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Research Article

Origin Estimation of Honey Samples by Using Constant and Discriminative Function Coefficients of Pure Honey and Honey Produced by Colonies Feeding with Different Sugars

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Abstract

In this study, 25 chemical characteristics of 60 pure and adulterated honey samples obtained from feeding honeybee colonies with different syrup levels (20 and 100 L/colony) of High Fructose Corn Syrup 85 (HFCS-85), High Fructose Corn Syrup 55 (HFCS-55) and sucrose (SS) were statistically analysed in order to determine their discriminative power using a Stepwise Method. Seven characteristics including C_4%, vitamin C, Fructose/Glucose (F/G), viscosity, invertase and the difference between the $\delta^{13}C$ value of honey and its protein ($\Delta\delta^{13}C_{p\text{-}h}$) were found to be discriminative. These seven characteristics allowed 60 honey samples to be grouped in their original groups with complete accuracy. The original sources of eight honey samples of unknown origin could be identified by using Standard Multivariate Canonical Discriminant Function and Constant Descriptive Coefficients (SMCDFCDC) belonging to the seven biochemical characteristics. It is possible to identify any honey sample of unknown origin taken from the market or brought to the laboratory for analysis as pure or adulterated by using these functions and descriptive coefficients.

Keywords: *Biochemistry, Colony, Commercial sugars, Discriminant analysis*

Saf ve Değişik Şekerlerle Beslenmiş Kolonilerden Üretilmiş Ballara Ait Sabit ve Ayrımsama Fonksiyonu Katsayıları İle Bal Örneklerinin Kaynağının Tahmini

Özet

Bu çalışmada, balarısı kolonilerinin farklı şurup seviyelerinde (20 l ve 100 l/koloni) Yüksek Früktoz Mısır Şurubu 85 (YFMŞ-85), Yüksek Früktoz Mısır Şurubu 55 (YFMŞ-55) ve Sukroz (SS) beslenmesi ile elde edilen 60 adet saf ve katkılı bal örneğinin 25 kimyasal karakteristiğinin ayrımsama gücünü belirlemek amacıyla adımsal yöntemle istatistiksel analize tabi tutulmuştur. $\%C_4$, Vitamin C, Früktoz/Glikoz (F/G), viskozite, İnvertaz ve bal ve bal proteinine ait $\delta^{13}C$ değeri farkı ($\Delta\delta^{13}C_{p\text{-}h}$) başta olmak üzere yedi özellik ayrımsayıcı olarak belirlenmiştir. Bu yedi özellik 60 bal örneğinin tam doğrulukla orijinal gruplarına ayrılabilmesini sağlamıştır. Kaynağı bilinmeyen sekiz bal örneğinin orijinal kaynağı yedi biyokimyasal özelliğe ait Standart Çoklu Kanonik Ayrımsama Fonksiyonu ve Sabit Tanımlama Katsayısı (SMCDFCDC) kullanılarak tanımlanabilmiştir. Marketlerden alınan ya da analiz için laboratuvarlara getirilen kaynağı bilinmeyen balların katkılı olup olmadığı bu çalışmada ortaya konulan fonksiyonlar ve tanımlayıcı katsayılar ile belirlenebilir.

Anahtar sözcükler: *Biyokimya, Koloni, Ticari şekerler, Ayrımsama analizi*

INTRODUCTION

Honey is vulnerable to various adulterations at each stage of production and processing [1-3]. Honey can be adulterated by adding different industrial sugar syrups (glucose and fructose) obtained from starch by heat, enzyme or acid treatment to the honey [4] or by feeding the bee colonies with excessive amounts of these syrups during the main nectar flow period [5,6]. These practices not only deteriorate honey quality but also lead to losses for

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unadulterated honey producers and cheat consumers [7,8].

Different methods are used to determine the botanical and geographical origins of honeys and also to detect whether honey samples are adulterated or not [9]. Many characteristics have been evaluated for different purposes. These characteristics are as follows: amino acid content [9], carbon isotope ratio ($\delta_{13}C/_{12}C$) and C_4% rate [1,4,6,10], protein profile, aroma, melissopanalogic analysis [11-13], organoleptic characteristics [14] and biochemical characteristics [2,5,15]. There has been discussion as to which characters or methods are reliable in distinguishing adulterated honey produced by adding sugar syrup (direct adulteration) or by excessively feeding bee colonies with industrial sugar syrup (indirect adulteration). Although carbon isotope ratio ($\delta_{13}C/_{12}C$) and C_4% rate have been accepted as the most reliable characteristics, they are not sufficient for discriminating honeys adulterated using sugars originating from C_3 plants such as sugar cane and wheat [4,6,16].

