aurum

AURUM MÜHENDİSLİK SİSTEMLERİ VE MİMARLIK DERGİSİ
AURUM JOURNAL OF ENGINEERING SYSTEMS AND ARCHITECTURE

Cilt 5, Sayı 1 | Yaz 2021
Volume 5, No 1 | Summer 2021, 139-148

ARAŞTIRMA MAKALESİ / RESEARCH ARTICLE

NATURALLY OCCURING NITRATE AND NITRITE İN NUTRIENTS: DETERMINATIONS İN ANALTOLIAN HONEY-BEE POLLENS BY CAPILLARY ELECTROPHORESIS

Zeynep KALAYCIOĞLU[1],
[1]Department of Chemistry, Istanbul Technical University, Istanbul, Turkey
kalaycioglu@itu.edu.tr ORCID No: 0000-0002-0967-0997

H. Sena KARADENİZ[2],
[2]Department of Chemistry, Istanbul Technical University, Istanbul, Turkey
haticesenakaradeniz@gmail.com ORCID No: 0000-0002-2309-6477

F. Bedia ERİM[3]
[3]Department of Chemistry, Istanbul Technical University, Istanbul, Turkey
erim@itu.edu.tr ORCID No: 0000-0001-9406-6681

Geliş Tarihi/Received Date: 01.05.2021 Revizyon Tarihi/Revision Date: 11.06.2021
Kabul Tarihi/Accepted Date: 14.06.2021

Abstract

The aim of this work is the simultaneous determination of nitrate and nitrite in a set of bee-pollens of Anatolia using a capillary electrophoretic technique. Separation and determination conditions were 30 mM formic acid/formate buffer at pH 4.0. In earlier steps with pollen samples, only nitrate anion was detected. Consequently, a sample stacking method was applied to increase the detection sensitivity of both anions for the pollen samples. In a stacking operation, the conductivity of separation buffer should be higher than the sample zone. The conductivity of the separation buffer was increased by the addition of 30 mM sodium sulfate. Injection condition was 160 s at 50 mbar. The calibration curves show linear dynamic ranges from 1.5 to 30 µmol/L and 1.5 to 10 µmol/L with correlation coefficients of 0.989 and 0.999 for nitrate and nitrite ions, respectively. All the pollen samples contained appreciable amounts of nitrate (from 2.880 ± 0.055 to 18.72 ± 0.46 mg/kg). Low levels of nitrite were found in all samples (from 0.048 ± 0.008 to 0.301 ± 0.001 mg/kg).

Keywords: Capillary electrophoresis, Pollen, Nitrate, Nitrite, Sample stacking

GIDALARDA DOĞAL OLARAK OLUŞAN NİTRAT VE NİTRİT: KAPİLER ELEKTROFOREZ ILE ANADOLU ARI POLENLERİNDEKİ TAYİNİ

Özet

Bu çalışmanın amacı, Anadolu'nun farklı bölgelerinden toplanan bir seri arı poleninde nitrat ve nitritin kapiler elektroforetik bir teknik kullanılarak bir arada tayin edilmesidir. Ayırma ortamı olarak 30 mM formik asit / format tamponu (pH: 4.0) kullanılmıştır. Ön çalışmalarda polen örneklerinde yalnızca nitrat anyonu tespit edilmiştir.

Polen numunelerinde her iki anyonun deteksiyon hassasiyetini artırmak için bir örnek sıkıştırma yöntemi uygulanmıştır. Örnek sıkıştırma yönteminde, ayırma tamponunun iletkenliği numune bölgesinden daha yüksek olmalıdır. Bu çalışmada, ayırma tamponunun iletkenliği, 30 mM sodyum sülfat ilavesiyle artırılmıştır. Enjeksiyon koşulu, 50 mbar'da 160 saniyedir. Kalibrasyon eğrileri, nitrat ve nitrit iyonları için sırasıyla 0.989 ve 0.999 korelasyon katsayıları ile 1.5-30 µmol / L ve 1.5-10 µmol / L arasında doğrusallık göstermiştir. Tüm polen örneklerinin önemli miktarda nitrat içerdiği görülmüştür (2.880 ± 0.055 - 18.72 ± 0.46 mg/kg). Nitrit içeren örneklerdeki nitrit seviyeleri ise 0.048 ± 0.008 ile 0.301 ± 0.001 mg/kg arasındadır.

