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ARTICLE

Dioxin analysis in pine honey from Turkey

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ABSTRACT The aim of the study is to determine concentrations of polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), dioxin-like polychlorinated biphenyls (dl-PCBs) and indicator PCBs (ind-PCBs) in pine (honeydew) honey, which is endemic and popular in Turkey. *Marchalina hellenica*, which lives on *Pinus brutia*, is the main source of honeydew, and *Apis mellifera* L. collects the honeydew for making the pine honey. Pine honey is a very important bee product due to the export all over the world. In this study, honey samples were collected from Muğla and were researched via microscope. The quality of honey samples was determined by correlating NHE (Number of Honeydew Elements) to NTP (Number of Total Pollen) ratio and the honey, which has NHE to NTP ratio higher than 4.5 was accepted as a high density-superior quality pine honey. According to identifications, which have been made via microscope, pooled high quality pine honey sample was selected and analysed for dioxin. All the dioxin results were found lower than the European Union regulatory limits.
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KEY WORDS

Apis mellifera
dioxin analysis
Marchallina hellenica
pine (Honeydew) honey
Pinus brutia

Introduction

The term "dioxin" generally refers to the polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls (dl-PCBs) with similar biological and toxicological properties, which are lipophilic contaminants and accumulate in lipids of biological systems (Fries 1995; Olanca et al. 2014).

The dioxin contamination incidents involving food and feedstuffs happened in recent years and the re-evaluation of the Tolerable Daily Intake (TDI) of dioxins (Van Leeuwen et al. 2000) have prompted wide-ranging efforts and the tightening of regulations to reduce dioxin release into the environment (Commission Regulation 2000). In 2006, the World Health Organization (WHO) re-evaluated the toxicity equivalent factors (TEFs) assigned to dioxins and dioxin-like PCBs (DL-PCBs) for the calculation of the toxic equivalent quantities (TEQs) (Van Den Berg et al. 2006) and the European Commission has recently established maximum permissible levels of dioxins and DL-PCBs in foods (Commission Regulation 2011).

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The International Agency for Research on Cancer (IARC) classified 2,3,7,8-TCDD, the most toxic of the dibenzo-p-dioxins, as a Group 1 carcinogen, meaning a "known human carcinogen". PCDD/Fs and PCBs cause a variety of health problems in organisms (Kodavanti et al. 1998; WHO 2016). Exposure of human populations to dioxins and PCBs occurs via the food chain (Hays and Aylward 2003).

Honey is a natural product that honeybees (*Apis mellifera L.*) make from the nectar of blossoms or from secretions coming from living parts of plants. The bees collect, transform and combine this material with specific substances of their own (enzymes), store and leave to ripen in the honeycombs of beehive (Council Directive 2001). Pine (Honeydew) honey is prepared from secretions of living parts of plants or excretions of plant-sucking insects on the living part of plants (Sanz et al. 2005). Honeydew is the origin of pine honey, which is a class of honeys. It refers to honey produced by honeybees collecting sweet substances, which are exuded from other insects such as aphids or scale insects (Zander and Koch 1994). Insects take essential nutrients from concentrated sugar solution in the floem and exude the remains. Honeybees take these remains and bring to the hive and convert into honey. This honey is called honeydew honey (Zander and Koch 1994).

Honeydew honey is generally characterized by honeydew elements composed of microscopic algae, fungus spores.

If a honey with the ratio "number of honeydew elements (NHE)"/"number of total pollens (NTP)" is greater than 3, is considered as honeydew honey (Louveaux et al. 1978; Soria et al. 2004).

Marchalina hellenica (syn. *Monophlebus hellenicus*) (Coccidea: Homoptera), which lives on *Pinus brutia*, is the main source of honeydew in Turkey. The habitats of this insect are only Turkey and Greece (Santas 1979). *Marchalina hellenica* is mainly found in Southern Marmara, the Aegean and West Mediterranean regions of Turkey (Gürkan 1989). Muğla is one of the best places for pine honey, which has been produced by *Marchalina hellenica*. About 30% of honey yield of Turkey is produced in the region of Muğla. Muğla, having nearly 60 000 hectares of *Pinus brutia* forest, is a very important site for the production of pine honey (Şahin 2000). From 25 to 30 tons of pine honeys are produced each year and majority of them are exported all over the world (Maybir 2015).

