Application Note
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Chemical Fingerprinting of Honey Using a Q-TOF LC/MS-Based Dilute-and-Shoot Approach

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Abstract

Honey is one of the most frequently adulterated foods globally, raising concerns about its authenticity, quality, and safety. This application note highlights a dilute-and-shoot approach combined with high-resolution mass spectrometry (HRMS) and advanced data processing tools to simultaneously evaluate honey's quality, safety, and authenticity. This workflow uses a 9-minute gradient with the Agilent 1290 Infinity II liquid chromatography (LC) system coupled with the Agilent 6545 quadrupole time-of-flight LC/MS (LC/Q-TOF) to generate complex chemical fingerprints that are challenging to replicate. This method enhances fraud detection, enabling a comprehensive analysis of honey's chemical composition and can be adapted for other liquid or semiliquid foods.

Introduction

Honey is a popular natural sweetener, and its global demand remains on the rise.[1] Honey contains an impressive diversity of natural molecules (for example, amino acids, polyphenols, vitamins, alkaloids), contributing to its nutritional and organoleptic properties. Regrettably, honey is also reported to be one of the most common food ingredients affected by food fraud worldwide. Issues related to honey authenticity have been on the rise, a concern for both consumers and honey professionals.[2,3] Furthermore, contaminants may also be present. Quality control of honey generally includes the analysis of hydroxymethylfurfural (HMF, a freshness indicator), pesticide and antibiotic residues, or novel adulterants. Therefore, efficient tools are needed to monitor the authenticity, as well as the quality and safety of honey.

This application note demonstrates the use of the "dilute-and-shoot" approach coupled with high-resolution mass spectrometry (HRMS) to concurrently explore the quality, safety, and authenticity of honey products. HRMS and advanced data processing tools can be used to investigate a broad range of quality attributes simultaneously (for example, contaminants, authenticity, and/or freshness markers). Due to their complexity, the resulting chemical fingerprints are virtually impossible to imitate for fraudsters. Recently, dilute-and-shoot methods in liquid chromatography (LC) coupled to MS (LC/MS) have been introduced for honey analysis. The chemical space investigated through the dilute-and-shoot approach is relatively unaltered, and therefore, more closely reflects the matrix of interest. Combining dilute-and-shoot methods with quadrupole time-of-flight LC/MS (Q-TOF LC/MS) can be used to simultaneously explore the chemical fingerprint of honey while inspecting specific chemicals of interest.

Experimental

Reagents and standards

Water, acetonitrile, ammonium acetate, and formic acid were used for the mobile phases. All reagents and solvents were HPLC or LC/MS grade.

A suite of pure compounds (Table 1) covering a wide range of molecular polarities (logP from –0.43 to 7.29) was purchased from MilliporeSigma (St. Louis, USA). These chemicals belong to families commonly reported in honey and were used to assess the performance of the method. A mixture of these 39 chemical standards was prepared in acetonitrile and later spiked (10 µg/L) into pure solvent (acetonitrile:water, 5:95, v/v) or into honey extracts to produce two reference samples. These reference samples are later referred to in the text as QCAS (solvent) and QCAM (matrix), respectively. Additionally, a standard mix (1 mg/L in acetonitrile) containing the seven mass-labeled compounds was prepared to test recoveries. These compounds included caffeine-d_3, cotinine-d_3, and TBBPA-d_4 (C/D/N isotopes, Pointe-Claire, Canada); methylparaben-d_4 and bentazon-d_7 (Toronto Research Chemical, Toronto, Canada); and imidacloprid-d_4, azoxystrobin-d_4 (MilliporeSigma).

Sample preparation

Honey samples, including 15 blueberry honeys, 15 clover honeys, and 15 buckwheat honeys, were collected from farms and markets in Canada. Approximately 0.2 g (± 0.01 g) of honey was weighed into a 15 mL polypropylene tube and spiked with 10 µL of the recovery standard mix. Following this, 2 mL of a mixture of acetonitrile and water (1:1, v/v) was added, and samples were vortexed for about 5 minutes, or until the honey was completely dissolved. Samples were then filtered through an Agilent 0.2 µm polytetrafluoroethylene (PTFE) filter (Agilent Technologies, Santa Clara, USA) and further diluted with water to a final concentration corresponding to 1% (m/v) of honey. Procedural blanks were prepared following the same procedure without any honey matrix.

