Application note | 001652

Mass spectrometry

A non-targeted metabolomics approach for the investigation of honey adulteration by HRMS

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Keywords

Honey, metabolomics, screening, identification and confirmation, HRMS, Orbitrap Exploris 240 mass spectrometer, Compound Discoverer software

Highlights

- Development of simple dilute and shoot UHPLC-MS methods for detection of sugar syrups added to honey
- Single acquisition offered full MS and ddMS^2 (MS^2) spectra
- High resolution enables more accurate identification and confirmation
- Fast acquisition rate (spectra/sec)
- Effective polarity switching
- Non-targeted data processing through Thermo Scientific™ Compound Discoverer™ software

Introduction

Honey, a sweet, flavorful, and nutritious food produced by honeybees, is widely consumed by humans as it has several therapeutic effects due to its antioxidant, antimicrobial, and anti-inflammatory properties.[1] Honey has been used in the treatment of wounds, burns, and gastric ulcers.[2] The high price, low production, and complex nature of honey have attracted more attention towards adulteration which can adversely affect consumer health. Also, the source of adulteration could be honey added from specific regions or flora, which result in a lower-value blended honey. Common honey adulterants are sugar syrups, such as corn syrup, high-fructose corn syrup, inverted syrup, and rice syrup added to replace pure honey.

As a result, there is a critical need to develop methods to identify and differentiate the adulterated honey to facilitate the control of the quality and safety of honey on the market and to protect consumers from fraud. Detection markers of honey adulteration include polysaccharides, di-fructose anhydrides (DFAs), and 2-acetylfuran-3-glucopyranoside (AFGP).[3] There are currently no simple and available LC-MS methods that can simultaneously detect all the common sugar syrups present in adulterated honey samples. Hence the need to develop simple, rapid, and sensitive detection methods for sugar syrups. To identify oligosaccharides, polysaccharides, and phytochemicals, a uniquely featured accurate, sensitive mass spectrometer for data acquisition, processing, and data mining is required.

The aim of this work was to create qualitative screening methods for honey by using a simple dilute-and-inject methodology and analysis by high-resolution accurate-mass (HRAM) MS with the Thermo Scientific™ Exploris™ Orbitrap 240 mass spectrometer.

Experimental

Sample preparation

Six honey samples (H-A02, H-A01, H-K02, H-82, H-105, and H-139) were collected from a local market. Individual honey samples were shaken and thoroughly mixed using a vortex mixer. A one-gram sub-sample was diluted with 10 mL water and 10 mL methanol in a 50 mL centrifuge tube. The whole mixture was vortex mixed for 2 mins and then centrifuged at 5000 rpm for 5 min at room temperature. The supernatant (0.5 mL) was diluted with water (0.5 mL) in an autosampler vial and 3 µL was injected into the LC-MS^2.

UHPLC-Orbitrap analysis

An ultra-high-performance liquid chromatography system (Thermo Scientific™ Vanquish™ Flex UHPLC system) was coupled with high-resolution mass-spectrometry (HRMS) (Orbitrap Exploris 240 mass spectrometer) equipped with heated electrospray ionization (HESI). Detailed instrument conditions are listed in Table 1.

Table 1. Optimized UHPLC-mass spectrometer instrument conditions.

UHPLC system		
Instrumentation: Vanquish Flex UHPLC system		
Parameters	**Method 1**	**Method 2**
Column	Thermo Scientific™ Accucore™ aQ column (100 × 2.1 mm × 2.6 µm) (P/N 27926-102130)	Accucore amide HILIC column (150 × 4.6 mm × 2.6 µm) (P/N 16726-154630)
Mobile phase A	Water + 0.1% HCOOH	Water
Mobile phase B	Methanol + 0.1% HCOOH	Acetonitrile
Gradient	0–1 min, 5% B; 1–4 min, 5–45% B; 4–11.5 min, 45-95% B; 11.5–15 min, 95% B; 15–15.5 min, 95–5% B; 15.5–20 min, 5% B phase	0–1.5 min, 95% B; 1.5–11 min, 95–30% B; 11–17 min, 30% B; 17–17.5 min, 30–95% B; 17.5–22 min, 95% B phase
Flow rate	0.3 mL/min	0.6 mL/min
Column oven	40 °C	40 °C
Total run	20 min	22 min
High-resolution mass spectrometer		
Instrumentation: Orbitrap Exploris 240 MS		
Parameters	**Method 1**	**Method 2**
Acquisition	Full scan—ddMS^2	Full scan—ddMS^2
Full scan mass range	*m/z* 100–1000	*m/z* 200–2000
Full scan resolution	R = 240,000	R = 240,000
ddMS^2 resolution	R = 15,000	R = 15,000
NCE	Stepped (10, 30, 55)	Stepped (10, 30, 55)
Ion spray voltage	3.5 kV (Positive) 2.5 kV (Negative)	3.5 kV (Positive) 2.5 kV (Negative)
Sheath gas	40.0 arb	50.0 arb
Aux gas	10.0 arb	10.0 arb
Sweep gas flow rate	1.0	1.0
Capillary temperature	300 °C	300 °C
Aux gas heater temperature	320 °C	350 °C
RF-lens	70%	70%
AGC target	Standard	Standard

Data processing

The data acquisition and processing were carried out by using Thermo Scientific™ Xcalibur™ v4.4 software and Compound Discoverer v3.2 software. These automated screening methods provided the identification of compounds in comparison with various libraries and databases (Thermo Scientific™ mzCloud™ spectral library, ChemSpider™ database, etc.). These databases provided compound-specific information in terms of chemical structure, molecular formula, molecular mass, isotopic pattern, and fragments with the associated mass error. Identification of the compounds was made according to the following criteria:

a. Retention time alignment

b. Evaluation of the mass error and isotopic pattern between the observed and the theoretical values

c. The mass error criteria were set at </= 5 ppm for precursor ion with a minimum of one fragment ion

d. The MS^2 spectra comparison between the observed and theoretical fragmentation followed by comparison with the mzCloud library and ChemSpider databases

e. The mzCloud library score should be higher than 65% along with </= 5 ppm mass error

f. Statistical analysis for differentiation

Results and discussion

Sample preparation and Orbitrap analysis

A simple 'dilute and inject' approach was preferred for all honey samples to avoid any losses of analytes during extraction. The data acquisition was performed in a full MS-ddMS^2 mode without an inclusion list. In this mode, the instrument collects one full MS spectrum (MS^1) for HRMS spectral information. Precursor ions that exceed a specified threshold in the MS^1 spectrum were selected for fragmentation in the higher-energy collision dissociation (HCD) cell. The resulting fragments were then transferred into the Orbitrap 240 mass spectrometer where they are measured. Parameters such as mass window and ion abundance required for triggering were defined in the instrument method. An AGC gain value of $1e^6$ was used during acquisition.[4] The chromatographic gradient elution conditions were optimized to obtain maximum coverage of analytes present in the honey samples. For polar analytes, the Accucore amide HILIC chemistry offered excellent retention and resolution along with symmetrical peak shapes with better sensitivity and selectivity for oligosaccharides and polysaccharides (Figures 1A and 1B). Also, reversed phase chemistry was used for the metabolomics approach to screen for natural components present in honey samples.

Impact of resolving power

The data was acquired on the high resolving power setting in the instrument (R = 240,000) and evaluated for the back-calculated (measured) resolution over the wide mass range used. Here, three different representative masses were selected to cover the wide range, i.e., maltooctadecaose, *m/z* 2934.9614 (high); maltotriose; *m/z* 503.1690 (middle); and 2-coumaric acid *m/z* 163.0401 (low). The observed resolution in negative polarity in honey samples were R = 93,003 for maltooctadecaose *m/z* 1466.4746 $[M-2H]^{2-}$, R = 159,107 for maltotriose *m/z* 503.1619 $[M-H]^-$, and R = 286,206 for 3-coumaric acid *m/z* 163.04007 $[M-H]^-$ (Figure 2).

