NMR profiling clarifies the characterization of Finnish honeys of different botanical origins

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Abstract

The Finnish varietal honeys are valued specialty foods with unique characteristics, owing to their botanical origin. ¹H NMR metabolomics was applied to examine the compositional differences and origin-related markers of naturally complex boreal honeys (buckwheat, clover, dandelion, heather, Himalayan balsam, honeydew, linden, lingonberry, multifloral). Special focus was on the metabolites in aqueous samples. Chloroform extracts of selected samples were also analyzed for additional metabolic evidence.

24 Qualitative profiling revealed 2-hydroxy-3-methylbutyric, 2-hydroxy-3-methylpentanoic, 3-methyl-
25 2-oxopentanoic and 4-methyl-2-oxopentanoic acids as specific markers for dandelion (*Taraxacum*
26 spp.) honeys. Full discrimination of buckwheat, dandelion, heather, and honeydew honeys was
27 achieved with principal component analysis (PCA) and orthogonal partial least squares
28 discriminant analysis (OPLS-DA). Melezitose, glucose, and fructose explained 83% of the variation
29 in the PCA. The OPLS-DA loadings highlighted the discriminatory effect of the origin-specific non-
30 saccharide metabolites.

31 This study is a platform for a national honey library and analysis service. The results will facilitate
32 and rationalize the characterization and quality control of the boreal varietal honeys.

33

34 **Keywords**

35 ¹H NMR; Botanical origin; Honey; Metabolomics

36 **1 Introduction**

37 Honey, as defined in the Codex Standard 12-1981 (Codex Alimentarius, 2011) and the Council
38 Directive 2001/110/EC (European Union, 2002), is a complex natural product made by honeybees
39 (*Apis mellifera* L.). The physicochemical characteristics and sensory properties of honey are
40 determined by the origin of the honey raw material – floral nectar or honeydew, the sugary
41 secretions of sap-sucking insects. Varietal or unifloral honeys mainly originate from one plant
42 source, indicated by the predominant pollen type (> 45%) (von der Ohe, Persano Oddo, Piana,
43 Morlot, & Martin, 2004). Determining the origin of honey is essential when assessing its quality,
44 authenticity, bioactive potential and commercial value. In the Arctic and boreal environment, the
45 production of pure unifloral honeys is a challenge because many plants flower coincidentally.
46 Therefore, Finnish varietal honeys, considered as valued specialty foods, are likely to contain
47 nectar and pollen from secondary sources, which complicates their characterization by commonly
48 used methods. The main source of nectar and the country of origin should be indicated in the
49 labeling as regulated by law (European Union, 2002; Codex Alimentarius, 2011).

50 Varietal honey types are generally identified based on sensory (Piana, Persano Oddo, Bentabol,
51 Bruneau, Bogdanov, & Gyuot Declerck, 2004) and melissopalynological (microscopic analysis of the
52 pollen present in the honey) analyses (Louveaux, Maurizio, & Vorwohl, 1978; von der Ohe et al.,
53 2004), and by physical and chemical analyses (Persano Oddo, Piazza, Sabatini, & Accorti, 1995).

54 The harmonized methods of the International Honey Commission (IHC) are used in routine
55 analysis of honey (International Honey Commission, 2009). Yet, several problems exist with
56 accurate determination of botanical origin, one important issue being the natural variability of
57 honeys (Persano Oddo & Bogdanov, 2004), especially in the case of boreal honeys.

58 In addition to the main components, fructose and glucose, other minor sugars, organic acids,
59 enzymes (invertase, diastase, and glucose oxidase), aroma compounds, and trace amounts of
60 minerals are found in honey. The consistency and composition vary between different botanical
61 origins, which also affect the taste and odor of the product. Highly aromatic honey type can
62 dominate in natural blends, even at small quantities (Piana et al. 2004). Different honey types
63 have varying physicochemical characteristics, such as crystallization and electrical conductivity
64 (Bogdanov, Ruoff, & Persano Oddo, 2004). The relative concentrations and the ratio of fructose
65 and glucose are also useful parameters in relation to botanical origin (Bogdanov et al., 2004).

66 The raw material of honeydew honeys is the sugary secretion of insects (*Hemiptera*) feeding on
67 plant sap. In melissopalynological analysis, elements like fungi and algae are used as indicators of
68 honeydew instead of pollen. Honeydew honeys are distinguished by their higher electrical
69 conductivity, lower concentrations of glucose and fructose, and the abundance of trisaccharides
70 (mainly melezitose) compared with floral honeys (Persano Oddo et al., 1995; Bentabol
71 Manzanares, Hernández García, Rodríguez Galdón, Rodríguez Rodríguez, & Díaz Romero, 2011).

72 The trisaccharide level is dependent on the insect species producing honeydew, the host plant,
73 and the presence of ants (Fischer & Shingleton, 2001).

