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Research Article

Distinguishing Pure Honey from Honey Adulterated with Added Sugar Using the LC–MS/MS Method

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Abstract
Honey adulteration through the addition of sugar such as liquid sugar, cane sugar, fructose, and glucose remains a major concern affecting honey quality and authenticity. This study aimed to differentiate pure honey, sugar-adulterated honey, and synthetic honey using a liquid chromatography tandem mass spectrometry (LC-MS/MS) fingerprinting approach. Samples included pure acacia (A), longan (K), and kapok/randu (N) honeys, their corresponding sugar-adulterated variants, and synthetic honey (S) as a negative control. The results demonstrated that LC-MS/MS fingerprints effectively distinguished pure honey from adulterated and synthetic samples. Pure honeys exhibited more complex and specific metabolite profiles, whereas sugar addition led to simplified chromatographic patterns dominated by sugar-related compounds. Fructose- and glucose-adulteration produced metabolite profiles most similar to synthetic honey, with randu honey showing the highest sensitivity to fructose addition. Fructosylated compounds such as N-(1-deoxy-1-fructosyl)phenylalanine (Fru-Phe) and isomaltol were identified as indicators of sugar addition and thermal processing. In contrast, biologically and botanically derived metabolites, including umbelliferone, indoleacrylic acid, hypoxanthine, O-glutarylcarnitine, cyclic melatonin, phenethyl tiglate, and indole-3-carboxaldehyde, reflected nectar origin and natural biological processes. The detection of phenanthrene-3,4-diol further indicated environmental influences on honey composition. Overall, integrating processing indicators with biological and botanical markers provides a comprehensive and reliable framework for honey authentication via LC-MS/MS fingerprinting.

Keywords: Authentication, honey, LC–MS/MS, sugar adulteration

1. INTRODUCTION

Honey is a natural sweetener produced by honeybees and is composed of various types of sugars, organic acids, amino acids, enzymes, and minerals [1]. However, honey is also recognized as one of the food products most frequently subjected to adulteration [2]. Honey adulteration can be carried out through several practices, including the deliberate addition of C4 sugars (derived from corn or sugarcane), C3 sugars (such as rice syrup), or invert sugar. In addition, sugar feeding of bees outside the nectar flow season, fermentation resulting from improper storage, and inappropriate processing methods may alter the chemical composition of honey. These conditions can potentially reduce the quality and natural benefits of honey. Therefore, efforts to determine honey authenticity using various analytical chemistry tools have been widely conducted [3].

Pure honey is defined as honey produced by bees from floral nectar without the addition of any external substances. Adulterated honey refers to pure honey that has been mixed with other substances, such

as sugar, to enhance sweetness or increase economic profit. Meanwhile, synthetic honey is an artificial product manufactured without the involvement of bees, typically consisting of solutions or syrups formulated to resemble natural honey.

Consequently, the identification of pure honey and honey adulterated with external substances such as sugar is an important research topic. One analytical instrument that can be applied for this purpose is liquid chromatography–tandem mass spectrometry (LC–MS/MS). LC–MS/MS is an analytical technique that combines the separation capability of liquid chromatography with the high sensitivity and specificity of mass spectrometry. The application of LC–MS/MS in honey analysis has been reported previously, demonstrating its capability to identify various bioactive compounds in honey with potential health benefits [4].

The advantages of LC-MS/MS include its ability to analyze a wide range of compounds, including thermolabile, highly polar, and high-molecular-weight compounds such as sugars. This method can also be used to elucidate the distribution and structural characteristics of oligosaccharides, including molecular weight, sugar sequence, and branching patterns [5], as well as to accurately quantify free sugars in samples [6]. Furthermore, LC–MS/MS has been employed to determine the concentration of the enzyme α-glucosidase III (HBG-III) from Apis mellifera as a marker of honey authenticity, since this enzyme is naturally produced by bees and is relatively difficult to counterfeit through the addition of sugar syrups [7].

2. RESEARCH METHODS

Instruments and Materials

The main instruments used in this study included a rotary evaporator (Heidolph), an LC–MS/MS system equipped with a Quadrupole Time-of-Flight (Q-TOF) mass spectrometer detector (ACQUITY UPLC® H-Class System and Xevo G2-S Qtof, Waters, USA), an analytical balance (KERN-Germany), and other standard laboratory glassware.

The materials used consisted of methanol (p.a., 99.8%), cane syrup (fresh sugarcane juice, commonly consumed as a snack in Ciputat, Indonesia), fructose syrup (liquid corn syrup purchased online), glucose syrup (BRIX 82, purchased online), and granulated sugar (Gulaku® Murni Premium).

Sample Collection

Three types of pure botanical honey were used in this study as control samples: acacia honey, longan honey, and kapok (*randu*) honey. All pure honey samples were obtained directly from Riau, Pati, and Ambarawa, Indonesia, respectively, on December 15, 2024.

Preparation of Adulterated Honey

To simulate honey adulteration, the pure honey samples were deliberately mixed with varying concentrations of cane syrup, fructose syrup, glucose syrups, and sugar liquid (prepared by dissolving the granulated sugar into water in a 1:1 (v/v) ratio (total volume 20 mL). The sugar solution was then transferred into 20 mL vials and mixed with honey at a 1:1 (v/v) ratio (total volume 20 mL). The mixture was homogenized by stirring and vortex mixing prior to LC-MS/MS analysis.

