Journal of Apiculture

2026.41(2) : 211-218
DOI: 10.17519/apiculture.2026.06.41.2.211

Original research article

Optimization of Protein Precipitation Using Phosphoric Acid for Stable Carbon Isotope Analysis of Honey Authenticity

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Abstract Stable carbon isotope ratio ($\delta^{13}C$) analysis of isolated honey protein serves as a robust internal standard method for authenticity assessment. However, conventional protein precipitation protocols rely on sulfuric acid (H_2SO_4), which poses severe safety hazards and risks acid catalyzed degradation of abundant sugars. This study evaluated a milder and safer alternative protocol by substituting sulfuric acid with phosphoric acid (H_3PO_4) and systematically optimized key pretreatment parameters including acid concentration, reaction temperature, reaction time, washing frequency, and drying methods using six distinct honey samples. The experimental results indicated that while the $\delta^{13}C$ values remained highly stable and consistent across most tested variables, the physical yield and procedural stability of protein recovery were significantly influenced by each parameter. Based on empirical findings, the optimal pretreatment configuration was established as 0.5 M phosphoric acid concentration, a reaction temperature of 60°C, a reaction duration of 30 min, a three-cycle washing procedure, and freeze-drying. Specifically, the 0.5 M concentration minimized the variation in protein recovery yields across samples to ensure maximum procedural stability. A temperature of 60°C provided the most favorable and robust protein yields, while a 30 min reaction duration maximized time efficiency without sacrificing recovery mass. For post precipitation refinement, three washing cycles effectively eliminated interfering matrix components while preventing excessive protein loss. Furthermore, compared to hot-air oven drying which induced minor thermal degradation and a scorched appearance, freeze-drying successfully preserved the physical quality and texture of the isolated proteins into a fine powder. Taken together, this optimized framework offers an efficient, reliable, and standardized pretreatment protocol that ensures both stable protein recovery and isotopic accuracy, making it highly suitable for routine honey authenticity testing via isotope ratio mass spectrometry.

Keywords Honey, Protein precipitation, Phosphoric acid, Stable carbon isotope ratio ($\delta^{13}C$)

INTRODUCTION

Honey is a natural sweet food produced by honey bees (*Apis mellifera*) through the enzymatic conversion and maturation of floral nectar (Anklam, 1998; De-Melo *et al*., 2017). It contains carbohydrates, proteins, amino acids, organic acids, minerals, and various bioactive substances (Alvarez-Suarez *et al*., 2010; da Silva *et al*., 2016). This composition varies depending on the botanical origin, climate, and geographical environment, which collectively determine the unique quality and characteristics of the honey (Kaškonienė and Venskutonis, 2010; da Silva *et al*., 2016). Recently, in the domestic and international honey markets, the increasing influx of inexpen-

http://journal.bee.or.kr/

Received 31 May 2026; Revised 19 June 2026; Accepted 21 June 2026
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sive imported honey and the persistent issue of illegal adulteration, including the distribution of counterfeit honey produced by artificially feeding sugar to bees, have heightened the demand for accurate quality evaluation and authenticity assessment technologies (Tosun, 2013; Guler *et al*., 2014).

Conventional indicators for honey authenticity, such as sugar composition, amino acid profiles, and enzyme activities, are highly susceptible to fluctuations caused by storage conditions and processing methods like heat treatment, making it difficult to guarantee the reliability of analytical results (Tosi *et al*., 2008; Wu *et al*., 2017). To overcome these limitations, stable carbon isotope ratio ($\delta^{13}C$) analysis has garnered significant attention as a novel alternative for scientific authenticity assessment (Förstel, 2007). Natural honey originates primarily from C3 plants, which typically exhibit lower $\delta^{13}C$ values, whereas sugar syrups derived from C4 plants, such as corn and sugarcane, show relatively higher values, enabling the detection of adulteration with derived sugars from C4 plants (Padovan *et al*., 2003).

