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Preliminary NMR and chemometric study of pine jams used as medicinal remedies

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Dedicated to Acad. Ivan Juchnovski on the occasion of his 80th birthday

Chemical profile of pine cone and pine bud jam is determined using ^{1}H and ^{13}C NMR spectra. Principal component and cluster analysis of 41 detected organic ingredients allow discrimination of jam from honey. Difference in the chemical profile of the two jams is found.

Key words: pine cone and bud; honey; ^{1}H nuclear magnetic resonance; ^{13}C NMR; cluster analysis, PCA

INTRODUCTION

Pine jam, also known as "pine honey" or pine elixir, does not originate from bee activity, but is made from pine cones or buds. Both products are delicious due to their unique aroma of a pine forest. Additionally they are healthy and a worthy substitute of honey. Pine jams are often used in traditional medicine as therapeutic agents against respiratory viral diseases and/or for strengthening of the immune system, very popular in Eastern Europe, Russia and Georgia. Despite abundant information on the chemical composition [1] and biological activities [2] of the *Pinus* species, jams are only poorly studied. We were able to find only two publications in the literature so far – one devoted to their antioxidant and antimicrobial properties [3] and one to characterization of the detected volatile components [4]. The traditional medical use of pine jams and honeys is very often quite close, however, their prize and actual activity differs considerably. That is why the aim of the present work is to determine the main components in pine jams from cones and buds using NMR spectroscopy and to test the suitability of unsupervised chemometric methods to distinguish pine jams from honey types.

EXPERIMENTAL

Sample preparation

Pine jams and honeys were bought from the local market. 0.5 g of jam or honey was dissolved in 0.5 ml D_2O, containing 0.02 v. % sodium salt of trimethylsilylpropionic acid-d_4 (TSPA) for internal standard and 0.02 v. % of NaN_3 as a preservative.

Spectral Parameters

^{1}H (600.01 MHz) and ^{13}C (150.88) NMR spectra have been acquired on an AVANCE AV600 II+ NMR spectrometer using topspin v.3 pl 6. All spectra have been recorded in D_2O at 293.0±0.1 K. TSPA-d_4 has been used as an internal reference with chemical shifts at 0.0 ppm and -2.63 ppm for ^{1}H and ^{13}C, respectively. Following acquisition parameters have been used for ^{1}H NMR: spectral width of 13.6 ppm (transmitter frequency at 4.84 ppm) on 64 K data points – FID resolution of 0.3 Hz and acquisition time 2.58 s; 60^0 pulses of 7.2 μs duration; relaxation delay of 4 s; 16 dummy and 256 scans. Zero filling by a factor of 2 and exponential multiplication by a line broadening of 0.3 Hz has been applied. Manual processing and careful manual phasing of the spectra ensured that the integrals have minimal distortion and thus contribute to the quantitative reproducibility. Standard ^{13}C NMR parameters have been used - spectral width 238.9 ppm, 32 K data points, 60^0 pulses of 6.5 μs duration; relaxation delay of 2.0 s; 4048 scans. Assignment of the signals has been made on the basis of the gradient enhanced versions of TOCSY, standard and semi-selective HSQC [5].

Preparation of data for chemometrics

A combined $^{1}H/^{13}C$ method was used to obtain reliable semi-quantitative data for characterization of the pine jams and their differentiation from the honey types. Quantitation relies on the intensities of the ^{13}C NMR signals utilizing the much higher

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dispersion (Fig. 2) of the signals in the carbon than in the ^{1}H spectra (Fig. 1). The region of the anomeric carbon atoms (106–83 ppm) contains a number of non-overlapped signals for most of the saccharides. Additionally, ^{13}C intensities for several typical honey ingredients as quercitol [6], butanediol [7] and proline [8] as well as for 15 unidentified constituents were determined (see Table S1). One carbon signal was chosen for every of the 41 components (Table 1). Reducing sugars were also represented by one non-overlapped signal taking into account the quantities of the corresponding epimers from the NMR spectra of the individual sugars in D_2O. The molar mass of all components was taken into account in order to determine the amount of the individual ingredients. Diffusion NMR spectra indicate that the unidentified components have diffusion coefficients in the range between mono- and disaccharides, and a tentative molar mass of 200 was ascribed to all of them. Additional adjustment of all carbon intensities was made via comparison with the integration results of several proton signals against TSPA.

