THE OCCURRENCE OF PESTICIDES AND PERSISTENT ORGANIC POLLUTANTS IN ITALIAN ORGANIC HONEYS FROM DIFFERENT PRODUCTIVE AREAS IN RELATION TO POTENTIAL ENVIRONMENTAL POLLUTION

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Abstract

Bee products, such as honey, are widely consumed as food and consumer interest is currently oriented towards organic foods. Regarding this, the European Commission establishes that the qualification of organic honey and other beekeeping products as being from organic production is closely bound with the characteristics of hive treatments as well as the quality of the environment. Agricultural contamination with pesticides is a challenging problem that needs to be fully addressed, in particular in the field of organic production systems. In this study, the occurrence of different classes of contaminants selected as representative of potential contamination sources were investigated in 59 organic honeys: organochlorines, OCs; organophosphates, OPs; polychlorobiphenyls, PCBs and polybromodiphenylethers, PBDEs. A method based on Accelerated Solvent Extraction with "in line" clean-up and GC-MS/MS detection was developed to detect contaminants. Residues of many pesticides were found in most of the samples investigated. The majority of honey samples contained at least one of the pesticides, even if their concentrations were found to be lower than its MRL. Diazinon, Mevinphos, Coumaphos, Chlorpyrifos and Quinoxyfen were the residues frequently detected in samples coming from the apple and citrus orchard areas. Furthermore, the results of the present study show that the presence of the residue in organic honey may also be affected by the geographical area (e.g. the presence of an agricultural system) confirming honey bee and beehive matrices as appropriate sentinels for monitoring contamination in the environment. The optimised method proved to be simple and rapid, requiring small sample sizes and minimising solvent consumption, due to the ASE having an "in line" clean-up step.

1. Introduction

Honey is a natural food product, made of nectar, secretions of livings parts of plants or excretions of insects sucking on the living parts of plants, which *Apis millifera* bees collect, transform by combining with specific substances and deposit in honeycombs (Giorgi et al., 2011; Wilczynska et al., 2007; Panseri et al., 2014). Honeybees (*Apis mellifera* L.) perform the vital task of pollinating agricultural crops and native species and are important for the commercial products of honey and beeswax. Honey composition mainly depends on the floral origin of nectar, climate conditions, bee physiology, honey harvesting and post-collection processing (Panseri et al., 2013). Today, consumer interest regarding honey and its derived products is oriented towards organic foods. Regarding this, the European Commission establishes that the qualification of organic honey and other beekeeping products as being from organic production is closely bound to the characteristics of hive treatments as well as the quality of the environment. This qualification also depends on the conditions of extraction, processing and storage of beekeeping products. The Council Regulation 1804/1999 EC is very restrictive with regard to 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, possibly leading to contamination, such as industrial areas, urban centres or motorways. Also, the use of veterinary medicinal products in beekeeping is regulated by the European Council (EC 1804/1999). Usually, beekeepers administered insecticides, fungicides, and acaricides to control some infestations such as *Varroa destructor*, *Acarapis woodi* and *Paenibacillus larvae* (López et al., 2014; Fell et

al., 2009; Genersch et al., 2010). According to the Council Regulation 1804/1999, the use of allopathic chemically-synthesised medicinal products for preventive treatments in organic beekeeping is prohibited, since these fat-soluble and non-volatile compounds can accumulate in the stored honey, where they are able to migrate from the wax comb (Panseri et al., 2014). In the cases of *Varroa* infestation, formic acid, acetic acid and oxalic acid can be used, as well as menthol, thymol, eucalyptol or camphor (Council Regulation 1804/1999 EC). Therefore, in organic honey production, direct pollution by beekeeping practices as well as indirect contamination from the environment must be prevented. Many pollutants in the environment may contaminate bee matrices, comprising bee, honey and pollen. Environmental pollutants include pesticides (Chauzat et al, 2011), heavy metals (Tuzen et al., 2007), bacteria and radioactive materials (Al-Waili et al., 2012). Honeybees are able to cover a wide area and come into contact with contaminated food sources, such as pollen, nectar and water during foraging. Therefore, honeybees and beehive products are considered potential indicators for environmental biomonitoring (Malhat et al., 2015; Kasiotis et al., 2014). Lambert et al. described the use of bees, honey and pollen as sentinels for environmental chemical contaminants in France (Lambert et al., 2012). Porrini et al. described the use of honey bees and bee products as bioindicators of pesticide, heavy metal and radionucleotide pollution (Porrini et al., 2003); Panseri et al. (2014) demonstrated the high direct relation between the contaminant source and pesticide residues found in honey samples. Among the environmental contaminants, different studies have documented the occurrence of organochlorines (OCs), polychlorobiphenyls (PCBs), organophosphates (OPs) and polybromodiphenylethers (PBDEs) in honey. In particular organochlorine, and to a minor extent organophosphorous pesticides, are highly stable, minimally volatile, lipophilic and persistent organic pollutants. Due to these characteristics, the

