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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

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ABSTRACT

Besides dermoprotective activity, honey also has a strong gastroprotective effect, from salivary reduction of nitrate (NO_3^-) to nitrite (NO_2^-) and intragastric formation of nitric oxide (NO), this lastly involved in the preservation of the gastric mucosa capillaries and in boosting mucous production. Aim of this work is to profile the distribution of NO metabolites (NO_3^-, NO_2^- and total N-nitroso-groups, N-NO) in a set of honeys ($n = 54$) of different botanical origins, using a chemiluminescence based technique (NO-analyzer, NOA). All the honeys contained appreciable amounts of NO_3^- (from 1.63 ± 0.04 to 482.98 ± 5.34 mg/kg), the highest in honeydew honeys (10–40 times than in nectar honeys). Low levels of NO_2^- were found in all samples (0.01 ± 0.00 to 0.56 ± 0.01 mg/kg). N-NO groups, at trace levels in some nectar honeys, were higher in honeydew samples (from 0.01 ± 0.00 to 0.29 ± 0.01 mg/kg). Total phenol content (TP) and total protein (TProt) were comparable to those in literature. Multivariate analysis indicated that N-NO groups were significantly associated with NO_2^- and TP thus to suggest an in situ environmental nitrosation of specific nitrosable substrates (lysine, proline) favored by high reducing conditions. The bee-smoking process can be an alternative or complementary explanation for N-NO contamination. Hence NO_3^- rich honeys intake may exert beneficial effects against NSAIDs-induced gastric injury. Finally NO_3^- is a potential reliable marker of a honey's origin and quality.

1. Introduction

Honey has become a focus of attention as a form of preventive medicine and for the treatment of wounds, burns or skin ulcers recalcitrant to conventional chemotherapy. It has also been used to relieve the pain associated with gastrointestinal diseases (dyspepsia and peptic ulcers) on account of its antiinflammatory and antibacterial activity. Mobarok and Al-Swayeh [1] demonstrated a protective effect of natural honey against ethanol-induced gastric hemorrhagic lesions in animals, and subsequently confirmed this intragastric protection against insult from ammonia [2,3]. They did not offer any possible mechanism against the gastric lesions and merely suggested a potential antioxidant action or a surface protective effect of sugars.

Subsequent studies on the gastroprotective effects of dietary nitrates indicate a beneficial role of intragastric nitric oxide (NO) metabolites in the rat against the gastrolesive effects of non-steroidal antiinflammatory drugs (NSAIDs) [4]. This is due to an enhancement of gastric mucosal blood flow and of mucous formation, and to an inhibition of thickening of the mucous layer, an effect which is abolished by antiseptic mouth-washes, confirming the oral bacterial microflora's involvement in intragastric NO formation [5].

In this context, Gladwin [6] reported that nitrate (NO_3^-) intake plays an important role in gastric mucosal defense, increasing mucosal blood flow, helping regulate platelet activity and stimulating intestinal motility and microcirculation. These results therefore suggest that the bioactivation of dietary NO_3^- to nitrite (NO_2^-) and NO may play a role in mucous protection.

From a pharmacokinetic point of view stable NO metabolites, i.e. NO_3^-, in rats and humans, are absorbed in the upper small intestine and approximately 70% is then excreted through the kidney; the remainder is actively concentrated in saliva to a concentration 10 times than in plasma. In the mouth, facultative anaerobic bacteria reduce nitrate to nitrite, resulting in high levels of nitrite both in saliva plasma, and in addition to bacterial nitrate reduction, a functional mammalian nitrate reductase contributes to circulating nitrite levels [5].

As regards NO metabolites in honey, to our knowledge there is no evidence of their analytical profiling: indirect evidence comes from Al-Waili [7] who reported increases of total plasma and uri-

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nary NO metabolites in sheep after i.v. infusion of some selected honeys. Thus, in the framework of our previous studies on the markers of honey quality and origin from floral or arboreal sources, and on their biological significance, this study determined and profiled a large set of honey samples (n = 54 of 20 different botanical origins). We employed a chemiluminometric approach, recently developed for the analysis of NO_2^-, NO_3^- and N-NO groups in low-molecular-weight heparins (LMWH) [8]. All the honeys contained appreciable levels of NO_3^-, significantly higher in honeydew than nectar honeys, small amounts of NO_2^- and trace contamination by N-NO, mainly in honeydew honeys.

In the second part of the study we investigated correlations between NO metabolites and total protein content (TProt), the latter as a potential source of NO metabolites during plant protein catabolism.

2. Materials and methods

2.1. Chemicals, reagents

Acetone, acetonitrile, acetic acid, hydrobromic acid, hydrochloric acid, heptafluorobutyric acid, formic acid, diethyl ether, glutathione (GSH), S-nitroso glutathione (GS-NO), sodium hydroxide (NaOH), 4-hydroxy-L-proline (Hyp), copper chloride (I) (CuCl), N-nitroso-diethanolamine, sodium nitrite ($NaNO_2$), sulphanilamide (SA), and vanadium chloride (VCl_3) were of analytical grade, purchased from Sigma–Aldrich Chemicals (Milan, Italy). Standard N-nitroso-4-hydroxy-L-proline (N-NO-Hyp) was synthesized as previously reported [9] and its purity was confirmed by HPLC–ESI-MS and NMR analyses. All reagents and test solutions were prepared with fresh Milli-Q® water. Helium 5.5 and NO_x/N_2 9.4 ppm were supplied by Sapio Industrie s.r.l. (Caponago, Milan, Italy).

