	297	30
					406	297	30
					564	**404***	20
100	2,2',4,4',6-Pentabromodiphenyl Ether	$C_{12}H_5Br_5O$	39.05	565	410	297	30
					406	297	30
					564	**404***	10
153	2,2',4,4',5,5'-Hexabromodiphenyl Ether	$C_{12}H_4Br_6O$	40.88	644	484	377	25
					642	**482***	10
154	2,2',4,4',5,6'-Hexabromodiphenyl Ether	$C_{12}H_4Br_6O$	41.76	644	484	324	30
					486	326	30
					644	**484***	20
–	3-fluoro-2,2,4,4,6-pentabromodiphenyl Ether[1]	$C_{12}H_4Br_5FO$	38.01	583	422	315	30
					424	315	30
					584	**424***	10

Table 7. SRM transitions for OCs ([1]internal standard).

Compound Name	Molecular Formula	Retention Time	Nominal Mass	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Collision Energy (eV)
α-HCH	$C_6H_6Cl_6$	15.27	297	181	**145***	10
				181	146	10
				219	183	10
Hexachlorobenzene	C_6Cl_6	15.45	285	284	**249***	20
				286	214	30
				286	251	20
β-HCH	$C_6H_6Cl_6$	16.69	297	181	**145***	10
				183	148	10
				219	183	10
Lindane (γ-HCH)	$C_6H_6Cl_6$	16.44	297	181	**145***	10
				183	145	10
				219	183	10
Heptachlor	$C_{10}H_5Cl_7$	19.27	373	272	**237***	10
				274	237	10
				274	239	10
Aldrin	$C_{12}H_8Cl_6$	20.84	365	261	**191***	30
				263	193	30
				265	193	30
Heptachlor epoxide	$C_{10}H_5Cl_7O$	22.77	389	353	**263***	10
				353	282	10
				355	265	10
trans-Chlordane	$C_{10}H_6Cl_8$	23.96	410	373	264	20
				373	**266***	20
				375	266	20
Endosulfan I	$C_9H_6Cl_6O_3S$	24.64	407	373	**266***	20
				375	266	20
				377	268	20
pp'-DDE	$C_{14}H_8Cl_4$	25.96	318	246	**176***	30
				248	176	30
				328	248	20
Endrin	$C_{12}H_8Cl_6O$	27.06	381	245	173	30
				263	**193***	30
				281	245	10
Endosulfan II	$C_9H_6Cl_6O_3S$	27.65	407	195	**159***	10
				241	206	10
pp'-DDD	$C_{14}H_{10}Cl_4$	28.18	320	235	**165***	20
				237	165	20
op-DDT	$C_{14}H_9Cl_5$	28.27	354	235	**165***	20
				237	165	20
Endosulfan sulfate	$C_9H_6Cl_6O_4S$	29.88	423	272	**237***	10
				274	237	10
				274	239	10
pp'-DDT	$C_{14}H_9Cl_5$	30.36	354	235	**165***	20
				237	165	20
4-Nonylphenol[1]	$C_{15}H_{24}O$	18.71	220	107	51	30
				107	77	20
				220	**107***	10

Table 8. SRM transitions for OPs (ⁱinternal standard).

Compound Name	Molecular Formula	Retention Time	Nominal Mass	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Collision Energy (eV)
Mevinphos	$C_7H_{13}O_6P$	11.28	224	127	109	10
				192	**109***	20
				192	127	10
Ethopropos	$C_8H_{19}O_2PS_2$	14.22	242	158	97	20
				158	**114***	10
				200	158	10
Dichlorvos	$C_4H_7Cl_2O_4P$	14.53	221	109	79	10
				145	109	10
				145	**113***	20
Phorate	$C_7H_{17}O_2PS_3$	15.19	260	121	65	10
				231	**129***	20
				231	203	10
Demephron (-O and -S)	$C_5H_{13}O_3PS_2$	15.75	216	115	**97***	10
				126	65	10
Diazinon	$C_{12}H_{21}N_2O_3PS$	17.01	304	179	121	30
				199	135	10
				304	**179***	10
Disulfoton	$C_8H_{19}O_2PS_3$	17.36	274	142	81	10
				142	**109***	10
Parathion-methyl	$C_8H_{10}NO_5PS$	19.09	263	125	79	10
				263	109	10
				263	**127***	10
Fenchlorphos	$C_8H_8Cl_3O_3PS$	19.58	321	285	240	20
				287	**242***	20
				287	272	10
Chlorpyrifos	$C_9H_{11}Cl_3NO_3PS$	20.99	351	125	79	10
				278	109	20
				278	**125***	20
Fenthion	$C_{10}H_{15}O_3PS_2$	21.17	278	245	125	10
				245	**213***	10
				278	109	20
Trichloronate	$C_{10}H_{12}Cl_3O_2PS$	21.76	334	109	81	10
				297	223	30
				297	**269***	10
Tetrachlorvinphos	$C_{10}H_9Cl_4O_4P$	24.43	366	109	79	10
				329	**109***	20
				331	109	20
Prothiofos	$C_{11}H_{15}Cl_2O_2PS_2$	25.55	345	267	239	10
				309	221	20
				309	**239***	10
Terbufos	$C_9H_{21}O_2PS_3$	26.17	288	169	113	10
				258	113	20
				258	**146***	10