Nowadays, bee products are being produced in an environment polluted by different sources of contamination, which can be transported by honey bees to the hive and incorporated into the honey (Tomasini et al. 2012). It is difficult to protect food and animal feed from the sources of toxic chemicals ubiquitous in the environment (Kim et al. 2013) particularly in the case of honey, since honeybees travel long distances and come close to many plants (Mohr et al. 2014a).

Residues of some Persistent Organic Pollutants (POPs) have been found in honey samples, such as organochlorine pesticides (Erdogrul 2007; Wang et al. 2010) and non-dioxin-like polychlorinated biphenyls (NDL-PCBs) (Herrera et al. 2005). There are very limited studies regarding PCDDs, PCDFs, and DL-PCBs' levels in honey (Mohr et al. 2014a; Wang et al. 2012).

In this study, we have determined amount of dioxin in pine honey, which is an important food and beekeeping product in Turkey. This paper presents the first results of analysis of honey samples for PCDDs, PCDFs, dioxin-like and indicator PCBs in Turkey. Therefore, the values obtained from this study are compared with the results reported in a few studies (Mohr et al. 2014a; Wang et al. 2012) and the EU standards.

As indicated by Devillers and Pharm-Delegue (2002) and Mohr et al. (2014b) during the process of gathering nectar, water, and pollen from flowers by the honeybee workers, various chemical particles, which are suspended in the air, are intercepted by these workers and retained in the hair of their body surface, or inhaled and attached to their trachea. Honey can be contaminated in an indirect way by industrial chemicals (Kujawski and Namieśnik 2008) and has been used as an environmental bioindicator in some studies (Kujawski et al. 2012; Wang et al. 2010; Rissato et al. 2007; Blasco et al. 2004; Blasco et al. 2011).

Materials and Methods

Microscopic analysis of honey samples

Honey samples were collected from 10 different hives in Muğla region in September to October 2014, pooled together, and then transferred to the laboratory. Upon collection, sample was given a unique laboratory reference number, and the sample details were logged into a database.

Preparates, to identify NTP and NHE in 10 grams of honey, were obtained as follows (Moar 1985; Sorkun 2008). Five-hundred grams of stock honey was well stirred with a sterile glass stick and 10 g of it was separated. Then 20 ml distilled water was added and the mixture was placed in a tube together with a tablet as positive control containing 12542 *Lycopodium* spores. To melt down the tablet, tubes were left in a water bath at 45 °C for 10-15 min. After the tablet fully melted, few drops of basic fuchsin were added for colouring pollens and spores and then the material was centrifuged in 3500 rpm for 45 min. Water was removed from the tubes and they were left upside down on a drying mat for full drainage. Homogenous mixing was ensured by adding 1 ml of 50% glycerine to each tube. From this mixture, 0.01 ml was taken and plated on a lam. The material was covered by a lamella of size 18 x 18 mm and two separate preparates were obtained for microscopic analysis.

Examination of the Number of Total Pollens (NTP)

Pollen and spore preparates were examined and counted under a Nikon Eclipse E400 light microscope. Objectives of 20x and 40x were used for counting pollens. During the counting process, the specimen was examined starting from the top left corner and by fully scanning the area of size 18 x 18 mm. The numbers of all pollens and *Lycopodium* spores in this area were taken separately. Counts of two separate samples were taken and the average was applied to the formula given below. The resulting figure is the total number of pollens in 10 g of honey.

NTP/10 gr = Number of pollens counted x 12542 / Number of *Lycopodium* spores counted

Examination of the Number of Honeydew Elements (NHE)

In the same preparates, in which NTP was counted, the number of honeydew elements (NHE) was also determined. During this process, starting from the top left corner and by fully scanning the area of size 18 x 18 mm the numbers of all spores, hyphae and, if there are any algae were taken. The

Table 1. Classification of honey samples by NHE/NTP ratio.