2.2. Standard solutions

NO_2^-, NO_x^- and N-NO-Hyp standard solutions were freshly prepared by diluting a 10 mM stock solution of $NaNO_2$, $NaNO_3$, or N-NO-Hyp in Milli-Q® water, and kept at 4 °C in the dark until analysis. These solutions were diluted with Milli-Q® water to obtain working standards in the concentration range of 1 μM–1 mM, and used to plot the calibration curves. The solutions were used to validate the method according to the Guidance for Industry, Bioanalytical Method Validation (http://www.fda.gov/cder/guidance/4252fnl.htm). A 250 nM solution of N-NO-Hyp was used as standard for the interday system suitability test.

2.3. Calibration curves

The calibration curves for different working days were obtained by triplicate injections of NO_2^-, NO_3^- or N-NO-Hyp at six concentration levels in the range 0.45–90 pmol (10 μL injected). The linearity of the calibration, in terms of correlation coefficient (R^2) obtained using a $1/x^2$ weighted quadratic fit, was always greater than 0.998. The calibration curve equations were $y = 27.06x$ (± 0.245) − 66.40 (± 23.26) for NO_2^-, $y = 62.24x$ (± 3.735) − 348.2 (± 211.3) for NO_3^-, $y = 297.2x$ (± 7.309) − 95.96 (± 211.4) for N-NO-Hyp.

2.4. Honey samples and preparation

Fifty-four samples of honey from different botanical origins were randomly purchased from various local stores in Milan or beekeepers in the surrounding area during the years 2008 and 2009, stored at 5 °C in the dark and processed as previously described [10].

NO_2^- and NO_3^- and N-NO groups were selectively determined by acidolytic and reductive denitrosation with different reagents and detection of NO released, by gas-phase chemiluminescence reaction with ozone, using a highly sensitive NO-analyzer (NOA) detector (CLD88 Exhalizer, Ecomedics, Dürten, Switzerland,) previously described by us and others [8,11,12]. Data were analyzed using Powerchrom™2.2.4 software (2001-11-29, ADI Instruments). The operative details of the instrumental set-up have been reported elsewhere (Beretta et al. [8]). The pre-analytical device comprised three sequentially connected, commercially available purge vessel units (Sievers, Boulder, CO).

Honey samples were diluted in Milli-Q® water to a concentration of 0.1 g/mL, stored in glass vials at 4 °C, then injected into the external purge vessel with a gas-tight syringe. For N-NO group analysis, 5 min before the injection, 100 μL of acidic sulphanilamide (SA, 20% in HCl 1N) were added to 900 μL of diluted honey to overcome the interference from NO_2^-. The specificity of N-NO determination was routinely checked by exposing diluted honey to UVB radiation [13].

Before analysis all the honeys were examined by standard analytical tests (physico-chemical tests: pH, electrical conductivity, titratable acidity, ash content; qualitative tests: Lugol test, diastases index for authenticity; quality test: hydroxymethylfurfural; quantitative test: reducing and non-reducing sugars).

2.5. NO_3^-, NO_2^-, and N-NO groups determination

NO_2^- and N-NO groups were independently and selectively determined in two sequential steps as NO [8,11]. Total NO metabolites (NO_3^-, NO_2^-, and N-NO groups) were quantified in the same apparatus by reductive treatment of diluted honey samples (VCl_3/HCl at 90 °C). In this condition each compound is quantitatively converted to gaseous NO, and total NO is determined chemiluminometrically. NO_3^- levels (method A) were calculated by subtracting from total NO the previously determined amounts of NO_2^- and N-NO groups.

The reliability of the method was confirmed by a second approach (method B): diluted honey (1:10 w/vol) was first depleted of NO_2^- by addition of SA (1:10, 5% in HCl 1N), and then of N-NO groups by exposing the same solution to UVB radiation (λ = 240 nm, 60 min, 7.14 J/cm^2, 4 °C) [13]. NO_3^- was determined by reduction with the reagent VCl_3/HCl at 90 °C. To determine NO_3^- and NO_2^- we injected 10 μL of the sample solution, equivalent to 1 mg of fresh honey, while for N-NO groups we used an amount in the range from 10 to 500 μL (0.9–45 mg of native honey). We plotted the calibration curves for the NO_3^-, NO_2^- and N-NO groups in a reconstructed honey blank using different dilutions of stock solutions, of respectively $NaNO_3$ (10 mM), $NaNO_2$ (10 mM) and N-NO-Hyp (10 mM). The reconstructed honey blank was obtained by mixing the typical sugars found in honey (40% fructose, 30% glucose, 10% maltose plus 20% water) with the prototype protein BSA (0.2–0.5%) and a set of the most representative amino acids present in honey (proline, phenylalanine, tyrosine, lysine, glutamic acid, tryptophan, leucine, arginine, and histidine). The reconstructed honey blank was spiked with different concentrations of the stock solution containing NO metabolites and recovery, accuracy, efficiency, linearity, stability, LOD and LLOQ were determined according to the FDA guidelines (http://www.fda.gov/cder/guidance/4252fnl.html). In particular, the LOD of the method was calculated from the LLOQ (0.83 pmol injected) determined by injecting a solution of reconstructed honey blank (10% w/vol) spiked with decreasing amounts (down to 0.5 pmol) of the analytes: NO_2^-, NO_3^- and N-NO-Hyp.