Table 8. SRM transitions for OPs ([1]internal standard). (continued)

Compound Name	Molecular Formula	Retention Time	Nominal Mass	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Collision Energy (eV)
Fensulfothion	$C_{11}H_{17}O_4PS_2$	27.82	308	156	141	10
				292	140	20
				292	**159***	10
Sulprofos	$C_{12}H_{19}O_2PS_3$	29.13	322	156	108	30
				156	**141***	10
				322	97	20
Azinphos-methyl	$C_{10}H_{12}N_3O_3PS_2$	34.95	317	132	51	30
				160	**51***	30
				160	77	20
Coumaphos	$C_{14}H_{16}ClO_5PS$	38.08	363	226	163	10
				226	**198***	10
				362	226	10
4-Nonylphenol[1]	$C_{15}H_{24}O$	18.71	220	107	51	30
				107	77	20
				220	**107***	10

Table 9. SRM transitions for additional agrochemicals ([1]internal standard).

Compound Name	Molecular Formula	Retention Time	Nominal Mass	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Collision Energy (eV)
Captan	$C_9H_8Cl_3NO_2S$	14.42	300	149	70	15
				149	**105***	5
Penconazole	$C_{13}H_{15}Cl_2N_3$	18.81	284	248	**192***	25
				248	206	15
Trifloxystrobin	$C_{20}H_{19}F_3N_2O_4$	18.86	408	116	89	15
				131	**130***	10
Quinoxyfen	$C_{15}H_8Cl_2FNO$	21.81	308	237	**208***	26
				272	237	12
Bupirimate	$C_{13}H_{24}N_4O_3S$	23.38	316	193	65	10
				193	**109***	10
Fluazinam	$C_{13}H_4Cl_2F_6N_4O_4$	27.37	465	387	359	10
				387	**324***	10
Boscalid	$C_{18}H_{12}Cl_2N_2O$	30.12	343	112	76	10
				140	76	20
				140	**112***	10
Iprodione	$C_{13}H_{13}Cl_2N_3O_3$	31.2	330	314	**245***	15
				314	271	15
Chlorantraniliprole	$C_{18}H_{14}BrCl_2N_5O_2$	31.91	483	278	**243***	15
				278	249	20
Spirodiclofen	$C_{21}H_{24}Cl_2O_4$	38.9	411	157	107	20
				157	**97***	10
Pyraclostrobin	$C_{19}H_{18}ClN_3O_4$	47.26	388	132	**104***	20
				132	77	20
4-Nonylphenol[1]	$C_{15}H_{24}O$	18.71	220	107	51	30
				107	77	20
				220	**107***	10

Results and discussion

A multiresidue method for the analysis of organic contaminants and pesticides was developed. The ASE extraction with inline cleanup was necessary for the removal of interfering substances (e.g., carbohydrates) from honey samples. Florisil proved to be very efficient for the cleanup of different foods as well as honey samples. The proposed method was optimized for the multiresidual analysis of 59 pesticides and persistent organic pollutants (POPs). Total ion current chromatograms (GC-MS/MS) of blank honey samples spiked with investigated compounds and a naturally contaminated sample are shown in Figures 4 and 5.

