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Mycotoxin Analysis in Pollen and Bee Bread Samples

Polen ve Arı Ekmeği Örneklerinde Mikotoksin Analizi

Sibel SİLİCİ[a], Uğur KESKİN[a]

[a]Erciyes University Faculty of Agricculture, Department of Agricultural Biotechnology, Kayseri, Türkiye

ABSTRACT Objective: Apitherapy is the use of bee products such as honey, pollen, bee bread, royal jelly, propolis and bee venom to protect against diseases and support healing. However, it is important that these products do not contain residues and contaminants. For this reason, this study aimed to determine the contaminant content by performing aflatoxin and ochratoxin analysis on pollen and bee bread samples obtained from different geographical regions. **Material and Methods:** In this study, moisture, dry matter, ash, protein and mycotoxin content of 18 pollen (Kırşehir, Nevşehir, Kayseri, Aydın, Silifke, Niğde, Bursa, Denizli, Isparta, Kütahya, İzmir, Afyon) and 2 bee bread samples (Kırşehir and Nevşehir) obtained from beekeepers were analyzed. **Results**: The moisture content of the samples is between 13.82% and 29.10% (average 17.80), dry matter content is between 70.90 and 86.18 (average 82.20), ash is between 1.60% and 3.32% (average 2.11) and protein content is between 15.06% and 27.95% (average of 20.49). Aflatoxin B1, Aflatoxin B2, Aflatoxin G1, Aflatoxin G2 and total Aflatoxin (B1+B2+G1+G2) levels were not detected above the detection limit (0.60 µg/kg) in any of the samples tested. **Conclusion:** According to the results obtained, the mycotoxin analysis values of pollen and bee bread samples obtained from different regions of our country were found to be in accordance with the Turkish Food Codex Limits.

Keywords: Pollen; bee bread; chemical analysis; aflatoxin; mycotoxin

ÖZET Amaç: Apiterapi bal, polen, arı ekmeği, arı sütü, propolis ve arı zehiri gibi arı ürünlerinin hastalıklardan koruma ve tedaviye destek amacıyla kullanımıdır. Ancak bu ürünlerin kalıntı ve bulaşan içermemesi önemlidir. Bu nedenle, bu araştırmada farklı coğrafik bölgelerden elde edilen polen ve arı ekmeği örneklerinde aflotoksin ve okratoksin analizi yapılarak bulaşan içeriğini belirlemek amaçlanmıştır. **Gereç ve Yöntemler:** Bu çalışmada, arıcılardan elde edilen 18 polen (Kırşehir, Nevşehir, Kayseri, Aydın, Silifke, Niğde, Bursa, Denizli, Isparta, Kütahya, İzmir, Afyon) ve 2 arı ekmeğinin (Kırşehir ve Nevşehir) nem, kuru madde, kül, protein ve mikotoksin içerikleri analiz edilmiştir. **Bulgular:** Numunelerin nem içeriği %13,82 ile %29.10 (ortalama 17,80), kuru madde içeriği 70,90 ile 86,18 (ortalama 82,20), kül içeriği %1,60 ile %3,32 (ortalama 2,11) arasında ve protein içeriği ise %15,06 ve %27,95 (ortalama 20,49) arasında bulunmuştur. Aflatoksin B1, Aflatoksin B2, Aflatoksin G1, Aflatoksin G2 ve toplam Aflatoksin (B1+B2+G1+G2) düzeyleri test edilen örneklerde tespit sınırının (0,60 µg/kg) üzerinde bulunmadı. **Sonuç:** Elde edilen sonuçlara göre ülkemizin farklı bölgelerinden alınan polen ve bee bread örneklerinin mikotoksin analiz değerlerinin Türk Gıda Kodeksi Limitlerine uygun olduğu tespit edilmiştir.

