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Separation of Polyphenols by Comprehensive 2D-LC and Molecular Formula Determination by Coupling to Accurate Mass Measurement

Application Note

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Abstract

This Application Note demonstrates the coupling of an Agilent 1290 Infinity 2D-LC Solution to an Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight LC/MS instrument for the measurement of polyphenolic compounds. The compounds separated by comprehensive 2D-LC are detected by the mass spectrometer and their accurate mass is measured. The compounds are identified by the LC Image LC × LC-HRMS software package. It is shown that their mass can be measured with high accuracy for the determination of their molecular formula.

Introduction

Polyphenolic compounds are secondary plant metabolites including, for example, phenolic acids, flavonoids, coumarins, and others. Many of these are present in grapes, berries, hops, and herbs, as well as in food and beverages such as fruit juices, beer, and wine. As plant metabolites, polyphenols typically occur in complex matrixes that require sophisticated separation techniques such as comprehensive 2D-LC[1]. In larger amounts, they can be detected by UV absorbance. However, their UV spectra can be very similar, as with flavonoids, because polyphenolic compounds often share the same aromatic carbon skeleton. Therefore, accurate mass measurement can help determine the formula of polyphenolic compounds. The accurate measurement of the mass of polyphenolic compounds can easily be done by coupling the comprehensive 2D-LC to a time-of-flight mass spectrometer.

Experimental

Equipment

The Agilent 1290 Infinity 2D-LC Solution comprises:

- Two Agilent 1290 Infinity Binary Pumps (G4220A)
- Agilent 1290 Infinity Autosampler (G4226A) with 1290 Infinity Thermostat (G1330A)
- Agilent 1290 Infinity Thermostatted Column Compartment (TCC) (G1316C) with Agilent 1200 Infinity Series Quick-Change 2-position/4-port-duo valve for 2D-LC (G4236A)
- Agilent 1290 Infinity Diode Array Detector (DAD) (G4212A) with 60-mm Max-Light flow cell (G4212-60007).
- Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS with Agilent Jet Stream electrospray ionization source or Agilent 6238 Accurate-Mass Time-of-Flight (TOF) LC/MS with Agilent Jet Stream electrospray ionization source. The mass spectrometer was connected to the second dimension column by a T-splitter. A 1:5 splitting ratio was generated by means of capillaries with an 0.12-mm id, and different length.

Software

- Agilent Open Lab CDS ChemStation Rev. C01.05 with 2D-LC add-on software.
- Agilent MassHunter software for TOF and Q-TOF data acquisition (Version B.05.01) and qualitative data analysis software (Version B.06.00).
- LCxLC Software for HiRes MS 2D-LC data analysis (V.2.4) from GC Image LLC., Lincoln, NE, USA.

Chemicals

All solvents used were LC grade. Acetonitrile and methanol were purchased from Merck, Germany. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22-µm membrane point-of-use cartridge (Millipak). All chemicals used as standard were purchased from Sigma-Aldrich, Germany. Polyphenols were dissolved in methanol to a concentration of 10 mg/100 mL as a stock solution. This solution was diluted to a final concentration of 10 ppb.

Instrument parameters

Columns	
1st dimension	Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 150 mm, 1.8 µm (p/n 959759-902)
2nd dimension	Agilent ZORBAX RRHD Eclipse Plus Phenyl-Hexyl, 3.0 × 50 mm, 1.8 µm. (p/n 959757-312)
Method	
1st Dimension pump	
Solvent A	Water + 0.1 % formic acid
Solvent B	Acetonitrile + 0.1 % formic acid
Flow rate	0.1 mL/min
Gradient	0 minutes 5 % B – 30 minutes 95 % B, 40 minutes – 95 % B
Stop time	40 minutes
Post time	15 minutes
2nd Dimension pump	
Solvent A	Water + 0.1 % formic acid
Solvent B	Methanol + 0.1 % formic acid
Flow rate	3 mL/min
Initial gradient	0 minutes - 5 % B, 0.4 minutes - 15 % B, 0.41 minutes - 5 % B, 0.5 minutes - 5 % B
Gradient modulation	0 minutes 5 % B to 30 minutes 50 % B, 0.4 minutes 15 % B to 30 minutes 95 % B, 0.41 minutes 5 % to 30 minutes 50 % B, 0.5 minutes 5 % B to 30 minutes 50 % B
TCC	
1st dimension column	On the left side at 25 °C
2nd dimension column	On the right side at 60 °C
Two 80-µL loops are connected to the 2-position/4-port-duo valve and are located on the left side.	
The valve is switched automatically after each 2nd dimension modulation cycle. In this case, the loops are used in a co-current manner (the loops are filled and eluted from the same sides).	
Autosampler	
Injection volume	5 µL
Sample temperature	8 °C
Needle wash	6 seconds in methanol
MS-TOF or Q-TOF in TOF mode	
Drying gas flow	9 L/min
Drying gas temperature	300 °C
Sheath gas temperature	400 °C
Sheath gas flow	12 L/min
Nebulizer pressure	45 psi
Capillary voltage	3,500 V
Nozzle voltage	300 V
Fragmentor voltage	175 V
Mass range	100–1,000 *m/z*
Data rate	10 Hz

