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Sample stacking – Capillary electrophoretic determination of nitrate and nitrite contents as nitric oxide metabolites in honey varieties originated from Anatolia

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ABSTRACT

Nitrate and nitrite ions taken from food are the sources of bioavailable nitric oxide (NO) in the nitrogen cycle. Some beneficial effects of honey on health are attributed to the ability of honey to increase NO production. The variation of nitrate and nitrite levels of honey samples collected from different Anatolia regions were clarified using capillary electrophoresis technique. The sensitivities of both anions were improved with the application of the sample stacking method. Separation buffer consisted of 30 mmol L^{-1} formic acid and 30 mmol L^{-1} sodium sulfate at a pH of 4.0. The CE technique revealed that 18 honey samples contained nitrate anion ranged between 2.53 and 31.8 mg kg^{-1}. Nitrite amounts were found in lower amounts in the honey samples as between non-detected and 0.533 mg kg^{-1}. The observed differences in nitrate levels between honey varieties may be a way to determine honey's origin.

KEYWORDS

capillary electrophoresis, honey, nitrate, nitrite, sample stacking

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1. INTRODUCTION

After nitric oxide (NO) has been shown to be a critical molecule for our cardiovascular system, it has been proven that NO deficiency is the cause of many human diseases (Loscalzo and Welch, 1995). NO is supplied by two pathways, the classical L-arginine-nitric oxide synthase pathway, and the later discovered nitrate-nitrite-nitric oxide pathway (Lundberg et al., 2008; Weitzberg et al., 2010). Dietary nitrate is one of the main sources of the nitrate-nitrite-nitric oxide pathway. Dietary nitrate is converted in the body to nitrites and then to NO (Cosby et al., 2003). Nitrate and nitrite compounds are taken with green leafy vegetables, fruits, and processed meat products (Hmelak Gorenjak and Cencic, 2013; Bryan and Ivy, 2015). The amounts of nitrate and nitrite in various foods consumed worldwide have been given in a review article in recent years (Kalaycıoğlu and Erim, 2019). The carcinogenic effects of nitrate and nitrite used as an additive, especially in cured meat products, have been discussed for many years. However, today this risk is considered to be very low (Parthasarathy and Bryan, 2012). On the contrary, due to the relationship between nitrate and nitrite with NO synthesis, the beneficial effects of nitrate and nitrite consumption on health have been revealed in a number research papers (Machha and Schechter, 2011; Kapil et al., 2014; Bryan and Ivy, 2015).

Honey is the most prominent and widely appreciated honey-bee product all over the world. Honey is mostly consumed for its nutritional value, but has also been used for therapeutic aims since ancient times (Al-Waili et al., 2011). Lately, it has been experimentally shown that honey consumption increases NO metabolite concentration in human fluids (Al-Waili and Boni, 2004). Nitrate and nitrite contents of different honey types have been reported in limited number of studies so far (del Nozal et al., 2000; Suarez-Luque et al., 2006; Beretta et al., 2010). On the other side, ingredients like phenolics, vitamins, and minerals of bee-products and their bioactivities are strictly dependent on their botanical origins (Tezcan et al., 2011; Can et al., 2015; Kaygusuz et al., 2016; Kalaycıoğlu et al., 2017; Kolaylı et al., 2020). Thereby, nitrate and nitrite levels in honey should be an additional factor in the classification of honey values. Due to its high plant biodiversity, chestnut and pine forests, Anatolia is one of the most diverse regions in terms of bee-products. For the first time, this study reports the nitrate and nitrite contents of 18 Anatolian honey samples of different botanical origin, using a fast and sensitive capillary electrophoretic analysis technique with sample stacking methodology that does not require a sample-preparation. The aim of this study is to find the nitrate and nitrite content of different honey varieties and in order to reveal their NO-dependent health values. It is also to clarify whether the differences of these two compounds in honey varieties could be an additional potential fingerprint for honey adulteration.