74 Honey's botanical origin plays an important role in defining its bioactive value. In several *in vivo*
75 (human and murine) and *in vitro* studies, honey has been shown to have a protective effect
76 against microbes, viruses, parasites, inflammation, oxidation, mutagenicity, and tumor
77 development (Bogdanov, Jurendic, Sieber, & Gallmann, 2008). In 2013, Huttunen, Riihinen,
78 Kauhanen, and Tikkanen-Kaukanen showed that Finnish honeys displayed antimicrobial activity
79 against the human pathogen cocci (*Streptococcus pneumoniae* and *S. pyogenes*, *Staphylococcus*
80 *aureus*) (Huttunen, Riihinen, Kauhanen, & Tikkanen-Kaukanen, 2013). The most notable effect was
81 achieved with willowherb (*Epilobium angustifolium*), heather (*Calluna vulgaris*), and buckwheat

82 (*Fagopyrum esculentum*) honeys, followed by lingonberry (*Vaccinium vitis-idaea*) and cloudberry
83 (*Rubus chamaemorus*).

84 Melissopalynological analysis, despite its ambiguity and prerequisite for expertise, can reveal the
85 main pollen type, the presence of honeydew elements, and the geographical origin through
86 pollens that are limited to a certain region (Louveaux et al., 1978; Anklam, 1998; von der Ohe et
87 al., 2004; Salonen, Ollikka, Grönlund, Ruottinen, & Julkunen-Tiitto, 2009). However, the pollen
88 content does not necessarily reflect the actual nectar source of the honey, because some types of
89 pollen can be over- (e.g. *Salix* spp. and Brassicaceae) or under-represented (e.g. *Taraxacum* spp.)
90 in relation to nectar or from wind-pollinated or non-melliferous plants (e.g. *Filipendula* spp.)
91 (Louveaux et al., 1978; von der Ohe et al., 2004; Salonen et al., 2009; Salonen, 2011). Salonen and
92 others have previously described the qualitative melissopalynological analyses of Finnish honeys
93 (Salonen et al., 2009). Together with pollen analysis, the electrical conductivity, diastase and
94 invertase activity, sugar composition, pH and acidity, and the contents of proline, water and 5-
95 hydroxymethylfurfural (HMF) are used in the quality assessment and characterization of unifloral
96 honeys (Bogdanov et al., 2004). Proline, one of the main amino acids in honey, indicates that the
97 honey is authentic and ripe (Bogdanov et al., 2004).

98 In addition to established methodologies in honey analysis (Persano Oddo et al., 1995), several
99 other approaches have been developed to characterize the botanical and geographical origins
100 more accurately by analyzing the sugar, amino acid, phenolics, and volatile compositions (Tomás-
101 Barberán, Ferreres, García-Vignera, Tomás-Lorente, 1993; Anklam, 1998; Conte, Miorini, Giomo,
102 Bertacco, & Zironi, 1998; Cotte, Casabianca, Chardon, Lhetier & Grenier-Loustalot, 2003;
103 Hermosín, Chicón, & Dolores Cabezudo, 2003; Consonni & Cagliani, 2008; Rebane & Herodes,
104 2008; Salonen, 2011; Consonni, Cagliani, & Cogliati, 2013). For example, different chromatographic
105 (Rebane & Herodes, 2008) and mass spectrometric methods (Tomás-Barberán et al., 1993;

106 Ampuero, Bogdanov, & Bosset, 2004; Zhou, Yao, Li, Chen, Wu, & Zhao, 2014), SDS-PAGE
107 immunoblotting (Baroni, Chiabrando, Costa, & Wunderlin, 2002), Fourier transform near-infrared
108 (FT-NIR) spectroscopy (Ruoff et al., 2006), and DNA barcoding (Bruni et al., 2015) have been used.
109 In recent years, honey analysis has evolved towards applying different nuclear magnetic resonance
110 (NMR) spectroscopic approaches. Having showed diverse applicability and accuracy in
111 quantitative/targeted analysis of metabolites (Sandusky & Raftery, 2005; Ohmenhaeuser,
112 Monakhova, Kuballa, & Lachenmeier, 2013; Spiteri et al., 2015), authentication (Bertelli, Lolli,
113 Papotti, Bortolotti, Serra, & Plessi, 2010; Boffo, Tavares, Tobias, Ferreira, & Ferreira, 2012; Spiteri
114 et al., 2015), and in determining geographical (Consonni & Cagliani, 2008; Consonni et al., 2013;
115 Donarski, Jones, & Charlton, 2008) and botanical origin (Ohmenhaeuser et al., 2013; Zieliński, Deja,
116 Jasicka-Misiak, & Kafarski, 2014; Spiteri et al., 2015), NMR metabolomic analysis of honey has
117 already led to commercial use of the methods (Bruker Corporation, 2015; Spiteri et al., 2015). Yet,
118 the applications have not extended to Nordic honey types until now. NMR metabolomics has been
119 used in characterizing some of the Central and Southern European honey types, most commonly
120 the acacia (more accurately, false acacia or black locust; *Pseudoacacia robinia*), buckwheat
121 (*Fagopyrum esculentum*), chestnut (*Castanea sativa*), citrus or orange blossom (*Citrus* spp.),
122 eucalyptus (*Eucalyptus* spp.), heather (*Calluna vulgaris* / *Erica* spp.), linden (*Tilia* spp.), and
123 honeydew honeys (Donarski et al., 2008; Lolli, Bertelli, Plessi, Sabatini, & Restani, 2008; Donarski,
124 Jones, Harrison, Driffield, & Charlton, 2010; Schievano, Peggion, & Mammi, 2010; Schievano,
125 Stocchero, Morelato, Facchin, & Mammi, 2012; Schievano, Morelato, Facchin, & Mammi, 2013;
126 Zieliński et al., 2014). NMR enables a rapid detection of fraudulent honeys, by indicating the
127 additions of sugar syrups diluting the honey (Bertelli et al., 2010; Spiteri et al., 2015). The presence
128 of ethanol from microbial fermentation and 5-hydroxymethylfurfural (HMF) from excess heating
129 and/or prolonged storage is also easily observed in the spectra (Spiteri et al., 2015). Both organic

