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Evaluation of the Botanical Origin of Estonian Uni- and Polyfloral Honeys by Amino Acid Content

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The free amino acid content of 61 honey samples from Estonia has been determined by HPLC-UV with precolumn derivatization with diethyl ethoxymethylenemalonate. Analyzed samples were seven types of unifloral honeys and polyfloral honeys. The main amino acids found in Estonian honeys were proline and phenylalanine. The resulting data have been analyzed by *t* test and principal component analysis (PCA). *t* Test revealed that some amino acids (α-alanine, β-alanine, asparagine, γ-aminobutyric acid, glutamine, glycine, histidine, ornithine, phenylalanine, proline, serine, and tryptophan) are more potent for assigning honey botanical origin than others. PCA enabled differentiation of some honey types by their botanical origin. In the space of the two first principal components, heather honeys form a cluster that is clearly separable from, for example, polyfloral honeys. It is concluded that analysis of the free amino acid profile may serve as a useful tool to assess the botanical origin of Estonian honeys.

KEYWORDS: Honey authenticity; free amino acid analysis; diethyl ethoxymethylenemalonate derivatization; HPLC

INTRODUCTION

The honey market is a large part of the economy for many countries, but honeys of various geographical and botanical origins are differently valued. Due to this difference, cheaper honeys are more commonly labeled as more expensive types of honeys. The traditional method that allows verification of honey botanical and geographical origin is melissopalynology. However, this kind of pollen analysis is not conclusive and is extremely tedious and time-consuming and requires trained analysts (*1*). Although European Union food laws establish composition and quality parameters for honey, such figures have no relationship with the botanical or geographical origin (*2*). Therefore, Directive 2001/L10 from The Council of European Communities lists a need to develop methods for honey verification (*3*).

Many studies have sought analytical markers of the botanical origin of honey, based on various honey components, one being amino acids. Twenty-six amino acids in honey account for 1% (w/w) of honey, the most abundant being proline. The origin of amino acids in honey is attributable to both animal and vegetal sources. Because pollen is the main source of honey amino acids, the amino acid profile of honey could be characteristic of their botanical origin (*2*).

It has been shown that there is a relationship between the amino acid composition of honey and its origin, most commonly botanical origin. Davies (*4*) analyzed honeys from 11 different countries and found that the ratios between certain honey amino acids were different depending on the geographical origin. More research has been done on amino acid composition and botanical origin. It has been found that Spanish lavendel and eucalyptus honeys can be distinguished by their amino acid composition and that the amino acid compositions of rosemary, thyme, and orange blossom honeys are more similar (*5*). Another study on Spanish honeys supports those conclusions (*6*). Amino acid analysis of Italian honeys by gas chromatography showed that the amino acid content of different unifloral honeys varies, but no statistical analysis was carried out (*7*).

Less work has been done about the relationship between the amino acid composition of polyfloral honeys and geographical origin. Analysis of Argentinian polyfloral honeys concluded that the amino acid composition of polyfloral honeys from different Argentinian regions varied (*8*).

To the best of our knowledge no research has been carried out to distinguish poly- and unifloral honeys from each other by amino acid composition.

For statistical treatment of amino acid composition, data may be used as absolute content of amino acid in honey or may be pretreated in several ways. The absolute concentration of amino acids in honey depends, for example, on the water content of honey, which may vary in the range of 16−20 g/100 g (*9, 10*). As a result, when honeys of the same botanical and geographical origin, but with different water contents, are considered, the absolute content of amino acids is different. In such situations the relative content of amino acids should better express the similarities between amino acid profiles. Which compound(s) should be used as a reference for relative amino acid content calculation? Ideally, the concentration of such a compound in honey should be independent of botanical and geographical

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origin and environmental parameters (such as water content) of the honey. Use of such a compound is similar to an internal standard calibration procedure. Davies (*4*) used logarithmic ratios of amides/phenylalanine and aspartic/proline for clustering. Cometto et al. (*8*) used relative amino acid content as percentage of total amino acid content (excluding proline) for Kruskal−Wallis analysis and principal component analysis (PCA). Amino acid concentration per dry matter has also been used (*4*).

Several authors (*5, 11*) have used absolute amino acid concentrations for statistical data treatment. This approach may be justified considering that a polyfloral nature of honeys brings about larger variation of amino acid concentrations than moisture content.