Chemometric analysis

Chemometric analysis [9, 10] was applied in order to test the possibility to differentiate pine jams from honey varieties. Taking into account the limited number of samples unsupervised pattern recognition via cluster analysis (CA) and principle component analysis (PCA) were applied using the algorithms offered by EXCEL [11], SIMCA14 [12] and Past3 [13] statistical software.

Cluster analysis allows grouping of a set of objects in such a way that objects in the same group are more similar to each other than to those in other groups. Very popular is the “connectivity model” that uses hierarchical clustering based on distance connectivity represented by a dendrogram (e.g. Fig. 4A). The x-axis marks the distance at which the clusters merge, while the investigated objects are disposed along the y-axis preventing cluster mixing, accompanied by a table presenting the distances between the objects. The Ward's minimum variance method was found most appropriate for the jam-honey discrimination. Principal components analysis is the most often applied procedure for identifying a smaller number of uncorrelated variables, called "principal components", from a large set of data. The goal of PCA is to explain the maximum amount of variance with the fewest number of principal components. These are linear combinations called usually factors being better descriptors than the original chemical or physical measurements, allowing easier visualization of the obtained results. In the case of a limited number of samples *n* as in our investigation (*n=5*) the number of derived factors is confined to *n-1*.

RESULTS AND DISCUSSION

NMR chemical profiling

The ^{1}H and ^{13}C NMR chemical shifts were obtained from the corresponding 1D NMR spectra of the five remedies. They are presented in Figs. 1 and 2 with annotation of the saccharide components in the corresponding anomeric spectral areas.

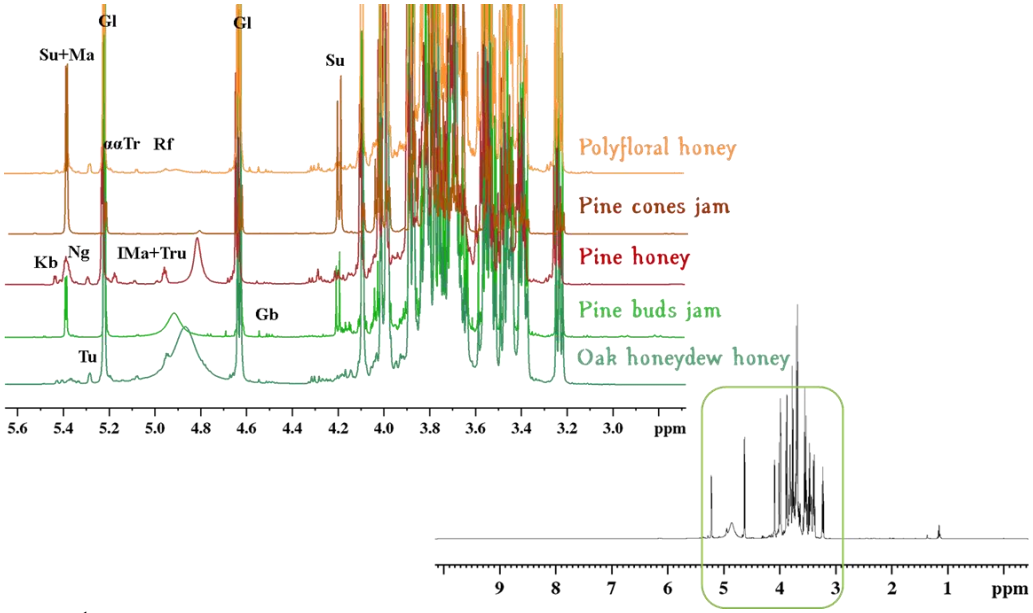


Fig. 1. ^{1}H NMR spectra of the investigated jams and honeys with expansion of the sugar region.