Preparation of Artificial Honey [8]

Artificial (synthetic) honey samples were formulated entirely without pure honey to serve as a negative control. The artificial honey was prepared by mixing 1 kg of granulated sugar with 500 mL of water, 1 thinly sliced lemon, and 2 cinnamon sticks (approximately 5 cm each). All ingredients were placed in a pot and heated over medium heat, stirring continuously until the sugar was completely dissolved. The mixture was then covered and simmered for 50 minutes. After heating, the solution was filtered to remove solid residues and then cooled to room temperature. The resulting artificial honey was stored in a refrigerator for 24 h before further analysis.

Table 1. Sample labels used in this study

No.	Code	Description
1	**A**	**Acacia honey**
2	A1	Acacia + liquid sugar
3	A2	Acacia + cane sugar syrup
4	A3	Acacia + fructose
5	A4	Acacia + glucose
6	**K**	**Longan honey**
7	K1	Longan + liquid sugar
8	K2	Longan + cane sugar syrup
9	K3	Longan + fructose
10	K4	Longan + glucose
11	**N**	**Kapok (randu) honey**
12	N1	Kapok + liquid sugar
13	N2	Kapok + cane sugar syrup
14	N3	Kapok + fructose
15	N4	Kapok + glucose
16	**S**	**Artificial (synthetic) honey**

Sample Extraction

The purpose of this stage was to isolate and concentrate the secondary metabolites from the complex honey matrix. An identical extraction protocol was systematically applied to all sample groups (pure honey, adulterated honey, and artificial honey). Exactly 100 g of each sample was mixed with 300 mL of methanol and stirred for 30 min using a

magnetic stirrer. The mixture was then allowed to stand in a fume hood for 24 h. Subsequently, the honey extract was separated into the precipitated residue and the filtrate using filter paper. The methanolic honey filtrate was then concentrated using a rotary evaporator at 40 °C to obtain a concentrated methanolic honey extract. Details of the sample variations are shown in **Table 1**.

Analysis LCMS/MS [9]

For the chromatographic analysis, 1 mg of the concentrated methanolic honey extract was accurately weighed and dissolved in 10 mL of methanol to ensure the concentration was within the instrument's sensitivity range. Then, 10 μL of the sample was injected into the LC-MS/MS system. Chromatographic separation was performed using an ACQUITY UPLC® BEH C18 column (2 x 150 mm) at a flow rate of 0.2 mL/min. The mobile phase consisted of acetonitrile and water, providing optimal separation.

The resulting chromatogram and mass spectra were analyzed using MassLynx software (Version 4.1). To identify the chemical structures of the compounds, the sample's base peak was compared against the MassBank database. Subsequently, suspected compounds were further searched and verified against the online databases, including the Human Metabolome Database (HMDB), and the MassBank of North America (MoNA). The detailed specifications and operating conditions of the LC–MS/MS system are summarized in **Tables 2 and 3**.