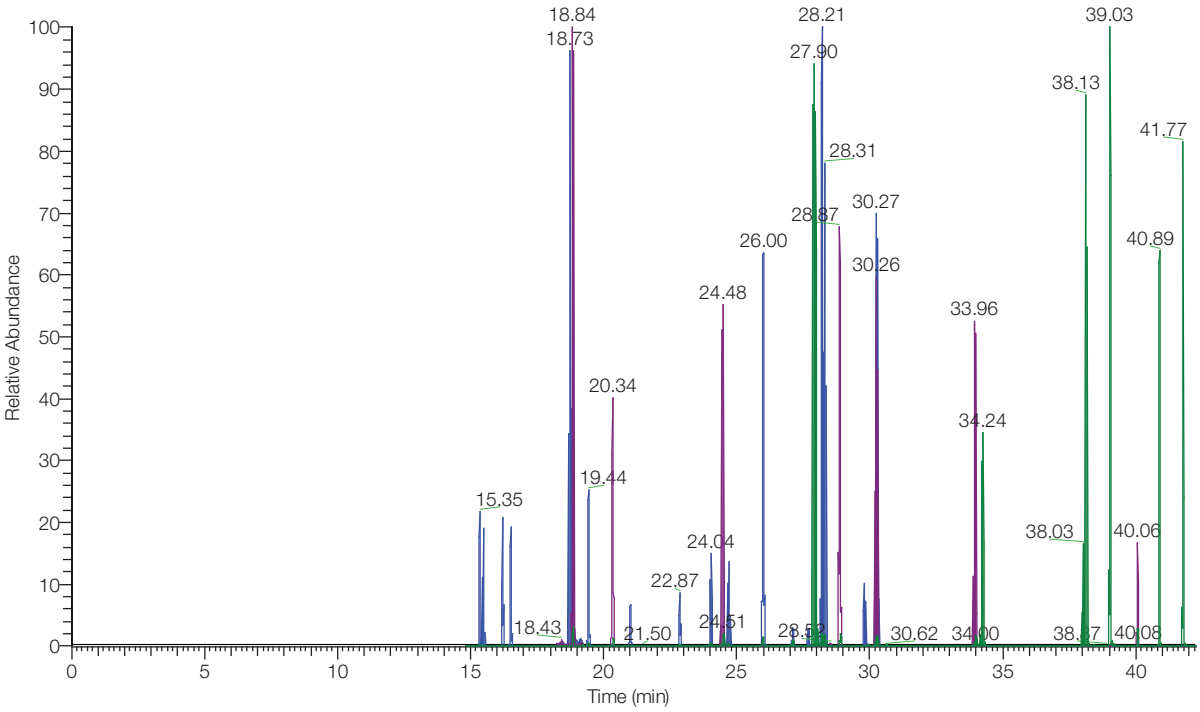

Figure 4. Total ion current (GC-MS/MS) chromatogram of a spiked honey sample.

