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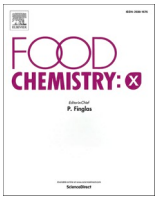

Antioxidant protein signatures in honey: botanical influence and proteomic variability

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ABSTRACT

Antioxidant capacity of honey derives from both polyphenols and proteins. We aimed to define putative antioxidant-protein signatures across honeys of differing botanical origin and relate them to pollen for authenticity assessment. Seven Lithuanian honeys were profiled by LC-MS proteomics, identifying 17 proteins with annotated antioxidant function (10 plant-, 6 bee-, 1 aphid-derived). Plant proteins, dominated by *Brassica napus*, were most abundant in monofloral *B. napus* honey. Bee proteins, glucose-methanol-choline oxidoreductases, were more variable and together comprised >75% of the summed antioxidant-protein signal. Correlations showed positive associations between *B. napus* pollen and most *B. napus*-derived proteins ($r = 0.815$–0.996) but not with bee-derived proteins; chalcone–flavonone isomerase correlated negatively. Fold-change analysis in comparison to monofloral *B. napus* honey confirmed up-regulation of *B. napus* proteins and down-regulation of several bee oxidoreductases. Altogether, plant proteins capture botanical origin, whereas bee proteins reflect apicultural factors, supporting a multi-indicator basis for honey authenticity and functional appraisal.

1. Introduction

The antioxidant properties of food are largely attributed to flavonoids, a diverse group of polyphenolic compounds known for their ability to neutralize free radicals. The general antioxidant potential of food is typically assessed by evaluating the free radical scavenging capacity of its components, including polyphenols and proteins. Honey, a unique natural product produced exclusively by bees, is primarily composed of sugars; however, its biological activity extends beyond the carbohydrate content (Vallianou et al., 2014). The therapeutic properties of honey are related to its diverse constituents - phenolic compounds, amino acids, vitamins, proteins, and minerals - of various concentrations (Valverde et al., 2022).

Among these, flavonoids exhibit notable antioxidant activity and have been shown to inhibit pro-inflammatory enzymes such as cyclooxygenase (COX), lipoxygenase (LOX), and inducible nitric oxide synthase (iNOS) (Silva et al., 2021). Antioxidants, whether enzymatic or non-enzymatic, protect cellular structures by scavenging free radicals and preventing oxidative damage. Key antioxidant enzymes include catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx), while small molecules such as vitamins C and E, glutathione, and uric acid further enhance defense mechanisms by chelating pro-oxidant metals (Kabel, 2014).

Research has primarily focused on the total antioxidant activity in bee products and the characterization of phenolic acids and flavonoids in honey, pollen, and beebread (Baltrušaityte et al., 2007). Pollen enters honey incidentally during nectar collection and processing in the hive. Each plant species contributes a unique flavonoid profile to its pollen, which influences its antioxidant capacity and plays a role in plant–pollinator interactions through specific light-reflective properties (Zerback et al., 1989). Antioxidants are broadly classified as endogenous produced through metabolic processes, and exogenous, acquired through diet. Endogenous antioxidants include both enzymatic (e.g., SOD, CAT, GPx, glutathione reductase [GRx]) and non-enzymatic (e.g., lipoic acid, coenzyme Q10, melatonin, bilirubin, uric acid) molecules (Pham-Huy et al., 2008). Exogenous antioxidants from plant-based foods include polyphenols (phenolic acids, flavonoids), vitamins, carotenoids, organosulfur compounds, and trace elements (Lobo et al.,

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2010).

The antioxidant capacity of honey is classically attributed to its phytochemical profile, in particular its phenolic compounds that also confer antimicrobial effects and remain active in topical formulations [Hossain M.A., et al., 2023]. The earlier studies targeting unifloral honeys link polyphenolic composition to measured antioxidant capacity, underscoring that phenolics, such as flavonoids and phenolic acids, are major radical scavengers and metal chelators (Zhang et al., 2021). Methodological factors can modulate these readouts: extraction/processing conditions influence the recovery and apparent activity of phenolics, with raw honeys and simulated digests often exhibiting stronger activity than processed counterparts (Alevia et al., 2021; Kačániová et al., 2022). Ecological context also matters as specific plant families contribute distinctive polyphenol patterns that correlate with antioxidant outcomes in derived honeys (Shakoori et al., 2024)(Alevia et al., 2021; Hossain et al., 2023; Kačániová et al., 2022; Kędzierska-Matysek et al., 2021; Shakoori et al., 2024; Zhang et al., 2021). Beyond phenolics, honey contains proteins and peptides, including antioxidant enzymes such as superoxide dismutase and catalase, as well as members of the Major Royal Jelly Protein family-that can contribute to redox activity and broader bioactivity (Ahmed et al., 2018; Yunus, 2023). Recent proteomic surveys have begun to catalog honey-specific proteins across botanical origins, but they typically stop short of partitioning the relative contributions of plant-derived versus bee-secreted antioxidant proteins or tying these systematically to pollen composition (Mureşan et al., 2022). Moreover, matrix effects-such as potential interactions between proteins and polyphenols-may alter stability, solubility, and net antioxidant readouts, suggesting that composite protein-phenolic assemblies could be functionally relevant yet remain underexplored in honey (Ahmed et al., 2018; Mureşan et al., 2022; Yunus, 2023).

In addition to honey, other products such as royal jelly, produced by the hypopharyngeal glands of bees, also contain antioxidants in particular include antioxidant enzymes such as peroxiredoxin and glutathione S-transferase, and antioxidant proteins - ferritin and transferrin and peroxiredoxin (Čeksterytė et al., 2023; Feng et al., 2009). Adenosylhomocysteinase (AHc) and its derivative peptide NL13 have shown promising antioxidant effects in various in vitro assays (Sarkar et al., 2020) as well as one of the stress-responsive proteins, detected in *Brassica napus*, honey samples of different botanical origins.

