applied sciences

MDPI

Article

Determination of the Carbohydrate Profile and Invertase Activity of Adulterated Honeys after Bee Feeding

Dimitrios Kanelis †, Vasilios Liolios †, Chrysoula Tananaki * and Maria-Anna Rodopoulou

Laboratory of Apiculture-Sericulture, Aristotle University of Thessaloniki, 57001 Thessaloniki, Greece; dkanelis@agro.auth.gr (D.K.); vliolios@agro.auth.gr (V.L.); rodopoum@agro.auth.gr (M.-A.R.)
* Correspondence: tananaki@agro.auth.gr; Tel.: +30-2310991753
† These authors contributed equally to this work.

Abstract: The higher demand for honey from consumers, combined with its limited availability, has led to different types of honey adulteration, causing substantial economic as well as negative impacts on consumers' nutrition and health. Therefore, a need has emerged for reliable and cost-effective quality control methods to detect honey adulteration to ensure both the safety and quality of honey. To simulate the process with those applied by beekeepers in real-time, bee colonies were fed with different types of bee feeding (sugar syrup, candy paste and commercial syrup). The produced samples were analyzed for their carbohydrate profile and their invertase activity with the aim to find the effects of bee feeding on the quality of the final product. Honey samples produced after feeding with commercial syrup presented low fructose (22.9 %) and glucose (31.7 %) concentrations and high content of maltose (20.1%), while the samples that came from bee feeding with sugar syrup and candy paste had high concentrations of sucrose (6.2 % and 3.2 %, respectively), exceeding in some cases the legislative limits. Moreover, the samples coming from sugar feeding had lower values of invertase activity, while the group with inverted syrup was clearly discriminated through multi-discriminant analysis. The invertase activity of control samples was found at 153.7 U/kg, which was significantly higher compared to the other groups. The results showed that bee feeding during honey production might lead to adulteration, which can be detected through routine analyses, including the carbohydrate profile and the invertase activity.

Keywords: bee feeding; syrup; honey adulteration; carbohydrate profile; HPLC; invertase activity

Citation: Kanelis, D.; Liolios, V.; Tananaki, C.; Rodopoulou, M.-A. Determination of the Carbohydrate Profile and Invertase Activity of Adulterated Honeys after Bee Feeding. *Appl. Sci.* **2022**, *12*, 3661. https://doi.org/10.3390/app12073661

Academic Editor: Jerzy Wilde

Received: 9 March 2022
Accepted: 3 April 2022
Published: 5 April 2022

1. Introduction

Honey is a foodstuff attracting more and more consumers due to its special sensory characteristics and its nutritional value. Its composition and quality depend on multiple factors such as production methods (beekeeping practices), beekeeping flora, soil and climatic conditions [1,2]. Generally, honey is a functional food with a number of health-promoting properties; however, it can be adulterated through bee feeding with different syrups and other sweeteners during the production season, leading to serious problems not only for beekeepers but also for consumers, causing negative effects on their nutrition and health [3–5].

Bee feeding is one of the basic treatments that beekeepers apply to help their colonies and strengthen their defense mechanisms, either in cases when honeybees are unable to collect the natural resources on their own or in cases of specific beekeeping practices, such as queen rearing, insertion of a new queen in a queenless colony, merging of colonies, etc. According to Goodwin [6], supplementary feeding of colonies improved their pollination capacity significantly, and also, newer research showed that bees' pollination capacity in crops of apples (apples, pears) increased by 130% when they were fed with artificial sugar syrup [7]. The supplements may contain honey, syrup, crystallized sugar, candy paste, pollen, pollen paste and pollen substitutes. Artificial sugar feeding, consisting of

syrups of high-fructose corn, glucose, sugar beet or cane sugar and inverted sugar, is the most common adulteration practice [2,8,9], with the use of inverted sugars remaining high in the preferences of beekeepers. They usually feed their colonies during the blooming season to pursue higher yield production per hive. In this case, this practice may affect the quality of the final product, resulting in the production of adulterated honey. Thus, during the blooming season, any artificial sugar feeding of bee colonies producing honey is unacceptable and is forbidden.

To detect possible adulteration of honey, additionally to microscopic analysis [10], various physicochemical parameters such as proline concentration, 13C/12C isotope analysis and laser-induced breakdown spectroscopy (LIBS) have also been used, while the investigation of the antimicrobial activity of honey has been proposed as a new qualitative parameter [2,5,9,11–13].

Because some of these methods require high technology equipment and are generally not economical, there is a need to develop more practical and less costly methods to detect honey adulteration. Given that the sugar composition of honey is affected by beekeeping treatments [14,15], apart from its botanical or geographical origin [16–18], honey's carbohydrate profile could also be a useful tool for the determination of its quality and the detection of its adulteration.