Recently, moving beyond the bulk stable carbon isotope analysis of honey, and analytical method utilizing the isotope ratio of isolated honey protein as an internal standard to compare the difference ($\Delta\delta^{13}C$) with the bulk honey isotope ratio has emerged as a promising alternative to supplement the limitations of existing discrimination methods (White and Winters, 1989; Elflein and Raezke, 2008). Honey proteins, which include enzymes secreted from the salivary and hypopharyngeal glands of honey bees as well as proteins derived from pollen, are less affected by processing and storage compared to the abundant sugar components, thus serving as a valuable indicator reflecting the unique biological origin of the honey (Lewkowski *et al*., 2019). However, for this approach to be established as a universally reliable authenticity assessment technique, ensuring the reproducibility and accuracy of the analytical process is a prerequisite. In particular, since honey is predominantly composed of sugars and proteins exist only in trace amounts (da Silva *et al*., 2016), the pure isolation of proteins is exceedingly challenging (Chua *et al*., 2013). Therefore, further research on establishing an efficient and standardized sample preparation process is essential for accurate and reliable stable isotope analysis (Akyıldız *et al*., 2022).

Currently, chemical precipitation using sodium tungstate (Na_2WO_4) and sulfuric acid (H_2SO_4) is predominantly utilized for the isolation of honey proteins (Güçlü *et al*., 2025; Li *et al*., 2026). However, sulfuric acid is a hazardous reagent with strong oxidizing and corrosive properties that poses safety risks to researchers (Rodrigues *et al*., 2025). Furthermore, when applied to samples with an overwhelmingly high sugar content like honey (Chen *et al*., 2019), especially under high temperature conditions, its strong dehydrating action carries the risk of potential side reactions, such as the degradation of sugar catalyzed by acid (Rosatella *et al*., 2011; Velasco *et al*., 2022). These harsh conditions necessitate highly precise control during the protein precipitation process and can act as hurdle in securing consistent sample preparation reproducibility for stable isotope analysis (Shen *et al*., 2018; Dunn and Skrzypek, 2023). Therefore, there is a need to apply an alternative reagent that effectively induces the tungstate protein precipitation reaction while significantly improving worker safety and providing a milder environment for the sample.

Accordingly, this study aimed to evaluate the applicability of a protein precipitation method substituting conventional sulfuric acid with phosphoric acid (H_3PO_4) as a sample preparation technique for the stable carbon isotope analysis of honey proteins. By utilizing phosphoric acid, which offers excellent buffering capacity and relatively safe handling, this study comparatively analyzed the variations in protein extraction yield and stable carbon isotope ratio ($\delta^{13}C$) according to variables such as phosphoric acid concentration, reaction temperature and time, number of washing cycles, and drying methods, ultimately proposing efficient, safe, and optimal pretreatment conditions that can enhance the reliability of stable isotope analysis.

MATERIALS AND METHODS

1. Honey samples

A total of six Rovenia honey samples collected in 2024 were used in this study. These included three Korean samples (KR-1, KR-2, and KR-3), two Chinese samples (CH-1, CH-2) provided by the Korea Honey Associa-

tion, and one Vietnamese sample (VT-1). All samples were stored in the dark at 20°C until analysis to ensure stability and integrity.

2. Modified protein precipitation with phosphoric acid

For the analysis of stable carbon isotope ratios ($\delta^{13}C$) in honey protein, a sodium tungstate precipitation method was employed. This approach is well-established in the literature for the effective isolation of protein fractions from honey matrices (Chen *et al*., 2019). Briefly, 15 g of honey was diluted with 4 mL of distilled water and mixed thoroughly. Protein precipitation was induced by adding 2 mL of 10% Na_2WO_4 (Samchun Chemical, Korea) and 2 mL of 0.3 M phosphoric acid solution. The mixture was reacted in thermostatic water bath at 80°C for 60 min. Subsequently, samples were centrifuged at 15,000 rpm for 5 min using centrifuge (Hanil Science, Korea), and the supernatant was discarded. To remove residual sugars and acidic residues, the precipitate was washed three times with 4 mL of distilled water; each washing step involved homogenization via vortex mixing followed by centrifugation. The final protein precipitate was dried in a dry oven (JSR, Korea) at 60°C for 24 hours.

