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2-Dimensional separation of polyphenols in beverages using the Agilent 1290 Infinity 2D-LC Solution and software-assisted 2D-LC data analysis for comparison of ingredients

Application Note

Food Testing & Agriculture

Authors

Stephen E. Reichenbach
University of Nebraska
Lincoln, NE, USA

Edgar Naegele
Agilent Technologies, Inc.
Waldbronn, Germany

Abstract

This Application Note presents a solution for the analysis of complex samples by an Agilent 1290 Infinity 2D-LC Solution in combination with dedicated software for data analysis. Polyphenols, inherent in several fruit juices and red wine, are analyzed by comprehensive two-dimensional liquid chromatography (LCxLC). The data are analyzed by recently developed software to compare the constituent compounds in the samples.

Introduction

Two-dimensional liquid chromatography (LC×LC) is a powerful tool for analyzing highly complex samples. Comprehensive two-dimensional liquid chromatography can be performed by online transfers of effluent from the first-dimension column to the second-dimension column. Ideally, both columns have orthogonal separation selectivity, thereby increasing the potential peak capacity to the product of the individual peak capacities.[1] In practice, the achievable separation in two dimensions is limited by the effective orthogonality.[2,3]

LC×LC data is typically complex, so routine analysis requires sophisticated software. This LC×LC software performs two-dimensional baseline correction by two-dimensional statistical modeling for accurate peak detection and quantification.[4,5] The two-dimensional peak detection is performed by the Drain algorithm[6] (based on the Watershed algorithm[7]) as well as deconvolution of co-eluting peaks by Parallel Factor Analysis (PARAFAC).[8] The most challenging task is nontarget, multisample analysis, which requires comparison of every constituent in every sample. This is done by a robust cross-sample feature matching (a feature is a chromatographic peak or chromatographic window, that is, an area or region for two-dimensional chromatography, that can be consistently identified across chromatograms and whose detector response can be reliably measured) and analysis based on automated compound identification by template matching.[9] The templates record *a priori* patterns including retention times and spectra. The template matching algorithm uses advanced pattern matching to recognize the same patterns in new chromatograms, and then the template meta-data including compound identities are applied to the new chromatograms.[10] For cross-sample comparisons, reliable peaks are identified by a pairwise matching and used to align and combine all chromatograms into a composite chromatogram. The composite chromatogram, with all peaks from all samples, is used to define peak-region features for the comprehensive comparison of samples.[11,12]

Polyphenols are a large and widely distributed class of natural compounds occurring in fruits, berries, and herbs.[13] From these sources, polyphenols are present in foods and beverages, especially fruit juice, red wine, and beer.[14, 15] Polyphenols are attributed with healthy, anti-oxidative effects. Hence, their analysis is important and cross-comparison of sample content is an important task.

In this Application Note we demonstrate how the Agilent 1290 Infinity 2D-LC Solution can be used to analyze complex samples and how dedicated software is used to analyze the acquired data. The compound class of polyphenols, inherent in some fruit juices and red wine, is analyzed by comprehensive two-dimensional liquid chromatography and the obtained data are analyzed and compared by GC Image LC×LC Edition Software showing compound comparison between the samples.

Experimental

Instrumentation

An Agilent 1290 Infinity 2D-LC Solution with the following configuration was used for the experiments.

Description	Model number
Agilent 1290 Infinity Pump (for 1st dimension)	G4220A
Agilent 1290 Infinity Pump (for 2nd dimension)	G4220A
Agilent 1290 Infinity Autosampler	G4226A
Agilent 1290 Infinity Thermostat (for autosampler)	G1330A
Agilent 1290 Infinity Thermostatted Column Compartment with built-in 2-position/4-port duo valve (G4236A) for 2D-LC	G1316C
Agilent1290 Infinity Diode Array Detector with 60-mm Max-Light flow cell (G4212-60007)	G4212A

Software

- Agilent OpenLAB CDS ChemStation revision C.01.04 with 2D-LC add-on software.
- GC Image LC×LC Edition Software for 2D-LC data analysis form 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. A standard solution of all compounds at a concentration of 100 µg/mL was prepared and used for all dilutions. Red wine and fruit juice samples were purchased in a local supermarket.

Sample Preparation

Red wine and fruit juice samples were filtered through a syringe filter (0.45 µm) directly into a vial and used for injection. Each sample was injected with an injection volume of 1, 2, 5 and 10 µL in duplicate.

