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Novel inspection of sugar residue and origin in honey based on the $^{13}\text{C}/^{12}\text{C}$ isotopic ratio and protein content



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ABSTRACT

Regarding the honey industry, market prices are strongly affected by the origin and composition of products. In particular, the adulteration of honey can be divided into cases of honey being mixed with artificial syrup, the different origin of the adulteration and the presence of cane sugar residue. Unfortunately, recent studies mentioned that most honey is mixed with artificial syrups. Thus, determining such unnaturally present sugar is necessary to maintain the trust of the consuming populations. To investigate the possibility of syrup augmentation, this study first clarifies two points of great importance. First, long-term feeding of cane sugar to honey bee colonies in winter and the continuous harvest of honey were investigated to evaluate the C_4 sugar ratio in spring through inspection of the $^{13}\text{C}/^{12}\text{C}$ isotopic ratio. As the results indicated, C_4 sugar was detected as “sugar residue” in honey samples when the honey bee colonies were fed with cane sugar in winter and when the honey was collected in the first and second harvests in March. As indicated from the samples of 89 Taiwanese longan honeys, 54 Thai longan honeys, and 20 Taiwanese non-longan honeys for analysis, such “sugar residues” were in 40% (8/20) of the Taiwanese non-longan honeys, 15% (3/20) of 2017 Taiwanese longan honeys and 20% (4/20) of 2017 Thai longan honeys; these samples were classified as adulterated honey ($\text{C}_4\% > 7$). Second, as revealed in the honeys' protein contents, statistically significant differences were found between Taiwanese (>1.00 mg/g) and Thai longan honeys (<1.00 mg/g). Apparently, this significant difference could be used to classify the difference in origins of longan honeys. This novel inspection of “sugar residue” and “origin” in honey could represent the first attempt for a protocol to guarantee both the quality and quantity assurance of honey in the marketplace.

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1. Introduction

Honey is a well-known, sweet and viscous substance produced by honey bees (e.g., *Apis mellifera*) from nectar (e.g., inside flowers or extrafloral nectaries) or honeydew secreted from insects. Compositions of honey usually contain 33–43% fructose, 25–35% glucose, and 2% sucrose with small amounts of proteins, pollen, acids, trace elements, enzymes, vitamins and flavonoids [1]. Moreover, honey provides myriad biological activities (e.g., anti-bacterial, anti-inflammatory, antioxidant and cytoprotective characteristics) significant to human health [2,3]. Honey can be divided into two major types, including uni-floral honey, which is defined as honey made from a single nectar source, and poly-floral honey, which is defined as honey made from a mixture of different nectars.

In Taiwan, the major honey harvest season is during March and April. In these months, uni-floral honeys (e.g., longan and litchi honey) are predominantly produced. Royal jelly and pollen harvest seasons are around June to February of the next year and October to February of the next year, respectively; from June to February of the next year, poly-floral honey can also be produced due to the specific external environment. As a matter of fact, during honey, royal jelly and pollen harvest seasons, honey bee colonies are sometimes fed with cane sugar syrup to increase production yields. Recently, due to climate change, continuous rainfalls usually occurred in the honey harvest seasons and winters; hence, beekeepers have had to feed honey bee colonies with cane sugar syrup. Under these circumstances, a “sugar residue” in the honey in the first and second honey harvest (after stopping cane sugar syrup feeding) has become a long-term problem. Of course, the issue of “sugar residue” in honey is an injustice for both consumers and pure honey producers.

In the 1990s, a group of Taiwanese beekeepers introduced beekeeping technology to Thailand and promoted the flourishing of the Thailand beekeeping industry there. Therefore, the honey bee breeding strategy in Thailand is similar to that of Taiwan. Due to the lack of nectar sources in the natural environment, honey bee colonies might be fed with cane sugar syrup. However, because of abundant demands from customers in Taiwan, most of the harvested longan honey has still been imported from Thailand. According to the record of the Customs Administration, Ministry of Finance in Taiwan, imported honey from Thailand substantially increased (i.e., 2399, 3692, 2867, 3800 and 4746 metric tons, respectively) from 2013 to 2017 (Fig. 1). Notably, honey imported from Thailand accounted for ca. 70% of the total honey imports (Fig. 1). As a matter of fact, the market price of honey fluctuated due to honey's purity and origin of production. Therefore, the identification and inspection of honey, which is imported to Taiwan, would be of great importance to the indigenous honey market.

In particular, honey was listed as the sixth most easily adulterated food by the EU in 2013. In Taiwan, most honey adulteration can be divided into cases of cane sugar residue and different origins of production; honey is mostly composed of sugars, and thus most of the honey adulteration occurs via supplementation with cane sugar or HFCS. To investigate such possible additions, methods are usually focused on the establishment of analytic techniques to classify sugar types in honey via gas chromatography (GC), liquid chromatography (LC) and

high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) [4–6]. Moreover, a technology has been established to determine sucrose, glucose, and fructose according to the $^{13}\text{C}/^{12}\text{C}$ isotope ratios by using liquid chromatography coupled to isotope ratio analysis (HPLC-IRMS) [7,8]. Cane sugar and HFCS from C_4 plants have usually been used as adulteration ingredients, as the honey bees collect nectar from C_3 plants; based on this character, the $\delta^{13}\text{C}$ value of C_3 plants were -23‰ to -28‰ but that of C_4 plants ranged between -9‰ to -15‰ . The $\delta^{13}\text{C}$ value of crassulacean acid metabolism (CAM) plants are between -11‰ to -13.5‰ [8–10]. Therefore, according to this concept, the stable isotopes of carbon could be adopted as a potential method to distinguish adulterated honey from C_4 plant syrup.