Results and Discussion

A mixture of 22 polyphenolic standard compounds was separated by the described comprehensive 2D-LC method. The first dimension column was a C18 column, and the second dimension column was a phenyl-hexyl column, which provided different selectivites for the separation of aromatic compounds. A fraction from the first dimension column effluent was collected in a loop and transferred to the second dimension column every 30 seconds.

The first eluting compound was gallic acid, a derivative of hydroxyl benzoic acid (1). Some compounds coeluting from the first dimension were separated on the second dimension column, such as luteolin (14) and quercetin (15), differing only by one hydroxyl group. Some compounds were clustered in the same retention time range (16–20) due to their structural similarity. Compounds 16–20 share the same flavone carbon skeleton. They only differ in the position or number of hydroxyl groups. The only difference between apigenin (16) and naringenin (17), is a C=C (double) bond versus a hydrogenated C-C bond, respectively (Figure 1, Table 1).

The second dimension column was connected to a time-of-flight mass spectrometer for accurate mass measurement. The connection was a t-piece splitter, and the appropriate flow rate to the ionization source was adjusted by capillaries of different length and the same 0.12-mm id.

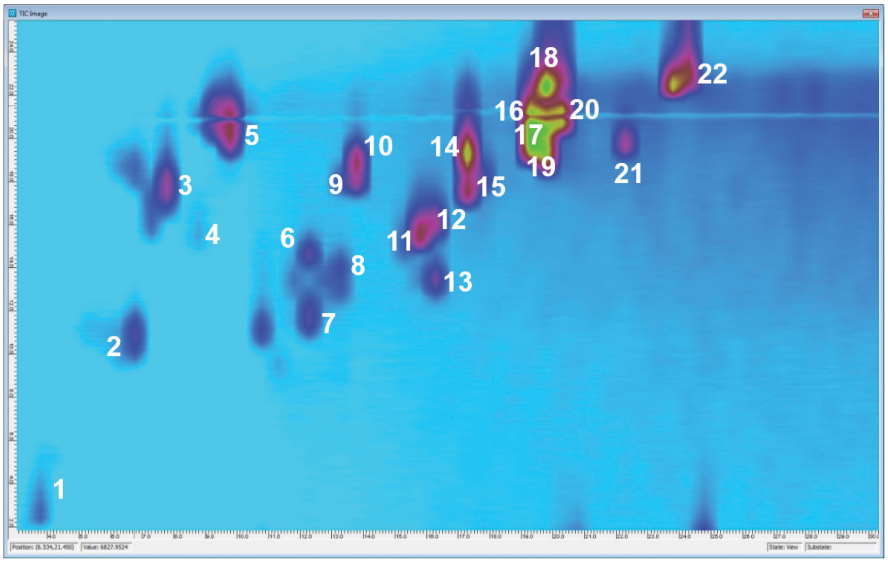

Figure 1. Separation of a mixture of 22 polyphenolic compounds by comprehensive 2D-LC and measurement of accurate mass by time-of-flight MS.

Table 1. Polyphenolic standard compounds separated by comprehensive 2D-LC and determined by accurate time-of-flight mass measurement.

