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Original Article

DIOXIN ANALYSIS OF BEE POLLEN PELLETS COLLECTED BY *APIS MELLIFERA* L. IN RURAL AREA OF TURKEY

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Abstract

Bee pollen, an important bee product, is harvested as a food supplement for humans, so it must be safe in terms of toxic components for consumption. The aim of this study is to determine the amounts of polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), dioxin-like polychlorinated biphenyls (dl-PCBs) and non dioxin-like PCBs (ndl-PCBs) in the bee pollen pellets of *Apis mellifera* L. collected from Çankırı, located in the central Anatolia region of Turkey, between June and July 2014. Six types of pollen belonging to four families: *Centaurea triumfettii* L. - Asteraceae family; *Brassica* spp. L. - Brassicaceae family; *Cistus* spp. L. - Cistaceae family; *Onobrychis* spp. L., *Hedysarum* spp. L. and *Trifolium* spp. L. - Fabaceae family, were determined through microscopic analysis. Dioxin and PCB congeners were determined in a pooled bee pollen sample and all the results were found lower than the European Union regulatory limits for other foods. To the best of our knowledge, this is among the first studies on dioxin analysis in bee pollen worldwide.

Keywords: *Apis mellifera*, bee pollen, dioxins, environmental contamination, polychlorinated biphenyls, Turkey.

INTRODUCTION

Honeybees store pollen in their combs for protein, essential vitamins and minerals supply (Crane, 1996). Harvested as a food supplement for humans, The pollen contains 7-40 % protein, essential amino acids, 2-8 % lipids and high levels of minerals and antioxidants, especially flavonoids, which improve the immune system and delay the aging process (Krell, 1996; D'Albore, 1997; Komosinska-Vassev et al., 2015). Furthermore, Kolankaya et al., (2006) reported about the use of pollen as an aid in protection against liver toxins.

Dioxins are hydrophobic contaminants and accumulate in lipids (Fries, 1995; Olanca et al., 2014). They refer to polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls (dl-PCBs). After a number of severe dioxin-contamination incidents in recent decades, the European Union has made wide-ranging efforts including tightening of regulations to decrease dioxin release into the environment (EC, 2000; 2011).

PCDD/Fs are undesired by-products of combustion processes in the presence of chlorine and also trace contaminants in various industrial processes (USEPA, 2012). PCBs are man-made products which had been used in many industrial fields such as transformers and capacitors before their production was banned

several decades ago (Babu-Rajendran et al., 2005). Both dioxin and PCBs are listed in the twelve most persistent organic pollutants by the Stockholm Convention (2017).

These days bee products are often produced in an environment polluted from different sources of contaminants which honey bees transport to the hive (Bogdanov, 2006; Tomasini et al., 2012). Since honeybees travel long distances and come in contact with many plants and also the sources of toxic chemicals in the environment (Kim et al., 2013; Mohr et al., 2014). Although residues of persistent organic pollutants (POPs) have been found in honey samples, such as organochlorine pesticides (Erdogrul, 2007; Wang et al., 2010; Kujawski, Pinteaux, & Namiesnik, 2012), non-dioxin-like polychlorinated biphenyls (ndl-PCBs) (Herrera, 2005; Lie, 2005; Erdogrul, 2007), PCDDs, PCDFs, and dl-PCBs (Wang et al., 2012; Mohr et al., 2014), only one study (Roszko et al., 2016) could have been found for pollen samples on non-dioxin like and dioxin like PCBs.

This study aimed to determine the PCDDs, PCDFs, dl-PCBs and ndl-PCBs levels in bee pollen. There is not much honey and pollen production in İzmit and Aliağa, industrialized parts of Turkey, because of the potential risk for dioxin pollution. Çankırı, a rural mid-Anatolia city, has a granite factory and an auto-tire factory around as well as a moderate level of honey and pollen production, and therefore it is a good sampling region to investigate the background contamination level found in pollen. To our knowledge, this is one of the first studies on dioxin analysis in bee pollen in the world.

