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Analysis of Amino Acid and Phenolic Content in Honey by UPLC-ESI-MS/MS

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Abstract

Honey is the very valuable natural animal product. It offers more than hundred nutritional substances to its consumers, human being and animals. Though major constituent of honey is sugar and water, honey also possesses amino acids, phenolic compounds, vitamins, minerals and enzymes. Amino acids are one of the important components of food. They provide the required building blocks and protein synthesis. Moreover, phenolic compounds in honey constitute the important quality parameter and account for its colour, sensory properties and antioxidant activity. Analysis of phenolic compound and amino acid is very important. They are generally used to identify the origin of honey. Amino acids in honey come from animals and vegetables. In the literature, there are several techniques concerning amino acid and phenolic compound identifications. In this chapter, usage of ultra-performance liquid chromatography with electrospray ionization coupled to tandem mass spectrometry (UPLC-ESI-MS/MS) techniques and methods for the determination of amino acids and phenolic compounds of honey is explained.

Keywords: honey, amino acids, phenolic compounds, liquid chromatography, UPLC-ESI-MS/MS

1. Introduction

Honey is highly well known and one of the earliest animal products. It has been appreciated throughout civilization and has been recently produced widely in the world [1]. Honey is yellowish or brownish viscid fluid produced by honeybees from the nectar of flowers or from the secretion obtained from the living parts of plants [2]. The honey can be made from a variety of different flowers, and its flavour, texture and chemical composition depend on the floral source from which it was collected. They store the nectar in their sac and enrich it

with some of their own substances, invertase enzyme, to introduce chemical changes. It is produced naturally, and when the honeybees return to the hive, they deposit the nectar in honeycombs for storage and ripening [2, 3]. Honey offered to people since the ancient times. It has been used as medicinal food and preservative having multiple tastes and flavours. In addition, the honeybees do not hesitate to use the needle sometimes at the expense of their own lives and that needle is a drug given to human suffering, even natural therapeutic. Besides being enjoyed as honey, it is used in baking or manufacturing of alcoholic beverages by mixing alcohol or by fermentation into honey-flavoured wine. Preparations containing honey, in combination with milk and cereals, are processed for children. Tobacco products are occasionally flavoured with honey. In medicine, honey is used in pure form or prescribed in preparations such as honey milk, fennel honey and ointments for wounds. It is used in cosmetics as glycerol-honey gels and tanning cream products [4].

Honey includes more than hundred substances. The major constituent of honey is sugar and water; however, amino acids, phenolic compounds, vitamins, minerals and enzymes are found in it [5, 6]. In addition, they directly contribute to the flavour of food and precursors of aroma compounds and formed during thermal or enzymatic reactions in production, processing and storage of food. Honey proteins are derived partly from plants and partly from honeybees.

Amino acids are the building blocks of our body. Amino acids help cells regenerate. Regular nutrition helps us avoid getting older as long as human take the necessary amino acids. It is usually very effective in the formation and progression of muscle tissue. And human-being owe to amino acids for healthy nails and hair. Regulation of brain function, balancing the mental health, muscle making and energy are related to the function of amino acids. Moreover, in the treatment of several illnesses amino acids are used regularly. Obesity is known as the most common diseases of today, and amino acids are used for fat-shattering in situations where obesity and weight control are required. Amino acids also help to treat cerebral diseases such as attention-deficit hyperactivity, dementia, Alzheimer's, Parkinson's, though treatment of those diseases is not fully possible. But amino acid supplements help keep the disease under control. Amino acids are also used in the mental development of school children and in particular stress control from exams. Amino acids are used effectively during the post-operative healing process, especially in burn and wound treatments. Repair and renewal of the tissues is accelerated by amino acids. Another area of use is to delay skin ageing effects and wrinkles, to regain the elastic structure of the drying skin and to prevent hair loss.

As the amino acids are important components of food, and supply the required building blocks or protein synthesis. Considering honey, the amino acids come from animals and vegetables. Amino acid analysis is very important and generally used to identify the origin of honey. There are several techniques concerning amino acid identification, which include multiple steps: sample extraction procedure, derivatization of amino acids, separation and confirmation/quantitation; and also gas chromatography [7] and liquid chromatography [8, 9] methods have been used. Most of the published studies on the determination of amino acids in honey have used derivatization agents and solid-phase extraction (SPE). Although chromatographic

separation of amino acids in honey has been confirmed in the literature, due to derivatization step, peaks appearing in residues and matrixes are still challenging, and frequently complete separation cannot be succeeded. It is considered that mass spectrometric (MS) detection is more selective than ultraviolet-visible (UV-Vis) or fluorescence [10]. It is very substantial to pay special attention to use of MS detection methods for amino acid analysis [1].

