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Monitoring Major Sugars in Greek Commercial Fir Honey and their Role in Geographical Differentiation, using Chemometrics

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Abstract

The aim of the present study was a) to provide information on fructose, glucose, sucrose and maltose content, along with sum of fructose and glucose content, fructose/glucose ratio, and sum of the four sugars, of a lees common type of honey produced in Greece namely fir, and b) investigate the possibility of geographical differentiation using above parameters in combination with chemometrics. For this purpose, 30 commercial fir honey samples were collected during the harvesting period 2011 from 4 different regions in Greece. The analysis of saccharides was performed by high pressure liquid chromatography coupled to a refractive index detector.Results showed that sugar content of fir honey was affected by geographical origin ($p<0.05$). Application of linear discriminant analysis (LDA) to sugar parameters resulted to the correct geographical differentiation of commercial fir honeys recording 80

Index terms— commercial fir honey; sugars; high pressure liquid chromatography; refractive index; differentiation.

I. Introduction ugars (saccharides) are the main components of honey. They are produced by honeybees from nectar sucrose, which is transformed through the action of several enzymes, mainly a-and b-glycosidase, a-and b-amylase and b-fructosidase (Huidobro et al.,1995;De la Fuente et al., 2011).

Fructose and glucose (monosaccharides) are the major constituents of honey, being the dominant components in almost all types, except for some honeys of dandelion (Taraxacum officinale), blue curl (Trichostema lanceolatum), and rape (Brassica napus) origin, where glucose is present in higher amounts (Cavia, et al., 2002). The content of fructose and glucose, as well as their ratio, has been considered as useful indicator for the classification of unifloral honeys (Oddo et al., 1995;Terrab et al., 2001;Oddo & Piro, 2004;De La Fuente et al., 2007;Manikis et al., 2011). Besides these two main constituents, there are also oligosaccharides (disaccharides, trisaccharides, and tetrasaccharides). These compounds are formed, mainly by the action of honey enzymes.

Author: Laboratory of Food Department of Chemistry, Section of Industrial and Food Chemistry, University of Ioannina, Ioannina Campus, 45110, Greece. e-mail: ikaraba@cc.uoi.gr Ruiz-Matute et al. (2010), reported 25 trisaccharides and 10 tetrasaccharides, for Spanish and New Zealand honeys. The trisaccharides planteose and ?-3-glucosylisomaltose were reported in honey for the 1st time by these authors.

Thus, new developments in analytical techniques enhance the possibilities of searching for more precise and representative geographical and botanical origin markers (De La Fuente et al., 2006). Dvash et al. (2002) used NIR spectroscopy for the analysis of avocado (Persea Americana Mill.) honey and found that carbohydrate alcohol perseitol (dglycerod-galacto-heptitol) in spite of its low content (average value 0.48g/100g) could be used as a marker of avocado honey. The same compound was reported in avocado honey by de La Fuente et al. (**??**006), at a higher amount of 0.75g/100g. Honey carbohydrate composition has been commonly determined by high performance liquid chromatography (HPLC) or by gas chromatography (GC). Since a high number of carbohydrate isomers are present in honey, resulting in very complex chromatograms with a high degree of overlapping, several methods have been proposed for their quantification (De La Fuente et al., 2006).

HPLC allows the determination of high molecular weight oligosaccharides (Swallow & Low, 1990;Weston & Brocklebank, 1999;Morales et al., 2006), while GC provides better resolution for many important minor sugars

as disaccharides and trisaccharides (Low & Sporns, 1988;Gómez-Bárez et al.„ 2000;Cotte et al., 2004;Sanz et al.„ 2004).

Carbohydrate derivatization is required for gas chromatography (GC) analysis, and when trimethylsilyl oximes are used, they produce two peaks for reducing sugars and only one for non reducing sugars (Gómez-Bárez et al., 2000;De La Fuente et al., 2011).