Overall, the antioxidant properties of honey can be attributed largely to its diverse array of phenolic compounds, along with enzymatic contributions from various proteins. Recent studies indicate the necessity of understanding the interrelationship between these components and lead us to the hypothesis, that the composition and amount of antioxidant proteins in honey depend not only on the botanical origin reflected by pollen, but also on proteins secreted by bees during hive processing; moreover, understudied flavonoid-protein complexes present in honey may exhibit stronger antioxidant activity than free flavonoids. Consequently, the degree of correlation between pollen content and antioxidant proteins should differ for plant-associated versus bee-associated proteins and can be evaluated by how these variables co-vary within the study system. To test this hypothesis, our study (i) profiles putative antioxidant proteins in honey of different botanical origins from Lithuania by LC–MSˆE proteomics, with functional roles assigned in silico, (ii) assigns plant versus bee origin, and (iii) quantifies their relationships with pollen composition to disentangle botanical from hive/physiological contributions-thereby extending phenolic-centric views and advancing from presence-absence catalogues to correlation-based interpretation.

2. Materials and methods

2.1. Collection of honey and determination of its botanical origin

Honey samples were collected from two districts in Lithuania: Kėdainiai district, from an apiary of Lithuanian Research Centre for Agriculture and Forestry and in Varėna district,Musteika village (Table 1).

The botanical origin of the honey samples was determined as described by Čeksterytė et al. (2024). Pollens were analysed using a Nikon Eclipse E600 light microscope (Nikon Corporation, Tokyo, Japan) at 400× magnification. For each sample, approximately 400 to 500 pollen grains were examined in triplicate to assess exine characteristics along with morphological characteristics and their botanical origin was confirmed using a reference pollen catalog (Čeksterytė, 2012). The botanical classification of honey was based on the relative abundance of pollen types: monofloral (predominant species >45%), secondary pollen (16–45%), minor important pollen (3–15%), and minor pollen (<3%).

2.2. Protein isolation and sample preparation

Protein extraction, enzymatic digestion, and LC-MS analysis were performed following protocols previously described in Čeksterytė et al. (2024). Briefly, proteins were isolated from honey-derived pollen using buffer-based homogenization, boiled for 5 min and centrifuged for 30 min, followed by precipitation with 5 vol of ice-cold 97.6% acetone, storing at −20 °C overnight and washing with twice with 96.6% ethanol. The resulting pellets were dissolved in 8 M urea buffer and subjected to tryptic digestion using the filter-aided sample preparation (FASP) method (Wiśniewski et al., 2009). Briefly, proteins were diluted in urea, alkylated and digested overnight with TPCK Trypsin 20,233 (Thermo Fisher Scientific, Lithuania), then centrifuged and additionally eluted using 20% CH_3CN. The solution was acidified with 10% CF_3COOH and lyophilized in a vacuum centrifuge. The lyophilized peptides were redissolved in 0.1% formic acid.

The peptides were purified and analysed using a Waters Acquity UPLC system (Waters Corporation, Wilmslow, UK) with ACQUITY UPLC HSS T3 250 mm analytical column coupled to a Synapt G2 mass spectrometer and Masslynx 4.1 software (Waters Corporation, Wilmslow, UK) in positive ion mode operating in data-independent acquisition (DIA) mode with ion mobility separation (IMS, *UDMS*^{E}) (Distler et al., 2013). For the survey scan, the mass range was set to 50–2000 Da with a scan time of 0.8 s.

2.3. Protein quantification and bioinformatic analysis

Raw data was lock mass-corrected using the doubly charged ion of [Glu1]-fibrinopeptide B (*m/z* 785.8426; [M + 2H]2+) and a 0.25 Da tolerance window and were processed with ProteinLynx Global Server (RRID:SCR_016664; Waters Corporation, Wilmslow, UK) to identify and quantify proteins. Apex3D and Pep3D algorithms to generate precursor mass lists and associated product ion mass lists for subsequent protein

Table 1
Characteristics of the analyzed honey samples, including collection site, bee subspecies, and honey classification.

Honey sample	Collection site	Bee subspecies	Classification of honey
S1	Akademija, Kėdainiai distr.	*Apis mellifera carnica*	Polyfloral summer honey
S2	Akademija, Kėdainiai distr.	*Apis mellifera carnica*	Monofloral oilseed rape honey
S3	Musteika, Varėna distr.	*Apis mellifera mellifera*	Polyfloral summer honey
S4	Akademija, Kėdainiai distr.	*Apis mellifera carnica*	Monofloral oilseed rape honey
S5	Akademija, Kėdainiai distr.	*Apis mellifera carnica*	Monofloral oilseed rape honey with honeydew and algae elements
S6	Akademija, Kėdainiai distr	*Apis mellifera carnica*	Monofloral oilseed rape honey with honeydew and algae elements
S7	Akademija, Kėdainiai distr	*Apis mellifera carnica*	Monofloral linden honey with honeydew and algae elements

identification and quantification. Peak lists were generated using the following parameters: (i) low energy threshold was set to 150 counts, (ii) elevated energy threshold was set to 50 counts, (iii) intensity threshold was set to 750 counts.