Statistical methods such as Canonical analysis, Principal Component analysis [17,18] and Multivariate Discriminant Analysis [8,10,19,20] have been used with the aim of classifying pure and adulterated honey samples. Each of these methods has advantages and disadvantages. The Multivariate Discriminant Analysis method is used to determine whether the origins of different biological units are different or not [21,22]. Furthermore, this method offers the opportunity to determine the origin of unknown honey samples using Standard Multivariate Canonical Discriminant Function and Constant Descriptive Coefficients (SMCDFCDC). Thus, Guler *et al*.[14] showed that this method was able to discriminate unadulterated honey samples from sucrose-adulterated ones using organoleptic characteristics.

In addition, greater cost and time are required to determine whether honey samples produced from many different sources are adulterated or not using 25-30 chemical characteristics. For this reason, our aim was to determine whether Multivariate Discriminant Analysis Stepwise Method (MDASM) can be used to discriminate adulterated and unadulterated honey samples using fewer biochemical characteristics. In the present study, the aims were: 1) to determine SMCDFCDC for each biochemical characteristic of pure and adulterated honey samples produced from 20 and 100 L/colony levels of HFCS.85, HFCS.55 and sucrose (SS) sugar syrups by analyzing 25 biochemical characteristics via the MDASM method, 2) to determine whether it is possible to discriminate adulterated honey samples, and 3) to estimate the origin of unknown honey samples by using these coefficients.

MATERIAL and METHODS

Materials

This study was carried out between 2011 and 2013 at the Apicultural Research and Application Unit of the Agricultural Faculty of Ondokuz Mayis University, Samsun, Turkey. Types, origins, compositions, forms, proportions and company's names of the industrial sugars used in the study are summarized in *Table 1*.

Methods

Colony management and honey production: Colonies with two aged queen bees of the same genetic origin were used in the study. All of the environmental factors (frames covered with adult bees, frames covered with brood, foundation comb, drugs, transport) were equalized, and all maintenance and control procedures were performed by the same staff. Honeys from all treatments group were produced by the shaking method [14]. After settling bees in the empty hives, cake and syrup were not further provided to the colonies and veterinary drugs were not used for any honeybee diseases. Levels of 20 and 100 L/colony of HFCS.85, HFCS.55 and SS were used first in the study. Syrup was applied at different intervals (eight times for the 20 L/colony and forty times for the 100 L/colony). Before new syrup application, the amount of unconsumed syrup (g/colony) was recorded on each colony's card. Sources and characteristics of the Industrial sugars used in the study are summarized in *Table 1*. A total of 60 honey samples {(HFCS.85-20 L/colony = 6 + HFCS.85-100 L/colony = 6 + HFCS.55-20 L/colony = 6 + HFCS.55-100 L/colony = 6 + SS-20 L/colony = 6 + SS-100 L/colony = 12 + pure honey = 18) = 60)} were analyzed using the analytical methods described below.

Analytical Methods: Honey samples were analysed for the characteristics given as quality criteria by the International Honey Commission (IHC) [2,15]. Moisture was measured at 20°C by an Abbe Refractometer by a refractive method [23]. Fructose, glucose, maltose, and sucrose were

Table 1. *Types, origins, compositions, forms and proportions of the industrial sugars used in the experiment*
Tablo 1. *Denemede kullanılan ticari şekerlerin tip, kaynak, kompozisyon, form ve oranları*

Sugar Type	Origin of Sugar	Form	Composition	Usage Proportion (water:sugar; w:w)	Company Name
HFCS.85	Corn *(Zea mays)*	Liquid	84.9% fructose 12.8% dextrose	1:3	Cargill
HFCS.55	Corn *(Zea mays)*	Liquid	55.6% fructose 39.6% dextrose	1:3	Cargill
SS	Beet sugar *(Beta vulgaris)*	Crystalline	99.5% sucrose	1:1.5	Turhal Sugar Company (Turkey)

identified and determined by high performance liquid chromatography (HPLC) according to DIN 10758 [24]. Hydroxymethylfurfural (HMF) was determined spectrophotometrically as outlined by Harmonization methods of the International Honey Commission (IHC). The diastatic activity was based on starch hydrolysis [23] as 300/time to a value of absorbance of 0.235 at 660 nm. A weighed sample was ignited in a muffle furnace at 550°C to a constant weight for ash determination [23]. Potassium was determined using an Atomic Absorbance Spectrophotometer (AAS) according to AOAC [23] method 985.35. Proline was determined spectrophotometrically using ninhydrin in methyl cellosolve, and the absorbance was read at 512 nm. A standard curve using pure proline was constructed according to AOAC [23] method 979.20. After calibrating the conductimeter, the electrical conductivity of each honey solution at 20% dry matter was measured at 20°C according to the Harmonised methods of the IHC [2]. Free acidity was determined photometrically by AOAC [23] method 962.19, and vitamin C and vitamin B_5 were quantified by R-Biopharm Vitafast Panthotenic Acid, Microbiological microtiter Plate Test. For pure blossom honey (control), and adulterated honey samples: $\delta^{13}C$ values were determined by isotope ratio mass spectrometry (EA-IRMS) after complete sample combustion to carbon dioxide, as described by AOAC [23] method 991.41. The C_4% sugar contents in honey samples were determined using the AOAC (998.12) standard [1,4,7].