Anahtar Kelimeler: Kapiler elektroforez, Polen, Nitrat, Nitrit, Örnek sıkıştırma

1. INTRODUCTION

Dietary nitrate and nitrite are consumed from vegetables, fruits or processed meats (Öztekin, Nutku and Erim, 2002). Nitrate is reduced to nitrite in mouth and gastrointestinal tract. It has been long believed that excessive nitrite increases the risk of gastrointestinal cancer. However, health benefit effects of nitrate and nitrite taken from natural products were revealed (Bondonno, Croft and Hodgson, 2016; d'El-Rei et al., 2016). The studies reporting the health benefits of dietary nitrate and nitrite were summarized in review articles (Bryan and Ivy, 2015; Kapil et al., 2014; Machha and Schechter, 2011). Moreover, nitrate and nitrite contents of food products from worldwide have been displayed considering their risk and benefit in our review article (Kalaycıoğlu and Erim, 2019).

Honey-bee products have gained a huge interest due to their many benefits to humans.

Bee pollen is a natural honey-bee product which is mostly consumed in terms of nutritional value. It has been also used for therapeutic aims since ancient times. It consists of the flower pollen which is collected by worker bees. Honey bee-pollen is promoted as a valuable nourishment source due to the secondary metabolites, enzymes and co-enzymes which are contained (Aylanc et al., 2021). The antioxidant, antibacterial, and antimutagenic activities of honey bee-pollen are reported (Dias et al., 2016; Kalaycıoğlu et al., 2017; Su et al., 2021). However, since the pollens come from different plants and flowers, the contents may significantly varied. The varying contents cause the difference in the biological activities and thus in therapeutic effects. Therefore, it is difficult to get a standardization in the application of bee-pollen in phytomedicine.

It is clearly shown that the contents such as vitamins, minerals, and phenolics of bee-products are certainly dependent on their botanical origins (Kalaycıoğlu et al., 2017; Kaygusuz et al., 2016). Hence, nitrate and nitrite levels in bee-pollen could be an additional factor in the classification.

Anatolia is one of the most extremely convenient region for the apicultural products in the world due to its geographical location, wide flora and a variety of fruits. The present study reports, for the first time, nitrate and nitrite contents of 10 Anatolian pollen samples from different botanical origins, using a capillary electrophoresis-sample stacking analysis technique.

2. EXPERIMENTAL SECTION

2.1 Chemicals and standard solutions

Analytical grade sodium nitrite, potassium nitrate, formic acid, sodium sulphate and sodium hydroxide were purchased from Merck (Darmstadt, Germany). Solutions were prepared using ultrapure water from a Milli-Q water system (Purelab Option Q).

The nitrate and nitrite stock solutions were prepared separately from each analyte at 10 mmol/L levels in deionized water and stored at 4°C. The stock solutions were gradually diluted to the working concentration levels with deionized water.

2.2 Pollen samples

Ten pollen samples were collected from beekeepers in different regions of Anatolia and given in Table 1 with the information on the geographical origins of the samples. The pollen samples collected from Anzer plateau where is famous for its many endemic flowers and the honeys, Kastamonu, Bayburt, and Balıkesir are heterofloral. Chestnut pollens were collected from Zonguldak and Ereğli region and oak pollen was from Kırklareli. Buckwheat rich pollen was collected from Konya.

2.3 Preparation of the samples for the analysis

Pollen samples were grounded in a pestle and then 500 mg of fine powdered samples were accurately weighed. Each sample was extracted twice with 2.5 mL of deionized water. The suspensions were stirred for 5 minutes at 85°C with vortex and then incubated for 30 minutes in water bath. The supernatants were combined after being filtered through a Whatman 41 filter paper and the final extract was diluted to 5 mL with deionized water. The resulting solution was filtered from 0.45 µm microfilter and directly injected. Pollen samples were analysed at least in three times.