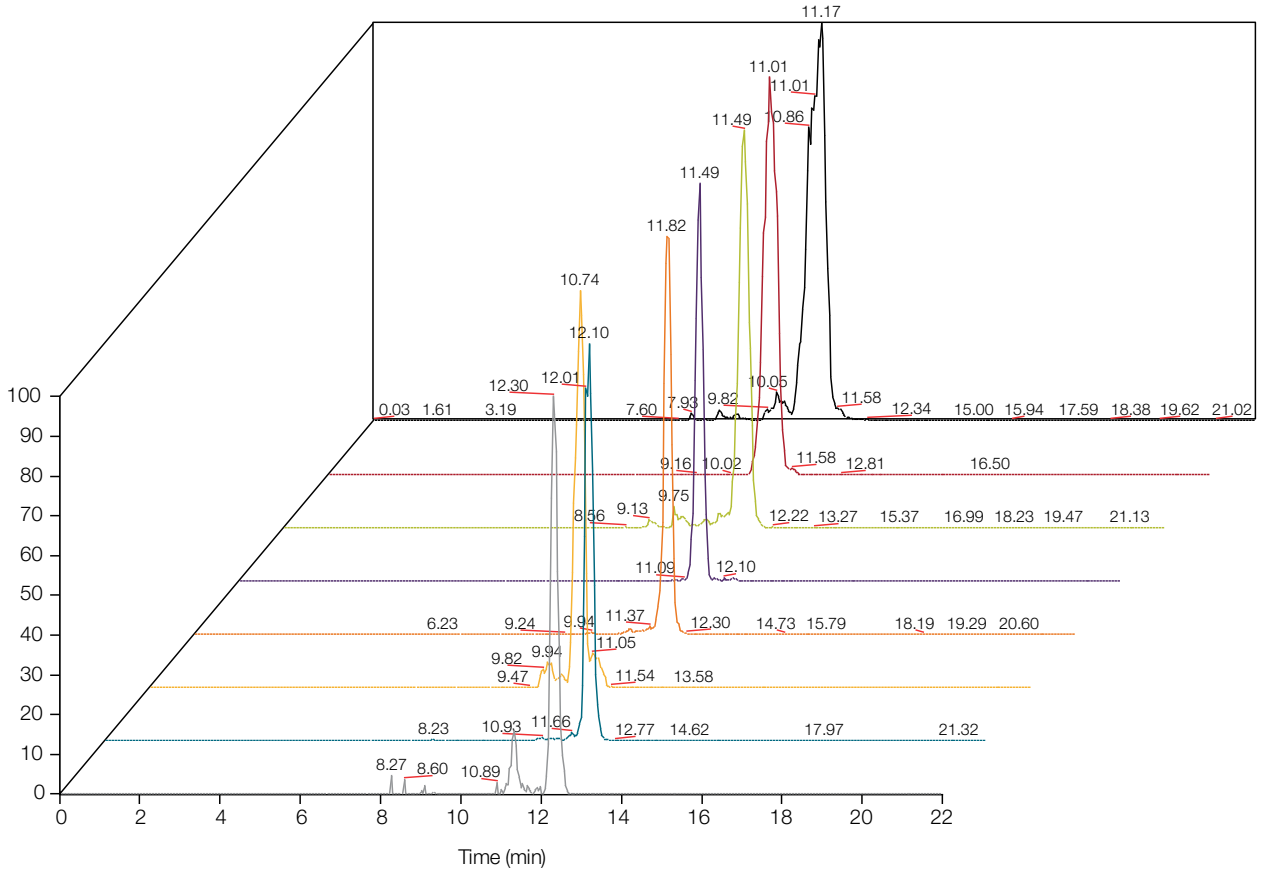


Figure 1A. A representative extracted ion chromatogram (EIC) for different oligosaccharides (DP 3–10) in honey.

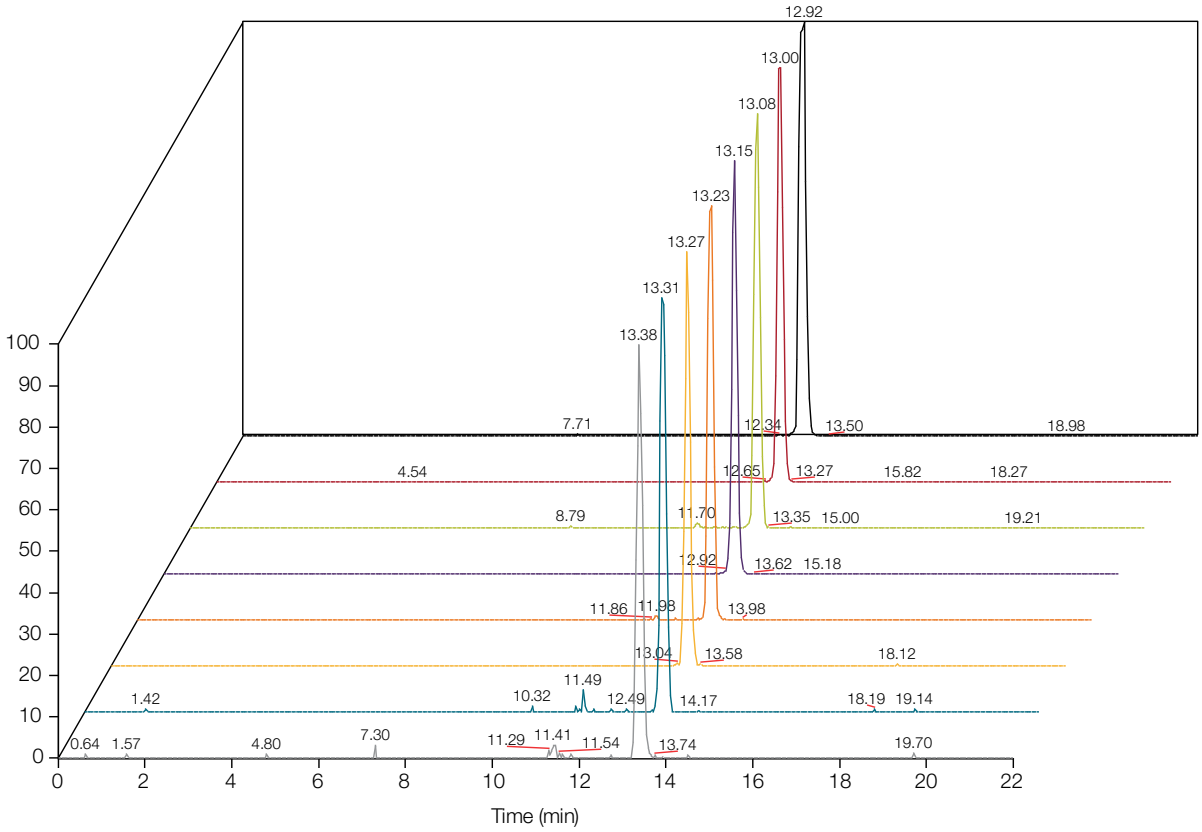
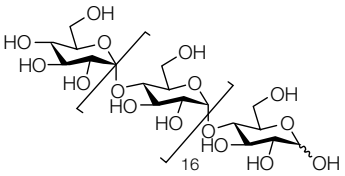
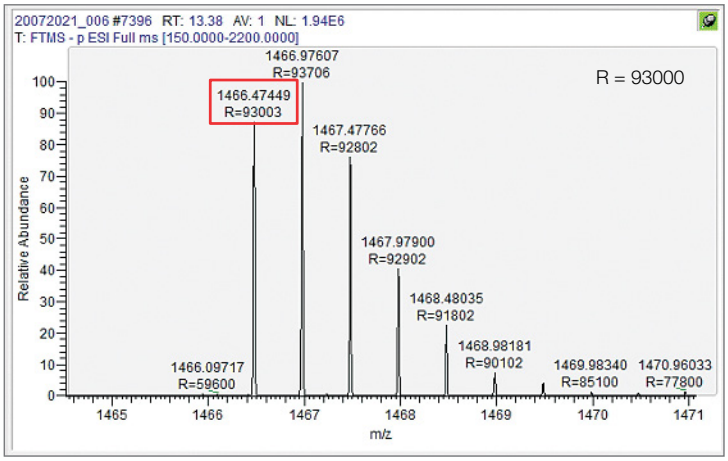
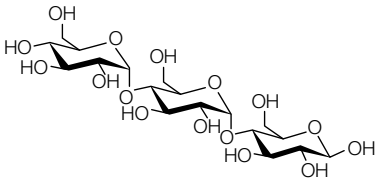


Figure 1B. A representative EIC for different polysaccharides (DP 11–18) in honey.

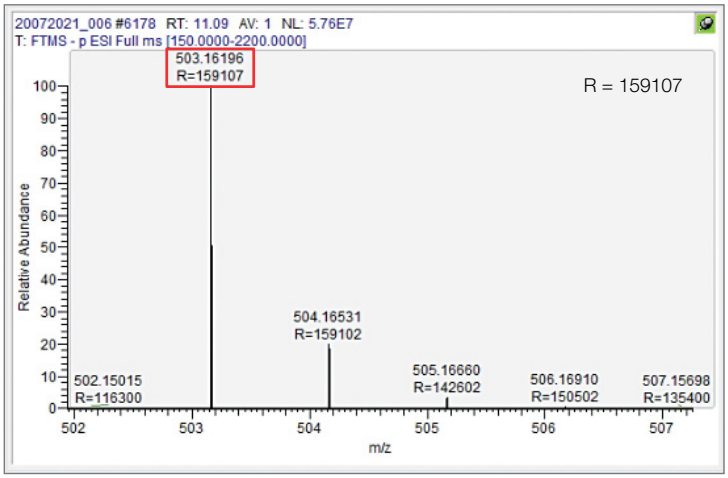


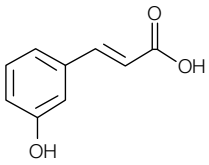
Maltooctadecaose (DP18)
m/z = 1466.4746 $[M-2H]^{2-}$





Maltotriose (DP3)
m/z = 503.1619 $[M-H]^{-}$





3-Coumaric acid
m/z = 163.04007 $[M-H]^{-}$

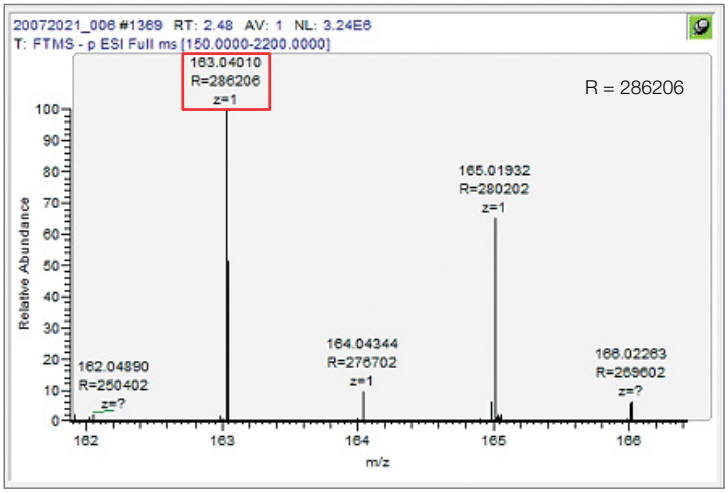


Figure 2. The actual resolution obtained for each compound is shown at the upper right corner in each spectrum and represent a wide mass range in the honey sample.