Anahtar Kelimeler: Polen; arı ekmeği; kimyasal analiz; aflatoksin; mikotoksin

Pollen grain is produced and released from anthers during pollination as the male gametophyte of flowering plants. One of the most important pollinators is honey bee (*Apis mellifera L.*) for entomophilous plants and wind for anemophilous plants.[1] Pollen is potentially a good dietary supplement for human nutrition. A mixture of flower pollen from different species is agglutinated by nectar and honeybee enzymes (e.g. amylase, catalase) secreted by salivary glands and pollen-loads are formed, which are recognised as bee pollen.[2] Bee bread (Perga), another important bee product, is a natural product obtained by fermenting bee pollen by mixing it with bee saliva and flower nectar in the honeycomb cells of the hive. Bee pollen and bee bread is considered a functional product, having various nutritional values and various bioactive molecules with imporatant biological effects.[3]

Correspondence: Sibel SİLİCİ
Erciyes University Faculty of Agricculture, Department of Agricultural Biotechnology, Kayseri, Türkiye
E-mail: silicis@erciyes.edu.tr

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Pollen grain contains a significant amount of secondary plant metabolites such as different phenolic compounds or carotenoids as part of the plant's defense mechanism and has antioxidant activity.[4,5] However, pollen may contain some contaminants.[4] Due to its ideal water (moisture) content, water activity and pH value, pollen often creates an ideal environment for the growth of different microorganisms (bacteria, mold and yeast). As a result of the presence of mold and yeast, mycotoxin production may occur. Mycotoxins are secondary metabolites of different fungal species that are toxic to vertebrates and can cause some diseases and even death. Various mycotoxins [aflatoxins (AFs) B, G and/or M, ochratoxin A (OTA), patulin] have been defined by the European Commission in different foods (nuts, cereals, dried fruit juices, milk, etc.).[6] Maximum tolerable levels were determined for fumonisin B1 and B2, deoxynivalenol and zearalenone. However, there is no information on bee products such as honey, pollen or bee bread. More than a hundred mycotoxins are known, most of which are produced by some species belonging to one of the three fungal species; *Aspergillus*, *Penicillium* and/or *Fusarium*. According to the available literature, it has been shown that the following mycotoxins can be found in pollen; AFs, ochratoxins, fumonisins, zearalenone, deoxynivalenol and its acetoxy derivative, T-2 toxin (T-2), HT-2 toxin, fusarenone-X, diacetoxycirpenol, nivalenol, neosolaniol, roridin A, verrucarrin A, α-β dehydrocurvularin, fomalactone, 6-(1-propenyl)-3,4,5,6-tetrahydro-5-hydroxy-4H-pyran-2-one, 5-[1-(1-hydroxybut-2-enyl)]-dihydrofuran-2-one and 5-[1-(1-hydroxybut-2-enyl)]-furan-2-one.[8-16]

AFs are the product of metabolism of *A. flavus* and *A. parasiticus* strains as *Aspergillus* spp. and different fungal species belonging to the genus *Aspergillus* spp. The most toxic and dangerous AFs are aflatoxins B1 (AFB1) and B2. Both AFB1 and B2 are carcinogenic to humans and animals and are listed as Group 1 carcinogens according to the International Agency for Research on Cancer.[17] It is important to carry out mycotoxicological analyzes together with the microbiological characterization of the pollen. Mycotoxins are secondary metabolites, and even though they occur at ppm and ppb levels, their prevalence and threat to human health has been the subject of research.[18,19]

AFs are toxic to mammals, common and the most effective mold-derived carcinogens.[20] It is stated that AFs cannot be completely eliminated by various processing methods and may pose a risk to human health even at a very small rate.[21] Therefore, in this study, it was aimed to determine the mycotoxin content of pollen and bee bread samples obtained from different geographical regions of Türkiye.