Table 1. Classification of the studied pollen samples

Pollen Samples	Botanical origin	Collected area of Turkey
Anzer 1	Heterofloral	Anzer (Low region)
Anzer 2	Heterofloral	Anzer (Middle region)
Anzer 3	Heterofloral	Anzer (Hill region)
Chestnut 1	Rich in chestnut pollen	Zonguldak
Chestnut 2	Rich in chestnut pollen	Ereğli
Buckwheat	Rich in buckwheat pollen	Konya
Oak	Rich in oak pollen	Kırklareli
Abana	Heterofloral	Kastamonu
Bayburt	Heterofloral	Bayburt
Balıkesir	Heterofloral	Balıkesir

2.4 Apparatus and operating conditions

All experiments were performed with an Agilent 1600 capillary electrophoresis system (Waldbronn, Germany). Separations were carried out in uncoated fused silica capillaries with 50 µm i.d. (Polymicro Technology, Phoenix, AZ, USA). The total length of capillary was 65 cm and the length to the detector was 50 cm. The separation electrolyte was 30 mmol/L formic acid buffer at pH 4.0 containing 30 mmol/L sodium sulphate. Samples were injected at 50 mbar for 160 s. The separation voltage was -25 kV, and measurements were performed at 25°C. The UV detection was carried out at 210 nm. Data collection and processing were carried out using Agilent ChemStation software. Before use, new capillaries were conditioned with 1 mol/L sodium hydroxide and water for 30 min. The capillary was flushed successively with 0.1 mol/L sodium hydroxide, water and running electrolyte between runs for 2 min each.

3. RESULTS AND DISCUSSION

3.1 Determination of nitrate and nitrite contents of pollen samples

Nitrate and nitrite anions were determined in the pollen samples by the sample stacking-capillary electrophoresis technique. Both anions were separated in 2 min using low pH running buffer. Since the electroosmotic flow (EOF) inside the capillary column is reduced at low pHs, the anions having high electrophoretic mobilies can rapidly move when the injections are applied from cathodic side. So, the polarity was changed to negative in this study. Formic acid/formate buffer at pH:4.0 was chosen as running buffer.

Pollen samples were applied to low volume sample injection with 50mbar 6s in the preliminary experiments. However, only nitrate was detected in the samples. Hence, in further step, a sample stacking technique was applied. Kalaycıoğlu and Erim (2016) developed this technique for the determination of nitrate and nitrite in fish products. In this study, it was applied for the analysis of both anions in the pollen samples. In the sample stacking technique, the conductivity of the running buffer is increased. Thus the overloaded peaks which the large volume injection cause, turns into sharpened analyte peaks. Therefore, 30 mmol/L sodium sulfate was added into the running buffer. The separation buffer presents lower electrical field than that of the sample zone due to the higher conducitivity of buffer zone. Both anions move fast in the sample zone. When they come across to buffer region, the suddenly slow down because of the low electrical field. Fig. 1 representatively shows the sample stacking technique in low pH separation medium. The sample is stacked at the sample and buffer zone boundary and sharp peaks are seen in the electropherogram. The detection sensitivites for nitrate and nitrite were enhanced 30-fold with this sample stacking technique.

