



# Suitability of sugar, amino acid, and inorganic ion compositions to distinguish fir and spruce honey

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## Abstract

Honeydew honey is produced by bees from excretions of plant-feeding insects, such as aphids and scale insects. Honeydew on conifers, like fir (*Abies alba*) or spruce (*Picea abies*), is produced by different species of the genera *Cinara* and *Physokermes*. This means that honeydew honey can stem from different botanical as well as zoological origins, but so far it is not possible to clearly distinguish the different types of honeys. In the attempt to identify distinguishing markers, 19 sugars, 25 amino acids and 9 inorganic ions were quantified in three groups of honeydew honey (fir/*Cinara*, spruce/*Cinara* and spruce/*Physokermes*) with 20 honey samples each. It could be demonstrated that the contents of isomaltose, raffinose, erlose, two undefined oligosaccharides, several amino acids, sulfate, and phosphate differed significantly between the three groups of honey. Furthermore, multivariate analyses resulted in a separation of spruce/*Physokermes* honey from spruce- or fir/*Cinara* honey due to its higher contents of phosphate, sulfate, erlose and two undefined oligosaccharides. Moreover, the amino acid composition and the isomaltose as well as the raffinose contents proved useful in the distinction between fir/*Cinara* and spruce/*Cinara* honey. In sum, the contents of sugars, amino acids, and inorganic ions in German fir and spruce honeys provide useful information about the botanical and zoological origin of honeydew honeys.

**Keywords** Honeydew honey · Fir honey · Spruce honey · Sugars · Free amino acids · Inorganic ions

## Introduction

Honeydew honey is a natural sweet substance produced by *Apis mellifera* from secretions of living parts of plants or from excretions of plant-feeding insects [1]. Different insects of the order Hemiptera, such as aphids and scale insects, feed on phloem sap of their host trees and utilize the contained amino acids as nutrients [2]. They excrete sugars that are not used for nutrition in the form of honeydew. The composition of honeydew varies between different insect species and it is also influenced by the host plant species and different environmental conditions [3–5]. The genera *Cinara* (Lachnidae) or *Physokermes* (Coccidae) feeding on conifers like spruce, fir, or pine are known to produce large amounts of honeydew. There are also species of other hemipteran

feeding on deciduous trees, like oak, chestnut or lime trees that produce honeydew [6]. Honey bees (*Apis mellifera*) collect honeydew for nutrition and produce a special kind of honey, known as honeydew-, forest-, fir- or spruce-honey. Therefore, the components of honeydew honey originate from the plant (botanical origin), from the honeydew producing insect (zoological origin), or they are added by the honey bees in the process of honey-production (additional zoological origin).

Honey contains mainly sugars (70–80%, w/w) and water (10–20%, w/w), but also a large number of minor components, such as amino acids, organic acids, minerals, phenolic compounds, enzymes, or proteins [1]. The chemical composition and the biochemical and physiochemical parameters of honeydew honey differ from that of flower honey (based on nectar) in parameters such as electrical conductivity, pH, sugar profile, or mineral content [7, 8]. The European legislation [1] states that flower honey (with exceptions) must have electrical conductivity values  $\leq 0.8 \text{ mS cm}^{-1}$ , whereas honeydew honeys must have electrical conductivity values  $\geq 0.8 \text{ mS cm}^{-1}$ . The sugar profile is one of the main parameters for the quality control of honey, and it proofs that

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honeydew honey contains less monosaccharides and more di- and oligosaccharides than flower honey [9–12]. However, few studies on the carbohydrate compositions of different honeydew-honey types have been carried out so far [8]. The total amino acid content in honey is about 0.3–2.0 g kg<sup>-1</sup> with proline being the dominating one [13, 14]. The amino acid composition in honeys have been used to characterize their geographic or botanical origin, but without highly conclusive results [13, 14]. The total mineral content in flower honey is generally lower than in honeydew honey (about 0.1–0.2% and 1% respectively) due to the higher amounts of K and P [15]. The mineral content was also used to determine the honey's botanical origin [16–18].

Authenticity of honeys, including honeydew honey, has become a major subject of investigation at an international level [8, 12, 19]. Honeydew honey is labelled on the basis of sensory judgements and electrical conductivity values, but there is no internationally accepted quality criterion for different types of honeydew honeys [7, 8]. As for flower honey, pollen analysis (melissopalynology) is used to identify the botanical origin; this method, however, is not useful for honeydew honey [8]. There are several studies dealing with the characterization of honeydew honey in general, but so far, only a small number of studies attempted to identify the botanical origin of honeydew honeys [8, 18].

Because of its strong malty-aromatic taste, honeydew honey is very popular and achieves a high market value [8]. Some compounds have been suggested to be specific botanical markers for several different honeydew honeys, such as quercitol for oak honey, 1-chloro-octane and tridecane for pine honey or protocatechuic acid for fir honey [20–25]. However, it was shown that the content of protocatechuic acid was similar in the honeys of different conifers, and protocatechuic acid can therefore only be used to differentiate between flower and honeydew honey [26, 27]. Additionally, there are no specific markers to differentiate between honeydew honey from spruce and fir and, to the best of our knowledge, there are no studies that investigated the characterization of the botanical as well as zoological origin of these kinds of honeys.