Database searching was performed with PLGS search engine using automatic peptide tolerance and fragment tolerance, minimum fragment ion matches of 1 per peptide and 3 per protein, and false discovery rate (FDR < 4%). Trypsin as the cleavage protease was used for data analysis, one missed cleavage was allowed, and fixed modification was set to carbamidomethylation of cysteines, the variable modification was set to oxidation of methionine. UniProtKB/SwissProt databases were used for protein identification and the identified ones were annotated for terms of gene ontology (GO) using AgBase (Version 2.0) [https://agbase.arizona.edu]. The statistical analysis was implemented in the open-source R statistical environment (version 4.5.1, (R Core Team, 2025)).

3. Results

3.1. Botanical composition of honey samples and their clasification

The pollen spectra revealed the principal plant species used by bees to produce honey and clustered honeys by botanical similarity (Fig. 1). According to the botanical composition, honey samples S2 and S3 were the most divergent: S2 was the monofloral oilseed rape honey, whereas other was polyfloral one with no *B. napus* pollen. The botanical profile of S3 consisted of pollen from 11 nectariferous plants, where pollen of raspberry and phacelia were categorized as of secondary importance reaching 35.3% and 26.6%, respectively, with minor contributions from alder buckthorn (*Frangula alnus*), heather (*Calluna vulgaris*), and a notable proportion (9.8%) of unidentified anemophilous pollen.

All studied samples, except S3, contained oilseed rape pollen with a mean value of 55.3%, ranging between 23.1% (S7) and 88.5% (S2) and classified as a predominant or secondary. Raspberry (*Rubus idaeus*) and willow (*Salix caprea*) pollens were present at lower levels across samples with a mean values of 7.1% and 4.5%, respectively (Fig. 2). The other commonly identified pollens were of linden tree (*Tilia cordata*) reaching the maximum percentage of 41.1% in S7 and categorized as a secondary pollen, while other samples contained linden pollen between 3.8% (S4) and 15.2% (S1). Faba bean (*Vicia faba)* appeared as secondary pollen in S1, S6, and S7 with a mean value of 23.9%, while S3 contained 26.6% of phacelia (*Phacelia tanacetifolia*) as secondary pollen. Important minor pollens included white clover (*Trifolium repens*), charlock (*Sinapis alba*), cornflower (*Centaurea cyanus*), willow (*Salix caprea*), caraway (*Carum carvi*), heather (*Calluna vulgaris*), alder (*Frangula alnus*), maple (*Acer platanoides*), buckwheat (*Fagopyrum esculentum*) and fruit tree (*Malus domestica*).

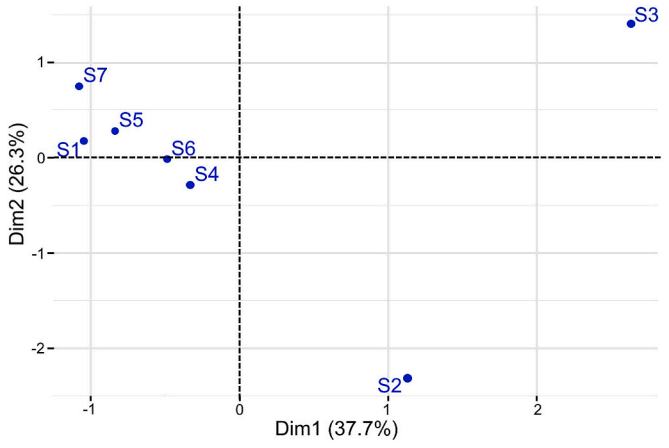

Fig. 1. Honey samples clustered according to botanical composition using multifactor analysis, with separation of monofloral and multifloral profiles and identification of honeydew-associated components

Notably, five of seven honeys contained honeydew elements ranging between 1% (S2) and 58,7% (S5), with substantial proportion in S1 (43.2%), S4 (32.9%), S5 (58.7%), S6 (28.0%), and S7 (32.9%), indicating mixed nectar–honeydew origin. In contrast, no honeydew elements were detected in samples S2 and S3.

3.2. Characterization of antioxidant protein composition in honey samples

A total of 17 putative antioxidant proteins were identified across seven honeys of diverse botanical and geographical origin: 10 plant-derived, 6 bee-derived, and 1 aphid-associated. A comprehensive list of all proteins identified across samples—beyond those annotated with antioxidant function is provided in Supplementary Material. Plant proteins were predominantly from *Brassica napus* L. ($n = 7$), with additional proteins from *Solanum tuberosum* L. ($n = 2$) and *Pisum sativum* L. ($n = 1$) (Table 2). Insect proteins comprised six from *Apis mellifera* and one peroxiredoxin from *Acyrthosiphon pisum*.

These proteins were detected in all samples, with substantial between-sample variability. The highest mean abundances were observed for adenosylhomocysteinase (A0A078FUB8) and catalase (A0A078GY74), both from *B. napus*, and were most pronounced in the monofloral oilseed rape honey S2 (Fig. 3). Their mean were 0.85 ± 1.23 ng and 0.64 ± 1.10 ng, respectively, reflecting high variation (Table 2). Isomeric forms showed distinct physicochemical features. Adenosylhomocysteinase isoforms (A0A078FUB8, A0A0781N20) differed in molecular weight (56.7 kDa vs 53.9 kDa) and sequence coverage (38.78% vs. 32.99%). Similarly, the catalase isomers (A0A078GY74, A0A078H2Z1) differed in isoelectric point (pI = 6.45 and 6.86), molecular weight (57.1 and 57.3 kDa), and sequence coverage (49.59% and 33.54%).