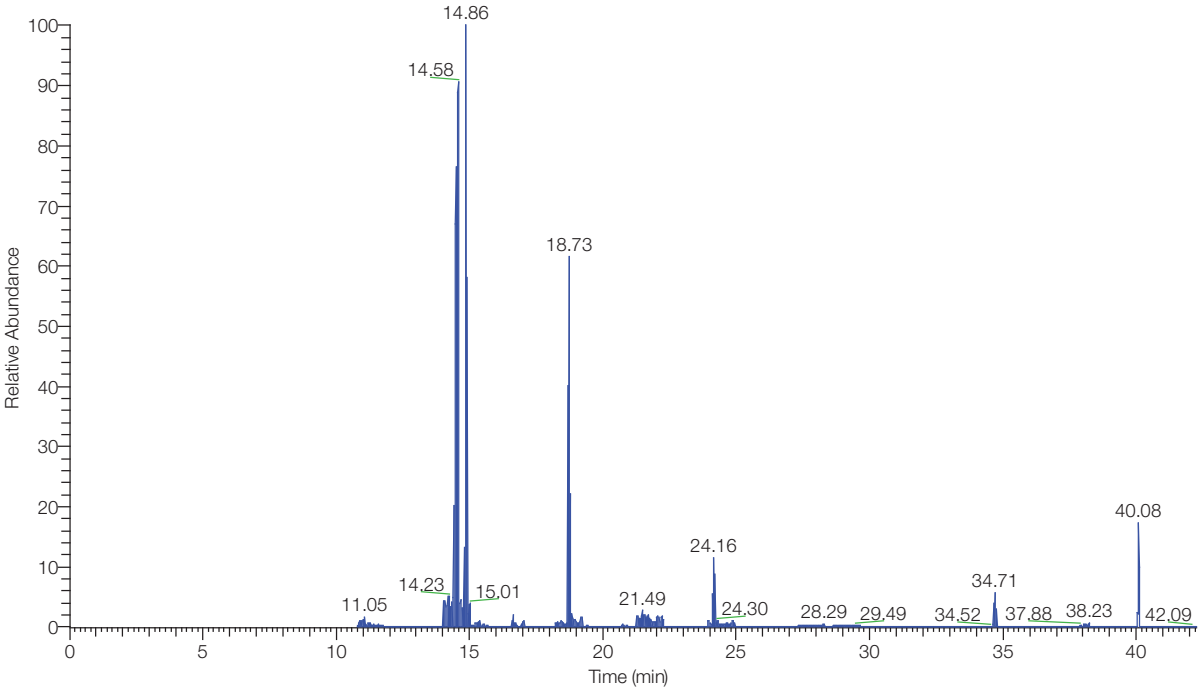

Figure 5. Total ion current (GC-MS/MS) chromatogram of a raw honey sample.

Table 10. Recoveries (%, RSD), LOD, LOQ and coefficient of determination (r^2)*

Contaminants	MRL* (ng/g)	LOD (ng/g)	LOQ (ng/g)	Recovery % (RSD)	Coefficient of Determination (r^2)
Polychlorobiphenyls (PCBs)					
PCB 28	-	0.08	0.24	102 (7)	0.9994
PCB 52	-	0.07	0.21	103 (7)	0.9999
PCB 101	-	0.04	0.12	97 (4)	0.9999
PCB 138	-	0.05	0.15	105 (4)	0.9999
PCB 153	-	0.02	0.06	102 (4)	0.9999
PCB 180	-	0.06	0.18	98 (9)	0.9999
Polybrominated Diphenyl Ethers (PBDEs)					
PBDE 28	-	0.01	0.03	100 (9)	0.9991
PBDE 33	-	0.02	0.06	98 (9)	0.9999
PBDE 47	-	0.02	0.06	97 (8)	0.9996
PBDE 99	-	0.03	0.09	102 (7)	0.9998
PBDE 100	-	0.01	0.03	103 (7)	0.9998
PBDE 153	-	0.03	0.09	97 (10)	0.9992
PBDE 154	-	0.02	0.06	100 (12)	0.9999
Organochlorines (OCs)					
α-HCH	-	0.99	2.97	78 (10)	0.9959
Hexachlorobenzene	-	1.26	3.78	80 (12)	0.9945
β-HCH	-	1.17	3.51	85 (12)	0.9995
Lindane (γ-HCH)	10	0.79	2.39	96 (10)	0.9985
Heptachlor	10[1]	0.95	2.84	93 (12)	0.9996
Aldrin	10	0.85	2.55	75 (14)	0.9991
Heptachlor Epoxide	10[1]	0.91	2.73	77 (14)	0.9994
trans-Chlordane	10[2]	1.48	4.44	92 (10)	0.9993
Endosulfan I	10[3]	1.13	3.38	80 (13)	0.9992
pp'-DDE	50[4]	0.85	2.55	97 (12)	0.9994
Endrin	10	0.99	2.98	88 (11)	0.9998
Endosulfan II	10[3]	1.14	3.42	90 (10)	0.9993
pp'-DDD	50[4]	0.91	2.74	87 (14)	0.9986
op-DDT	50[4]	0.94	2.83	82 (14)	0.9963
Endosulfan Sulfate	10[3]	1.07	3.22	85 (12)	0.9921
pp'-DDT	50[4]	0.91	2.74	95 (12)	0.9992

Table 10. Recoveries (%, RSD), LOD, LOQ and coefficient of determination (r^2)* (continued)