MATERIAL AND METHODS

Collection of samples

Pollen samples were collected in June-July 2014 from an apiary with 300 hives located near the granite factory in the Kurşunlu-Çankırı region. Pollen traps (Fig. 1) were fitted on ten Langstroth hives for *Apis mellifera* L. to collect the samples. Pollen accumulated in the hives' pollen traps was put into glass jars, previously decontaminated with a hexane- toluenesolvent washing, and then transported in a refrigerated container to the laboratory. Pollen pellets were firstly separated according to their colors for microscopic analysis. Coinciding flowers of plants

Fig. 1. Pollen trap with drawer (Photographed by Aslı Özkök)

which had also been collected in the field and their pollen were made as reference preparats.

Pollen preparats

The Güler & Sorkun (2010) method was followed for preparing the pollen preparats. According to this method basic, the fuchsin-glycerin-gelatine mixture was gathered with the edge of a sterile needle and mixed with the pollen pellet on a microscope slide. This mixture was melted on a hotplate at 40-50°C, and then 18 x 18 mm cover slips were placed on the preparats.

Microscopic analysis

The slides were researched via a Nikon Eclipse E400 microscope, and immersion objective (x100) was used for to identify the pollens. Studies by Özkök Tüylü & Sorkun (2007), Sorkun (2008), Avcı et al., (2013) and reference preparats were used in the identification of pollen samples. Pollen taxa were determined in six pooled pollen samples with a Nikon Eclipse E400 microscope at a x40 magnification and prepared for dioxin analysis.

Analytical procedure

After microscopic analysis, a pooled pollen sample was placed in a polyethylene box and brought to the National Food Reference Laboratory. Sample details were coded and the sample was stored in a freezer (-20°C).

Standards, solvents, and quality control

Cambridge Isotope Laboratories' standards for dioxin, furan and PCBs analysis were used: Native dioxin, furan congeners mixture, native non-ortho PCBs mixture, native ndl-PCBs mixture, ^{13}C labelled dioxin, furan congeners mixture, ^{13}C labelled non-ortho PCBs mixture, ^{13}C labelled ndl-PCBs mixture, and recovery standards. Dioxin/Furan and non-ortho PCBs' standard solutions were prepared in toluene, and mono-ortho and ndl-PCBs' standard solutions were prepared in iso-octane. A seven-point calibration curve was plotted for the quantification of dioxins and furans in the range of 0.02–20 pg µl-1And an eight-point calibration curve for that of non-ortho, mono ortho and ndl-PCBs in the range of 0.10–50 pg µl-1. ^{13}C-labelled internal, recovery and clean-up standards were also added to all calibration standards. Procedure blanks and a quality control sample (vegetable oil) were also analysed with the samples.

Extraction and clean-up

A homogenised pooled sample (9 g) was taken and mixed with diatomaceous earth (5-6 g)(Merck) in order to homogenise it and increase the surface area, and then spiked with labelled dioxin and PCB standards. Dioxins, furans, and PCBs were extracted through accelerated solvent extraction (ASE) by using toluene:ethanol (90:10) (Dionex Technical Note, 2006). Moreover, 30 g of homogenised pooled sample was taken and spiked with labelled dioxin and PCB internal standards. The Smedes & Thomasen (1996) method, which includes mechanical extraction via ultra-turrax using solvents with a different polarity, was applied to extract the lipids together with dioxins, furans and PCBs from the sample. An additional extraction technique for pollen sample was also carried out.

The pollen extracts were dissolved in n-hexane, and after the ^{13}C-labelled clean-up standard addition, aliquots were introduced to the PowerPrepTM system (FMS Inc.,USA) in which all the sample extracts were cleaned on silica, alumina and carbon columns (Focant et al., 2005). For the elution of the columns, hexane, dichloromethane, ethylacetate and toluene were used (Traag et al., 2008). Two fractions were collected, one containing all mono-ortho and ndl-PCBs, and the other containing the non-ortho PCB and dioxin congeners. After solvent evaporation, the congeners were transferred to injection vials by adding recovery standards (^{13}C-1,2,3,4-TCDD and ^{13}C-1,2,3,7,8,9-HxCDD). The samples were then injected into a gas chromatography-high-resolution mass spectrometry (GC-HRMS-Micromass Autospec/Ultima) system (USEPA, 1994, 1999; Traag et al., 2003).