NHE/NTP	Identification	Honey type
0-1,5	Low density	Floral honey
1,5-3,0	Medium density	Pine + floral honey
3,0-4,5	Dense	Pine honey
>4,5	High dense	Superior quality pine honey

NHE content in 10 g of honey was calculated by using the following formula:

NHE/10 g = Number of spores + hyphae + algae counted x 12542 / Number of *Lycopodium* spores counted

NHE/NTP ratio

According to the obtained results, by using NHE/NTP ratio, a pine honey can be classified as High Density-Superior Quality Pine Honey, Dense Pine Honey, Floral Honey Added Pine Honey and Low Density Floral Honey (Louveaux et al. 1978; Şahin 2000; Sawyer 1988). Table 1 displays honey types and classes based on NHE/NTP ratio values.

The microscopic view of a High Dense-Superior Quality Pine Honey sample (Fig. 1) exhibits the presence of honeydew elements (spores and hyphae) and few pollens. Following microscopic examinations, honey sample was determined as high density-superior quality pine honey sample and prepared for dioxin analysis.

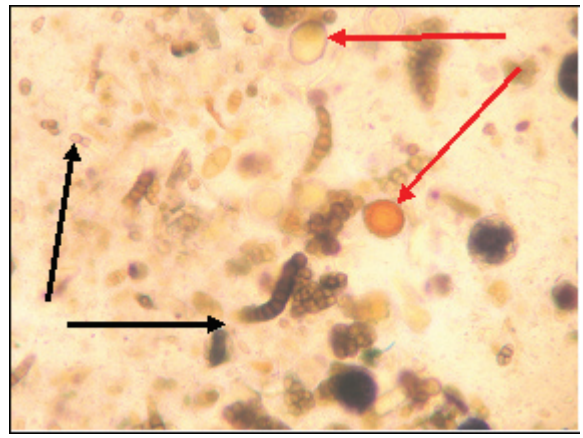

Figure 1. Microscopic view of high density-superior quality pine honey. Spores and hyphae: Black arrows. Pollens: red arrows.

Analytical procedure

Standards

All standards were bought from Cambridge Isotope Laboratories (Tewksbury, MA). A seven-point calibration curve was plotted for dioxins with a concentration range of 0.02-20 pg μl^{-1} and an eight-point calibration curve was used for PCBs, with a concentration range of 0.10-50 pg μl^{-1}. ^{13}C-labelled standards were also added to calibration standards. Laboratory has participated in every proficiency tests coordinated by EURL Dioxin Laboratory since 2011.

Extraction and clean-up

A 50-51 g of homogenised pooled pine honey sample was taken and spiked with ^{13}C-labelled internal standards. After that, extraction was performed with Smedes and Thomasen method (1996). Extracted honey sample was dissolved in hexane before clean-up step. After that, ^{13}C-labelled clean-up standard was added. In Power-PrepTM system, all samples were treated with the silica, alumina and carbon columns (Focant et al. 2005). For elution of the columns, hexane, hexane/dichloromethane, ethyl acetate/toluene and toluene were used (Traag et al. 2008). Two fractions were collected, one containing all mono-ortho and indicator PCBs, and the other containing the non-ortho PCB and dioxin congeners. The solvents were then evaporated in a TurboVap system. Hexane was added to the TurboVap tubes and then pipetted to smaller tubes. These tubes were concentrated to dryness under a gentle nitrogen stream in a heating mantle. For the PCDD/Fs 10 µl and PCB congeners 200 µl, labelled recovery standards were added to the tubes, after vortexing, and were pipetted into the vials to be injected into the HRMS system (USEPA 1994; USEPA 1999; Traag et al. 2003).