2.6. Stability (0–30 min)

Of the NO metabolites studied, NO_2^- is the most labile analyte that can be easily converted to NO_3^- after dilution of the honey, so we carried out a stability study on this analyte after spiking it in both the reconstructed honey and the native honey, working between 0 and 4 °C in an 0–30 min interval of analysis, and processing it as described above.

2.7. Ion chromatography: NO_3^- and NO_2^- analysis

The analyses were carried out using an ion-exchange chromatograph (Dionex ICS1000) equipped with an isocratic pump, a sample injection valve with a 50 μL sample loop, and a conductivity system equipped with a ASRS-ULTRA suppression mode (Dionex) as detector.

Anions analysis was carried out using an Ion Pac AS14A (Dionex) column using 8 mM Na_2CO_3/1 mM $NaHCO_3$ as eluent at 1 mL/min flow rate. The Chromeleon software was employed for the system control and data analysis. The instrument was daily calibrated with standard solutions.

2.8. Bee-smoking simulation test

Contamination of honey with NO metabolites by bee-smoking was simulated in three separate experiments where 3 g of acacia honey (free from basal N-NO groups) was exposed for 30 s to the smoke from burning jute (sackcloth) or cardboard, the most commonly used materials for bee-smoking operations. Acacia samples were then left to stand in the dark for 24 h and NO_2^-, NO_3^- and N-NO groups determined as described.

2.9. Proteins

The protein content was determined by the method reported by Bradford [14]. Honey samples were diluted to 1 g/mL and 1.5 mL of Brilliant Blue G was added to 50 μL of solution. After 5 min incubation, the absorbance was measured at λ = 595 nm, against a standard solution of bovine serum albumin, 10–100 μg/0.1 mL in 0.15 M NaCl.

2.10. TP (total phenols)

TP were determined as previously reported experimental conditions [10] and expressed as mg of gallic acid equivalent (GAE)/Kg of honey.

2.11. Data presentation and statistics

Data are presented as original recordings or the means ± standard deviation of four individual experiments with buffer and reagent blank. Assay reproducibility was determined by comparison of the peak area from 10 repeated measurements of standards obtained on the same day (intraday variation) and expressed as the coefficient of variation (%).

All statistical analysis was done using the SPSS Version 17.0 software for Windows (SPSS, Chicago, IL, USA). Relationships between variables were examined by the Pearson parametric correlation. To reduce skewness, data were log-transformed before analysis. Multivariable linear regression (stepwise) was used to identify variables influencing the level of N-NO in honey. Differences between groups were determined using the *t*-test, and the difference was considered statistically significant when $P < 0.05$.

3. Results and discussion

3.1. Chemiluminescence

In the first part of the study we investigated whether the ozone-based chemiluminescence approach previously used by us and by others [8,11] was suitable for profiling the NO metabolites in the particular matrix of honey. Briefly, the method seemed highly satisfactory in terms of efficiency of recovery of the total and single analytes. All the parameters were calculated on reconstructed honey blank and on three different native honeys (strawberry-tree, multiflora and acacia) working on a matrix amount of one and spiked with the reported concentrations of N-NO, NO_2^- and NO_3^-. The interday precision (CV%) ranged from 5 to 10% (<20% at the lower limit of quantification – LLOQ), the interday accuracy (expressed as BIAS%) from −5.83 to +17.50% (<18% at the LLOQ), and linearity, evaluated on reconstructed honey, was from 0.8 to 250 pmol of injected $NaNO_3$, $NaNO_2$, *N*-NO-H-Hyp.

The limit of detection (LOD) of the method in honey blank was 500 fmol (not shown), and the LLOQ was 800 fmol. The results are summarized in Table 1. There was satisfactory coincidence between the values on reconstructed and native honey, and consequently the instrumental LOD of 500 fmol of NO meant we could detect 30 pg of N-NO and 23 pg of NO_2^- in a sample of diluted honey equivalent to 1 mg of native honey.

The intraday stability of NO_2^- spiked to native honey and reconstructed honey was close to the nominal spiked content within 30 min (Table 1). Interference from native honey components was excluded by evaluation of the recovery of the three analytes spiked to the matrix and determined after subtraction of the basal values of NO metabolites present in the native honey.

3.1.1. Profiles of NO metabolites

NO_3^-, NO_2^- and N-NO groups were determined in 54 honey samples of different botanical origins, and the individual results are reported in Table 2 and summarized in Fig. 1.

3.1.2. NO_2^-

All honeys contained very small amounts of NO_2^- (mean ± SD: 0.23 ± 0.20 mg/kg), ranging from 0.01 ± 0.00 (in Chestnut A) to 0.58 ± 0.01 (Heather 1). The wide SD might indicate an intrinsic lability of this unstable intermediate metabolite, due to storage or handling conditions, making it useless as a marker. However, the NO_2^- content of all the honeys was very low, confirming the dietary safety of this functional food.