Honey is frequently subjected to adulteration or to mislabeling of the origin. Therefore, it is necessary to determine reliable parameters to verify the botanical and zoological authentication of honeydew honey [8, 12]. In Germany, the main proportion of coniferous honeydew honey originates from spruce or fir [6]. The main objective of this study, therefore, was to investigate whether it is possible to differentiate between honeydew honey of fir (*Abies alba* Mill.) and spruce [*Picea abies* (L.) H. Kast.]. For that purpose, the contents of sugars, amino acids, and inorganic ions in honeydew honey in relation to the host tree species, either fir or spruce (botanical origin), as well as the hemipteran species which produced the honeydew, either *Cinara* or

*Physokermes* (zoological origin), were analyzed. The honeydew honeys' contents of sugar, amino acid, and inorganic ion were conflated with multivariate analyses.

## Materials and methods

### Sample collection

Honeydew honeys of silver fir (*Abies alba* Mill.) and spruce [*Picea abies* (L.) H. Kast.] were obtained from selected beekeepers by the Apicultural State Institute, University of Hohenheim (Stuttgart, Germany). All honeys were collected from several parts of Baden-Württemberg (Germany) between 2017 and 2019. The samples were marked by beekeepers and classified by the botanical and zoological origin, indicated by the location where the beehives were positioned. The honeydew producers were *Cinara* spec. on fir and *Cinara* spec. or *Physokermes* spec. on spruce, which has been verified by the Apicultural State Institute, University of Hohenheim (Stuttgart, Germany).

Furthermore, human sensorial analyses of the aromas were carried out by the Apicultural State Institute, University of Hohenheim (Stuttgart, Germany) to confirm the honeydew nature of all honey samples. For fir/*Cinara* honeys, there were warm and caramelized aromas, reminding of dates, toffee, fresh butter, and vanilla with a subtle note of lemon; for spruce/*Cinara* honeys, there were spicy, resinous and a little balsamic aroma; and for fir/*Physokermes* honeys, the aromas were woody like leafy wood dust.

Twenty samples of each honey type (fir/*Cinara*, spruce/*Cinara*, and spruce/*Physokermes*) were analyzed. Samples were stored at about 18–20 °C and dry and dark conditions. All honeydew honey samples had electrical conductivity values  $\geq 0.8$  mS cm<sup>-1</sup>. The average water content was  $16.3 \pm 0.8\%$  for fir/*Cinara*,  $16.0 \pm 1.6\%$  for spruce/*Cinara*, and  $15.7 \pm 1.8\%$  for spruce/*Physokermes* honeydew honey; no significant differences between the three honey groups were found.

### Honey preparation for analyses

1 g of honey was dissolved in 40 mL ultrapure water. The solution was centrifuged for 10 min at 5,000 rpm to separate pollen and other material. The solutions were stored at –80 °C until analyses. All honey samples were analyzed in the year of collection.

### Analyses of sugars

High performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was used for the analysis of mono-, di-, and oligosaccharides in honey

according to Shaaban et al. [5]. Honey samples were diluted with ultrapure water so that they provide signals within the linear range of the detector response, for each sample two different dilutions were analyzed (1:1000 for glucose and fructose, and 1:40 for di- and oligosaccharides). Sugars were separated with an anion-exchange column (Carbopac™ PA10 4 mm × 250 mm; Dionex corp, Sunnyvale, CA, US), eluted isocratically with 80 mM sodium hydroxide, and detected with a pulse amperometric detector (ESA Model 5200, Coulochem II, Bedford, MA, US). Pulse setting was at 50, 700, and – 800 mV for 500, 540, and 540 ms, respectively. Standards for glucose, fructose, sucrose, trehalose, turanose, kojibiose, gentiobiose, maltose, isomaltose, maltulose, isomaltulose, melezitose, erlose, raffinose, 1-kestose, isomaltotriose, maltotriose, nigrose, stachyose were measured in parallel (0–250 µmol/L) and for each sugar, a calibration curve was created. Sugars were quantified by comparing their chromatographic peak area to that of the standard calibration curve. Furthermore, the obtained results were checked regularly with the standard addition method. Long-chain oligosaccharides [degree of polymerization (DP) ≥ 5] were analyzed with the same system, with the difference that the column was eluted isocratically with 200 mM NaOH instead of 80 mM NaOH.

### Limit of detection and limit of quantification of the identified sugars

The calibration curves were prepared in triplicates ( $n=3$ ) at five different dilutions (0–250 µmol/L). The determination coefficients for the calibration curves of the different sugars were each  $R^2 > 0.99$ . Limit of detection (LOD) and limit of quantification (LOQ) values were determined using regression parameters from the calibration curve ( $3.3 S_{y/x}/a$  and  $10 S_{y/x}/a$ , respectively; where  $S_{y/x}$  is the standard deviation of the residues and  $a$  is the slope) [19]. With the HPAEC-PAD system described above, the aforementioned LOD was the best for glucose (0.6 µmol/L) and the worst for erlose (1.0 µmol/L) and the corresponding LOQ were 1.8 µmol/L (glucose) and 3.0 µmol/L (erlose). The values for the other sugars were distributed between these two limits.