Various methods for sugar profile analysis are proposed in the literature to determine honey quality [4,14,19,20]. Mainly, high-performance liquid chromatography (HPLC) or gas chromatography (gas chromatography-mass spectrometry -GC-MS) is applied [21,22], while nuclear magnetic resonance (NMR) spectroscopy has recently been used [23]. In addition, the multivariate statistical approach has shown promising results regarding distinguishing between adulterated and non-adulterated honey [3,20].

According to the present literature, samplings come mostly from admixtures of non-adulterated honey with different types of syrup in laboratory tests [3] and not from feeding experiments in the field. For this reason, the aim of this study was to investigate the effect of the type of supplementary feeding and the applied quantity throughout honey's production on the carbohydrate profile and invertase activity of the final product. The field experiments regarding bee feeding occurred in an experimental apiary to simulate the process with those applied by beekeepers in real-time.

2. Materials and Methods

We produced honey samples from bee colonies located in the experimental apiary of Apiculture-Sericulture, AUTH, under different feeding conditions; we determined the concentration of 16 different sugars and the invertase activity of the samples.

2.1. Production and Sampling

For the production of the honey samples, we applied different feeding protocols commonly used by beekeepers in bee colonies throughout the production season. Specifically, the examined groups, from which two collections per group were conducted, are presented in Table 1. The feeding protocols that followed included candy paste, commercial inverted syrup and sugar syrup (1:1) in different quantities per colony. There was also a control group without any artificial feeding; the colonies were fed only through natural nectar collection.

In each colony, two empty frames were placed before the beginning of the experiment, and the honey samples were collected immediately after the sealing of honey cells. The artificial feeding was supplied in colonies gradually, allowing bees to enrich the final product with nectar from the blooming plants and other substances (e.g., enzymes). The second collection was performed one month later, but in group E (candy paste), it was impossible to collect the samples, as the sampling took place after the blooming season, and the colonies consumed the reserves of honey. All the samples were obtained by pressing honeycombs and were filtered to remove possible wax fragments. The samples were placed

in a freezer (−18 °C) until their analysis. In total, 66 samples were derived from the two different samplings and further analyzed.

Table 1. Groups, feeding protocols per group and number of samples collected.

Group (n * = 6)	Feeding Type	Samples (1st Collection)	Samples (2nd Collection)	Quantity per Colony (L/day)
A	Inverted syrup (Commercial syrup)	6	6	2.0
B	Inverted syrup (Commercial syrup)	6	6	0.5
C	Sugar syrup production ratio 1:1	6	6	2.0
D	Sugar syrup production ratio 1:1	6	6	0.5
E	Candy paste (commercial feed)	6	-	Constant presence
F	Control–No artificial feeding	6	6	Nectar collection by bees

* n: Number of colonies per group.

2.2. Carbohydrate Analysis

To determine the carbohydrate profile, high-performance liquid chromatography (HPLC) with a refractive index detector system (RID) [24] was used (Agilent, 1200 series).

Sample preparation and analysis: Honey samples (5.0 g) were mixed with methanol:water (25:75, v/v) solution in a final volume of 50 mL, and the mixture was filtered through a disposable syringe filter 0.45 μm before the injection. The sugars were separated into two columns: the Zorbax Carbohydrate Analysis column (4.6 mm ID × 150 mm × 5 μm) coupled in series, protected by a guard column (NH2 Guard Cartridge, 4.6 mm × 12.5 m). In the mobile phase, a mixture of acetonitrile:water (75:25, v/v) at a flow rate of 1.8 mL min^{-1} was used. The column and the refractive index detector were maintained at 35 °C. The injection volume was 10 μL. For the quantification, a five-point calibration curve was created and evaluated for each sugar.

Calibrations curves: In the present research, we studied 16 different sugars: D(-)-Fructose, D-(+)- Glucose, D(+)- Saccharose, D-maltose monohydrate, D-(+)-Turanose, D-(+)-Trehalose dehydrate, Isomaltose, D(+)-Maltotriose, D-(+)-Melezitose hydrate, Erlose, D-Raffinose pentahydrate, Melibiose, D-panose, Maltulose, Maltotetraose and Isomaltotriose in HPLC grade. To create the calibration curve for each sugar, stock solutions in a concentration of 100 mg mL^{-1} were prepared, diluted in a methanol:water solution (25:75, v/v) and kept in the freezer. Five-point calibration curves in different concentrations were created for each sugar using the stock solutions. Specifically, for fructose and glucose, the concentrations ranged from 0.5 to 40 mg mL^{-1} (0.5, 2, 10, 25, 40 mg mL^{-1}), for sucrose 0.1 to 20 mg mL^{-1} (0.1, 2, 5, 12.5, 20 mg mL^{-1}) and for the rest of the sugars 0.1 to 10 mg mL^{-1} (0.1, 2, 5, 7, 10 mg mL^{-1}). Each mixture of standards (point of calibration curves) was analyzed five times, and the mean value was applied in the calibration curve.