Method

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)
1st Dimension pump	
Solvent A	Water + 0.1% formic acid
Solvent B	Acetonitrile + 0.1% formic acid
Flow rate	0.1 mL/min
Gradient	5 % B at 0 minutes; 95 % B at 30 minutes; 95 % B at 40 minutes
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	5% B at 0 minutes; 15% B at 0.5 minutes; 5% B at 0.51 minutes; 5% B at 0.65 minutes
Gradient modulation	5% B at 0 minutes to 50% B at 30 minutes; 15% B at 0.5 minutes to 95% B at 30 minutes; 5% B at 0.51 minutes to 50% B at 30 minutes; 5% B at 0.65 minutes to 50% B at 30 minutes
Post time	15 minutes
Thermostatted column compartment	
1st Dimension column	25 °C at left side
2nd Dimension column	60 °C at right side
Loops	Two 80-µL loops are connected to the 2-position/4-port duo valve and are located on the left side.
Valve	The valve is switched automatically after each 2nd dimension modulation cycle (0.65 minutes).
Autosampler	
Injection volume	1, 2, 5, 10 µL
Sample temperature	8 °C
Needle wash	6 seconds in methanol
Diode array detector	
Wavelength	260/4 nm
Slit	4 nm
Data rate	80 Hz
Flow cell	60-mm Max-Light flow cell

Data Analysis

The automated, nontargeted, multi-sample data analysis was performed in a sequence of steps.

1. Create a project with vials for each of the samples.
2. Import the DAD data.
3. Configure the settings for baseline correction, peak detection, and template matching.
4. Process each of the chromatograms.
5. Use the *Image Investigator* to analyze and compare all 24 chromatograms. The Composite Chromatogram is the sum of all 24 chromatograms after alignment, based on the reliable peaks. The feature regions are the outlines of the peaks detected in the Composite Chromatogram. The *Image Investigator* characterizes each chromatogram based on these feature areas.
6. Export the *Summary Table* for Areas (that is, the feature regions) and the *Attribute Table* for Percent Response for Areas.
7. Combine the two tables and create the comparative figures.

Results and Discussion

A large set of polyphenol and flavonoid standard compounds were used to design the two-dimensional separation method.[16] This set of 26 compounds was separated in 35 minutes in the first dimension with a modulation of 39 seconds in the second dimension. The compounds and their first and second dimension retention times are shown in Table 1.

Compound	Compound name	Peak I (min)	Peak II (s)
1	Gallic acid	7.15	7.56
2	Esculin	9.75	18.63
3	3,4 HO benzoic acid	9.75	9.74
4	HO Phenacetic acid	11.70	13.55
5	6,7 HO coumarin	12.35	19.66
6	HO Benzoic acid	12.35	16.51
7	Syringic acid	13.00	25.78
8	Rutin	13.65	33.63
9	Naringin	14.95	32.43
10	Coumaric acid	14.95	25.44
11	Hesperidin	15.60	34.89
12	Ferulic acid	15.60	27.63
13	Myricetin	17.55	30.20
14	Morin	18.85	30.20
15	Resveratrol	18.85	26.65
16	Salicylic acid	19.50	18.55
17	Luteolin	19.50	32.79
18	Quercetin	20.15	30.20
19	Kaempferol	21.45	30.96
20	Apigenin	22.10	25.84
21	Naringenin	22.10	27.65
22	Hesperetin	22.75	28.74
23	7 HO Flavone	22.75	34.29
24	Pinosylvin	24.70	18.81
25	Chrysin	27.30	27.49
26	Flavone	28.60	26.33

Table 1
Compounds used in the standard solution, retention times in the first and second dimension.

The compounds were detected by two-dimensional peak detection in the LC×LC software and the compound numbers and names were assigned manually (Figure 1A, Table 1). From one of the obtained data files, a template was generated to record the retention times and spectra of each compound in the standard. Most of the yellow template spots are matched when the template is applied to the other standard sample files analyzed with this separation method (Figure 1B). Template matching automates identification of the compounds in real samples of unknown composition.