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Greece holds a leading position internationally in hives and honey production, regarding its population and area. While in all European countries the quantity of colonies decreased or remained constant, over the last twenty years in Greece have increased, by approximately two colonies per km. What is interesting, is that there are a quite few studies in Greece dealing with the characterization of fir honey based on sugar profile (Manikis et al., 2011;Spilioti et al., 2014).

2 II. Materials and Methods

3 a) Honey samples

Thirty fir honey samples were collected from professional beekeepers during the harvesting period 2011 from 4 different geographical regions: Messinia (8 samples), Lakonia (10 samples), Arkadia (8 samples), Karditsa (4 samples). Samples were stored in glass containers, shipped to the laboratory and maintained at 4±1 °C until analysis.

4 b) Standards and chemicals

Fructose, glucose, sucrose and maltose, were obtained from Merck (Darmstadt, Germany). All chemicals used in the present study were of analytical grade and deionized water was used to prepare all solutions. Acetonitrile (HPLC grade), methanol (HPLC grade), ammonium hydroxide and ethylenediamine were also obtained from Merck (Darmstadt, Germany).

5 c) Preparation of standards

The preparation of the standard solutions of saccharides was carried out based on the method described by Bogdanov and Baumann (1988). d) Preparation of honey samples 5g of honey was weighed into a beaker and dissolved in 40 mL deionized water. Then, 25 mL of methanol was pipetted into a 100 mL volumetric flask and the honey solution was quantitatively transferred into the flask. It was filled to the mark, with deionized water. Finally, the obtained solution was filtered through a 0.45?m membrane filter prior to HPLC analysis (IHC, 1997). Each sample was run in duplicate (n=2). e) Saccharides were quantified by comparison their chromatographic peak areas with the calibration curves of the standards. The calibration curves were made in triplicate (n=3) for each individual standard at five different concentrations (100-20000 mg/L). The determination coefficients for the calibration curves were: R 2 =0.993 for fructose, R 2 =0.996 for glucose, R 2 =0.995 for sucrose, and R 2 =0.996 for maltose respectively. Limit of detection (LOD) and limit of quantification (LOQ) were: LOD= 0.11 and LOQ = 0.37 mg/Kg for fructose, 0.21 and 0.71 mg/Kg for glucose, 0.06 and 0.19 mg/Kg for sucrose, 0.05 and 0.18 mg/Kg for maltose, respectively. Figure **??** shows a representative chromatogram of a mixture of the four standard sugars. f) HPLC Analysis i. Apparatus HPLC analysis was performed with a SHIMADJU LC solution (Kyoto, Japan), consisting of a quaternary pump (LC-20AD), a thermostated column oven (CTO-10A), a 20 ?L loop injector and a SHIMADJU chemstation for data analysis. Detection was carried out using a SHIMADJU refractive index (RID-10A).

ii. HPLC conditions A separation column (Zorbax Rx-SIL, 250 mm x 4.6 mm i.d., 5 ?m, Hewlett-Packard, USA) was used. The column temperature was held at 25 °C. The mobile phase for isocratic elution was a mixture of water/acetonitrile (1:2.6 v/v) containing 0.03% (v/v) ethylenediamine as a modifier and ammonium hydroxide (0.05%, v/v), which was used to adjust the pH to 9-10. The flow rate was 1.0 mL/min. Before analysis, a mixture of water/acetonitrile (1:2.6, v/v) containing 0.3% (v/v) ethylenediamine was run through the column forming a dynamic coating layer on the silica surface (Wei & Ding, 2000).

6 g) Statistical analysis

Data processing was performed using the SPSS 20.0 statistics software (SPSS Inc., 2012). Comparison of the means was achieved using multivariate analysis of variance (MANOVA), while correct classification ability according to the production area of fir honey was performed using LDA to sugar data collected at the confidence level $p<0.05$ (Karabagias et al., 2014).