Impact of resolution and polarity switching on sensitivity

This system has an excellent scan speed and polarity switching (700 ms) in full MS mode. The polarity switching experiment was evaluated using three different resolving powers (i.e., R = 60,000, 120,000, and 240,000).

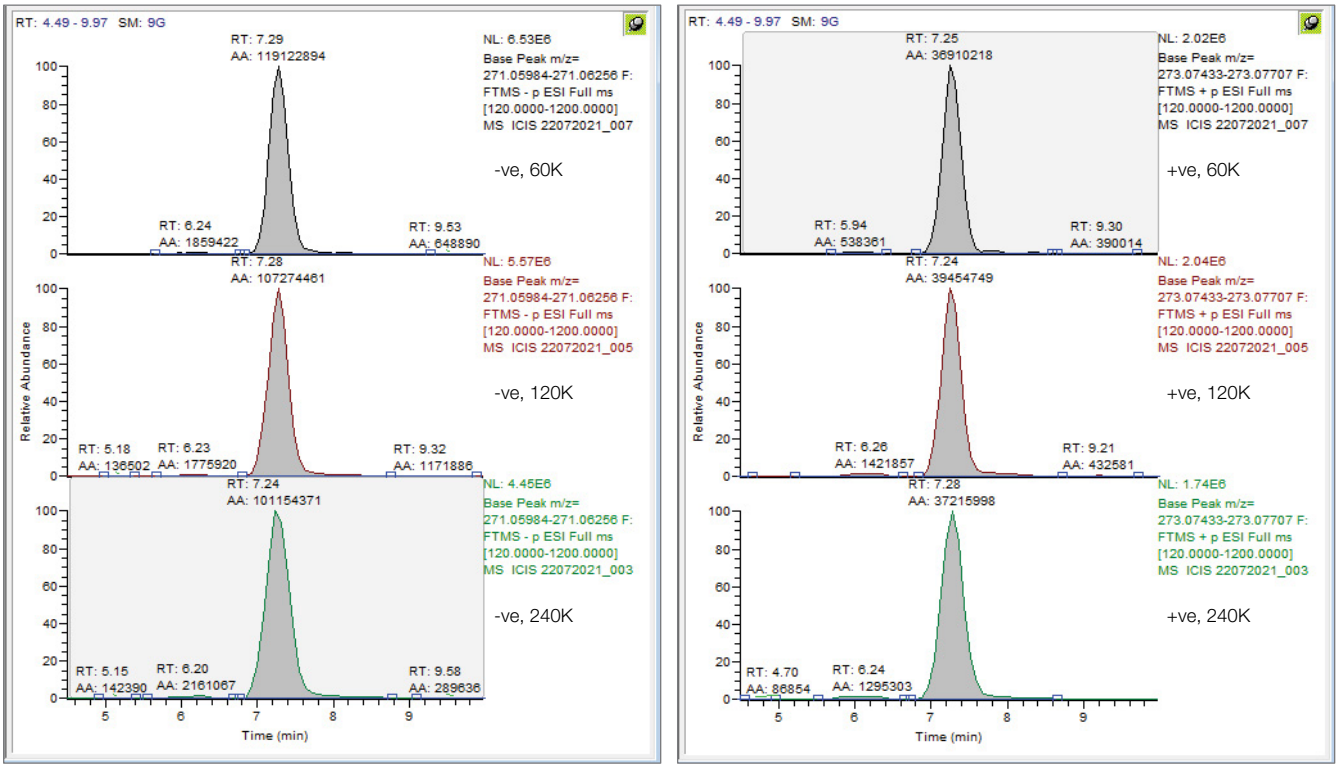


Figure 3. Impact of polarity switching and resolution on the sensitivity of naringenin.

Maltohexaose > 20 scans/peak

Maltododecaose > 15 scans/peak

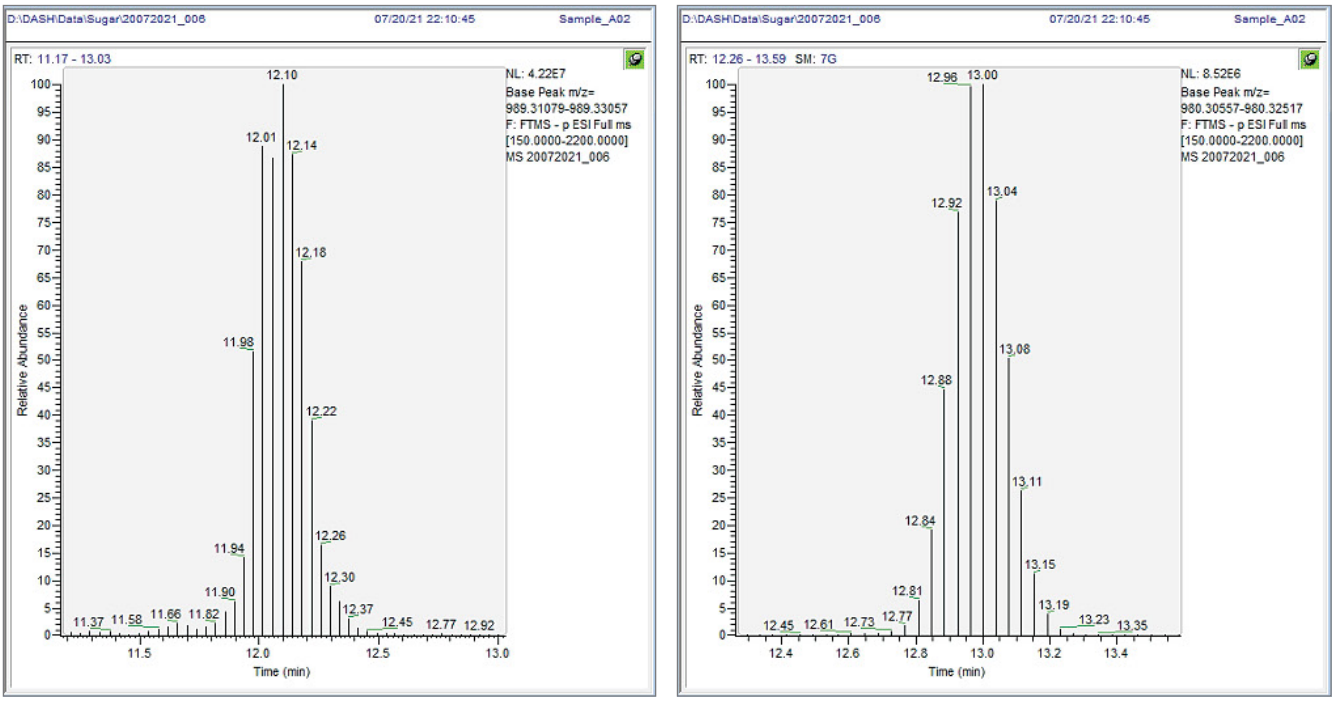


Figure 4. Impact of high resolution on the peak quality (scans/peak).

The impact of polarity switching as well as resolving power on sensitivity was evaluated for Naringenin, which is an analyte that occurs naturally in the honey samples. The negative polarity offered a better signal (4×) for naringenin over positive polarity. The area variation observed at three different resolutions was less than 8.35% in negative and 3.66% in positive polarity mode which indicates that there is no significant change observed in the sensitivity of naringenin as a function of resolution (Figure 3). In addition to this, the impact of resolution was evaluated for the peak quality in terms of scans/peak (data points). The data acquired in high resolution (R = 240,000) showed greater than 15 scans/peak (Figure 4). The high-resolution data therefore provides sufficient scans/peak which enables accurate quantitation and reproducibility, which are critical for relative analyte comparisons between samples.

Identification of compounds

Data processing was performed using Compound Discoverer v3.2 software as a non-target screening approach. This software is a unique platform that enables peak picking based on accurate mass and mass accuracy, retention time alignment, MS, and MS^2 spectral match with online databases like ChemSpider database, mzCloud library, etc. (Figure 5).