Phenolic compounds constitute the important quality parameter of honey and account for their colour, sensory properties and antioxidant activity. Phenolic compounds present in honey can be used as indicators of floral origins and botanical resources, such as pollens, nectars, resins and oils, and the quality of honey [2]. In addition, relatively little is known about honey colour pigments. As an example, the amber colour appears to originate from phenolic compounds and from products of non-enzymatic browning reactions between amino acids and fructose of honey. The health implications also warrant further knowledge of flavonoid contents of the food supply such as honey [13]. It is known that flavonoids and phenolic acids of honey are responsible for significant antioxidant capacity, and other beneficial pharmacologic properties of honey include wound healing, anti-inflammatory, anti-mutagenic and anti-tumoural, protection of skin cells and tissues from oxidative damage and food preservation. Therefore, it is highly demanding to analyse honey and find out which polyphenols are present and in what amount [2, 11, 12, 14, 15].

In this chapter, usage of ultra-performance liquid chromatography with electrospray ionization coupled to tandem mass spectrometry (UPLC-ESI-MS/MS) techniques and methods for the determination of amino acids and phenolic compounds of honey will be explained. In the eighteenth and nineteenth centuries, extremely important advances were made in the development of qualitative and quantitative methods for analysing organic substances. Hence, to check the authenticity and quality control of honey, it is necessary to establish a simple, fast and accurate method to perform extensive honey compositional analysis that will help to identify its most characteristic constituents.

2. Methods

2.1. Instrumental conditions of UPLC-ESI-MS/MS for the amino acid analysis and the phenolic compound analysis

The free amino acids and the phenolic compounds are identified in honey. The amino acid analysis method [1, 12, 16] and the phenolic compound analysis method [2, 11, 12] are easy, fast and reliable procedures without sample clean-up and without derivatization steps. The analyses were performed using an UPLC-ESI-MS/MS instrument, consisting of ultra-performance liquid chromatography with a column manager and heater/cooler, binary system manager, sample manager coupled to a triple quadrupole mass spectrometer equipped with electro spray ionization (ESI). The mass spectrometry parameters, confirmation and quantification mass transition (m/z), and their collision energies are listed in **Tables 1** and **2** for amino acids and phenolic compounds, respectively. Separation operations are accomplished using a C_{18} column, and gradient mobile phase conditions are given in **Tables 3** and **4**, respectively.

Amino acid	Retention time (min)	Quantification transition (m/z)	Confirmatory transition (m/z)	Collision energies (V)
Glycine	0.58	76.00	30, 44, 76	8, 8, 3
Alanine	0.59	90.00	57.1, 71	8, 8
Serine	0.58	106.00	60, 88	9, 10
Proline	0.67	116.10	43.3, 70,1	22, 12
Valine	0.83	118.10	55, 72	18, 10
Threonine	0.61	120.10	56.1, 74, 84, 102.1	15, 10, 12, 9
4-hydroxy Proline	0.61	132.10	68.11, 86.08	14, 12, 8
Leucine	1.67	132.10	69.2, 86	20, 10
Isoleucine	1.54	132.20	69.2, 86,1	20, 9
Asparagine	0.59	133.10	74, 87.13, 115.1	15, 10, 10
Aspartic acid	0.60	134.10	74, 88	14, 10, 8
Lysine	0.58	147.00	84, 115, 130.1	20, 12, 10
Glutamine	0.58	147.10	84.1, 130,1	16, 10
Glutamic acid	0.61	148.10	84, 102.1, 130.2	15, 12, 8
Methionine	1.00	150.20	56.1, 104.1, 133.2	15, 10, 9
Histidine	0.56	156.10	83.1, 93.1, 110.19	22, 20, 15
Phenylalanine	3.41	166.20	77, 91.2,103.1,120	30, 30, 25, 14
Arginine	0.57	175.20	60, 70, 116	15, 20, 15
Tyrosine	1.35	182.16	123.1, 136.1, 165.06	15, 15, 9
Tryptophan	4.27	205.10	91, 118.1, 188.16	35, 25, 10
Cystine	0.58	241.30	74, 120, 152	25, 20, 12

Table 1. Chromatographic and MRM method parameters for free amino acids using UPLC-ESI-MS/MS [1].