Statistical analysis: The Multivariate Discriminant Stepwise Analysis Method (MDASM), which determines differences and grouping levels in terms of biochemical characteristics between more than two biological sources, was used to determine the SMCDFCDC of pure and adulterated honey samples produced from 20 and 100 L/colony syrup levels of HFCS.85, HFCS.55 and SS [22]. The territorial regions of honeys in a Coordinate system were determined and standardized using these SMCDFCDC *(Fig. 1)*. Then, eight samples were randomly selected from a total of 60 honey samples. The origins of these eight samples were kept confidential. The real groups of these eight unknown samples were confirmed using the SMCDFCDC *(Table 4)*. To achieve this aim, the Score Function 1 (SF1) and Score Function 2 (SF2) were calculated [21,22].

Data were evaluated in two steps. First, MDASM was applied to the data to determine the differences between honeys produced with different commercial sugar syrup levels (20 and 100 L/colony) in terms of a great number of biochemical properties and to determine the descriptive SMCDFCDC of the biochemical properties of seven original honeys. Second, a model for predicting unknown honey samples was developed using the SMCDFCDC of biochemical properties of these original honeys [21]. All analysis was executed using SPSS [25] with licence of Ondokuz Mayis University.

RESULTS

The results of the ANOVA are presented in *Table 2*. Except for F+G, there were significant differences ($P<0.001$) between sugar types and syrup levels in terms of the investigated 25 biochemical characteristics. As shown in *Table 2*, it was rather difficult to discriminate adulterated honey samples from pure samples by assessing many biochemical characteristics according to ANOVA. For this reason MDASM was used for that purpose.

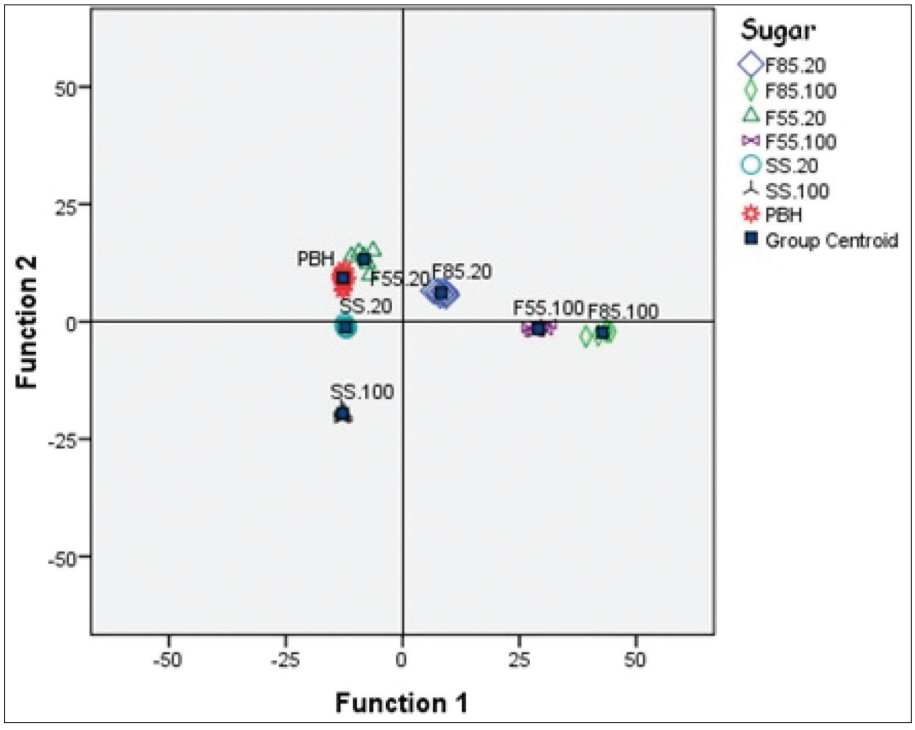

Fig 1. The distribution regions of 60 pure and adulterated honey samples in coordinate system according to the Multivariate Discriminant Stepwise Method. The horizontal axis, the vertical axis and square icon represent 1st canonical function, 2nd canonical function and the centre of each honey group, respectively