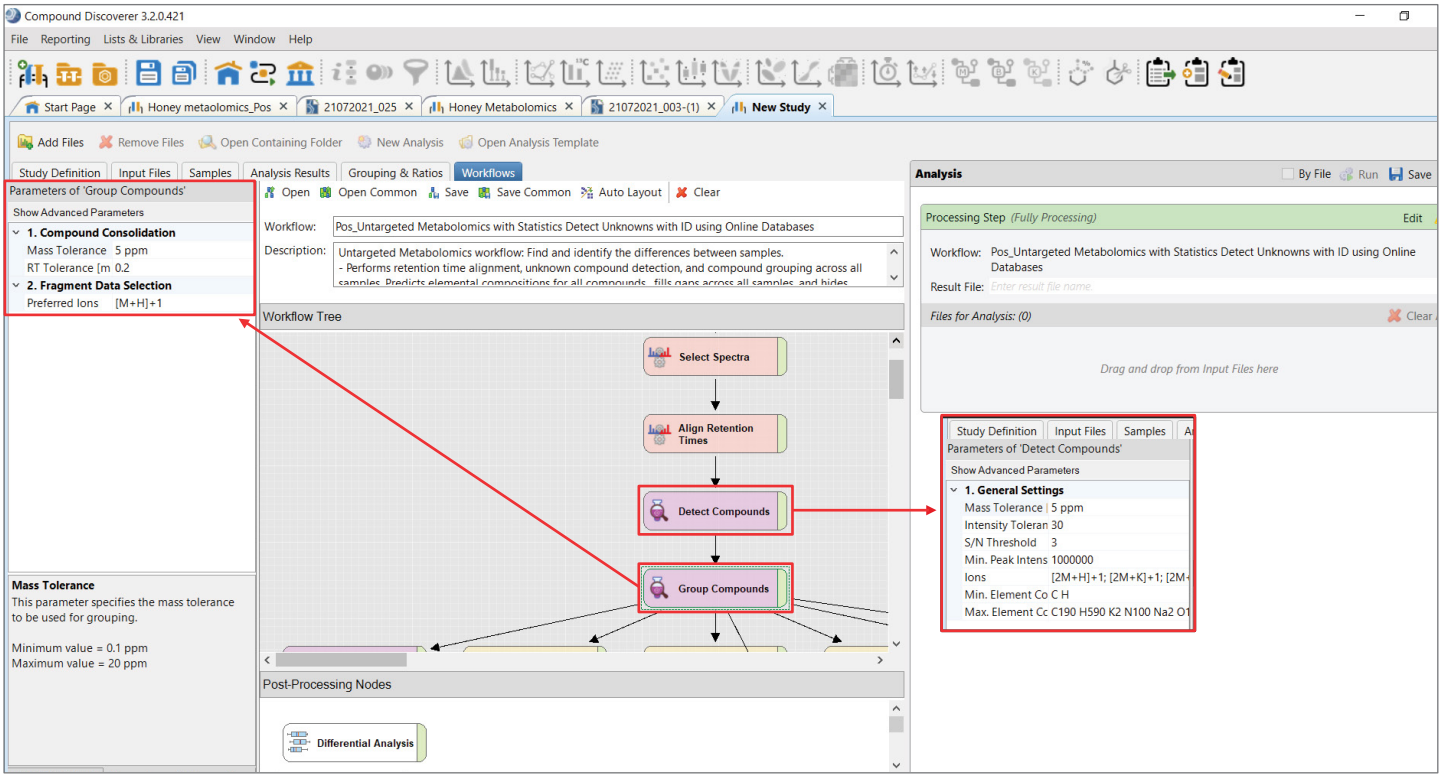
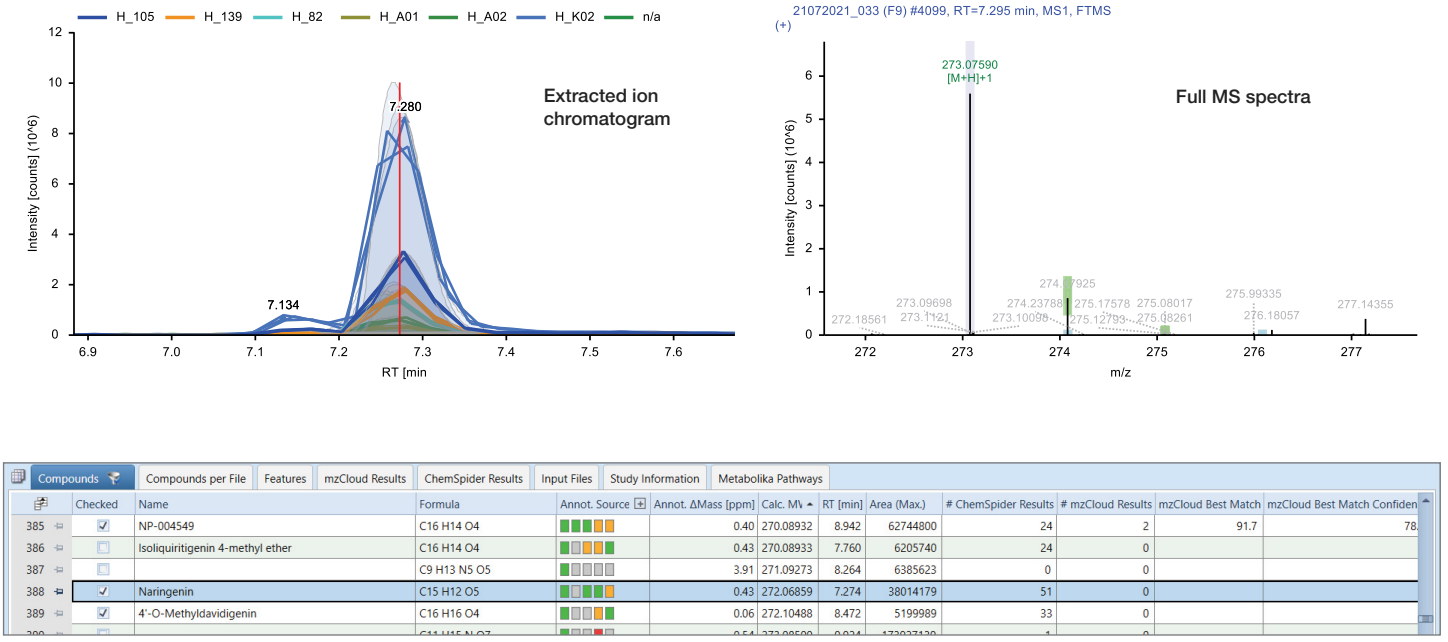


Figure 5. A workflow for non-targeted data processing using Compound Discoverer software.

A total of ninety-seven compounds were identified using the HILIC method in honey samples, including higher molecular weight sugars (oligosaccharides and polysaccharides) and other polar derivatives. One hundred and ten compounds, including polyphenols and amino acids, were detected using the reverse phase method and are presented in supplementary Tables 2 and 3.

Tentative identification of compounds was based on a library score (>65%), and a precursor ion with a minimum of one fragment ion both with mass accuracy (</= 5 ppm). The identification of naringenin was based on the protonated ion *m/z* 273.0753 with 0.43 ppm mass error which was further confirmed through the MS^2 spectral interpretation (Figure 6).



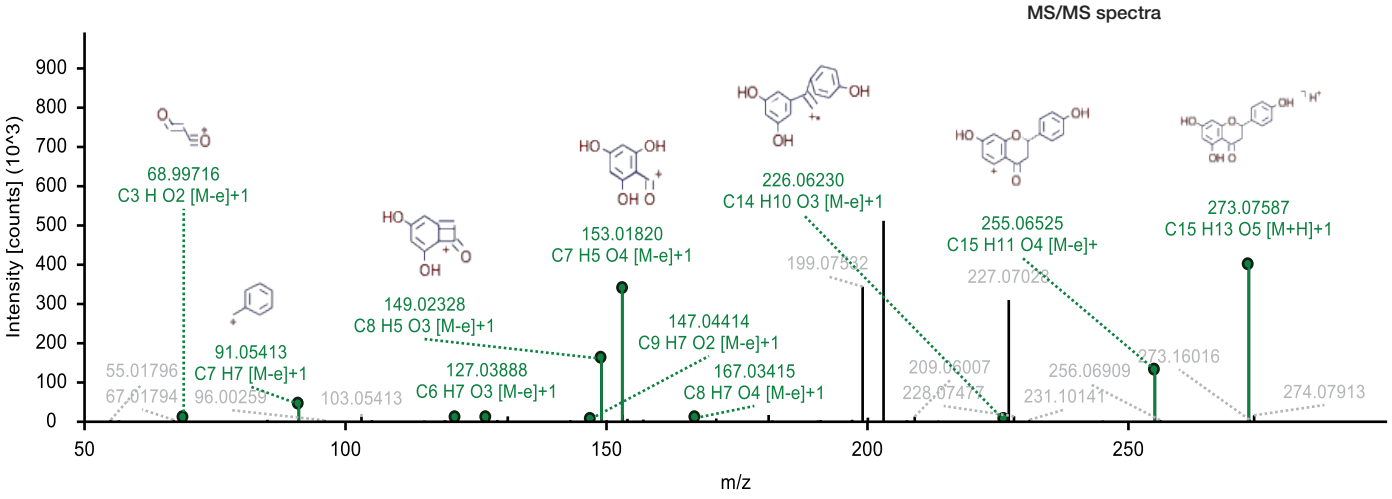


Figure 6. Demonstration of identification and confirmation of naringenin in honey samples through Compound Discoverer software.

Pinobanksin and naringenin have the same elemental composition, retention time (7.30 min), and exact molecular weight. Due to the difference in the hydroxyl group position, the MS^2 spectral interpretation confirmed the fragment ions *m/z* 227.07028 and *m/z* 199.07532 are matching with the pinobanksin structure and not with naringenin (Figure 7). The information is very useful in this example to distinguish the positional isomeric compounds. Also, retrospective analysis through data mining can identify significant molecular markers which help differentiate sample groups.