Phenolic compounds	Quantification > Confirmatory transition (m/z)	Cone (V)	Collision energies (V)	Mode
Pyrogallol	125.01 > 69.10, 79.04, 81.02	20	17, 17, 14	ESI (-)
Homogentisic acid	167.03 > 123.03, 122.08, 108.00	10	20, 20, 10	ESI (-)
Protocatechuic acid	153.06 > 108.00, 81.01, 91.01	10	20, 25, 20	ESI (-)
Gentisic acid	153.05 > 109.04, 108.03, 81.00	10	20, 20, 12	ESI (-)
Pyrocatechol	153.06 > 81.01, 108.00, 109.04	8	20, 25, 20	ESI (-)
Galantamine	288.10 > 198.00, 213.09, 230.95	20	32, 23, 17	ESI (+)

Phenolic compounds	Quantification > Confirmatory transition (m/z)	Cone (V)	Collision energies (V)	Mode
p-hydroxy benzoic acid	136.98 > 93.03, 65.10	10	25, 14	ESI (-)
3,4-dihydroxybenzaldehyde	137.00 > 91.93, 107.94, 136.00	8	21, 20, 18	ESI (-)
Catechin hydrate	288.88 > 109.15, 124.99, 245.26	30	25, 20, 15	ESI (-)
Vanillic acid	166.98 > 151.97, 108.03, 123.03	20	18, 12, 14	ESI (-)
Caffeic acid	179.10 > 135.14, 107.10, 133.9	32	23, 23, 24	ESI (-)
Syringic acid	197.20 > 123.00, 167.00, 182.00	15	22, 18, 14	ESI (-)
Vanillin	150.95 > 135.94, 91.90, 107.97	30	20, 20, 14	ESI (-)
p-coumaric acid	189.18 > 151.00, 203.00, 205.00	20	20, 20, 20	ESI (-)
Ferulic acid	163.01 > 119.04, 93.00, 117.01	5	27, 27, 15	ESI (-)
Epicatechin	193.03 > 134.06, 178.00, 149.02	20	16, 12, 13	ESI (-)
Catechin gallate	441.00 > 168.98, 288.97	30	20, 20	ESI (-)
Rutin	609.00 > 254.99, 270.93, 299.90	17	55, 55, 40	ESI (-)
trans-2-hydroxy cinnamic acid	163.04 > 119.04, 117.01, 93.07	10	25, 22, 13	ESI (-)
Myricetin	316.90 > 107.07, 137.01, 150.97	30	30, 25, 25	ESI (-)
Resveratrol	227.01 > 143.01, 159.05, 185.03	30	25, 18, 18	ESI (-)
Trans-cinnamic acid	146.98 > 103.03, 62.18	30	10, 10	ESI (-)
Luteolin	284.91 > 107.01, 133.05, 151.02	20	30, 33, 30	ESI (-)
Quercetin	303.00 > 137.00, 153.00, 229.00	20	30, 32, 30	ESI (+)
Naringenin	270.98 > 107.00, 119.04, 150.97	20	25, 25, 20	ESI (-)
Genistein	271.00 > 153.00, 215.00, 243.00	20	27, 25, 24	ESI (+)
Apigenin	269.10 > 107.00, 117.00, 149.00	20	30, 30, 25	ESI (-)
Kaempferol	284.90 > 158.97, 117.10, 227.14	10	34, 40, 30	ESI (-)
Hesperetin	301.02 > 108.01, 136.00, 163.99	20	36, 30, 24	ESI (-)
Chrysin	252.99 > 63.05, 107.05, 142.99	20	30, 25, 25	ESI (-)

Table 2. Chromatographic and MRM method parameters for the analysis of phenolic compounds using UPLC-ESI-MS/MS [2].

Total ion chromatograms (TIC) of each analyte are displayed in **Figures 1** and **2** for amino acids and phenolic compounds, respectively.

2.2. Extraction procedure for amino acid analysis

To prepare 10% (m/v) water honey solutions, 20% methanol solution (v/v) (20 mL), initially acidified with 0.1% formic acid (v/v), is added to 2.0 g honey samples. The resulting mixtures are placed in an ultrasonic bath at 36°C for 10 min to completely mix the