7 Quantification analysis

Volume XVI Issue II Version I Thus, the aim of the present study was to characterize and investigate the possibility of differentiating fir honey according to geographical origin based on its major sugars determined with HPLC, and by using chemometrics.

Figure **??**: A typical HPLC-RI chromatogram of a standard mixture (100 mg/L) of sugars obtained with the applied method.

8 III. Results and Discussion

a) Sugar content and sugar parameters of commercial fir honey according to geographical origin Fructose (g/100g), ranged between 21.87 (sample no.5 from Arkadia) and 42.48 (sample no 1. from Messinia). Glucose (g/100g), ranged between 6.56 (sample no.2 from Arkadia) and 39.21 (sample no.1 from Messinia). Maltose (g/100g), ranged between 0.21 (sample no.4 from Lakonia) and 5.69 (sample no.10 from Lakonia), while it was not detected in two samples. Finally, sucrose (g/100g) ranged between 0.27 (sample no.1 from Arkadia) and 7.81 (sample no. 9 from Lakonia).

According to directive 127/2004 of the Greek Ministry of Agricultural Development and Food ("Classification of monofloral honeys"), the sum of fructose and glucose (F+G) must be?45g/100g. In most of the fir honey samples analyzed, (F+G) was higher than 45g/100g. All the Arcadia samples (Menalon fir honey) gave (F+G) ?45g/100g. This is in great agreement with **??**anikis et **??**004) determined the predominant disaccharides in several types of honeys from France: maltose and turanose in acacia; maltulose and turanose in chestnut and linden; turanose and trehalose in fir; and sucrose, maltose in lavender honey. In the same study, these authors characterized 37 fir honey samples reporting mean values of fructose 31.49 (g/100g), glucose 24.17 (g/100g), sucrose 0.04 (g/100g) and maltose 0.17 (g/100g), respectively. The reported values for fructose and glucose are in very good agreement with present results regarding fir honeys from Messinia, Karditsa, and Lakonia regions. The lower glucose content reported in the present study for fir honeys from Arkadia, may be attributed to these samples were collected from mountain Menalon. It is widely known that this region gives the only PDO honey in Greece, and it is characterized by its low glucose content as compared to other types of honey (Manikis et al., 2011). that fructose and glucose were the main sugars in all samples analyzed with a mean value of 37.14 (g/100g) and 30.02 (g/100g), respectively. Such values are higher than those obtained in the present study (Table **??**).

Table **??**: Sugar content (g/100g) of commercial fir honeys according to geographical origin

The results are the mean of two replicates (n=2). MANOVA in comparison of means ($p<0.05$), nd: not detected. b) Classification of commercial fir honeys according to geographical origin based on sugar data MANOVA analysis was applied to the sugar data of the thirty commercial fir honey samples in order to point out which sugar parameters are significant for the differentiation of honeys from the four different geographical origins. Dependent variables included the independent variable. Pillai's trace= 1.806 (F=4.752, df=21, p-value=0.000<0.05) and Wilk's Lambda= 0.018 (F=8.445, df=21, p-value=0.000<0.05) index values showed the existence of a significant multivariable effect of geographical origin on the identity of fir honey sugar data. Four sugar parameters (Table 2) were found to be significant ($p<0.05$) for the F/G: fructose/glucose ratio, F+G: sum of fructose and glucose (g/100g), F+G+M+S: sum of fructose, glucose, maltose, and sucrose (g/100g).

differentiation of fir honeys. Thus, these 4 sugar parameters were subjected to LDA. Cotte et al. (2004), using a much larger number of honey samples (280) produced in the wider area of France, and belonging to 7 botanical origins (acacia, chestnut, rape, lavender, fir, linden, sunflower) reported that the 17 carbohydrates determined along with fructose/glucose ratio, resulted to a classification rate of 72.1% according to honey type, after the application of principal component analysis. Nozal et al. (2005) characterized 77 honeys belonging to several botanical origins (ling, spike lavender, French lavender, thyme, forest, and multifloral) from a single (identical) geographical area, the Province of Soria (Spain), using 14 carbohydrates in combination with chemometrics. These authors, managed to classify above types of honey, reporting an overall classification rate of 90%.