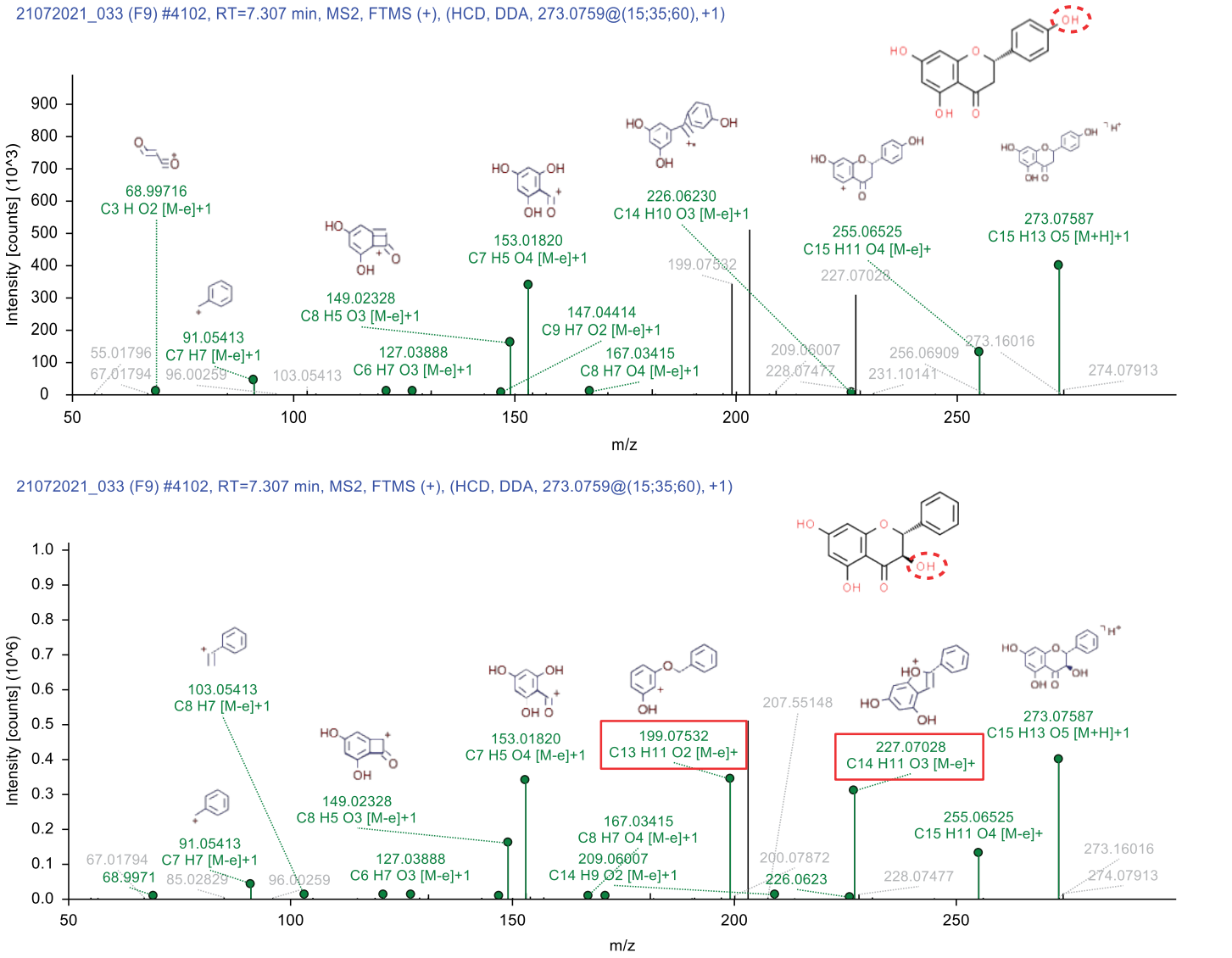


Figure 7. Product ion (MS^2) spectral confirmation of isomeric compounds naringenin and pinobanksin.

The six honey samples were compared using Principal Component Analysis (PCA) with a scores plot. The PCA clearly showed differentiation between the samples containing the externally added sugars (ID 82) as compared to the pure honey (ID 105; unadulterated). These results were in alignment with isotope ratio mass spectrometry (IRMS) measurements for these same samples. The box-and-whisker plot indicated that DP 6 and DP 16 sugars are detected in A02, A01 and H82 with very high amount (Figure 8).

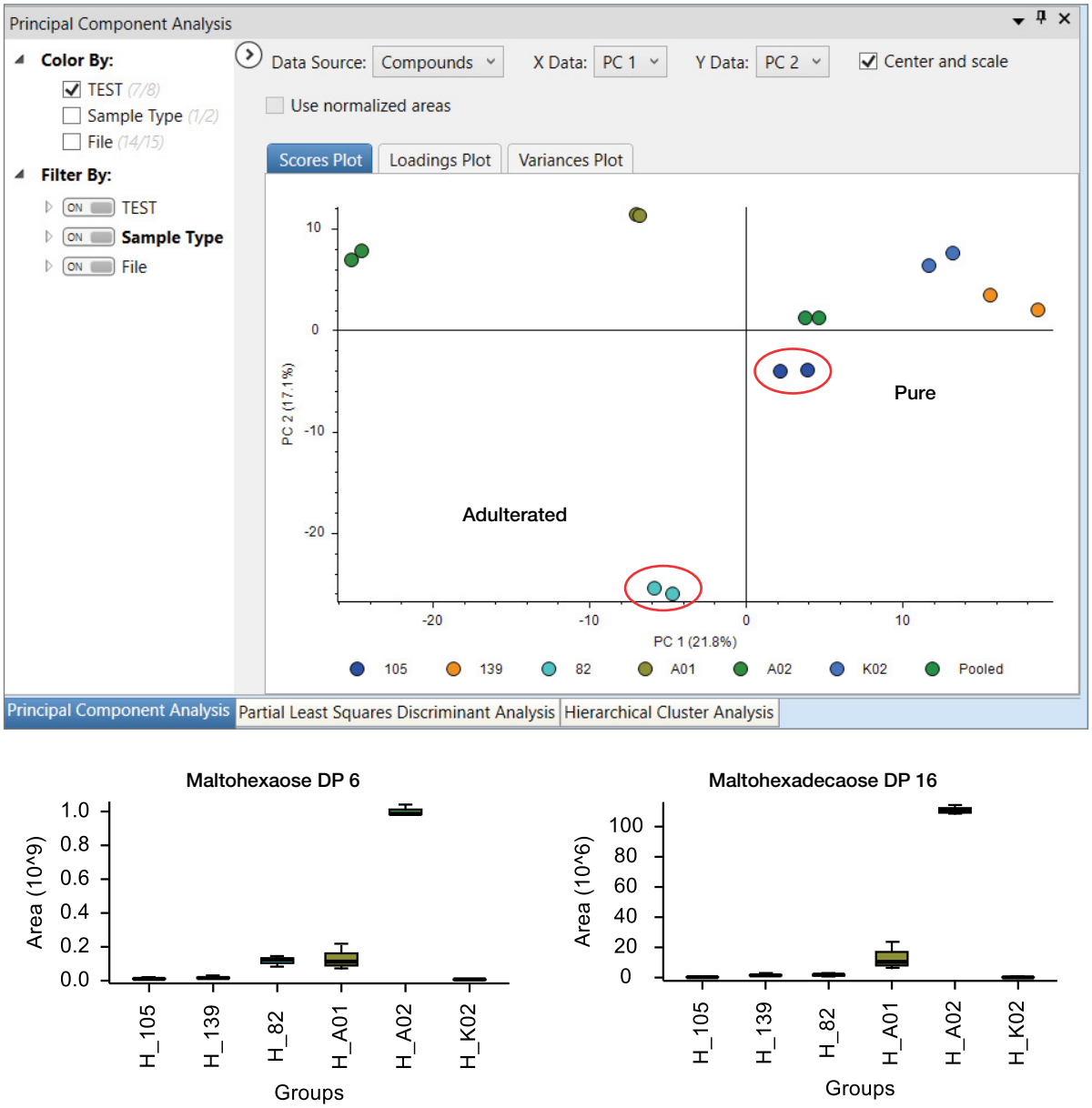


Figure 8. Principal Component Analysis (PCA) for honey samples (top), and box-and-whisker plots (bottom) for maltohexaose (DP6) and maltohexadecaose (DP16).

Conclusion

- A fit-for-purposed UHPLC-HRMS methodology using both reversed phase and HILIC approaches was shown to effectively screen honey samples for native compounds and metabolites. By applying stringent mass accuracy (</= 5 ppm) criteria, over 200 analytes in honey samples were detected.
- The methodology combined with Compound Discoverer software allowed clear differentiation of the six honey samples through principal component analysis. The PCA analysis helped to identify adulteration of some samples due to externally added sugars. This complete workflow not only provided information regarding adulteration but also offers the chemical name of the adulterant.
- The Orbitrap Exploris 240 mass spectrometer provides excellent response for analytes at 60,000, 120,000, and 240,000 resolution settings. In addition, quantitation ability with high number of scans/peak is maintained when performing polarity switching experiments, improving laboratory productivity and/or confidence in the data.

Supplementary Table 2. Identified compounds in honey samples using amide HILIC column chromatography with negative polarity.