compounds tend to accumulate and bioaccumulate, representing important groups of dangerous organic contaminants, since they can contaminate foodstuffs if not directly treated (Panseri et al., 2014). Organophosphorus pesticides (OPs) represent important environmental and food contamination sources, as they are widely used in agriculture for the control and protection of crop-eating insects. In addition, OPs are acetylcholinesterase inhibitors leading to acute poisoning via food consumption (He et al., 2015). Recently EFSA (European Food Safety Authority) has realised scientific opinion on the risks to public health related to the presence of brominated flame retardants in food (EFSA, 2010). Thus, the Commission used the Recommendation of 3 March 2014 ask European countries to monitor traces of brominated flame retardants in food. Brominated flame retardants (BFRs), especially polybromodiphenyethers (PBDEs), are organobromine compounds applied to products in order to reduce their flammability. They contaminate the environment and food chain because of their persistent, lipophilic, bioaccumulative and toxic nature, and are suspected of causing neurobehavioral effects and endocrine disruption (Mohr et al., 2014). In general, the European Commission set the maximum residue levels values (MRLs) for feed as well as for food of animal origin (Commission Regulation 396/2005; Commission Regulation 839/2008).

Critical steps in the determination of contaminants residues in food are the extraction from matrices and the following sample clean-up (Rissato et al., 2007; LeDoux et al., 2011). Among the many extraction techniques, accelerated solvent extraction (ASE) is characterised by shorter extraction times and reduced solvent consumption. The accelerated solvent extraction utilises high temperatures combined with high pressure. A 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 (Beyer et al., 2008). Recently, the ASE technique has also been tentatively used combining the clean-up step during the extraction process, generating an "in line" extraction-clean-up method in which the sample purification is directly performed in the ASE cell. Until now, only three studies reported the use of ASE for the extraction of pesticides from honey without "in line" clean-up (Kort et al., 2002; Wang et al., 2010, Lambert et al., 2012).

Considering the lack of information in the literature about the presence of pesticides and other contaminants in organic bee products, the aim of the present study was to investigate the presence of POPs in organic honeys arising from different Italian regions. Our attention was focused on the residues of pesticides used in citrus and apple orchards for crop protection [organochlorines (OCs) and organophosphates (OPs)] as well as other POPs present in the environment as a possible consequence of antrophic activities [polychlorobiphenyls (PCBs) and polybromodiphenylethers (PBDEs)]. Lastly, this paper presents a rapid, accurate and sensitive method to evaluate multiple residues by using the accelerated solvent extraction (ASE) sample preparation method with "in line" clean-up purification followed by GC–MS/MS (triple quadrupole – QqQ) analysis.

2. Material and methods

2.1 Chemicals and reagents

Mixtures of PCB congeners (PCB 28; PCB 52; PCB 101; PCB 138; PCB 153 and PCB 180) and PBDE congeners (PBDE 28; PBDE 33; PBDE 47; PBDE 99; PBDE 100; PBDE 153 and PBDE 154), PCB 209, internal standard (IS) for PCBs, and 3-fluoro-2,2,4,4,6-pentabromodiphenyl ether (FBDE), and IS for flame retardants, were purchased from AccuStandard (New Haven,