Instrumental analysis

PCDD/Fs and PCBs were determined via GC-HRMS (EI+ mode) equipped with a DB5MS column. At a 10 000 resolution, native and labelled compounds were selected through ion monitoring (SIM). The source temperature and detector voltage were 260°C and 350 V, respectively. Mass calibration was done with Perfluorokerosen. Injections injected in splitless mode at 280°C. Temperature ramps of the oven programme were set between 110°C and 300°C (USEPA, 1994; 1999). Congeners were determined via the isotope dilution technique, and concentrations calculated according to relative response factors results were expressed in pg TEQ g^{-1} fresh weight calculated with appropriate $WHO\text{-}TEFs_{(2005)}$ (Van den Berg et al., 2006).

RESULTS

In this study six pollen taxa belonging to four families were determined via microscopic analysis. These were *Centaurea triumfettii* L. - Asteraceae family; *Brassica* spp. L. - Brassicaceae family; *Cistus* spp. L.- Cistaceae family: and *Onobrychis* spp. L., *Hedysarum* spp. L. and *Trifolium* spp. L. - Fabaceae family (Fig. 2).

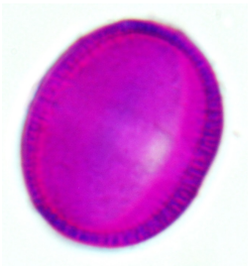
As a result of the morphologic analysis of the pollen samples, *Centaurea triumfettii* L. *was* determined to have echinate ornemantation and tricolporate pollen type, *Cistus* spp. L., *Trifolum* spp. L. to have reticulate ornemantation and tricolporate pollen type, *Onobrychis* spp. L., and *Hedysarum* spp. L. to have reticulate ornemantation and tricolpate pollen type. The

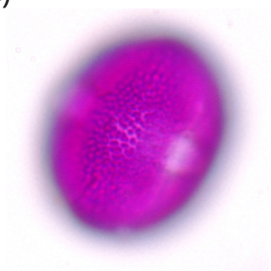
fat contents of pollen, the concentrations and limit of quantifications on a fresh weight basis, and the recoveries of each dioxin and PCB congener are given in Tab. 1. The calculated concentrations of each congener was multiplied by the corresponding WHO toxic equivalent factor (WHO-TEF_{2005}) (Van den Berg et al., 2006) to obtain toxic equivalents (TEQ) values.

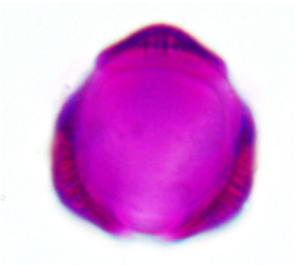
The TEQ results of the pollen samples for the PCDDs/PCDFs and dl-PCBs calculated according to the upper bound principle, in which LOQ levels were used in calculation when the congener concentration was below LOQ, are shown in Tab. 1. The fat percentages in the samples were

Pollen taxa

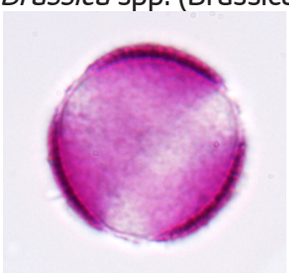
Centaurea triumfetti (Asteraceae)

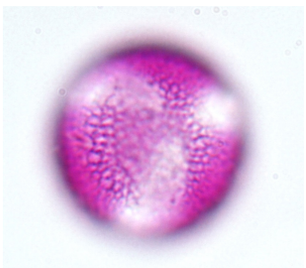

equatorial view

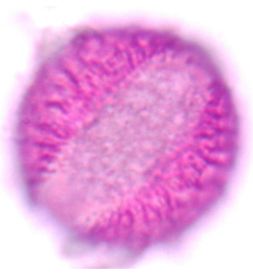

aperture and ornemantation view


polar view

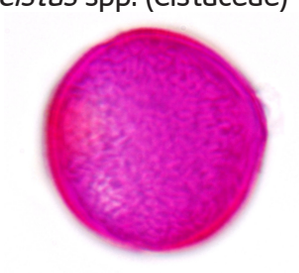
Brassica spp. (Brassicaceae)