Sr. no.	Name of compound	Formula	Delta mass (ppm)	Calc. MW	RT (min)	Area (max)	mzCloud best match
1	Gluconic acid	C6H12O7	0.26	196.05835	8.44	3.39E+10	99.2
2	2-Deoxyribose 5-phosphate	C5H11O7P	0.81	214.02441	6.38	1.85E+10	81.4
3	α, α-Trehalose	C12H22O11	-0.22	342.11614	10.02	1.30E+10	86.9
4	2-Deoxyribose 5-phosphate	C5H11O7P	0.92	214.02444	6.58	1.45E+10	81.4
5	(±)-Abscisic acid	C15H20O4	-0.07	264.13614	2.96	5.92E+09	97.3
6	D- (+)-Maltose	C12H22O11	-0.12	342.11617	10.74	3.76E+09	79.3
7	δ-Gluconic acid δ-lactone	C6H10O6	-0.11	178.04772	6.63	2.97E+09	70.3
8	Citric acid	C6H8O7	0.08	192.02702	7.19	2.23E+09	99.3
9	3-Isopropylmalic acid	C7H12O5	-0.02	176.06847	3.11	1.64E+09	92.4
10	Galacturonic acid	C6H10O7	-0.18	194.04262	7.57	1.32E+09	98.1
11	D-Saccharic acid	C6H10O8	0.14	210.03760	7.61	1.38E+09	95.8
12	D-Raffinose	C18H32O16	0.41	504.16924	10.94	4.10E+09	93.3
13	α-Lactose	C12H22O11	-0.21	342.11614	9.25	8.79E+08	97.5
14	D- (-)-Quinic acid	C7H12O6	0.05	192.06340	7.95	6.03E+08	95.5
15	5,7-Dihydroxy-4-methylcoumarin	C10H8O4	0.06	192.04227	2.80	2.75E+07	99.5
16	3-Phenyllactic acid	C9H10O3	-0.02	166.06299	3.10	5.83E+08	96.1
17	Naringenin	C15H12O5	0.14	272.06851	2.77	5.37E+08	97.0
18	Ethyl-β-D-glucuronide	C8H14O7	-0.49	222.07384	6.25	4.96E+08	94.9
19	L-Ascorbic acid 2-sulfate	C6H8O9S	0.21	255.98896	6.81	4.87E+08	77.4
20	Phloretin	C15H14O5	0.14	274.08416	2.64	4.57E+08	85.6
21	2-Dehydro-D-gluconate	C6H10O7	-0.21	194.04261	5.01	5.58E+08	ChemSpider DB
22	3-Hydroxy-3-methylglutaric acid	C6H10O5	0.19	162.05285	7.08	4.35E+08	99.1
23	Oleic acid	C18H34O2	-0.04	282.25587	2.59	3.92E+08	97.8
24	Glucose 1-phosphate	C6H13O9P	0.05	260.02973	7.42	3.70E+08	97.8
25	Maltooctadecaose DP 18 ([M+5H2O-2H]2-	C108H192O96	0.42	3025.01549	11.12	2.98E+08	ChemSpider DB
26	β-D-Glucopyranuronic acid	C6H10O7	0.05	194.04266	7.32	6.74E+08	97.0
27	Maltodecaose, DP10	C60H102O51	0.26	1638.53922	12.86	2.95E+08	ChemSpider DB
28	Maltoundecaose, DP11	C66H112O56	0.34	1800.59223	12.97	2.89E+08	ChemSpider DB
29	Maltononaose, DP9	C54H92O46	0.11	1476.48614	12.72	2.78E+08	ChemSpider DB
30	Dodecyl sulfate	C12H26O4S	0.00	266.15518	1.70	2.74E+08	99.1
31	Maltododecaose, DP12	C72H122O61	0.29	1962.64501	13.07	2.57E+08	ChemSpider DB
32	Maltopentadecaose, DP15 [M+2H2O-2H]2-	C90H156O78	0.10	2484.82430	11.90	2.50E+08	ChemSpider DB
33	Ethyl-β-D-glucuronide	C8H14O7	-0.05	222.07394	10.70	2.51E+08	94.9

Supplementary Table 2. Identified compounds in honey samples using amide HILIC column chromatography with negative polarity. *(continued)*

Sr. no.	Name of compound	Formula	Delta mass (ppm)	Calc. MW	RT (min)	Area (max)	mzCloud best match
34	2-Acetyl-3-furyl alpha-D-glucopyranoside	C12H16O8	0.46	288.08465	7.12	2.28E+08	71.9
35	Maltotridecaose, DP13	C78H132O66	0.40	2124.69811	13.16	2.18E+08	ChemSpider DB
36	Maltohexaose DP 6	C36H62O31	0.27	990.32778	12.19	1.04E+09	ChemSpider DB
37	3-Phenyllactic acid	C9H10O3	-0.14	166.06297	6.45	1.92E+08	99.0
38	Bis-D-fructose2'_1:2_1'-dianhydride	C12H20O10	0.30	324.10574	10.78	1.89E+08	ChemSpider DB
39	{(1R,2R)-2-[(2Z)-5-(Hexopyranosyloxy)-2-penten-1-yl]-3-oxocyclopentyl} acetic acid	C18H28O9	0.00	388.17333	7.05	1.80E+08	97.2
40	Maltooctadecaose, DP18 [M+2H2O-2H]2-	C108H186O93	0.73	2970.98469	12.16	1.78E+08	ChemSpider DB
41	Maltotetradecaose, DP14	C84H142O71	0.23	2286.75061	13.24	1.72E+08	ChemSpider DB
42	Luteolin	C15H10O6	0.24	286.04781	2.98	1.71E+08	96.3
43	Isocitric acid (tentative)	C6H8O7	0.18	192.02704	6.71	2.19E+08	95.3
44	Maltoheptaose, DP7 [M+H2O-H]	C42H74O37	0.18	1170.39110	11.36	1.68E+08	ChemSpider DB
45	Maltooctaose DP8	C48H82O41	0.16	1314.43336	12.57	4.48E+08	ChemSpider DB
46	Methyl 2-O-beta-L-arabinofuranosyl-beta-L-arabinofuranoside	C11H20O9	0.00	296.11073	2.84	1.37E+08	ChemSpider DB
47	5,7-Dihydroxy-4-(4-methoxyphenyl)-2-chromanone	C16H14O5	-0.02	286.08412	2.92	1.36E+08	73.7
48	L-Phenylalanine	C9H11NO2	0.46	165.07905	9.32	1.36E+08	96.7
49	Maltopentadecaose, DP15	C90H152O76	0.22	2448.80345	13.30	1.33E+08	ChemSpider DB
50	α-Lactose	C12H22O11	-0.49	342.11605	3.24	1.29E+08	84.8
51	Uridine	C9H12N2O6	0.04	244.06955	7.46	1.23E+08	96.1
52	3-tert-Butyladipic acid	C10H18O4	0.04	202.12052	3.05	2.40E+08	73.6
53	Maltoheptaose, DP7	C42H72O36	0.27	1152.38064	12.41	4.59E+08	ChemSpider DB
54	Maltohexadecaose, DP16	C96H162O81	0.36	2610.85667	13.37	1.14E+08	ChemSpider DB
55	Maltohexaose, DP6	C44H55N10O17P	0.04	1026.34868	10.07	1.71E+08	ChemSpider DB
56	Linoleic acid	C18H32O2	0.11	280.24026	2.62	1.11E+08	97.8
57	2-Norbornaneacetic acid	C9H14O2	0.11	154.09940	2.97	1.11E+08	84.2
58	Chlorogenic acid	C16H18O9	-0.30	354.09498	6.61	1.17E+08	98.2
59	3,8,9-trihydroxy-10-propyl-3,4,5,8,9,10-hexahydro-2H-oxecin-2-one	C12H20O5	-0.45	244.13096	4.01	1.63E+08	93.5
60	Dimethyl (3-oxocyclohexyl) malonate	C11H16O5	0.01	228.09978	3.31	3.70E+08	ChemSpider DB
61	2-Deoxyribose 5-phosphate	C5H11O7P	0.86	214.02442	7.99	1.01E+08	97.8
62	Myristyl sulfate	C14H30O4S	0.16	294.18653	1.69	1.00E+08	78.8
63	1,2,4-Benzenetricarboxylic acid	C9H6O6	0.05	210.01645	2.59	9.57E+07	98.8
64	Quercetin	C15H10O7	0.27	302.04273	3.09	9.55E+07	97.3
65	Maltoheptadecaose DP 17	C102H172O86	-0.13	2772.90819	13.43	9.09E+07	ChemSpider DB
66	Uridine 5'-diphosphogalactose	C15H24N2O17P2	-0.09	566.05497	7.11	8.00E+07	98.1
67	Myristyl sulfate	C14H30O4S	0.26	294.18656	2.21	7.89E+07	92.9
68	L-Phenylalanine	C9H11NO2	0.36	165.07904	9.83	7.52E+07	95.1
69	UDP-N-acetylglucosamine	C17H27N3O17P2	0.07	607.08161	7.09	7.39E+07	84.8
70	Maltooctadecaose, DP18	C108H182O91	0.79	2934.96369	13.48	7.36E+07	ChemSpider DB
71	(-)-Quebrachitol	C7H14O6	-0.63	194.07892	7.62	7.07E+07	ChemSpider DB
72	Pantothenic acid	C9H17NO5	-0.18	219.11063	5.00	6.98E+07	91.8
73	Ethyl-β-D-glucuronide	C8H14O7	-0.04	222.07394	10.06	6.87E+07	76.6