Şekil 1. Adımsal Çok Değişkenli Ayrımsama Yöntemine göre 60 adet saf ve katkılı bal örneğinin dağılım bölgeleri. Yatay eksen, dikey eksen ve kare simge sırasıyla 1. kanonik fonksiyonu, 2. kanonik fonksiyonu ve her bal grubunun merkezini göstermektedir

Table 2. *The means ($\bar{X}$) and pooled standard error (PSE) values of biochemical characteristics of pure (PBH) and adulterated honeys produced by feeding bee colonies with HFCS.85, HFCS.55 and sucrose syrups (SS)*

Tablo 2. *YFMŞ.85, YFMŞ.55 ve Sukroz şurubu (SS) ile beslenmiş balarısı kolonilerinden elde edilen saf ve katkılı ballara ait biyokimyasal özelliklerin ortalama ($\bar{X}$) ve bileşik standart hata (PSE) değerleri*

Sugar Syrup Level	HFCS.85		HFCS.55		SS		PBH	PSE
	20	100	20	100	20	100		
Water	19.20^{e}	16.77^{b}	17.07^{bc}	18.40^{d}	18.20^{d}	15.72^{a}	17.93^{c}	0.04
pH	14.7^{c}	9.2^{a}	16.0^{d}	11.0^{b}	15.5^{d}	8.0^{a}	16.8^{e}	0.02
HMF	6.27^{a}	10.68^{b}	6.27^{a}	10.93^{b}	4.67^{a}	4.68^{a}	3.71^{a}	0.99
Proline	530.00^{c}	279.17^{a}	618.17^{d}	348.67^{b}	704.50^{e}	249.33^{ab}	768.2^{e}	7.98
EC	0.201^{cd}	0.117^{a}	0.203^{cd}	0.138^{b}	0.195^{c}	0.130^{b}	0.213^{d}	0.006
Diastase	7.70^{b}	7.70^{b}	7.14^{a}	7.70^{b}	7.70^{b}	7.70^{b}	7.70^{b}	0.00
Invertase	58.15^{ab}	57.65^{a}	70.20^{d}	62.57^{c}	60.23^{b}	58.53^{ab}	59.33^{b}	0.58
α- Glucosidase	27.43^{b}	27.64^{b}	31.23^{c}	26.94^{b}	26.40^{b}	22.38^{a}	27.36^{b}	0.83
Fructose	44.02^{b}	57.07^{c}	37.78^{a}	37.20^{a}	38.95^{a}	36.07^{a}	35.49^{a}	0.66
Glucose	24.00^{b}	17.08^{a}	29.35^{cd}	27.85^{c}	30.27^{de}	30.40^{e}	30.35^{e}	0.47
Sucrose	0^{b}	0 ^{b}	0 ^{b}	0 ^{b}	0 ^{b}	3.05^{a}	0 ^{b}	0.01
F+G	68.02^{a}	74.15^{b}	67.13^{a}	65.05^{a}	69.22^{a}	66.47^{a}	65.84^{a}	1.05
F/G	1.84^{c}	3.35^{d}	1.29^{b}	1.34^{b}	1.29^{b}	1.19^{a}	1.17^{a}	0.02
G/water	1.250^{b}	1.019^{a}	1.719^{de}	1.514^{c}	1.663^{d}	1.934^{f}	1.693^{e}	0.03
Vit C	1.89^{d}	0.20^{ab}	3.70^{f}	0.34^{b}	1.30^{c}	0.19^{a}	2.94^{e}	0.03
Vit B_5	0.077^{c}	0.062^{b}	0.084^{cd}	0.059^{b}	0.086^{d}	0.050^{a}	0.094^{e}	0.002
Ash	0.113^{c}	0.056^{a}	0.098^{bc}	0.055^{a}	0.082^{ab}	0.070^{a}	0.109^{bc}	0.006
Na	0.792^{d}	0.585^{ab}	0.809^{bcd}	0.465^{a}	0.699^{bcd}	0.746^{cd}	0.603^{bc}	0.05
K	16.88^{b}	6.81^{a}	20.9^{b}	7.6^{a}	15.05^{b}	7.34^{a}	18.11^{b}	0.42
K/Na	19.69^{b}	12.87^{c}	26.67^{a}	16.35^{c}	21.60^{b}	9.86^{c}	30.75^{a}	1.41
δ^{13}Cprotein	-24.82^{d}	-23.38^{e}	-25.07^{c}	-23.38^{e}	-25.4^{b}	-25.57^{a}	-25.97^{a}	0.06
δ^{13}C honey	-21.70^{c}	-15.87^{e}	-24.52^{b}	-17.2^{d}	-25.75^{a}	-25.75^{a}	-26.07^{a}	0.09
$\Delta\delta^{13}C_{p\text{-}h}$	-3.12^{c}	-7.52^{a}	-0.55^{d}	-6.18^{b}	0.35^{e}	0.18^{e}	0.10^{e}	0.09
C_4%	20.62^{c}	54.77^{e}	3.67^{b}	45.2^{d}	0^{a}	0^{a}	0.09^{a}	0.39
Viscosity	5111.17^{a}	14605.42^{d}	15650.08^{d}	7611.08^{b}	8888.83^{b}	33111.0^{e}	10773.2^{c}	312.2