MATERIAL AND METHODS

Pollen (n=18; Kırşehir, Nevşehir, Kayseri, Aydın, Silifke, Niğde, Bursa, Denizli, Isparta, Kütahya, İzmir, Afyon) and bee bread samples (n=2; Kırşehir and Nevşehir) used in the research were obtained from beekeepers. The collected fresh samples were labeled and stored in the freezer at-20 °C in moisture-proof packages until analysis.

MOISTURE ANALYSIS

For analysis, 5 g of the ground sample was weighed and put in an oven at 130 °C. After the moisture was evaporated, the remaining material was weighed. Moisture content was calculated according to the formula: X=(m-mo)/mx100 (m; initial weight of sample (g), mo; weight of dried sample (g)

ASH ANALYSIS

The sample was burned in the muffle furnace at 550±10 °C and turned into ashes and the residue was weighed and the ash amount was calculated.

PROTEIN ANALYSIS

Protein analysis of the samples was determined using a fully automatic protein analyzer from Dumas (Elementar Analysis Systeme GmbH, Germany). The nitrogen released as a result of burning the sample with pure oxygen (99.9%) at high temperature (850-950 °C) was measured with the help of thermal conductivity and expressed as % protein by multiplying with the appropriate protein factor.[22] Calculation; It was made according to the formula % crude protein (m/m)=%NxF protein (N conversion factor 6.25).

AFLATOXIN ANALYSIS

High performance liquid chromatography (HPLC), HPLC Pump (Shimadzu LC-20AD), RF-10AXL Fluorescence detector (FLD) and HPLC column (ODS3-250 mm-5µm-4.6 mm) were used in the analysis. Certified mixed standard with a total aflatoxin concentration of 2,500 ng/mL was used as aflatoxin B1, B2, G1, G2 standard. Each toxin concentration contained 1,000 ng/mL B1, 250 ng/mL B2, 1,000 ng/mL G1, 250 ng/mL G2. AOAC Official Method 991.31 was used for aflatoxin analysis. For the determination of aflatoxin, the quantitative analysis method was applied after the pre-treatments (extraction, cleaning of the extract, immunoaffinity stage, injection into HPLC).[23,24]

Briefly, 25 g sample was weighed and 5 g NaCl was added, 125 mL of 70% methanol (MeOH) was added and mixed at high speed for 5 minutes. It was filtered through coarse filter paper and Whatman no: 4 filter paper using a glass funnel. 5 mL of the filtrate was taken with a pipette and 20 mL of distilled water was added to it. The column was fixed to the injector, 25 mL of extract and water mixture was transferred to the 20 mL injector with a pipette. Then, washing was done by passing 15 mL of distilled water. The flow rate did not exceed 5 mL/min. 1 mL of MeOH is added and slowly passed through the column. Methanol is collected in a 2.5 mL flask. The process is repeated with 1.5 mL of distilled water and the water is taken into the same flask. The total volume is made up to 2.5 mL. Mix thoroughly by vortexing. It is taken into a 2 mL vial and injected. 100 µL is injected from each sample once. HPLC conditions; Wavelength Ex: 360 nm; Em: 430 nm, Temperature 40 °C, Pump Flow Rate: 1 mL/min, Pressure<180 bar, Injection Volume 100 µL, Column ODS3 column 250 mm 5 µm-4.6 mm. Aflatoxin G2 recovery rate=90.72%, G1 recovery rate=94.04%, B2 recovery rate=92.70%, B1 recovery rate=92.24%, Total Aflatoxin recovery rate was 92%.

OTA ANALYSIS

Shimadzu brand HPLC device was used in the analysis of ochratoxin according to the method of AOAC Official Method 2000.03, R-BIOPHARM Rice Ochraprep Application Note, Ref no: A23-P14.V2.[25] According to the HPLC-FLD analysis method used for the determination of ochratoxin, its principle is to capture OTA and take it from the IAK with methanol-acetic acid (98:2), under high pressure with the help of a mobile liquid phase, while passing through the HPLC column at certain wavelengths (Ex: 333 nm, Em: 443 nm) was read with a FLD.