Finally, de la Fuente et al. (**??**011) in a study dealing with the characterization of 59 Spanish floral honeys (citrus, rosemary, heather, rosaceae, eucalyptus, and echium) in terms of carbohydrate composition, reported that the carbohydrates determined did not allow an unambiguous classification of honeys according to their type, after application of chemometric analyses (correct classification rate < 70%).

F: Fisher's linear discriminant functions, p: probability, F/G: fructose/glucose ratio Results showed that two statistically significant discriminant functions were formed: Wilk's Lambda= 0.028, X 2 =89.523, df=12, p-value=0.000<0.05 for the first function, and Wilk's Lambda= 0.322, X 2 =28.334, df=6, p-value=0.000<0.05 for the second. These significant values of Wilk's Lambda index shows that the discriminant functions created were basic for the differentiation of the investigated regions.

The first discriminant function accounted for 84.7% of total variance, the second accounted for 14.4%. Both accounted for 99.1% of total variance, an excellent rate.

In Figure 2 it is shown that fir honeys from Arkadia are fully separated. Fir honeys from Karditsa and Messinia are close, the latter seems to be not well separated. Honeys from Lakonia are also separated, as compared to honeys from Arkadia.

The overall correct classification rate was 80% using the original and 76.7% the cross validation method, a quite satisfactory value especially for the second method. Correct classification (100%) was obtained for honey samples from Arkadia, followed by those of Karditsa (correct classification 75%), Lakonia (correct classification 70%) and Messinia (correct classification 62.5%) (Table **??**). Table **??**: Differentiation ability of the proposed chemometric model using sugar data (g/100g) of commercial fir honey *Pooled within-groups correlations between

discriminating variables and standardized canonical discriminant functions Variables ordered by absolute size of correlation within function.

9 IV. Conclusion

In the present study results showed that sugar content of commercial fir honey is affected by geographical origin ($p<0.05$). This is the first attempt to differentiate fir honeys produced in different regions in Greece, using selected sugar parameters, this constituting the novelty of the present work. The classification rate obtained is within the range reported previously in the literature (Cotte et al., 2004;Nozal et al., 2005;De La Fuente et al., 2011). It is worth mentioning that the sugar content of the honeydew secretions is greatly variable and depends strongly on the insect and plant species, as well as on the climate in a specific area (Salvucci and Crafts-Brander, 2000), affecting thus fir honey sugar content.

Thus, the classification rate presented in the present study will be further evaluated by collecting honeydew secretions from the same regions. In that sense, a more sophisticated differentiation model will be constructed for fir "honeydew" honey.