HFCS: *High Fructose Corn Syrup,* ***$\Delta\delta^{13}C_{p\text{-}h}$:*** *Difference between the δ^{13}C value of honey and its protein,* ***HMF:*** *Hydroxymethylfurfurol,* ***EC:*** *Electrical conductivity,* ***δ^{13}C:*** *Carbon, ●values within rows with different superscripts differ significantly at P<0.05*

Determination of Discriminating Biochemical Characteristics Using the Stepwise Method

The SMCDFCDC of seven biochemical characteristics that were found to be significant (P<0.001) in classifying honey samples according to step order are given in *Table 3*. The C_4%, Vit C, F/G, viscosity, invertase, $\Delta\delta^{13}C_{p\text{-}h}$ and proline were found to be significant (P<0.001) in discriminating honey samples. In addition, 60 honey samples were classified in their original groups with 100% accuracy when they were evaluated according to these seven biochemical characteristics *(Fig. 1)*.

In total, 6 functions were found to be significant in classification. However, while the 1st Discriminant Function defined the 66.4% of the total variance, the 2nd and 3rd functions defined 21.7 and 8.4%, respectively. These functions altogether defined 96.5% of the total variance. The 4th, 5th and 6th functions defined only 2.9, 0.4 and 0.3% of the total variance, respectively. Furthermore, while C_4% and $\Delta\delta^{13}C_{p\text{-}h}$, which were successful in 1st step, were represented by the 1st discriminant function, Vit C, F/G ratio, viscosity, invertase and proline were represented by the 2nd, 3rd, 4th, 5th and 6th functions, respectively.

Determination of Standard Original Distribution Areas of Honeys Produced with Different Sugar Syrups in the Coordinate System

The projection and intersection regions of pure and adulterated honey samples are shown in coordinate system *(Fig. 1)*. The first discriminant function was able to differentiate the adulterated honey samples produced by the 100 L/colony of HFCS.55 from; i) pure honey samples, ii) 20 L/colony of HFCS.55 and iii) 20 and 100 L/colony of SS. This function represented C_4% and Δδ13Cp-h. This

Table 3. *The unstandardised canonical discriminant functions and constant coefficients of biochemical characteristics to be used for classification of pure and adulterated honeys*

Tablo 3. *Saf ve katkılı balların sınıflandırılması için kullanılan biyokimyasal özelliklerin standardize edilmemiş kanonik ayrımsama fonksiyonları ve sabit katsayıları*

Characters	Canonical Discriminant Function Coefficients					
	1	2	3	4	5	6
C_4%	0.819	0.345	-0.538	0.386	-0.183	1.046
Vit C	-0.104	6.652	1.321	3.375	-2.269	0.077
F/G	2.975	0.914	15.588	-3.329	2.821	-0.370
Viscosity	0.000	0.000	0.000	0.000	0.000	0.000
Invertase	0.097	0.104	-0.006	0.304	0.634	0.016
$\Delta\delta^{13}C_{p\text{-}h}$	-0.399	1.241	-1.605	0.751	-0.142	6.697
Proline	-0.003	0.007	0.004	0.000	0.001	0.011
Constant coefficients	-18.867	-19.218	-27.185	-30.988	-33.969	-11.309