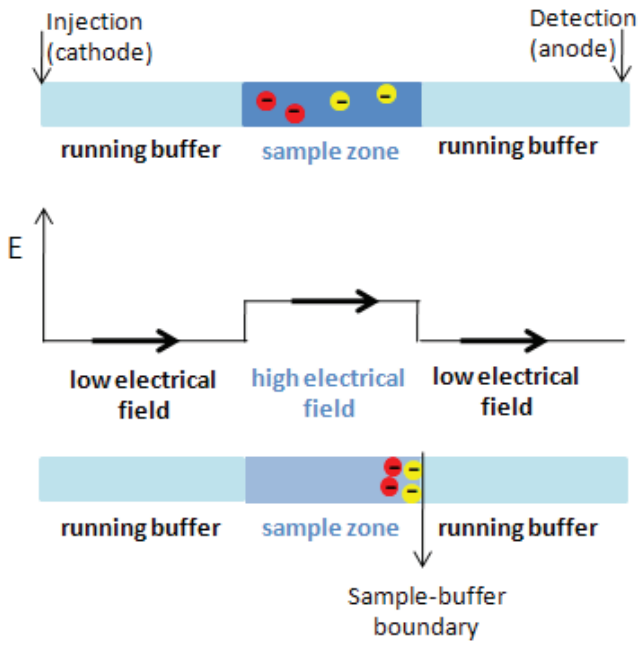

Figure 1. Sample stacking technique in low pH separation medium

3.2 Method validation

The calibration curve showed linear dynamic ranges from 1.5 to 30 µM with correlation coefficient of 0.989 for nitrate. For nitrite, the calibration curve was constructed between 1.5-10 µM with the calibration coefficient of 0.998. The precision of the method was performed in terms of inter-day and intra-day repeatability as RSD%. Intra-day analyses were determined by injecting the anions seven times in the same day. Inter-day repeatability was calculated by injecting both anions on three different days, seven injections in each day. The relative standard deviation was smaller than 3.70 for intra-day analysis and inter-day analysis for both anions as seen in Table 2. The limit of detection, LOD (S/N=3) was calculated as three times the average noise taken for three different baseline areas. The limit of quantification, LOQ (S/N=10) was given as ten times the average noise. LOD values of nitrate and nitrite were 0.21 µM and 0.31 µM and LOQ values were respectively. Analytical method validation values are given in Table 2.

Table 2. Analytical parameters of the method applied

Parameter	Nitrate	Nitrite
Intra-day precision		
Corrected peak area (RSD, %)	1.70	2.12
Migration time (RSD, %)	1.34	1.60
Inter-day precision		
Corrected peak area (RSD, %)	2.14	3.61
Migration time (RSD, %)	1.68	1.82
Linearity		
Linear range (μM)	1.5-30	1.5-10
Regression equation	y=0.0077x+0.0016	y=0.0064x+0.0015
Correlation coefficient	0.989	0.998
LOD (μM)/(mg/L)	0.21/0.013	0.31/0.014
LOQ (μM)/(mg/L)	0.71/0.044	1.03/0.047

Recoveries of the anions were calculated by using the standard addition method (Table 3). A pollen extract (Anzer 1) was fortified with the analyte ions, each at three different concentrations corresponding to 50, 100 and 200 % of the real sample concentrations. The percentage of recovery was calculated with the formula (1):

Recovery (%)= $[(C_1 - C_2)/C_3]$ x100

where C_1 is the concentration determined in fortified sample, C_2 is the concentration determined in unfortified sample and C_3 is the concentration of added standard. Good results of mean recoveries were obtained, with values ranging between 90.2 and 107 %.

Table 3. Recovery values of nitrate and nitrite for a pollen sample (Anzer 1) at three concentration levels

	Sample content (μM)	Anion added (μM)	Recovery% ± SD (n=2)
Nitrate	19.72	9.86	95.3 ± 2.5
		19.72	102 ± 3.7
		39.45	107 ± 2.3
Nitrite	1.63	0.82	90.2 ± 1.4
		1.63	97.8 ± 2.9
		3.26	105 ± 3.8

3.3 Nitrate and nitrite concentrations of pollen samples

Nitrate and nitrite were determined in 10 bee-pollen samples using sample stacking capillary electrophoresis method. Sample solutions were prepared as described in the experimental part. Each sample was injected directly for nitrite and nitrate analysed at least three times. One representative electropherogram of a pollen sample is given in Fig. 2. For nitrate determination, each sample was diluted 10-fold (Fig. 3).

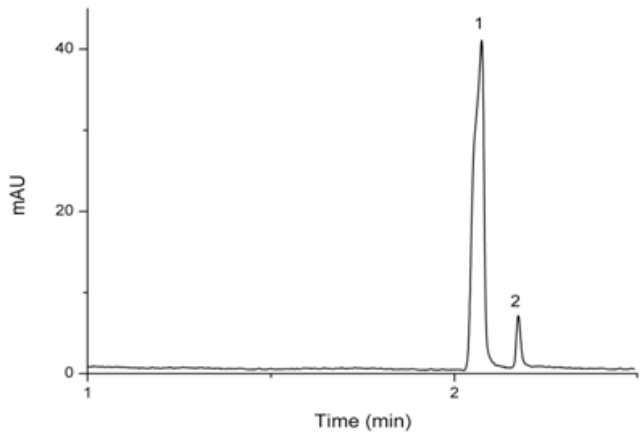

Figure 2. Electrophoregram of a pollen sample (Anzer 1). Buffer: 30 mM formic acid, 30 mM sodium sulfate, pH:4.0. Capillary column: 65 cm (50 cm to dedector)x50 µm i.d. Potential: -25 kV, Injection: 50 mbar, 160 s. Detection: λ:210 nm. Peaks: (1) Nitrate; (2) Nitrite