Instrumental analysis

PCDD/Fs and PCBs' determination was carried out via GC-HRMS (EI + mode) equipped with a DB-5MS column. The source temperature and detector voltage were 260 °C and 350 V, respectively. Perfluorokerosen was used as the mass reference. Inlet temperature of the GC method used in the determination of the dioxin and non-ortho PCB congeners was 280 °C. For determination of the congeners; the injection volume is 2 µl, and the temperature ramps of the oven programme was started with 110 °C, in increments of 20 °C min^{-1} up to 200 °C, 4 °C min^{-1} up to 280 °C, holding time 20 min, 5 °C min^{-1} up to 300 °C and holding time 8 min (USEPA 1994; USEPA 1999). Carrier and make-up gas was helium with a flow of 1.2 ml min^{-1}. Compounds were acquired by selected ion monitoring with the resolution being maintained at 10.000 (10%).

Table 2. Concentrations of PCDD/F, dl-PCB (Upper boundary WHO-TEQ$_{(2005)}$ pg g^{-1} fresh weight and also pg g^{-1} fresh weight) and indicator PCB congeners (pg g^{-1} fresh weight) in honey sample. Source: Mu la-Turkey. Sample weight: 50.12 g. Collection time: September-October 2014. NHE: 47363. NTP: 6855. NHE/NTP: 6.91. Fat: 0.35 g. Fat (%): 0.698.

Congener IUPAC no.	Concentration (pg/g fresh weight)	pg WHO-TEQ/g fresh weight (u.b.)	LOQ (pg/g fresh weight)	Recovery (%)
2,3,7,8-TCDF	0.0040	0.0004	0.004	72.9
1,2,3,7,8-PeCDF	0.0060	0.0002	0.006	56.3
2,3,4,7,8-PeCDF	0.0040	**0.0012**	0.004	77.7
1,2,3,4,7,8-HxCDF	0.0030	0.0003	0.003	79.9
1,2,3,6,7,8-HxCDF	0.0030	0.0003	0.003	78.2
2,3,4,6,7,8-HxCDF	0.0030	0.0003	0.003	91.6
1,2,3,7,8,9-HxCDF	0.0030	0.0003	0.003	88
1,2,3,4,6,7,8-HpCDF	**0.0036**	0.0000	0.002	86.7
1,2,3,4,7,8,9-HpCDF	0.0030	0.0000	0.003	89.3
OCDF	0.0060	0.0000	0.006	88.8
2,3,7,8-TCDD	0.0050	**0.0050**	0.005	78.8
1,2,3,7,8-PeCDD	0.0050	**0.0050**	0.005	74.6
1,2,3,4,7,8-HxCDD	0.0030	0.0003	0.003	83.4
1,2,3,6,7,8-HxCDD	0.0030	0.0003	0.003	88.2
1,2,3,7,8,9-HxCDD	0.0030	0.0003	0.003	103.9
1,2,3,4,6,7,8-HpCDD	0.0040	0.0000	0.004	84.4
OCDD	**0.0101**	0.0000	0.005	88.8
PCB81	0.0100	0.0000	0.01	28
PCB77	**0.0447**	0.0000	0.007	36.7
PCB126	**0.0090**	**0.0009**	0.006	76.4
PCB169	**0.0054**	0.0002	0.004	91.8
PCB 123	0.0920	0.0000	0.092	80.4
PCB 118	**0.3712**	0.0000	0.097	78.4
PCB 114	0.0960	0.0000	0.096	80.5
PCB 105	**0.1210**	0.0000	0.097	79.1
PCB 167	0.0940	0.0000	0.094	69.6
PCB 156	0.0840	0.0000	0.084	75.8
PCB 157	0.0960	0.0000	0.096	71.3
PCB 189	0.0680	0.0000	0.068	75.5
PCB 028	**1.6419**	**1.6419**	0.196	27.5
PCB 052	**0.7975**	0.7975	0.094	44.3
PCB 101	**0.6873**	0.6873	0.111	89.3
PCB 153	**0.8361**	0.8361	0.086	87.9
PCB 138	**0.5092**	0.5092	0.09	84.9
PCB 180	**0.3784**	0.3784	0.064	86.8
Σ WHO-PCDD/Fs-TEQ (2005)	**0.014**			
Σ WHO-Non-ortho PCBs-TEQ (2005)	**0.001**			
Σ WHO-Mono-ortho PCBs-TEQ (2005)	**0.000**			
Σ Indicator PCBs	**4.850**			
Σ WHO-DL-PCBs-TEQ (2005)	**0.001**			
Σ WHO-PCDD/F-PCB-TEQ (2005)	**0.015**			
Dioxin-Furan/dL-PCB ratio	**12.72**			