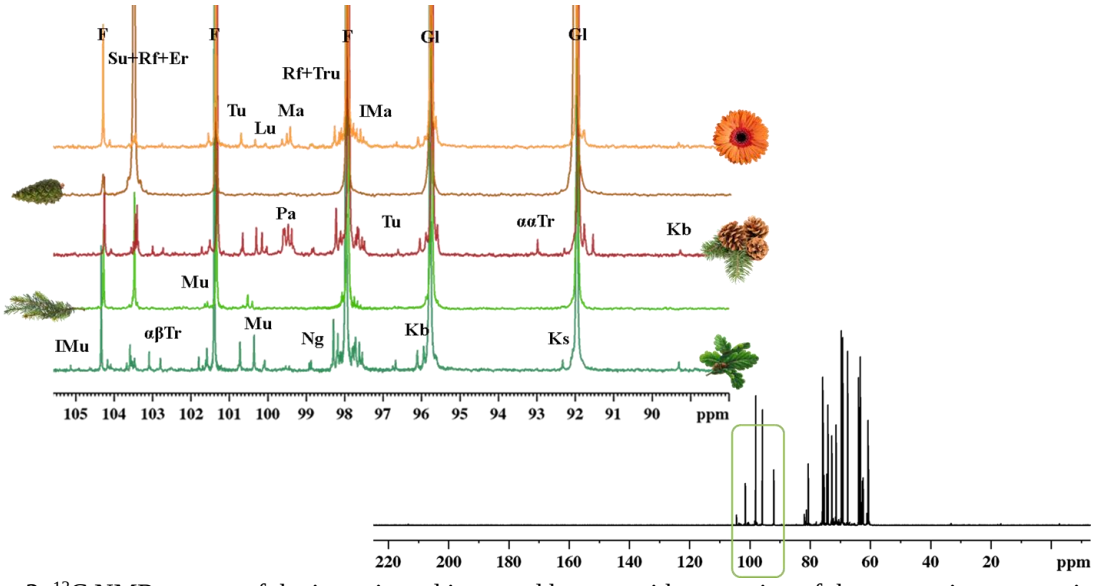


Fig. 2. ^{13}C NMR spectra of the investigated jams and honeys with expansion of the anomeric sugar region.

Use of the gradient version of the HSQC technique with high resolution in the indirect dimension and comparison with previously made sugar profiling [14, 15] and literature data [16] assures the unambiguous identification of the organic ingredients - carbohydrates, amino acids and some other detectable components. The profile of two jams is compared with the data of three previously studied types of honey [14, 15] – pine honeydew, oak honeydew and polyfloral honey. The chemical shifts used for quantitation of the detected organic ingredients are presented in Table 1 and the chemical profiles are visualized in Fig. 3.

Chemometric analysis

The input data set with normalized intensities for 41 detected organic ingredients in the five investigated natural remedies is presented in Table S1. The data is first standardized by the z-transform procedure to eliminate the parameter dimension impact on the classification and interpretation results. For hierarchical aggregation of the samples into a cluster the Ward`s method is used. The standardized component quantities, presented in Fig. 3, cluster in two statistically significant groups for the five studied samples. One is for the studied honeys and the other - for the jams (Fig. 4A) with Euclidean distances between the different elements presented on Fig. 4B. Principle component analysis identifies two factors responsible for 75% of the total variance in the chemical composition of honey and jam. The results are visualized in a 3D PCA plot on Fig. 4C and listed in Table 2S. The first factor, accounting for 51% of the explained system variance correlates with the main di- and trisachacharides characteristic for honey, quercitol, 2,3-butanediol and several unidentified components while the second factor, responsible for 23% is connected to the quantities of proline, several less common saccharides and the unrecognized components U1 and U10.