The aim of this work is to analyze honeys from Estonia as so far no data on amino acid profiles of honeys from northern Europe are available. Honeys from northern Europe are considered to be more valuable (*1*) and are therefore likely to be mislabeled. Moreover, because unifloral honeys are more expensive than polyfloral ones, the possibility of distinguishing between uni- and polyfloral honeys within a small geographic region was examined.

MATERIALS AND METHODS

Honey Samples. Sixty-one honey samples were analyzed. Seventeen samples were obtained from the laboratory of the Estonian Environmental Research Centre. Results of pollen analysis were obtained from the same laboratory. Forty-four samples were obtained directly from beekeepers, and the botanical origin declared by the beekeepers was used. The botanical origins of the samples were as follows: heather (*Calluna vulgaris*, 19 samples); dandelion (*Taraxacum officinale*, 5 samples); linden (*Tilia* L., 3 samples); rape (*Brassica napus*, 7 samples), willow (*Salix* L., 5 samples); phacelia−sweetclover (*Phacelia Juss.*−*Melilotus Mill.*, 2 samples); rosacean (Rosaceae, 3 samples); and polyfloral (17 samples). Samples were kept in the dark at room temperature (<25 °C).

Reagents and Standards. Acetonitrile (J. T. Baker) was of HPLC grade. Ultrapure water was prepared by using a Milli-Q system (Millipore, Bedford, MA). An essential L-amino acids kit and γ-aminobutyric acid were from Sigma. β-Alanine and L-ornithine monohydrochloride were purchased from Fluka. Solid phase extraction cartridges (styrene−divinylbenzene polymeric strong cation exchange resin, 500 mg) were purchased from Alltech Associates, Inc. All other reagents were of analytical grade.

Sample Preparation. The amino acid isolation procedure was adapted from the method described by Bouseta et al. (*12*) and Chen et al. (*13*). Twenty-five milliliters of phosphate buffer (0.03 M, pH 2.12) was added to 1 g of honey. A solid phase extraction cartridge was first conditioned with 10 mL of HCl (0.1 M). The buffered honey sample was applied to the cartridge at ~1.5 mL/min flow rate. The analytes were eluted with 15 mL of 2.5 M ammonium hydroxide containing 10% of acetonitrile. The eluate was evaporated to dryness using nitrogen flow and redissolved in 1 mL of ultrapure water.

Amino Acid Analysis. The derivatization procedure for amino acids was as follows: 30 μL of diethyl ethoxymethylenemalonate, 1.5 mL of methanol, and 3.5 mL of borate buffer (0.75 M, pH 9.0) were added to 1 mL of the solution of isolated amino acids (*5*).

For chromatographic analysis, an Agilent series 1100 HPLC system was used with a Phenomenex Synergy 4u Hydro-RP 80A 250 mm × 4.60 mm analytical column. The detection wavelength of the UV detector was 280 nm, and the column temperature was maintained at 45 °C. The flow rate was 0.9 mL/min. Elution solvents were acetate buffer (A) and acetonitrile (B) with the following gradient program: 0−12 min, 20−25% B; 12−20 mi,n 25% B; 20−50 min, 25−60% B.

Statistical Analysis. Statistical analysis was performed using the R statistical software (*14*).

To compare the amino acid profiles, the absolute value of each amino acid concentration in honey was used; concentrations were not corrected for water content or method recovery. For data analysis PCA and the *t* test were used.

PCA. PCA provides a method of constructing from a multiple-variable data set new variables that are pairwise uncorrelated and have maximum possible variance. Each principal component is a linear combination of the observed variables, and these linear functions are chosen to be orthogonal. The first principal component is defined as the linear combination of variables, which has the maximum variance of all linear functions derivable from the given variables. Graphical representation of principal components provides a picture that allows recognition of systematic patterns of data that are otherwise difficult to deduce from the original data matrix (*15*).

t Test. The *t* test is a statistical test that compares the means of the two groups of observations. The results show if differences between groups are coincidental or due to actual differences between groups (i.e., statistically significant). If the calculated *p* value is below the statistical significance threshold (0.05), two groups differ. The *t* test assumes normal distribution of data and equality of variances (*16*).