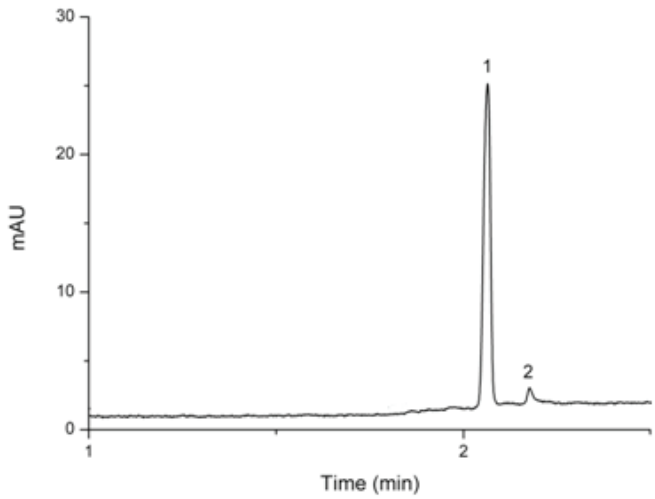

Figure 3. Electrophoregram of a 1:10 diluted pollen sample (Anzer 1). Conditions are the same as Fig.2

Nitrate and nitrite contents of pollen samples analyzed are shown in Table 4. Nitrate amounts varied from 2.880 to 18.72 mg/kg in pollen samples. As seen from Table 4, the nitrate contents of oak pollen are significantly higher than those of other pollens. Anzer-3 pollen (13.47 mg/kg) and the pollen sample collected from Bayburt (13.40 mg/kg) follow the oak pollen. Except the pollen sample collected from Balıkesir (2.880 mg/kg), the other pollen samples seem to have more or less the same amount of nitrate.

Nitrite values were between 0.048 mg/kg and 0.301 mg/kg. The nitrite amount of two pollen samples (Abana and Balıkesir) was found to be lower than limit of quantification (LOQ) of the method. The Pearson correlation analysis was performed between nitrate and nitrite contents. The value of R is 0.8732 ($p <$

0.05). This is a strong positive correlation, which means that high nitrate contents go with high nitrite contents and (vice versa).

This is the first report on the nitrate and nitrite contents in pollen samples, as far as we know. For the other apicultural products, there is a limited number of articles and all of them are centered on honey. Nitrate contents of Spanish honey were determined by capillary electrophoresis and found to be between 2.4 and 9.0 mg/kg (Suarez-Luque et al. 2006). Gao et al. (2016) reported the only nitrite ion concentration in honey as 38.65 µM. There are only two study which reported both the nitrate and nitrite anion. Beretta et al. (2010) determined these anions in different originated honey samples by ion-chromatography technique. Nitrate amount was changed between 1.63 and 482.98 mg/kg. Nitrite was found to be between 0.01 and 0.23 mg/kg which is very small amount. Kaygusuz (2020) reported the nitrate and nitrite anion amount as 3.47 and 19.05 mg/kg in a rare honey sample from Iğdır region, respectively.

Table 4. Nitrate and nitrite contents of the studied pollen samples

	Nitrate (mg/kg ± SD)	**Nitrite (mg/kg ± SD)**
Anzer 1	11.69 ± 0.53	0.073 ± 0.003
Anzer 2	11.83 ± 0.68	0.098 ± 0.006
Anzer 3	13.47 ± 0.48	0.117 ± 0.016
Chestnut 1	8.375 ± 0.413	0.048 ± 0.008
Chestnut 2	11.65 ± 0.57	0.072 ± 0.007
Buckwheat	12.73 ± 0.96	0.106 ± 0.010
Oak	18.72 ± 0.46	0.301 ± 0.001
Abana	6.271 ± 0.396	<LOQ
Bayburt	13.40 ± 0.61	0.115 ± 0.004
Balıkesir	2.880 ± 0.055	<LOQ

4. CONCLUSION

In this study, nitrite and nitrate contents of 10 pollen samples from Anatolia were clarified using a simple, rapid, and efficient capillary electrophoresis technique. The sensitivities of ions were enhanced by sample stacking application. Pollen is found having appreciable amounts of nitrate. The nitrite, both as an ingredient of pollen and coming from reduction of nitrate, is rapidly protonated to nitric acid, which decomposes to NO and other nitrogen oxides. NO can exert a vasodilation effect when comes into contact with the gastric vasculature even in very low amounts. The nitrite content of all the pollens is very low, confirming the dietary safety of this functional food product.