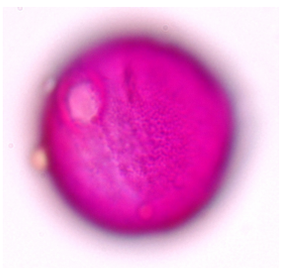

polar view

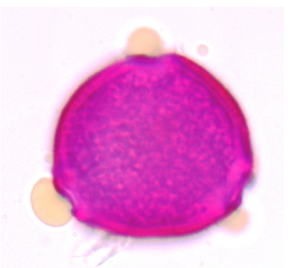

aperture and ornemantation view


equatorial view

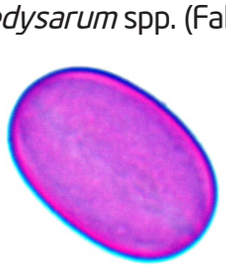
Cistus spp. (Cistaceae)

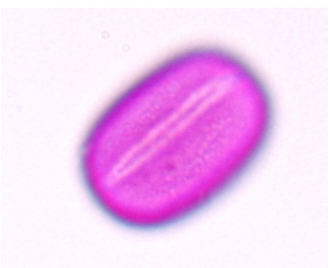

quatorial view

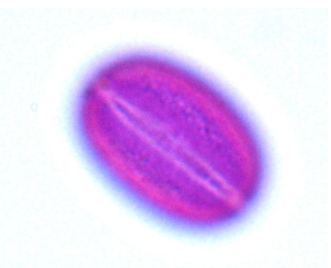

aperture and ornemantation view


polar view

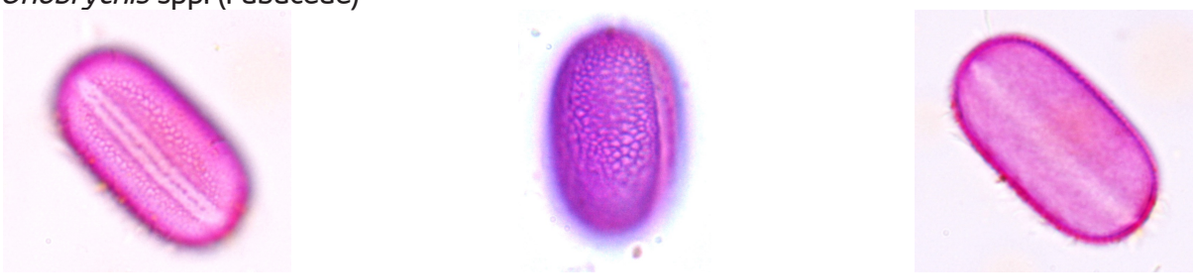
Hedysarum spp. (Fabaceae)


equatorial view


aperture and ornemantation view

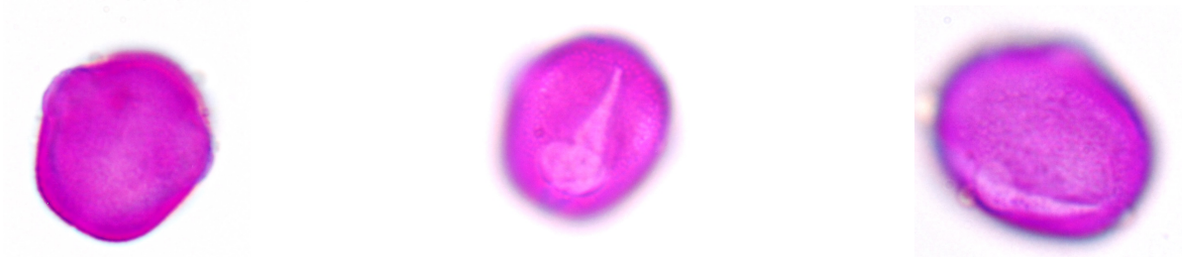

equatorial view

Onobrychis spp. (Fabaceae)