It has been reported that the $\delta^{13}\text{C}$ value of honey and protein can be determined by stable carbon isotope ratio analysis (SCIRA) for the detection of honey adulteration [11–14]. Moreover, the origin of honey adulteration is also an important issue, and the differences among honeys of various geographical origins could be identified via fingerprints of carbon and nitrogen stable isotopes [15]. Another method was to use the elemental content through total reflection X-ray fluorescence spectrometry (TXRF), combined with the stable carbon and nitrogen isotope ratios [16].

This study attempted to investigate honey samples from Taiwan and Thailand by SCIRA, including Taiwanese longan honeys, Taiwanese honeys from beekeepers and Thai longan honeys. The moisture of honey was reduced to 20% or less via rotary evaporator without mixing with any processed substances, thereby following the beekeepers' production standard for honey. Moreover, inspection of cane sugar residue in samples was performed during March and April of 2014, using the raw honey collected from three apiaries monitored during nectar flow periods. All of these collected samples were tested by $^{13}\text{C}/^{12}\text{C}$ isotope ratio analysis. The findings provided not only the figures of cane sugar residues but also origins of honey adulteration in Taiwan. Such information could be helpful in establishing a protocol to disclose the possible existence of honey adulteration in the current market.

2. Materials and methods

2.1. Honey samples

A total of 201 samples, randomly selected via beekeepers, were subjected to the analysis process, including 89 samples of 2012, 2013 and 2017 Taiwanese longan honeys (Table 1), 54 samples of 2012, 2013 and 2017 Thai longan honeys (Table 1) and 20 samples of 2012 Taiwanese non-longan honeys (Table 2). The honey was only pretreated via rotary evaporator to remove moisture to less than 20%. Thirty-eight samples of 2014 raw honeys were collected from the monitoring of three apiaries in the nectar flow periods of the spring season.

To have a better understanding of the sugar residues of the honeys, testing of “sugar residues” was performed during February–April, and experimental samples of sugar residues

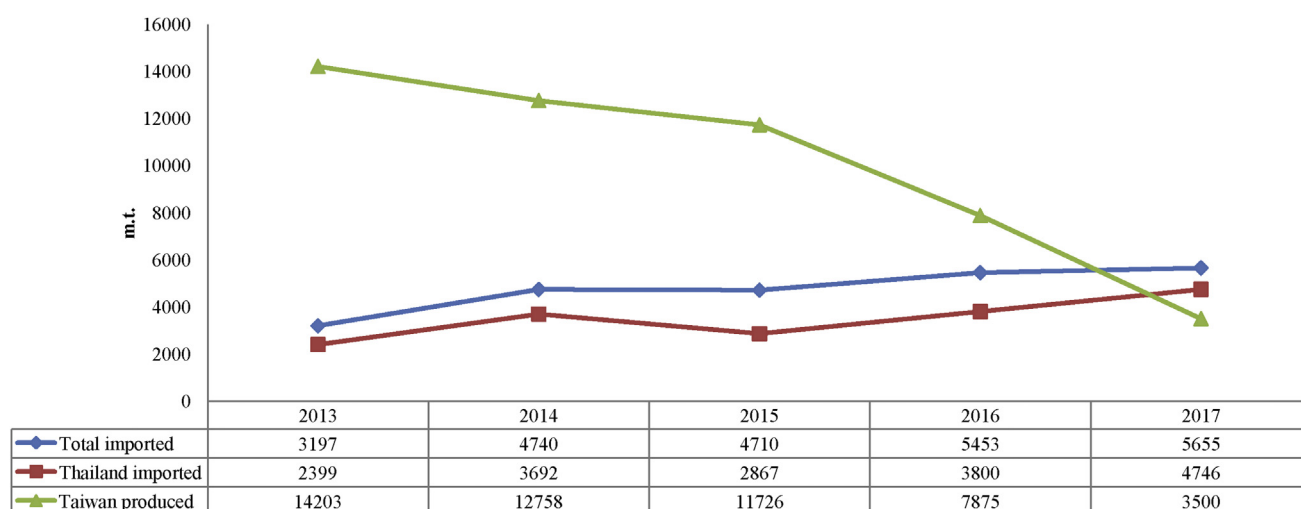


Fig. 1 – Data of locally produced and imported honeys in Taiwan over the past five years. Taiwanese locally produced honey reveals a dramatic decrease in 2016 and 2017. In 2017, honey imported from Thailand was more prevalent than local honey produced in Taiwan.

were collected from three apiaries (denoted as apiaries A, B and C). Apiary A was located in New Taipei City (24°50'41.2"N 121°26'53.4"E), at an altitude of 297 m; the flora surrounding it consisted of pilose beggarticks and ivy trees. In apiary A, a total of 100 honey bee colonies were fed. Apiary B was located in Miaoli (24°33'07.8"N 120°48'12.7"E), at the altitude of 99 m; its surrounding flora included pilose beggarticks, Chinese tallow trees and longan. In apiary B, a total of 120 honey bee colonies were fed. Apiary C was located in Hsinchu (24°53'41.5"N 121°03'13.2"E), at an altitude of 91 m; the flora surrounding it was predominantly pilose beggarticks. In apiary C, a total of 300 honey bee colonies were fed. The cane sugar syrup was prepared as 1 kg water: 1.5 kg cane sugar, and the degree of Brix was ca. 61%. The amount of sugar syrup, which was fed to honey bee colonies since early 2014 before the honey harvest, was recorded. Different frequencies of cane sugar syrup feeding were performed in each apiary as follows: once (1.5 L) every three days in apiary A (high), once

every five days in apiary B (middle) and once every seven days in apiary C (low). The dates of the last cane sugar syrup feeding performed in each apiary were Mar. 19 (apiary A), Mar. 17 (apiary B) and Mar. 15 (apiary C). Three days before the first harvest (first honey harvest after stopping cane sugar syrup feeding), all colonies were intentionally moved to Taichung during the longan and litchi nectar flow period. The honey samples in this sugar residues trial were collected immediately and preserved at –20 °C without any pretreatment via rotary evaporator.