147 USA). A mixture of 19 standard OCs (α-HCH; Hexachlorobenzene; β-BHC; Lindane;
148 Heptachlor; Aldrin; Heptachlor epoxide; Trans Chlordane; 4,4'-DDE; Endosulphan I; 2,4'-DDT;
149 Endrin; 4,4'-DDD; Endosulphan II; 4,4'-DDT and Endosulphan sulphate, Dieldrin, Endrin
150 Aldehyde and Methoxychlor) was purchased from Restek (Bellefonte, PA, USA). OP pesticide
151 standards of Mevinphos, Ethoprophos, Phorate, Diazinon, Disulphoton, Methyl Paration,
152 Fenchlorphos, Chlorpyriphos, Fenthion, Sulprofos, Coumaphos, Tetrachlorpirophos, Protiofos,
153 Tribuphos, Anzifos metile, Chlorpyriphos, Penconazol, Captan, Bupiramate, Quinoxyfen,
154 Fluazinam, Trifloxystrobin, Iprodion, Chlorantraniliprol, Spirodiclofen, Boscalid, and
155 Pyraclostrobin were purchased from Sigma-Aldrich, St Louis, Mo, USA. Florisil (100–200 96
156 mesh) was provided by Promochem (Wesel, Germany). Hexane, isooctane, acetone, ethyl acetate
157 (special grade for pesticide residue analysis (Pestanal)) and 4-nonylphenol (IS for OCs and OPs)
158 were purchased from Fluka (Sigma-Aldrich, St.Louis, MO, USA). Working solutions were
159 prepared by diluting the stock solution in hexane for pesticides and then stored at -40°C. Mixed
160 compound calibration solution, in hexane, was prepared daily from the stock solutions (10µ mL^{-1})
161 and the proper volume was used as a spiking solution as well.
162
163 *2.2 Sample collection*
164 Fifty-nine organic honey samples were provided by the beekeepers from three different Italian
165 regions: Calabria, South Italy (14 samples); Trentino Alto Adige, North Italy (18 samples) and
166 Lombardia, North Italy (27 samples), as summarised in Table 1. All samples were stored at -
167 20°C until analysis to prevent any possible matrix alteration (fermentation phenomena).
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2.3 Extraction and clean-up

The extraction was performed using an ASE 350 (Thermo-Fisher Scientific, Waltham, MA, USA). The extraction conditions are shown in Table 2. Here, 33 mL cells for accelerated solvent extraction (ASE) were used for the analysis. A 2 g sample of honey was homogenised with an equal weight of Diatomaceous earths, sodium sulphate and transferred into the cell. Then, 1 mL of isooctane solution containing the three ISs was added. In order to fill the remaining empty part of the cell, Diatomaceous earths were added. The cells were finally packed with a cellulose filter at the bottom followed by Florisil (5 g). The dried samples were transferred to the ASE cells. Temperature (80°C), pressure (1500 psi), number of static cycles (3 min each), and purging time (90 s with nitrogen) were fixed throughout the study. The extraction solvent was a mixture of hexane/ethyl acetate (4:1, v/v). Organic extracts were finally collected in 66 mL vials and treated with sodium sulphate to remove any possible humidity. Afterwards, the extract was collected and dried under vacuum in a centrifugal evaporator at 30°C. The residue was dissolved in 200 µL of isooctane and submitted to analysis by GC/MS-MS. An uncontaminated honey sample used as control was selected for the optimisation 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^{-1} for PCBs, from 0.5 to 10 ng g^{-1} for PBDEs, and from 5 to 100 ng g^{-1} for OCs and OPs, and also in relation to pesticide MRLs when available in order to realise the matrix-matched calibration curves.

2.4 GC-MS/MS analysis of Pesticides and POPs

Triple quadrupole mass spectrometry (QqQ) in electronic impact (EI) mode was used for the simultaneous detection and quantification of pesticides and POPs in honey samples.

A GC Trace 1310 chromatograph coupled to a TSQ8000 triple quadrupole mass detector (Thermo Fisher Scientific, Palo Alto, CA, USA) was used to confirm and quantify residues in honey samples by using a fused-silica capillary column Rt-5MS Crossbond-5% diphenyl 95% dimethylpolysiloxane (35 m x 0.25 mm i.d., 0.25 μm film thickness, Restek, Bellefonte, PA, USA). The oven temperature program was as follows: initial temperature of 80°C, held for 3 min, and increased to 170°C at 10°C min^{-1}; then, increased from 170°C to 190°C at 3°C min^{-1}, and raised to 240°C at 2°C min^{-1}, before being ramped to 280°C at 3°C min^{-1} and finally from 280°C to 310°C at 10°C min^{-1} and held at this temperature for 5 min. The carrier gas (helium, purity higher than 99.999%) was in constant flow mode at 1.0 ml min^{-1}. A volume of 1 μL was injected using a programmed temperature vaporiser injector (PTV) in splitless mode with a 1-min splitless period and the following inlet temperature programme: 80°C (0.05 min), 14.5°C s^{-1} to 200°C (1 min) and 4.5°C s^{-1} to 320°C (12 min – cleaning phase). A baffle liner (2 mm × 2.75 mm × 120 mm, Siltek-deactivated; Thermo Fisher Scientific) was used. The transfer line was maintained at 270°C and the ion source at 250°C. The electron energy and emission current were set to 70 eV and 50 μA, respectively. The scan time was 0.3 s and the peak width of both quadrupoles was 0.7 Da full widths at half maximum. Argon was used as a collision cell gas at a pressure of 1.5 mTorr. The QqQ mass spectrometer was operated in selected reaction monitoring mode (SRM) detecting two-three transitions per analyte, which are listed together with the particular collision energies in Table 3. 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.