### Analyses of amino acids

The analysis of free amino acids by HPLC was performed according to Göttinger et al. [28]. Amino acids with a primary amino group were processed by precolumn derivatization with *o*-phtaldialdehyde, amino acids with a secondary amino group (e.g., proline) with fluorenylmethoxycarbonyl. The separation of amino acids was performed on a reversed-phase column (LiChroCart 125–4, Superphere 100 RP-18 endcapped; Merck, Darmstadt, Germany) with an acetonitrile gradient in 18 mM potassium phosphate, pH

7.1. The derivatives were detected by fluorescence using wavelengths of excitation and emission at 330 and 405 nm (*o*-phtaldialdehyde) or 265 and 305 nm (fluorenylmethoxycarbonyl). Amino acid standards (Sigma-Aldrich, Germany) were measured in parallel (0–20 µM) and for each amino acid a calibration curve was created.

### Limit of detection and limit of quantification of the identified amino acids

The calibration curves were prepared in triplicates ( $n=3$ ) at four different dilutions (0–20 µmol/L). The determination coefficients for the calibration curves of the different amino acids were between  $R^2 = 0.99–0.93$ . Limit of detection (LOD) and limit of quantification (LOQ) values were determined using regression parameters from the calibration curve ( $3.3 S_{y/x}/a$  and  $10 S_{y/x}/a$ , respectively; where  $S_{y/x}$  is the standard deviation of the residues and  $a$  is the slope) [19]. With the HPLC system described above, the aforementioned LOD was the best for glutamate (0.17 µmol/L) and the worst for tryptophane (0.30 µmol/L) and the corresponding LOQ were 0.51 µmol/L (glutamate) and 0.90 µmol/L (tryptophane). The values for the other amino acids were distributed between these two limits.

### Analyses of inorganic ions

Anions ( $\text{Cl}^-$ ,  $\text{PO}_4^{3-}$ ,  $\text{SO}_4^{2-}$ ,  $\text{NO}_3^-$ ) and cations ( $\text{K}^+$ ,  $\text{Na}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{NH}_4^+$ ) were analyzed separately by ion chromatography connected with a conductivity detector (DX 500, Dionex, Idstein, Germany) according to Göttinger et al. [28]. Anions were separated using an IonPac anion exchange column (AS4, 4 × 200 mm, Dionex Corp, Sunnyvale, CA, USA) and the ions were eluted with 1.8 mM  $\text{Na}_2\text{CO}_3$  and 1.7 mM  $\text{NaHCO}_3$ . Cations were separated using an IonPac cation exchange column (CS12A, 4 × 200 mm, Dionex Corp, Sunnyvale CA, USA) and the ions were eluted with 15 mM  $\text{H}_2\text{SO}_4$ . Inorganic ion standards (Sigma-Aldrich, Germany) were measured in parallel (0–750 µM) and for each anion or cation a calibration curve was created.

### Statistical analyses

Sugar, amino acid, or ion contents in honeydew honey are shown as means ( $\pm$  SD). The means of each of the sugars, amino acids, or ions in the three groups of honey (fir/*Cinara*, spruce/*Cinara*, and spruce/*Physokermes*) were compared separately to check for significant differences. Skewness and kurtosis were calculated to capture the distribution of the dataset; normal distribution was assumed if skewness values were less than two and kurtosis values were less than seven [29]. Moreover, Levene's tests were applied to test for homogeneity of variances for the data of each metabolite

or ion. When data conformed to the normality assumption but failed on homogeneity of variances, analysis of variance was performed using the Welch's test followed by post-hoc test (Games-Howell test). For the cases where both normality and homogeneity assumptions were confirmed, one-way ANOVA was performed. Subsequently, post-hoc tests (Tukey's HSD) were carried out ( $p$  value < 0.05).

In order to investigate whether the differences in sugar, amino acid, and ion contents could be explained by the zoological and/or the botanical origin of the honeydew honey samples, a Powered Partial Least Squares-Discriminant Analysis (PPLS-DE) was performed. All values for sugars, amino acids, and ions were taken for the PPLS-DE and values were then autoscaled. For PPLS-DA, the performance of the model must be assessed before any interpretation of score plots is possible. Therefore, the number of misclassifications (or error rate), i.e., the number of samples that do not belong to the group predicted by the model, was calculated (outer loop: sevenfold CV, inner loop: sixfold CV). Then, a permutation test based on the classification error rate was used to determine the statistical significance of the differences between groups. Lastly, pairwise comparisons of the zoological origins were performed. PPLS-DE analysis was performed using the 'pls' and 'RVAideMmoire' packages with the *cppls*, *MVA.cmv*, *MVA.test*, *pairwise.factorfit* and *MVA.plot* routines [30–35].

All statistical analyses were performed using R (version 3.5.1, [www.r-project.org](http://www.r-project.org)) and SPSS (version 24.0, IBM, Cooperation).

## Results

### Sugars in honeydew honey

Honeydew honey samples were analyzed for sugars, amino acids and ions. The monosaccharides fructose and glucose were the dominant sugars in all samples (about 60 g/100 g honey), and the fructose content was higher than that of glucose in both fir and spruce honey (Table 1). No significant differences between the three groups of honey were found. Different disaccharides were detected in all honeydew honey samples, with trehalose and isomaltose showing the highest contents and the isomaltose content was significantly higher in fir honey than in both types of spruce honey. It must be stated, however, that the isomaltose peak was not separated from the maltulose peak. Melezitose and erlose were the dominant trisaccharides. Melezitose was the most abundant trisaccharide in fir/- and spruce/*Cinara* honey, whereas the erlose content was significantly higher in spruce/*Physokermes* honey compared to the other honey samples. The raffinose content was significantly higher in fir/*Cinara* honey than in

**Table 1** Sugar contents in three groups of honeydew honey (*Abies alba/Cinara* spec., *Picea abies/Cinara* spec., and *Picea abies/Physokermes* spec.)