130 and aqueous extracts of honey have been used in NMR studies. Chloroform extracts of nonvolatile
131 organic components that are useful fingerprint compounds (Schievano et al., 2010), but aqueous
132 samples are also adequate in discriminating honey types based on the botanical origin (Consonni
133 & Cagliani, 2008; Zieliński et al., 2014).

134 Here, ¹H NMR metabolomics was applied to analyzing boreal varietal honeys for the first time. Our
135 aim was to determine the botanical origin and the key markers of a set of Finnish honeys. The
136 described methodology will serve as platform for more unequivocal characterization and
137 authentication of the botanical origins of the honeys, benefiting the consumers, beekeeping
138 industry, and honey trade through accurate labeling and pricing of the products.

139 **2 Materials and Methods**

140 *2.1 Honey samples*

141 The botanical origins of the Finnish honey samples in focus were buckwheat (*Fagopyrum*
142 *esculentum*; *n* = 3), dandelion (*Taraxacum officinale*; *n* = 4), and heather (*Calluna vulgaris*; *n* = 4).
143 Samples of Himalayan balsam (*Impatiens glandulifera*; *n* = 1), linden (*Tilia* spp.; *n* = 1), lingonberry
144 (*Vaccinium vitis-idaea*; *n* = 1), and clover (*Trifolium repense/hybridum*; *n* = 1), together with three
145 samples of honeydew honeys and two multifloral honeys were included in the study (**Table 1**). The
146 samples were from eleven different beekeeper from the harvests of 2012 and 2013. The honeys
147 were harvested prior the end of flowering of the main plant. Dandelion and lingonberry honeys
148 were extracted (harvested) at the end of June. All the other honey types, excluding heather, were
149 extracted during late July–early August. Heather honey was extracted in mid-August. The honeys
150 were handled and stored according to each beekeeper’s customary practices, yet using as gentle
151 conditions as possible. Samples 1–17 originated directly from the producers (of which 3–16 via the
152 Finnish Beekeepers’ Association), while samples 18–20 were bought. The melissopalynological

analyses were performed on approximately 400 pollen particles and the percentages of pollen types were calculated (supplementary **Table S1**). The honey samples were stored in the dark at room temperature prior to sample preparation.

2.2 Chemicals

Internal standard 2,2,3,3,4,4- d_6 -3-(trimethylsilyl)-1-propane sulfonic acid (DSS- d_6) and sodium azide (NaN_3) in D_2O was from Chenomx Inc. (Edmonton, Alberta, Canada). Sodium hydroxide and hydrochloric acid solutions were from Fisher Scientific (Fair Lawn, NJ). Chloroform (CHCl_3) and chloroform- d (CDCl_3 , 99.8% atom D) were purchased from Sigma–Aldrich (St. Louis, MO).

2.3 Preparation of NMR samples

After thorough mixing, 100 mg of honey was dissolved in 800 μL of Type I ultrapure H_2O from Millipore Synergy UV system (Millipore, Billerica, MI). The samples were centrifuged for 1 min with $14,000 \times g$ at 20°C and the supernatant was filtered through a 3 kDa molecular weight cut-off Amicon ultracentrifugal filter unit (Millipore). An aliquot of 207 μL of the filtrate was combined with 23 μL of internal standard DSS- d_6 (at 4.6187 mM in D_2O , with 0.2% NaN_3). The pH of the sample containing DSS- d_6 standard was adjusted to 6.55 ± 0.05 with small additions of NaOH and HCl. 180 μL of the sample was analyzed in 3-mm NMR-tubes (Bruker Biospin Corporation, Billerica, MA). Three technical replicates per sample were performed to minimize possible matrix inhomogeneity.

In addition to the aqueous samples, few samples were prepared according to the chloroform–water extraction method by Schievano and others (Schievano et al., 2010). The chloroform layer was concentrated until dry with MiVac Duo concentrator (Genevac Inc., Stone Ridge, NY). The residue was re-dissolved in 600 μL CDCl_3 and transferred to a 5-mm NMR tube.