Table 2. Conditions and details instrumentation

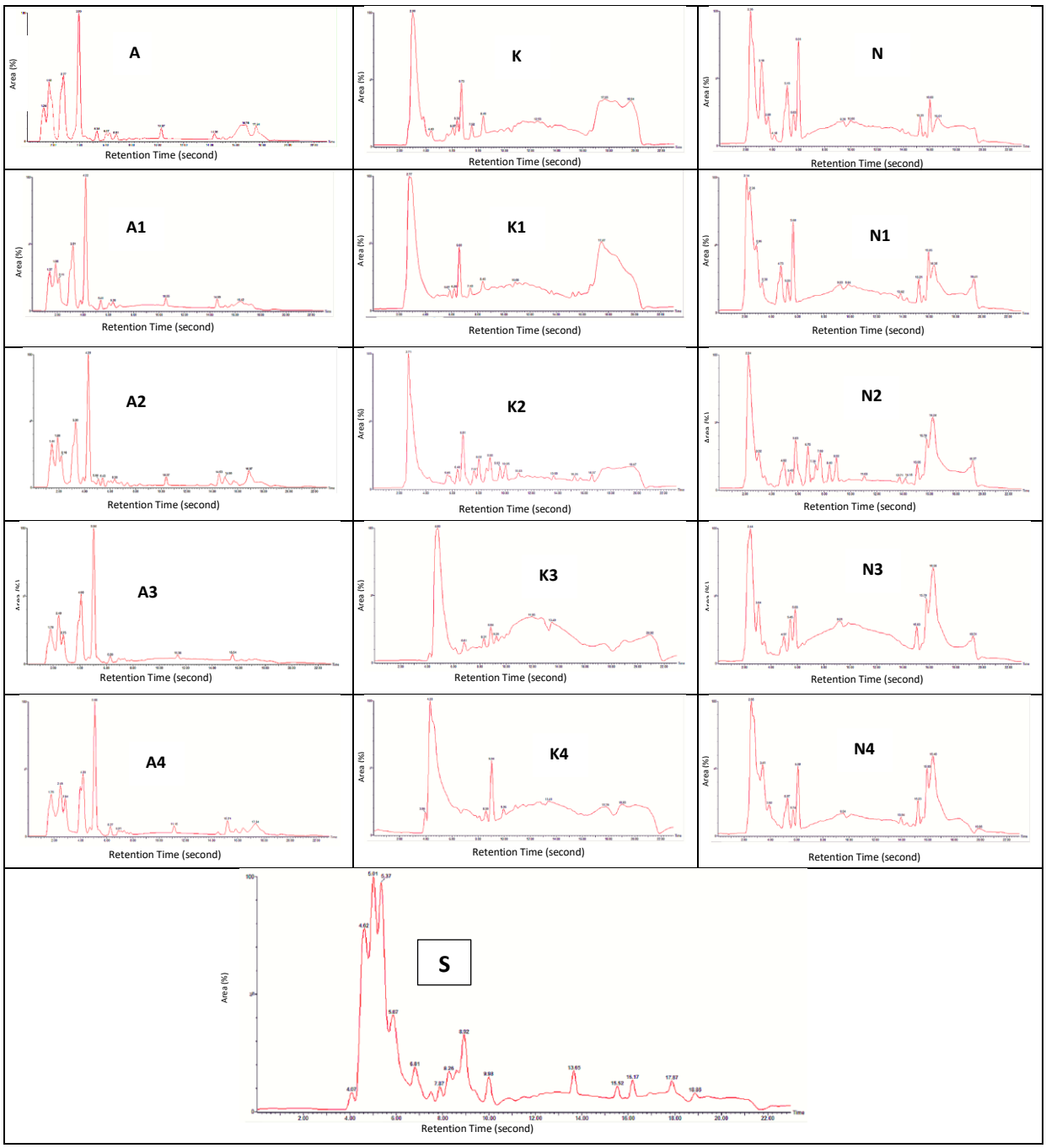
Conditions	Details
Chromatographic Separation:	
LC System	Ultra Performance Liquid Chromatogphy (UPLC)
Column	C18 (1.8 µm 2.1x100 mm) HSS and see informasi at: (http://www.waters.com/1/3/acquity-uplc-hss-t3-column)
Temperature	50ºC (Column), 25ºC (room)
Mobile phase	Water + 5 mM Amonium Formic (A) and Acetonitril + 0,05 % Formic acid (B)
Flow rate	0.2 mL/min (step gradien) running 23 minute • This is a stepwise (discrete) gradient method in which the mobile phase composition is changed incrementally rather than continuously (non-linear gradient). • Function: To achieve sharper separation of compounds with differing polarities. • Suitable for: Complex matrices (e.g., biological samples, honey, and herbal extracts) and multi-analyte targets
Injection Volume	5 µL (filter through 0.2 µm syring filter first)
Mass Spectrometry:	
System	ESI (electrospray ionization)
Mode	Positive mode
Mass analysis range	50 – 1200 m/z
Source Temperature	100ºC
Desolvation Temperature	350ºC
Cone gas flow	0 L/hr
Desolvation gas flow	793 L/hr
Collision energy	4 Volt (low energy)
Rampt Colision energy	25-60 volt (high energy)

Table 3. LC-MS analysis detail

Instrument	Specification	Details
LC System	ACQUITY UPLC®H-Class System (waters, USA)	UPLC (Ultra Performance Liquid Chromatography)
LC Column	ACQUITY UPLC®HSS C18 (1.8 µm 2.1x50 mm) (waters, USA)	UPLC Column HSS (high Streght Silica)
Mass Spectrometer	Xevo G2-S Qtof (waters, USA)	Two Generation Stepwave Quadrupole time-of-flight mass spectrmetry

3. RESULTS AND DISCUSSION

The LC–MS/MS fingerprinting revealed clear differences in the compound profiles, particularly in the secondary metabolites present in each honey sample. The addition of simple sugar biomolecules to honey resulted in distinct changes in the chromatographic patterns compared to those of pure honey (**Figure 1**).



Description:
A: pure acacia honey; A1: acacia honey + liquid sugar; A2: acacia honey + cane sugar syrup; A3: acacia honey + fructose solution; A4: acacia honey + glucose solution.
K: pure longan honey; K1: longan honey + liquid sugar; K2: longan honey + cane sugar syrup; K3: longan honey + fructose solution; K4: longan honey + glucose solution.
N: pure kapok (randu) honey; N1: kapok honey + liquid sugar; N2: kapok honey + cane sugar syrup; N3: kapok honey + fructose solution; N4: kapok honey + glucose solution.
S: synthetic honey.

Figure 1. LC–MS/MS fingerprints of pure honey samples and honey samples adulterated with added sugar

The main bioactive compounds or their derivatives were still detected in the sugar-adulterated honey samples. However, the presence of added sugars could be detected by changes in peak intensities and the emergence of specific compound patterns distinct from those of pure honey. Although no definitive marker compounds have been conclusively established, the distinct LC–MS/MS fingerprint profiles observed between authentic and sugar-adulterated honey samples clearly indicate significant compositional differences. Notably, 6-(α-D-Glucosaminyl)-1D-myo-inositol was detected in acacia honey samples adulterated with cane sugar and glucose, while it was absent in authentic acacia honey

(Table 5). This compound is not typically reported as a dominant constituent of natural honey, which is generally characterized by a high abundance of simple sugars [10]. Therefore, its presence may serve as a potential indicator of exogenous sugar addition. In addition, several other distinct compounds identified in Table 5 further support the differentiation between authentic and adulterated samples. However, these compounds cannot yet be considered definitive chemical markers, as further validation using targeted analysis and a broader sample set is required. The complexity of bioactive compounds in pure honey, sugar-adulterated honey, and synthetic honey differed significantly. This was reflected in the distinct LC–MS/MS fingerprint patterns obtained and was further supported by the compound identification results presented in **Table 4**.