2.2. Reagents and standards

Sodium tungstate dehydrate (Merck, Germany) and a 10% aqueous solution of $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ and 0.67 N sulfuric acid (Sigma, Germany) were used to extract protein. Bovine serum albumin (BSA) was used as a standard to measure the protein content of honey using the Bradford assay.

Table 1 – $\delta^{13}\text{C}$ values and protein content of pure longan honeys from Taiwan and Thailand.

Type of honey	n	$\delta^{13}\text{C}_{\text{protein}}$ (‰)	$\delta^{13}\text{C}_{\text{honey}}$ (‰)	$\delta^{13}\text{C}_{\text{protein-honey}}$ (‰)	Protein content (mg/g)	Range of protein content (mg/g)	Note
Taiwan 2012	39	-24.76 ± 0.67	-26.18 ± 0.71	1.42 ± 0.71	$1.37 \pm 0.15\text{a}^*$	1.05 to 1.73	Honey from beekeepers via Miaoli District Agricultural Research and Extension Station, Council of Agriculture, Taiwan Honey from monitoring Apiary (A: No 9–14, B: No 9–14 and C: No 8–10)
Taiwan 2013	30	-25.63 ± 1.10	-26.27 ± 1.52	1.14 ± 0.42	$1.43 \pm 0.13\text{a}$	1.12 to 1.70	
Taiwan 2014	15	-25.29 ± 0.48	-26.34 ± 0.60	1.04 ± 0.73	$1.22 \pm 0.13\text{b}$	1.03 to 1.47	
Taiwan 2017	17	-25.25 ± 0.41	-26.13 ± 0.78	0.88 ± 0.56	$1.29 \pm 0.20\text{b}$	0.93 to 1.63	
Thailand 2012	19	-24.40 ± 0.47	-25.07 ± 0.61	0.67 ± 0.73	$0.64 \pm 0.08\text{d}$	0.48 to 0.89	Thailand local honey buyers Thailand local honey buyers Thailand local honey buyers, 4/20 sample were detected adulterated
Thailand 2013	15	-25.69 ± 0.49	-26.57 ± 0.99	0.88 ± 0.68	$0.80 \pm 0.08\text{c}$	0.71 to 0.96	
Thailand 2017	16	-24.60 ± 0.38	-24.53 ± 0.76	-0.07 ± 0.55	$0.75 \pm 0.08\text{c}$	0.61 to 0.86	

*Mean $\pm$ s.d. in each same column followed by different letters were significantly different by LSD test ($P < 0.05$).

Table 2 – $\delta^{13}\text{C}$ values and protein content of Taiwanese non-longan honeys collected from local beekeepers in 2012.

Sample No.	Floral source	$\delta^{13}\text{C}_{\text{protein}}$ (‰)	$\delta^{13}\text{C}_{\text{honey}}$ (‰)	$\delta^{13}\text{C}_{\text{protein-honey}}$ (‰)	Protein content (mg/g)	C ₄ -sugar (%)	Honey quality*
2012-NL-1	Beggar-ticks	−27.31	−27.88	0.57	0.58	0	Pure
2012-NL-2	Tallow tree	−24.77	−26.35	1.59	0.31	0	Pure
2012-NL-3	Poly-flora	−23.88	−23.22	−0.66	0.66	4.6	Pure
2012-NL-4	Cinnamon	−26.22	−26.48	0.26	0.56	0	Pure
2012-NL-5	Avocado	−24.61	−21.46	−3.15	0.82	21.1	Adulterated
2012-NL-6	Litchi	−24.29	−21.66	−2.63	0.86	18.0	Adulterated
2012-NL-7	Beggar-ticks	−26.52	−25.73	−0.79	0.33	4.7	Pure
2012-NL-8	Poly-flora	−27.52	−25.47	−2.05	0.74	11.5	Adulterated
2012-NL-9	Poly-flora	−23.60	−21.84	−1.77	0.76	12.7	Adulterated
2012-NL-10	Poly-flora	−24.91	−23.62	−1.29	0.70	8.5	Adulterated
2012-NL-11	Poly-flora	−26.23	−26.60	0.38	0.64	0	Pure
2012-NL-12	Poly-flora	−25.22	−24.82	−0.40	0.69	2.6	Pure
2012-NL-13	Poly-flora	−26.00	−26.80	0.80	1.23	0	Pure
2012-NL-14	Poly-flora	−25.41	−22.27	−3.14	0.71	20.0	Adulterated
2012-NL-15	Poly-flora	−24.99	−25.12	0.13	0.55	0	Pure
2012-NL-16	Poly-flora	−26.59	−23.95	−2.63	1.38	15.6	Adulterated
2012-NL-17	Litchi	−24.90	−23.62	−1.29	0.84	8.5	Adulterated
2012-NL-18	Poly-flora	−27.16	−26.77	−0.40	0.71	2.3	Pure
2012-NL-19	Aglaia	−24.97	−24.26	−0.71	0.87	4.7	Pure
2012-NL-20	Beggar-ticks	−26.61	−27.01	0.40	0.71	0	Pure

* Adulterated was C₄-sugar > 7%.