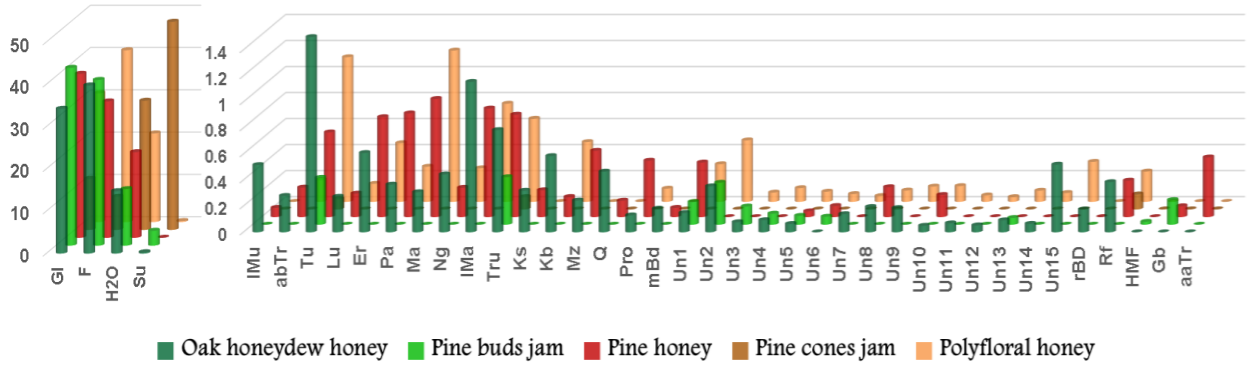
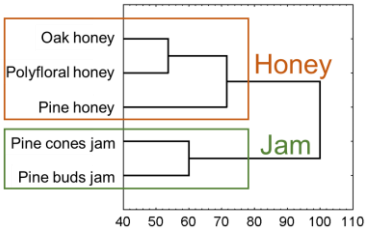


Fig. 3. Chemical profile of the studied natural remedies (in g/100g jam or honey), representing the semi-quantitative data obtained from the ^{1}H and ^{13}C NMR spectra.

Table 1. Chemical shifts of the carbon signals used for quantitation and their attached protons (in ppm), acronyms of the different organic ingredients, α/β ratio of the reducing sugars and proton signals used for adjustment of the carbon intensities.

Component	*δ ^{13}C*	*δ ^{1}H*	*Acronym*	*α:β Ratio*	*Used δ ^{1}H*
Isomaltulose	104.64	-	IMu	0.164	
αβ-Trehalose	102.79	4.63	αβTr		
Gentiobiose	102.49	4.47	Gb	0.364	
Turanose	100.72	5.29	Tu	0.444	5.29
Leucrose	100.11	5.09	Lu		5.09
Erlose	99.67	5.38	Er		
Panose	99.64	5.38	Pa	0.615	
Maltose	99.54	5.38	Ma	0.417	
Nigerose	98.87	5.33	Ng	0.583	
Isomaltose	97.73	4.95	IMa	0.636	
Trehalulose	97.71	-	Tru		
αα-Trehalose	93.00	5.17	ααTr		
Isokestose	92.31	5.41	1-Ks		
Kojibiose	89.30	5.43	Kb	0.415	5.43
Melezitose	83.24	4.29	Mz		
Sucrose	76.21	4.21	Su		
Raffinose	76.14	4.22	Rf		4.99
Glucose	75.75	3.45	Gl	0.600	4.64
Fructose	67.45	3.78	F	0.688	4.10
Quercitol	33.20	1.98/1.80	Q		
Proline	23.71	1.99	Pro		
meso-Butanediol	16.72	1.13	mBd		
racemic Butanediol	17.80	1.13	rBd		
Unidentified compounds*	103.46	-	U1		
	97.79	-	U2		
	101.69	-	U3		
	101.22	5.23	U4		
	96.74	5.48	U5		
Hydroxymethyl-furfural	-	9.44	HMF		9.44