Based on the LC–MS/MS fingerprint patterns shown in **Figure 1**, a heatmap representation was constructed to illustrate the suspected compounds identified in pure honey, sugar-adulterated honey, and synthetic honey samples, as presented in **Table 5**.

Table 4. LC–MS/MS fingerprint analysis of pure honey and sugar-adulterated honey samples shown in Figure 1

No.	Honey Group	Description
1.	Pure Honey	• Pure honey samples (A, K, and N) exhibited complex LC–MS/MS fingerprint patterns with a higher number of peaks and characteristic intensity distributions. • Each pure honey type showed specific peaks at distinct retention times, which may serve as botanical markers. • Variations among A, K, and N indicate that nectar source influences the secondary metabolite profiles of honey.
2.	Acacia Honey (A–A4)	• Pure acacia honey (A) exhibited the most complex LC–MS/MS fingerprint among acacia samples. • A1 (acacia + liquid sugar) and A2 (acacia + cane sugar syrup) showed reduced intensities of characteristic honey peaks and the presence of dominant peaks associated with simple sugars. • A3 (acacia + fructose) and A4 (acacia + glucose) displayed simplified fingerprint patterns with major peaks at early retention times, indicating monosaccharide dominance and reduced honey-specific minor compounds. • These pattern changes clearly indicate sugar addition and distinguish adulterated samples from pure acacia honey.
3.	Longan Honey (K–K4)	• Pure longan honey (K) exhibited an LC–MS/MS fingerprint distinct from acacia and kapok honeys, with characteristic peaks at specific retention times. • K1–K4 showed pattern changes similar to the acacia group, characterized by reduced honey-specific peaks and increased sugar-related peaks. • K3 and K4 (fructose and glucose addition) displayed highly similar fingerprint patterns, indicating that different monosaccharides produce relatively uniform LC–MS/MS fingerprints.
4.	Kapok (randu) Honey (N–N4)	• Pure kapok honey (N) exhibited a unique LC–MS/MS fingerprint distinct from acacia and longan honeys. • N1 and N2 still retained some of the randu honey peaks, although with reduced intensities. • N3 and N4 showed simplification of chromatographic patterns, approaching those of pure sugar profiles.
5.	Synthetic Honey (S)	• Synthetic honey (S) exhibited the simplest LC–MS/MS fingerprint, characterized by dominant peaks at specific retention times representing simple sugars. • No characteristic peaks of minor metabolites typically found in natural honey were detected. • This profile was clearly distinct from all pure honey samples and served as a negative reference.

Based on the results presented in **Table 5**, a total of 28 compounds were identified in the honey samples, indicating the presence of a mixture of phenolic metabolites, modified sugars, aroma-related compounds, and nitrogen-containing metabolites. These compounds were further classified into several categories as follows:

1. Bioactive compounds: genipin, isogingerenone B, demethoxyshogaol, umbelliferone, and perlolyrine.
2. Antioxidant and aromatic compounds: gamma-terpinene, phenethyl tiglate, and indole-3-carboxaldehyde.
3. Modified sugar compounds: N-(1-deoxy-1-fructosyl)phenylalanine and isomaltol.
4. Organic acid compounds: cis,cis-muconic acid and indoleacrylic acid.
5. Nitrogen-containing metabolites: hypoxanthine, His–Glu–Lys, and O-glutarylcarnitine.

A further analysis was conducted to evaluate the impact of sugar addition on the LC–MS/MS fingerprints of pure honey. The changes observed in compound presence, intensity, and pattern complexity were interpreted to assess how different types of added sugar affect honey authenticity. A comparative summary of these effects across acacia, longan, kapok, and synthetic honey samples is presented in **Table 6**.

Amadori compounds and sugar reaction products, such as N-(1-deoxy-1-fructosyl)phenylalanine (Fru-Phe) **(Figure 2a)** and isomaltol **(Figure 2b),** were predominantly detected in honey samples with added sugars, including liquid sugar, cane sugar, fructose, and glucose (A1–A4, K1–K4, and N1–N4). This pattern is consistent with the behavior of 5-hydroxymethylfurfural (HMF), another well-established thermal and quality indicator in honey, whose levels have been reported to increase significantly following heat treatment due to the dehydration of reducing sugars such as glucose and fructose [11]. Similarly, Fru-Phe (N-(1-deoxy-1-fructosyl)phenylalanine) has been reported to occur at low levels in naturally mature honey but to increase significantly in honey subjected to artificial heating (typically above 60 °C). These findings suggest that Fru-Phe has strong potential as an indicator of thermal processing and/or sugar adulteration in honey [12].

The percentage of peak area (% area) was used as a semi-quantitative parameter in the LC–MS/MS analysis to compare relative changes in metabolite profiles among samples. This parameter is commonly applied in metabolomics and chemical fingerprinting studies [13,14]. However, it does not represent absolute concentrations, as it is influenced by differences in ionization efficiency, fragmentation behavior, and detector response among compounds [15,16].