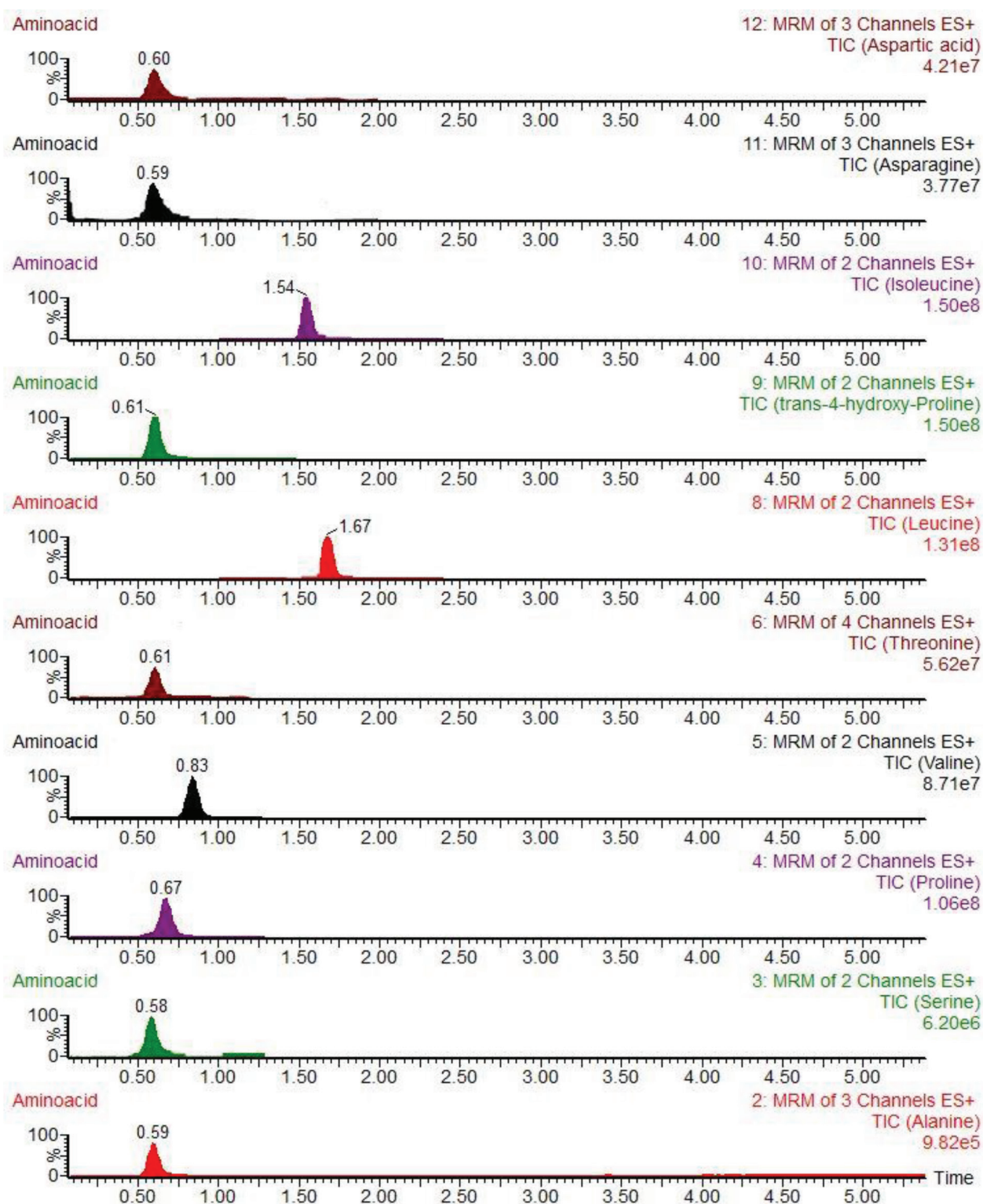
Column	C₁₈ column (1.7 µm 2.1 × 100 mm)			
Mobile Phase A	0.1% aqueous formic acid			
Mobile phase B	methanol/water (50:50, v/v) containing 0.1% formic acid			
Column oven temp.	40°C			
Injection volume	1 µL			
Gradient	Time (min)	Flow (mL/min)	Mobil phase A (%)	Mobil phase B (%)
	0.00	0.400	99.00	01.00
	2.00	0.400	99.00	01.00
	8.00	0.400	30.00	70.00
	9.00	0.400	99.00	01.00
	10.00	0.400	99.00	01.00

Table 3. Chromatographic conditions for free amino acid analysis [1, 12, 16].

Column	C₁₈ column (1.7 µm 2.1 × 100 mm)			
Mobile phase A	0.5% (v/v) acetic acid in ultrapure water			
Mobile phase B	0.5% (v/v) acetic acid in acetonitrile			
Column oven temp.	40°C			
Injection volume	2 µL			
Gradient	Time (min)	Flow (mL/min)	Mobile phase A (%)	Mobile phase B (%)
	0.00	0.650	100.00	00.00
	1.00	0.650	99.00	01.00
	10.00	0.650	70.00	30.00
	12.00	0.650	5.00	95.00
	13.00	0.650	99.00	01.00
	14.00	0.650	100.00	00.00

Table 4. Chromatographic conditions for phenolic compound analysis [2, 11, 12].

extracts of analysed honey samples and subsequently centrifuged at 4000 rpm and 4°C, then the supernatant is filtered through 0.20-µm-pore diameter polytetrafluoroethylene (PTFE) membranes to remove any solid particles, and added to vials and injected into UPLC-ESI-MS/MS [1].