3.1.3. NO_3^-

NO_3^-, the final metabolites from NO oxidation, was more stable than NO_2^-, as evidenced by the narrower SD. NO_3^- seems to be prominent in flowers from arboreal species, and in particular in honeydew honeys, where it was 10–40 times higher than in other nectar honeys. This might be because of the particular origin of honeydew, the secretion produced by plant-sucking insects (aphids) that feed by piercing the leaves and ingesting the phloem sap emitted by the plant. After passing through the insect's gut, the surplus is excreted as sugar-rich droplets of honeydew, which are collected by the honeybees to produce honey. In view of its substantial exposure to air, honeydew droplets might conceivably be susceptible to contamination from environmental NO, NO_2, N_2O, and N_2O_4 which, once absorbed, are converted to NO_2^- and, after further oxidation mediated by molecular oxygen (O_2), to NO_3^-. The higher NO_3^- content in honey from arboreal species could be due to natural blending of the honey from the arboreal flowers with that from honeydew lying on the surface of leaves of arboreal plants.

Table 1
Determination of NO metabolites in native and reconstructed honey. Recovery, precision and accuracy of the method.

Analyte	Nominal added (pmol)	Mean ± SD	Precision (CV%)	Accuracy (BIAS%)	Recovery (%)
NO_3^-					
Honey blank	0.80	0.91 ± 0.14	15.38	−0.28	116.58
	10.00	10.45 ± 0.92	8.80	4.57	104.50
	100.00	100.90 ± 3.25	3.22	0.90	100.90
Spiked honey (sample 22)	0.8.0	0.96 ± 0.05	5.21	5.90	117.50
	10.00	9.81 ± 0.14	1.45	−1.89	98.13
	100.00	99.00 ± 3.18	3.21	−0.96	99.00

Analyte	Nominal added (pmol)	Mean ± SD	Precision (CV%)	Accuracy (BIAS%)	Recovery (%)	Short-term stability %
NO_2^-						
Honey blank	0.80	1.03 ± 0.16	15.53	9.17	109.17	96.51 ± 4.78
	10.00	9.61 ± 0.92	9.57	−3.84	96.10	99.46 ± 1.82
	100.00	99.19 ± 3.34	3.37	−0.75	99.19	100.54 ± 3.81
Spiked honey (sample 7)	0.80	1.03 ± 0.13	12.62	6.70	108.33	97.65 ± 2.91
	10.00	9.98 ± 0.84	8.46	0.13	99.80	101.67 ± 0.94
	100.00	99.77 ± 2.19	2.20	−0.20	99.77	99.08 ± 1.11

Analyte	Nominal added (pmol)	Mean ± SD	Precision (CV%)	Accuracy (BIAS%)	Recovery (%)
N-NO					
Honey blank	0.80	1.07 ± 0.12	11.21	9.96	115.83
	10.00	10.36 ± 0.63	6.08	3.57	103.57
	100.00	97.33 ± 7.38	7.58	−2.71	97.33
Spiked honey (sample 35)	0.80	0.98 ± 0.13	13.26	840	94.17
	10.00	10.26 ± 0.52	5.07	2.58	102.63
	100.00	99.31 ± 2.67	2.69	−0.67	99.31

In particular, the different distribution of NO_3^- in nectar and non-nectar honeys highlights the potential utility of this parameter as a marker of contamination, adulteration or blending of nectar and honeydew honeys. It could also be used to distinguish nectar honeys from herbal and arboreal flower types and for the accurate identification of botanical origin, when properly correlated with other characteristics such as honey color, antioxidant activity, presence of specific structural markers protein content, etc.

3.1.4. N-NO groups

In contrast to nectar honey, honeydew honeys contained quantifiable traces of N-NO groups (0.018 ± 0.040 mg/kg), at concentrations too low to be considered harmful to human health. The N-NO groups were below the LOQ of the method in 39 honeys (e.g., linden, rosemary, acacia, cardoon, sunflower, lavender, multiflora, thymus and dog-rose), but there were substantial amounts in 13 others, with the highest content in a chestnut honey (0.051 mg/kg). The chemical identity of the N-NO