Sugars [g/100 g]	<i>Abies alba</i>	<i>Picea abies</i>	
	<i>Cinara</i> sp.	<i>Cinara</i> sp.	<i>Physokermes</i> sp.
Fructose (fru)	30.6 ± 3.1 <sup>a</sup>	31.0 ± 2.9 <sup>a</sup>	29.7 ± 2.7 <sup>a</sup>
Glucose (glu)	28.5 ± 2.2 <sup>a</sup>	27.7 ± 3.0 <sup>a</sup>	27.4 ± 3.2 <sup>a</sup>
Trehalose (tre)	1.9 ± 0.7 <sup>a</sup>	1.3 ± 0.6 <sup>a</sup>	1.8 ± 0.7 <sup>a</sup>
Isomaltose* (iso)	<b>2.4 ± 1.1<sup>a</sup></b>	<b>1.5 ± 0.7<sup>b</sup></b>	<b>1.5 ± 0.4<sup>b</sup></b>
Maltose (mal)	1.1 ± 0.5 <sup>a</sup>	1.0 ± 0.7 <sup>a</sup>	1.3 ± 0.8 <sup>a</sup>
Kojibiose (koj)	1.3 ± 0.5 <sup>a</sup>	1.3 ± 0.8 <sup>a</sup>	1.4 ± 0.7 <sup>a</sup>
Turanose** (tur)	1.5 ± 0.7 <sup>a</sup>	1.4 ± 0.8 <sup>a</sup>	1.1 ± 1.1 <sup>a</sup>
Sucrose (suc)	0.9 ± 0.5 <sup>a</sup>	0.8 ± 0.5 <sup>a</sup>	1.0 ± 0.6 <sup>a</sup>
Melezitose (mel)	3.0 ± 1.6 <sup>a</sup>	4.1 ± 3.7 <sup>a</sup>	2.6 ± 1.8 <sup>a</sup>
Erlose (erl)	<b>1.5 ± 1.5<sup>a</sup></b>	<b>1.1 ± 0.9<sup>a</sup></b>	<b>3.2 ± 2.7<sup>b</sup></b>
Raffinose (raf)	<b>1.9 ± 1.1<sup>a</sup></b>	<b>0.8 ± 0.7<sup>b</sup></b>	<b>1.1 ± 0.8<sup>b</sup></b>
1-Kestose*** (kes)	0.3 ± 0.5 <sup>a</sup>	0.7 ± 0.5 <sup>a</sup>	1.1 ± 1.2 <sup>a</sup>
Undef 1	0.1 ± 0.1 <sup>a</sup>	0.1 ± 0.1 <sup>a</sup>	0.1 ± 0.1 <sup>a</sup>
Undef 2	0.2 ± 0.1 <sup>a</sup>	0.1 ± 0.1 <sup>a</sup>	0.2 ± 0.1 <sup>a</sup>
Undef 3	0.3 ± 0.3 <sup>a</sup>	0.4 ± 0.7 <sup>a</sup>	0.2 ± 0.3 <sup>a</sup>
Undef 4	<b>0.9 ± 0.6<sup>a</sup></b>	<b>0.7 ± 0.5<sup>a</sup></b>	<b>1.5 ± 0.8<sup>b</sup></b>
Undef 5	0.1 ± 0.1 <sup>a</sup>	0.1 ± 0.1 <sup>a</sup>	0.1 ± 0.1 <sup>a</sup>
Undef 6	<b>0.7 ± 0.5<sup>a</sup></b>	<b>0.7 ± 0.7<sup>a</sup></b>	<b>2.1 ± 1.0<sup>b</sup></b>
Undef 7	0.1 ± 0.2 <sup>a</sup>	0.1 ± 0.2 <sup>a</sup>	0.1 ± 0.2 <sup>a</sup>

All values are means of  $n = 20$  independent measurements ± SD

Different letters represent significant differences between the sugars in the tree groups of honeydew honey. Significantly different values were also marked in bold

\*Peak of isomaltose was not completely separated from maltulose peak

\*\*Peak of turanose was not completely separated from isomaltulose and gentiobiose peak

\*\*\*Peak of kestose was not completely separated from nigerose and stachyose peak

Undef 1 and 2 = undefined monosaccharides

Undef 3, 4, 5, 6, and 7 = undefined oligosaccharides

spruce/*Cinara* or spruce/*Physokermes* honey. Several further sugars were detected in the honey samples, named as *undefined* 1–7. Undefined sugar 4 and 6 are probably oligosaccharides and their contents were significantly higher in spruce/*Physokermes* honey than in fir/- or spruce/*Cinara* honey.

### Amino acids in honeydew honey

The total contents of all free amino acids were low in all honey samples (less than 0.1 g/100 g honey; Table 2). Proline accounted for up to 90% of the amino acids and with that it was the dominant amino acid in all samples. Its content was significantly higher in *Cinara* honey than in *Physokermes* honey. All other proteinogenic as well as

**Table 2** Amino acid contents in three groups of honeydew honey (*Abies alba*/*Cinara* spec., *Picea abies*/*Cinara* spec., and *Picea abies*/*Physokermes* spec.)