Results

Concentrations were determined with isotope dilution and the results were expressed in pg TEQ g^{-1} fresh weight calculated with appropriate WHO-TEFs (Van Den Berg et al. 2006). Table 2 shows that the concentrations of PCDD/F, dl-PCB (Upper boundary WHO-TEQ$_{(2005)}$ pg g^{-1} fresh weight and pg g^{-1} fresh weight) and indicator PCB congeners (pg g^{-1} fresh weight) in honey sample. The results are obtained with comparison according to calibration table. These results are in mass/volume concentration. By multiplying with user divisor, dilution factor, these results are converted into mass/mass concentration.

Discussion

The percentage of fat was determined according to Smedes and Thomasen (1996). The result for each congener was multiplied by the appropriate WHO toxic equivalent factor

(WHO-TEF) (Van Den Berg et al. 2006) and summed (TEQ) for all PCDDs/PCDFs and DL-PCBs congeners. TEQ results of the honey samples for the PCDDs/PCDFs and dl-PCBs calculated according to upper bound principle, in which LOQ levels were used in calculation when the concentration of a congener is below LOQ, are shown in Table 2. All the results are given in fresh weight basis. Dry weight of honey is not taken into account. Thus, on dry weight basis the results may differ slightly.

The summed concentration of indicator PCBs is the sum of the concentrations of the congeners measured. All indicator PCBs analysed for were detected above the LOQs. The concentration of fat in the sample was 0.698%. Most of the concentrations of PCDDs/PCDFs and dioxin-like PCBs in honey sample were at LOQs level except 1,2,3,4,6,7,8-HpCDF, OCDD, PCB 77,126,169,118 and 105.

Among the dioxin and furan congeners detected and quantified, highest concentrations (TEQ_{2005}, Upper-bound calculation) were found for 2,3,4,7,8-PeCDF, 2,3,7,8-TCDD, and 1,2,3,7,8-PeCDD. The highest dioxin concentrations detected in the sample were 2,3,7,8-TCDD and 1,2,3,7,8-PeCDD with the concentration of 0.0050 WHO-$TEQ_{(2005)}$ pg/g fresh weight. The main dioxin-like and indicator PCBs detected were PCB 126 and 28. The highest dioxin-like PCB concentration detected in the samples was PCB 126 with a concentration of 0.0009 WHO-$TEQ_{(2005)}$ pg/g fresh weight. The highest indicator PCB concentration detected in all samples was PCB 28 with a concentration of 1.6419 pg/g fresh weight. Although, there is no limit for honey indicated in the EU regulation, all the results were significantly lower than the EU regulation limits for all types of food (Commission Regulation 2011). There is no data on contamination level of the region where this honey was collected, and as mentioned before, these results are the first results of analysis of honey samples for PCDDs, PCDFs, and dioxin-like and indicator PCBs in Turkey. In order to compare the results of this study, a few studies could be found in the literature (Mohr et al. 2014a; Wang et al. 2012). In terms of PCDDs/PCDFs and dioxin-like PCBs concentration, the values found in this study are lower than those found in honey samples from Brazil and Spain (Mohr et al. 2014a). The most remarkable findings found in Mohr et al. (2014a) study were the large contribution of the highly chlorinated PCDD/Fs, and PCBs 105 and 118 to the total PCDD/Fs and DL-PCBs in the honey samples were similar with this study. Very low contamination levels in honey sample show, in terms of PCDDs/PCDFs and dioxin-like PCBs concentration, the values are like those found by Wang et al. (2012) in honey from Taiwan and Mainland China.

According to the results of this study, it can be mentioned that honey sample from Muğla-Turkey is safe for the consumers. Beside these studies, further studies are essential for evaluating relationship between dioxin and honey or other bee products (*e.g.*, pollen, propolis)

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