2.3. Instrumentation

The $\delta^{13}\text{C}$ values of honey and protein samples were determined by an elemental analyzer (ECS-4010, Costech, Valencia, CA, USA) coupled to a Picarro Liaison Universal Interface and a Cavity Ring Down Spectroscopy isotopic CO₂ gas analyzer (CRDS, Picarro G2121-i, Picarro Inc., CA, USA). The temperature setting of the combustion module was 980 °C. The flow rates of the carrier gas (N₂) in the combustion module and gas analyzer were 90 and 25 mL/min, respectively. The definition of $\delta^{13}\text{C}$ values is listed as below:

$$\delta^{13}\text{C} (\text{‰}) = \left\{ \left[\frac{(^{13}\text{C}/^{12}\text{C})_{\text{sample}}}{(^{13}\text{C}/^{12}\text{C})_{\text{standard}}} \right] - 1 \right\} \times 1000$$

Calibration standards included urea ($\delta^{13}\text{C} = -47.8 \pm 0.18\text{‰}$, in house standard, internal precision), benzoic acid ($\delta^{13}\text{C} = -28.81 \pm 0.19\text{‰}$), atropine ($\delta^{13}\text{C} = -22.05 \pm 0.04\text{‰}$), acetanilide ($\delta^{13}\text{C} = -34.02 \pm 0.1\text{‰}$), and L-glutamic acid ($\delta^{13}\text{C} = -26.39 \pm 0.04\text{‰}$). Each sample was run twice to assess precision. The analytical precision (standard deviation) for these standards were within 0.5‰ (n = 7–20) [17]. All samples were measured and reported against the working standards listed above, which were previously characterized relative to their respective IAEA $\delta^{13}\text{C}$ values using a linear regression. All stable isotope values of the samples are expressed in 'delta' (δ) notation. Delta values for carbon are expressed relative to Pee Dee Belemnite (PDB) [18]. The instrumental precision was better than <0.11‰ [19].

2.4. Extraction of protein

The $\delta^{13}\text{C}$ value of honey samples were determined according to the Official Methods of Analysis 998.12 (AOAC, 2005) [20]. Ten milliliters of each honey sample was transferred into a clear 50-mL centrifuge tube with 4.0 mL dH₂O added and well-mixed via vortex. A freshly prepared solution (2.0 mL) with

10% sodium tungstate and 0.67 N sulfuric acid was then added to the centrifuge tube with well-mixing via vortex. The final mixture was incubated for 10 min at 80 °C until a clear solution was obtained with visible flotation or precipitation. Then, 30 mL dH₂O was added to the centrifuge tube and mixed. The mixture was centrifuged for 7 min at 1500× g and then the supernatant was removed. The pellet was washed with 40 mL dH₂O and centrifuged for 7 min at 1500× g. The wash step was repeated 5 times. The pellet was dried in an oven at 50 °C for 12 h and used for measuring $\delta^{13}\text{C}$ in proteins.

2.5. Measured $\delta^{13}\text{C}$ value of honey and honey protein

Prior to analysis, honey and honey protein were placed in a vacuum suction machine to remove water. Approx. 0.9–1.1 mg of honey and honey protein were weighed and wrapped in tin capsules for $\delta^{13}\text{C}$ isotope analysis. Then, the $\delta^{13}\text{C}$ value of honey and honey protein was measured by elemental analyzer (ECS-4010, Costech, Valencia, CA, USA) coupled to a Picarro Liaison Universal Interface and a Cavity Ring Down Spectroscopy isotopic CO₂ gas analyzer (CRDS, Picarro G2121-i, Picarro Inc. CA).

2.6. Formula of C₄ sugar (%)

The formula of C₄ sugar (%) was determined by $\text{C}_4 \text{ sugar (\%)} = [(\delta^{13}\text{C}_{\text{protein}} - \delta^{13}\text{C}_{\text{honey}}) / (\delta^{13}\text{C}_{\text{protein}} - \delta^{13}\text{C}_{\text{sweetener}})] \times 100$. In addition, the $\delta^{13}\text{C}$ value of sweetener was -9.7‰ , which is the mean value for HFCS [8,11].

2.7. Determination of protein content of honey

The protein content of honey was determined via Bradford assay [21] using bovine serum albumin (BSA) as the standard. A 2.0 g honey sample was transferred into a clear 50 mL centrifuge tube with 10.0 mL dH₂O added and mixed via vortex. This procedure of adding 10 mL dH₂O and vortexing was

Table 3 – $\delta^{13}\text{C}$ values and protein content of 2017 longan honeys from Taiwan (TA) and Thailand (TH).