Contaminants	MRL* (ng/g)	LOD (ng/g)	LOQ (ng/g)	Recovery % (RSD)	Coefficient of Determination (r^2)
Organophosphorus (OPs)					
Mevinphos	-	0.75	2.25	75 (12)	0.9996
Ethopropos	-	0.44	1.32	86 (10)	0.9991
Dichlorvos	-	0.33	0.99	93 (10)	0.9997
Phorate	10[5]	0.52	1.56	75 (13)	0.9993
Demephron (-O and -S)	-	1.12	3.36	77 (14)	0.9992
Diazinon	10	1.10	3.30	90 (10)	0.9994
Disulfoton	10[6]	0.90	2.70	80 (14)	0.9998
Parathion-methyl	10[7]	0.83	2.49	97 (8)	0.9993
Fenchlorphos	-	1.12	3.36	88 (11)	0.9986
Chlorpyrifos	50	0.95	2.85	90 (9)	0.9963
Fenthion	10[8]	0.78	2.34	87 (12)	0.9996
Tricloronat	-	0.98	2.94	85 (14)	0.9998
Tetrachlorpyrifos	-	1.12	3.36	85 (12)	0.9998
Prothiofos	-	0.75	2.25	92 (12)	0.9992
Terbufos	-	0.68	2.04	90 (12)	0.9963
Fensulphotion	-	1.09	3.27	88 (10)	0.9921
Sulprofos	-	0.98	2.94	87 (10)	0.9992
Azinphos-methyl	-	1.07	3.21	87 (12)	0.9978
Coumaphos	-	0.78	2.34	84 (14)	0.9962
Other Agrochemicals					
Captan	50[9]	0.52	1.56	92 (3)	0.9963
Penconazole	50	0.75	2.25	85 (4)	0.9962
Trifloxystrobin	50	0.75	2.25	102 (1)	0.9991
Quinoxyfen	50	0.18	0.54	98 (3)	0.9981
Bupirimate	50	0.23	0.69	75 (3)	0.9954
Fluazinam	50	0.15	0.45	85 (2)	0.9938
Boscalid	50	0.41	1.23	92 (3)	0.9989
Iprodione	50	0.39	1.17	94 (1)	0.9969
Chlorantraniliprole	50	0.77	2.31	98 (1)	0.9999
Spirodiclofen	50	0.56	1.68	87 (6)	0.9988
Pyraclostrobin	50	0.32	0.96	90 (2)	0.9995

*Fortification level 10 ppb. MRLs according to the European Pesticide Database, Part A of Annex I to Reg. 396/2005.

[1]Sum of heptachlor and heptachlor epoxide expressed as heptachlor;

[2]Sum of *cis*- and *trans*-Chlordane;

[3]Sum of α- and β-isomers and endosulfan-sulphate expressed as endosulfan;

[4]Sum of *p,p'*-DDT, *o,p'*-DDT, *p,p'*-DDE and *p,p'*-TDE (DDD) expressed as DDT;

[5]Sum of phorate, its oxygen analogue and their sulfones expressed as phorate;

[6]Sum of disulfoton, disulfoton sulfoxide and disulfoton sulfone expressed as disulfoton;

[7]Sum of parathion-methyl and paraoxon-methyl expressed as parathion-methyl;

[8]Fenthion and its oxigen analogue, their sulfoxides and sulfone expressed as parent;

[9]Sum of captan and THPI, expressed as captan

Optimization of the MS/MS method consisted of (1) acquisition of respective MS spectra in full-scan mode (*m/z* 100–1000 mass range), (2) selection of precursor ions, (3) product ion scans at different collision energies (10, 20 and 30 eV) and (4) data collection in SRM mode. For each compound, two MS/MS transitions were chosen to fulfill the identification criteria. According to the SANTE 2015 (guidance document on analytical quality control and method validation procedures for pesticides residues analysis in food and feed), one precursor ion with two product ions or two precursor ions with one product ion should be available for the unbiased identification of the target analyte. In general, MS/MS allows for minimal matrix component interferences, and, due to the possibility of selecting suitable precursor and product ions, makes identification and quantification of the above mentioned contaminants possible even at ultra-trace concentrations. A highly selective triple quadrupole mass spectrometer was used, because GC-MS instruments are generally somewhat intolerant to nonvolatile matrix impurities. The choice of an appropriate sample preparation strategy is also critical in order to avoid poor ionization, background noise, and contamination of the whole GC-MS system. All results obtained confirm the efficacy of the present method for the determination of multiresidue pollutants in honey.