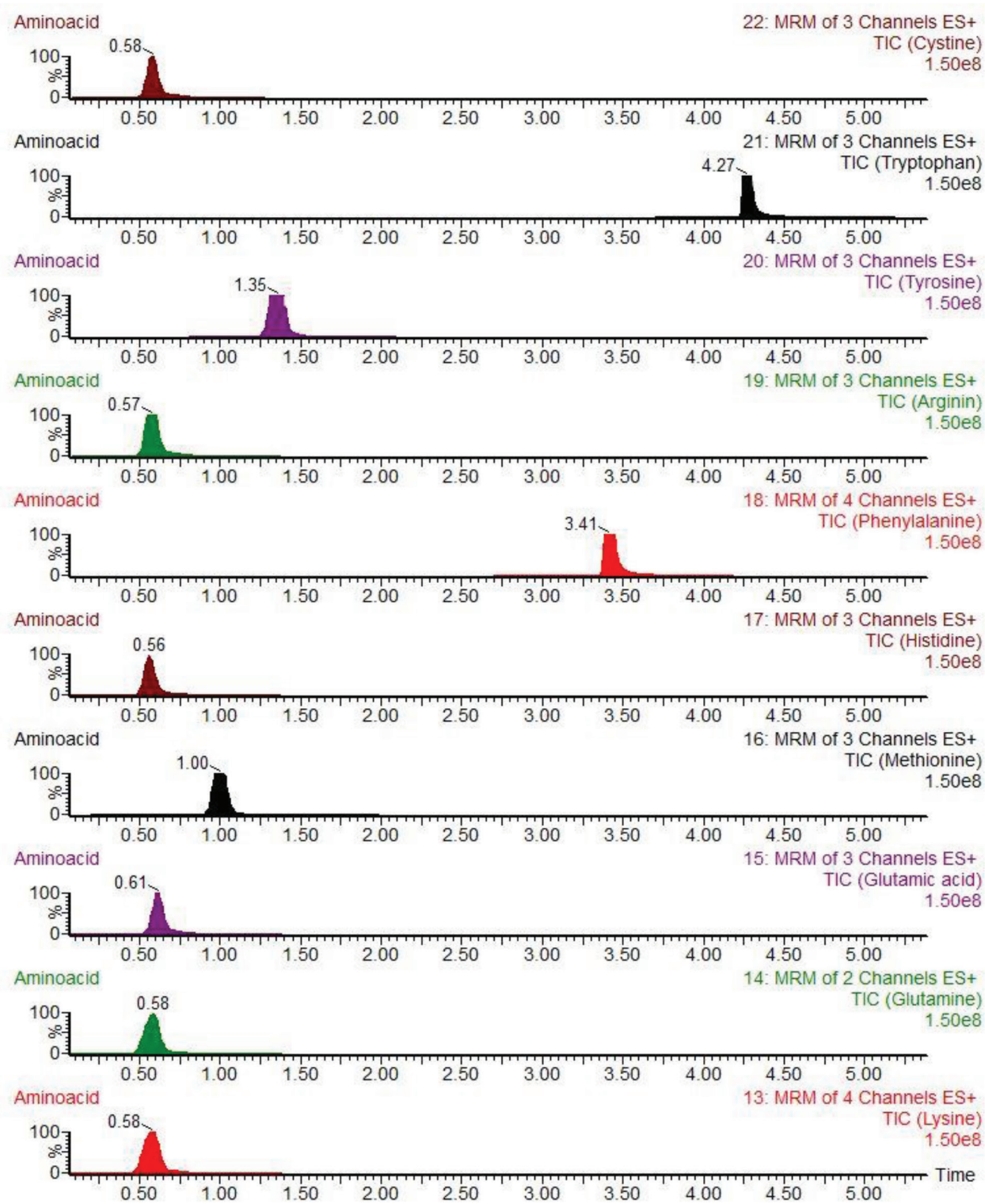