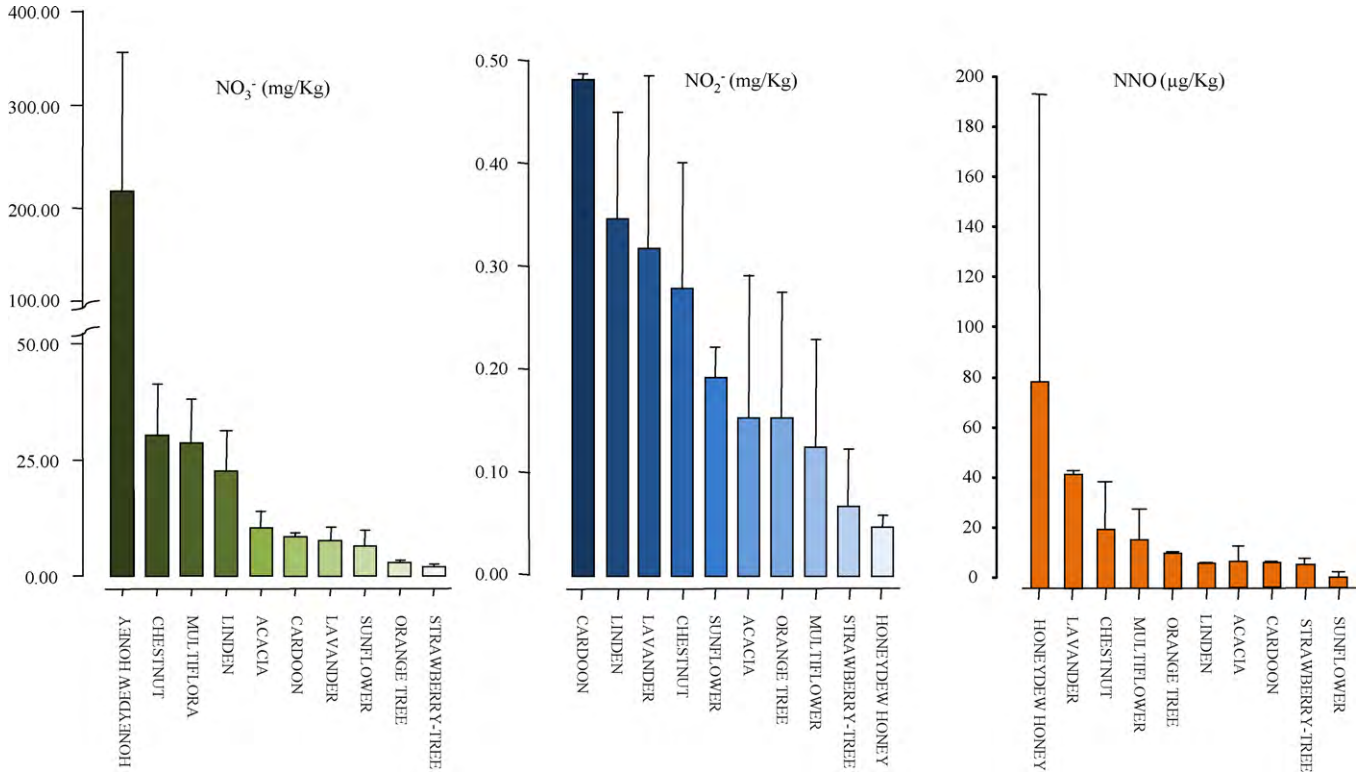

Fig. 1. Mean content of NO metabolites in nectar honeys and honeydew honeys.

Table 2
NO_3^-, NO_2^-, N-NO, total proteins (TProt) and total phenol content (TP) in 54 honeys from 20 different botanical origins. NO_3^-, NO_2 and TNG are expressed as mg/kg, TProt as mg_{BSA}/kg and TP as mg_{GAE}/kg. Data are mean of $n = 4$ independent measurements ± standard deviation (SD). n.d.: not detectable.