A catalase from *P. sativum* (C0STY9) was detected at 0.15 ± 0.10 ng on average. Two antioxidants of *S. tuberosum*, glycine-rich protein 2 (M1B8D0) and multicopper oxidase (M0ZKA2), were found at lower concentrations (0.08 ± 0.10 ng and 0.14 ± 0.00 ng, respectively). *B. napus* peroxiredoxin (A0A078CG85) was 11.75-fold more abundant than the aphid homolog (J9JQE9) showing a slightly higher molecular weight of 21.7 kDa and had the sequence coverage of 12.95%. In the monofloral oilseed rape honey S2, concentrations were highest for adenosylhomocysteinase (A0A078FUB8; 3.64 ng) and catalase (A0A078GY74; 3.12 ng). Catalase amounted to 86.01% of the adenosylhomocysteinase level, and the difference between the two proteins was statistically significant ($p \leq 0.05$). In S2, all other antioxidant proteins individually represented only 2.61–24.84% of the adenosylhomocysteinase abundance ($p \leq 0.05$).

Among bee-derived proteins, three glucose-methanol-choline oxidoreductase (GMC_OxRdtase_N domain-containing proteins) isoforms were identified: two ferritin and one transferrin. Although plant-origin proteins were more numerous, their cumulative abundance was lower (25.04%) than that of insect-origin proteins (74.96%). A GMC_OxRdtase_N isoform from *A. mellifera* (A0A088A031) was particularly abundant and highly consistent (CV = 26.41%) across all honeys, with concentrations ranging from 3.64 to 9.07 ng and had the highest sequence coverage (58.54%; Fig. 4).

In the polyfloral summer honey S3, GMC_OxRdtase_N protein A0A087ZVX2 reached 30.03% of the A0A088A031 level but remained lower in other samples. Ferritin isoforms (A0A087ZXU0 and A0A087ZXW2) differed in molecular weight (25.4 kDa and 15.2 kDa) with comparable sequence coverage (38.25% and 34.09%); each accounted only for 1.32–5.93% of GMC_OxRdtase_N (A0A088A031). Similarly, transferrin (A0A088AFH7) was detected at 2.34 to 5.37% of A0A088A031 level ($p \leq 0.05$).

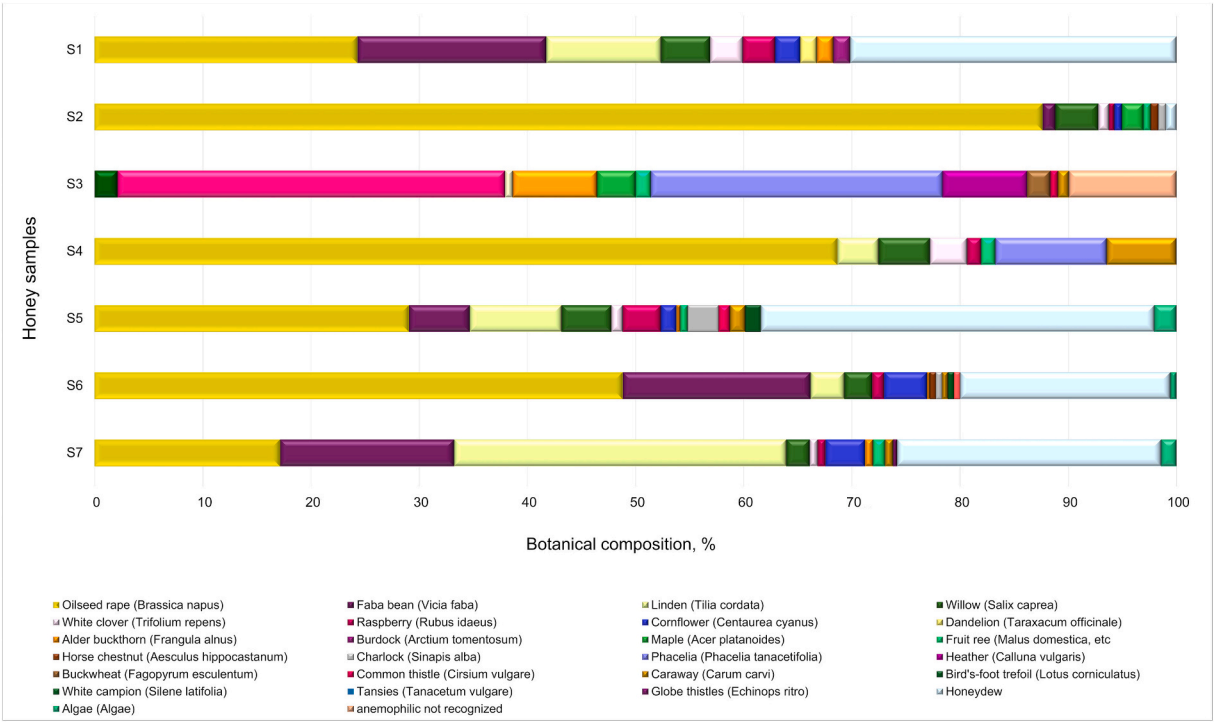

Fig. 2. Botanical composition (%) of the honey samples analyzed in this study

3.3. The influence of oilseed rape (Brassica napus L.) pollen on the antioxidant protein content in honey

The influence of oilseed rape pollen was assessed by correlating pollen content with protein abundance across all samples except S3, because it contained no *B. napus* pollen. For plant-derived putative antioxidant proteins, most oilseed rape-associated candidates showed strong positive correlation with *B. napus* pollen (r = 0.815 to 0.996, Fig. 5). An exception was chalcone-flavonone isomerase (A0A0 78I7K6), which exhibited a strong negative correlation (r = −0.746 to −0.825) across samples.