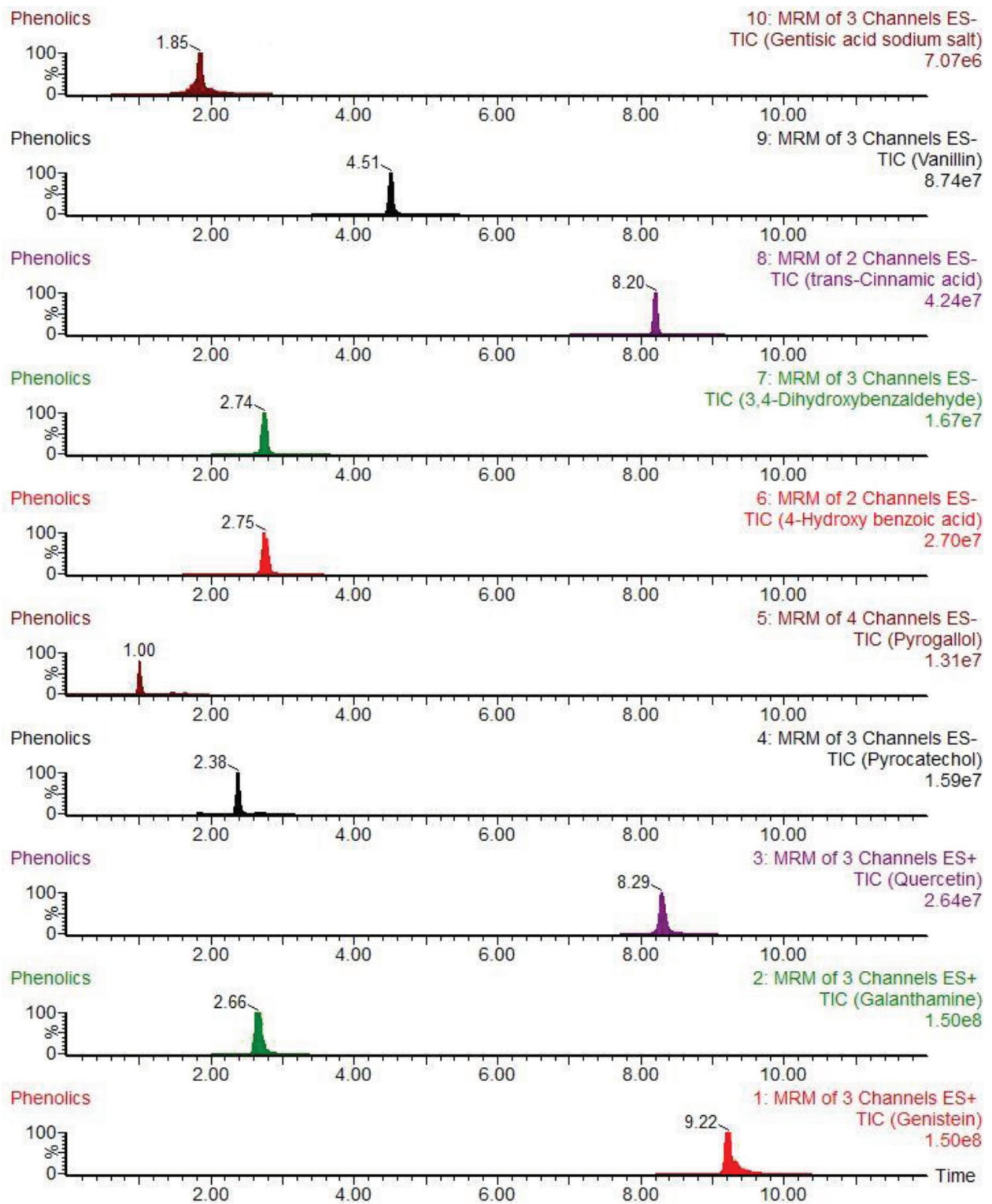