Method Validation: For the determination of the sugars, the HPLC method was validated for linearity, limit of detection (LOD) and limit of quantification (LOQ). Linearity was calculated by least squares linear regression analysis of the calibration curve. The LOD and LOQ values were calculated using the equations from JRC Technical Reports [25]. Ten blank samples containing the mobile phase were analyzed, and the standard deviation of the blank signals was calculated. The equations are given below:

$$\mathrm{LOD} = 3.9 \times \frac{S}{b}$$

where: S: Standard deviation of the blank signals; b: Slope of the calibration curve.

$$\mathrm{LOQ} = 3.3 \times \mathrm{LOD}$$

2.3. Invertase Activity

Procedure: The analysis of the enzyme was performed using a spectrophotometer (Genesys, V10 UV-Vis) and measured at 400 nm [24]. The chemical compound p-

Nitrophenyl-a-D-glycopyranoside (pNPG) was used as a substrate to determine the amount of enzyme; it was divided into glucose and p-Nitrophenol in the presence of invertase. By adjusting the pH to 9.3, the activity of the enzyme inhibits while the nitrophenol is converted to nitrophenol anion, which corresponds to the percentage of the converted substrate, and it is determined spectrophotometrically at 400 nm.

Reagents: The reagents used to determine the invertase enzyme were potassium dihydrogen phosphate (KH_2PO_4), sodium bicarbonate dihydrate ($Na_2HPO_4.2H_2O$) for the preparation of the working solution A, p-Nitrophenyl-aD-glycopyranoside (pNPG) for the working solution B, tris-(hydroximethyl) aminomethane for the working solution C and HCl (3 M) to adjust the pH of solution C. Working solutions based on the above-mentioned reagents were prepared for the analysis of the samples. Specifically, for solution A, reagents KH_2PO_4 and $Na_2HPO_4.2H_2O$ in proportions of 11.66 g L^{-1} and 2.56 g L^{-1}, respectively, were used, to which deionized water was added. Then, a quantity of solution A was mixed with pNPG to a final concentration of 7.5 g L^{-1} to form solution B, which was heated at 40 °C for about half an hour until it was completely dissolved, with continuous stirring. Finally, an aqueous solution of tris-(hydroximethyl) aminomethane with a concentration of 181.71 g L^{-1} was prepared, the pH of which was adjusted to 9.3 using 3 M HCl (solution C).

Sample analysis: Honey samples (2 g) were diluted with solution A to a final volume of 10 mL. After being placed in a test tube, a volume of 5 mL of solution B was heated to 40 °C for five minutes. Then, 0.5 mL of the honey solution was placed in the test tube with solution B and mixed well, while this mixture remained in the water bath for an additional 20 min. After the 20 min, 0.5 mL of solution C were added to the mixture to stop the enzyme reaction, and the test tubes were placed at room temperature for about 20 min until the absorbance of the mixture was measured at 400 nm. For the blank, 5 mL of solution B were placed in a test tube and kept at 40 °C for five minutes. Finally, 0.5 mL of solution C and 0.5 mL of honey solution were added to solution B, and the mixture was kept for 20 min at room temperature until it was measured at 400 nm. Total invertase activity for each sample was calculated using the equation [24]:

$$\text{Invertase} = 158.94 \times (\text{A400} - \text{Ablank}) \text{ (U kg}^{-1}\text{)}$$

2.4. Statistical Analysis

The resulting sugar concentrations were processed by multivariate analysis of variance (MANOVA) to determine statistical significances between the groups and by multi-discriminant analysis (MDA) to visualize class separation via dimensionality reduction. We also applied *t*-tests to examine the effect of feeding amount and sampling on the carbohydrate profile and the invertase activity of the groups. The significance level of the statistical tests was set at $\alpha \leq 0.05$. The analyses were carried out using the Minitab v.20.1.0 software (Minitab, Coventry, UK).

3. Results and Discussion

Calibration Curves: Analyzing the standard stock solutions (100 mg mL^{-1}) of sugars, five times the average retention times of the 16 examined sugars were determined (Table 2). The standard solutions were further analyzed five times and the mean of the repetitions was used to create the calibration curves. The equations of the standard curves of the studied sugars are given in Table 3. All calibration curves showed good linearity in the concentration range of 0.2 to 40 mg mL^{-1} for fructose and glucose, 0.1 to 20 mg mL^{-1} for sucrose and 0.1 to 10 mg mL^{-1} for the other sugars, as they all had a high correlation coefficient (R) ($\geq$0.965) (Figure S1).

Table 2. Average retention time (±sd), equations, correlation coefficient, LOD (mg mL^{-1}) and LOQ (mg mL^{-1}) of the 16 studied sugars.