For bee-derived antioxidant proteins, correlations with *B. napus* pollens were more variable (Fig. 6). Very strong within-bee correlations were observed between two *Apis mellifera* ferritin isomers (r = 0.981) and between two GMC oxidoreductase _N domain-containing isoforms (r = 0.960). And opposite, correlations between *B. napus* pollen and bee-derived antioxidants were weak or negative. Moderate positive within-bee associations were also noted, for instance transferrin versus GMC_OxRdtase_N, r = 0.685 and ferritin versus GMC_OxRdtase_N, r = 0.694. Importantly, no significant positive correlation was detected between *B. napus* pollencontent and any bee-origin antioxidant protein.

3.4. Evaluation of protein expression levels relative to monofloral oilseed honey

Protein expression levels differed across samples. Computational analysis showed that the monofloral oilseed rape honey (S2) containing 88.5%, displayed a distinct expression profile of the identified putative antioxidant proteins compared with the other samples S1–S7 that had lower or mixed pollen composition (Fig. 7).

Monofloral rapeseed honey (S2) was used as a reference for fold-change (FC) calculations across all samples tested. In pairwaise comparison with S2, 40 proteins showed positive FCs and 55 proteins exhibited negative FCs. Positive FC counts by comparison were: S2/S1–11; S2/S3–6; S2/S4–4; S2/S5–6; S2/S6–5; S2/S7–8 (Fig. 7). Overall, relative to S2, most samples displayed more downregulated than upregulated proteins. Negative FCs relative to the control S2 were observed for 55 proteins: S2/S1–5; S2/S3–9; S2/S4–12; S2/S5–10; S2/S6–11; S2/S7–8. Two plant-derived proteins, in particular, adenosylhomocysteinase of OS=*B. napus* (A0A0 78FUB8) and a catalase isomer OS=*Brassica napus* (A0A0 78GY74), showed high positive FCs across all comparisons with S2, ranging 3.269–5.874 and 4.184–19.137 respectively. The highest positive FCs was observed for peroxiredoxin OS=*Brassica napus* (A0A0 78CG85_BRANA) in S2/S1 with a value of 30.66, while catalase OS=*Brassica napus* (A0A0 78GY74_BRANA) reached 19.137. In contrast, the strongest negative FCs involved GMC_OxRdtase_N domain-containing protein OS = *Apis mellifera* (A0A0 088A030) between samples S2/S1 and S2/S7 (−11.651) and (−12.524) respectively.

Achieved for the protein between samples while for the The highest negative FC for the GMC_OxRdtase_N domain-containing protein OS = *Apis mellifera*: A0A0 87ZVX2 showed −14.134 (S2/S3) and −10.166 (S2/S7) and A0A0 88A030 showed −11.651 (S2/S1) and −12.524 (S2/S7). Ferritin OS = *Apis mellifera* OX = 7460 (A0A0 887ZXU0) was negative in all comparisons (− 2.778 to - 6.047), whereas ferritin (A0A0 887ZW2) was positive only in S2/S1 with a value of 2.379 and negative for the remaining pairs (−2.778 to −6.047).

4. Discussion

In recent years, there has been growing interest in bee products for their antioxidant properties and health-promoting effects, with honey standing out as a natural source of antioxidants that contribute to the prevention of coronary heart disease and support the treatment of gastrointestinal, respiratory, and degenerative diseases (Hossen et al., 2017; Olas, 2020). The antioxidant components of honey include not only well-known compounds such as polyphenols and flavonoids, but also proteins originating from plants, bee tissues, and even aphids (Baltrušaityte et al., 2007; Čeksterytė et al., 2023). Building on this foundation, our study provides new insights into the botanical and protein-based antioxidant composition of honey, emphasizing the influence of floral sources—particularly oilseed rape (*Brassica napus* L.)—on antioxidant protein diversity and abundance. We identified a variety of plant and bee-specific putative antioxidant proteins with potential utility as biomarkers of botanical origin and indicators of honey

Table 2
Characterization and amount of candidate antioxidant proteins identified in honey samples (ng),