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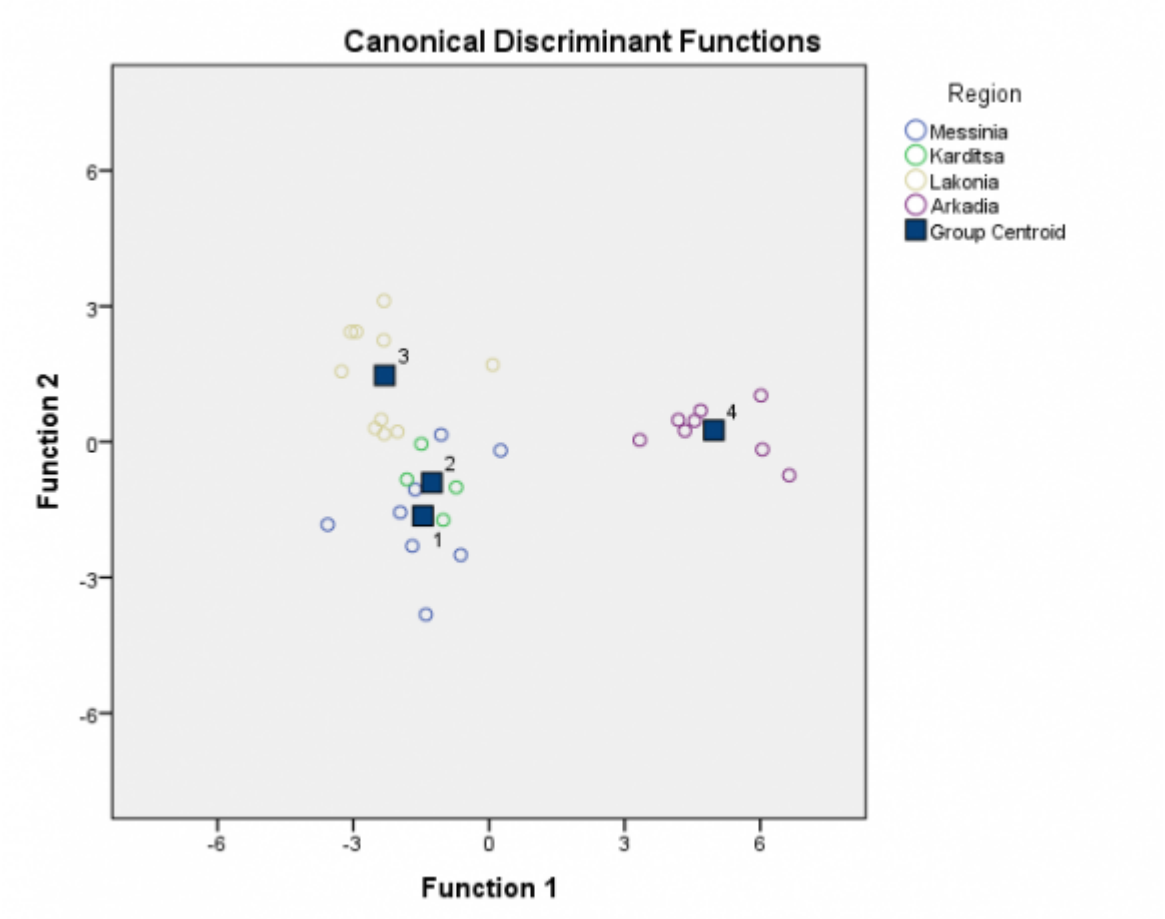

Figure 1:

al. (2011) who reported
(F+G) ranging between 32.60-38.20 g/100g for Menalon fir honey. In the same directive the sucrose content must be ? 5g/100g. Only five samples from Lakonia (nos. 5-9) exhibited this upper limit. No limits have been set for the maltose content in fir honey by the Greek Ministry of Agricultural Development and Food or the European Council Directive relating to honey (110/EC, 2001).

[Note: Oddo et al., (1995) in 52 honeydew honeys (Abies spp.) analyzed reported fructose, glucose, sucrose and maltose values (g/100g) ranging between: 24.50 and 35.80, 18.0 and 28.60, 0.4 and 1.8 and 0.4 and 1.60, respectively.Mateo and Bosch-Reig (1997), in an effort to characterize honeydew Spanish honeys reported values (g/100gMaltose was the major disaccharide present in 80 genuine Brazilian honey samples (mostly Eucalyptus spp., extra-floral, and multifloral honeys) with a mean value of 3.05g/100 g (Da CostaLeite et al., 2000). In this case, maltose was considered as marker for the geographical classification of honey. These reported values for maltose are in very good agreement with present results regarding fir honeys collected from Lakonia and Arkadia regions (Table1).Cotte et al. ()]

Figure 2:

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[2]Monitoring Major Sugars in Greek Commercial Fir Honey and their Role in Geographical Differentiation, using Chemometrics