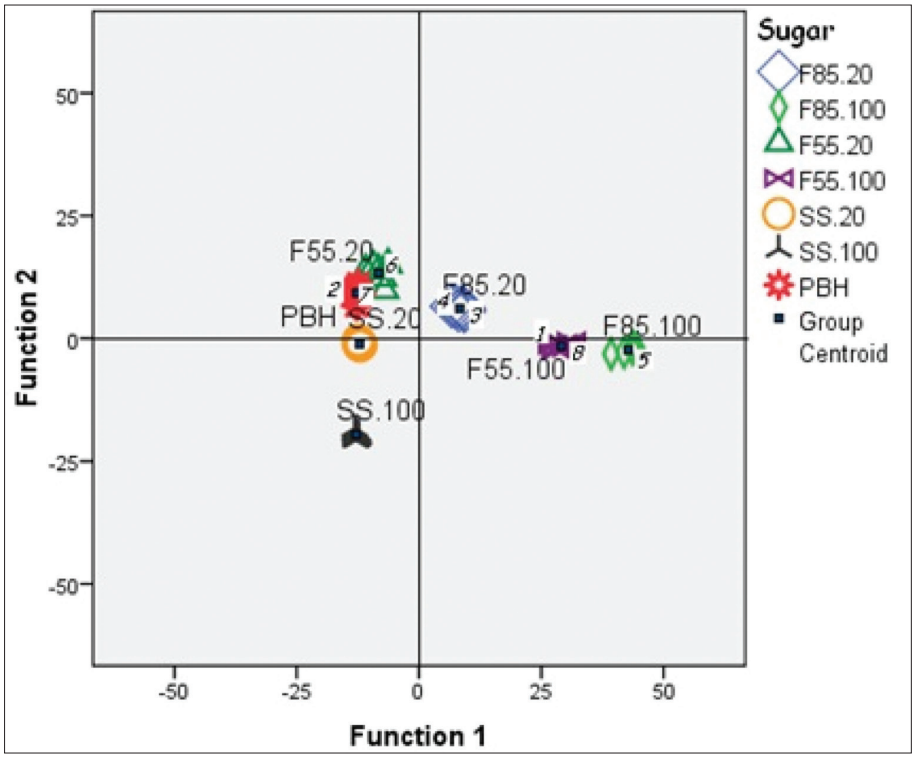

Fig 2. Real grouping regions of unknown eight honey samples in coordinate system

Şekil 2. Koordinat sisteminde kaynağı bilinmeyen sekiz bal örneğine ait gerçek gruplama bölgeleri

function was also accepted as a differentiation function that can differentiate adulterated honey samples produced from C_3 and C_4 plants.

Furthermore, the 2nd discriminant function was found to be effective in discriminating adulterated honey samples produced using the 100 L/colony of HFCS.85 and HFCS.55 and the 20 and 100 L/colony of SS from pure honey samples. Also, the honey samples produced from the 20 L/colony of HFCS.55, SS and PBH were grouped in the same coordinate axis although they all were completely different from each other. The adulterated honey samples produced from the 100 L/colony of HFCS.85, HFCS.55 and SS were grouped in the farthest region of the coordinate axis. Furthermore, adulterated honey samples produced using the 100 l/colony of HFCS.85 and HFCS.55 were located along the same axis.

Verification Test

For the verification test eight honey samples were selected randomly from the 60 samples. The production method, honey type (pure or adulterated) and number of these 8 samples were kept secret and the source of these 8 samples was unknown during analysis. Prior to analysis the samples were coded as UnS_1, UnS_2,...,UnS_8. The region of each unknown honey sample was determined using SMCDFCDC *(Table 4)*. Two score functions (SFs) were calculated with the aim of determining the groups. While calculating these functions, the standard first discriminant

Table 4. *Standardised canonical classification functions and constant descriptive coefficients for seven biochemical characteristics to be used for classification of pure and adulterated honey samples, and calculation of score functions related to the unknown samples*

Tablo 4. *Saf ve katkılı bal örneklerinin sınıflandırılmasında kullanılan yedi biyokimyasal özellik için standardize edilmiş kanonik sınıflama fonksiyonu ve sabit tanımlama katsayıları ve bilinmeyen örnekler için skor fonksiyonlarının hesaplanması*

Characteristic	Canonical Classification Coefficients						Unknown Sample (X_i)	SCORE Func.1	SCORE Func.2
	F1(α_1)	F2(α_2)	F3(α_3)	F4(α_4)	F5(α_5)	F6(α_6)			
C_4	0.819	0.345	-0.539	0.386	-0.183	1.046	44.5	36.4455	15.3525
Proline	-0.003	0.007	0.004	0.000	0.001	0.011	341	-1.023	2.387
Viscosity	0.000	0.000	0.000	0.001	0.000	0.000	9500	0	0
$\Delta\delta^{13}C_{p\text{-}h}$	-0.399	1.241	-1.605	0.751	-0.142	6.697	-6.1	2.4339	-7.5701
Vit C	-0.104	6.652	1.321	3.375	-2.269	0.077	0.34	-0.03536	2.26168
Invertase	0.097	0.104	-0.006	0.304	0.634	0.016	63.5	6.1595	6.604
F/G	2.975	0.914	15.588	-3.329	2.821	-0.370	1.35	4.01625	1.2339
Constant (α_0. β_0)	-18.87	-19.22	-27.19	-30.99	-33.97	-11.31		48.00	20.27
Coordinate scores of sample								29.13	1.05

$\Delta\delta^{13}C_{p\text{-}h}$: *The difference between the $\delta^{13}C$ value of honey and its protein*

Table 5. *Analysis results of eight unknown honey samples related to the seven biochemical characteristics*

Tablo 5. *Yedi biyokimyasal özelliğe bağlı olarak sekiz bilinmeyen bal örneğine ait analiz sonuçları*