Entry	Uniprot Accession Number	Antioxidant Proteins Specific to Plant, Microbiota, and Bee, Determined in Honey Samples	pI (pH)	MW (Da)	Sec. Cov. (%)	AVG +SD	CV, (%)
Proteins Associated with Plants							
A0A078FUB8_BRANA Ex 20	A0A078FUB8	Adenosylhomocysteinase OS=B. napus OX=3708 GN=BnaA01g23510D PE=3 SV=1	5.49	56699.1	38.78	0.85 ±1.23	145.3
A0A078IN20_BRANA Ex 26	A0A078IN20	Adenosylhomocysteinase OS=B. napus OX=3708 GN=BnaCnng19320D PE=3 SV=1	5.48	53885.8	32.99	0.48 ±0.21	43.78
A0A078IJ86_BRANA Ex 247	A0A078IJ86	Germin-like protein OS=B. napus OX=3708 GN=BnaC03g34440D PE=3 SV=1	7.4	23313.84	21.1	0.12 ±0.08	66.66
A0A078GY74_BRANA Ex 72	A0A078GY74	Catalase OS=B. napus OX=3708 GN=BnaA06g14520D PE=3 SV=1	6.45	57075.2	49.59	0.64 ±1.10	172.44
A0A078H2Z1_BRANA Ex 201	A0A078H2Z1	Catalase OS=B. napus OX=3708 GN=BnaC07g15270D PE=3 SV=1	6.86	57296.54	33.54	0.15 ±0.19	124.99
A0A078CG85_BRANA Ex 29	A0A078CG85	Peroxiredoxin OS=B. napus OX=3708 GN=BnaA09g54370D PE=3 SV=1	6.35	17717.5	55.56	0.47 ±0.65	137.81
A0A078I7K6_BRANA Ex429	A0A078I7K6	Chalcone-flavonone isomerase family protein OS=B. napus OX=3708 GN=BnaC06g06390D PE=3 SV=1	9.6	31339.7	9.93	0.04 ±0.03	78.87
C0STY9_PEA Ex 253	C0STY9	Catalase OS=Pisum sativum OX=3888 GN=PCAT1 PE=2 SV=1	6.77	57518.6	18.62	0.15 ±0.10	69.53
M1B8D0_SOLTU Ex 273	M1B8D0	GRP 2 OS=S. tuberosum OX=4113 GN=102600369 PE=4 SV=1	5.39	40187.86	21.2	0.08 ±0.10	123.51
M0ZKA2_SOLTU Ex 403	M0ZKA2	Multicopper oxidase OS=S. tuberosum OX=4113 GN=102600371 PE=4 SV=1	8.92	62707.46	4.14	0.14 ±0.00	0.50
Protein associated with aphis							
J9JQE9_ACYPI EX 428	A0A0J9JQE9	Peroxiredoxin OS= *Acyrthosiphon pisum* OX=7029 GN=100161272 PE=3 SV=1	5.54	21736.7	12.95	0.04 ±0.02	44.07
Proteins associated with *Apis mellifera*							
A0A088AFH7_APIME Ex 380	A0A088AFH7	Transferrin OS=Apis mellifera OX=7460 PE=3 SV=1	6.73	80055.1	35.25	0.22 ±0.16	74.72
A0A087ZXU0_APIME Ex 167	A0A087ZXU0	Ferritin OS=Apis mellifera OX=7460 GN=Fer2LCH PE=3 SV=1	6.09	25356.88	38.25	0.31 ±0.10	31.89
A0A087ZXW2_APIME Ex 226	A0A087ZXW2	Ferritin OS=Apis mellifera OX=7460 PE=3 SV=1	6.08	15199.71	34.09	0.11 ±0.04	34.49
A0A088A031_APIME	A0A088A031 Ex 27	GMC_OxRdtase_N domain-containing protein OS=Apis mellifera OX=7460 PE=4 SV=1	6.37	68296.2	58.54	6.93 ±1.83	26.41
A0A087ZVX2_APIME Ex 100	A0A087ZVX2	GMC_OxRdtase_N domain-containing protein OS=Apis mellifera OX=7460 PE=4 SV=1	5.82	73778.9	55.38	1.25 ±0.70	56.01
A0A088A030_APIME 318	A0A088A030 Ex 318	GMC_OxRdtase_N domain-containing protein OS=Apis mellifera OX=7460 GN=Gld2 PE=4 SV=1	5.50	69976.9	25. 47	0.55 ±0.50	91.21

authenticity.

The antioxidant properties of honey are increasingly recognized as meaningful indicators of both quality and authenticity. Recent work of Hossain et al. (2023) highlights the central role of nectar-derived phenolic compounds, which strongly influence antioxidant activity and can serve as chemical markers for authentication. While phenolics remain central to antioxidant capacity, our proteomic data indicate that plant- and bee-derived proteins provide complementary, source-specific

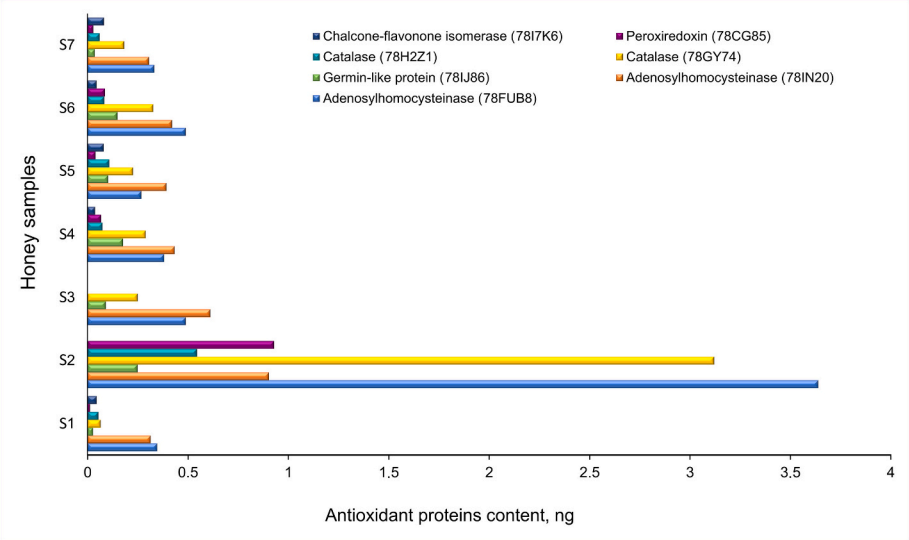

Fig. 3. Antioxidant protein content in honey samples associated with *Brassica napus*