3. Optimization of experimental conditions

To optimize the efficiency of the protein precipitation process, the effects of various experimental parameters were independently evaluated. The phosphoric acid concentration was assessed at 0.3, 0.5, and 0.7 M, while the reaction temperature and duration were investigated at 40, 60, and 80°C and 30, 60, and 120 min, respectively. Furthermore, the number of washing cycles was tested at 1, 3, and 5 repetitions to determine the optimal removal of residual sugars and acids. Finally, the effects of the drying method were compared by evaluating hot-air drying at 60°C for 24 h against freeze-drying. For each experimental condition, the protein yield (mg) and the stable carbon isotope ratio ($\delta^{13}C$) were measured to systematically compare and establish the optimal pretreatment conditions for honey protein analysis.

4. Carbon stable isotope analysis

The stable carbon isotope ratio ($\delta^{13}C$) of the honey protein samples was determined using an elemental analyzer (EA; vario PyroCube, Elementar, Germany), coupled with an isotope ratio mass spectrometer (IRMS; vision, Elementar, UK). Approximately 100–200 μg of the dried protein sample was weighed into a tin capsule (Elemental Microanalysis, UK) and introduced into the system via an autosampler. The samples were completely combusted in a high-temperature oxidation furnace at 1150°C under an oxygen atmosphere for 60 s. the resulting CO_2 gas was purified through a reduction furnace and absorption column before being introduced into the IRMS. To ensure analytical accuracy and instrument stability, the following international reference materials were used: IAEA-CH-6 (sucrose, $\delta^{13}C = -10.449‰$), IAEA-600 (caffeine, $\delta^{13}C = -27.771‰$), and a urea working standard ($\delta^{13}C = -36.54‰$).

5. Statistical analysis

All experimental procedures were performed in triplicate ($n = 3$), and the results are expressed as the mean ± standard deviation (SD). Statistical analyses were conducted using the Jamovi software (Version 2.7; The jamovi project, 2025). To evaluate the statistical significance of the treatment effects across different conditions, a one way analysis of variance (ANOVA) was performed. Subsequently, significant differences among the group means were determined using Tukey's multiple range test, with a significance level established at $p < 0.05$.

RESULTS

1. Effect of phosphoric acid concentration

The effect of phosphoric acid concentration (0.3, 0.5, and 0.7 M) on the protein precipitation efficiency was evaluated to establish the optimal pretreatment conditions for stable carbon isotope analysis. As summarized in Table 1, the $\delta^{13}C$ values remained consistent across all tested concentrations, confirming that the acid concentration did not significantly influence the isotopic integrity of the honey protein samples.

Regarding protein recovery, the average yield was 19.3 ± 3.8 mg at 0.3 M, which increased to an optimal 19.1 ± 1.7 mg at 0.5 M. Although the 0.7 M condition also showed a comparable yield of 19.0 ± 4.0 mg, the 0.5 M

Table 1. Protein yield (mg) according to phosphoric acid concentration

Concentration	Korea			China and Vietnam		
	KR-1	KR-2	KR-3	CH-1	CH-2	VT-1
0.3 M	11.6 ± 3.8[a]	24.7 ± 3.1[a]	34.9 ± 7.6[a]	9.5 ± 1.7[a]	3.4 ± 0.3[a]	31.6 ± 5.4[a]
0.5 M	20.5 ± 1.4[a]	23.8 ± 1.3[a]	28.7 ± 3.0[a]	11.2 ± 0.5[a]	4.6 ± 0.4[a]	25.9 ± 3.9[a]
0.7 M	20.1 ± 1.7[a]	21.6 ± 4.4[a]	32.9 ± 2.1[a]	10.8 ± 0.6[a]	4.1 ± 1.2[a]	34.8 ± 15.7[a]

*All values are expressed as mean ± standard deviation of three technical replicates (n = 3).
*Different superscript letters within a column indicate significant differences according to Tukey's multiple range test ($p < 0.05$).