2. MATERIALS AND METHODS

2.1. Chemicals and standard solutions

Formic acid, sodium sulphate, sodium nitrite, potassium nitrate, and sodium hydroxide were obtained from Merck (Darmstadt, Germany). All solutions were prepared using ultrapure water obtained from a Milli-Q water system (Purelab Option Q). All reagents used were of analytical grade.

Nitrate and nitrite stock solutions were prepared at 10 mmol L^{-1} levels in deionised water and stored at 4 °C until the analyses. Calibration solutions were prepared by diluting the stock solutions gradually with deionised water. The pH of the buffer solution containing 30 mmol L^{-1} formic acid and 30 mmol L^{-1} conductivity enhancing salt, sodium sulphate, was adjusted to 4.0.

2.2. Honey samples

Monofloral honey varieties with the highest palynological scores were selected for the study. With the Turkish Beekeepers Association's help, 5 rhododendrons, 3 heathers, 3 oaks, 4 pines, and 3 chestnuts honey varieties were collected from the local forest beekeepers. The regions where the honey samples were collected are given in Table 1. The honey samples of the same plant species collected from different producers were individually numbered.

2.3. Preparation of the samples for the analysis

Honey samples were weighed to the nearest 0.5 g, dissolved in 10 mL of deionised water, and stirred for 15 min. Before injection into the capillary column, the solution was filtered with a 0.45 µm pore-sized regenerated cellulose membrane filter. The filtrates were directly injected. When needed, the extracts were diluted for nitrate determination. The samples were stored in darkness at room temperature until analysis.

2.4. Apparatus and operating conditions

Analyses were carried out with an Agilent 1600 capillary electrophoresis system (Waldbronn, Germany). The data processing was carried out with the Agilent ChemStation software. Separations were performed in silica capillaries with 50 µm i.d. (Polymicro Technology, Phoenix, AZ, USA). The total length of the capillary was 58 cm, and the length to the detector was 50 cm. The new fused-silica capillary was conditioned prior to use by rinsing with 1 mmol L^{-1} NaOH for 30 min and with water for 10 min. The capillary was flushed with 0.1 mmol L^{-1} NaOH for 2 min, water for 2 min, and buffer for 5 min between runs. The temperature was set at 25 °C. Sample injections were made at 50 mbar for 160 s. The applied voltage was –25 kV and the UV detection was carried out at 210 nm.

2.5. Statistical analysis

The results were statistically evaluated using IBM SPSS 23.0 statistical software program for Windows. Significant differences of the mean values at $P < 0.05$ were compared using one-way analysis (ANOVA) and the Duncan's new multiple range test.

Table 1. Areas where honey samples were collected and number of honeys collected from different producers from the same region

Honey samples	Collecting area in Turkey
Rhododendron (5)	Trabzon
Heather (3)	Muğla
Oak (3)	Kırklareli
Pine (4)	Muğla
Chestnut (3)	Trabzon

3. RESULTS AND DISCUSSION

3.1. Determination of nitrate and nitrite contents of honey samples

In this study, nitrate and nitrite anions were separated, detected, and quantified with the sample stacking-capillary electrophoresis technique. The capillary electrophoresis method used based on the migration of nitrite and nitrate anions using a separation medium at low pHs, where electroosmotic flow (EOF) inside the capillary to the cathodic side is reduced. Nitrate and nitrite anions have high electrophoretic mobilities, and they can easily migrate across reduced EOF when they are injected from the cathodic side. The pH of the medium does not affect the mobility of the nitrate ion. In contrast, the electrophoretic mobility of nitrite, a conjugate base of a weak acid, is affected by pH. Since the pKa of the nitrous acid was 3.15, the pH of the separation medium was not selected far from this value. Since increasing the pH of the separation medium will accelerate the electroosmotic flow in the capillary, the movement of both ions against the electroosmotic flow would be difficult. For this reason, formic acid was chosen as the separation buffer, and pH optimisation of buffer solution was made around pKa (3.74) of formic acid. pH 4.0 was determined as the optimal separation medium pH based on both resolutions and arrival times. Both ions were negatively charged and rapidly migrated against the electroosmotic flow in the capillary at this pH.