n.	Honey type	NO_3^-	NO_2^-	N-NO	TP	TProt
1	Acacia	6.76 ± 0.28	0.16 ± 0.02	0.03±0.00	97.54 ± 4.56	2212 ± 46
2	Acacia	14.15 ± 0.34	0.11 ± 0.01	0.02±0.00	322.74 ± 15.76	2213 ± 54
3	Acacia	10.57 ± 0.41	0.02 ± 0.00	0.00±0.00	504.77 ± 22.31	2520 ± 74
4	Acacia	4.48 ± 0.06	0.01 ± 0.00	0.01±0.00	388.07 ± 16.4	1980 ± 33
5	Acacia	15.94 ± 0.71	0.56 ± 0.01	n.d.	127.16 ± 6.98	1845 ± 26
6	Acacia	18.91 ± 1.11	0.54 ± 0.01	n.d.	144.70 ± 5.32	1763 ± 15
7	Acacia	7.7 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	302.89 ± 12.88	2020 ± 51
8	Acacia	5.12 ± 0.01	0.04 ± 0.00	0.01 ± 0.00	190.24 ± 7.82	2123 ± 34
9	Orange	3.44 ± 0.94	0.31 ± 0.01	0.01 ± 0.00	203.00 ± 8.79	1870 ± 22
10	Orange	2.43 ± 0.72	0.01 ± 0.00	0.01 ± 0.00	252.42 ± 9.44	1675 ± 19
11	Cardoon	9.32 ± 0.42	0.50 ± 0.02	0.01 ± 0.00	391.28 ± 14.53	2213 ± 42
12	Cardoon	7.81 ± 0.33	0.49 ± 0.02	n.d.	476.67 ± 19.59	2410 ± 81
13	Chestnut	11.14 ± 0.48	0.01 ± 0.00	0.05 ± 0.00	602.45 ± 13.11	2221 ± 56
14	Chestnut	13.48 ± 0.53	0.25 ± 0.01	0.02 ± 0.00	880.85 ± 17.49	2350 ± 49
15	Chestnut	49.51 ± 2.01	0.31 ± 0.01	0.01 ± 0.00	788.94 ± 14.01	2312 ± 64
16	Chestnut	31.72 ± 1.28	0.50 ± 0.01	0.01 ± 0.00	312.29 ± 13.83	2140 ± 35
17	Chestnut	64.46 ± 2.57	0.46 ± 0.01	0.03 ± 0.00	545.42 ± 21.40	2432 ± 66
18	Chestnut	28.79 ± 1.36	0.49 ± 0.01	n.d.	694.73 ± 31.33	2630 ± 61
19	Chestnut	14.26 ± 0.65	0.03 ± 0.00	0.03 ± 0.00	474.04 ± 20.21	2600 ± 55
20	Chicory	14.83 ± 0.65	0.03 ± 0.00	0.02 ± 0.00	516.64 ± 23.65	2610 ± 59
21	Strawberry	2.55 ± 0.12	0.14 ± 0.00	n.d.	280.94 ± 14.21	2070 ± 51
22	Strawberry	1.63 ± 0.04	0.00 ± 0.00	0.01 ± 0.00	657.86 ± 31.32	2800 ± 77
23	Eucalyptum	18.12 ± 0.87	0.15 ± 0.00	0.00 ± 0.00	313.92 ± 14.37	2190 ± 45
24	Sunflower	2.37 ± 0.11	0.19 ± 0.00	0.00 ± 0.00	322.11 ± 15.83	2210 ± 39
25	Sunflower	4.41 ± 0.19	0.25 ± 0.01	0.01 ± 0.00	710.27 ± 33.45	2235 ± 50
26	Lavender	10.78 ± 0.43	0.54 ± 0.02	n.d.	26.19 ± 12.29	1976 ± 24
27	Lavender	4.62 ± 0.22	0.13 ± 0.00	0.05 ± 0.00	507.13 ± 26.87	2132 ± 41
28	Liquorice	59.61 ± 0.30	0.42 ± 0.02	0.02 ± 0.00	654.66 ± 31.37	2134 ± 37
29	Honeydew	162.28 ± 7.43	0.04 ± 0.00	0.04 ± 0.00	583.39 ± 21.95	2570 ± 36
30	Honeydew	482.98 ± 5.34	0.05 ± 0.00	0.03 ± 0.00	752.29 ± 33.19	2570 ± 48
31	Honeydew	243.59 ± 10.66	0.08 ± 0.00	0.29 ± 0.01	31.35 ± 1.11	2790 ± 70
32	Honeydew	221.37 ± 10.75	0.02 ± 0.00	0.03 ± 0.00	721.56 ± 15.33	2980 ± 75
33	Sulla	2.39 ± 0.11	0.05 ± 0.00	0.00 ± 0.00	426.16 ± 15.38	1946 ± 22
34	Multiflora	4.03 ± 0.18	0.13 ± 0.00	0.00±0.00	249.22 ± 10.77	2324 ± 26
35	Multiflora	10.75 ± 0.43	0.50 ± 0.02	n.d.	837.10 ± 25.25	2650 ± 28
36	Multiflora	34.97 ± 1.53	0.08 ± 0.00	0.01 ± 0.00	593.58 ± 26.78	2430 ± 27
37	Multiflora	20.47 ± 0.81	0.06 ± 0.00	0.04 ± 0.00	673.88 ± 28.61	2543 ± 32
38	Multiflora	250.22 ± 11.49	0.48 ± 0.02	n.d.	414.18 ± 18.4	2710 ± 69
39	Multiflora	50.55 ± 1.30	0.02 ± 0.00	0.02 ± 0.00	497.89 ± 20.44	2420 ± 45
40	Multiflora	36.63 ± 1.58	0.11 ± 0.00	0.01 ± 0.00	406.93 ± 17.40	2600 ± 77
41	Multiflora	9.39 ± 0.37	0.02 ± 0.00	0.01 ± 0.00	465.17 ± 18.68	3310 ± 98
42	Multiflora	18.85 ± 0.83	0.07 ± 0.00	0.02 ± 0.00	973.18 ± 32.16	2134 ± 43
43	Rododhendron	5.18 ± 0.22	0.44 ± 0.02	0.00 ± 0.00	254.83 ± 10.33	2112 ± 56
44	Rosemary	5.48 ± 0.26	0.30 ± 0.01	n.d.	391.64 ± 16.75	2390 ± 56
45	Taraxacum	7.54 ± 0.32	0.06 ± 0.00	0.01 ± 0.00	629.18 ± 25.88	2201 ± 75
46	Lime tree	28.44 ± 1.37	0.44 ± 0.02	0.01 ± 0.00	273.69 ± 12.56	2540 ± 47
47	Lime tree	29.31 ± 1.31	0.53 ± 0.02	n.d.	285.56 ± 17.34	2446 ± 77
48	Lime tree	14.26 ± 0.65	0.41 ± 0.01	n.d.	507.13 ± 22.54	2340 ± 87
49	Lime tree	44.53 ± 2.19	0.39 ± 0.01	n.d.	402.04 ± 8.89	2420 ± 65
50	Lime tree	7.43 ± 0.33	0.02 ± 0.00	0.01 ± 0.00	507.34 ± 23.31	2312 ± 78
51	Lime tree	12.59 ± 0.35	0.35 ± 0.01	0.02 ± 0.00	514.48 ± 19.40	2590 ± 45
52	Trifolium	8.27 ± 0.38	0.12 ± 0.00	0.02 ± 0.00	540.80 ± 22.57	2540 ± 66
53	Pluvial forest	42.65 ± 1.95	0.55 ± 0.02	n.d.	1116.13 ± 42.86	2690 ± 43
54	Forest	19.76 ± 0.76	0.27 ± 0.01	0.03 ± 0.00	332.82 ± 11.82	2143 ± 64

Table 3
Pearson coefficients of correlation between NO_3^-, NO_2^-, N-NO, TP and TProt and multivariate regression analysis of N-NO.