Table 2. Impact of phosphoric acid concentration on $\delta^{13}C$ values (‰) of isolated protein fractions

Concentration	Korea			China and Vietnam		
	KR-1	KR-2	KR-3	CH-1	CH-2	VT-1
0.3 M	−24.66 ± 0.11	−22.91 ± 0.09	−24.13 ± 0.07	−23.05 ± 0.04	−23.53 ± 0.08	−24.07 ± 0.34
0.5 M	−27.70 ± 0.06	−23.08 ± 0.02	−24.13 ± 0.04	−23.53 ± 0.01	−23.36 ± 0.08	−23.94 ± 0.40
0.7 M	−24.73 ± 0.08	−23.06 ± 0.05	−24.10 ± 0.02	−23.94 ± 0.08	−24.20 ± 0.33	−24.08 ± 0.17

*All values are expressed as mean ± standard deviation of three technical replicates (n = 3).

Table 3. Effect of reaction temperature on honey protein yield (mg)

Temperature	Korea			China and Vietnam		
	KR-1	KR-2	KR-3	CH-1	CH-2	VT-1
40°C	18.7 ± 3.2[a]	34.3 ± 2.5[a]	41.7 ± 4.2[a]	13.6 ± 0.1[a]	3.7 ± 0.2[a]	54.3 ± 2.0[a]
60°C	24.8 ± 2.2[a]	35.6 ± 1.5[a]	39.1 ± 5.0[a]	19.6 ± 4.2[a]	6.1 ± 0.1[b]	74.3 ± 24.8[a]
80°C	22.0 ± 0.9[a]	26.9 ± 0.2[a]	34.3 ± 1.6[a]	8.7 ± 1.7[a]	5.1 ± 0.6[b]	22.5 ± 1.6[a]

*All values are expressed as mean ± standard deviation of three technical replicates (n = 3).
*Different superscript letters within a column indicate significant differences according to Tukey's multiple range test ($p < 0.05$).

concentration provided the most reliable recovery efficiency and isotopic consistency across all honey sample tested (Table 2). Consequently, 0.5 M phosphoric acid was selected as the optimal concentration for this protocol.

2. Effect of reaction temperature

The influence of reaction temperature (40, 60, and 80°C) on the protein precipitation process was investigated to determine the optimal thermal condition. Analytical results indicated that the protein recovery yield exhibited a notable dependency on the temperature. On average, the reaction at 60°C achieved the most favorable recovery yield, demonstrating the most stable and robust protein precipitation across all tested honey samples (Table 3).

While the stable carbon isotope ratios remained consistent regardless of the temperature variations, confirming that the thermal conditions did not induce isotopic fractionation, the 60°C conditions provided the most reliable and consistent mass for subsequent analysis. Consequently, 60°C was selected as the optimal reaction temperature to ensure stable protein isolation while maintaining isotopic integrity (Table 4).

3. Effect of reaction time

The effect of reaction time (30, 60, and 120 min) on protein precipitation efficiency was evaluated to deter-

Table 4. Effect of reaction temperature on stable carbon isotope ratio ($\delta^{13}C$) of honey proteins (‰)

Temperature	Korea			China and Vietnam		
	KR-1	KR-2	KR-3	CH-1	CH-2	VT-1
40°C	−24.46 ± 0.08	−22.49 ± 0.04	−24.22 ± 0.02	−23.95 ± 0.10	−23.60 ± 0.02	−24.42 ± 0.06
60°C	−24.82 ± 0.01	−22.22 ± 0.07	−24.22 ± 0.03	−23.85 ± 0.24	−24.27 ± 0.01	−24.12 ± 0.11
80°C	−24.32 ± 0.11	−22.96 ± 0.01	−23.91 ± 0.16	−24.23 ± 0.08	−24.14 ± 0.19	−23.51 ± 0.14

*All values are expressed as mean ± standard deviation of three technical replicates (n = 3).