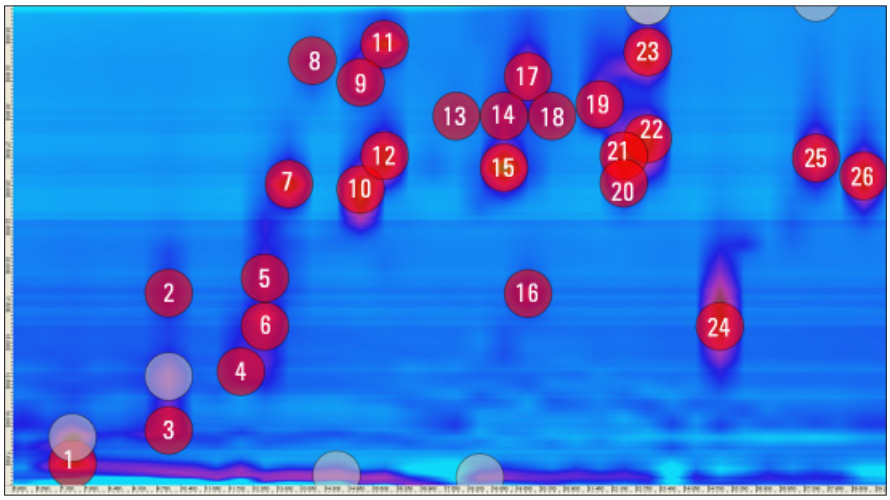

Figure 1A
An Agilent 1290 Infinity 2D-LC Solution plot of the optimized separation of 26 polyphenolic compounds with software detected peak annotation.

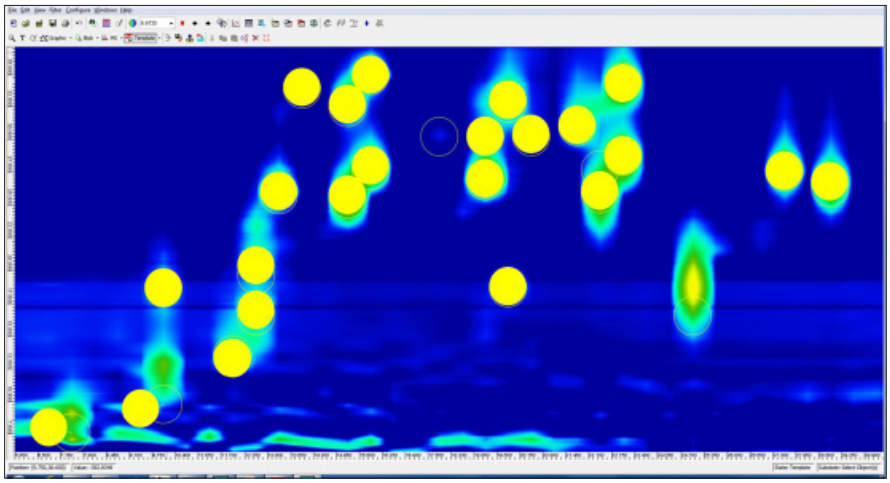

Figure 1B
Template generated from the detected compounds of the Agilent 1290 Infinity 2D-LC Solution separation. Undetected peaks are due to low concentration, co-elution, distances in retention time or spectrum. Settings for template matching can be configured to improve performance.

As an example, phenolic compounds could be identified from the analyzed beverage samples (red grape juice, antioxidant juice, and merlot red wine) by application of the template and comparison of retention times and spectra to the standard compound separation (Figures 2 and 3). The example for red grape juice shows more simple phenolic compounds such as gallic acid (Figure 2) and the analysis of the antioxidant juice shows some additional, more complex glycoside compounds, such as rutin in another area of the two-dimensional plot (Figure 3). This approach offers the possibility to compare and distinguish different sources of beverages containing polyphenol compounds by more sophisticated software based analysis. For this analysis, a larger set of data is required, which was generated by replicative sample injection of different concentration levels (by volume, 1, 2, 5, and 10 µL). As a premise from the instrumental side, it is necessary to have little variation between the individual runs. As demonstrated in earlier work, the performance of the 1290 Infinity 2D-LC Solution fulfills this requirement.[17] For instance, the retention time precision in the second dimension separation is typically below 1% RSD and for the peak volume, the RSD is also typically below 1%.

First, each individual beverage sample was compared to a composite average data file of all runs (Figure 4). There is a bubble for each feature area. Each bubble is positioned at the retention times in first and second dimension and the size (area) of the bubble is proportional to the difference. Positive differences are blue; negative differences are white. These figures give a sense of where (in the chromatographic plane) each beverage sample has more or less of the different features, for example, more small polar compounds in the red grape juice and more hydrophobic compounds in the antioxidant juice and red wine compared to the average.

The second comparison shows the pairwise differences between each kind of beverage (Figure 5). There are differences between antioxidant juice and merlot, antioxidant juice and red grape juice, and merlot and red grape juice. The blue circles indicate first average is greater than second average and white circles indicate second average is greater than first average.