Standard Solution	Retention Time	Calibration Curve	R	LOD (mg mL^{-1})	LOQ (mg mL^{-1})
Fructose	5.688 (±0.002)	y = 42967x + 2390	0.999	0.022	0.074
Glucose	6.355(±0.006)	y = 39267x + 2667	0.999	0.030	0.099
Sucrose	8.557(±0.001)	y = 43333x + 3347	0.998	0.017	0.058
Turanose	9.311(±0.005)	y = 51050x − 24388	0.986	0.006	0.021
Maltulose	9.689(±0.002)	y = 43706x − 6540	0.991	0.007	0.025
Maltose	10.001(±0.012)	y = 40876x − 7818	0.995	0.007	0.024
Trehalose	10.907(±0.009)	y = 48163x − 28102	0.989	0.009	0.031
Isomaltose	11.811(±0.016)	y = 33258x − 18194	0.984	0.010	0.033
Melibiose	12.858(±0.024)	y = 33214x − 21648	0.985	0.018	0.060
Erlose	13.127(±0.015)	y = 58367x − 46836	0.967	0.005	0.017
Melezitose	14.308(±0.034)	y = 46332x − 13694	0.990	0.007	0.025
Raffinose	16.343(±0.038)	y = 2592x − 26104	0.997	0.016	0.053
Maltotriose	16.836(±0.023)	y = 38501x − 17603	0.998	0.012	0.040
Panose	19.238(±0.031)	y = 59823x − 80025	0.965	0.024	0.080
Isomaltotriose	23.126(±0.028)	y = 25926x − 26104	0.975	0.029	0.096
Maltotetraose	27.909(±0.039)	y = 42102x − 45354	0.979	0.025	0.085

The LOD values were 0.022 and 0.030 mg mL^{-1} for fructose and glucose, respectively, while for the other sugars ranged from 0.005 mg mL^{-1} to 0.029 mg mL^{-1}. The LOQ values were 0.074 and 0.099 mg mL^{-1} for fructose and glucose, respectively, whereas for the other sugars ranged from 0.017 mg mL^{-1} to 0.099 mg mL^{-1}.

Supplementary feedings: Analyzing the syrups and the candy paste, we found that the inverted syrup was composed of fructose, glucose and maltose in concentrations of 19.7%, 17.7% and 28.1%, respectively. In turn, the sugar syrup had fructose, glucose and melezitose in small quantities (1.6%, 4.8% and 5.8%, respectively) and sucrose in high concentrations (34.9%). Finally, the candy paste was composed of fructose, glucose and sucrose in concentrations of 2.3%, 3.2% and 72.1%, respectively. Thus, Groups A and B were fed with syrup with concentrations of maltose, while Groups C, D and E received supplements with high concentration of sucrose.

Effect of feeding quantity: Comparing through *t*-test the honey samples of Group A with Group B (commercial inverted syrup) and Group C with Group D (sugar syrup) (Table 1), which received the same type of feeding but a different amount, we found a similar carbohydrate profile between the compared groups. The p_{value} of the detected sugars ranged from 0.082 to 0.695 and from 0.509 to 0.580 in groups with inverted syrup and groups with sugar syrup, respectively, showing that the amount of feeding (0.5 and 2.0 L per day) for the two different kinds, seem not to affect the sugar content of the produced honey samples significantly.

Effect of sampling time: A *t*-test was also applied in the examined groups for the effect of sampling time on honey's adulteration, where non- significant differences were observed between the first and the second collection regarding the studied characteristics (p: 0.134–0.739, $>\alpha = 0.05$).

Effect of feeding type: Given that the feeding quantity had no effect on the carbohydrate composition, the groups were examined only for the effect of feeding type. Analyzing the samples (Figure S2), significant differences were observed between the feeding groups ($p = 0.00 < \alpha = 0.05$), highlighting the effect of the supplementary feeding on the carbohydrate profile of the honey and the invertase units (Table 3).

The main sugars fructose and glucose were found in all honey samples, with the control and candy paste groups showing significantly higher concentrations compared to the other groups. Moreover, samples derived from groups fed with the invert syrup showed significantly higher levels of maltose, which may be explained by the fact that most commercial invert syrups are made from isoglucose or other composite sugars, such as maltose, which was contained in the inverted syrup used in this study. Likely,

Cordella et al. [3] found that the adulteration with glucose–fructose–maltose syrups resulted in modifications of the honey samples' sugar composition. Furthermore, Al-Mahasneh et al. [26] noted that increasing sucrose syrup in the feeding resulted in a significant decrease in glucose and fructose contents. On the other hand, according to Paradkar and Irudayaraj [27], feeding colonies with small amounts of inverted syrups had a significant effect on the indicated honey's fructose and glucose levels.

Table 3. Average (±sd) concentration of sugars (%) and invertase activity (U kg^{-1}) in different honey samples produced after the application of different feeding types.