The Xcalibur™ processing and instrument control software program and Trace Finder 3.0 for data analysis and reporting (Thermo Fisher Scientific) were used.

2.5 Validation parameters and quality control

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^{-1}, while the method repeatability (expressed as coefficient of variation, CV, %) was evaluated analysing six replicates each by adding known quantities of POPs standard solution (10 ng g^{-1}) to 2 g of honey (SANTE/11945/2015; Panseri et al., 2011).

2.6 Statistical analysis

As residue concentrations in honey do not follow a normal distribution, the non-parametric Kruskal–Wallis ANOVA test was used to evaluate the differences of contaminants in samples among the investigated regions. The level of significance was set as $p \leq 0.05$ throughout this study. Data were analysed using SPSS 15.0 software (SPSS, Inc., Illinois, USA). In addition, it must be pointed out that, for the calculations, ½ LOD was used for those compounds whose concentration was below LOD.

3. Result and discussion

3.1 Method development and validation

A multi-residue method for the analysis of organic contaminants and pesticides was developed. The ASE procedure with clean-up in a single step with an “in line” was necessary for the removal of interfering substances from honey samples. For this purpose, Florisil was used since it proved to be very efficient for the clean-up of different foods (Sun, Gea, Lva & Wang, 2012) as well as for honey samples (Rissato, Galhiane, Knoll & Apon, 2004; Amendola et al., 2010; Panseri et al., 2014).

A total ion current (GC-MS/MS) chromatograms of blank honey samples spiked with investigated compounds and a naturally contaminated sample are shown in figures S1 and S2 (supplementary materials section). Optimisation of the MS/MS method consisted of (1) acquisition of respective MS spectra in full-scan mode (m/z 100 – 1,000 mass range), (2) selection of precursor ions, (3) product ion scans at different collision energies (10, 20 and 30 eV) and (4) final tuning of the collision energy in selected reaction monitoring mode. For each compound, two MS/MS transitions were chosen to fulfill the generally applied identification criteria: according to the SANTE document, 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. An overview of the quantitative and confirmation MS/MS transitions and collision energies selected for each compound in EI mode are given in Table S1.

The method showed good linearity with determination coefficients equal to or higher than 0.99 for all of the compounds investigated; there was also good repeatability, demonstrating that it is useful for monitoring compounds belonging to different chemical classes (Table 2). The recoveries ranged from 97 to 102% for PCBs and PBDEs, from 75 to 95% for OCs and from 75 to 97% for OPs. The CVs were all in the range from 4 to 14%. The one-step ASE method using Florisil as an interference retainer is both rapid and cost-effective and minimises waste

generation compared to the classic methods. The time required in the laboratory is reduced to half by combining the extraction and the two clean-up steps (i.e., GPC and SPE) in one single ASE step (Panseri et al., 2014).

Our results are in accordance with Lambert et al. (2012), who used Florisil as an interference retainer to extract and clean-up polycyclic aromatic hydrocarbons (PAHs) from bees using ASE extraction techniques combined with in line clean-up obtaining good validation parameters in term of recovery and precision for all PAHs. At present this research represents the first ASE application using an in line clean-up step to screen the presence of different pesticides and organic contaminants from honey.