175 2.4 NMR analysis and spectral processing

176 The ^1H NMR spectra of the aqueous samples were acquired at 298 K on a Bruker 600 MHz NMR
177 spectrometer (Bruker BioSpin AG, Fällanden, Switzerland) equipped with a TCI cryoprobe and a
178 SampleJet using the *noesypr1d* pulse programs. A number of 128 scans were collected over 32 k
179 data points, with 16 dummy scans, sweep width of 12 ppm, acquisition time of 2.5 s, relaxation
180 delay of 2.5 s, and mixing time 100 ms. The receiver gain was set to 32. Water presaturation
181 during relaxation delay and mixing time was achieved with a 70 dB irradiation. The spectra were
182 zero-filled to 128 k data points. Chenomx NMR Suite 8.0 (Chenomx Inc., Edmonton, Canada) was
183 applied to manually correct the phase and baseline. Shim correction to 0.5 Hz and line broadening
184 of 0.5 Hz were applied. Prior multivariate data analyses, the spectra were normalized to total
185 spectral area and binned using 0.02 ppm bin width. The water peak at 4.88–4.92 ppm was
186 excluded.

187 The acquisition parameters for the qualitative ^1H NMR experiment (*zg30*) of the organic extracts in
188 CDCl_3 at 298 K were: number of scans, 512; number of dummy scans, 8; data points, 32 k; spectral
189 width, 14 ppm. The chemical shifts (δ) were referenced to the CHCl_3 residual peak at 7.26 ppm.

190 2.5 Data analysis

191 The chemical shifts were assigned with literature, databases (HMDB, YMDB), the Chenomx
192 Profiler, and an in-house library of saccharide standard compounds. In the multivariate data
193 analyses, The SIMCA-P+ v12.0.0.0 software (Umetrics AB, Umeå, Sweden) was used. The primary
194 dataset of 62 observations and 458 *X*-variables (bins) were Pareto-scaled prior principal
195 component analysis (PCA) and orthogonal partial least squares–discriminant analysis (OPLS-DA)
196 (Eriksson, Byrne, Johansson, Trygg, & Wikström, 2013).

197 **3 Results and Discussion**

198 *3.1 Melissopalynological analysis*

199 The melissopalynological analyses (supplementary **Table S1**) highlighted the ambiguity in the
200 characterization of Finnish honeys (**Table 1**). The most common pollen types in the samples were
201 Brassicaceae, Apiaceae, *Rubus* spp., *Trifolium* spp., and *Salix* spp. However, many of these pollen
202 types were over-represented, while the pollen from a plant source giving the honey its main
203 characteristic may be present in very minor proportions, which was the case with buckwheat
204 (*Fagopyrum esculentum*), heather (*Calluna vulgaris*), dandelion (*Taraxacum* spp.), and linden (*Tilia*
205 spp.) (von der Ohe et al., 2004; Salonen, 2011). Dandelion honeys usually feature a high
206 proportion of pollen from *Salix* spp., which was also seen in the present samples (Persano Oddo et
207 al., 1995; Salonen, 2011). Although being a non-melliferous plant, *Filipendula* spp. are a common
208 source of pollen in Finnish honey (Salonen et al., 2009). Pollen from the Brassicaceae family (most
209 likely from the turnip rape, *Brassica rapa*) was the most abundant group in the samples. Often the
210 nectar source defining the characteristic properties was present only at minor levels. Dandelions
211 produce little or no pollen, which may explain the fact that some of the dandelion honeys
212 contained *Taraxacum* pollen only at levels below 3%.

213 *3.2 Qualitative analysis of aqueous NMR samples*

214 The NMR metabolomic profiles are presented in **Fig. 1**. Highlighted fingerprints of selected
215 samples in different spectral regions are shown in **Fig. 2**. The fingerprints were consistent within
216 the sample groups but their levels differed. The aqueous extracts of buckwheat honeys had higher
217 levels of amino acids isoleucine, leucine, threonine, tyrosine, valine, and 4-hydroxybenzoic acid (d
218 7.80 and d 6.91 ppm) (**Fig. 2A–B** and **Fig. 2E**). Also, the unassigned resonances at 5.04 (broad s),

219 6.67 (d), 6.70 (dd), and 6.80 ppm (s) could serve as potential indication of the origin in buckwheat
 220 honeys. Tyrosine and 4-hydroxybenzoic acid have been reported as buckwheat markers earlier
 221 (Steeg & Montag, 1987; Zieliński et al., 2014). Phenylalanine and tyrosine have also been reported
 222 to be markers for lavender (*Lavandula*) honey (Hermosín et al., 2003). The levels of isoleucine,
 223 leucine, and valine (δ 1.1–0.9 ppm) were relatively high in buckwheat honeys (**Fig. 2A**).
 224 2-hydroxy-3-methylbutyric acid (2-hydroxyisovaleric acid; δ 0.82, 0.96 ppm), 2-hydroxy-3-
 225 methylpentanoic acid (2-hydroxy-3-methylvaleric acid; δ 0.86, 0.93 ppm), 3-methyl-2-
 226 oxopentanoic acid (3-methyl-2-oxovaleric acid; δ 0.88, 1.09 ppm), and 4-methyl-2-oxopentanoic
 227 acid (2-oxoisocaproic acid; δ 0.93, 2.60 ppm) were assigned with the Chenomx Profiler specifically
 228 to dandelion honeys (**Fig. 2A–B**). Their chemical structures with proton assignments are shown in
 229 **Fig. 3**. The doublets at δ 0.78 and 1.11 ppm were also characteristic to these honeys. These
 230 compounds may be products of microbial fermentation, but still specific for this origin, as was the
 231 high glucose content (Salonen, 2011).
 232 In addition to melezitose and trehalose, distinctive honeydew markers were found from the
 233 aliphatic and aromatic regions. The honeydew honeys were characterized by fumaric, lactic, malic,
 234 and succinic acids, and the presence of asparagine and aspartic acid at detectable levels (**Fig. 2A–B**
 235 and **Fig. 2E**). Other markers were observed at δ 5.58 and 6.43 ppm (**Fig. 2E**). Clover honey lacked
 236 any apparent signature markers, but was distinguished by its low levels of glucose. The known
 237 linden marker, 1-*O*- β -gentiobiosyl (6-*O*-(β -D-glucopyranosyl)- β -D-glucopyranosyl) ester of 4-(1-
 238 hydroxy-1-methylethyl)cyclohexa-1,3-diene-1-carboxylic acid was present (Naef, Jaquier, Velluz, &
 239 Bachofen, 2004; Schievano et al., 2013). Gentiobiose was the most abundantly present in the
 240 linden honey. Additional unidentified fingerprints for linden honey are shown in green in **Fig. 2B–**
 241 **C**.