Table 5. Suspected compounds detected in pure honey and sugar-adulterated honey samples

No	Potential Compound	RT	Mass Error (ppm)	A	A1	A2	A3	A4	K	K1	K2	K3	K4	N	N1	N2	N3	N4	S
1	Genipin	9.63	1.32						■	■	■			■	■	■			
2	Isoamyl 2-furonpropionate	10.88	11.37					■	■	■	■								
3	Isogingerenone B	14.37	1.81									■	■	■	■	■			
4	2-(1,3-Dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-isopropylphenyl)acetamide	7.83	3.37	■	■	■													
5	3-Methylcrotonylglycine	5.35	2.53	■	■	■	■	■											
6	6-(alpha-D-Glucosaminyl)-1D-myo-inositol	1.37	0.00	■	■	■	■	■									■	■	
7	6'-O-Formylmarmin	12.24	1.94						■	■	■			■	■	■			
8	beta-D-Galactopyranosyl-(1->4)-2-amino-2-deoxy-beta-D-glucopyranosyl-(1->6)-D-mannose	5.87	1.19														■	■	
9	cis,cis-Muconic acid	5.8	4.89		■	■	■	■							■	■	■		
10	cyclic Melatonin	7.89	2.60	■	■	■	■	■											
11	Demethoxyshogaol	15.54	1.62	■	■	■	■	■				■	■	■	■	■	■	■	
12	Epiisopiloturine	7.65	2.79	■	■	■	■	■											
13	Ethyl aconitate	20.00	7.88	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	
14	Gamma-Terpinene	0.84	0.73		■	■		■	■	■	■	■	■	■	■	■	■	■	■
15	Gerberinol	7.81	9.86	■	■			■	■	■	■	■	■	■					
16	His-Glu-Lys	4.20	4.60							■	■	■	■	■					
17	Indole-3-carboxaldehyde	8.40	3.42				■	■	■	■	■	■	■	■					
18	Indoleacrylic acid	5.08	2.13	■	■	■			■	■	■			■	■	■			
19	Isogingerenone B	15.74	1.29					■	■	■	■	■	■		■	■	■	■	
20	Isomaltol	3.81	1.83	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	
21	Lotaustralin	1.85	3.43	■	■	■	■	■		■	■	■	■	■	■	■	■	■	
22	Mono-trans-p-coumaroylmesotartaric acid	6.07	9.00				■	■	■	■	■	■	■		■	■	■	■	■
23	N-(1-Deoxy-1-fructosyl)phenylalanine	4.40	0.61	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	
24	O-Glutarylcarnitine	2.77	5.07	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	
25	Perlolyrine	8.99	4.15	■	■	■													
26	Phenanthrene-3,4-diol	10.27	3.32	■	■	■	■	■											
27	Phenethyl tiglate	13.49	1.46				■	■	■	■	■	■							
28	Umbelliferone	14.72	4.29	■	■			■	■	■	■	■	■	■	■	■	■	■	

Description:

A: pure acacia honey; A1: acacia honey + liquid sugar; A2: acacia honey + cane sugar syrup; A3: acacia honey + fructose solution; A4: acacia honey + glucose solution.

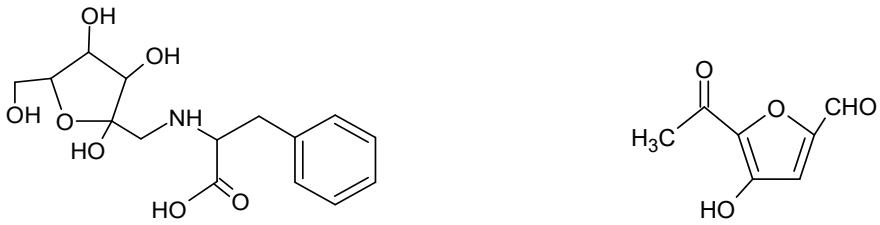
K: pure longan honey; K1: longan honey + liquid sugar; K2: longan honey + cane sugar syrup; K3: longan honey + fructose solution; K4: longan honey + glucose solution.

N: pure kapok (randu) honey; N1: kapok honey + liquid sugar; N2: kapok honey + cane sugar syrup; N3: kapok honey + fructose solution; N4: kapok honey + glucose solution.

S: synthetic honey. RT : Retention Time.

Table 6. Effect of sugar adulteration on LC–MS/MS analysis of pure honey

No.	Honey Group	Impact of Adulteration
1.	Acacia Honey (A1–A4)	• Strong appearance of Isomaltol, a modified sugar compound (A1) • Increase in fructosyl-sugar compounds (A2) • Increase in N-(1-Deoxy-1-fructosyl)phenylalanine (A3) • Dominance of Isomaltol coupled with a decrease in phenolic compounds (A4)
	Interpretation	Adulteration of acacia honey consistently enhanced sugar reaction–derived compounds, while honey-specific phenolic compounds were suppressed. Fructose and glucose addition promoted the formation of early Maillard reaction products, clearly distinguishing adulterated samples from pure acacia honey.
2.	Longan Honey (K1–K4)	• K1–K4 showed a sharp increase in isomaltol and fructosyl compounds. • Several longan-specific marker compounds decreased in intensity or were no longer detected. • O-Glutarylcarnitine remained detectable, indicating partial preservation of the natural honey matrix.
	Interpretation	The longan honey profile remained identifiable as adulterated natural honey rather than synthetic honey, indicating that the natural metabolite structure was not completely lost.
3.	Kapok (randu) honey (N1–N4)	• Increased reactive sugar–derived products • Reduction of natural indole compounds. • The N3 LC–MS/MS fingerprint was highly similar to S
	Interpretation	Kapok honey adulterated with fructose (N3) showed the highest susceptibility to transformation toward a synthetic-like LC–MS/MS profile.
4.	Synthetic Honey (S)	• Exhibited minimal to nearly absent natural phenolic compounds. • No nectar-derived metabolites were detected, including natural indol compounds or plant-specific phenolics.
	Interpretation	The S profile was distinctly different from A, K, and N, and showed the highest similarity to fructose and glucose adulterated honey samples.