The method showed good linearity with determination coefficients equal to or higher than 0.99 for all of the compounds investigated. The method also showed good repeatability, demonstrating that it is effective for monitoring compounds belonging to different chemical classes (Table 10). The recoveries ranged from 97 to 102% for PCBs and PBDEs, from 75 to 95% for OCs, from 75 to 97% for OPs and from 75% to 102% for the additional agrochemicals. The CVs ranged from 4 to 14%. The one-step ASE method, using Florisil as an interference retainer, is rapid, cost-effective, and minimizes waste generation compared to the classic methods. Combining the extraction and the two clean-up steps (i.e., GPC and SPE) in a single ASE step reduced laboratory by half. At present, this is the first ASE application using an inline cleanup step to screen for the presence of different pesticides and organic contaminants in honey.[10]

Polychlorinated biphenyls

The six PCBs examined were detected in all samples, with similar concentrations for each molecule in the three different regions ranging from 0.27 to 0.92 ng/g. These data show that there are no significant differences in concentrations among the three areas, therefore, for these samples, the PCB contamination of honey is not influenced by the sample origin. The regions from which these samples originated were characterized by the former presence of several polluting industries.

Polybrominated diphenyl ethers

No PBDEs were detected. This is probably due to the fact that the samples are labeled as organic honey, so the environment and conditions of production have probably significantly reduced the presence of this class of pollutants.

Organochlorine pesticides

Based on historic use of these now-banned compounds, several OC pesticides were detected; all honey samples from Calabria showed the presence of Eldrin, with a concentration ranging from 1.95 to 18.9 ng/g. Samples from Trentino Alto Adige contained a greater number of OCs. This situation is probably related to the fact that Trentino Alto Adige, in particular Trento Province, is one of the major apple growing areas of Europe. Intensively cultivated apple plantations are subject to the extensive use of pesticides to control most agricultural pests, even if the integrated pest management system is applied during the growing season. Aldrin and Endrin were detected again, with a frequency of 5% and 44% and a maximum concentration of 1.17 ng/g and 13.34 ng/g, respectively. In addition, Dieldrin was found. The prevalence of this compound was 5% and the maximum concentration was 0.94 ng/g; with the same frequency, Heptachlor was detected at a concentration levels up to 0.15 ng/g. *pp*'-DDT and its metabolite *pp*'-DDE were also present, both with a prevalence of 17%, but with a maximum concentration of 0.09 ng/g for DDT and 1.47 ng/g for DDE. Endosulfan sulfate was found, with a frequency of 22% and a maximum concentration of 5.43 ng/g. Although many OC pesticides are prohibited, the presence of their residues further highlights the persistent nature of these compounds; it also shows that they can enter the food chain not only via fatty products, but also via non-fatty products such as honey. The concentrations of OC pesticides in all samples from Trentino Alto Adige are lower than the MRLs. The situation is analogous for the honey

samples from Lombardia, in which all of the concentration values were lower than MRLs. Here, *pp*'-DDT and its metabolites *pp*'-DDD and *pp*'-DDE, were present at concentrations up to 1.99 ng/g and with prevalence of 41%, 22%, and 33% respectively. Heptachlor was detected with a frequency of 11% and a maximum concentration of 1.19 ng/g; Dieldrin was also found, with a prevalence of 41% and a maximum concentration of 2.93 ng/g.

Organophosphorus pesticides

Organophosphorus pesticides are typically used for crop protection in the geographical area characterized by extensive apple orchards. Many of them have been found in honey samples, especially those from Trentino Alto Adige, where 12 different pesticides were detected. In particular, Quinoxifen, usually employed in the control of oidium infections, was detected with a prevalence of 100% and a concentration ranging from 3.09 to 4.23 ng/g. Diazinon was always found in samples from Trentino Alto Adige at concentrations ranging from 1.13 ng/g to 1.15 ng/g, while in samples from Calabria it was detected with a prevalence of 64% and a maximum concentration of 1.14 ng/g. Mevinphos was found in honey from both Trentino Alto Adige and Calabria, with a prevalence of 67% and 86%, respectively. The samples from Lombardia showed the fewest number of OPs; the highest prevalence (37%) was for Captan, a fungicide that is mainly used for diseases of apples during the growing season, with a maximum concentration of 20.56 ng/g. Coumafos was detected with high and similar frequencies in honey from Calabria and Trentino (78% and 79%, respectively). Coumafos followed by Amitraz and Carbendazim are the most commonly used fungicide and acaricide, used by beekeepers to control *Varroa destructor*.