Sample No.	$\delta^{13}\text{C}_{\text{protein}}$ (‰)	$\delta^{13}\text{C}_{\text{honey}}$ (‰)	$\delta^{13}\text{C}_{\text{protein-honey}}$ (‰)	Protein content (mg/g)	C ₄ -sugar (%)	Honey quality [*]
2017-TA-1	–24.88	–24.59	–0.29	1.17	1.9	Pure
2017-TA-2	–25.40	–25.64	0.24	1.10	0	Pure
2017-TA-3	–25.97	–26.95	0.98	0.99	0	Pure
2017-TA-4	–24.99	–25.52	0.54	1.47	0	Pure
2017-TA-5	–24.65	–22.58	–2.07	1.01	13.9	Adulterated
2017-TA-6	–25.52	–26.89	1.38	1.33	0	Pure
2017-TA-7	–25.19	–26.85	1.65	1.50	0	Pure
2017-TA-8	–24.76	–25.01	0.26	1.10	0	Pure
2017-TA-9	–25.43	–26.98	1.55	1.21	0	Pure
2017-TA-10	–25.03	–25.88	0.85	1.63	0	Pure
2017-TA-11	–26.32	–27.21	0.89	0.95	0	Pure
2017-TA-12	–24.98	–25.55	0.57	1.47	0	Pure
2017-TA-13	–24.80	–25.87	1.07	1.47	0	Pure
2017-TA-14	–25.17	–26.06	0.90	1.25	0	Pure
2017-TA-15	–25.31	–26.91	1.59	1.22	0	Pure
2017-TA-16	–24.99	–25.89	0.89	1.31	0	Pure
2017-TA-17	–25.32	–25.62	0.30	1.21	0	Pure
2017-TA-18	–25.21	–26.71	1.51	1.54	0	Pure
2017-TA-19	–25.61	–23.22	–2.39	0.78	15.0	Adulterated
2017-TA-20	–25.40	–23.63	–1.77	0.76	11.3	Adulterated
2017-TH-1	–24.70	–24.64	–0.05	0.61	0.4	Pure
2017-TH-2	–24.34	–24.64	0.30	0.70	0	Pure
2017-TH-3	–24.52	–25.04	0.52	0.63	0	Pure
2017-TH-4	–24.69	–25.44	0.75	0.80	0	Pure
2017-TH-5	–23.80	–22.39	–1.40	0.57	10.0	Adulterated
2017-TH-6	–24.44	–24.15	–0.29	0.76	2.0	Pure
2017-TH-7	–24.36	–23.81	–0.56	0.67	3.8	Pure
2017-TH-8	–25.29	–25.77	0.48	0.77	0	Pure
2017-TH-9	–24.71	–24.01	–0.70	0.71	4.7	Pure
2017-TH-10	–25.19	–24.89	–0.30	0.76	1.9	Pure
2017-TH-11	–24.68	–23.96	–0.72	0.67	4.3	Pure
2017-TH-12	–24.62	–25.06	0.45	0.82	0	Pure
2017-TH-13	–24.56	–23.22	–1.34	0.67	9.0	Adulterated
2017-TH-14	–25.06	–24.88	–0.19	0.86	1.2	Pure
2017-TH-15	–24.63	–25.48	0.85	0.86	0	Pure
2017-TH-16	–25.53	–21.21	–4.31	0.58	27.2	Adulterated
2017-TH-17	–24.75	–21.18	–3.57	0.83	23.7	Adulterated
2017-TH-18	–24.07	–23.52	–0.56	0.73	3.9	Pure
2017-TH-19	–24.42	–24.00	–0.42	0.83	2.9	Pure
2017-TH-20	–23.84	–23.13	–0.70	0.77	5.0	Pure

^{*} Adulterated was C₄-sugar > 7%.

repeated four times to guarantee completion. The final volume was then adjusted to 50 mL. An aliquot of this (50 μL) was transferred to a cell on a 96-well plate with 200 μL of Bradford reagent (Sigma, USA) and was mixed carefully to prevent bubble formation. To ensure data reproducibility, each sample was prepared in triplicate. Absorbance at 595 nm using a microplate reader (Thermo) was measured. Protein concentration was calculated by using a standard curve with BSA at concentrations of 100, 80, 60, 40, 20 and 0 $\mu\text{g/mL}$.

3. Results

3.1. $\delta^{13}\text{C}$ value of honey and protein

3.1.1. Taiwan and Thailand longan honeys

The highest and the lowest negative $\delta^{13}\text{C}$ values of pure longan honey ($\delta^{13}\text{C}_{\text{honey}}$) of $-24.53 \pm 0.53\%$ and

$-26.57 \pm 0.60\%$ were found for a 2013 and a 2017 Thailand longan honey, respectively (Table 1). The $\delta^{13}\text{C}$ values of protein ($\delta^{13}\text{C}_{\text{protein}}$) were between $-24.40 \pm 0.47\%$ and $-25.69 \pm 0.49\%$ (Table 1) in 151 pure longan honey samples from Taiwan and Thailand. The $\delta^{13}\text{C}_{\text{honey}}$ values of adulterated longan honey ranged from $-21.18 \sim -23.63\%$ (Table 3), while the $\delta^{13}\text{C}_{\text{protein}}$ values ranged from $-23.80 \sim -25.61\%$ (Table 3) for Taiwanese and Thai adulterated longan honeys.

3.1.2. Taiwanese non-longan honeys

Regarding the 12 samples of Taiwanese pure honeys, the $\delta^{13}\text{C}_{\text{honey}}$ and $\delta^{13}\text{C}_{\text{protein}}$ values were between $-23.22 \sim -27.88\%$ and $-23.88 \sim -27.31\%$, respectively (Table 2). Moreover, the $\delta^{13}\text{C}_{\text{honey}}$ and $\delta^{13}\text{C}_{\text{protein}}$ values were between $-21.46 \sim -25.47\%$ and $-23.60 \sim -27.52\%$, respectively, for the 8 samples of Taiwan adulterated honeys (Table 2).

3.2. Difference between the $\delta^{13}\text{C}$ value of honey and protein

3.2.1. Taiwanese and Thai longan honeys

The average highest and the lowest $\delta^{13}\text{C}_{(\text{protein} - \text{honey})}$ values of pure longan honey observed for Taiwanese longan honey in 2012 and Thai longan honey in 2017 were $1.42 \pm 0.71\text{‰}$ and $-0.07 \pm 0.55\text{‰}$ (Table 1), respectively. In contrast, the $\delta^{13}\text{C}_{(\text{protein} - \text{honey})}$ values of adulterated longan honey were between $-1.40 \sim -4.31\text{‰}$ (Table 3).