Table 5. Effect of reaction time on protein yield (mg)

Time	Korea			China and Vietnam		
	KR-1	KR-2	KR-3	CH-1	CH-2	VT-1
30 min	24.3 ± 4.2[a]	25.5 ± 1.5[a]	40.5 ± 3.2[a]	9.3 ± 0.4[a]	3.3 ± 0.7[a]	49.2 ± 5.8[a]
60 min	31.9 ± 0.3[a]	28.4 ± 7.3[a]	36.3 ± 1.9[a]	9.8 ± 0.8[a]	4.2 ± 0.2[a]	34.5 ± 4.9[a]
120 min	24.2 ± 0.4[a]	28.9 ± 0.1[a]	31.6 ± 7.3[a]	7.8 ± 0.0[a]	7.9 ± 2.8[a]	36.0 ± 3.1[a]

*All values are expressed as mean ± standard deviation of three technical replicates (n = 3).
*Different superscript letters within a column indicate significant differences according to Tukey's multiple range test ($p < 0.05$).

Table 6. Effect of reaction time on stable carbon isotope ratio ($\delta^{13}C$) of honey proteins (‰)

Time	Korea			China and Vietnam		
	KR-1	KR-2	KR-3	CH-1	CH-2	VT-1
30 min	−24.28 ± 0.01	−23.03 ± 0.01	−24.10 ± 0.06	−23.68 ± 0.09	−23.80 ± 0.06	−24.29 ± 0.04
60 min	−24.55 ± 0.15	−22.64 ± 0.04	−24.11 ± 0.20	−24.39 ± 0.02	−24.03 ± 0.25	−24.36 ± 0.08
120 min	−24.18 ± 0.09	−21.84 ± 0.20	−24.13 ± 0.02	−24.29 ± 0.01	−24.62 ± 0.19	−24.37 ± 0.19

*All values are expressed as mean ± standard deviation of three technical replicates (n = 3).

mine the optimal reaction duration (Table 5).

The average protein recovery yields were 25.1 ± 2.8 mg for 30 min, 21.6 ± 3.4 mg for 60 min, and 22.7 ± 2.3 mg for 120 min. While the protein recovery showed slight variations, statistical analysis indicated no significant improvement in protein mass as the reaction time was extended beyond 30 min. Furthermore, the stable carbon isotope ratios ($\delta^{13}C$) remained constant regardless of the reaction duration, confirming that the reaction time had no significant impact on the isotopic analysis (Table 6). Consequently, to maximize the procedural efficiency and throughput of the overall analysis, 30 min was selected as the optimal reaction time for honey protein precipitation.

4. Effect of washing frequency

The number of washing cycles (1, 3, and 5 times) was evaluated to optimize the removal of residual sugars and acidic matrix components from the recovered protein precipitate. Analytical observations indicated that a single washing cycle was insufficient, leaving behind substantial interfering matrix components that could potentially compromise the purity of the sample (Table 7).

Meanwhile, the stable carbon isotope ratios ($\delta^{13}C$) remained closely consistent across all tested washing cycles, demonstrating that the repetition of the washing process did not inherently alter or distort the isotopic integrity of the honey protein (Table 8). However, extending the protocol to five washing cycles did not yield a

Table 7. Protein yield obtained after various washing cycles (mg)

Washing cycle	Korea			China and Vietnam		
	KR-1	KR-2	KR-3	CH-1	CH-2	VT-1
1 time	198.7±6.1[a]	184.9±24.6[a]	181.6±39.6[a]	36.5±5.2[a]	31.7±24.9[a]	118.6±47.8[a]
3 time	26.5±3.5[b]	37.4±8.8[b]	44.8±5.0[b]	8.8±1.9[b]	37.4±8.8[a]	22.9±0.2[a]
5 time	19.1±0.2[b]	24.9±1.7[b]	24.0±3.1[b]	7.7±0.6[b]	25.0±1.8[a]	19.6±1.6[a]

*All values are expressed as mean±standard deviation of three technical replicates (n=3).
*Different superscript letters within a column indicate significant differences according to Tukey's multiple range test ($p<0.05$).