The most prominent feature of the capillary zone electrophoresis technique is that the analyte is introduced into the capillary column as a narrow zone of a few nL in size. However, in the preliminary experiments by small volume injection, nitrate peak was detected, while the amount of nitrite in honey samples was found to be below the detection limit of the method for nitrite ion. One way to increase the peak sensitivity in the capillary zone electrophoresis is to apply sample stacking methodology (Öztekin et al., 2002; Szökő et al., 2004; Kalaycıoğlu and Erim, 2016; Slampova et al., 2019). In this online sample preconcentration method, detection sensitivities of the analytes are increased by large volume sample injection. Since the large volume injection causes overloaded peaks, the conductivity of the separation buffer should be increased in order to obtain sharp analyte peaks. For that purpose, 30 mmol L^{-1} sodium sulphate was added to the separation buffer. Higher conductivity in the separation buffer presents a lower electrical field than that of the sample zone. Both anions moving rapidly in the sample zone suddenly slow down due to the fall of the electric field when they reach the buffer region with high conductivity. Thus, the sample is condensed at the sample and buffer zones boundary and displayed as sharp peaks in the detector. With this sample stacking method, the detection limits of both ions are significantly reduced.

3.2. Method validation

The calibration curves were drawn in the range of 1.5–30 μmol L^{-1} for nitrate and nitrite with the correlation coefficients of 0.999 and 0.998, respectively. The limits of detections (LODs) were 0.21 μmol L^{-1} and 0.31 μmol L^{-1} for nitrate and nitrite, respectively. The precision of the applied method was tested with intraday ($n = 7$) and interday reproducibility ($n = 21$). The reproducibility values as % RSD were lower than 5% for both intraday and interday experiments. Recovery studies were performed by spiking the honey samples with three different standard nitrate and nitrite concentrations corresponding to 50, 100, and 200% of the real sample

concentration. Satisfactory recovery values by percentage were obtained between 90.2 ± 1.4 and 107 ± 3 levels for nitrate and nitite, respectively.

3.3. Nitrate and nitrite concentrations of honey samples

The electropherogram of a honey sample (Pine 1) is given in Fig. 1. As seen from the electropherogram, the migration times of nitrate and nitrite ions are very short, around 2.2 min. There are no matrix peaks in the region of the arrival of the two peaks. This is due to the fact that the electrophoretic mobility of possible phenolic substances and sugar molecules is lower than that of nitrate and nitrite ions. Nitrate and nitrite contents of honey samples are shown in Table 2. Nitrate amounts varied from 2.53 mg kg^{-1}–31.8 mg kg^{-1} in honey samples.

The graphical distribution of the nitrate content of honey varieties is shown in Fig. 2. Nitrate contents of pine and chestnut honey samples were higher than of other honey varieties. When the one-way ANOVA test is applied to the nitrate values in Table 2, statistically significant differences between these honeys and others are seen. All pine honeys differ significantly from other honeys in terms of nitrate level, only one of the chestnut honeys is at the level of pine honeys in terms of nitrate content. The nitrate contents of other chestnut honeys are statistically different from both pine honeys and other honeys in the table. Except for pine and chestnut honey, there is no significant difference between other honey varieties in terms of nitrate content. With an informed estimate, the significantly higher nitrate content of pine honey compared to other honeys may possibly be due to the fact that pine honey is a honeydew honey.