242 Heather honeys contain aromatic carboxylic acids, namely benzoic, 3-phenyllactic and
243 phenylacetic acids, which contribute to the aroma profile and their honey-like odor (Steeg &
244 Montag, 1987). The levels of formic acid were also highest in heather honeys. A doublet at δ 1.92
245 ppm, partially overlapping with the acetic acid singlet, was found specific to heather (**Fig. 2B**). 3-
246 phenyllactic acid (PLA) may be a contributor to the high antimicrobial activity reported in heather
247 honeys (Mu, Yu, Zhu, Zhang, & Jiang, 2012; Huttunen et al., 2013).

248 As with the dandelion-specific markers, the PLA could be a microbial metabolite of the lactic acid
249 bacteria naturally present in the honey (Mu et al., 2012). The thixotropic nature of heather honey
250 makes it prone to exhibit broad NMR peaks, especially those belonging to acids. In the aliphatic
251 region, two peaks at δ 1.03 and 1.06 ppm were characteristic to the heather honeys (**Fig. 2A**).

252 Any distinctive water-soluble markers were not identified for multifloral, Himalayan balsam or
253 lingonberry honey. However, the aromatic region of the lingonberry spectra contained
254 unidentified signals at low levels (**Fig. 2E**). Their chemical shifts in the high frequency region
255 suggest (hetero)aromatic nature.

256 Several di- and trisaccharides were assigned for each honey type, most abundant ones being
257 kojibiose, nigerose, turanose, and isomaltotriose, kestose, melezitose, raffinose, respectively (**Fig.**
258 **2C–D**). Due to the matrix effect, signal overlapping, abundance of isomers and the co-suppression
259 of signals near the water peak, the accurate quantitative profiling of several saccharides was not
260 possible. Trigonelline and uridine were common metabolites in all the honeys, at low
261 concentrations. Ethanol and acetic acid were found in some of the studied honeys, indicating
262 fermentation. Both heather and dandelion honeys are known to be prone for excess moisture,
263 enabling the microbial fermentation. Heather honey has a relatively high moisture content of 18%
264 (Salonen, 2011), while dandelion honey may also contain excess moisture if centrifuged too early.
265 In addition to the fermentation products, the ^1H NMR analysis allowed the detection of 5-

hydroxymethylfurfural (HMF) at δ 9.45 ppm (Spiteri et al., 2015). The levels of HMF, a marker of quality defect due to heating and prolonged storage, were generally minute in every honey (**Fig. 2E**). Therefore, the doublets at δ 7.53 and 6.67 ppm were not detected in many cases.

The advantage of analyzing water extracts is above all cost-efficiency: the sample handling and the NMR experiment require fairly little time per sample. Also, the amount of costly deuterated solvents can be reduced to 10% of the total volume without loss of spectral quality. For routine and high-throughput analyses of determining honey origin the water extraction is recommended, even though Schievano and others recommend chloroform for its NMR-applicable properties (Schievano et al., 2010). The use of a chemical shift standard is not a necessity because the chemical shifts can be adjusted e.g. to the α -anomer of glucose at δ 5.23 ppm. For qualitative screening of honeys, ^1H NMR of samples diluted with D_2O is simple and fairly rapid, provided that characteristics of different honey varieties are known. However, quantitative analysis of aqueous extracts and extraction of organic compounds require more intricate sample preparation techniques to overcome the aforementioned challenges. Viscosity of the NMR samples is an issue especially with thixotropic heather honeys. The reduction of sample viscosity by dilution of 1:6 (w/w) was not adequate for heather honeys (Sandusky & Raftery, 2005). The linewidth at peak half-height for heather honeys was up to 4 Hz, which is highly undesirable in quantitative analysis when narrow lineshapes (peak half-width of the chemical shift indicator ≤ 1.5 Hz) are required. By increasing the dilution factor, the heather honeys reached the linewidths below 1 Hz. Yet, the peak belonging to acids were broad.