5. REFERENCES

Aylanc, V., S.I. Falcao, Ş. Ertosun, and M. Vilas-Boas. 2021. From the hive to the table: Nutrition value, digestibility and bioavailability of the dietary phytochemicals present in the bee pollen and bee bread. Trends in Food Science and Technology (109), 464-481.

Beretta, G., F. Gelmini, V. Lodi, A. Piazzalunga, and R. Maffei Facino. 2010. Profile of nitric oxide (NO) metabolites (nitrate, nitrite and N-nitroso groups) in honeys of different botanical origins: Nitrate accumulation as index of origin, quality and of therapeutic opportunities. Journal of Pharmaceutical and Biomedical Analysis (53), 343–349.

Bondonno, C.P., K.D. Croft, and J.M. Hodgson. 2016. Dietary nitrate, nitric oxide, and cardiovascular health. Critical Reviews in Food Science and Nutrition (56), 2036–2052.

Bryan, N.S. and J.L. Ivy. 2015. Inorganic nitrite and nitrate: evidence to support consideration as dietary nutrients. Nutrition Research (35), 643– 654.

d'El-Rei, J., A.R. Cunha, M. Trindade, and M.F. Neves. 2016. Beneficial effects of dietary nitrate on endothelial function and blood pressure levels. International Journal of Hypertensions (2016), 6791519.

Dias, L.G., G. Tolentino, A. Pascoal, and L.M. Estevinho. 2016. Effect of processing conditions on the bioactive compounds and biological properties of bee pollen. Journal of Apicultural Research (55), 357-365.

Gao, M., W. Fang, J. Ren, A. Shen, and J. Hu. 2016. Reliable SERS detection of nitrite based on pH and laser irradiance-dependent diazotization through a convenient sampling micro-chamber. Analyst (141), 5195–5201.

Kalaycıoğlu, Z., and F.B. Erim. 2016. Simultaneous determination of nitrate and nitrite in fish products with improved sensitivity by sample stacking-capillary electrophoresis. Food Analytical Methods (9), 706-711.

Kalaycıoğlu, Z., and F.B. Erim. 2019. Nitrate and nitrites in foods: worldwide regional distribution in view of their risks and benefits. Journal of Agricultural and Food Chemistry (67), 7205-7222.

Kalaycıoğlu, Z., H. Kaygusuz, S. Döker, S. Kolaylı, and F.B. Erim. 2017. Characterization of Turkish honeybee pollens by principal component analysis based on their individual organic acids, sugars, minerals, and antioxidant activities. LWT-Food Science and Technology (84), 402-408.

Kaygusuz, H., F. Tezcan, F.B. Erim, O. Yıldız, H. Şahin, Z. Can, and S. Kolaylı. 2016. Characterization of Anatolian honeys based on minerals, bioactive components and principal component analysis. LWT-Food Science and Technology (68), 273-279.

Kaygusuz, H. 2020. Analysis of a rare honey sample from Tuzluca/Iğdır region. Iğdır Üniversitesi Fen Bilimleri Enstitüsü Dergisi (10), 1139-1145.

Kapil, V., E. Weitzberg, J.O. Lundberg, and A. Ahluwalia. 2014. Clinical evidence demonstrating the utility of inorganic nitrate in cardiovascular health. Nitric Oxide (38), 45–57.

Machha, A., and A.N. Schechter. 2011. Dietary nitrite and nitrate: a review of potential mechanisms of cardiovascular benefits. European Journal of Nutrition (50), 293–303.

Öztekin, N., M.S. Nutku, and F.B. Erim. 2002. Simultaneous determination of nitrite and nitrate in meat products and vegetables by capillary electrophoresis. Food Chemistry (76), 103-106.

Su, J., X. Yang, Q. Lu, and R. Liu. 2021. Antioxidant and anti-tyrosinase activities of bee pollen and identification of active components. Journal of Apicultural Research (60), 297-307.

Suarez-Luque, S., I. Mato, J.F. Huidobro, J. Simal-Lozano, and M.T. Sancho. 2006. Capillary zone electrophoresis method for the determination of inorganic anions and formic acid in honey. Journal of Agricultural and Food Chemistry (54), 9292-9296.

Acknowledgments

The authors would like to thank Prof. Dr. Sevgi Kolaylı for supplying the pollen samples and the Research Foundation of Istanbul Technical University for financial support. The authors have declared no conflict of interest.