Figure 1. Total ion chromatograms (TIC) of free amino acids using UPLC-ESI-MS/MS.



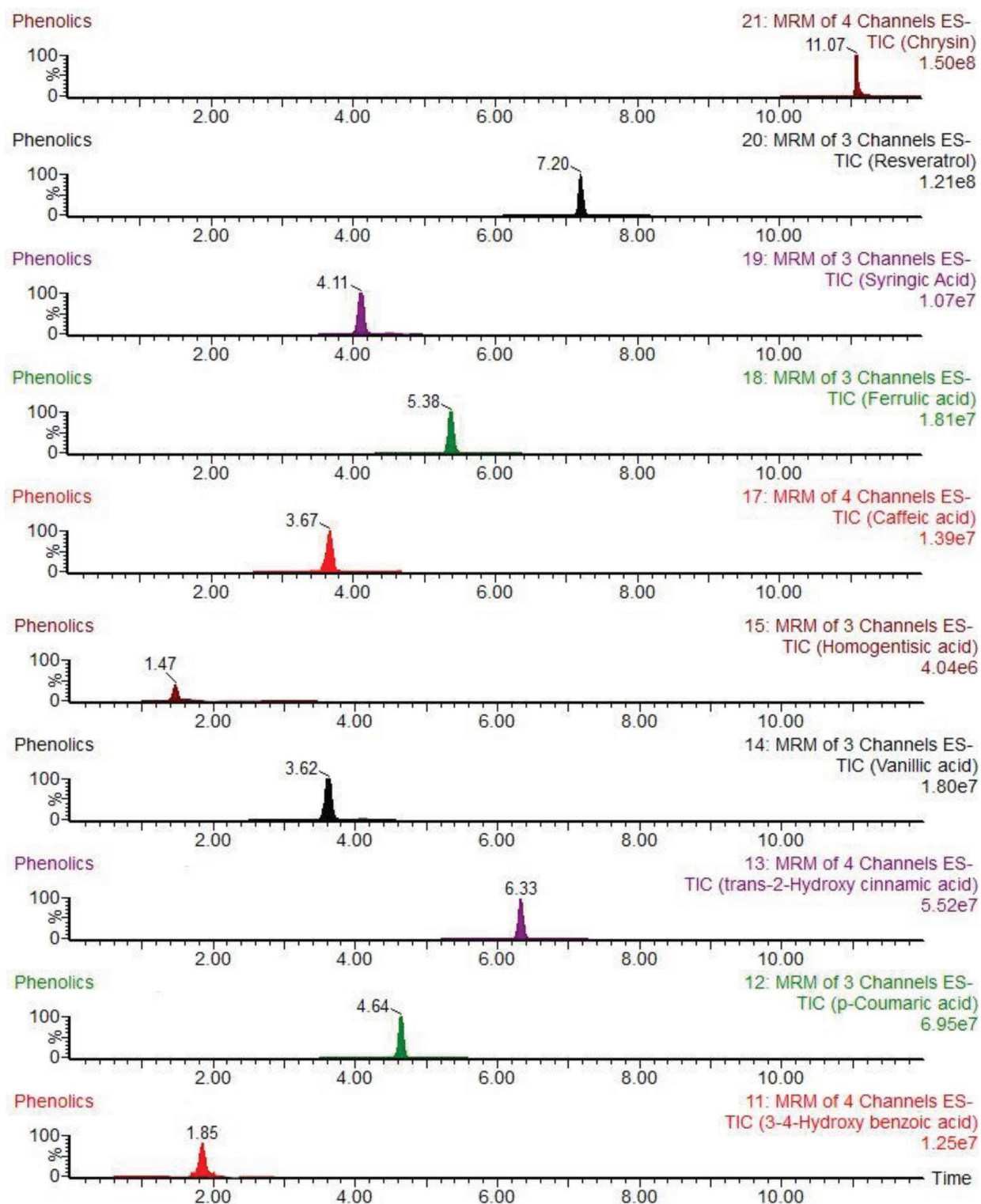


Figure 2. Total ion chromatograms (TIC) of phenolic compounds using UPLC-ESI-MS/MS.

2.3. Extraction procedure for phenolic compound analysis

Honey sample (10 g) is dissolved in ultrapure water (50 mL) and mixed 5 min via vortex. Then ethyl acetate (50 mL) is added into solution flask and the flask is placed on a shaker for 30 min.

After then, the flask settles for the phase separation for 180 min. The water phase is extracted two more times with ethyl acetate, and the combined ethyl acetate extract is evaporated under vacuum at 36°C. The residue is redissolved in methanol (5 mL) and filtered from polytetrafluoroethylene (PTFE) membrane 0.20 µm and added to vials, and 2 µL of the solution is injected into UPLC-ESI-MS/MS [2].

3. Discussion

In the analysis of amino acids and phenolic compounds, an extraction procedure is important. Extraction of amino acids and phenolic compounds depends on their chemical properties such as natural matrix and molecular structure together with their polarity, concentration, aromatic chain number and variation in their hydroxyl groups, etc. Protein, carbohydrate and other complex structures are hindered to extract several phenolic compounds. Differences in the chemical structure of phenolics in the sample are related to concentration of the functional groups, simple and complex polyphenolic structures and phenolic acid and flavonoid in different proportions. In the literature, more than one methods and techniques are needed to be used for the extraction.