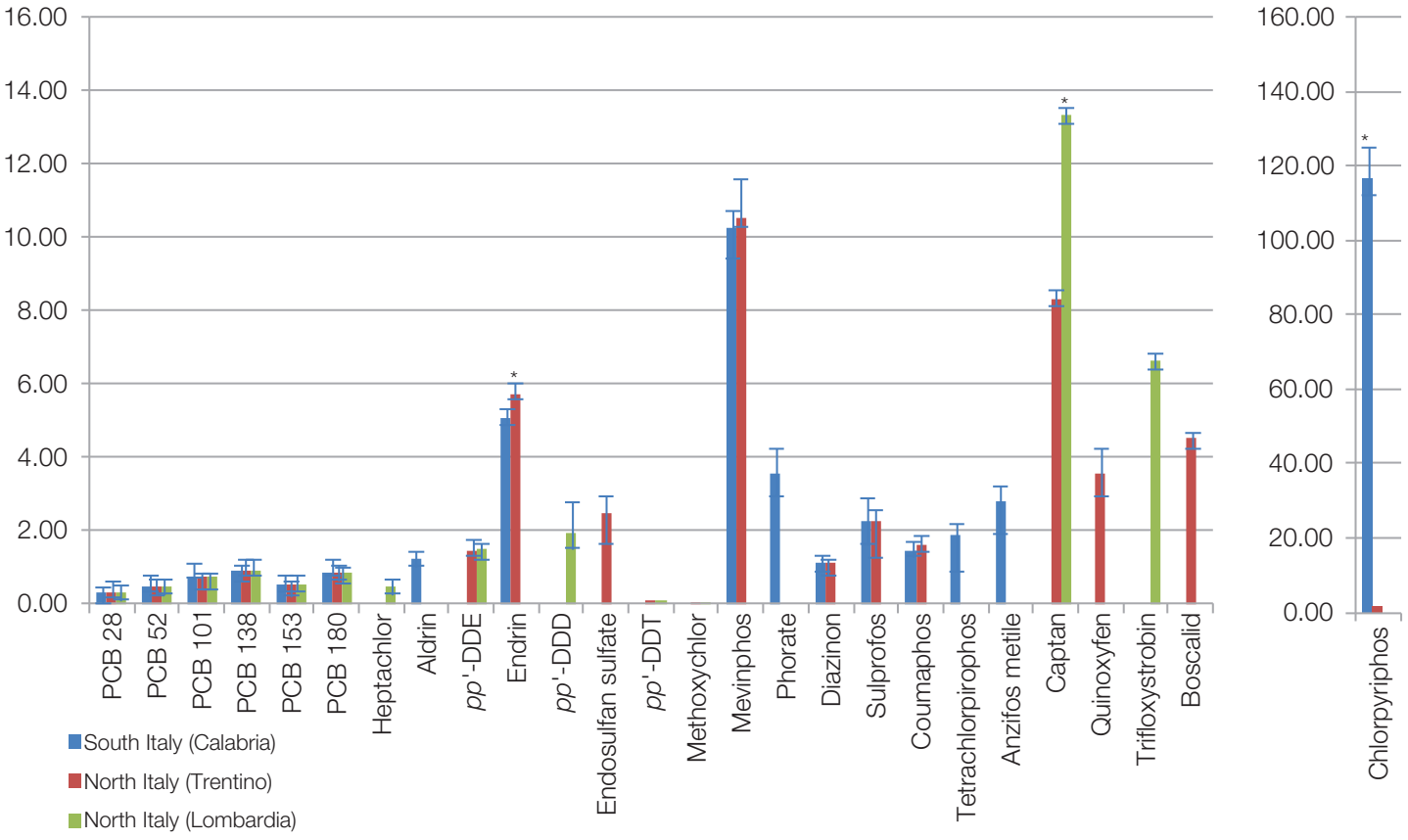

Figure 6. Distribution of detected contaminants (ng/g) in organic honey samples according to their sampling area.

Summary

An analytical method was developed and successfully applied to evaluate pesticides and POP residues in organic honey samples produced in three different Italian regions that are characterized by different contamination sources. The method proved to be simple and rapid, requiring small sample sizes, and minimizing solvent consumption, due to the ASE with an inline cleanup step. MS/MS detection provided both quantitative information and the confirmation of POP residues in honey, confirming the one-step ASE method as a valid alternative to classical extraction methods.

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