RESULTS AND DISCUSSION

General Observations. From a comparison of average amino acid concentrations in analyzed honeys, sulfur-containing amino acids (Met and Cys) were not present in any of the Estonian honeys and very low amounts of Trp and Orn were found. Cotte et al. (*17*) found low amounts of Met, Cys, and Leu in French honeys. Similarly, Hermosín found low amounts of Met and Cys in Spanish honeys, as well as Thr. Major amino acids in honeys were Pro and Phe, just as in French (*17*) and Spanish (*5*) honeys. Lower but still substantial amounts of α-Ala, Asp, Gln, Glu, and Lys were present (**Table 1**). Comparing Estonian honey amino acids concentrations with those of other countries showed that Estonian honeys have higher concentrations of Pro than some French (*17*) and Italian (*7*) honeys; Estonian rape and linden honeys have higher contents of Phe, Ser, Leu, and Gly and also higher total amino acid content, but lower content, for example, of His and Trp (*17*), than French honeys.

Nozal et al. (*6*) and González Paramás et al. (*18*) have determined amino acid profiles of Spanish honeys. The relative contents of Phe detected in heather honeys (relative to the total concentration of amino acids) are 3.0% (this work), 12.9% (*18*), and 16.2% (*6*). Although the relative Phe content in Estonian heather honeys is lower, it is still the second most abundant amino acid after Pro, similar to Spanish honeys. The third most abundant amino acid is Glu, 2.6% (this work) and 11.1% (*6*), but Trp in the work of González Paramás (*18*).

Amino acid analysis reveals that heather honey has higher Arg and Pro concentrations and also total amino acid content than other Estonian uni- and polyfloral honeys. Orn was not detected in any of the dandelion honey samples. Rape honey has a higher concentration of Glu than other analyzed honeys. On the other hand, even though heather honeys have high total amino acid content, they have lower Phe content than other Estonian honeys.

***t* Test.** It could be deduced from the application of the *t* test that differences among the group values of arithmetical means were found to be significant ($p < 0.05$) for α-Ala, β-Ala, Asn, GABA, Gln, Gly, His, Orn, Phe, Pro, Ser, and Trp (**Table 2**). This shows that it is not necessary to analyze all amino acids to identify honeys. *t* Test results show that heather honey can be distinguished from other honeys except linden by using Gly and Phe concentrations. Dandelion and linden honeys can be distinguished from each other using Asn, Gln, His, Orn, and Pro concentrations. Linden and willow honeys can be distinguished from each other using β-Ala, GABA, and His concentrations.

Table 1. Distribution of Amino Acid (AA) Concentrations (as Milligrams per Kilogram of Honey) for Estonian Honeys: Mean Value (MV) and Standard Deviation (SD)

	heather ($n = 19$)		dandelion ($n = 5$)		linden ($n = 3$)		rape ($n = 7$)		willow ($n = 5$)		polyfloral ($n = 17$)	
AA	MV	SD	MV	SD	MV	SD	MV	SD	MV	SD	MV	SD
His	3.8	1.4	3.8	0.6	2.7	0.4	3.9	1.3	4.2	1.0	5.0	2.3
Arg	9.8	4.1	7.4	2.1	8.2	2.2	7.7	3.6	7.2	2.3	7.2	3.0
Asn	7.9	4.0	6.7	0.9	4.5	1.1	8.4	5.8	11.7	5.6	10.0	5.1
Gln	11.2	5.5	16.3	3.7	8.3	3.7	14.4	7.5	17.2	7.2	20.8	10.2
Ser	9.9	3.4	6.7	1.1	6.0	1.7	6.9	1.7	7.7	1.5	9.6	4.9
Asp	12.2	4.6	7.6	1.8	8.7	1.7	8.6	1.5	9.1	1.7	10.5	5.1
Glu	17.3	7.5	11.1	2.2	11.8	3.5	13.7	6.3	14.0	4.1	17.5	8.6
Thr	5.3	2.6	3.5	0.4	2.7	0.7	3.5	1.0	3.8	1.0	4.7	2.0
Gly	5.5	1.5	3.1	0.3	3.9	0.6	3.6	1.0	3.5	0.5	4.3	1.7
β-Ala	6.9	1.6	5.6	0.7	5.1	1.0	6.3	1.5	7.0	1.0	7.4	1.8
GABA	4.1	1.6	3.2	0.8	2.3	0.3	3.3	0.8	3.9	0.7	3.7	0.9
α-Ala	13.8	5.2	6.6	0.9	8.4	2.4	7.6	2.2	8.6	0.9	10.0	3.8
Pro	487.4	136.7	246.4	13.2	345.3	63.5	327.0	148.2	281.9	80.9	382.5	153.7
Tyr	8.7	3.1	6.8	3.5	5.2	1.6	6.7	3.8	7.5	3.5	7.0	2.2
Val	8.0	2.8	6.3	0.7	5.2	1.3	5.7	1.6	6.5	0.9	7.2	2.2
Trp	0.0	0.0	0.6	1.3	0.0	0.0	0.4	0.8	0.4	0.9	1.0	1.6
Orn	0.0	0.0	0.5	0.1	0.2	0.2	0.2	0.3	0.3	0.3	0.4	0.3
Phe	19.7	5.8	32.6	19.5	17.6	7.1	33.4	19.8	39.2	31.5	34.7	20.5
Ile	5.5	2.2	3.8	0.4	3.4	0.9	4.1	1.1	4.7	1.2	5.0	1.3
Leu	7.0	3.0	5.0	2.6	3.9	1.0	5.6	3.0	6.8	2.1	6.1	2.0
Lys	12.0	4.7	12.7	2.8	7.2	1.9	12.6	4.5	13.5	5.9	16.5	8.2
total	656.1	183.5	396.4	35.5	460.6	89.3	483.5	193.6	458.6	83.3	571.2	209.5