equatorial view | aperture and ornemantation view | equatorial view

Trifolium spp. (Fabaceae)



equatorial view | aperture and ornemantation view | equatorial view

Fig. 2. Microscopic pollen analysis (x40) (Photographed by Aslı Özkök)

detected as 4.7 % and 6.7 % for ASE and the Smedes -Thomasen extraction, respectively.

It can be concluded that the Smedes - Thomasen extraction worked well for fat extraction, but ASE extraction was better for the recovery of extracted congeners. Although the concentrations were lower in the Smedes-Thomasen extraction, the congener profiles were almost the same with ASE. All of the ndl-PCB congeners were detected above the LOQs. The concentrations of PCDDs/PCDFs and dl-PCBs in the pollen sample were at the LOQ level except for 2,3,7,8-TCDF, 1,2,3,4,6,7,8-HpCDF, 1,2,3,4,6,7,8-HpCDD, OCDD, PCB 81, 77, 126, 118, 105 and 156.

Among the detected and quantified dioxin and furan congeners, the highest TEQ levels (TEQ_{2005}, upper-bound calculation) were found for 1,2,3,7,8-PeCDD, 2,3,7,8-TCDD and 2,3,4,7,8-PeCDF. The highest TEQ levels (TEQ_{2005}, upper-bound calculation) were 1,2,3,7,8-PeCDD and 2,3,7,8-TCDD with the values of 0.030 and 0.024 WHO-$TEQ_{(2005)}$ pg/g f.w., respectively. The main dioxin-like and ndl-PCBs were determined to be PCB 126 and 28. The highest dl-PCB contribution to TEQ was found after ASE extraction of the sample for PCB 126 with 0.005 WHO-$TEQ_{(2005)}$ pg/g f.w. The highest ndl-PCB concentration was found after ASE extraction of the sample for PCB 28 with 17.3 pg/g f.w.

DISCUSSION

Bee pollen is a sold in mixed colors. The color of the pollen varies from bright yellow to black. Bees usually collect pollen from the same plant but sometimes from many different plant species. The pollen grains depend on the plant species and differ in shape, color, size and weight. Most families of bee pollen are the Asteraceae, Brassicaceae, Cistaceae and Fabaceae, which are the ones determined in this study (D'Albore, 1997; Sorkun, 2008). For this reason it is important to know what are the types and contents of these mixed pollens, which are sold in the markets and people use as food supplement. As mentioned by Kasiotis et al. (2014), Oliveira et al. (2016), Roszko et al. (2016), bee products and pollens are potential bioindicators of the presence of contaminants in the environment since bees travel long distances. In our study we determined six types of pollen belonging to four families and the pollen morphology results matched those described by Sorkun (2008) and Paldat (2016). The Smedes-Thomasen (1996)

Table 1.

Concentrations of individual PCDD/F, dl-PCB (pg g^{-1} fresh weight upper bound WHO-TEQ (2005), also pg g^{1} fresh weight) and indicator PCB congeners (pg g^{1} fresh weight) in a pooled pollen sample, extracted (1) using ASE, and (2) according to Smedes and Thomason (1996)