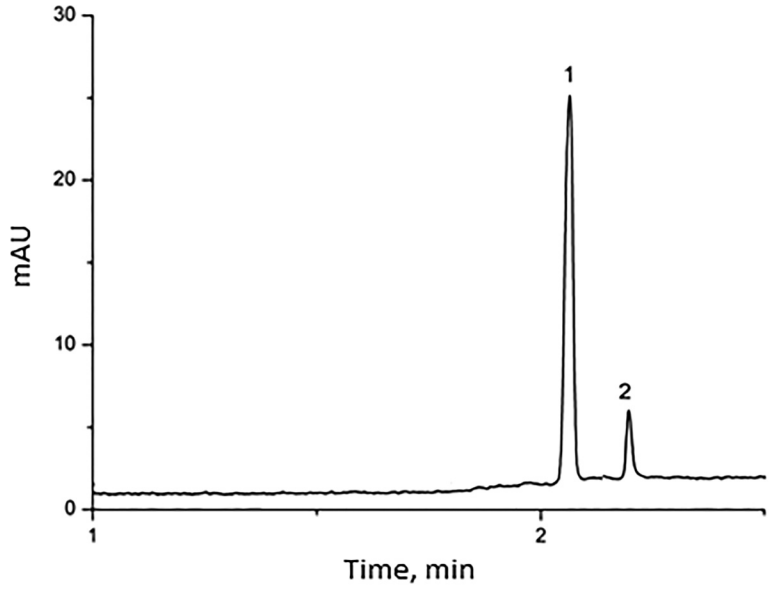

Fig. 1. Electropherogram of a honey sample (Pine 1). Injection: 50 mbar, 160 s. Running potential, −25 kV; capillary, 58 cm (50 cm to detector) × 50 µm i.d.; 210 nm, buffer: 30 mmol L^{-1} formic acid, 30 mmol L^{-1} Na_2SO_4, pH 4.0. Peaks: 1) NO_3^-, 2) NO_2^-

Table 2. Nitrate and nitrite contents of the studied honey samples (mg kg^{-1})

Samples	Nitrate	Nitrite
Rhododendron 1	3.33 ± 0.05^{a}	0.101 ± 0.050^{ab}
Rhododendron 2	3.16 ± 0.05^{a}	0.202 ± 0.078^{abc}
Rhododendron 3	3.89 ± 0.15^{a}	0.0891 ± 0.0012^{a}
Rhododendron 4	2.53 ± 0.00^{a}	0.208 ± 0.066^{abc}
Rhododendron 5	4.96 ± 0.26^{ab}	0.233 ± 0.031^{abc}
Heather 1	3.31 ± 0.04^{a}	0.336 ± 0.069^{cd}
Heather 2	3.79 ± 0.12^{a}	nd
Heather 3	3.75 ± 0.09^{a}	nd
Oak 1	4.77 ± 1.85^{ab}	nd
Oak 2	4.41 ± 0.13^{a}	nd
Oak 3	2.84 ± 0.52^{a}	nd
Pine 1	30.4 ± 2.1^{e}	0.328 ± 0.045^{bcd}
Pine 2	31.8 ± 3.1^{e}	0.325 ± 0.012^{bcd}
Pine 3	22.9 ± 0.9^{d}	0.298 ± 0.025^{abc}
Pine 4	20.8 ± 2.3^{d}	0.308 ± 0.085^{abc}
Chestnut 1	21.9 ± 2.6^{d}	0.533 ± 0.149^{d}
Chestnut 2	15.7 ± 0.4^{c}	0.216 ± 0.025^{abc}
Chestnut 3	8.82 ± 0.26^{b}	nd

Results are expressed as mean ± standard deviation (n = 2).
Different letters in the same column show significant differences at the 5% level ($P < 0.05$).
nd: non-detected.

Bees use a sweet liquid secreted by some tiny insects such as aphids, which suck the tree's sap, to produce pine honey. The significantly higher nitrate content in pine honey than in other varieties could be due to the contribution of the insect's secretion. In terms of nitrate amount, the chestnut kind follow the pine kind of honeys. Though the chestnut honey is classified as mainly blossom honey, a small part of this honey contains honeydew honey due to honeydew left on the tree. Bees collect this honeydew together with chestnut nectar and pollen.

Nitrite values were between 0.0891 mg kg^{-1} and 0.336 mg kg^{-1}. Some honey samples contained no nitrite. Nitrite levels of honey samples did not differ significantly ($P > 0.05$).