3.2 Application to honey samples

In the present study, the developed method was applied for the analysis of 59 honey samples produced in different Italian geographic areas in order to screen and tentatively relate the presence of pesticide residues to their potential contamination source, also confirming organic honey as a suitable indicator of environmental pollution as well as an indicator of the presence of pesticides utilised in crop protection management. This topic is crucial, especially for organic productions in which the use of allopathic chemically synthesised medicinal products for preventive bee treatments is prohibited and specific guidelines are given in order to minimise the impact of environmental pollution on bee products like honey (e.g. siting of the apiaries). Overall, the results of detection frequency, concentration levels and distribution of pesticide residues found in organic honey samples according to their sampling area are presented in Table 3 and Fig 1. This research represents the first investigation on the presence of different classes of pesticides and POPs in organic honey. As a consequence, it is difficult to compare our results

284 with those obtained from other monitoring programs, because only a few are published, and the
285 range of pesticides considered is different.
286 The six PCBs examined were detected in all samples, with similar concentrations for each
287 molecule in the three different regions ranging from 0.27 to 0.92 ng g^{-1}. These data show that
288 there are no significant differences in concentrations among the three areas; therefore, the PCB
289 contamination of honey is not influenced by the sample origin. Our results reflect the fact that
290 these regions were characterised by the presence of several harmful industries in the past.
291 Moreover, our data, even at higher concentrations, agree with those of Erdogul (2006), who
292 found PCBs in honey samples from Kahramanmarao, Turkey. Concerning flame retardants, no
293 PBDEs were detected. Unfortunately, there are not many studies regarding the concentration of
294 PBDEs in organic honey, so few data are available. The study by Wang et al. (2010), which
295 focused on the presence of PBDEs in developing and developed countries, detected all of the
296 investigated PBDEs and showed that the average concentration of PBDEs in developed regions
297 is always higher than the corresponding PBDE in developing countries, except for PBDE 209,
298 which was not considered in our study. Mohr et al. (2014) also provided data on the presence of
299 PBDEs; according to our data, PBDE 28 and 154 were not detected in their samples, while
300 PBDE 33, 99, 100 and 153 were detected at concentrations in the order of pg g^{-1}. This
301 incongruity is probably due to the fact that our samples are made of organic honey, so the
302 environment and conditions of production have probably significantly reduced the presence of
303 this class of pollutants.
304 Several OC pesticides were present; all honey samples from Calabria showed the presence of
305 Eldrin, with a concentration ranging from 1.95 to 18.9 ng g^{-1}. In one sample, the concentration
306 value was higher than the MRL, while in the other two, the values were close to the MRL.

Aldrin, whose prevalence was 50%, was also found at concentrations up to 1.24 ng g^{-1}. Honey is considered unfit for human consumption if residues surpass the maximum residue level (MRL) [Regulation (EC) No 396/2005].

Samples from Trentino Alto Adige are those in which there was 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 (Marini, Quaranta, Fontana, Biesmeijer, & Bommarco, 2012). 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 (Berrie & Cross, 2005; Tresnik et al., 2007). Aldrin and Endrin were detected again, with a frequency of 5% and 44% and a maximum concentration of 1.174 ng g^{-1} and 13.343 ng g^{-1}, respectively. In addition, Dieldrin, an Aldrin metabolite produced by insects, was found. The prevalence of this compound was 5% and the maximum concentration was 0.94 ng g^{-1}; with the same frequency, Heptachlor was detected at a concentration levels up to 0.15 ng g^{-1}. 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^{-1} for DDT and 1.47 ng g^{-1} for DDE. Endosulphan sulphate was found, with a frequency of 22% and a maximum concentration of 5.43 ng g^{-1}. Although many OC pesticides are prohibited, the presence of their residues further underlines 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 of 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