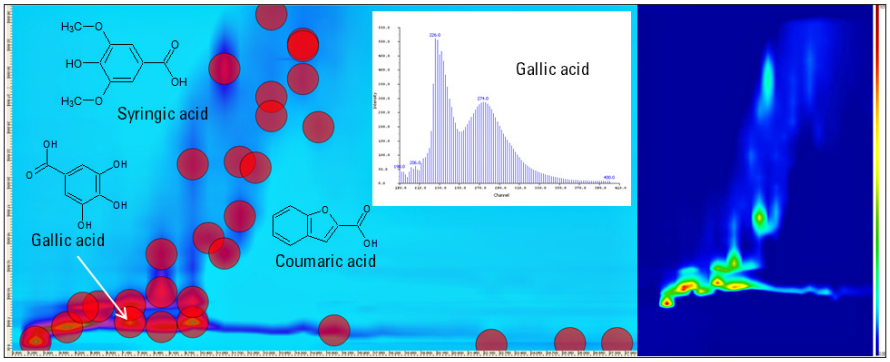

Figure 2
Sample of red grape juice separated by an Agilent 1290 Infinity 2D-LC Solution. The main components are typically hydroxyllic benzoic acid compounds like gallic acid (insert: UV spectrum of gallic acid). Right side: 2D-plot without peak detection.

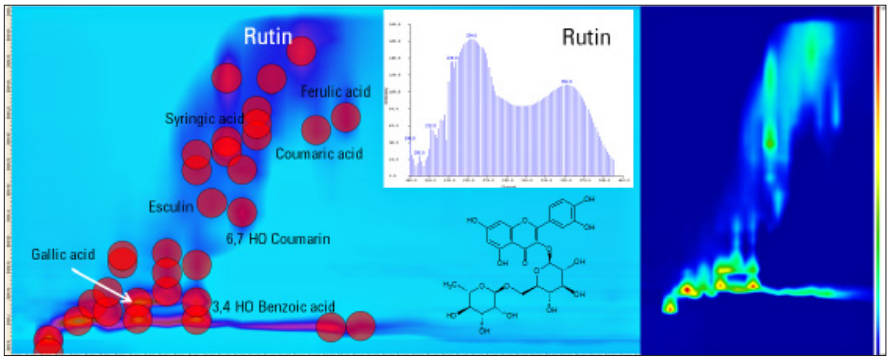

Figure 3
An Agilent 1290 Infinity 2D-LC Solution separation of a mixed antioxidant juice containing red and green grape, apple, black current, cherry, cranberry, pomegranate, bilberry. Besides the simple hydroxy benzoic acid derivatives, more complex glycosidic compounds such as rutin and esculin were identified. Right side: 2D-plot without peak detection.

Taken as a group, these figures show that the red grape juice has relatively larger responses in the lower left of the separation space and that antioxidant juice and merlot have relatively larger responses in the upper right of the separation space. Merlot has a larger response in the upper right than does antioxidant juice. In terms of increasing responses in the upper right, the mixes would be ordered: 1) Red grape juice, 2) Antioxidant juice, 3) Merlot.

These results could indicate more complex rutin-like compounds in merlot and in the antioxidant juice compared to red grape juice. For identification, the retention times and UV spectra could be used. In an improved experiment, the information from a connection to a single quadrupole MS or even a time-of-flight MS could be used for compound identification.

Conclusions

This Application Note demonstrates the capability of the Agilent 1290 Infinity 2D-LC Solution to unravel differences in sample groups in combination with a dedicated 1290 Infinity 2D-LC Solution data analysis software package. It was shown that, with the demonstrated highly accurate repetitive run-to-run performance of the 1290 Infinity 2D-LC Solution, differences of polyphenol constituents in beverages can be explored.