(a) (b)

Figure 2. Chemical structures of (a) *N-(1-deoxy-1-fructosyl)phenylalanine* (Fru-Phe) and (b) isomaltol.

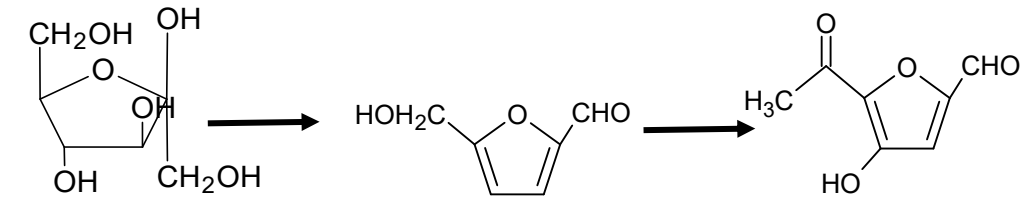
The data in **Table 7** show a relative increase in the peak area of Fru-Phe in acacia honey samples with added sugar (A1) compared to pure acacia honey (A). This increase in relative intensity indicates the accumulation of Amadori compounds resulting from sugar addition, which is consistent with the occurrence of early-stage Maillard reactions. Although Fru-Phe was also detected in pure honey, changes in its relative intensity can be used as an early indicator of honey processing or sugar adulteration.

Another compound abundantly detected in sugar-adulterated honey samples was isomaltol (**Table 5**). Chemically, isomaltol is a sugar degradation product commonly associated with heating, caramelization, and advanced stages of the Maillard reaction. Isomaltol (6-methyl-3-hydroxy-2-pyrone) is a heterocyclic compound belonging to the pyrone group, primarily formed through thermal degradation of carbohydrates and transformation of reducing sugars (**Figure 3**), particularly fructose, under non-natural processing or heating conditions [17,18].

Several studies have reported that isomaltol can be used as an indicator of quality changes in sweet products subjected to heating or intensive processing [22]. The increased frequency of occurrence and relative peak area of isomaltol in samples A1–A4, K1–K4, and N1–N4 indicate that sugar-based adulteration and/or thermal treatment generate metabolite profiles that are clearly distinct from those of pure honey. To further clarify the differences between pure honey and honey subjected to processing or adulteration, a comparison of the relative peak area (%) of isomaltol in each sample is presented in **Table 8**.

Table 7. Relative peak area of Fru-Phe in pure acacia honey and sugar-adulterated acacia honey based on LC–MS/MS analysis

Sampel	RT (min)	Relative Area (%)	Measured mass	Calculated mass	Formula	Potential Compound
A	4.4	0.94	3.281.398	3.281.396	$C_{15}H_{21}NO_7$	N-(1-Deoxy-1-fructosyl)phenylalanine
A1	4.64	1.01	3.281.395	3.281.396	$C_{15}H_{21}NO_7$	N-(1-Deoxy-1-fructosyl)phenylalanine



Fructose 5-Hydroxymethylfurfural (5-HMF) Isomaltol

Figure 3. Transformation of fructose into isomaltol [19–21].

Table 8. The relative peak area (%) of isomaltol in honey samples based on LC-MS/MS analysis

Sampel	RT (min)	Relative Area (%)	Measured mass	Calculated mass	Formula	Potential Compound
A1	3.81	1.35	2.730.400	2.730.395	$C_6H_6O_3$	Isomaltol
A2	3.9	0.88	1.270.398	1.270.395	$C_6H_6O_3$	Isomaltol
A3	4.64	0.47	1.270.399	1.270.395	$C_6H_6O_3$	Isomaltol
A4	4.7	0.75	1.270.399	1.270.395	$C_6H_6O_3$	Isomaltol
K	6.38	1.49	1.270.390	1.270.395	$C_6H_6O_3$	Isomaltol
K1	6.2	0.52	1.270.390	1.270.395	C_6H_6O3	Isomaltol
K2	6.4	1.1	1.270.389	1.270.395	$C_6H_6O_3$	Isomaltol
K3	8.84	2.28	1.270.389	1.270.395	$C_6H_6O_3$	Isomaltol
K4	8.59	0.57	1.270.389	1.270.395	$C_6H_6O_3$	Isomaltol
N	5.63	1.2	1.270.390	1.270.395	$C_6H_6O_3$	Isomaltol
N1	5.25	1.18	1.270.389	1.270.395	$C_6H_6O_3$	Isomaltol
N2	5.43	0.91	1.270.390	1.270.395	$C_6H_6O_3$	Isomaltol
N3	5.45	1.84	1.270.391	1.270.395	$C_6H_6O_3$	Isomaltol
N4	5.74	1.01	1.270.390	1.270.395	$C_6H_6O_3$	Isomaltol

Phenolic and aromatic compounds, including umbelliferone, were more frequently detected in pure honey samples than in honey adulterated with added sugar in this dataset (**Table 5**). Comprehensive studies on minor honey constituents indicate that phenolic compounds and volatile organic compounds (VOCs) are commonly used as chemical fingerprints to determine the botanical and geographical origin of honey. The reduction or loss of these compounds in adulterated honey samples reflects dilution of botanical signatures caused by sugar addition or substitution with non-natural ingredients [23]. Therefore, the relative presence of phenolic and aromatic compounds supports honey authenticity, whereas decreased intensity or absence of these compounds may serve as an early indicator of processing or adulteration.