3.3 Qualitative analysis of the organic extracts

Qualitative analysis of CDCl_3 extracts for nonpolar compounds was also performed. Honeydew honey marker diacylglycerol ether supported the identification of this honey type (Schievano et al.,

289 2012; Schievano et al., 2013). Dehydrovomifoliol (4-hydroxy-3,5,5-trimethyl-4-(3-oxo-1-butenyl)-2-
290 cyclohexen-1-one), a flavor-related (but odorless) carbonyl compound, was present in heather
291 honey as expected (Häusler & Montag, 1989). The *Calluna* honeys can contain dehydrovomifoliol
292 190–260 mg/kg (Häusler & Montag, 1989). It has also been reported to be a component in
293 eucalyptus and chestnut honeys, up to levels of 6 mg/kg (Häusler & Montag, 1989; Schievano et
294 al., 2012).

295 The chloroform extract of linden honey contained the respective aglycon of the marker found in
296 the aqueous sample, the 4-(1-hydroxy-1-methylethyl)cyclohexa-1,3-diene-1-carboxylic acid
297 (Frérot, Velluz, Decorzant, & Naef, 2006; Schievano et al., 2012; Zieliński et al., 2014). Lingonberry
298 honey was characterized by relatively high methyl syringate (singlets at δ 7.33, 3.95 and 3.90 ppm)
299 content. Methyl syringate was also detected at varying but low levels in all the tested honeys
300 except honeydew. Another (unidentified) marker present in lingonberry honey had signals at δ
301 8.60 (singlet) and 8.52 (triplet) ppm. The singlet at δ 8.78 ppm and the two doublets at δ 8.41 and
302 8.18 ppm were detected only in the buckwheat honey. The Himalayan balsam honey did not have
303 any characteristic signals in the chloroform extract to suggest botanical origin. Himalayan balsam
304 (*Impatiens glandulifera*) is considered as a pernicious alien plant widely spread outside gardens
305 and recommended to be eradicated, yet its late flowering and abundant production of high-sugar
306 nectar benefits honey bees. Thus far, little is known about this honey type. Due the lack of
307 biomarkers and distinctive aroma or color, the differentiation of *Impatiens* honey from multifloral
308 or other mild honeys can be challenging.

309 3.4 Multivariate data analysis

310 The general sugar composition in relation to other honey types played an important part in the
311 discrimination. Principal component analysis (PCA) of the primary dataset (**Fig. 4A**) showed how

312 the first principal component ($R^2X = 0.50$) clearly separated honeydew honeys from the floral
 313 honeys. This separation was due to melezitose, the most obvious honeydew marker, as seen in the
 314 loadings plot in **Fig. 4B** (bins 3.85, 3.63, 4.29). Trehalose had also an influence on the first principal
 315 component through bins 3.63 and 3.85. The second component ($R^2X = 0.33$), on the other hand,
 316 signified the distribution of floral honeys and was mainly explained by the most abundant sugars,
 317 fructose (bins 3.79, 3.57, 3.55) and glucose (3.73, 3.45). Therefore, the second component may
 318 serve as an indicator of the fructose/glucose ratio and a valuable indicator of probable nectar
 319 source. The subsequent principal components did not resolve the issue of overlapping among the
 320 floral honeys. Since the NMR spectral data did not reveal any significant markers for Himalayan
 321 balsam honey, the positioning of this honey type in the center of the PCA model (**Fig. 4A**) could
 322 serve as an indicator of the potential botanical source through the sugar composition. In addition,
 323 lingonberry honey should have a relatively high fructose/glucose ratio, mainly due to its low
 324 glucose content, making it also liquid (Salonen, 2011). Here, however, the presence of *Rubus* spp.
 325 (**Table S1**) may have counterbalanced the sugar ratio. Yet, the low-intensity signals in both the
 326 aqueous and chloroform extracts are likely to originate from the high proportion of *Vaccinium* spp.
 327 However, since we only had one sample of clover, lingonberry and Himalayan balsam honeys, with
 328 limited previous information available, any advanced conclusions on their metabolic
 329 characteristics without further examination cannot be drawn.

330 Accentuated discrimination of a certain sample or a class can be achieved by using supervised
 331 multivariate methods, and special approaches, like *one-versus-all* and pairwise comparisons,
 332 where the differences between the samples in question are purposefully enhanced (Schievano et
 333 al., 2010; Consonni, Cagliani, & Cogliati, 2012; Zieliński et al., 2014). Therefore, to emphasize the
 334 class discrimination, orthogonal partial least-squares discriminant analysis (OPLS-DA) was applied.
 335 OPLS enhances relevant variation in *X* that correlates with the *Y* variable. The three largest sample

336 classes of floral honeys, the buckwheat, dandelion and heather, were chosen for this examination
337 to improve their model visualization and interpretation (**Fig. 5**). Also, by using this supervised
338 method, identification of putative biomarkers was facilitated. The samples originated from
339 different locations and all classes included one commercial sample (**Table 1**).