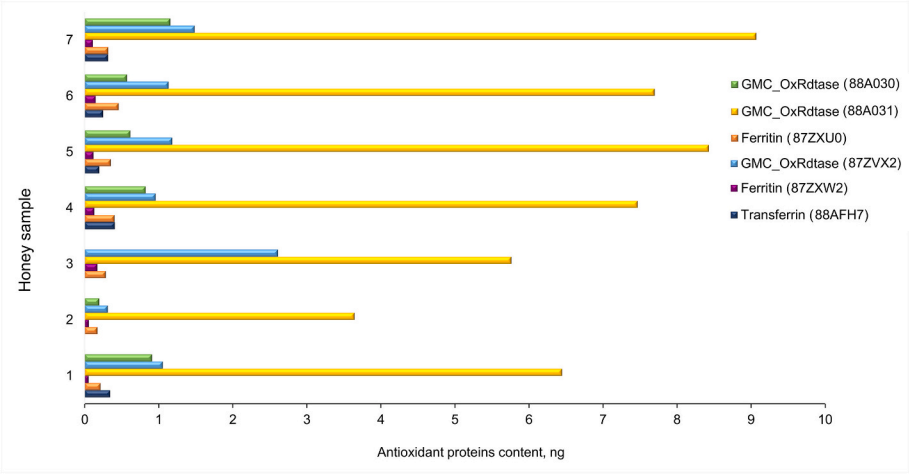

Fig. 4. Antioxidant protein content in honey samples associated with *Apis mellifera*

signals that are informative for authenticity. Consistent with this framework, our datasets reveal source-linked patterns: plant proteins track floral origin, whereas bee proteins primarily reflect hive-intrinsic contributions. Numerous bee-derived proteins were detected in rapeseed honey, indicating a prominent *Apis mallifera* signal supporting bee-origin authenticity; although individual rapeseed proteins were not specified, bee proteins are affirmed as useful authenticity markers for distinguishing genuine honey from adulterated products (Bong et al., 2023; Borutinskaitė et al., 2017; Čeksterytė et al., 2023). In buckwheat honey, antioxidant enzymes were observed, most notably catalase and glucose oxidase (GOD), a quality and freshness indicators; and non-peroxide mechanisms also contribute to antimicrobial efficacy (Chin & Sowndhararajan, 2020; Kretavičius et al., 2010; Wang et al., 2022). Because enzyme activities are modulated by storage and processing, stable proteotypic peptides of these proteins offer a more reproducible route for authenticity assessment than single-enzyme readouts. Moreover, several bee-associated proteins, including MRJPs, serve as authenticity markers that confirm bee origin and help exclude counterfeit products (Koulis et al., 2021; Lippolis et al., 2020). In parallel, the identification of plant-origin proteins and specific phenolics illuminates floral source and supports authenticity claims (Alevia et al., 2021; Dzugan et al., 2018; Zhang et al., 2021). Studies also emphasize the antioxidant and broader bioactive properties of honey proteins, underscoring their value for quality evaluation (Kačániová et al., 2022; Nyarko et al., 2023; Shakoori et al., 2024). Reviews further support diverse protein signatures as indicators of botanical origin and authenticity, including discrimination of monofloral versus polyfloral honeys, and show that nectar-derived peptide profiles enable authentication (Bong et al., 2023; Song et al., 2019; Yunus, 2023). Taken together, these findings support a multi-indicator approach—combining plant- and bee-derived proteins—and indicate that antioxidant proteins are practical biomarkers for authenticity and functional assessment, while no single antioxidant protein can yet be recommended as a universal marker across honey types (Pocol et al., 2022; Wu et al., 2024).

Botanical composition of honey, as revealed by pollen analysis and multifactor classification, showed considerable variability between samples. Monofloral honey, such as the oilseed rape-dominant samples, was clearly distinguishable from multifloral honey, containing a diverse array of nectariferous species and anemophilous pollen while biochemical profiles of several honey samples were influenced by significant proportions of honeydew elements. These botanical differences likely contribute to the variability in antioxidant protein diversity and abundance and thus understanding these influences is essential for interpreting functional and biochemical quality. Interestingly, despite

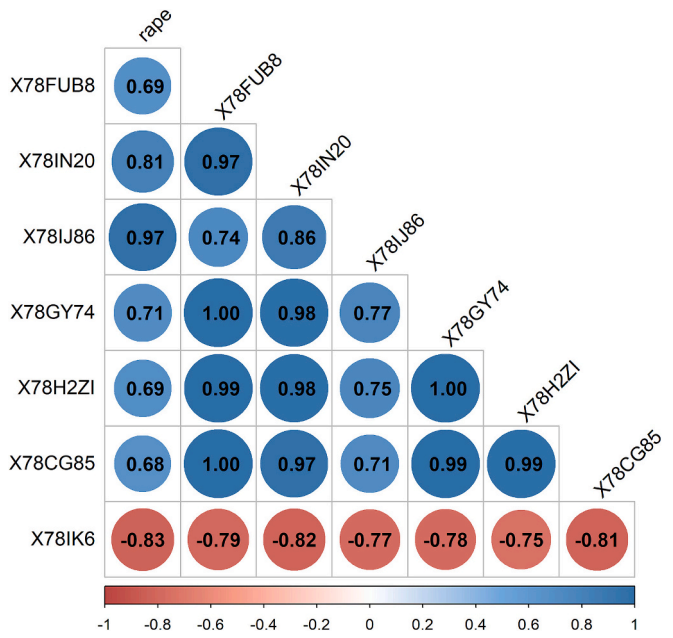

Fig. 5. Pearson correlations between the oilseed rape (*Brassica napus*) pollen content and protein content of plants in honey samples, where rape stands for oilseed rape pollen content, 78FUB8 – adenosylhomocysteinase, 78IN20 – adenosylhomocysteinase, 78IJ86 – germin-like protein, 78GY74 – catalase, 78H2Z1 – catalase, 78CG85 – peroxiredoxin, 78I7K6 – chalcone-flavonone isomerase. Positive correlations are presented in blue, while negative correlations are depicted in red circles