Amino acids [mg/100 g]	<i>Abies alba</i>	<i>Picea abies</i>	
	<i>Cinara</i> sp.	<i>Cinara</i> sp.	<i>Physokermes</i> sp.
Proline (PRO)	<b>54.3 ± 17.5<sup>a,b</sup></b>	<b>61.7 ± 33.0<sup>b</sup></b>	<b>40.9 ± 19.6<sup>a</sup></b>
Glutamate (GLU)	<b>4.6 ± 4.5<sup>a</sup></b>	<b>8.4 ± 10.3<sup>b</sup></b>	<b>2.4 ± 2.6<sup>a</sup></b>
Glutamine (GLN)	<b>2.6 ± 1.7<sup>a</sup></b>	<b>6.4 ± 4.9<sup>b</sup></b>	<b>1.9 ± 2.1<sup>a</sup></b>
Aspartate (ASP)	<b>1.7 ± 1.5<sup>a</sup></b>	<b>4.5 ± 4.6<sup>b</sup></b>	<b>1.6 ± 2.5<sup>a</sup></b>
Asparagine (ASN)	<b>0.7 ± 0.6<sup>a</sup></b>	<b>2.3 ± 2.7<sup>b</sup></b>	<b>0.8 ± 2.3<sup>a</sup></b>
Glycine (GLY)	0.3 ± 0.1 <sup>a</sup>	0.5 ± 0.3 <sup>a</sup>	0.2 ± 0.1 <sup>a</sup>
Serine (SER)	0.5 ± 0.3 <sup>a</sup>	1.0 ± 0.9 <sup>a</sup>	0.6 ± 0.4 <sup>a</sup>
Threonine (THR)	<b>0.2 ± 0.1<sup>a</sup></b>	<b>1.0 ± 1.0<sup>b</sup></b>	<b>0.3 ± 0.3<sup>a</sup></b>
Alanine (ALA)	<b>0.9 ± 0.4<sup>a</sup></b>	<b>2.1 ± 1.4<sup>b</sup></b>	<b>1.0 ± 1.0<sup>a</sup></b>
Valine (VAL)	<b>0.5 ± 0.4<sup>a</sup></b>	<b>1.7 ± 1.4<sup>b</sup></b>	<b>0.6 ± 0.4<sup>a</sup></b>
Isoleucine (ILE)	<b>0.3 ± 0.3<sup>a</sup></b>	<b>1.6 ± 1.9<sup>b</sup></b>	<b>0.6 ± 0.7<sup>a</sup></b>
Leucine (LEU)	0.1 ± 0.2 <sup>a</sup>	0.5 ± 0.3 <sup>a</sup>	0.3 ± 0.3 <sup>a</sup>
Phenylalanine (PHE)	<b>0.4 ± 0.2<sup>a</sup></b>	<b>2.4 ± 1.6<sup>b</sup></b>	<b>0.8 ± 0.7<sup>a</sup></b>
Tyrosine (TYR)	<b>0.6 ± 0.4<sup>a</sup></b>	<b>2.0 ± 1.4<sup>b</sup></b>	<b>1.0 ± 0.6<sup>a</sup></b>
Tryptophan (TRP)	0.1 ± 0.1 <sup>a</sup>	0.1 ± 0.3 <sup>a</sup>	0.1 ± 0.1 <sup>a</sup>
Arginine (ARG)	<b>0.1 ± 0.1<sup>a</sup></b>	<b>0.8 ± 0.5<sup>b</sup></b>	<b>0.2 ± 0.2<sup>a</sup></b>
Lysine (LYS)	<b>0.1 ± 0.1<sup>a</sup></b>	<b>1.1 ± 0.8<sup>b</sup></b>	<b>0.3 ± 0.2<sup>a</sup></b>
Histidine (HIS)	0.1 ± 0.3 <sup>a</sup>	0.3 ± 0.5 <sup>a</sup>	0.3 ± 0.5 <sup>a</sup>
Methionine (MET)	0.1 ± 0.1 <sup>a</sup>	0.1 ± 0.1 <sup>a</sup>	0.1 ± 0.1 <sup>a</sup>
β-Alanine (β-ALA)	<b>0.5 ± 0.2<sup>a</sup></b>	<b>1.2 ± 0.6<sup>b</sup></b>	<b>0.5 ± 0.2<sup>a</sup></b>
γ-Amino-butyric acid (GABA)	0.1 ± 0.1 <sup>a</sup>	0.6 ± 0.3 <sup>a</sup>	0.3 ± 0.4 <sup>a</sup>
Non-proteinogenic amino acids (NP)	0.4 ± 0.4 <sup>a</sup>	1.2 ± 0.9 <sup>a</sup>	0.4 ± 0.3 <sup>a</sup>

All values are means of  $n=20$  independent measurements  $\pm$  SD

Different letters represent significant differences between the sugars in the tree groups of honeydew honey. Significantly different values were also marked in bold

Further non-proteinogenic amino acids: homoserine, phosphoserine, ornithine, and taurine

further non-proteinogenic amino acids were also found in the honey samples, but their contents were low. Nevertheless, the contents of glutamate, glutamine, aspartate, asparagine, threonine, alanine, valine, isoleucine, phenylalanine, tyrosine, arginine, lysine, and β-alanine differed significantly between the three groups of honey. The contents of these amino acids were higher in spruce/*Cinara* honey than in fir/*Cinara* and spruce/*Physokermes* honey.