STATISTICAL ANALYSIS

Values are given as the means±standard deviation of five measurements.

RESULTS

The amount of dry matter, ash amount, ash amount in dry matter, moisture content and protein values in the pollen and bee bread samples used in the research are shown in Table 1. The moisture content of the samples varied between 13.82 and 29.10%. The mean moisture content was found to be 17.80±3.19%. Dry matter content of pollen samples varied between 70.90 and 86.18 values. The average dry matter content was found to be 82.20±3.19%. The ash content of the samples varied between 1.60% and 3.32%. The average ash content was found to be 2.11±0.42%. The mean protein content was found to be 20.49±3.53%.

As a result of the analyzes, AFB1, Aflatoxin B2 (AFB2), Aflatoxin G1 (AFG1), Aflatoxin G2 (AFG2) and total Aflatoxin (B1+B2+G1+G2) and OTA not detected (Table 1).

DISCUSSION

Evaluation of the microbiological quality of bee pollen is very important as it is used as a supplement in the human diet. Determining the microbiological quality of pollen is as important as determining its chemical content. Although studies of the analysis of mycotoxins in pollen have mostly started in the last decade, the identification of different microorganisms (bacteria, mold and yeast) in pollen samples started with Gilliam's work in the late 1970s.[26]

Although there are Tolerable Daily Intake values for some mycotoxins, there is no information on recommended limits for mycotoxins in pollen. In 2008, Campos et al. suggested that in the case of

TABLE 1: Chemical content and mycotoxin analysis of pollen and bee bread samples.

Sample	Dry matter %	Ash %	Ash in dry matter %	Moisture %	Protein %	Total aflatoxin/ ochratoxin A
Pollen 1	70.90	1.82	2.56	29.10	17.22	ND
Pollen 2	84.61	2.24	2.64	15.39	25.84	ND
Pollen 3	84.58	2.76	3.26	15.42	24.44	ND
Pollen 4	80.54	2.12	2.64	19.46	21.40	ND
Beebread 1	78.08	1.86	2.39	21.92	16.48	ND
Pollen 5	83.52	1.74	2.09	16.48	25.89	ND
Beebread 2	84.71	2.24	2.64	15.29	17.04	ND
Pollen 6	85.79	2.31	2.69	14.21	19.81	ND
Pollen 7	86.18	1.76	2.05	13.82	19.83	ND
Pollen 8	84.86	1.74	2.05	15.14	20.81	ND
Pollen 9	82.36	1.72	2.08	17.64	18.57	ND
Pollen 10	84.54	1.96	2.31	15.46	15.11	ND
Pollen 11	84.83	1.94	2.29	15.17	20.12	ND
Pollen 12	77.10	3.32	4.31	22.90	20.72	ND
Pollen 13	84.23	2.11	2.50	15.77	18.83	ND
Pollen 14	85.21	2.19	2.57	14.79	18.86	ND
Pollen 15	83.39	2.05	2.45	16.61	27.95	ND
Pollen 16	84.02	2.26	2.69	15.98	15.06	ND
Pollen 17	78.83	2.46	3.12	21.17	22.06	ND
Pollen 18	75.80	1.60	2.10	24.20	23.80	ND
$\bar{X}$±SD	82.20±3.19	2.11±0.42	2.57±0.56	17.80±3.19	20.49±3.53	-
Minimum	70.90	1.60	2.05	13.82	15.06	-
Maximum	86.18	3.32	4.31	29.10	27.95	-

ND: Not detected; SD: Standard deviation.

AFB1 formation in pollen, the MPC value should be 2 µg/kg, ie 4.2 µg/kg for total AFs.[27] The Tolerable Weekly Intake for ochratoxins as required by the European Food Safety Authority is 0.12 µg/kg bw.[28] In these regulations, there is no information about the recommended limits for mycotoxins in pollen. In our study, the detection limit was determined as 0.60 µg/kg in pollen and bee bread samples. As a result of the analyzes, AFB1, AFB2, AFG1, AFG2 and total aflatoxin (B1+B2+G1+G2) and OTA could not be detected (Table 1).