Table 8. Impact of washing repetitions on $\delta^{13}C$ values (‰) of isolated protein fractions

Washing cycle	Korea			China and Vietnam		
	KR-1	KR-2	KR-3	CH-1	CH-2	VT-1
1 time	−23.34±0.01	−21.18±0.01	−23.56±0.22	−23.60±0.07	−25.30±0.09	−22.95±0.01
3 time	−24.19±0.32	−22.57±0.08	−23.82±0.01	−23.93±0.07	−25.01±0.01	−24.49±0.08
5 time	−24.12±0.07	−23.31±0.23	−24.11±0.05	−23.89±0.01	−24.06±0.01	−24.69±0.05

*All values are expressed as mean±standard deviation of three technical replicates (n=3).

Table 9. Stable carbon isotope ratios ($\delta^{13}C$) of honey proteins obtained by two drying methods (‰)

Drying method	Korea			China and Vietnam		
	KR-1	KR-2	KR-3	CH-1	CH-2	VT-1
Oven dry	−24.36±0.01	−22.70±0.02	−24.00±0.04	−23.86±0.10	−24.82±0.01	−24.62±0.10
Freeze dry	−24.72±0.01	−22.86±0.06	−24.07±0.02	−24.28±0.07	−24.92±0.03	−24.81±0.01

*All values are expressed as mean±standard deviation of three technical replicates (n=3).

statistically significant improvement compared to three cycles. Instead, it unnecessarily increased the processing time and the potential risk of physical protein loss due to repeated centrifugation and supernatant decanting. Ultimately, a three-cycle washing procedure was adopted as the optimal condition, as it effectively eliminated impurities while minimizing unnecessary protein loss, thereby achieving an ideal balance between sample purity and yield.

5. Comparison of drying methods

The effects of different drying techniques on the physical characteristics and quality of the recovered honey protein were evaluated by comparing hot-air oven drying and freeze-drying. Visual and physical observations revealed a distinct difference in the final state of the dried samples between the two methods. When subjected to hot-air oven drying, the protein samples exhibited a slight scorched appearance, suggesting potential thermal degradation or minor carbonization of residual components. Conversely, freeze-drying produced a significantly superior outcome, yielding a finely texturized, well-dried powder. Meanwhile, the stable carbon isotope ratios ($\delta^{13}C$) showed no significant differences between the two drying methods (Table 9), confirming that neither technique induced isotopic fractionation or altered the isotopic integrity of the honey protein. These results indicate that while both methods maintain isotopic consistency, freeze-drying is more advantageous for preserving the physical quality and appearance of the isolated proteins for stable isotope analysis.