### Inorganic ions in honeydew honey

Honeydew honey samples contained several inorganic ions, and the total content of inorganic ions was about 0.5 g/100 g honey (Table 3). Potassium (K<sup>+</sup>) was the main cation, and phosphate (PO<sub>4</sub><sup>3-</sup>) was the most abundant anion. Phosphate and sulfate differed significantly between the three groups of honey. The phosphate content was about twofold higher in spruce/*Physokermes* honey than in *Cinara* honey.

**Table 3** Inorganic anion and cation contents in three groups of honeydew honey (*Abies alba*/*Cinara* spec., *Picea abies*/*Cinara* spec., and *Picea abies*/*Physokermes* spec.)

Ion [mg/100 g]	<i>Abies alba</i>	<i>Picea abies</i>	
	<i>Cinara</i> sp.	<i>Cinara</i> sp.	<i>Physokermes</i> sp.
<b>Cations</b>			
Potassium (K <sup>+</sup> )	219 ± 51 <sup>a</sup>	187 ± 62 <sup>a</sup>	234 ± 82 <sup>a</sup>
Sodium (Na <sup>+</sup> )	11 ± 15 <sup>a</sup>	10 ± 13 <sup>a</sup>	10 ± 12 <sup>a</sup>
Ammonium (NH <sub>4</sub> <sup>+</sup> )	5 ± 5 <sup>a</sup>	4 ± 4 <sup>a</sup>	4 ± 4 <sup>a</sup>
Magnesium (Mg <sup>2+</sup> )	13 ± 11 <sup>a</sup>	14 ± 8 <sup>a</sup>	19 ± 13 <sup>a</sup>
Calcium (Ca <sup>2+</sup> )	14 ± 24 <sup>a</sup>	12 ± 13 <sup>a</sup>	14 ± 18 <sup>a</sup>
<b>Anions</b>			
Chloride (Cl <sup>-</sup> )	32 ± 14 <sup>a</sup>	36 ± 15 <sup>a</sup>	40 ± 34 <sup>a</sup>
Phosphate (PO <sub>4</sub> <sup>3-</sup> )	<b>104 ± 44<sup>a</sup></b>	<b>104 ± 31<sup>a</sup></b>	<b>212 ± 42<sup>b</sup></b>
Sulfate (SO <sub>4</sub> <sup>2-</sup> )	<b>25 ± 8<sup>a,b</sup></b>	<b>20 ± 6<sup>a</sup></b>	<b>31 ± 16<sup>b</sup></b>
Nitrate (NO <sub>3</sub> <sup>-</sup> )	2 ± 2 <sup>a</sup>	1 ± 2 <sup>a</sup>	1 ± 3 <sup>a</sup>

All values are means of  $n=20$  independent measurements  $\pm$  SD

Different letters represent significant differences between the inorganic ion content in the tree groups of honeydew honey. Significantly different values were also marked in bold

## Honey composition in relation to the zoological and botanical origin

In order to investigate whether the differences in sugar, amino acid, and ion contents could be explained by the zoological and/or botanical origin of the honeydew honey samples, a Powered Partial Least Squares-Discriminant Analysis (PPLS-DA) was performed. All sugar, amino acid, and inorganic ion values were used and autoscaled.

For PPLS-DA, the performance of the model must be assessed before any interpretation of score plots is possible. Therefore, the number of misclassifications (or error rate), i.e., the number of samples that do not belong to the group predicted by the model, was calculated. The classification error rate was about 0.259 using cross model validation (outer loop: sevenfold cross validation, inner loop: sixfold cross validation). Then, a permutation test based on the classification error rate was used to determine the statistical significance of the differences between groups. Because the  $p$  value was 0.001, the interpretation of the score plots can follow (Fig. 1). It was possible to visually separate three groups of honey (fir/*Cinara*, spruce/*Cinara*, and spruce/*Physokermes*; Fig. 1a), with erlose, undefined sugar 6, sulfate, and phosphate being responsible for the separation of spruce/*Physokermes* honey samples (Fig. 1b). Additionally, raffinose and isomaltose were the differentiating variables for the separation of fir/*Cinara* honey from the other two, and tyrosine, arginine, lysine, isoleucine, and phenylalanine caused the separation of spruce/*Cinara* from other honeys (Fig. 1b). Lastly, pairwise comparisons showed that within the zoological origins, differs significantly from the other (Table 4).

An additional cross model validation was carried out for the same PPLS-DE according to the botanical origin, fir *versus* spruce. The classification error rate (CER) was 0.14583 and  $p$  value = 0.001\*\* for 999 permutations. This indicates that fir and spruce honey can be separated

**Table 4**  $p$  values of pairwise comparisons for each pair of zoological origin

	Fir/ <i>Cinara</i>	Spruce/ <i>Cinara</i>
Spruce/ <i>Cinara</i>	0.001	–
Spruce/ <i>Physokermes</i>	0.001	0.001

according to the sugar, amino acid, and inorganic ion content.