Supplementary Table 2. Identified compounds in honey samples using amide HILIC column chromatography with negative polarity. *(continued)*

Sr. no.	Name of compound	Formula	Delta mass (ppm)	Calc. MW	RT (min)	Area (max)	mzCloud best match
74	Jasmonic acid	C12H18O3	-0.03	210.12559	2.80	6.75E+07	81.6
75	α-Lactose	C12H22O11	-0.38	342.11608	4.00	8.64E+07	85.3
76	2,4-Dihydroxybenzoic acid	C7H6O4	0.12	154.02663	1.84	6.29E+07	96.9
77	3-Coumaric acid	C9H8O3	0.27	164.04739	2.59	6.27E+07	98.8
78	Maltooctadecaose, DP18 [M+HCOO+OH-2H]2-	C109H188O96	0.75	3032.98518	12.17	5.88E+07	ChemSpider DB
79	Chrysin	C15H10O4	0.24	254.05797	2.71	5.70E+07	98.4
80	Maltononadecaose DP 19	C114H192O96	0.58	3097.01602	13.53	5.36E+07	ChemSpider DB
81	α-Eleostearic acid	C18H30O2	0.11	278.22461	2.62	5.10E+07	98.0
82	D-Saccharic acid	C6H10O8	-0.08	210.03755	7.92	5.92E+07	95.8
83	Orotic acid	C5H4N2O4	0.27	156.01715	6.84	7.64E+07	81.4
84	Caffeic acid	C9H8O4	-0.02	180.04225	3.11	4.87E+07	89.1
85	Isorhamnetin	C16H12O7	0.28	316.05839	2.91	4.74E+07	93.2
86	1,5-Anhydro-D-glucitol	C6H12O5	0.17	164.06850	7.36	4.56E+07	79.4
87	3,4,5-trihydroxycyclohex-1-ene-1-carboxylic acid	C7H10O5	-0.06	174.05281	7.27	4.19E+07	92.3
88	Stearic acid	C18H36O2	-0.12	284.27150	2.59	3.95E+07	67.4
89	2,3-Dihydro-1-benzofuran-2-carboxylic acid	C9H8O3	0.32	164.04740	3.05	5.33E+07	90.3
90	3,8,9-trihydroxy-10-propyl-3,4,5,8,9,10-hexahydro-2H-oxecin-2-one	C12H20O5	-0.12	244.13104	3.10	6.11E+08	80.7
91	N-Acetyl-α-D-glucosamine 1-phosphate	C8H16NO9P	0.23	301.05634	7.24	1.73E+07	98.3
92	Ethyl-β-D-glucuronide	C8H14O7	-0.05	222.07394	10.46	8.64E+07	95.0
93	5-Sulfosalicylic acid	C7H6O6S	-0.20	217.98847	1.72	1.67E+07	87.9
94	Erucic acid	C22H42O2	0.40	338.31862	2.55	1.36E+07	97.6
95	Glycitein	C16H12O5	0.40	284.06859	3.00	6.75E+06	75.7
96	Luteolin 7-sulfate	C15H10O9S	-0.39	366.00441	1.70	6.49E+06	ChemSpider DB

Supplementary Table 3. Identified compounds in honey samples using reverse phase chromatograpy with both polarities.

Sr. no.	Name of compound	Formula	Delta mass (ppm)	Calc. MW	RT (min)	Area (max)	mzCloud best match
Positive polarity							
1	Bis-D-fructose2'_1:2_1'-dianhydride	C12H20O10	-0.03	324.10564	0.79	1.91E+10	ChemSpider DB
2	L-Phenylalanine	C9H11NO2	0.01	165.07898	1.90	9.15E+09	95.9
3	Pyrogallol	C6H6O3	0.40	126.03174	0.89	6.24E+09	73.3
4	Citral	C10H16O	0.29	152.12016	5.03	4.12E+09	88.2
5	1,5-Anhydro-1-(2,4,6-trihydroxyphenyl) hexitol	C12H16O8	0.22	288.08458	0.80	2.41E+09	ChemSpider DB
6	(±)-Abscisic acid	C15H20O4	0.46	264.13628	6.30	1.61E+09	83.0
7	Phenyl D-glucopyranosiduronic acid	C12H14O7	0.42	270.07407	0.80	1.35E+09	ChemSpider DB
8	5-hydroxy-4-methoxy-5,6-dihydro-2H-pyran-2-one	C6H8O4	0.39	144.04231	1.46	1.25E+09	78.9
9	5-hydroxy-4-methoxy-5,6-dihydro-2H-pyran-2-one	C6H8O4	0.39	144.04231	1.20	1.15E+09	65.0
10	10-HDA	C10H18O3	-0.04	186.12559	4.75	6.65E+08	76.6
11	Citral	C10H16O	0.13	152.12013	4.91	1.39E+09	88.4
12	Citral	C10H16O	0.29	152.12016	7.64	4.99E+08	88.7

Supplementary Table 3. Identified compounds in honey samples using reverse phase chromatograpy with both polarities.
(continued)

Sr. no.	Name of compound	Formula	Delta mass (ppm)	Calc. MW	RT (min)	Area (max)	mzCloud best match
Positive polarity							
13	2-Succinylbenzoate	C11H10O5	0.26	222.05288	0.80	4.67E+08	ChemSpider DB
14	Carvone	C10H14O	0.27	150.10451	5.72	3.22E+08	73.9
15	L-Tyrosine	C9H11NO3	-0.15	181.07387	1.07	4.68E+08	99.2
16	DIHYDROCONIFERIN	C16H24O8	-0.15	344.14706	5.85	2.55E+08	ChemSpider DB
17	Isoleucine	C6H13NO2	0.49	131.09469	1.18	2.41E+08	99.5
18	Pulegone	C10H16O	0.35	152.12017	5.34	2.83E+08	88.3
19	Citral isomer (n)	C10H16O	0.31	152.12016	7.35	1.82E+08	91.6
20	2,3,4,9-Tetrahydro-1H-β-carboline-3-carboxylic acid	C12H12N2O2	0.31	216.08994	4.21	1.79E+08	93.7
21	Lacinilene C 7-methyl ether	C16H20O3	0.34	260.14133	8.14	1.52E+08	ChemSpider DB
22	Dimethyl sebacate	C12H22O4	0.07	230.15182	7.44	1.51E+08	64.8
23	Butopyronoxyl	C12H18O4	0.05	226.12052	5.20	1.64E+08	ChemSpider DB
24	(+/-)-Sakuranetin	C16H14O5	0.14	286.08416	7.00	1.62E+08	ChemSpider DB
25	Adenine	C5H5N5	0.50	135.05456	0.90	1.37E+08	99.1
26	Limonene-1_2-diol	C10H18O2	-0.11	170.13066	5.57	1.28E+08	ChemSpider DB
27	5-Hexyl-2-oxotetrahydro-3-furancarboxylic acid	C11H18O4	0.32	214.12058	7.00	1.9E+08	ChemSpider DB
28	n-Propyl Gallate	C10H12O5	0.23	212.06852	6.16	1.47E+08	ChemSpider DB
29	Pipecolic acid	C6H11NO2	0.20	129.07900	0.76	1.15E+08	99.0
30	5-Hydroxyferulate	C10H10O5	0.02	210.05283	0.81	1.14E+08	ChemSpider DB
31	3-(3,4,5-trimethoxyphenyl) propanoic acid	C12H16O5	0.39	240.09987	4.17	1.13E+08	80.2
32	Trigonelline	C7H7NO2	0.28	137.04772	0.73	1.14E+08	99.7
33	L-Pyroglutamic acid	C5H7NO3	0.37	129.04264	0.94	1.25E+08	95.9
34	Picrocrocin	C16H26O7	0.12	330.16789	5.80	1.58E+08	ChemSpider DB
35	N-Glycosyl-L-asparagine	C10H18N2O8	0.08	294.10634	0.73	85099224	ChemSpider DB
36	Pinocembrin	C15H12O4	0.33	256.07364	9.54	79481260	98.6
37	Bis(4-ethylbenzylidene) sorbitol	C24H30O6	-0.57	414.20400	11.18	77064210	95.1
38	(±)-Abscisic acid	C15H20O4	0.48	264.13629	7.69	75762652	85.0
39	Carvone	C10H14O	0.27	150.10451	4.93	74844106	76.1
40	6-Hydroxy-8-methoxy-3-methyl-3,4-dihydro-1H-isochromen-1-one	C11H12O4	0.16	208.07359	6.30	68966002	64.5
41	4-Phenylbutyric acid	C10H12O2	-0.10	164.08371	6.99	78420099	85.0
42	Carvone	C10H14O	0.13	150.10448	4.76	74037773	75.6
43	3-Succinoylpyridine	C9H9NO3	0.15	179.05827	3.53	51872876	83.2
44	Dimethyl sebacate	C12H22O4	-0.15	230.15178	9.20	48334364	70.4
45	Jasmonal	C14H18O	-0.07	202.13575	6.66	46711453	ChemSpider DB
46	7-hydroxy-5-methoxy-2-phenyl-3,4-dihydro-2H-1-benzopyran-4-one	C16H14O4	0.34	270.08930	8.26	45144089	88.9
47	4-Phenylbutyric acid	C10H12O2	-0.19	164.08370	5.55	1.08E+08	92.4
48	Reboxetine	C19H23NO3	0.11	313.16783	8.37	41109020	ChemSpider DB
49	N-Acetyl-L-tyrosine	C11H13NO4	0.22	223.08451	1.96	45660417	ChemSpider DB
50	8-Hydroxyquinoline	C9H7NO	0.29	145.05281	4.41	38414796	91.5
51	Pinobanksin	C15H12O5	0.43	272.06859	7.27	38014179	ChemSpider DB
52	Pantothenicacid (Vitamin B5)	C9H17NO5	0.22	219.11072	2.78	36318726	ChemSpider DB
53	6-Pentyl-2H-pyran-2-one	C10H14O2	-0.20	166.09935	4.43	30222524	78.4
54	4-Methoxycinnamaldehyde	C10H10O2	-0.26	162.06804	6.66	22238307	68.6
55	Jasmonic acid	C12H18O3	0.08	210.12561	7.51	21144687	65.3