Umbelliferone (7-hydroxycoumarin) (**Figure 4**) is a phenolic compound belonging to the coumarin derivative group that originates from plant metabolites and is transferred from nectar into honey. The presence of this compound reflects contributions from specific botanical origins and ecosystems, particularly those dominated by plants from the Apiaceae family [24]. In addition to its role as a botanical marker, umbelliferone is known to exhibit a wide range of biological activities, including antioxidant and anti-inflammatory properties, as well as pharmacological potential in the prevention and treatment of various diseases such as neurodegenerative disorders, diabetes, cancer, and microbial infections [25–27]. Nevertheless, the absence of umbelliferone alone cannot be used as definitive evidence of honey adulteration, as not all honey types naturally contain this compound.

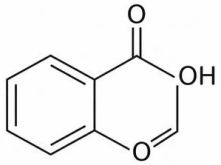


Figure 4. Chemical structure of umbelliferone

In *randu* honey, suspect screening analysis and a non-targeted metabolomics approach revealed that several organic acids and indole derivatives, particularly indoleacrylic acid (IAA), were consistently detected (**Table 5**) and may serve as potential markers associated with nectar type characteristics. Previous metabolomics studies have reported that indole metabolites, including IAA, can serve as candidate markers for the classification of certain monofloral honeys, such as chestnut and citrus honeys [28].

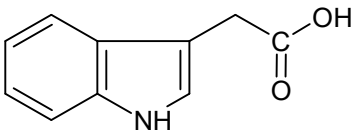


Figure 5. Chemical structure of indoleacrylic acid

Several commensal bacteria are known to produce indoleacrylic acid (IAA) (**Figure 5**) via tryptophan metabolism (**Figure 6**), including those in the genus Peptostreptococcus. Microbiota studies have shown that the tryptophan-to-IAA metabolic pathway has biological implications for the host, thereby enabling the accumulation of IAA in food matrices associated with microbial activity, including honey [29]. In addition, IAA exhibits important biological activities, such as interactions with xenobiotic receptors (AhR/PXR) and modulatory or anti-inflammatory effects in host models. These mechanisms contribute to the biological activity of indole compounds detected in natural food products such as honey [29].

Hypoxanthine is a purine derivative originating from biological metabolism and is not associated with sugar components. An LC-QqQ-MS/MS study demonstrated that hypoxanthine is consistently detected in monofloral honeys and contributes to honey classification based on nitrogen-containing metabolite profiles [34]. This compound is known to originate from purine catabolic pathways in plants, particularly from nectar and pollen, and has therefore been proposed as a novel parameter with potential utility for authenticating the botanical origin of honey. Hypoxanthine has been reported at relatively high levels in Phacelia and fir honeys, further supporting its association with specific botanical sources [35].

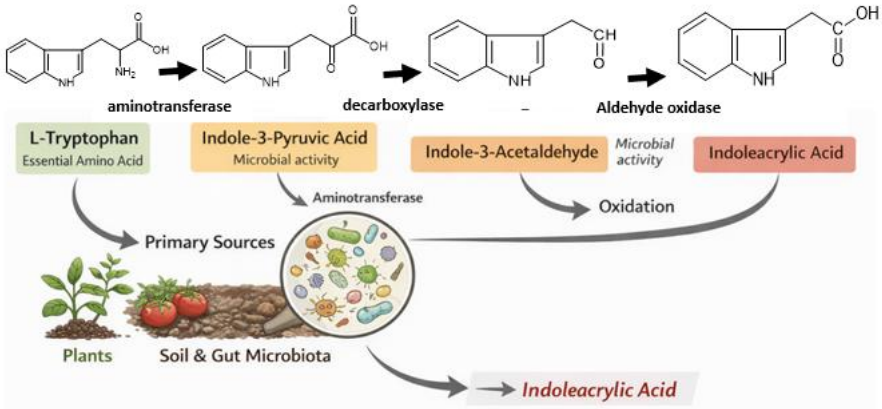


Figure 6. Schematic of indoleacrylic acid transformation involving the tryptophan metabolic pathway and microbiota contributions [30–33]

In the present study, hypoxanthine was detected only in pure kapok/randu honey (N) and randu honey adulterated with cane sugar (N2), but not in synthetic honey or honeys derived from other botanical origins (**Table 5**). This distribution pattern suggests that hypoxanthine has potential as a botanical marker for randu honey. However, its interpretation should be combined with other biological markers to improve the reliability of honey authentication.