330 DDE, were present at concentrations up to 1.99 ng g^{-1} and with prevalence of 41%, 22% and
331 33% respectively. These results are due to the metabolic degradation of DDT after microbial
332 catabolism, even if the mechanisms have not yet been clarified (Panseri et al, 2014). Heptachlor
333 was detected with a frequency of 11% and a maximum concentration of 1.19 ng g^{-1}; Dieldrin was
334 also found, with a prevalence of 41% and a maximum concentration of 2.93 ng g^{-1}.
335 Some OP pesticides were also investigated. They are insecticides that are typically used for crop
336 protection in the geographical area characterised by intensive apple orchards (Panseri et al,
337 2014). Many of them have been found in honey samples, especially those from Trentino Alto
338 Adige, where 12 different pesticides were detected. In particular, Quinoxifen, usually employed
339 in the control of oidium infections, was detected with a prevalence of 100% and a concentration
340 ranging from 3.09 to 4.23 ng g^{-1}. Agricultural activities can be a source of contamination by a
341 variety of pesticides. The pesticide pollution in intensively cultivated areas represents a matter of
342 concern because these products accumulate in vegetation, water and soil and cause damage to
343 beneficial organisms such as honey bees (*Apis mellifera* L.) (Porrini et al., 2002; Wallner, 1999).
344 Diazinon was always found in samples from Trentino Alto Adige at concentrations ranging from
345 1.13 ng g^{-1} to 1.15 ng g^{-1}, while in samples from Calabria this was detected with a prevalence of
346 64% and a maximum concentration of 1.14 ng g^{-1}. Mevinphos was found in honey from both
347 Trentino Alto Adige and Calabria, with a prevalence of 67% and 86%, respectively. The samples
348 from Lombardia showed the fewest number of OPs; the highest prevalence (37%) was for
349 Captan, a fungicide that is mainly used for diseases of apples during the growing season (Berrie
350 & Cross, 2005; Blasco et al., 2003; Blasco et al., 2008), with a maximum concentration of 20.56
351 ng g^{-1}. All of the values of pesticides are lower than their MRLs. Only Chlorpyrifos has been
352 detected in some samples of all three regions, showing the highest prevalence (29%) and the

353 highest concentration (389.5 ng g^{-1}) in honey from Calabria: as it is one of the most commonly
354 used insecticides worldwide (Environmental Protection Agency, 738-R-01-007, 2002), such high
355 concentrations are justified. Furthermore, no MRLs are provided for this compound (Cutler et
356 al., 2014). Intensively cultivated apple and citrus plantations are subject to an extensive use of
357 pesticides to control most agricultural pests, even if the integrated pest management system is
358 applied during the growing season, leading to the contamination of bee products (Berrie & Cross,
359 2005; Ponikvar et al., 2005).
360 Also, Coumafos was detected with high and similar frequencies in honey from Calabria and
361 Trentino (78% and 79%, respectively). Coumafos followed by amitraz and carbendazim are the
362 most commonly used fungicide and acaricide, used by beekeepers to control *Varroa destructor.*
363 This result is surprising considering that the use of allopathic chemically synthesised medicinal
364 products for preventive bee treatments is prohibited for organic system productions. Several
365 other studies have previously demonstrated that the chemicals used by beekeepers inside the
366 hives are frequently found in the apicultural matrices (Pedersen et al., 2006; Lambert et al., 2013,
367 Garry et al., 2016). Coumaphos, another acaricide extensively used against *Varroa* in recent
368 decades, was also frequently detected in apicultural matrices (Haarmann et al., 2002). In
369 addition, many studies indicate that coumaphos was persistent in wax and diffused from wax to
370 honey in high proportions (Haarmann et al., 2002; Blasco et al., 2011).
371

372 **4. Conclusion**

373 An analytical method was developed and successfully applied to evaluate pesticides and POP
374 residues in organic honey samples produced in three different Italian regions characterised by
375 different contamination sources. The method proved to be simple and rapid, requiring small

sample sizes, minimising solvent consumption, due to the ASE with an “in line” clean-up step. MS/MS detection provides 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 because the analytical quality is comparable. The determination of chemical residues in the environment and foods is necessary to ensure that human exposure to contaminants, especially by dietary intake, does not exceed tolerable levels for health. The presence of residues of a number of pesticides in the honey samples and organic contaminant residues indicate that bee colonies in the investigated regions are probably exposed to chronic impacts of pesticides. Furthermore, the results of the present study showed that the presence of the residue in organic honey may also be affected by the contaminant’s geographical area (e.g. the presence of an agricultural system) confirming honey bee and beehive matrices as appropriate sentinels for monitoring contamination in the environment. In agricultural areas with developed apiculture, useful information about the occurrence and distribution of pesticide residues due to crop protection treatments can be obtained from the analysis of collected honey samples, which were used as bioindicators. This approach is pivotal and could help beekeepers to select production areas, in particular if dedicated for organic honey production.

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11. Commission Regulation (EC), pp No 839/2008 of 31 July 2008 amending Regulation (EC), pp No 396/2005 of the European Parliament, of the Council as regards Annexes II, III, IV setting maximum residue levels of pesticides in or on certain products.
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