DISCUSSION

In this study, a comprehensive evaluation of key pretreatment parameters including phosphoric acid concentration, reaction temperature, reaction time, washing frequency, and drying methods was conducted to establish the optimal protocol for isolating honey protein for stable carbon isotope analysis. While conventional precipitation protocols predominantly utilize sodium tungstate and sulfuric acid (Güçlü *et al*., 2025; Li *et al*., 2026), alternative precipitants and optimized physical parameters are necessary to mitigate issues such as acid catalyzed degradation of abundant sugars under harsh conditions (Rosatella *et al*., 2011; Velasco Calderón *et al*., 2022). Our experimental results demonstrated that while the stable carbon isotope ratios ($\delta^{13}C$) remained robust and consistent across most tested conditions, the physical efficiency and stability of protein recovery were highly dependent on each parameter. This highlights the critical need to fine tune the precipitation environment to overcome the challenges of isolating trace amounts of protein from an overwhelmingly sugar rich matrix (Chua *et al*., 2013; da Silva *et al*., 2016). Based on the empirical findings the optimal pretreatment configuration was established as 0.5 M phosphoric acid concentration, a reaction temperature of 60°C, a reaction duration of 30 min, a three cycle washing procedure, and freeze drying. Specifically, 0.5 M phosphoric acid minimized the variation in protein recovery yields across samples and provided a mild, safe buffering environment compared to sulfuric acid which poses potential side reactions and safety hazards (Rodrigues *et al*., 2025). Regarding thermal conditions, while excessive heat treatment can cause fluctuations in conventional indicators like enzyme activities (Tosi *et al*., 2008), our controlled heating at 60°C for 30 min successfully promoted sufficient protein denaturation without inducing noticeable thermal degradation, thereby maximizing procedural efficiency. Furthermore, to ensure the accuracy of the internal standard method which relies on the isotopic difference between protein and bulk sugar to detect C4 sugar adulteration (White and Winters, 1989; Elflein and Raezke, 2008), thorough removal of residual matrix is essential. In this regard, the three cycle washing protocol successfully eliminated impurities while preventing the excessive protein loss observed with five cycles. Finally, unlike hot-air oven drying which caused minor thermal degradation and a scorched appearance, freeze-drying preserved the physical quality of the isolated proteins, ensuring a reliable sample preparation process for accurate stable isotope analysis (Akyıldız *et al*., 2022). In conclusion, the established combination of 0.5 M acid concentration, 60°C temperature, 30 min reaction time, 3 washing cycles, and freeze-drying offers an efficient, safe, and standardized pretreatment framework for honey protein isolation. By effectively addressing potential methodological flaws such as residual sugar contamination and thermal degradation, this optimized protocol ensures both stable protein recovery and strict isotopic accuracy. Consequently, the phosphoric acid based protein pretreatment method established in this study ensures worker safety while enhancing the accuracy of isotope analysis, thereby suggesting a crucial methodological foundation for the advancement of honey authenticity verification and quality control systems.

ACKNOWLEDGEMENTS

This study was supported by a research project (PJ 01745704) and 2026 the RDA Fellowship Program of National Institute of Agricultural Science, Rural Development Administration, Republic of Korea.

LITERATURE CITED

Akyıldız, İ. E., Ö. Erdem, S. Raday, T. Daştan, S. Acar, D. Uzunöner, G. Düz and E. Damarlı. 2022. Elucidating the false positive tendency at AOAC 998.12 C-4 sugar test for pine honey samples: Modified sample preparation method for accurate $\delta^{13}C$ measurement of honey proteome. J. Food Compos. Anal. 114: 104787.

Alvarez-Suarez, J. M., S. Tulipani, S. Romandini, E. Bertoli and M. Battino. 2010. Contribution of honey in nutrition and human health: a review. Med J Nutrition Metab. 3: 15-23.

Anklam, E. 1998. A review of the analytical methods to determine the geographical and botanical origin of honey. Food Chem. 63(4): 549-562.

Chen, C. T., B. Y. Chen, Y. S. Nai, Y. M. Chang, K. H. Chen and Y. W. Chen. 2019. Novel inspection of sugar residue and

origin in honey based on the $^{13}C/^{12}C$ isotopic ratio and protein content. J. Food Drug Anal. 27(1): 175-183.

Chua, L. S., J. Y. Lee and G. F. Chan. 2013. Honey protein extraction and determination by mass spectrometry. Anal. Bioanal. Chem. 405(10): 3063-3074.

da Silva, P. M., C. Gauche, L. V. Gonzaga, A. C. Oliveira Costa and R. Fett. 2016. Honey: Chemical composition, stability and authenticity. Food Chem. 196: 309-323.

De-Melo, A. A. M., L. B. Almeida-Muradian, M. T. Sancho and A. Pascual-Maté. 2017. Composition and properties of *Apis mellifera* honey: A review. J. Apic. Res. 57(1): 5-37.