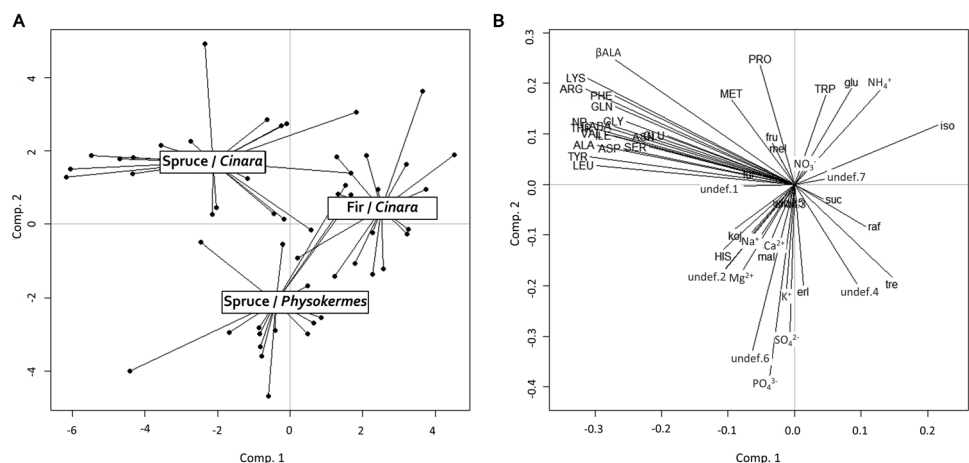
## Discussion

Honeydew honey belongs to the main unifloral honey types produced in European countries and, because of its strong malty-aromatic taste, it is very popular [36]. In Germany, fir and spruce honey are the most common honeydew honeys [6], but until now it is hardly possible to distinguish between both types of honeydew honey [8]. Furthermore, honeydew honey compared with floral honey can not only have different botanical origins but also different zoological origins (honeydew producer). Therefore, we focused on three groups of honeydew honey (fir/*Cinara*, spruce/*Cinara*, and spruce/*Physokermes*) and analyzed the sugar, amino acid, and inorganic ion contents in order to examine whether these compounds are useful to distinguish between the different types of honeydew honey.

## Differences in the sugar composition

Fructose and glucose make up about 60% (w/w) of fir and spruce honey and with that they are the dominant compounds (Table 1). This content is similar to values of other fir honeys from France (measured with HPAEC-PAD), Spain (measured with GC-MS), or Germany (measured by  $^1\text{H-NMR}$ ), but it is lower than that of Greek pine and

**Fig. 1** Powered Partial Least Squares-Discriminant Analysis (PPLS-DA). **a** score plot of the sugar, amino acid, and inorganic ion content observed in three groups of honeydew honey (Fir/*Cinara*, Spruce/*Cinara*, Spruce/*Physokermes*). **b** Loading plot of sugar, amino acid, and inorganic ion content. CER (classification error rate) = 0.259.  $p$  value = 0.001, number of permutations = 999



fir honeys (measured  $^1\text{H-NMR}$ ) [19, 37–39]. The monosaccharides in honeydew honey can stem from different sources; one part can stem directly from the monosaccharides in the honeydew collected by the bees [5]. The bees themselves can also contribute to the monosaccharides in honeydew honey by cleaving the honeydew's di- or oligosaccharides, probably by the activity of  $\alpha$ -glucosidases [40]. The trehalose content in the honeydew honey of fir and spruce is about 2% (w/w), which is higher than that of several flower honeys [8, 37]. Trehalose was also found in the honeydew of hemipteran species feeding on fir or spruce [5]. In insects, trehalose serves as a haemolymph-sugar and it is the major carbohydrate energy source used by insects to facilitate flight [41].

Fir and spruce honeys contained up to 10% (w/w) of oligosaccharides, mainly melezitose and erlose (Table 1). In general, honeydew honey contains more di- and oligosaccharides than flower honey [8] and the trisaccharide melezitose was reported to be a marker for honeydew honey [42]. There does not seem to be evidence of melezitose formation in honey bees, but the sugar was found in the honeydew of different hemipteran species [4, 5]. Erlose was also found in the honeydew of different hemipteran species [5] and a smaller proportion of erlose could also be produced by the action of honey bee invertase on sucrose [9]. As the phloem sap of most plant species, including fir or spruce, contains only sucrose [5], melezitose and erlose must be produced by the phloem feeding insects or honey bees and not by the plants.

The concentrations of five di- and oligosaccharides differed significantly between the three groups of honeydew honey (Table 1). The content of erlose, *undef4*, and *undef6* was about twofold–threefold higher in spruce/*Physokermes* honey than in spruce/- or fir/*Cinara* honey. Von der Ohe and von der Ohe [43] reported the detection of particular oligosaccharides (named L1 and L2) in *Physokermes* honey; the structures of these sugars, however, have not yet been resolved. It can be assumed that *undef4* and *undef6* as well as L1 and L2 are the same oligosaccharides, further analyses are necessary for a final clarification. The sugar composition in the honeydew honey samples also allow for the distinction between fir and spruce honey (Table 1). The content of isomaltose and raffinose was higher in fir/*Cinara* honey than in spruce/*Cinara* or spruce/*Physokermes* honey. Similar contents of raffinose were also found in French and Greek fir honeys [19, 37]. Moreover, the raffinose content was higher in Greek fir honeys compared to pine honeys [19]. Because the sugar composition of honeys is also influenced by the geographic origin and the high biological variability within different honey samples [19], a number of studies is needed to characterize honeydew honeys of different botanical origins only on the basis of their carbohydrate compositions [8].

## Differences in the amino acid composition

Although numerous amino acids were found in fir and spruce honeys, the total amino acid content was less than 0.1% (w/w). The amino acids in honeydew honey can stem from different sources; for example, the honeydew alone already contains small amounts of amino acids [2, 5]. However, the sugar-to-amino acid ratio is higher in honeydew than in nectar, which means that a honeydew diet for honey bees is less rich in nitrogen than a nectar diet [44, 45]. The bees themselves can also contribute to the amino acids in honey, particularly to the amount of proline, and ripe honey should contain at least 180 mg proline  $\text{kg}^{-1}$  honey [46].