Characters	Samples of Unknown Origin							
	UnS_1	UnS_2	UnS_3	UnS_4	UnS_5	UnS_6	UnS_7	UnS_8
$\%C_4$	44,50	0.00	21.20	0.00	54.20	3.90	0.00	44.10
Vit C	0.34	2.86	1.97	1.37	0.22	3.84	0.16	0.31
F/G	1.35	1.16	1.87	1.29	3.19	1.29	1.21	1.32
Viscosity	9500.0	11266.5	5300.0	8900.0	15066.5	16733.5	32166.5	8700.0
Invertase	63.50	59.60	57.4	58.4	58.20	69.3	57.20	62.40
$\Delta\delta^{13}C_{p\text{-}h}$	-6.10	0.10	-3.20	0.50	-7.70	-0.60	0.00	-6.00
Proline	341,00	748.00	516.00	736.00	282.00	642.00	247	358.00

$\Delta\delta^{13}C_{p\text{-}h}$: *The difference between the $\delta^{13}C$ value of honey and its protein*

function coefficient (a_i) of each property was multiplied by the value of this property given by the analysis of (X_1, X_2,…, X_n) additional samples. Then this value added to the constant coefficient of Function 1 and so SF1 was calculated. SF2 was calculated in a similar way *(Table 4)*. In the coordinate system *(Fig. 1)* SF1 is the apsis and SF2 is the ordinate [21,22]. For each sample two score functions were calculated using equations 1 and 2 given below.

Score Function 1=$a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_4x_4 + a_5x_5 + a_6x_6 + a_7x_7$ (1st correlation)

Score Function 2=$\beta_0 + \beta_1x_1 + \beta_2x_2 + \beta_3x_3 + \beta_4x_4 + \beta_5x_5 + \beta_6x_6 + \beta_7x_7$ (2nd correlation)

The calculated values of these SFs were located in places of F1 and F2 in the standard clustering diagram *(Fig. 1)* and so the real group of this sample was determined. The SF1 and SF2 were calculated for samples of unknown origin and then the clustering regions of these samples were determined in the coordinate system *(Fig. 2)*. Furthermore, the Excel Programme was used for easy calculation of SF1 and SF2 and then the method was standardized *(Table 4)*.

The 1st and 2nd SFs of 8 randomly selected honey samples from 60 of unknown origin were calculated as follows:

UnS_1: SF1=29.13; SF2=1.05

UnS_2: SF1= -12.22; SF2=12.43

UnS_3: SF1=9.15; SF2=8.52

UnS_4: SF1=11.91; SF2=2.92

UnS_5: SF1=42.86; SF2=2.33

UnS_6: SF1= -7.20; SF2=19.81

UnS_7: SF1= -10.48; SF2= -9.37

UnS_8: SF1=28.52; SF2=0.81

When the SF1 and SF2 values were inserted in the coordinate system *(Fig. 2),* the UnS1 coded sample overlapped with the 100 L/colony of HFCS.55. Similarly, UnS_2 overlapped with pure honey (PBH), UnS_3 with the 20 L/colony of HFCS.85, UnS_4 with the 20 L/colony of HFCS.85, UnS_5 with the 100 L/colony of HFCS.85, UnS_6 with the 20 L/colony of HFCS.55, UnS_7 with pure honey (PBH) and UnS_8 with the 100 L/colony of HFCS.55. Thus, the origins

of all 8 unknown origin honey samples were determined by using a confirmation test. More importantly, there was no overlap among the samples. The analysis results for the 8 unknown origin honey samples in relation to the 7 biochemical characteristics are shown in *Table 5*.

DISCUSSION

Many biochemical characteristics of pure and adulterated honey samples produced by feeding bee colonies with different syrup levels of various industrial sugars are significantly different. These differences are greater in syrup level when compared to sugar type. The biochemical characteristics of pure and adulterated honey samples determined in the present study are compatible with previous studies, and also with international standards [1,3,6,9].

The Stepwise method could group the 60 honey samples with 100% accuracy. The honey samples were clustered in different regions in the coordinate system. The grouping levels, clustering regions and importance of discriminant functions all indicated that the honey samples originated from different sugar sources. Thus correct classification level of the 60 samples was 100% and values of Wilks' λ indicating the importance of the first and second discriminant functions were found to be λ=0.002 and λ=0.000, respectively. The high (100%) classification ability of the Stepwise method has been reported previously by many authors [8,10,20,26,27].