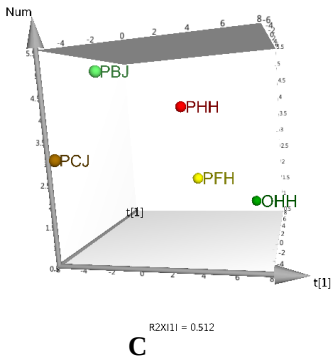
* Additional compounds U6-U15 (102.26; 104.08; 103.66; 103.55; 103.44; 103.38; 102.86; 100.77; 99.26; 98.17).



A

Case No.	OHH	PFH	PHH	PCJ	PBJ
OHH	0.0	6.7	9.2	11.8	10.8
PFH	6.7	0.0	7.6	9.5	7.5
PHH	9.2	7.6	0.0	9.7	8.9
PCJ	11.8	9.5	9.7	0.0	7.5
PBJ	10.8	7.5	8.9	7.5	0.0

B



C

Fig. 4. Visualization of the multivariate analysis. A) Hierarchical dendrogram for clustering of 41 ingredients; B) Table of distances between different objects; C) PCA 3D score plot for: PCJ – pine cone jam; PBJ – pine bud jam, PHH – pine honeydew honey; PFH – polyfloral honey; OHH - oak honeydew honey.

The observed differences in the chemical profile of the two studied jams can be best visualized by the Nightingale's "rose diagram".

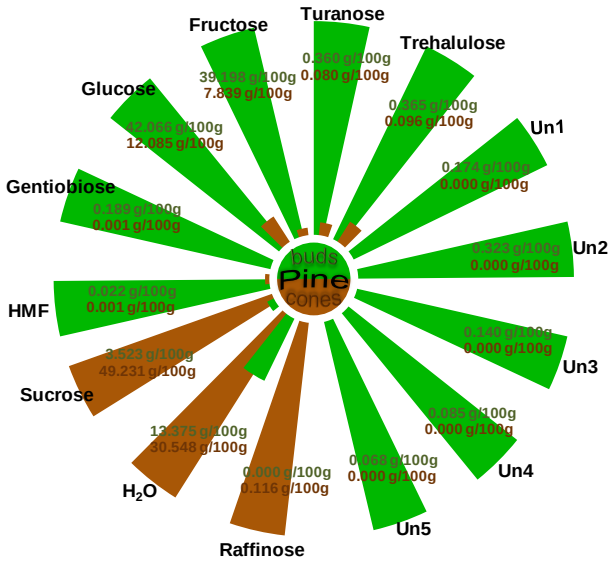


Fig. 5. Nightingale's diagram.

CONCLUSION

Significant difference in the carbohydrate profile of the studied jams has been detected. The jams are characterized generally by a lower content of di- and trisaccharides compared to honeys. Higher amounts in the jams only of saccharose and gentiobiose were determined. Pine cone jam contains appreciable amount of sucrose, while pine bud jam is rich in gentiobiose. The combination of NMR spectroscopy with chemometric methods is a power tool not only to detect adulteration, to distinguish geographical and botanical origin of honey but also to discriminate jam from homey, provoking more detailed analysis of pine jams.

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Electronic Supplementary Data available here.

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ИЗСЛЕДВАНЕ НА БОРОВИ СЛАДКА ИЗПОЛЗВАНИ В НАРОДНАТА МЕДИЦИНА ПОСРЕДСТВОМ ЯМР И ХЕМОМЕТРИЯ

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(Резюме)

Проведено е начално изследване на химичния профил на сладко от борови шишарки и от борови връхчета посредством ^{1}H and ^{13}C ЯМР спектроскопия. Анализ на главните компоненти и клъстерен анализ на 41 органични съставки позволява разграничаване на сладко от пчелен мед. Открити са разлики в химичния профил на двете борови сладка.