Table 2. *t* Test Results of Pairwise Comparisons of Mean Amino Acid Contents of Heather (H), Dandelion (D), Linden (L), Rape (R), Willow (W), and Polyfloral (P)

AA	H−D	H−L	H−R	H−W	D−L	D−R	D−W	L−R	L−W	H−P	D−P	L−P	R−P	W−P
His	1.00	0.18	0.98	0.63	0.02	0.98	0.52	0.17	0.05	0.07	0.27	0.10	0.22	0.43
Arg	0.23	0.52	0.24	0.19	0.63	0.89	0.87	0.82	0.56	0.04	0.90	0.61	0.77	0.97
Asn	0.54	0.18	0.80	0.10	0.02	0.54	0.09	0.30	0.08	0.18	0.18	0.09	0.52	0.53
Gln	0.07	0.41	0.24	0.06	0.03	0.62	0.81	0.22	0.10	0.00	0.35	0.05	0.15	0.47
Ser	0.06	0.08	0.04	0.20	0.48	0.83	0.25	0.46	0.18	0.85	0.21	0.23	0.17	0.42
Asp	0.05	0.24	0.06	0.18	0.41	0.36	0.20	0.86	0.76	0.33	0.23	0.57	0.33	0.56
Glu	0.09	0.24	0.27	0.37	0.74	0.41	0.20	0.64	0.46	0.95	0.12	0.28	0.30	0.40
Thr	0.15	0.11	0.10	0.23	0.08	0.88	0.53	0.21	0.15	0.44	0.19	0.10	0.15	0.34
Gly	0.00	0.09	0.00	0.01	0.04	0.36	0.20	0.54	0.26	0.02	0.14	0.73	0.29	0.30
β-Ala	0.12	0.09	0.42	0.90	0.40	0.38	0.04	0.25	0.04	0.42	0.04	0.05	0.17	0.66
GABA	0.23	0.07	0.22	0.73	0.13	0.78	0.19	0.07	0.01	0.31	0.28	0.02	0.37	0.65
α-Ala	0.01	0.10	0.01	0.04	0.17	0.36	0.01	0.64	0.85	0.02	0.06	0.48	0.13	0.42
Pro	0.00	0.10	0.02	0.01	0.01	0.26	0.36	0.85	0.29	0.04	0.07	0.69	0.43	0.18
Tyr	0.26	0.08	0.20	0.47	0.49	0.98	0.76	0.52	0.33	0.08	0.87	0.18	0.82	0.70
Val	0.23	0.13	0.06	0.30	0.16	0.43	0.67	0.65	0.14	0.38	0.40	0.16	0.12	0.54
Trp	0.05	*a*	0.02	0.05	0.48	0.79	0.82	0.39	0.48	0.01	0.60	0.31	0.38	0.46
Orn	0.00	0.00	0.01	0.00	0.03	0.04	0.06	0.85	0.81	0.00	0.44	0.24	0.07	0.26
Phe	0.02	0.59	0.01	0.01	0.26	0.95	0.70	0.23	0.30	0.00	0.84	0.18	0.89	0.71
Ile	0.13	0.15	0.15	0.51	0.41	0.57	0.14	0.36	0.15	0.45	0.06	0.06	0.13	0.72
Leu	0.20	0.10	0.28	0.85	0.51	0.77	0.29	0.40	0.08	0.31	0.33	0.08	0.58	0.56
Lys	0.78	0.11	0.77	0.56	0.02	0.99	0.78	0.09	0.13	0.05	0.33	0.07	0.26	0.46

[a] Trp is not present in dandelion honey.