340 The OPLS-DA models in **Fig. 5** demonstrate high explained variation ($R^2 > 0.9$) and predictive ability
341 ($Q^2 > 0.9$). In the score overview of the first ($R^2X[1] = 0.46$) and second ($R^2X[2] = 0.06$) components
342 in **Fig. 5A**, the three classes are clearly separated. To highlight the class separation and learn the
343 discriminatory variables, pairwise OPLS-DA models were also created (**Fig. 5B–D**). The between-
344 class variations were explained by the respective loadings in **Fig. 5E–G**. The OPLS-DA models were
345 validated by subjecting the corresponding PLS-DA models to permutation analysis (**Fig. 6**).

346 Although the low observation-to-variable ratio and the use of (O)PLS-DA models are prone to
347 overfitting, the permutation tests exhibit valid models in all cases except in **Fig. 6D**, where the R^2Y -
348 intercept of 0.48 slightly exceeds the 0.3–0.4 threshold (Eriksson et al., 2013).

349 The most significant loadings to discriminate the honeys corresponded to glucose (e.g. bins 5.23,
350 4.63, 3.83, 3.73, 3.43, 3.23) and fructose (e.g. bins 4.11, 3.89, 3.79, 3.69, 3.55). The low-magnitude
351 loadings, specific to buckwheat honeys, represented tyrosine (7.19, 6.89), unknown singlet (5.05),
352 and leucine, isoleucine and valine (1.03–0.95). Tyrosine was suggested as a marker for buckwheat
353 honey (36), as was formic acid, which indeed was separating the buckwheat from the dandelion
354 honeys (**Fig. 5E**), but not from the heather honeys (**Fig. 5G**). Low levels of formic acid (8.45) were
355 characteristic to dandelion honeys, while the strongest contribution of this compound was
356 observed for heather honeys. A bin at 1.91, which is mainly acetic acid, also holds an additional
357 peak in heather honey that may be related to an acetylated metabolite. Other heather-related
358 markers were benzoic acid (7.87), phenyllactic acid (7.37, 7.31, 3.09, 2.87), dehydrovomifoliol
359 (6.43, 6.07), pyruvic acid (2.37) and proline (2.35). The OPLS-DA loadings of the models in **Fig. 5**

360 confirmed what was visually observed in the spectral data that the low-intensity non-saccharide
361 compounds are discriminatory.

362 **4 Conclusions**

363 The results of this study demonstrated the applicability of NMR-based metabolomics in
364 characterizing naturally complex Finnish honeys. Authentication and correct labeling is of utmost
365 importance to both the honey producers and the customers seeking for quality and value.
366 Producers gain advantage by higher pricing and smoother trade. The potential bioactivities and
367 the specific taste and aroma of varietal honeys are sought-after attributes. Therefore, appropriate
368 screening methods and gained knowledge of the Finnish honey types are crucial. The boreal
369 climate, short summer season, and subsequently narrow window of flowering amongst plants
370 complicate the harvest of pure varietal honeys in Finland. Hence, the varietal honeys are likely to
371 contain nectar and pollen from several botanical origins, and thus cause ambiguity in product
372 classification when using sensory, physicochemical, or melissopalynological analysis. A categorical
373 classification according to the level of varietal purity is therefore proposed.

374 Here, ^1H NMR metabolomics allowed a clear discrimination of Finnish buckwheat, dandelion,
375 heather, and honeydew honeys. Compositional features that would allow NMR-based
376 classification were also characterized for clover, linden, and lingonberry honeys. Qualitative
377 profiling revealed specific fingerprints were for clover, dandelion, and heather honeys. The effect
378 of the natural variation in honey composition was minimized by using the OPLS-DA, which allowed
379 the clear separation of the three honey classes: buckwheat, dandelion, and heather. Yet, more
380 work is needed to profile some of the rarest and most valued Finnish honey types, such as
381 cloudberry (*Rubus chamaemorus*), bilberry (*Vaccinium myrtillus*), and willowherb (*Epilobium*
382 *angustifolium*) honey, while aiming towards a comprehensive national honey library.

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513 **Figure captions**

514 **Figure 1.** ^1H NMR spectra of the aqueous honey samples. (A) Superimposed spectra (δ 10.0–0.6
515 ppm). (B) Overlaid spectral fingerprint regions (δ 10.0–5.6 ppm and 2.9–0.6 ppm). From bottom to
516 top: buckwheat (samples 1, 2, 20; blue), dandelion (3–5, 18; yellow), honeydew (6, 7, 13; brown),
517 heather (10–12, 19; purple), clover (8; dark green), linden (9; green), Himalayan balsam (14; pink),
518 multifloral (15, 17; gray), and lingonberry (16; red) honeys.

519 **Figure 2.** Highlighted fingerprints for buckwheat (blue), dandelion (yellow), heather (purple),
520 honeydew (brown), linden (green), and lingonberry (red) honeys. Spectral regions (A) 1.50–0.75
521 ppm, (B) 2.94–1.50 ppm, (C) 4.70–4.10 ppm, (D) 5.50–4.93 ppm, and (E) 9.50–5.60 ppm.
522 Abbreviations: Ala, alanine; HMF, 5-hydroxymethylfurfural; Ile, isoleucine; Leu, leucine; Phe,
523 phenylalanine; Pro, proline; Tyr, tyrosine; Val, valine. Peak multiplicities: s, singlet; d, doublet; dd
524 doublet of doublets; m, multiplet; t, triplet.