	Region	Fructose	Glucose	Maltose	Sucrose	F/G	F+G	F+G+M+S
	Messinia	42.48	39.21	nd	0.28	1.08	81.70	81.97
	Messinia	38.01	38.00	nd	2.68	1.00	76.01	78.69
	Messinia	25.02	11.91	0.33	1.04	2.10	36.94	38.30
	Messinia	34.18	36.93	1.37	1.84	0.93	71.11	74.32
	Messinia	25.43	24.25	0.61	0.83	1.05	49.68	51.13
	Messinia	37.24	24.25	0.58	0.97	1.54	61.49	63.03
Year	Messinia	35.15	23.64	0.41	2.10	1.49	58.79	61.30
2016	Messinia	38.23	24.40	0.45	1.97	1.57	62.63	65.06
	Mean ±SD	34.47 6.21	27.82	0.47	1.46	1.34	62.29	64.23
			9.44	0.43	0.80	0.40	14.43	14.54
	Karditsa	30.38	28.60	0.21	1.16	1.06	58.98	60.35
	Karditsa	27.62	27.05	0.73	1.37	1.02	54.67	56.77
Volume	Karditsa	34.55	25.91	0.68	1.19	1.33	60.46	62.33
XVI	Karditsa	31.39	25.40	0.99	1.07	1.24	56.79	58.86
Is-	Mean ±SD	30.99	26.74	0.65	1.20	1.16	57.73	59.58
sue	Lakonia	2.86 28.45	1.42	0.33	0.13	0.15	2.53 56.34	2.35 59.69
II	Lakonia	26.75	27.89	0.82	2.53	1.02	53.13	55.98
Ver-	Lakonia	26.30	26.38	0.62	2.23	1.01	50.66	52.52
sion	Lakonia	26.87	24.36	0.31	1.55	1.08	52.27	54.23
I	Lakonia	28.77	25.40	0.21	1.76	1.06	49.64	57.06
	Lakonia	32.53	20.88	2.23	5.19	1.38	58.38	70.76
	Lakonia	32.64	25.85	5.38	6.99	1.26	58.62	71.42
	Lakonia	32.99	25.98	5.27	7.53	1.26	60.38	73.36
	Lakonia	29.77	27.39	5.30	7.68	1.20	49.64	63.11
			19.87	5.65	7.81	1.50		
D	Lakonia	30.96	22.55	5.69	4.92	1.37	53.51	64.13
D	Mean	29.60	24.65	3.15	4.82	1.21	54.26	62.23
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(	±SD	2.57	2.71	2.50	2.61	0.17	3.93	7.57
	Arkadia	25.51	8.46	4.35	0.27	3.01	33.97	38.59
	Arkadia	26.04	6.56	3.61	0.45	3.97	32.60	36.66
	Arkadia	22.21	7.76	3.66	0.78	2.86	29.96	34.40
	Arkadia	22.89	7.90	3.39	0.82	2.90	30.79	35.01
	Arkadia	21.87	7.55	5.00	0.83	2.90	29.42	35.25
	Arkadia	23.94	9.56	3.72	0.82	2.51	33.49	38.03
	Arkadia	24.86	9.50	3.72	0.82	2.62	34.36	38.90
	Arkadia	27.04	13.24	3.72	0.82	2.04	40.28	44.82
	Mean	24.30	8.82	3.90	0.70	2.85	33.11	37.71
	±SD	1.88	2.05	0.52	0.22	0.55	3.44	3.35

Figure 3:

2

Sugar data (g/100g)	Discriminant function 1 84.7%	Discriminant function 2 14.4%	F	p
Sucrose	-1.496	0.694	12.343	<0.001
Maltose	1.370	0.589	9.472	<0.001
F/G	-0.067	-0.809	37.949	<0.001
Fructose			9.665	<0.001

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Figure 4: Table 2 :

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