The C_4%, vitamin C, F/G ratio, viscosity, invertase, $\Delta\delta^{13}C_{p\text{-}h}$ were found to be successful in discrimination of honey samples in the Stepwise method, which was applied to 25 biochemical characteristics. The C_4% ranked first (1st step) and the classification ability of this characteristic is evident from its relationship with the 1st discriminant function, because the relationship of this function with total variation was found to be very high (r=0.995). Furthermore, this function could define the differences among 60 honey samples at a 66.4% level. The C_4% ratio was determined to be the most important criterion for determining whether sugar or syrups originating from C_4 plants were added to honey directly or indirectly by bee feeding as in the present study. This ratio has been reported to not be higher than 7% [1,16]. For this reason, this characteristic, which is considered a formal method, creates the basis for many standards [2,15,28]. The finding that the C_4 ratio ranked first in the Stepwise method confirms the importance of this characteristic as mentioned in previous studies [1,4,16]. Similarly, the difference between the $\delta^{13}C$ value of honey and its protein, which is used to determine the C_4 plant-derived adulteration, was found to be significant in the Stepwise method.

In the present study, discriminative biochemical characteristics (C_4%, Vitamin C, F/G, Viscosity, Invertase, $\Delta\delta^{13}C_{p\text{-}h}$ and proline) were different from those reported by Devillers *et al*.[27], Ruoff *et al*.[10], and Guler *et al*.[5]. These differences might be attributed to the differences in sugar types and research methods used in these studies. In the present study, we evaluated adulterated honey samples produced from different syrup levels (20 and 100 L/colony) of HFCS (derived from corn) or sucrose (derived from sugar beet). However, other researchers evaluated pure honey samples produced using different plants [10], monofloral honey samples [9] and polyfloral honey samples [5,10,29]. Consequently, the inconsistent results between the studies are not surprising. For instance, the fructose and glucose ratios of HFCS.85 used in the present study were 84.9% and 12%, respectively. However, many plant nectars do not contain fructose at this level.

So far, proline [5,11], K/Na ratio [10,11], electrical conductivity [5,26] and sugar contents [2,15,28] have been among the characteristics used to discriminate adulterated honey samples produced by using sucrose. Whereas in the present study, electrical conductivity and any sugar did not present in the Stepwise, and proline was found significant only in the 7th step. Vitamin C, viscosity and invertase have taken their place in the upper row in the Stepwise. The inefficiency of proline might be attributed to the fact that the sugars (SS, HFCS.85 and HFCS.55) used to produce adulterated honey samples were derived from C_3 (sugar beet) and C_4 (corn or sugar cane) plants. Thus, the average C_4% sugar content of HFCS.85 originating from corn, and SS originating from sugar beet were found to be significantly different (54.77±0.71 and 0.0±0.0, respectively). However, the average proline contents of these adulterated honey samples (100 L/colony of HFCS.85 and SS) were close to each other *(Table 2)*.

When we evaluated only sucrose-adulterated (20 and100 L/colony of SS) and pure honey samples by stepwise discriminant analysis in terms of 24 biochemical characteristics, proline ranked first in the Stepwise. The relationship among the 24 biochemical characteristics underlines the importance of proline. There were significant relationships ($P<0.001$) between proline and acidity (r=0.969), $\Delta\delta^{13}C_{p\text{-}h}$ (r=0.662), electrical conductivity (r=0.906), vitamin C (r=0.823), vitamin B5 (r=0.966), and K/Na ratio (r=0.742). In addition, there were negative relationships between proline and characteristics causing loss of quality such as the $\delta^{13}C$ value of honey (r=- 0.659), the $\delta^{13}C$ value of protein (r= - 0.588), C_4% ratio r= -0.641, and sucrose (r= - 0.589). This is confirmed by the high multiple regression coefficient of this relationship (R^2=0.922). All these findings showed that proline is more efficient in discriminating adulterated honey samples produced by using sugars originating from C_3 plants compared to C_4 plants.

Similarly, the lack of discriminating effect of sucrose and glucose sugars in discriminating pure and adulterated honeys produced using strong syrups might be attributed to the fact that some biochemical characteristics have extremely different values depending on the type of sugar

(C_3 or C_4). For example, the C_4% ratio of honey coming from the 100 L/colony of HFCS.85 (54.77±0.71%) was significantly different from the value coming from the same syrup level of SS (0.0±0.0%). Similar findings have been reported by other researchers [7,9,11,29]. However, F/G ratio was found to be significant in the 3rd step. The fact that the highest F/G ratio was determined for the 100 L/colony of HFCS.85 (3.35±0.07) confirms this finding. All these results indicated that distinctive biochemical characteristic(s) can change depending on the plant source, honey production method, sugar type (C_3 or C_4), sugar content and amount of sugar syrup given to the colony.

In the present study, the origin of 8 unknown origin honey samples was estimated with 100% accuracy by using biochemical characteristics SMCDFCDC. Hence, through this method it is possible to determine; i) whether honeys found in the market are pure or not, ii) whether they are produced from HFCS.85, HFCS.55 and sucrose (SS), and iii) the syrup levels with which they are produced.

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