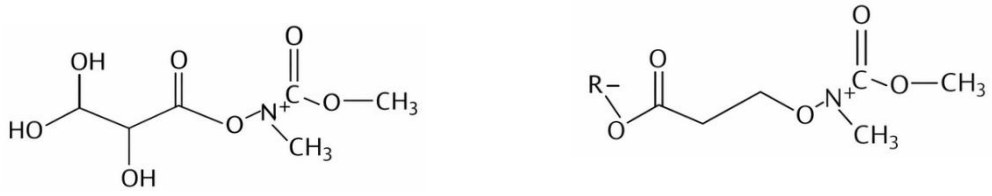
In contrast, analysis of **Table 5** shows that cyclic melatonin and phenanthrene-3,4-diol were consistently detected only in acacia honey samples (A and A1–A4), but not in longan honey, randu honey, or synthetic honey. Cyclic melatonin is a biologically derived indoleamine metabolite originating from plant metabolism, bee activity, and associated microbiota, and cannot be replicated through simple sugar adulteration. As reported previously, the presence of melatonin and its cyclic derivatives in honey reflects natural biological processes, supporting their role as auxiliary biological markers linked to botanical origin[36].

Conversely, phenanthrene-3,4-diol is a polycyclic aromatic hydrocarbon (PAH) derivative not produced by plants or bees, but it enters honey as an environmental contaminant, originating from combustion sources such as smoke and industrial emissions. Previous studies have reported PAHs in honey as indicators of environmental exposure [37]. Therefore, the occurrence of phenanthrene-3,4-diol in acacia honey in this study likely reflects environmental and geographical conditions of nectar collection rather than honey authenticity or adulteration.

A different botanical pattern was observed for longan honey, where analysis of **Table 5** shows that phenethyl tiglate and indole-3-carboxaldehyde were predominantly detected in longan honey samples, while being absent in acacia honey, randu honey, and synthetic honey. Phenethyl tiglate is an aromatic ester derived from plant secondary metabolism and has been reported as a characteristic floral compound associated with nectar-derived products [38]. The presence of phenethyl aromatic structures has also been linked to sweet–floral sensory properties in naturally derived compounds [39], supporting its botanical origin in honey.

Complementing this aromatic signature, indole-3-carboxaldehyde is an indole-derived aromatic compound originating from plant tryptophan metabolism and has recently been identified as a key biomarker for verifying jujube honey authenticity [40]. In this study, the consistent presence of indole-3-carboxaldehyde and phenethyl tiglate in longan honey, including samples subjected to sugar addition, indicates that both compounds reflect botanical metabolic contributions rather than sugar adulteration. Their combined presence therefore supports their role as supporting botanical markers for longan honey within a metabolite fingerprint-based authentication framework.

In addition to botanical and aromatic markers, the occurrence of O-glutarylcarnitine, which belongs to the acyl-carnitine group, in natural honey samples indicates the presence of intrinsic biological metabolites that are not easily replicated through the addition of simple sugar syrups. The Human Metabolome Database (HMDB) and numerous metabolomics studies report acyl-carnitines as common biological metabolites detected across a wide range of biogenic matrices. The relatively consistent presence of O-glutarylcarnitine in pure honey samples (A, K, and N) suggests that a combination of biological markers, such as acyl-carnitines (**Figure 7a**), together with phenolic and aromatic markers, may serve as a robust approach to distinguish natural honey from synthetic honey or honey subjected to extensive processing.



(a) (b)

Figure 7. Chemical structures of (a) acyl-carnitines and (b) O-glutarylcarnitine

LC–MS/MS approaches, both untargeted and targeted, are capable of detecting tens to hundreds of acyl-carnitine species. Therefore, if O-glutarylcarnitine (**Figure 7b**) is present at concentrations above the instrumental detection limit, it will be observable in the metabolomic profile. Consistent with this, several honey metabolomics studies have reported that LC-HRMS or LC-QTOF profiling is effective for detecting lipid metabolites and other minor molecules that reflect the natural biological processes of honey[41].

In contrast, synthetic honey (S) exhibited a metabolite profile dominated by sugars and Amadori reaction products, with a relative lack of phenolic compounds and other biological metabolites. This pattern is consistent with the characteristics of artificial honey, which is typically formulated from sugar mixtures without contributions from nectar or honeybee-mediated biological processes. Honey samples adulterated with fructose or glucose (A3/A4, K3/K4, and N3/N4) showed metabolite profiles most closely resembling synthetic honey (**Table 5**),

particularly characterized by the accumulation of Fru-Phe and isomaltol. These findings indicate that the addition of simple monosaccharides tends to generate metabolomic profiles that closely resemble those of synthetic honey.

4. CONCLUSIONS

This study demonstrates that LC-MS/MS fingerprinting effectively distinguishes pure honey, sugar-adulterated honey, and synthetic honey, confirming its strong potential as a method for honey authentication. Pure honeys (A, K, and N) exhibited more complex chromatographic profiles than adulterated samples, in which sugar addition (particularly fructose and glucose) simplified the metabolite patterns, closely resembling synthetic honey, with randu honey (N3) showing the highest susceptibility. Fructosylated compounds such as N-(1-deoxy-1-fructosyl)phenylalanine (Fru-Phe) and isomaltol were identified as key indicators of sugar addition and thermal processing, while biologically and botanically derived compounds, including umbelliferone, indoleacrylic acid, hypoxanthine, O-glutarylcarnitine, cyclic melatonin, phenethyl tiglate, and indole-3-carboxaldehyde, served as supporting markers of honey authenticity reflecting nectar origin and botanical characteristics. The detection of phenanthrene-3,4-diol further indicated environmental contributions to honey's chemical profile. Overall, an integrated approach combining processing indicators, biological markers, and botanical environmental markers provides a more comprehensive and reliable framework for honey authentication than reliance on a single marker.

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