3.2.2. Taiwanese non-longan honeys

Regarding the 12 samples of Taiwanese pure non-longan honeys, the $\delta^{13}\text{C}_{(\text{protein} - \text{honey})}$ values ranged between -0.79 and 1.59‰ (average ca. $0.10 \pm 0.72\text{‰}$, Table 2). In contrast, the $\delta^{13}\text{C}_{(\text{protein} - \text{honey})}$ values of the 8 samples (sample No. 2012-NL-5, 2012-NL-6, 2012-NL-8, 2012-NL-9, 2012-NL-10, 2012-NL-14, 2012-NL-16, 2012-NL-17) were between -1.29 and -3.15‰ (Table 2); these were defined as cane sugar residue honeys, since their $\delta^{13}\text{C}_{(\text{protein} - \text{honey})}$ values were less than -1.0‰ . In addition, two of them were litchi honey, one was avocado honey, and 5 were poly-flora honey.

3.3. C_4 sugar % in the longan honeys

3.3.1. Taiwanese and Thai longan honeys

According to the formula used to indicate the adulterated cases, C_4 sugar was possibly not detected in the Taiwanese longan honeys in 2012 and 2013. However, C_4 sugar was positively detected in four of the Taiwan longan honey samples in 2017 (ca. 1.9–15.0%), and three samples (2017-TA-5, 2017-TA-19, and 2017-TA-20) were identified as adulterated (Table 3). For Thai longan honeys, C_4 sugar was also found in 3 of the 19 samples in 2012, ranging from 1.5 to 2.9%. In addition, 1 of 15 samples of the Thai longan honeys in 2013 contained C_4 sugar at 4.4%. However, it was found that 14 of 20 honey samples in 2017 ranged from 0.4 to 27.2%. Four of these samples (2017-TH-5, 2017-TH-13, 2017-TH-16, and 2017-TH-17) were identified as adulterated cases (Table 3).

3.3.2. Taiwanese non-longan honeys

According to C_4 sugar analysis, 13 of 20 Taiwan non-longan honey samples ranged from 2.3 to 21%. That is, 8 of the Taiwanese non-longan honey samples were adulterated with high C_4 sugar above 7% to be classified as adulterated (Table 3).

3.4. Monitoring the sugar residue in bee hives

To obtain a better understanding of the correlation of sugar feed and the resultant “sugar residue” in field studies, dose-

response studies of sugar feeding and honey harvest were applied to the three apiaries, A, B and C. As the results revealed, the concentration of sugar residue was strongly associated with the frequency of sugar feeding. For apiary A, the sugar residue (C_4 -sugar) was 34.3% at the first harvest, and the sugar residue had decreased to 12.1% at the second harvest (Supplementary Table 1). Regarding apiary B, the C_4 -sugar was 27.8% at the first harvest time and had decreased to 3.0% at the second harvest (Supplementary Table 2). The results of the low-dose fed apiary C indicated that the C_4 -sugar was 24.1% at the first harvest and had decreased to an undetectable 0% at the second harvest (Supplementary Table 3).

3.5. Protein content of honey samples

Comparing the protein content of different Taiwanese longan honey samples, the highest average protein content was observed in 2013 (1.43 ± 0.13 mg/g average, range 1.12–1.70 mg/g), followed by 2012 (average 1.37 ± 0.15 mg/g and range 1.05–1.73 mg/g), 2017 (average 1.29 ± 0.20 mg/g and range 0.93–1.63 mg/g) and 2014 (average 1.22 ± 0.13 mg/g and range 1.03–1.47 mg/g) (Table 1). Furthermore, 12 cases of the Taiwanese pure non-longan honeys from beekeepers ranged between 0.31 and 1.28 mg/g (Table 2) (average 0.65 ± 0.24 mg/g).

The highest average protein content of the Thai longan honeys was found in 2013 (0.80 ± 0.08 mg/g average, range 0.71–0.96 mg/g), followed by 2017 (0.75 ± 0.08 mg/g average, range 0.61–0.86 mg/g) and 2012 (0.64 ± 0.08 mg/g average, range 0.48–0.89 mg/g) (Table 1).

Overall, the protein content of the Taiwanese longan honeys (1.35 ± 0.17 mg/g, $n = 101$) was apparently higher than that of the Taiwanese litchi honeys (0.87 ± 0.16 mg/g, $n = 14$) (Table 4). Note that the Thai longan honeys (0.72 ± 0.10 mg/g, $n = 50$) and Taiwanese poly-flora honeys (0.65 ± 0.24 mg/g, $n = 12$) showed significantly lower protein content ($P < 0.05$).

4. Discussion

According to the official methods of AOAC 978.17 [22], pure honey should have a value of $\delta^{13}\text{C}_{\text{honey}} < -23.5\text{‰}$; all pure longan honeys were in accordance with this condition. Compared to pure honey, the increased $\delta^{13}\text{C}_{\text{honey}}$ values of adulterated honeys were also observed, and this result was consistent with previous findings [12,13]. According to the AOAC 991.41 standard method [23], the $\delta^{13}\text{C}_{(\text{protein} - \text{honey})}$ values of pure honey should be greater than -1‰ . Based on the standard criteria, this study indicated that the 20 samples of Taiwanese longan honeys in 2017 contained three cases

Table 4 – $\delta^{13}\text{C}$ values and protein content of pure honeys from Taiwan and Thailand.