The contents of several amino acids (proline, glutamate, glutamine, aspartate, asparagine, threonine, alanine, valine, isoleucine, phenylalanine, tyrosine, arginine, lysine,  $\beta$ -alanine) were significantly higher in spruce/*Cinara* honey than in fir/*Cinara* or in spruce/*Physokermes* honey. Proline and phenylalanine contents in honey have already been shown in previous studies to be suitable to characterize the botanical origin of flower honeys or to differentiate between flower honeys and honeydew honey [13]. It was possible to identify samples of lavender honey on the basis of high phenylalanine contents [13]. However, similarly high contents of phenylalanine were also detected in honeys of other Lamiaceae, like *Rosmarinus* or *Thymus* [47]. One reason for this could be the high content of phenylalanine in the nectar of some Lamiaceae species [48], probably because phenylalanine has a phagostimulatory effect on potential pollinators, like honey bees [49]. The proline content in honey also depends on the botanical origin [13] but the variation of the proline content in different unifloral honeys is quite high and it seems not to be possible to classify unifloral honey on this basis [13, 50]. The ripeness of honey also has an influence on the proline content [46]. A high degree of variability in the phenylalanine and proline content was shown for Greek fir honeys from different geographical regions [19]. Moreover, the amino acid content in honey decreases with storage time and the decrease-rate is not identical for the individual amino acids [51]. Therefore, the differentiation between different honeydew honeys solely on the basis of the amino acid composition appears to be insufficient [8].

## Differences in the inorganic ion composition

Honeydew honey contains more inorganic ions than flower honey [15] and this can be a reason for the higher electrical conductivity of honeydew honey [52]. Potassium is the main inorganic ion in all honey samples (Table 3) and it is also the dominant ion in the honeydew of hemipteran species feeding on fir or spruce as well as in the phloem exudates of these tree species [5]. Yet, not only potassium, but the complete inorganic ion composition (with the exception of phosphate)

in honey roughly reflects the composition in honeydew or phloem exudates of fir and spruce. The proportion of phosphate was higher in all honey samples than it is in honeydew or phloem exudates of fir and spruce (Table 3) [5]. As inorganic ions are taken up from the soil by the plant's roots, their content and composition in honeys are indirectly dependent on the soil composition and the geographic area [15, 18, 52]. However, the honeydew honeys used in this study were all from the same state (Baden-Württemberg) in Germany to minimize variation due to different geographic areas. Differences in the inorganic ion contents in honeys are also used to identify their botanical origin, but the major differences were found between flower and honeydew honey [15, 18, 52, 53].

As for the inorganic ions, only sulfate and phosphate differed significantly between the three groups of honeydew honey (Table 3). The content of both ions is about 1.5—twofold higher in spruce/*Physokermes* honey than in spruce/*Cinara* or fir/*Cinara* honey. This corresponds to the results on the ion composition in honeydew, as the proportion of phosphate was particularly high in the honeydew of *Physokermes* species located on spruce compared to *Cinara* species located on spruce or fir [5]. Therefore, the inorganic ion composition seems to be more useful for the determination of the honeys' zoological origin than its botanical origin.

### Suitability of the sugar, amino acid, and inorganic ion composition for the distinction between different honeydew honeys

A significant variation of the sugar, amino acid, and ion contents for the three groups of honey (fir/*Cinara*, spruce/*Cinara*, spruce/*Physokermes*) was visible in the PPLS-DA (Fig. 1). Moreover, permutation tests based on the cross-validation model were performed and  $p$  values  $< 0.05$  support the visual separation of the honey groups. In addition, pairwise comparisons showed that within the zoological origins, each differs significantly from the other (Table 4). Fir honey can be separated from spruce honey mainly due to its higher contents of raffinose and isomaltose. Spruce/*Physokermes* honey can be separated from spruce/*Cinara* or fir/*Cinara* honey mainly due to its higher contents of sulfate, phosphate, erlose, and two undefined oligosaccharides (*undef4* and *undef6*), and spruce/*Cinara* honey can be separated from fir/*Cinara* or spruce/*Physokermes* honey mainly due to its higher contents of several amino acids.

In sum, the three groups of honeydew honey could be separated by their sugar, amino acid, and ion composition and not by single chemical markers. Moreover, it should be noted that further factors may influence the honey's chemical composition, such as geographical origin, collection

season, weather conditions, conditions of honey harvest and storage [8, 54].

### Conclusion

Honeydew honey belongs to the main unifloral honey types produced in European countries and, because of its strong malty-aromatic taste, it is very popular. Honeydew honey can stem from different botanical as well as zoological origins, but so far it is not possible to clearly distinguish the different types of honeys. Therefore, a metabolomics approach associated with multivariate analysis was applied to authenticate the most common honeydew honeys in Germany (fir/*Cinara*., spruce/*Cinara*, and spruce/*Physokermes*). In sum, the three groups of honeydew honey could be separated by their sugar, amino acid, and ion composition. Therefore, the metabolite compositions provide useful information about the botanical and zoological origin of honeydew honeys. This contributes to a more accurate labeling of honeydew honeys.

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### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Compliance with ethics requirements** The article does not contain any studies with human participants or animal subjects.

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