	Feeding Type	Average Concentration (±sd)			Feeding Type	Average Concentration (±sd)	
Fructose	Inverted syrup	22.9 ^{a*}	±1.5	Melibiose	Inverted syrup	0.06	±0.3
	Candy paste	38.0 ^{b}	±1.2		Candy paste	n.d.	
	Control	41.2 ^{c}	±1.3		Control	n.d.	
	Sugar syrup	37.0 ^{b}	±2.7		Sugar syrup	n.d.	
Glucose	Inverted syrup	31.7 ^{a}	±3.0	Erlose	Inverted syrup	n.d.	
	Candy paste	34.8 ^{b}	±2.4		Candy paste	1.4 ^{b}	±1.6
	Control	39.9 ^{c}	±1.6		Control	0.6 ^{ab}	±0.6
	Sugar syrup	33.9 ^{ab}	±3.0		Sugar syrup	3.0 ^{c}	±1.3
Sucrose	Inverted syrup	n.d. **		Melezitose	Inverted syrup	n.d.	
	Candy paste	3.2 ^{b}	±2.9		Candy paste	n.d.	
	Control	0.6 ^{a}	±1.0		Control	0.3	±0.3
	Sugar syrup	6.2 ^{c}	±4.5		Sugar syrup	n.d.	
Turanose	Inverted syrup	0.1 ^{a}	±0.3	Raffinose	Inverted syrup	3.7 ^{b}	±4.5
	Candy paste	1.3 ^{bc}	±0.8		Candy paste	n.d.	
	Control	1.5 ^{c}	±0.4		Control	n.d.	
	Sugar syrup	1.2 ^{b}	±0.3		Sugar syrup	0.05 ^{a}	±0.23
Maltulose	Inverted syrup	n.d.		Maltotriose	Inverted syrup	n.d.	
	Candy paste	0.5 ^{bc}	±0.5		Candy paste	n.d.	
	Control	0.7 ^{c}	±0.5		Control	n.d.	
	Sugar syrup	0.3 ^{ab}	±0.5		Sugar syrup	n.d.	
Maltose	Inverted syrup	20.1 ^{a}	±3.4	Panose	Inverted syrup	0.4	±0.7
	Candy paste	2.5 ^{b}	±0.6		Candy paste	n.d.	
	Control	2.6 ^{b}	±1.0		Control	n.d.	
	Sugar syrup	2.2 ^{b}	±0.9		Sugar syrup	n.d.	
Trehalose	Inverted syrup	0.1 ^{a}	±0.2	Isomaltotriose	Inverted syrup	n.d.	
	Candy paste	n.d.			Candy paste	n.d.	
	Control	0.5 ^{b}	±0.8		Control	n.d.	
	Sugar syrup	0.03 ^{a}	±0.1		Sugar syrup	n.d.	
Isomaltose	Inverted syrup	0.4 ^{a}	±0.5	Maltotetraose	Inverted syrup	n.d.	
	Candy paste	n.d.			Candy paste	n.d.	
	Control	0.5 ^{a}	±0.5		Control	n.d.	
	Sugar syrup	n.d.			Sugar syrup	n.d.	
Invertase	Inverted syrup	32.4 ^{a}	±13.9				
	Candy paste	129.9 ^{c}	±17.6				
	Control	153.7 ^{c}	±46.4				
	Sugar syrup	68.9 ^{b}	±47.2				

* Different letters in the same column show significant differences among the groups, based on Duncan's multiple range test (a = 0.05). ** n.d.: not detected.

Additionally, supplements with a high concentration of sucrose (candy paste and sugar syrup) resulted in samples with a significant amount of the specific sugar, which many times exceeded the established limit of 5% [28]. Similar results are reported by Ozcan et al. [29]. Regarding the other sugars, the control group showed significantly higher concentrations of trehalose and melezitose. Samples from groups with inverted syrup had

no or very low concentrations of the sugars turanose, maltulose and erlose compared to the others, and only in these samples, some sugars such as melibiose, raffinose and panose were detected. The sugars maltotriose, isomaltotriose and maltotetraose were not in any sample of any group.

As for invertase activity, supplementary feeding during the production led to lower invertase values in the collected samples. This may be attributed to the fact that bees, when they are fed with syrup, collect it quickly and have no time to enrich the product with enzymes, resulting in honey samples with low enzymatic content. According to the International Honey Commission, honey with invertase values greater than 10 IN or 73.43 U kg^{-1} is characterized as fresh [30]. In the present study, the samples produced after feeding with inverted syrup and sugar syrup presented values lower than 73.43 U Kg^{-1} (32.4 and 68.9 U Kg^{-1}, respectively) and they were characterized as adulterated (Table 3).