No.	Compound	Formula	MW	$[M-H]^-$	RT I (min)	RT II (sec)	*m/z*	ppm
1	Gallic acid	$C_7H_6O_5$	170.0215	169.0142	3.53	2.51	169.0142	0.28
2	3,4-Hydroxyl benzoic acid	$C_7H_6O_4$	154.0266	153.0193	6.53	10.73	153.0195	1.10
3	Esculin	$C_{15}H_{16}O_9$	340.0794	339.0722	7.53	17.78	339.0714	2.23
4	Hydroxyl benzoic acid	$C_7H_6O_3$	138.0317	137.0244	8.53	15.56	137.0245	0.60
5	6,7-Hydroxyl coumarin	$C_9H_6O_4$	178.0266	177.0193	9.53	20.48	177.0197	2.08
6	Rutin	$C_{27}H_{30}O_{16}$	610.1534	609.1461	12.03	14.59	609.1467	0.97
7	Coumaric acid	$C_9H_8O_3$	164.0473	163.0401	12.03	11.69	163.0399	1.03
8	Ferulic acid	$C_{10}H_{10}O_4$	194.0579	193.0506	13.03	14.01	193.0511	2.42
9	Naringin	$C_{27}H_{32}O_{14}$	580.1792	579.1719	13.53	17.78	579.1726	1.16
10	Hesperidin	$C_{28}H_{34}O_{15}$	610.1898	609.1825	13.53	18.84	609.1822	0.48
11	Salicylic acid	$C_7H_6O_3$	138.0317	137.0244	15.53	15.30	137.0243	0.86
12	Morin	$C_{15}H_{10}O_7$	302.0427	301.0354	16.03	16.14	301.0360	2.07
13	Reservatrol	$C_{14}H_{12}O_3$	228.0786	227.0714	16.03	13.43	227.0711	1.18
14	Luteolin	$C_{15}H_{10}O_6$	286.0477	285.0405	17.03	19.23	285.0404	0.22
15	Quercetin	$C_{15}H_{10}O_7$	302.0427	301.0354	17.03	17.59	301.0358	1.41
16	Apigenin	$C_{15}H_{10}O_5$	270.0528	269.0455	19.03	21.26	269.0450	2.03
17	Naringenin	$C_{15}H_{12}O_5$	272.0685	271.0612	19.03	19.52	271.0607	1.83
18	7-Hydroxyl flavone	$C_{15}H_{10}O_3$	238.0630	237.0557	19.53	22.42	237.0559	0.77
19	Kaempferol	$C_{15}H_{10}O_6$	286.0477	285.0405	19.53	19.04	285.0412	2.59
20	Hesperetin	$C_{16}H_{14}O_6$	302.0790	301.0718	20.03	20.67	301.0720	0.79
21	Pinosylvin	$C_{14}H_{12}O_2$	212.0837	211.0765	22.03	19.71	211.0768	1.64
22	Chrysin	$C1_5H_{10}O_4$	254.0579	253.0506	23.53	22.42	253.0511	1.85

The accurate profile mass spectra were extracted from the 2D-LC TOF data by means of the 2D-LC imaging software, and mass accuracies were calculated. For instance, the $[M-H]^-$ ion from the small gallic acid molecule (1) was measured with a mass accuracy of 0.28 ppm. The large molecule, hesperidin (10), was measured with a mass accuracy of 0.48 ppm. Resveratrol (13) and its dehydroxylated relative pinosylvin (21) were measured with mass accuracies of 1.18 ppm and 1.64 ppm, respectively (Figure 2). In a formula range of $C_{3\text{-}35}H_{7\text{-}72}O_{1\text{-}20}$ and a required mass accuracy of 5 ppm, the calculated formulae were the single hit. The retention time range in the first dimension was between 3.53 and 23.35 minutes, and in the second dimension between 2.51 and 22.42 seconds. The achieved mass accuracies were typically below 2 ppm (Table 1).