525 **Figure 3.** ^1H NMR assignments (600 MHz, H_2O + 10% D_2O , pH 6.55, 298 K) for the dandelion honey
526 markers. (A) 2-Hydroxy-3-methylbutyric acid, (B) 2-hydroxy-3-methylpentanoic acid, (C) 3-methyl-
527 2-oxopentanoic acid, and (D) 4-methyl-2-oxopentanoic acid. (Structures: Chenomx NMR Suite 8.0
528 Profiler).

529 **Figure 4.** PCA model ($t[2]$ vs. $t[1]$). The $R^2X_{(\text{cum})}$ and $Q^2_{(\text{cum})}$ for the model were 0.99 and 0.96,
530 respectively. (A) Scores plot ($R^2X[1] = 0.50$ and $R^2X[2] = 0.33$). The legend indicates the honey type.
531 (B) Loadings plot.

532 **Figure 5.** OPLS-DA comparison of buckwheat, dandelion and heather honeys. (A) Score overview of
533 the of the three classes ($t[2]$ vs. $t[1]$); $R^2X_{(\text{cum})} = 0.94$, $R^2Y_{(\text{cum})} = 0.98$, $Q^2_{(\text{cum})} = 0.96$). (B) Buckwheat
534 and dandelion honey ($to[1]$ vs. $t[1]$); $R^2X_{(\text{cum})} = 0.91$, $R^2Y_{(\text{cum})} = 1.00$, $Q^2_{(\text{cum})} = 0.98$). (C) Dandelion

535 and heather honey ($to[1]$ vs. $t[1]$; $R^2X_{(cum)} = 0.93$, $R^2Y_{(cum)} = 0.98$, $Q^2_{(cum)} = 0.97$). (D) Buckwheat and
536 heather honey ($to[1]$ vs. $t[1]$; $R^2X_{(cum)} = 0.95$, $R^2Y_{(cum)} = 1.00$, $Q^2_{(cum)} = 0.99$). (E) Loadings
537 distribution between buckwheat and dandelion. (F) Loadings distribution between dandelion and
538 heather. (G) Loadings distribution between buckwheat and heather.

539 **Figure 6.** Validation tests of the OPLS-DA models in Fig. 5A–D, respectively, by permutation ($n =$
540 20) of corresponding PLS-DA models (R^2 , green triangle; Q^2 , blue square). Intercepts: (A) $R^2 = (0.0,$
541 $0.26)$, $Q^2 = (0.0, -0.66)$, (B) $R^2 = (0.0, 0.26)$, $Q^2 = (0.0, -0.66)$, (C) $R^2 = (0.0, 0.38)$, $Q^2 = (0.0, -0.47)$,
542 and (D) $R^2 = (0.0, 0.48)$, $Q^2 = (0.0, -0.48)$.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Table 1

Table 1. Honey samples. The exact geographical origin of the samples 18–20 is not known.

Sample no.	Botanical origin		Geographical origin	Harvest year
1	Buckwheat	<i>Fagopyrum esculentum</i>	60°N 23°E, Loimaa (southwestern Finland)	2012
2	Buckwheat	<i>Fagopyrum esculentum</i>	62°N 25°E, Jyväskylä (central Finland)	2013
3	Dandelion	<i>Taraxacum</i> spp.	60°N 21°E, Parainen (southwestern Finland)	2012
4	Dandelion	<i>Taraxacum</i> spp.	64°N 24°E, Ylivieska (central Finland)	2013
5	Dandelion	<i>Taraxacum</i> spp.	62°N 30°E, Ilomantsi (eastern Finland)	2013
6	Honeydew		62°N 29°E, Joensuu (eastern Finland)	2013
7	Honeydew		62°N 25°E, Jyväskylä (central Finland)	2013
8	Clover	<i>Trifolium repens/hybridum</i>	62°N 29°E, Joensuu (eastern Finland)	2012
9	Linden	<i>Tilia</i> spp.	60°N 24°E, Helsinki (southern Finland)	2012
10	Heather	<i>Calluna vulgaris</i>	60°N 25°E, Porvoo (southern Finland)	2013
11	Heather	<i>Calluna vulgaris</i>	61°N 21°E, Rauma (southwestern Finland)	2012
12	Heather	<i>Calluna vulgaris</i>	63°N 23°E, Kaustinen (central Finland)	2013
13	Honeydew		64°N 26°E, Vaala (central Finland)	2013
14	Himalayan balsam	<i>Impatiens glandulifera</i>	64°N 24°E, Ylivieska (central Finland)	2013
15	Multifloral		60°N 24°E, Hämeenlinna (southern Finland)	2013
16	Lingonberry	<i>Vaccinium</i> spp.	63°N 28°E, Rautavaara (eastern Finland)	2013
17	Multifloral		60°N 24°E, Turenki (southern Finland)	2013
18	Dandelion	<i>Taraxacum</i> spp.	not specified	2013
19	Heather	<i>Calluna vulgaris</i>	not specified	2013
20	Buckwheat	<i>Fagopyrum esculentum</i>	not specified	2013