In a study on this subject, Xue et al. reported that they did not find ochratoxin contamination in Chinese pollen samples, and that this may be due to the collection time and dry weather conditions.[29] Our results was found to be consistent with this study. González et al. detected *Aspergillus* spp. and *Penicillium* spp. in bee pollen from Spain.[30] Ochratoxin contamination has been reported in bee pollen collected in Slovakia[14]. Medina et al. found contamination in bee pollen added to yeast extract sucrose juice inoculated with *A. ochraceus* spores.[9] In a study conducted in Serbia, Kostić et al. determined that there was AFB1 contamination.[31] In another study conducted in Serbia, the number of fungi, incidence of fungi and AFB1 contamination were determined in bee pollen. Examples include *Acremonium* spp. *Alternaria* spp., *Aspergillus* spp., *Cladosporium* spp., *Epiccocum* spp., *Fusarium* spp., *Mucor* spp., *Penicillium* spp. and *Rhizopus* spp. In their studies, the dominance of fungi of the genus *Aspergillus* spp. and *Alternaria* spp. has been shown.[32] Pitta and Markaki showed in their study that mycelial growth was not observed and AFB1 was not detected in bee pollen samples containing natural microbiota.[33]

In this study, mycotoxin analyzes were performed on eighteen different pollen and two different bee bread samples obtained from different regions in Türkiye, and the risks and reliability of the samples were determined. Analysis results showed that

no aflatoxin or ochratoxin derivatives were found in any of the samples. According to the Turkish Food Codex Contaminants Regulation and other international regulations, there is no set limit for pollen and bee bread yet. However, the maximum limits determined for foodstuffs such as dried fruits, peanuts, oil seeds, nuts, hazelnuts, almonds, pistachios and apricot kernels are between 4-15 µg/kg for total aflatoxin and 3-80 µg/kg for OTA. Therefore, the results obtained are below the Turkish Food Codex limits and are safe.[34]

Aflatoxin and ochratoxin results obtained by Campos et al. suggested that the MPC value in pollen is below the value, indicating that the samples can be easily used as a suitable supplementary food.[27]

In addition, since bee pollen is an important risk factor for the formation of ochratoxin and aflatoxin in the food chain, it is important that the samples do not contain harmful formations in the light of the data obtained as a result of the study.

CONCLUSION

As a result, pollen quality can be significantly affected by the presence of toxigenic fungi. Mycotoxicological analyzes should be included as a regular control measure along with microbiological testing, as the absence of microbial contamination in pollen has not been shown to exclude the presence of mycotoxins. Since AFs and ochratoxins have been proven to be carcinogenic substances, their presence in pollen is extremely undesirable. Therefore, it is important to monitor mold and mycotoxin levels in feed/food to avoid adverse health effects. It would be beneficial to use pollen as a food supplement in the current legislation. It should cover the tolerable daily/weekly intake for different mycotoxins as well as the sum of the recommended quality parameters. Further studies on the toxicological effect of mycotoxin combinations are needed to obtain reliable and accurate recommendations for pollen quality control. Conducting and disseminating such studies and analyzes is important for the reliability of bee products produced in our country.

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Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea/Concept: *Sibel Silici;* ***Design:*** *Sibel Silici;* ***Control/Supervision:*** *Sibel Silici;* ***Data Collection and/or Processing:*** *Uğur Keskin;* ***Analysis and/or Interpretation:*** *Uğur Keskin;* ***Literature Review:*** *Uğur Keskin;* ***Writing the Article:*** *Sibel Silici;* ***Critical Review:*** *Sibel Silici;* ***References and Fundings:*** *Sibel Silici;* ***Materials:*** *Sibel Silici.*

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