Samples	**Pollen-1-ASE extraction**				**Pollen-2-Smedes and Thomasen extraction**			
Source	Kurşunlu-Çankırı				Kurşunlu-Çankırı			
Collection time	June-July, 2014				June-July, 2014			
% Fat	4.7				6.7			
Congener IUPAC no.	Concentration (pg/g f.w.)	pg WHO-TEQ/g f.w. (u.b.)	LOQ (pg/g f.w.)	Recovery (%)	Concentration (pg/g f.w.)	pg WHO-TEQ/g f.w. (u.b.)	LOQ (pg/g f.w.)	Recovery (%)
Furans								
2,3,7,8-TCDF	0.075	0.008	0.039	70.6	<LOQ	0.001	0.014	61.2
1,2,3,7,8-PeCDF	<LOQ	0.002	0.057	43.8	<LOQ	0.001	0.025	46.0
2,3,4,7,8-PeCDF	<LOQ	0.008	0.027	77.7	<LOQ	0.005	0.016	56.1
1,2,3,4,7,8-HxCDF	<LOQ	0.002	0.019	80.2	<LOQ	0.001	0.01	63.5
1,2,3,6,7,8-HxCDF	<LOQ	0.002	0.018	83.1	<LOQ	0.001	0.01	64.5
2,3,4,6,7,8-HxCDF	<LOQ	0.002	0.02	96.2	<LOQ	0.001	0.011	65.2
1,2,3,7,8,9-HxCDF	<LOQ	0.003	0.027	75.7	<LOQ	0.002	0.017	39.3
1,2,3,4,6,7,8-HpCDF	0.022	0.000	0.016	93.4	0.02	0.000	0.01	64.3
1,2,3,4,7,8,9-HpCDF	<LOQ	0.000	0.023	92.9	<LOQ	0.000	0.017	60.7
OCDF	<LOQ	0.000	0.042	72.8	<LOQ	0.000	0.02	63.0
Dioxins								
2,3,7,8-TCDD	<LOQ	0.024	0.024	67.6	<LOQ	0.024	0.024	50.2
1,2,3,7,8-PeCDD	<LOQ	0.030	0.03	75.7	<LOQ	0.021	0.021	50.8
1,2,3,4,7,8-HxCDD	<LOQ	0.003	0.028	90.0	<LOQ	0.002	0.015	63.7
1,2,3,6,7,8-HxCDD	<LOQ	0.003	0.028	95.0	<LOQ	0.001	0.014	67.8
1,2,3,7,8,9-HxCDD	<LOQ	0.003	0.025	99.4	<LOQ	0.001	0.012	66.9
1,2,3,4,6,7,8-HpCDD	0.028	0.000	0.02	92.7	0.016	0.000	0.01	69.6
OCDD	0.108	0.000	0.054	72.8	0.040	0.000	0.018	63.0
NonOrtho PCBs								
PCB81	0.067	0.000	0.051	27.4	<LOQ	0.000	0.037	17.5
PCB77	1.196	0.000	0.036	37.2	0.321	0.000	0.025	31.0
PCB126	0.053	0.005	0.032	77.5	<LOQ	0.003	0.027	68.6
PCB169	<LOQ	0.001	0.026	101	<LOQ	0.001	0.025	64.9
MonoOrtho PCBs								
PCB 123	<LOQ	0.000	0.512	96.2	<LOQ	0.000	0.114	94.6
PCB 118	5.992	0.000	0.513	93.5	1.840	0.000	0.115	90.2
PCB 114	<LOQ	0.000	0.456	84.0	<LOQ	0.000	0.228	38.5
PCB 105	2.700	0.000	0.549	90.8	0.623	0.000	0.121	89.6
PCB 167	<LOQ	0.000	0.445	82.0	<LOQ	0.000	0.142	74.0
PCB 156	0.432	0.000	0.421	86.3	<LOQ	0.000	0.13	74.5
PCB 157	<LOQ	0.000	0.433	87.5	<LOQ	0.000	0.149	72.8
PCB 189	<LOQ	0.000	0.334	74.4	<LOQ	0.000	0.198	46.2
Ind. PCBs								
PCB 028	17.3	17.3	0.289	112	11.19	11.2	0.086	102
PCB 052	13.2	13.2	0.325	109	10.50	10.5	0.06	107
PCB 101	7.86	7.86	0.514	119	3.754	3.75	0.113	120
PCB 138	2.61	2.61	0.445	96.5	1.381	1.38	0.126	100
PCB 153	5.96	5.96	0.408	104	3.15	3.15	0.132	106
PCB 180	2.30	2.30	0.286	96.9	1.234	1.23	0.107	93.9
ΣWHO-PCDD/Fs-TEQ (2005)		0.089				0.062		
ΣWHO-Non-ortho PCBs-TEQ (2005)		0.006				0.003		
ΣWHO-Mono-ortho PCBs-TEQ (2005)		0.000				0.000		
ΣIndicator PCBs		49.30				31.20		
ΣWHO-DL-PCBs-TEQ (2005)		0.006				0.003		
ΣWHO-PCDD/F-PCB-TEQ (2005)		0.095				0.065		
Dioxin-Furan/dL-PCB ratio		14.80				20.70		