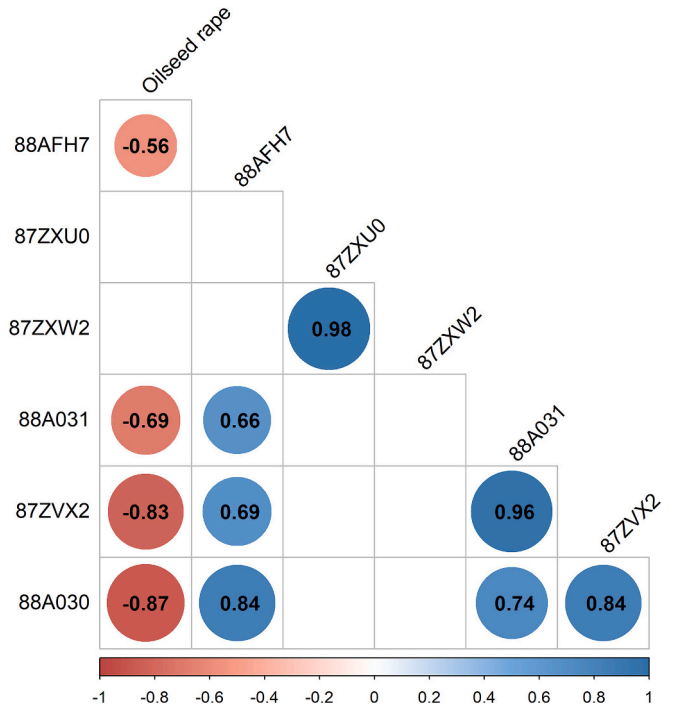

Fig. 6. Pearson correlations between the oilseed rape (*Brassica napus*) pollen content and the content of bee-related proteins in honey samples, where rape stands for oilseed rape pollen content, while antioxidants associated with *Apis mellifera* are coded as 88AFH7 – transferrin, 87ZXU0– ferritin, 87ZXW2 – ferritin, 88A031 – GMC_OxRdtase_N domain-containing protein, 87ZVX2 – GMC_OxRdtase_N domain-containing protein, 88A030 – GMC_OxRdtase_N domain-containing protein. Positive correlations are presented in blue, while negative correlations are depicted in red circles

the diversity in pollen content, no significant positive correlation was found between *Brassica napus* pollen levels and the abundance of antioxidant proteins indicating that expression is not driven by the composition alone. Taken together, the divergent correlations for plant- versus bee-derived proteins support a multi-indicator strategy, in which botanical attribution and apicultural provenance are addressed by distinct but complementary markers. Physiological and environmental stressors, namely intensive agriculture, pesticides, climate change, pathogens are plausible drivers of bee-derived protein variation (Lambert et al., 2013; Osterman et al., 2021). As a discovery LC–MS^E workflow, coverage varies across batches and the tryptic-peptide focus can underrepresent glycoforms and glycosylation-dependent activities; nonetheless, recurring source-linked proteins across samples support practical relevance, and these constraints do not affect protein-level identifications or the correlation/fold-change patterns used for provenance.

Protein-antioxidant interactions and microbiota-derived metabolites may further modulate honey's bioactivity. Complex formation between proteins and antioxidants can enhance capacity, though effects are interaction-specific (Gligorijević et al., 2020), and storage can attenuate enzyme activity via protein-polyphenol binding (Brudzynski & Maldonado-Alvarez, 2015). Framed within authenticity testing, these nuances reinforce the value of peptide-level markers and panels rather than single analytes. Overall, our findings delineate two complementary information layers, one describes plant-derived antioxidant proteins that capture botanical origin and the other – bee-derived antioxidant proteins that attest to apicultural provenance/physiology. Together with phenolic markers, these proteins provide a practical, reproducible basis for authenticity and functional appraisal.

Our data delineate two complementary information layers in honey proteomes, where one describes plant-derived antioxidant proteins that capture botanical origin and the other – bee-derived antioxidant proteins that reflect apicultural provenance and physiology. Consistent enrichment of *Brassica napus* proteins, namely adenosylhomocysteinase, catalase, peroxiredoxin in oilseed-rape honey, alongside the absence of positive pollen correlations for bee proteins, such as ferritin, glucose-methanol-choline oxidoreductases, supports our *a priori* hypothesis that botanical and hive drivers contribute differently to the antioxidant protein profile. Together with established phenolic markers, these findings argue for a multi-indicator approach to authenticity and functional appraisal rather than reliance on any single protein.

Because enzyme activities vary with storage and processing, proteotypic peptide-level measurements of the above proteins offer a more reproducible basis for routine assessment, especially in monofloral honeys, suggesting antioxidant protein profiles can serve as potential indicators of botanical origin and authenticity. While discovery proteomics cannot predetermine which proteins will be recovered in every batch, the recurring detection of source-linked proteins across samples strengthens their practical relevance as biomarkers. Overall, this study expands current understanding of antioxidant system of honey by identifying both plant- and bee-derived proteins with redox-regulating functions, clarifying their attribution to botanical versus hive factors, and providing a stable foundation for future work on peptide panels, bioavailability, and interactions with other honey components during storage and digestion.

CRediT authorship contribution statement

Violeta Čeksterytė: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Algirdas Kaupinis:** Writing – review & editing, Formal analysis. **Gabrielė Gvazdauskė:** Writing – review & editing. **Rūta Navakauskienė:** Writing – review & editing, Formal analysis. **Kristina Jaškūnė:** Writing – review & editing, Writing – original draft, Visualization.

Fig. 7. The logarithmic ratio and average abundance of proteins, where the vertical axis represents the difference between the logarithms of protein abundance and the horizontal axis represents the average of protein abundance. Red dots indicate an increase in protein abundance, while protein decrease is shown in blue

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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