	Correlation analysis (Pearson coefficients R)					Multiple regression analysis (N-NO independent variable)	
	NO_3^-	NO_2^-	N-NO	TP	TProt	B estimate (SE)	P value
NO_3^-	1	0.133	126	0.449*	0.419**	0.107	0.416
NO_2^-		1	−0.490**	0.033	−0.191	−0.499	0.000
N-NO			1	0.237	0.291*	–	
TP				1	0.363**	−0.253	0.000
TProt					1	0.126	0.330

* $P < 0.05$.
** $P < 0.01$.

Table 4
Comparison of the NO_3^- and NO_2^- contents in honeys determined by ion-exchange HPLC coupled to conductimetric detection and by the chemiluminescence based methodology. n.d.: not detectable.

Honey samples	NO_3^- content (mg/kg)		NO_2^- content (mg/kg)	
	Ion chromatography	Chemiluminescence	Ion chromatography	Chemiluminescence
Sample 4	6.263 ± 0.19	4.48 ± 0.24	n.d.	0.01 ± 0.00
Sample 7	6.16 ± 0.51	7.70 ± 0.47	n.d.	0.00 ± 0.00
Sample 16	29.73 ± 2.74	31.72 ± 2.18	n.d.	0.50 ± 0.01
Sample 17	62.82 ± 1.31	64.46 ± 2.11	n.d.	0.46 ± 0.01
Sample 22	1.57 ± 0.07	1.63 ± 0.04	n.d.	0.00 ± 0.00
Sample 37	21.32 ± 1.72	20.47 ± 0.06	n.d.	0.06 ± 0.00
Sample 38	257.52 ± 5.93	250.20 ± 3.74	n.d.	0.48 ± 0.01

groups was confirmed by the complete disappearance of their chemiluminescent response after UVB exposure, as described above.

Statistical analysis (Table 3) indicated significant correlations between the levels of N-NO and those of NO_2^- levels ($R = -0.490$, $P < 0.01$) and TP ($R = 0.291$, $P < 0.05$).

The fact that honey N-NO was independently and negatively related to honey NO_2^- levels ($\beta_{NO_2^-} = -0.499$, $P < 0.01$) and to TP ($\beta_{TP} = 0.253$, $P < 0.01$) was confirmed by multiple regression analysis using NO_2^-, NO_3^-, TP and TProt as independent variables. Despite the limited predictability of the calculated model ($R^2_{ad} = 0.277$, $P < 0.01$, ANOVA), it indicated that most of the N-NO variability was due to unknown factors. This suggested that N-NO may originate first from some modification of the nitrosable amino acid residues (lysine, proline) present in the protein backbones, and (ii) from the greater honey-reducing capacity (increasing TP) due to the presence of antioxidant species (flavonoids, phenolic acids, Maillard reaction products) which in the honey acidic medium may stabilize NO_2^-, that in turn generates the nitrosating species NO^+.

An alternative or complementary explanation for the greater formation of N-NO in honeydew honey might be the longer exposure to bee-smoking, a procedure used by beekeepers to collect honey. Honeydew honey is collected late in the year (October–November), when the majority of floral nectars are no longer available to the bees, and this makes them highly aggressive. The exposure to smoke can lead to direct contamination of honey by N-NO or free radical NO metabolites present in the smoke itself. As depicted in the bee-smoking simulation test (Fig. 2), exposing 3 g of native acacia honey, intentionally selected because it is free from N-NO contamination, to cardboard and jute smoke substantially increases the N-NO groups in both sources, with no effects on NO_3^- levels and with an increase in NO_2^- only after exposure to jute smoke.

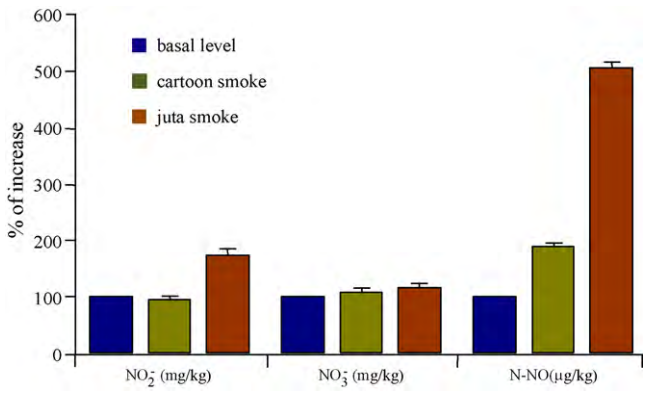

Fig. 2. Bee-smoking simulation test.