There are a limited number of articles on the nitrate and nitrite contents of honey samples in the literature. Suarez-Luque et al. (2006) reported the nitrate amounts varying in the range of 2.4 and 9.0 mg kg^{-1} in 10 honey samples from Spain determined by a capillary zone electrophoresis method with a LOD value of 0.03 mg kg^{-1}. Nitrate contents in the range of 0.5–2.6 mg kg^{-1} were reported in several honey and honeydew samples determined by an ion chromatographic technique with a LOD value of 0.122 μg g^{-1} (del Nozal et al., 2000). Only one study was found that simultaneously determined both nitrate and nitrite ions; Beretta et al. (2010) investigated nitrate, nitrite, and N–NO groups in honey samples from different botanical origins by ion chromatography. All honey samples contained appreciable amounts of nitrate between 1.63 and 482.98 mg kg^{-1}, whereas nitrite anion occurred in very small amounts ranging from 0.01 to 0.23 mg kg^{-1}.

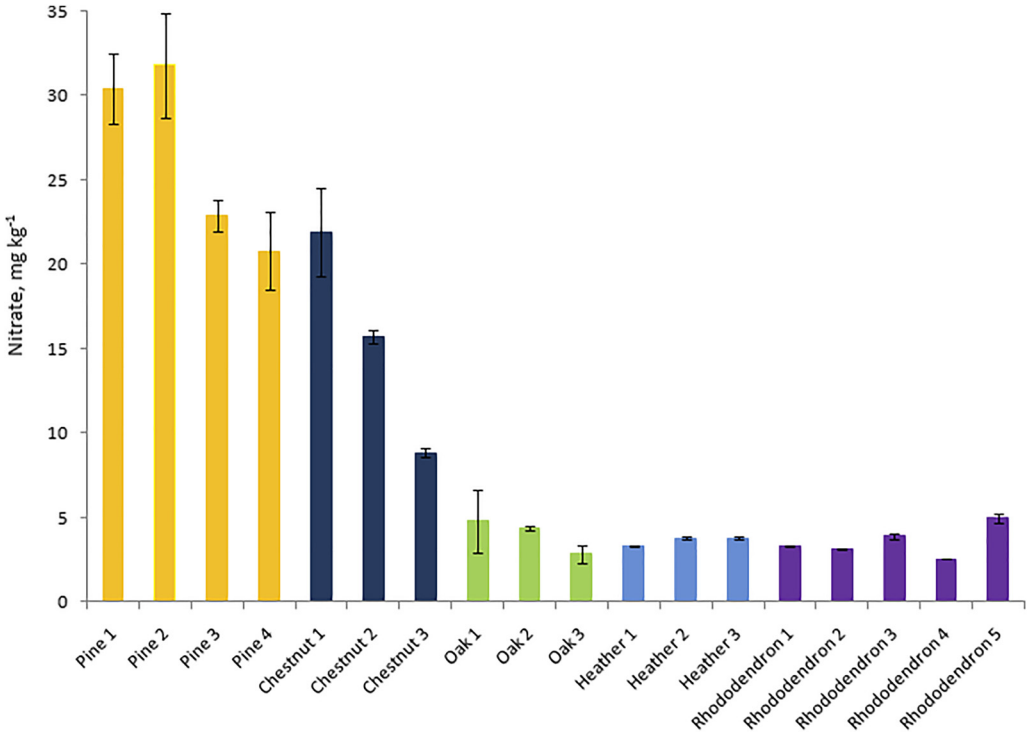

Fig. 2. The graphical distribution of the nitrate content of honey varieties

4. CONCLUSIONS

In this study, nitrite and nitrate contents of 18 honey samples from Anatolia were determined simultaneously using a simple, rapid, and efficient capillary electrophoresis technique. The sensitivities of ions were enhanced by sample stacking application. Pine honey samples have significantly higher nitrate contents among all honey samples investigated. Chestnut honey samples contained a considerable amount of nitrate, as well. It was found that the nitrate levels of honey varieties differ according to their botanical origin.

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