Equipment

Separation was performed using the Agilent 1290 Infinity II LC system consisting of the following modules:

- Agilent 1290 Infinity II high-speed pump (G7120A)
- Agilent 1290 Infinity II multisampler with sample cooler (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)

The LC system was coupled to the Agilent 6545 quadrupole time-of-flight LC/MS (LC/Q-TOF) equipped with the Agilent Jet Stream technology ion source (G1958-60470). Agilent MassHunter acquisition software for LC/MS systems (v. 10.1) was used for data acquisition.

Table 1. Mass measurement errors for a range of chemicals in honey matrix (QCAM).

Chemical	Chemical Class	RT (min)	*m/z* Theoretical	*m/z* Measured in Honey Extract (QCAM)	Mass Measurement Errors (ppm)	Ionization Mode
5-(Hydroxymethyl) Furfural (HMF)	Honey freshness marker	2.93	127.0395	127.0392	2.4	ESI+
L-Glutamic Acid	Amino acid	1.39	148.0609	148.0608	0.7	ESI+
Thiamethoxam	Pesticide	3.49	292.0271	292.0263	2.7	ESI+
Imidacloprid	Pesticide	3.78	256.0601	256.0596	2.0	ESI+
Caffeine	Alkaloid	3.11	195.0882	195.0878	2.1	ESI+
Acetaminophen	Drug residue	3.06	152.0711	152.0707	2.6	ESI+
Clothianidin	Pesticide	3.71	250.0165	250.0162	1.2	ESI+
Genistin	Natural product	2.88	433.1134	433.113	0.9	ESI+
Sulfamethazine	Veterinary drug	3.58	279.0915	279.0911	1.4	ESI+
Sulfamethoxazole	Veterinary drug	3.98	254.059	254.0597	2.7	ESI+
Bis (Butoxyethyl) Phosphate (BBOEP)	Contaminant	5.71	299.1623	299.1616	2.3	ESI+
Tylosin A	Veterinary drug	3.32	916.5264	916.527	0.7	ESI+
Atrazine	Herbicide	4.67	216.1015	216.1012	1.4	ESI+
Diuron	Herbicide	4.66	233.0248	233.0242	2.6	ESI+
Metolachlor	Herbicide	5.35	284.1417	284.1414	1.1	ESI+
Carbamazepine	Drug residue	4.18	237.1027	237.1024	1.3	ESI+
Propiconazole	Pesticide	5.35	342.0776	342.0774	0.6	ESI+
Fluoxetine	Drug residue	3.53	310.1418	310.1414	1.3	ESI+
Syringaldehyde	Natural product	3.73	183.0657	183.0655	1.1	ESI+
Tris(2-Butoxyethyl) Phosphate (TBOEP)	Contaminant	5.72	399.2511	399.2511	0.0	ESI+
Azoxystrobin	Pesticide	4.99	404.1247	404.1244	0.7	ESI+
Bis(2-Ethylhexyl) Phthalate	Contaminant	6.05	391.2848	391.2839	2.3	ESI+
Florfenicol	Veterinary drug	3.94	355.9926	355.9926	0.0	ESI–
Vanillin	Natural product	3.78	151.0395	151.0398	2.0	ESI–
Diphenyl Phosphate (DPHP)	Contaminant	3.31	249.0316	249.0321	2.0	ESI–
4-Chlororesorcinol	Contaminant	3.97	142.9899	142.9899	0.0	ESI–
Bisphenol S	Contaminant	4.06	249.0221	249.0223	0.8	ESI–
Monomethyl Terephthalate	Contaminant	2.83	179.0344	179.0346	1.1	ESI–
trans-Cinnamic Acid	Natural product	2.71	147.0446	147.045	2.7	ESI–
2,4-Dichlorophenoxyacetic Acid	Contaminant	3.32	218.9615	218.9617	0.9	ESI–
Bisphenol AF	Contaminant	5.11	335.0506	335.0507	0.3	ESI–
Chrysin	Natural product	5.12	253.0500	253.0506	2.4	ESI–
Ibuprofen	Drug residue	5.17	205.1228	205.1228	0.0	ESI–
Bisphenol A	Contaminant	4.71	227.1072	227.1069	1.3	ESI–
Gemfibrozil	Drug residue	5.74	249.149	249.1492	0.8	ESI–
Bisphenol AP	Contaminant	5.09	289.1228	289.1231	1.0	ESI–
Bisphenol BP	Contaminant	5.46	351.1385	351.1376	2.6	ESI–
Triclosan	Contaminant	5.98	286.9433	286.9429	1.4	ESI–
Perfluorooctane Sulfonic Acid (PFOS)	Contaminant	4.43	498.9296	498.9303	1.4	ESI–

Method

The LC/MS conditions were optimized from a previous, peer-reviewed work[4] and are provided in Table 2.