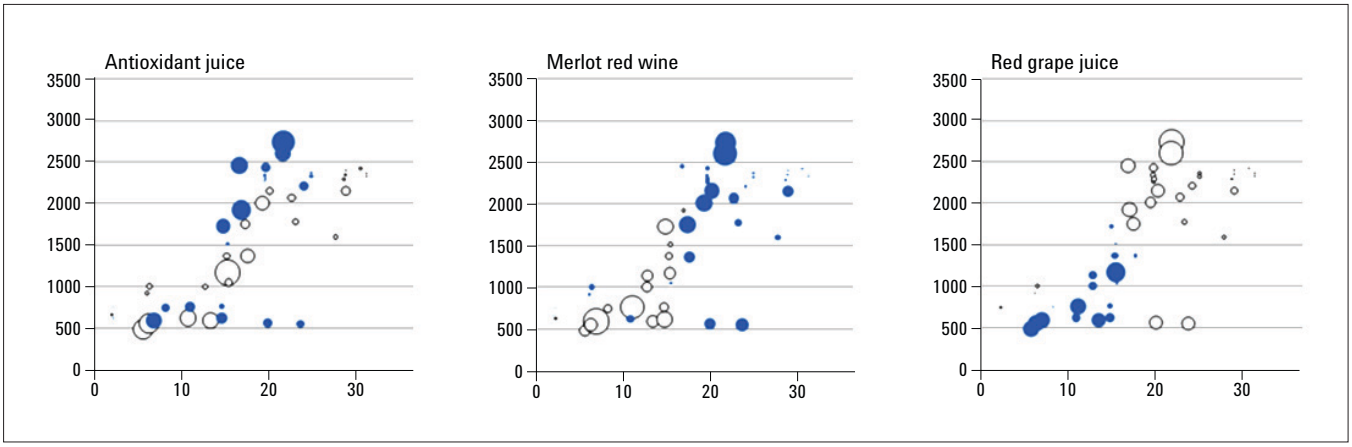

Figure 4
Differences for peak-region features between drink-specific averages and overall averages. (Blue circles indicate drink-specific average is greater than overall average; white circles indicate drink-specific average is less than overall average; areas indicate differences.)

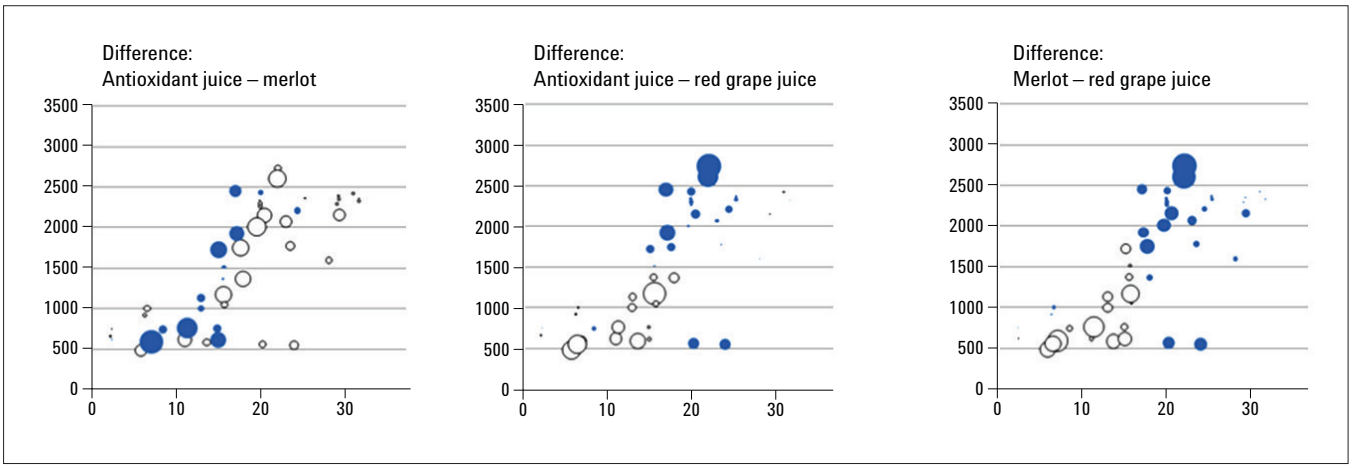

Figure 5
Pairwise differences for peak-region features between drink-specific averages show that the beverages have notably different compositions.
(Blue circles indicate first average is greater than second average; white circles indicate second average is greater than first average; areas indicate differences.)

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Supplier of LCXLC software

Headquarters

JSB International
Tramstraat 15
5611 CM Eindhoven
T +31 (0) 40 251 47 53
F +31 (0) 40 251 47 58

Zoex Europe
Tramstraat 15
5611 CM Eindhoven
T +31 (0) 40 257 39 72
F +31 (0) 40 251 47 58

Sales and Service

Netherlands
Apolloweg 2B
8239 DA Lelystad
T +31 (0) 320 87 00 18
F +31 (0) 320 87 00 19

Belgium
Grensstraat 7
Box 3 1831 Diegem
T +32 (0) 2 721 92 11
F +32 (0) 2 720 76 22

Germany
Max-Planck-Strasse 4
D-47475 Kamp-Lintfort
T +49 (0) 28 42 9280 799
F +49 (0) 28 42 9732 638

UK & Ireland
Cedar Court,
Grove Park Business Est.
White Waltham, Maidenhead
Berks, SL6 3LW
T +44 (0) 16 288 220 48
F +44 (0) 70 394 006 78

info@go-jsb.com
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