Type of honey	n	$\delta^{13}\text{C}_{\text{protein}} (\text{‰})$	$\delta^{13}\text{C}_{\text{honey}} (\text{‰})$	$\delta^{13}\text{C}_{\text{protein-honey}} (\text{‰})$	Protein content (mg/g)	Range of protein content (mg/g)
Taiwan longan	101	$-25.18 \pm 0.84\text{b}^*$	$-26.37 \pm 0.87\text{a}$	$1.19 \pm 0.64\text{a}$	$1.35 \pm 0.17\text{a}$	0.93 to 1.73
Taiwan litchi	14	$-24.33 \pm 0.44\text{d}$	$-25.09 \pm 0.85\text{b}$	$0.76 \pm 0.70\text{b}$	$0.87 \pm 0.16\text{b}$	0.64 to 1.13
Taiwan poly-flora	12	$-25.82 \pm 1.05\text{a}$	$-25.92 \pm 1.33\text{ab}$	$0.10 \pm 0.72\text{c}$	$0.65 \pm 0.24\text{c}$	0.31 to 1.23
Thailand longan	50	$-24.85 \pm 0.71\text{c}$	$-25.35 \pm 1.14\text{b}$	$0.50 \pm 0.76\text{bc}$	$0.72 \pm 0.10\text{c}$	0.48 to 0.96

*Mean $\pm$ s.d. in each same column followed by different letters were not significantly different by LSD test ($P < 0.05$).

that were adulterated (e.g., sample 2017-TA-5, 207-TA-19, and 2017-TA-20 with values of -2.07 , -2.39 and -1.77‰ , respectively). For the 20 Thailand honey samples in 2017, four were found to be adulterated (e.g., sample 2017-TH-5, 2017-TH-13, 2017-TH-16, and 2017-YH-17 with values of -1.40 , -1.34 , -4.31 and -3.57‰ , respectively). Although the Thai pure longan honeys still had negative values of $-0.07 \pm 0.55\text{‰}$ (Table 1) (range $-0.05 \sim -0.72$ (Table 3)) and 10/16 were negative, they were not considered to be adulterated due to the $\delta^{13}\text{C}_{(\text{protein} - \text{honey})}$ values being much greater than -1‰ . Apparently, beekeepers sometimes used cane sugar syrup to feed honey bee colonies when there was a lack of external nectar in Taiwan and Thailand, as discussed above. Cane sugar feeding of honey bee colonies of course could alter the values of $\delta^{13}\text{C}_{\text{honey}}$; however, it seemed not to change the $\delta^{13}\text{C}_{\text{protein}}$ values. Either adulterated honey or honey produced by honey bee colonies fed with cane sugar syrup could be detected through the values of $\delta^{13}\text{C}_{(\text{protein} - \text{honey})}$, as stated elsewhere [10,11,13,21].

The cane sugar syrup was fed to maintain honey bee colonies in the winter. Although the honey was harvested from the bee hives the following spring, the honey still contained residual compositions of cane sugar syrup (ca. $< 30\%$) (i.e., “sugar residue”). Therefore, 13 Taiwan non-longan honey samples, including No. 2012-NL-3, 5, 6, 7, 8, 9, 10, 12, 14, 16, 17, 18, and 19, with C_4 sugar ($< 30\%$) were very likely due to “sugar residue”, strongly implying cane sugar syrup was fed to honey bee colonies prior to honey production. Attention to the first and second harvests (after cane sugar syrup feeding of the honey bee colonies) for possible sugar residue should be increased. To provide more indications for possible adulteration, tests of adulterated honey produced by honey bee colonies fed with cane sugar syrup via $^{13}\text{C}/^{12}\text{C}$ isotopic ratio were later implemented.

Regarding the experiments on sugar residue, the highest level of C_4 -sugar% were found in the first harvest at apiary A (34.3%), which was evidently much higher than others reported elsewhere (e.g., for 60% honey + 40% glucose syrup (C_4 : $32.80 \pm 1.41\%$) and 60% honey + 40% HFCS (C_4 : $29.85 \pm 0.77\%$) [13]; 75% pine honey + 25% HFCS (C_4 : 25.5%) [24]; 40% honey + 60% HFCS (C_4 : 30.6%) [11]). Some points could be elucidated to be possible reasons behind such a significant difference. First, cane sugar syrup was fed to honey bee colonies could thus be metabolized or processed by honey bees. Some other studies also mentioned that HFCS or other sugar syrups could not be sufficiently metabolized by honey bees.

However, as some literature also pointed out, when different syrups were fed to the honey bee colonies, $20.62 \pm 0.54\%$ and $54.77 \pm 0.71\%$ of C_4 sugar HFCS-85 were found in the honey at the 20 and 100 L/colony levels, respectively. Moreover, the case of 100 L/colony of HFCS-55 was $45.2 \pm 0.58\%$ [25]. Based on the data of comparative studies, it is revealed that all of the C_4 sugar was found for the first harvest, but in particular, the C_4 sugar was still found in apiaries A and B at the second harvest in the 3 levels of cane sugar syrup-fed cases (e.g., 24 L/colony of cane sugar syrup (apiary A), 15 L/colony of cane sugar syrup (apiary B) and 10.5 L/colony of cane sugar syrup (apiary C)). This study also was the first

attempt to have long-term monitoring on a practical field for nectar flow. The results clearly showed that if cane sugar was fed to honey bee colonies before honey harvest, pure honey would be obtained after one or two harvests. Since sugar residue is one of the major existing problems with honey, it should be of concern in the standard inspectional procedure of honey (i.e., CNS1305) to the aim for justice for both consumers and pure honey producers.