extraction and ASE extraction results were found to be different. Moreover, the PCB 126 concentration in extracts from ASE was twice as high as the Smedes extraction, a finding which could not be explained. Thus an efficient and more consistent extraction procedure should be established in future studies. There is no regulatory limit for dioxins/furans and PCBs for bee products in the EU Regulations. However, all measured concentrations were found to be lower than the smallest ML (Maximum Limit) given for various foods in EU legislation (EC, 2011; 2016). No previous data is available on contamination levels of the region where this pollen was collected, and as noted before, these outcomes are the first results of analysed pollen samples for PCDDs, PCDFs, and dioxin-like PCBs and ndl-PCBs in Turkey. The levels of PCDDs/PCDFs concentration were similar to those found in honey samples from Brazil and Spain (Mohr et al., 2014), while the dl-PCBs concentration values were lower. The most remarkable finding described in the study by Mohr et al., (2014) is the large contribution of the OCDD/Fs, PCBs 105 and 118 congeners to the total of PCDD/Fs, and of dl-PCBs. A similar congener pattern was also found in this study. Indicator PCBs' results found in this study are much lower than the results found in Herrera et al. (2005), Erdoğrul (2007), and lower than Roszko et al. (2016) findings. Moreover, dl-PCBs' results in our study are found lower than Roszko et al. (2016). Since Çankırı is a rural mid-Anatolia city with a few industrial plants, the results may be a good indicator showing background contamination of pollen in Turkey. According to the Turkish Ministry of Health Publishment No: 931 (TBSA, 2014), the average daily consumption of honey, jam and pekmez (traditional Turkish food) is 9.97g. Since there is no separate figure for honey, all of 9.97g is thought to result from honey as the worst scenario in this study. Thus, daily exposure to dioxin and dl-PCBs from honey is approximately 0.015 pgTEQ/kg b.w. assuming 65 kg as an average body weight, and the calculated daily exposure is much lower than 2 pgTEQ/kg b.w. set by the EU Scientific Committee on Food (SCF) (SCF, 2001) as a tolerable daily intake.

The outcomes of this study clearly show that tested bee pollen types are safe in terms of the environmental pollutants dioxins and PCBs, but future studies including more bee pollen taxa from different regions should be performed to further evaluate dioxin and PCB levels in bee pollen in Turkey. Morever further studies are needed in order to improve the extraction method efficiency.

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ABBREVIATIONS

ASE: Accelerated solvent extraction
dl-PCBs: Dioxin-like polychlorinated biphenyls
GC-HRMS: Gas chromatography-high-resolution mass spectrometry
HpCDF: Heptachlorodibenzofuran
HxCDD: Hexachlorodibenzo-p-dioxin
HpCDD: Heptachlorodibenzo-p-dioxin
LOQ: Limit of detection
ndl-PCBs: Non-dioxin-like polychlorinated biphenyls
OCDD: Octachlorodibenzodioxin
PCB: Polychlorinated biphenyls
PCDDs: Polychlorinated dibenzo-p-dioxins.
PCDFs: Polychlorinated dibenzofurans
PCDD/Fs: Polychlorinated dibenzodioxins
PeCDD: Pentachlorodibenzo-p-dioxin
PeCDF: Pentachlorodibenzofuran
POPs: Persistent organic pollutants
TCDD: Tetrachlorodibenzo-p-dioxin
TCDF: Tetrachlorodibenzofuran
TEQ: Toxic Equivalents
USEPA: United States Environmental Protection Agency
WHO-TEQ: World Health Organization – Toxic Equivalents