Dunn, P. J. H. and G. Skrzypek. 2023. Perspective: Hidden biases in isotope delta results and the need for comprehensive reporting. Rapid Commun. Mass Spectrom. 37(20): e9623.

Elflein, L. and K.-P. Raezke. 2008. 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 elemental analyzer - isotope ratio mass spectrometry and liquid chromatography - isotope ratio mass spectrometry ($\delta^{13}C$-EA/LC-IRMS). Apidologie 39(5): 574-587.

Förstel, H. 2007. The natural fingerprint of stable isotopes-use of IRMS to test food authenticity. Anal. Bioanal. Chem. 388: 541-544.

Guler, A., H. Kocaokutgen, A. V. Garipoglu, H. Onder, D. Ekinci and S. Biyik. 2014. 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. 155: 155-160.

Güçlü, H., P. Yücel, O. Oktar, N. Yazıcı and S. B. Ocak. 2025. Carbon isotope ratios in honey and proteins: A novel method for regional differentiation. J. Food Compos. Anal. 144: 107676.

Kaškonienė, V. and P. R. Venskutonis. 2010. Floral markers in honey of various botanical and geographic origins: A review. Compr. Rev. Food Sci. Food Saf. 9: 620-634.

Lewkowski, O., C. I. Mureşan, D. Dobritzsch, M. Fuszard and S. Erler. 2019. The effect of diet on the composition and stability of proteins secreted by honey bees in honey. Insects 10(9): 282.

Li, A., J. Yang, Y. Ju, S. Niu, S. Liu, S. D. Kelly, C. Vlachou and M. Wang. 2026. Multiple stable isotopes characterization as a tool for determination of the geographical origin of honey in Northern China. J. Food Compos. Anal. 149: 108800.

Padovan, G. J., D. De Jong, L. P. Rodrigues and J. S. Marchini. 2003. Detection of adulteration of commercial honey samples by the $^{13}C/^{12}C$ isotopic ratio. Food Chem. 82: 633-636.

Rodrigues, W. D., A. C. Zanatta, T. C. Borelli, R. R. da Silva, C. L. Cardoso and N. P. Lopes. 2025. Sustainable extraction of alkaloids from *Worsleya procera*: Improving the method with green chemistry. ACS Omega 10: 54834-54849.

Rosatella, A. A., S. P. Simeonov, R. F. M. Frade and C. A. M. Afonso. 2011. 5-Hydroxymethylfurfural (HMF) as a building block platform: Biological properties, synthesis and synthetic applications. Green Chem. 13: 754-793.

Shen, S., B. An, X. Wang, S. P. Hilchey, J. Li, J. Cao, Y. Tian, C. Hu, L. Jin, A. Ng, C. Tu, M. Qu, M. S. Zand and J. Qu. 2018. A surfactant cocktail-aided extraction/precipitation/on-pellet digestion (SEPOD) strategy enables rapid, efficient and reproducible sample preparation for large-scale quantitative proteomics. Anal. Chem. 90(17): 10350-10359.

Tosi, E., R. Martinet, M. Ortega, H. Lucero and E. Ré. 2008. Honey diastase activity modified by heating. Food Chem. 106: 883-887.

Tosun, M. 2013. Detection of adulteration in honey samples added various sugar syrups with $^{13}C/^{12}C$ isotope ratio analysis method. Food Chem. 138: 1629-1632.

Velasco Calderón, J. C., J. S. Arora and S. H. Mushrif. 2022. Mechanistic investigation into the formation of humins in acid-catalyzed biomass reactions. ACS Omega 7: 44786-44795.

White, J. W. and K. Winters. 1989. Honey protein as internal standard for stable carbon isotope ratio detection of adulteration of honey. J. Assoc. Off. Anal. Chem. 72(6): 907-911.

Wu, L., B. Du, Y. Vander Heyden, L. Chen, L. Zhao, M. Wang and X. Xue. 2017. Recent advancements in detecting sugar-based adulterants in honey - A challenge. Trends Anal. Chem. 86: 25-38.