The extraction step of amino acids and phenolic compounds is the very critical step after sample preparation. Organic and inorganic solvents are commonly used in the extraction. The efficiency of the extraction is affected from including extraction temperature, time, solvent-sample ratio and solvent types.

Furthermore, treatment time and temperature together with a selection of solvent ratio is very crucial for optimum recovery of amino acids and phenolic compounds. Generally, increasing time and temperature is preferable for the solubility of analytes; however, undesirable enzymatic oxidation arising from high temperature and extended extraction time may cause degradation of amino acids and phenolic compounds. The solvent-sample ratio and repetition number of extraction affect the recovery of phenolic compounds for each sample [17].

Sample matrix and particle size are highly affected the extraction of amino acids and phenolics. Issue of diffusion is related to particle size. Diffusion becomes easier as particle size gets smaller and efficiency of extraction gets higher. However, this increasement continues to some level and after that point it stops or decreases. That situation shows up with the reduction of the mass transfer rate caused by small particles. More solvent is needed in this stage.

Phenolic substance can bound to organic bodies such as carbohydrate and protein in the sample materials. And thus, bounded phenolics can be liberated by hydrolysis with the addition of enzyme.

According to literature survey, there is a lack of knowledge about the profiles of amino acids and phenolic contents in honey to evaluate the quality of the product.

Several studies have been revealed that honey serves as a source of natural antioxidants with the anti-microbial, anti-inflammatory, anti-mutagenic, anti-tumour and anti-oxidative activity, which are effective in reducing the risk of heart disease, immune-system decline, different

inflammatory processes, etc. [2]. Honey species also possess antibacterial activities and are scavengers of active oxygen radicals [15]. Among the components present in honey which are responsible for its anti-oxidative effect are phenolic compounds (flavonols, flavones, flavanones, benzoic and cinnamic acids) [17].

Thereby, as honey is a very complex product. Depending on the nectar-providing plant species, bee species, geographical area, season and a method of storage demand a comprehensive analysis of constituents, such as volatile compounds, phenolic acids, flavonoids, carbohydrates and amino acids, for its characterization [2, 18].

According to literature survey, arginine, tryptophan, phenylalanine, tyrosine and lysine are found in considerable amounts in honey. And also in various studies, they are qualified as a characteristic of some floral types of honey [18, 19].

Phenylalanine, proline, tyrosine, isoleucine, and leucine are revealed as the main amino acids [1]. The studies indicate, on the basis of honey activity, a better differentiation, considering free amino acid contents instead of physicochemical honey characteristics [19]. Moreover, amino acid composition may also be a suitable method to determine honey botanical origin [1, 20].

Around 200 substances have been reported in this complex natural liquid but the composition especially its secondary metabolites and quality of honey may be influenced by some external factors such as environmental and seasonal factors, processing, handling and storage [5, 6].

The determination and evaluation of phenolic constituents in honey appeal high attention by consumers and researchers owing to a health-promoting feature that is accompanied by bioactivity [21].

Botanical origin of honey is classified according to phenolic ingredients [21, 22] and this consequently implies that as honeybees collect nectar from plants which contain bioactive components. These phytochemical ingredients can be transferred to honey by honeybees [23, 24]. Numerous flavonoids (such as apigenin, kaempferol, quercetin, chrysin and luteolin) and phenolic acids (caffeic, gallic, cinnamic, protocatechuic, p-coumaric and chlorogenic acids) are identified in various honey samples [2].

4. Conclusion

The studies displayed that the UPLC-ESI-MS/MS instrument demonstrates to be reliable for the unambiguous detection of a large number of compounds, by enabling the determination of amino acids and phenolic profiles of honey.

Currently, most studies that provide information on honey are directly related to the quality parameters, there are not many studies that analyse chemical compounds present in the honey. Therefore, this research is needed to found a control system that evaluates maintenance of the characteristics and levels of those compounds provided by honey to human nutrition and health. Thereby increasing the levels of security in quality, generating reliability for consumers and ensuring honey consumption devoid of toxic compounds for human health.

The effective technique for identifying the natural nature of a honey is the amino acid analysis. Amino acid analysis of honey is a promising technique in the evaluation of the botanical origin. Thus, honey is described with a good level of complacency.

The rapid, accurate determination and identification phenolic compounds in honey are provided by an improved and easy analytical. Both of the methods proved to be effective for determining honey quality.

Nevertheless, apart from the delicious sweet taste of honey, being the crucial source of free amino acids and phenolic compounds, honey can also be consumed as supplementary materials for food products and applied in nutrients, cosmetics, and pharmaceutical industries.

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