QCAS and QCAM were used to assess the mass measurement errors and matrix effects for the 39 spiked chemicals during the instrumental analysis. The matrix effect was evaluated by comparing the response of QCAM (honey extracts) and QCAS (solvent). Absolute recovery was evaluated with the recovery standard mix using the standard addition method. The relative standard deviation (RSD) for the interday precision was calculated based on the analysis of three replicates of the same sample on different days.

Results and discussion

Method performance

The mass measurement error for the 39 molecules of interest is lower than 3 ppm in the honey extract matrix (spiked QCAM standards, see Table 1). Analyte recoveries range from 93 to 102% for all the spiked recovery compounds, confirming that no significant loss of chemicals from the honey samples occurs during the sample preparation steps (dilution and filtration). Mild matrix effects (≤ 20% of signal enhancement in honey matrices) are observed for all the spiked compounds (QCAS and QCAM). The interday RSDs are lower than 15%.

Table 2. LC/MS working parameters.

Agilent 1290 Infinity II LC System	
Column	Agilent InfinityLab Poroshell 120 CS-C18, 3.0 × 100 mm, 2.7 µm
Column Temperature	20 °C
Injection Volume	20 µL
Autosampler Temperature	4 °C
Mobile Phase A	Water with 0.1% formic acid (electrospray ionization, ESI+), water with 5 mM ammonium acetate (ESI−)
Mobile Phase B	Acetonitrile with 0.1% formic acid (ESI+), acetonitrile with 5 mM ammonium acetate (ESI−)
Flow Rate	0.3 mL/min
Gradient Program	Time (min) %A %B 0.0 95 5 0.2 95 5 4.0 0 100 6.0 0 100 6.1 95 5
Post Time	Off
Stop Time	9 min
Agilent 6545 LC/Q-TOF	
Ion Source	Agilent Jet Stream (AJS)
Polarity	Positive and negative
Gas Temperature	275 °C
Drying Gas (Nitrogen)	10 L/min
Nebulizer	30 psi
Sheath Gas	325 °C
Fragmentor Voltage	125 V
Nozzle Voltage	250 V
Acquisition Mode	MS

Honey fingerprints

The total ion chromatograms (TICs) obtained for different types of honeys (clover, buckwheat, and blueberry honeys) are illustrated in Figure 1A. These TICs are extremely rich compared to the procedural blanks, reflecting the chemical diversity encountered in honeys. Additionally, the honey fingerprints can be explored to extract molecular features unique to specific honeys (for example, feature *m/z* 96.0445 at 2.45 minutes, which is only present in buckwheat honeys; Figure 1B). Taking advantage of both chromatographic and high-resolution mass spectrometric separations, signals for individual molecules can also be isolated. Examples of extracted ion chromatograms (EICs) are provided for 5-(hydroxymethyl) furfural (Figure 1C), showing detectable traces of this freshness marker in all honeys, but not in the procedural blank. These results illustrate how the present LC/Q-TOF data can be seamlessly processed to obtain information relevant to honey quality and authenticity.