MDA was applied for all estimated parameters to investigate the possibility of discriminating the groups based on feeding type. The analysis revealed four discriminant functions: The first of which corresponded to 96.6% of variance and it was determined by the presence of fructose and glucose, the second to 3.0% and it was related to the presence of sucrose and melezitose and the third to 0.4% of variance and it was determined by the presence of erlose and invertase (Table 4).

Table 4. Percentages of variance, cumulative and cumulative variance and discriminant functions as derived from the multi-discriminant analysis (MDA).

	Discriminant Function		
	1	**2**	**3**
Fructose	−1.430	0.314	0.144
Glucose	1.288	0.722	0.922
Sucrose	−0.232	0.529	0.116
Turanose	−0.303	−0.084	−0.536
Maltulose	0.274	−0.333	0.316
Maltose	0.944	0.438	0.193
Trehalose	0.180	−0.235	−0.135
Isomaltose	−0.264	0.338	0.328
Erlose	0.119	−0.438	0.755
Melezitose	0.089	0.668	0.597
Raffinose	−0.141	0.000	0.082
Panose	0.079	−0.257	0.027
Invertase	0.023	0.357	−0.655
Eigenvalue	72.946	2.265	0.328
% Variance	96.6	3.0	0.4
% Cumulative variance	96.6	99.6	100.0

Additionally, as shown in Figure 1, samples of the same group appear in the same quadrant grouped around the center of the corresponding group, emphasizing the large correlation within the same group. The discrimination of the group fed with the commercial inverted syrup is obvious, while the groups fed with the syrup and the control seem to stand out as they appear in a different quadrant.

Indirect honey adulteration involving feeding bee colonies with commercial sugars is extremely difficult to detect [27,29]. It seems from the present study that honey adulteration, especially with inverted syrup, can be detected through low-cost analysis.

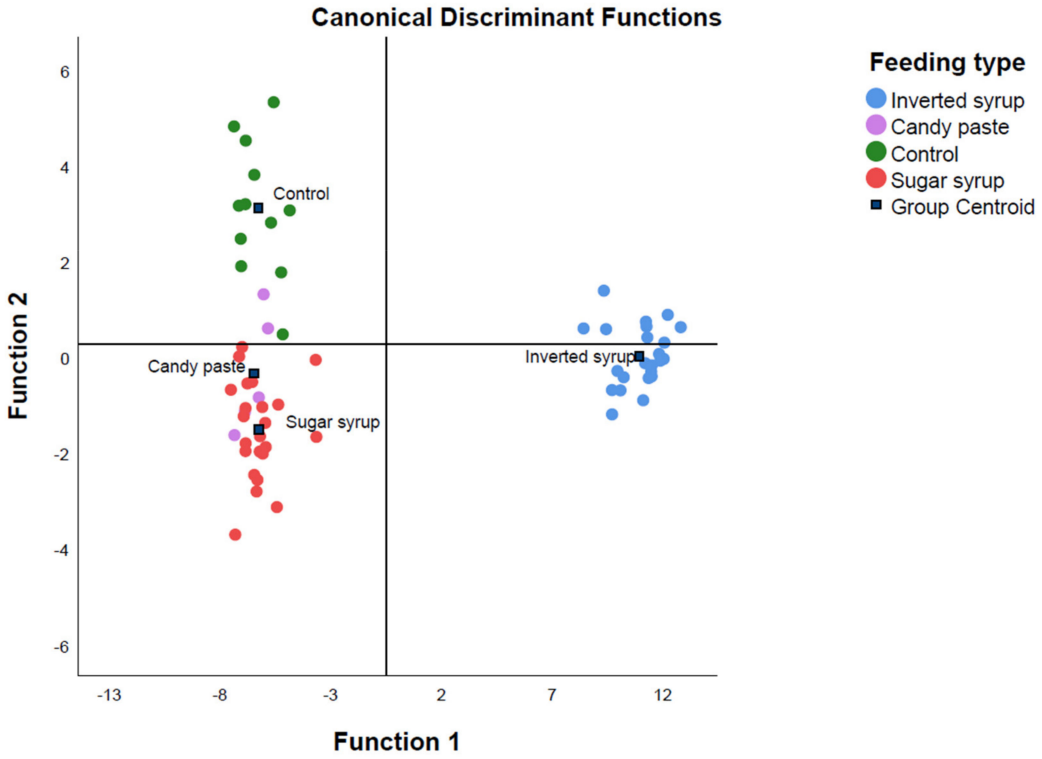

Figure 1. Scatterplot of the canonical discriminant scores obtained from the two first discriminant functions.

4. Conclusions

The feeding of honeybees during production season seems to affect the quality of the final product, leading to its indirect adulteration, which is extremely difficult to detect. We found in the present study that this type of adulteration can be detected through its carbohydrate profile and invertase activity, even in cases when the honey was collected after a longer time. The presence of sucrose or maltose in artificial syrup increases the concentration of these sugars in the final product, while all the other sugars may be affected. Moreover, the most common beekeeping practice (feeding with inverted syrups) may lead to honey with lower invertase activity, refuting the allegations of beekeepers that the adulteration, in this case, cannot be detected. The study showed that honey adulteration can be detected through economical routine analyses.