PCA. PCA was applied to all honey samples to discover natural groupings. PCA is used due to the difficulties of interpretation of large data sets. In **Table 3** is the loading matrix for the first four principal components, and the percent of overall variance described by each of them is given. The first principal component (PC1) accounts for 55.3% of the variance. The cumulative variance for two components is 67.4%, and four principal components account for 81.5% of total variability. The cumulative variance explained by the four first principal components is larger than in the case of Spanish honeys (78.25%) (*6*).

A component loading is a correlation coefficient that reflects how strongly each of the variables correlates with the principal components. The higher the absolute value of a component loading, the more the respective variable contributes to that principal component (*16*). PC1 is a function of all amino acid concentrations almost equally (**Table 3**). Hermosín et al. (*5*) and Nozal et al. (*6*) found that the contents of some amino acids are strongly correlated. Examples are Val, Glu, and α-Ala in ref *5* and Asn, Asp, and Glu in ref *6*. In the present work, all amino acids must be taken into account for PCA.

In **Figure 1** botanical origins are presented according to the first two principal components, PC1 and PC2. Honeys form groups (clusters) according to their botanical origin in the PC1 versus PC2 graph (**Figure 1**). It can be seen that polyfloral honeys are rather widespread and vague in distribution and overlapped with all unifloral honeys except heather.

One of the reasons heather honeys are different from other honeys could be that heather blossoms late in the summer when most of the other honey plants are no longer blooming.

Table 3. Component Loadings Matrix for the First Four Factors and the Variance Explained by Each

AA	PC1	PC2	PC3	PC4
His	0.2258	0.2302	−0.0695	0.2570
Arg	0.2140	−0.1489	0.0026	−0.2809
Asn	0.1858	0.2114	−0.2338	0.1001
Gln	0.1992	0.3726	0.0346	0.1048
Ser	0.2727	−0.0396	−0.0345	−0.0028
Asp	0.2442	−0.1561	−0.2519	−0.0673
Glu	0.2673	0.0309	−0.1515	−0.0705
Thr	0.2802	−0.0613	0.0161	0.1182
Gly	0.2613	−0.2003	−0.0738	−0.1957
β-Ala	0.2636	0.1096	−0.0090	0.0708
GABA	0.2322	−0.0896	−0.0709	0.2418
α-Ala	0.2412	−0.3066	0.0154	−0.0878
Pro	0.2368	−0.1786	−0.1006	−0.2887
Tyr	0.1979	−0.0211	0.3399	−0.1961
Val	0.2800	−0.0733	0.1056	−0.0176
Trp	0.0911	0.3699	0.1171	−0.3994
Orn	0.0834	0.4916	−0.0803	−0.2381
Phe	0.0517	0.2034	0.5979	−0.1747
Ile	0.2394	−0.0680	0.2998	0.1282
Leu	0.0947	−0.1359	0.4602	0.4381
Lys	0.1937	0.2754	−0.1564	0.3520
% variance	55.3	12.1	7.96	6.11
cumulative % variance	55.3	67.4	75.35	81.47

Moreover, heather plants grow close together and have more nectar than most other plants (*19*). Therefore, heather honeys contain a larger percentage of one type of nectar, whereas other unifloral honeys usually contain various types of pollen. Both the *t* test and PCA show that the amino acid composition of heather honeys differs from those of other types of honeys analyzed in this work.

PCA shows that honeys from the same honeykeeper from different years are separated on the graph. Heather honeys from 2007 (**Figure 1**, point 1) and 2006 (**Figure 1**, point 2) are more likely to have positive PC1, whereas honeys from 2005 (**Figure 1**, point 3) and 2004 (**Figure 1**, point 4) are more likely to have negative PC1. This observation can have two possible explanations. The amino acid content of honeys may differ from year to year. This has been demonstrated in the case of polyfloral honeys (*20*), but we are not aware of similar research on unifloral honeys. Alteration of the amino acid content during storage could also cause the year to year differences.