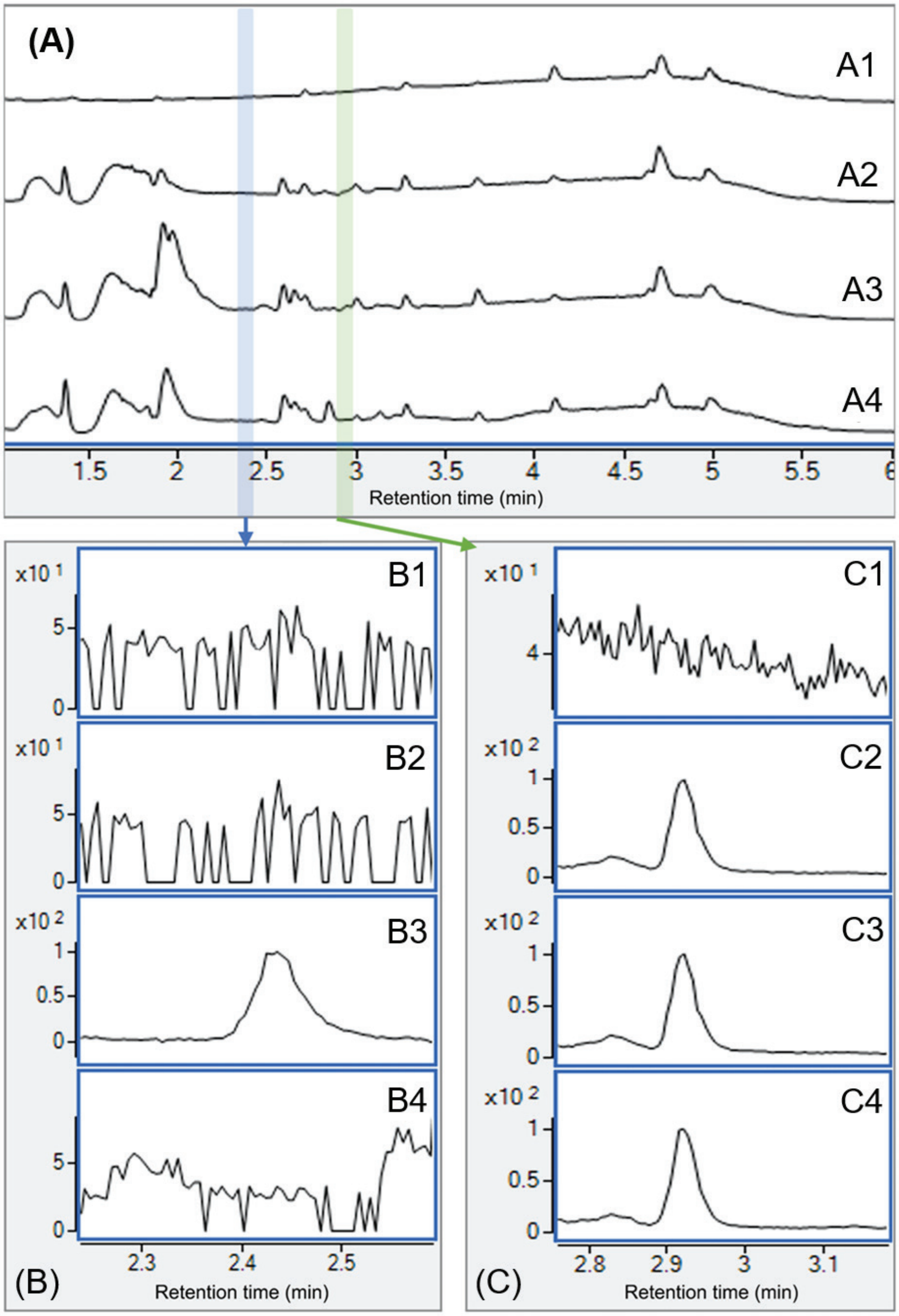

Figure 1. (A) Total ion chromatograms for different samples (1. procedural blank, 2. clover, 3. buckwheat, 4. blueberry). (B) Extracted ion chromatograms for feature *m/z* 96.0445 (1. procedural blank, 2. clover, 3. buckwheat, 4. blueberry). (C) Extracted ion chromatograms for 5-(hydroxymethyl) furfural (1. procedural blank, 2. clover, 3. buckwheat, 4. blueberry). Data were acquired under positive mode.

Simultaneous and rapid analysis of multiclass chemicals

Dilute-and-shoot with LC/MS has been deployed as an effective approach to screen chemicals related to food safety and food quality.[4] Methods in the literature have illustrated the capacity of the approach for analyzing specific classes of chemicals in honey.[4–6] In the present study, a Q-TOF LC/MS-based dilute-and-shoot approach was set up for the detection of chemicals in honeys across multiple families of compounds in the same data set (Table 2 and Figure 2). These include antibiotic residues (for example, sulfamethazine), human drug residues (for example, carbamazepine), pesticides (for example, clothianidin), plastic-related chemicals (for example, bisphenols), environmental contaminants (for example, perfluorooctane sulfonic acid). Substances relevant to honey quality can also be detected, including freshness markers like 5-(hydroxymethyl) furfural, amino acids (for example, L-glutamic acid), and phenolic compound like vanillin. In other words, the chemical fingerprint obtained in one single analysis contains information relevant to honey quality, safety, and authenticity.