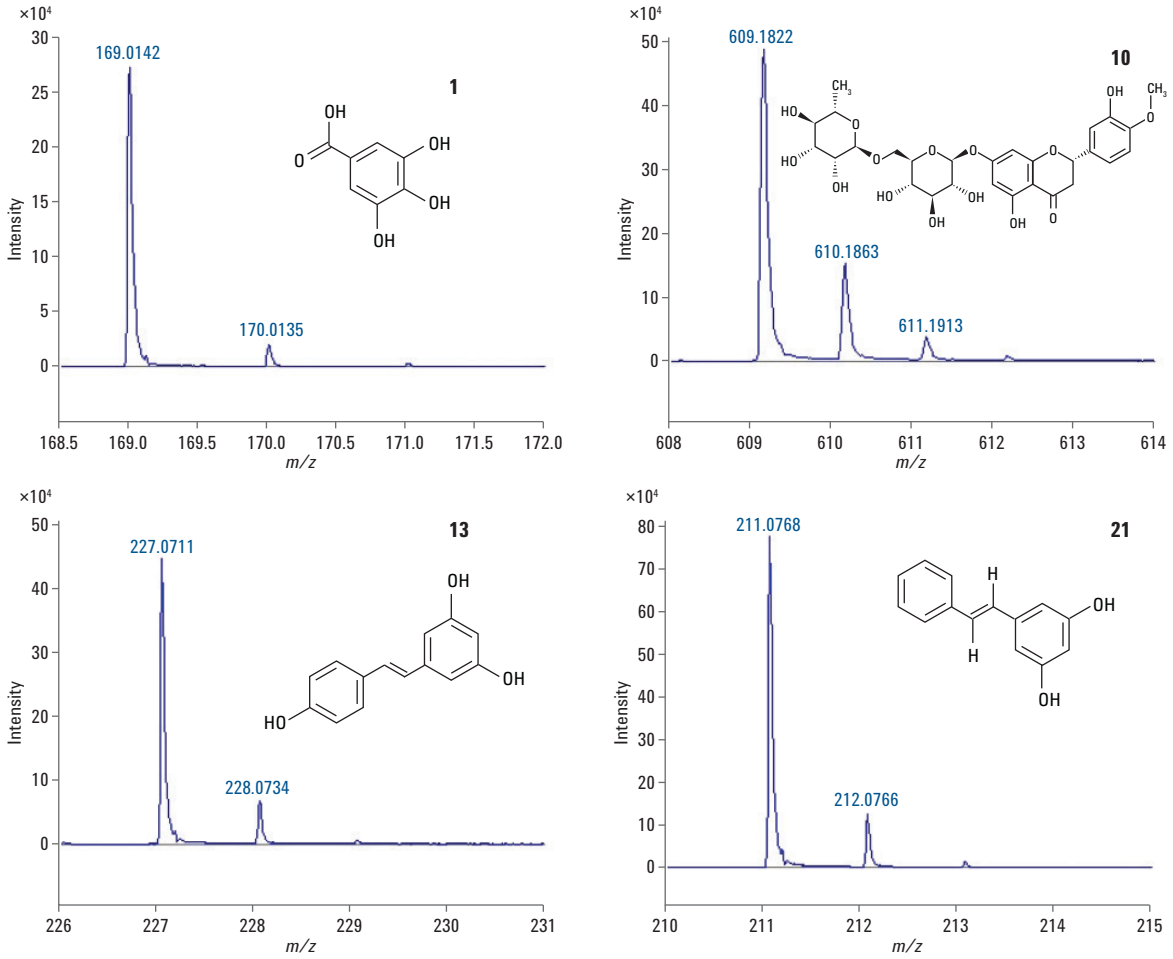

Figure 2. Accurate profile mass spectra from comprehensive 2D-LC coupled to time-of-flight mass measurement. Compounds: (1) Gallic acid, $C_7H_6O_5$, $[M-H]^-$ calc.:169.0142, $[M-H]^-$ measure.: 169.0142, mass accuracy: 0.28 ppm, (10) Hesperidin, $C_{28}H_{34}O_{15}$, $[M-H]^-$ calc.: 609.1825, $[M-H]^-$ measure.: 609.1822, mass accuracy: 0.48 ppm, (13) Resveratrol, $C_{14}H_{12}O_3$, $[M-H]^-$ calc.: 227.0714, [M-H]- measure.: 227.0711, mass accuracy: 1.18 ppm, (21) Pinosylvin, $C_{14}H_{12}O_2$, $[M-H]^-$ calc.: 211.0765, $[M-H]^-$ measure.: 211.0768, mass accuracy: 1.64 ppm.

Conclusion

This Application Notes demonstrates the ease of coupling an Agilent 1290 Infinity 2D-LC Solution to an Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight LC/MS. The connection was made with a T-piece, and flow rates were adjusted accordingly by the restriction of capillaries of different length. From the 2D-LC TOF data, a two dimensional imaging plot was created by means of the HRS MS version of the LCxLC Imaging software. From the two dimensional image, the mass spectra were extracted directly. The used polyphenolic compounds could be determined with high mass accuracy, and their formulae could be calculated with high confidence.

Reference

1. Naegele, E., Qualitative and quantitative determination of phenolic antioxidant compounds in red wine and fruit juice with the Agilent 1290 Infinity 2D-LC Solution, *Agilent Technologies Application Note*, publication number 5991-0426EN, **2012**.

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