Moreover, PCA shows some interesting results for rape honeys. As compared to other honeys, they form more than one

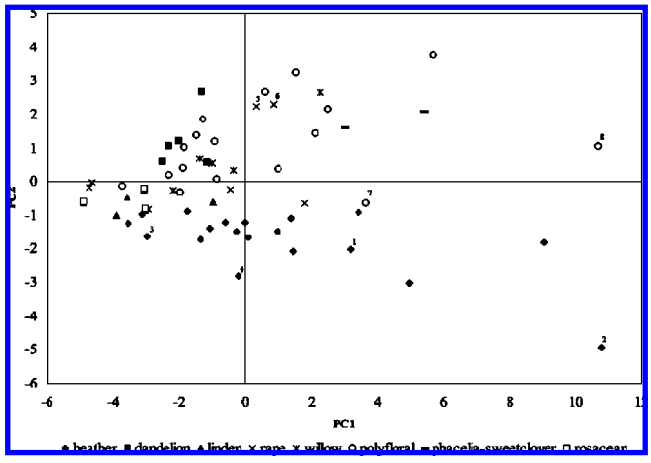

Figure 1. Two first-component scores of honeys from the studied botanical origins. (See text for comments on numbered points).

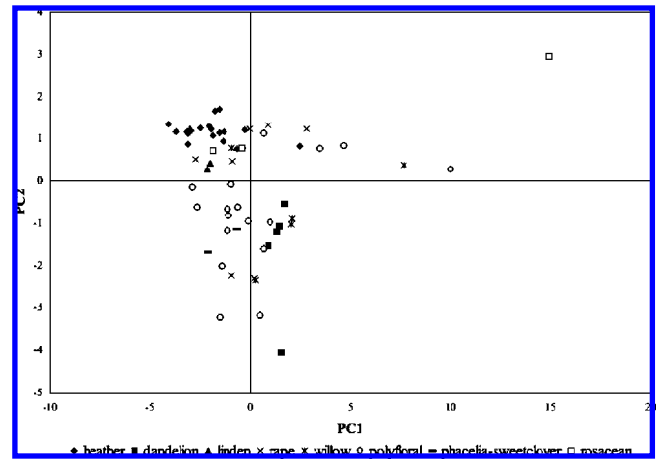

Figure 2. Two first-component scores of honeys from the studied botanical origins (relative amino acid concentrations).

group that situate far apart on the graph. Pollen analysis gives some explanation. Rape honeys with PC2 >1 are rape honeys that contain 55% (**Figure 1**, point 5) and 61% (**Figure 1**, point 6) of rape pollen, that is, near the borderline (>50% of most abundant pollen) between uni- and polyfloral honeys. Therefore, PCA classified those honeys more closely to polyfloral honeys than rape honeys.

It can be seen (**Figure 1**) that unifloral honeys other than heather overlap with polyfloral honeys. However, dandelion, willow, rosacean, and phacelia−sweetclover honeys group together by botanical origin. There are some samples that deviate from each of the groups, but this might be explained by the content of certain pollens in honey. As rape honey analysis discussed in the previous paragraph shows, the percentage of pollen plays an important role in amino acid concentration and thus grouping.

If we would disregard polyfloral honeys on the PCA graph (**Figure 1**), overlapping of unifloral honeys is less likely. Polyfloral honeys are a combination of many different nectars, and therefore amino acid compositions vary largely. This makes a distinction between poly- and unifloral (except heather) honeys more difficult. Close analysis of different polyfloral honeys shows that honeys from Saaremaa (the largest island in Estonia) are situated far from other polyfloral honeys (**Figure 1**, points 7 and 8). This may be attributed to a unique plant community of Estonian islands.

In the present work relative amino acid concentrations were also subjected to PCA (**Figure 2**). All amino acid concentrations were used relative to proline content. Proline was regarded as a reference amino acid because bees add it to the honey (but it may also be of botanical origin), and this analysis showed some change compared to PCA of absolute amino acid concentrations. For example, heather honeys grouped more closely together and rape honeys as well. In general, both approaches gave similar results.

In conclusion, determination of the amino acid composition of various Estonian honeys shows that even though honeys have similar amino acid profiles, the *t* test and PCA can bring out some differences. Heather honeys have amino acid compositions that differentiate them from other types of honeys. The *t* test makes it possible to distinguish some honey types from each other. PCA may become a useful tool for botanical origin assessment if larger data sets are analyzed.

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