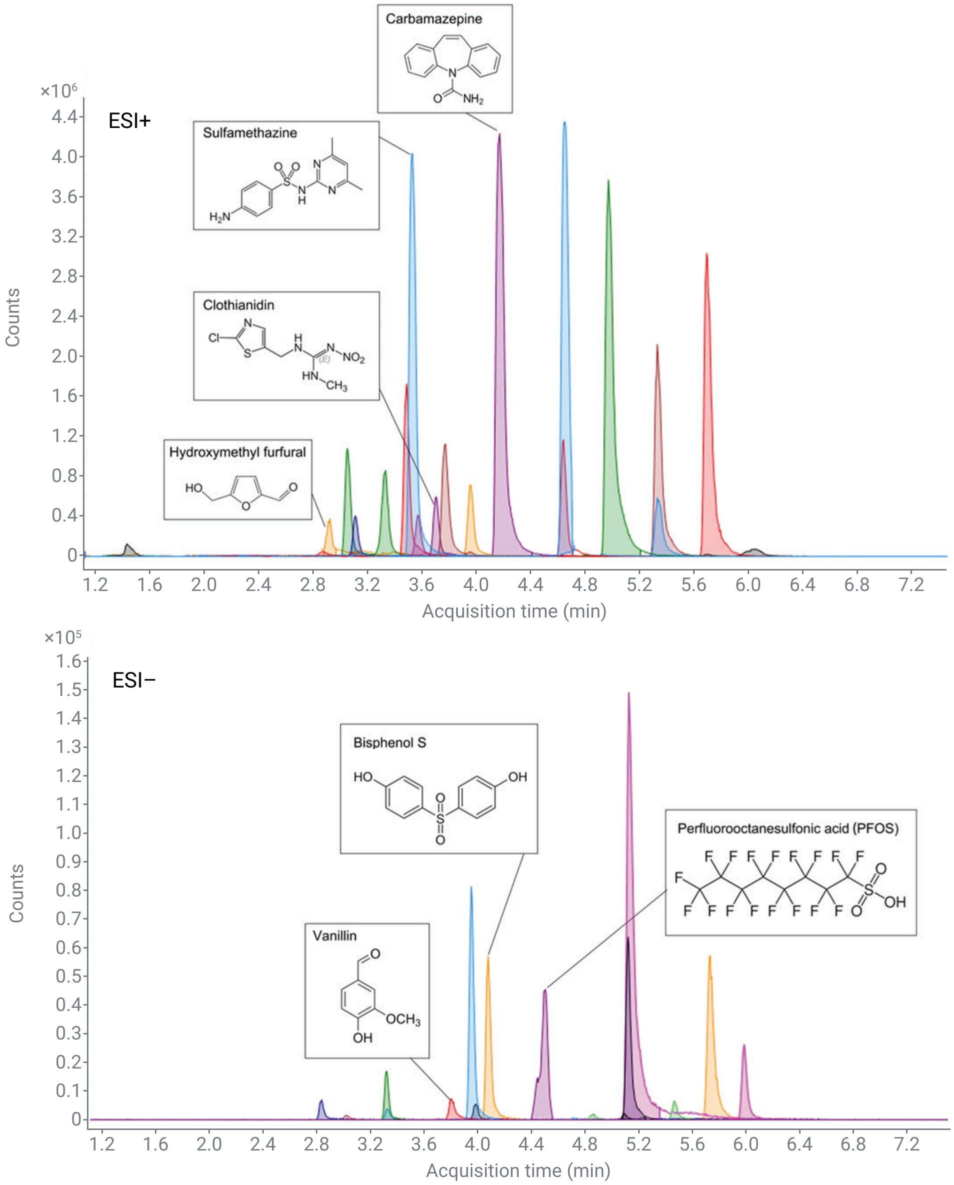

Figure 2. Extracted ion chromatograms for QCAM (honey extract) standards (200 pg injected for each substance).

Conclusion

This application note demonstrates the capacity of Q-TOF LC/MS as a method to explore the composition of honey products. This analytical workflow is extremely simple: (i) dilution of honey, (ii) filtration of honey with an Agilent PTFE filter, followed by (iii) injection into the Agilent Q-TOF LC/MS system. Analysis with an Agilent 1290 Infinity II LC system coupled to an Agilent 6545 LC/Q-TOF allowed for the simultaneous screening of multiple classes of chemicals in honey, including antibiotic residues, human drugs, pesticides, plastic-related chemicals, environmental contaminants, and other chemicals relevant to honey quality and authenticity.

The 9-minute LC gradient method using an Agilent InfinityLab Poroshell 120 CS-C18 column provided effective chromatographic separation across a wide range of polarities. The final analytical workflow in this study is rapid, reproducible, and automatable. This method can be used to delve into honey authenticity, safety, and quality simultaneously and could be adapted to study other liquid/semiliquid foods.

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