Supplementary Table 3. Identified compounds in honey samples using reverse phase chromatograpy with both polarities.
(continued)

Sr. no.	Name of compound	Formula	Delta mass (ppm)	Calc. MW	RT (min)	Area (max)	mzCloud best match
Positive polarity							
56	Apocynin	C9H10O3	-0.05	166.06299	4.21	20972889	81.1
57	Zaleplon	C17H15N5O	-3.66	305.12654	5.20	18041244	82.0
58	Formononetin	C16H12O4	0.52	268.07370	9.00	16954410	85.6
59	Glycitein	C16H12O5	0.21	284.06853	7.19	16947586	82.8
60	3-Amino-2-naphthoic acid	C11H9NO2	0.04	187.06334	4.28	16881303	80.6
61	DL-Tryptophan	C11H12N2O2	0.06	204.08989	3.60	16285948	97.6
62	Indole-3-acrylic acid	C11H9NO2	0.04	187.06334	4.08	15201754	80.8
63	Atagabalin	C10H19NO2	0.07	185.14159	7.57	14063980	ChemSpider DB
64	Dithranol	C14H10O3	0.38	226.06308	7.11	13554190	ChemSpider DB
65	3,7,15-Trihydroxy-12,13-epoxytrichothec-9-en-8-one	C15H20O6	-0.08	296.12596	6.39	11954558	ChemSpider DB
66	7-Ethoxycoumarin	C11H10O3	-0.22	190.06295	6.66	11733430	ChemSpider DB
67	2-coumarate	C9H8O3	-0.15	164.04732	5.35	10782788	ChemSpider DB
68	4-methoxy-6-(prop-2-en-1-yl)-2H-1,3-benzodioxole	C11H12O3	0.06	192.07866	8.32	10811647	84.9
69	Isophorone	C9H14O	0.63	138.10455	7.12	9301853	68.5
70	Kynurenic acid	C10H7NO3	-0.01	189.04259	4.31	9120856	86.8
71	4-Phenylbutyric acid	C10H12O2	-0.06	164.08372	8.61	6731184	80.2
72	4'-O-Methyldavidigenin	C16H16O4	0.06	272.10488	8.47	5199989	94.0
73	2-Succinylbenzoate	C11H10O5	0.23	222.05287	5.49	4718860	95.0
Negative polarity							
1	D- (-)-Fructose	C6H12O6	0.11	180.06341	0.90	2.6E+10	99.0
2	L-Threonic acid	C4H8O5	-0.06	136.03717	0.75	8.42E+09	83.0
3	Gluconic acid	C6H12O7	-0.04	196.05829	0.75	8.61E+09	98.0
4	D- (+)-Maltose	C12H22O11	-0.11	342.11617	0.81	5.38E+09	92.0
5	(±)-Abscisic acid	C15H20O4	-0.05	264.13615	6.68	4.16E+09	98.0
6	Citric acid	C6H8O7	0.04	192.02701	0.94	1.04E+09	98.0
7	Amylose	C14H26O11	-0.18	370.14744	1.32	4.83E+08	ChemSpider DB
8	δ-Gluconic acid δ-lactone	C6H10O6	0.56	178.04784	0.88	4.79E+08	77.0
9	3-tert-Butyladipic acid	C10H18O4	0.35	202.12058	7.44	4.18E+08	81.0
10	Dendromoniliside C	C21H32O10	-0.19	444.19946	6.67	3.82E+08	ChemSpider DB
11	(±)-Abscisic acid	C15H20O4	-0.07	264.13614	6.30	1.22E+09	92.0
12	L-Phenylalanine	C9H11NO2	0.35	165.07904	1.94	3.71E+08	96.0
13	D- (+)-Malic acid	C4H6O5	0.59	134.02160	0.80	2.79E+08	95.0
14	3-Phenyllactic acid	C9H10O3	0.03	166.06300	5.21	2.39E+08	95.0
15	2-Norbornaneacetic acid	C9H14O2	0.10	154.09940	6.68	1.69E+08	89.0
16	Citraconic acid	C5H6O4	0.73	130.02670	0.81	1.77E+08	78.0
17	6-Hydroxycaproic acid	C6H12O3	0.31	132.07868	4.91	1.43E+08	93.0
18	β-D-Glucopyranuronic acid	C6H10O7	0.38	194.04273	0.74	1.23E+08	75.0
19	Citraconic acid	C5H6O4	0.71	130.02670	1.13	1.22E+08	95.0
20	Naringenin	C15H12O5	0.00	272.06847	7.27	1.12E+08	97.0
21	5,7-Dihydroxy-4-(4-methoxyphenyl)-2-chromanone	C16H14O5	0.57	286.08429	7.00	1.1E+08	81.0
22	Lusitanicoside	C21H30O10	0.01	442.18390	4.90	1.06E+08	ChemSpider DB
23	Taurine	C2H7NO3S	0.35	125.01471	0.71	90771852	89.0
24	{(1R,2R)-2-[(2Z)-5-(Hexopyranosyloxy)-2-penten-1-yl]-3-oxocyclopentyl} acetic acid	C18H28O9	-0.13	388.17328	4.92	70084142	91.0

Supplementary Table 3. Identified compounds in honey samples using reverse phase chromatograpy with both polarities.
(continued)

Sr. no.	Name of compound	Formula	Delta mass (ppm)	Calc. MW	RT (min)	Area (max)	mzCloud best match
Negative polarity							
25	D- (-)-Ribose	C5H10O5	-0.16	150.05280	0.70	8.69E+09	97.0
26	(±)-Abscisic acid	C15H20O4	-0.03	264.13615	7.33	67499556	89.0
27	cis-Aconiticacid	C6H6O6	0.23	174.0165	0.98	5.52E+07	ChemSpider DB
28	Biflorin	C16H18O9	-0.20	354.095	4.41	4.26E+07	ChemSpider DB
29	Aucubin	C15H22O9	-0.34	346.1263	5.22	4.54E+07	ChemSpider DB
30	3-Methylglutaric acid	C6H10O4	0.16	146.0579	4.27	3.80E+07	89.0
31	Sedoheptulose	C7H14O7	0.77	210.0741	0.77	3.80E+07	ChemSpider DB
32	[3-(Hydroxymethyl)-3-methyl-2-oxobicyclo [2.2.1] hept-1-yl] methyl hexopyranoside	C16H26O8	-0.36	346.1626	6.10	3.71E+07	ChemSpider DB
33	Suberic acid	C8H14O4	0.04	174.0892	5.57	6.75E+07	89.0
34	Jasmonic acid	C12H18O3	-0.22	210.1256	7.52	2.63E+07	70.0
35	2-Isopropylmalic acid	C7H12O5	0.35	176.0685	4.18	2.26E+07	87.0
36	Chlorogenic acid	C16H18O9	-0.18	354.095	4.59	1.86E+07	99.0
37	Mevalonic acid	C6H12O4	0.25	148.0736	1.71	1.67E+07	70.0
38	2,3-Dihydro-1-benzofuran-2-carboxylic acid	C9H8O3	0.15	164.0474	5.37	2.32E+07	61.0
39	Nigrescin	C15H12O7	0.15	304.0584	5.43	1.61E+07	ChemSpider DB
40	Ethyl-β-D-glucuronide	C8H14O7	0.12	222.074	1.38	3.73E+07	79.0
41	3-Hydroxy-1-(8-hydroxy-6-methoxy-3-methyl-1-oxo-3,4-dihydro-1H-isochromen-7-yl)-3-methyl-2-butanyl acetate	C18H24O7	-0.17	352.1521	5.45	1.55E+07	ChemSpider DB
42	5,7-Dihydroxy-4-methylcoumarin	C10H8O4	-0.05	192.0423	6.52	1.28E+07	82.0
43	Pantothenic acid	C9H17NO5	-0.08	219.1107	2.81	2.57E+07	81.0
44	Scopoletin acetate	C12H10O5	-0.19	234.0528	5.25	9.09E+06	ChemSpider DB
45	2-[(2R,4aS,6S,8aS)-6-Hydroxy-4a-methyl-8-methylenedecahydro-2-naphthalenyl]-2-propanyl beta-D-glucopyranoside	C21H36O7	-0.22	400.246	11.67	7.76E+06	ChemSpider DB
46	Terephthalic acid	C8H6O4	-0.09	166.0266	4.22	7.59E+06	65.0

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