Supplementary Materials: https://www.mdpi.com/article/10.3390/app12073661/s1, Figure S1: Calibration Curves; Figure S2: Indicative chromatograms.

Author Contributions: Conceptualization, C.T. and D.K.; methodology, D.K., V.L. and C.T.; investigation, D.K., V.L. and M.-A.R.; writing—original draft preparation, D.K., V.L., C.T. and M.-A.R.; visualization, D.K. and M.-A.R.; supervision, C.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded from Greek national funds through the Public Investments Program (PIP) of General Secretariat for Research and Innovation (GSRI), under the Emblematic Action "The bee routes" (project code: 2018ΣΕ01300000).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data supporting reported results are restored at Laboratory of Apiculture-Sericulture, AUTH.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Anklam, E. A review of the analytical methods to determine the geographical and botanical origin of honey. *Food Chem.* **1998**, *63*, 549–562. [CrossRef]
2. Guler, A.; Kocaokutgen, H.; Garipoglu, A.V.; Onder, H.; Ekinci, D.; Biyik, S. Detection of adulterated honey produced by honeybee (*Apis mellifera* L.) colonies fed with different levels of commercial industrial sugar (C3 and C4 plants) syrups by the carbon isotope ratio analysis. *Food Chem.* **2014**, *155*, 155–160. [CrossRef] [PubMed]
3. Cordella, C.B.Y.; Militao, J.S.L.T.; Clement, M.C.; Drajnudel, P.; Cabrol-Bass, D. Detection and quantification of honey adulteration via direct incorporation of sugar syrups or bee—feeding: Preliminary study using high—performance anion exchange chromatography with pulse damperometric detection and chemometrics. *Anal. Chim. Acta* **2005**, *531*, 239–248. [CrossRef]
4. Elflein, L.; Raezke, K.P. Improved detection of honey adulteration by measuring differences between $^{13}C/^{12}C$ stable carbon isotope ratios of protein and sugar compounds with a combination of element alanalyzer-isotope ratio mass spectrometry and liquid chromatography-isotope ratio mass spectrometry (δ13C-EA/LCIRMS). *Apidologie* **2008**, *39*, 574–587.
5. Nisbet, C.; Kazak, F.; Ardali, Y. Determination of Quality Criteria that Allow Differentiation between Honey Adulterated with Sugar and Pure Honey. *Biol. Trace Elem. Res.* **2018**, *186*, 288–293. [CrossRef]
6. Goodwin, M.R. Feeding sugar syrup to honey bee colonies to improve pollination: A review. *Bee World* **1997**, *78*, 56–62. [CrossRef]
7. Geslin, B.; Aizen, M.; Garcia, N.; Pereira, A.J.; Vaissiere, B.; Garibaldi, L. The impact of honey bee colony quality on crop yield and farmers' profit in apples and pears. *Agric. Ecosyst. Environ.* **2017**, *248*, 153–161. [CrossRef]
8. Li, S.; Shan, Y.; Zhu, X.; Zhang, X.; Ling, G. Detection of honey adulteration by high fructose corn syrup and maltose syrup using Raman spectroscopy. *J. Food Compos. Anal.* **2012**, *28*, 69–74. [CrossRef]
9. Tosun, M. Detection of adulteration in honey samples added various sugar syrups with $^{13}C/^{12}C$ isotope ratio analysis method. *Food Chem.* **2013**, *138*, 1629–1632. [CrossRef]
10. Kerkvliet, J.D.; Shrestha, M.; Tuladhar, K.; Manandhar, H. Microscopic detection of adulteration of honey with cane sugar and cane sugar products. *Apidologie* **1995**, *26*, 131–139. [CrossRef]
11. Stefas, D.; Gyftokostas, N.; Kourelias, P.; Nanou, E.; Tananaki, C.; Kanelis, D.; Liolios, V.; Kokkinos, V.; Bouras, C.; Couris, S. Honey discrimination based on the bee feeding by Laser Induced Breakdown Spectroscopy. *Food Control* **2022**, *134*, 108770. [CrossRef]
12. Cavrar, S.; Yildiz, O.; Sahin, H.; Karahalil, F.; Kolayli, S. Comparison of physical and biochemical characteristics of different quality of Turkish honey. *Uludag Bee J.* **2013**, *13*, 55–62.
13. Majtan, J.; Bucekova, M.; Kafantaris, I.; Szweda, P.; Hammer, K.; Mossialos, D. Honey antibacterial activity: A neglected aspect of honey quality assurance as functional food. *Trends Food Sci. Technol.* **2021**, *118*, 870–886. [CrossRef]
14. Bertelli, D.; Lolli, M.; Papotti, G.; Bortolloti, G.; Serra, G.; Plessi, M. Detection of honey adulteration by sugars yrups using one-dimensional and two-dimensional high-resolution nuclear magnetic resonance. *J. Agric. Food Chem.* **2010**, *58*, 8495–8501. [CrossRef]
15. Siddiqui, A.J.; Musharraf, S.G.; Choudhary, M.I.; Rahman, A. Application of analytical methods in authentication and adulteration of honey. *Food Chem.* **2017**, *217*, 687–698. [CrossRef]
16. Cotte, J.F.; Casabianca, H.; Giroud, B.; Albert, M.; Lheritier, J.; Grenier-Loustalot, M.F. Characterization of honey amino acid profiles using high-pressure liquid chromatography to control authenticity. *Anal. Bioanal. Chem.* **2004**, *378*, 1342–1350. [CrossRef]
17. Consonni, R.; Cagliani, L.R.; Cogliati, C. Geographical discrimination of honeys by saccharides analysis. *Food Control* **2013**, *32*, 543–548. [CrossRef]
18. She, S.; Chen, L.; Song, H.; Lin, G.; Li, Y.; Zhou, J.; Liu, C. Discrimination of geographical origins of Chinese acacia honey using complex $^{13}C/^{12}C$, oligosaccharides and polyphenols. *Food Chem.* **2019**, *272*, 580–585. [CrossRef]
19. Ruiz-Matute, A.I.; Soria, A.C.; Martinez-Castro, I.; Sanz, M.L. A new methodology based on GC-MS to detect honey adulteration with commercial syrups. *J. Agric. Food Chem.* **2007**, *55*, 7264–7269. [CrossRef]
20. Cordella, C.B.Y.; Moussa, I. Identication of fraud in honey: The use of microscopies and mass spectrometry of 13-carbon. *Actual. Chim.* **2009**, *330*, 7–13.
21. Ouchemoukh, S.; Amessis-Ouchemoukh, N.; Gómez-Romero, M.; Aboud, F.; Giuseppe, A.; Fernández-Gutiérrez, A.; Segura-Carreteroc, A. Characterisation of phenolic compounds in Algerian honeys by RP-HPLC coupled to electrospray time-of-flight mass spectrometry. *LWT—Food Sci. Technol.* **2017**, *85*, 460–469. [CrossRef]
22. Pascual-Mate, A.; Oses, S.M.; Fernández-Muiño, M.; Sancho, T. Analysis of Polyphenols in Honey: Extraction, Separation and Quantification Procedures. *Sep. Purif. Rev.* **2018**, *47*, 142–158. [CrossRef]
23. Schievano, E.; Sbrizza, M.; Zuccato, V.; Piana, L.; Tessari, M. NMR carbohydrate profile in tracing acacia honey authenticity. *Food Chem.* **2020**, *309*, 125788. [CrossRef] [PubMed]
24. Bogdanov, S.; Martin, P.; Lüllmann, C. Harmonized methods of the European Honey Commission. *Apidologie* **1997**, *(extra issue)*, 1–59.
25. Wenzi, T.; Haedrich, J.; Schaechtele, A.; Robouch, P.; Stroka, J. *Guidance Document on the Estimation of LOD and LOQ for Measurements in the Field of Contaminants in Feed and Food*; EUR 28099; Publications Office of the European Union: Geel, Luxembourg, 2016; ISBN 978-92-79-61768-3. [CrossRef]