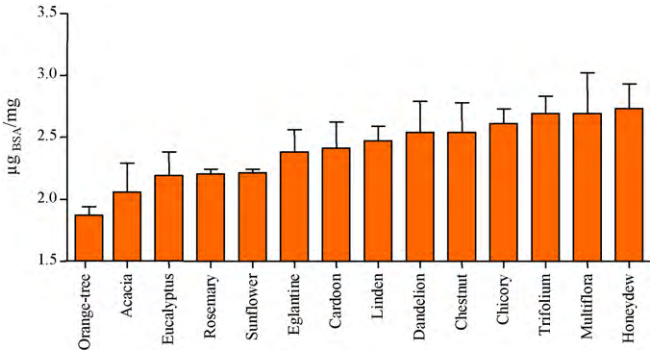

Fig. 3. Total protein content (TProt) in honeys divided by botanical origin.

3.1.5. Ion chromatography

The results obtained using the ion-exchange HPLC technique gave for NO_2^- undetectable values for all the honeys (Table 4). This is probably due to the lower instrumental sensitivity (in terms of LOD of the method). For what concerns the NO_3^- values, there was a strict correlation between the values obtained with the two techniques (see Table 4, where a subset of honey samples have been analyzed, $n = 7$).

3.2. TProt

The honey TProt (expressed in $\mu g_{BSA}/g$) agreed with the results of previous studies. TProt was in the range from 1.84 ± 0.07 μg/g to 3.31 ± 0.02 μg/g. As shown in Fig. 3 the highest levels were in honeydew honeys compared to the nectar and arboreal flowers.

3.3. TP

The mean TP and its range in the honeys analyzed were in accordance with those previously reported in the literature [10,15] and range from 1116 mg_{GAE}/kg to 26 mg_{GAE}/kg.

4. Conclusions

This study unequivocally demonstrates that the method previously established for determining NO_2^- and N-NO contamination in a relatively simple aqueous matrix (injectable preparations of LMWH) can be successfully applied to a more complex, viscous, multi-component matrix such as honey. In addition, the sensitivity of the chemiluminometric response meant we did not encounter any difficulties in detecting trace levels of NO_2^- and N-NO groups. The LOD for NO_3^-, equivalent to 0.001 μM (obtained injecting 500 μL of diluted native honeys), is comparable to those obtained with the conventional, highly selective and universally adopted ion chromatography methodology. The LOD can therefore be attained

starting from a sample, equivalent to 5 mg of native honey. This gives reliable results with only minimal amounts of diluted matrix. In addition the chemiluminometric approach sounds promising for the determination of NO metabolites in honey since it does not require sample preparation (i.e. pre-purification), but only dilution and injection, so acquisition takes only a few minutes, with no interference from other nitrogen-containing compounds (as demonstrated by the experiment on honey blank).

The results clearly show that honeydew honeys contain traces of N-NO that may arise from (i) nitrosation of suitable substrates (such as proteins and/or amino acids) mediated by NO_2^- from environmental contamination, or (ii) from the bee-smoking procedure. From the biological point of view, the results provide strong evidence that honeys from honeydew and arboreal flowers contain appreciable levels of NO_3^- which, after salivary reduction, can be converted to intragastric NO. This could well explain honey's preventive effect against gastric lesions induced by ethanol and ammonia [1,2] and therefore strongly supports the dietary intake of selected varieties of this functional food rich in NO_3^- as a means of overcoming gastric injury induced by NSAIDs, in particular during chronic therapy almost always accompanied by gastric inflammation, and sometimes bleeding.

If we consider that the dietary intake on NO_3^- rich honey can easily attain 5/10 g daily (3–5 mg of NO_3^-), although much of the NO_3^- is excreted in the urine up to 70%, the remaining taken up by salivary glands, and due to entero-salivary circulation, concentrated up to 20-fold in saliva [6]. The nitrate levels drop to one-tenth by NO_3^- reduction to NO_2^-, and when saliva enters the acidic environment of the stomach (pH 1–1.5), much of NO_2^- is rapidly protonated to HNO_2, which decomposes to NO and other nitrogen oxides. Hence even if NO comes into contact with the gastric vasculature in very low amounts (few ppm), it can exert a vasodilation effect since active at nanogram levels, while HNO_2 accumulates within the mucous where it acts as a further reservoir of vasoprotective NO.

In conclusion, the dietary $NO_3^-/NO_2^-/NO$ pathway from high NO_3^- honey can be viewed as a complementary source of NO, or as an alternative to the classical L-arginine–NO-synthases pathway when molecular oxygen availability is reduced and NO signaling decreases and open interesting therapeutic opportunities limited not only to gastroprotection but also to systemic vasodilation.

Finally, for what concerns food composition, such striking difference in NO_3^- content in nectar honeys from herbal flowers compared to that from arboreal species and from honeydew makes this parameter a reliable marker of honey origin and quality. The presence of low or sustained levels of NO_3^- could be an indicator of the botanical origin of honey and a marker of fraudulent or involuntary adulteration, this latter due a blending carried out by bees themselves which blend nectar and honeydew.

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