In addition, not only honey adulteration but also the honey country and flora of origin are of great importance to the market. For protein content, our data was consistent with the prior findings [3] (e.g., the longan honey protein content of 1.56 ± 0.03 was higher than litchi honey at 1.20 ± 0.04). The results showed that the protein content could effectively distinguish honey from different floral origins (Table 4). As previous research indicated, the protein content varies across different floral origins [26,27]. In fact, honey protein was mainly obtained from pollen, and the protein content did not decrease when filtered honey was compared to unfiltered honey [28]. Honey contains amino acids, and proline is the most abundant. Therefore, protein content or proline can be used to identify different floral and country origins. The major proteins of honey could be further identified by proteomic methods, such as SDS-PAGE, 2-dimensional SDS-PAGE, and MALDI-TOF MS [29,30]. It has been reported that the pollens' protein in honeys could be used as a honey floral markers [30]. In conclusion, for the cases in this study, the major proteins of honey in either Taiwan or Thailand should be further investigated in the future.

According to official reports of the Customs Administration, Ministry of Finance in Taiwan, the imported honey from Thailand significantly increased from 2013 to 2017 (e.g., 2399, 3692, 2867, 3800 and 4746 metric tons, respectively). However, the imported Thai honey was very likely to be sold as honey originating from Taiwan, and thus market demand and supply were significantly affected. This would be an urgent issue to be resolved without dispute. Here, comparison of the protein contents of Taiwanese and Thai longan honey samples indicated a statistically significant difference (Table 4). In total, 158 samples were specifically located on four quadrants (x-axis and y-axis denoted $\delta^{13}\text{C}_{\text{protein-honey}}$ and protein content, respectively) in Fig. 2 and divided into quadrants I–IV. As protein content indicated, most (99/101) of the Taiwan pure longan honeys were higher than 1.00 mg/g and located in the quadrant I (Fig. 2), although there were two samples (No. 2017-TA-3 and 11) lower than 1.00 mg/g (Table 3). This might suggest that at least external mixing with litchi honey had taken place, resulting in decreased protein content. All (50/50) of the Thailand pure longan honeys were lower than 1.00 mg/g and located in quadrant IV. In addition, most (6/7) of the Thailand and Taiwan adulterated longan honeys were lower than 1.00 mg/g and located in quadrant III. Only one sample (2017-TA-5) of Taiwanese adulterated longan honey contained more protein (1.01 mg/g) than 1.00 mg/g (Table 3). Thus, this study could propose criteria based on protein content as a potential indicator to classify different origins of longan honeys, in particular to distinguish honeys with origins from Taiwan and Thailand.

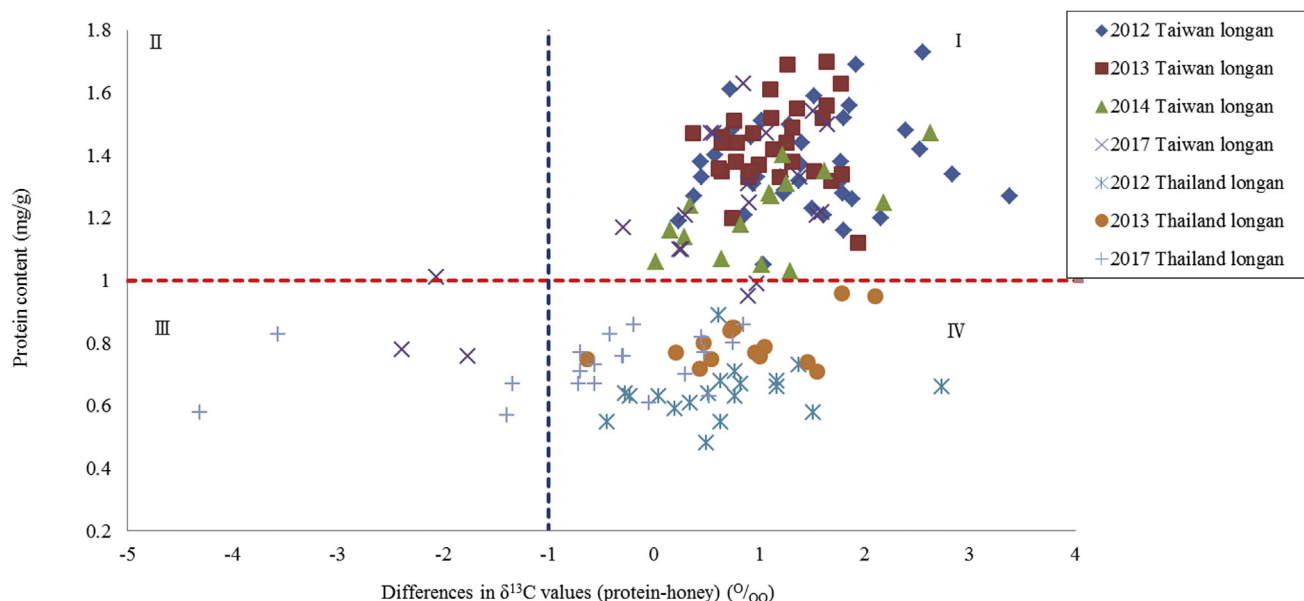


Fig. 2 – Protein content in the Taiwanese and Thailand longan honeys. The red line divides the Thai and Taiwanese longan honeys, and the blue line distinguishes pure and adulterated honeys. Most of the pure Taiwan and Thailand longan honeys were located in regions I and IV, respectively, while the adulterated honeys were in region III. No longan honey was in region II.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.jfda.2018.08.004>.

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