26. Al-Mahasneh, M.; Al-U'datt, M.; Rababah, T.; Al-Widyan, M.; Abu Kaeed, A.; Al-Mahasneh, A.J.; Abu-Khalaf, N. Classification and Prediction of Bee Honey Indirect Adulteration Using Physiochemical Properties Coupled with K-Means Clustering and Simulated Annealing-Artificial Neural Networks (SA-ANNs). *J. Food Qual.* **2021**, 6634598. [CrossRef]
27. Paradkar, M.M.; Irudayaraj, J. Discrimination and classification of beet and cane inverts in honey by FT-Raman spectroscopy. *Food Chem.* **2001**, *76*, 231–239. [CrossRef]
28. European Economic Community (EEC). Council directive of 20 December 2001 relating to honey. *Off. J. Eur. Commun. Legis.* **2002**, *110*, 47–50.
29. Ozcan, M.; Arslan, D.; Ceylan, D.A. Effect of inverted saccharose on some properties of honey. *Food Chem.* **2006**, *99*, 24–29. [CrossRef]
30. Bogdanov, S.; Lüllmann, C.; Martin, P.; von der Ohe, W.; Russmann, H.; Vorwohl, G.; Flamini, C. Honey quality and international regulatory standards: Review by the International Honey Commission. *Bee World* **1999**, *80*, 61–69. [CrossRef]