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Qualitative and quantitative determination of phenolic antioxidant compounds in red wine and fruit juice with the Agilent 1290 Infinity 2D-LC Solution

Application Note

Food Testing

Author

Edgar Naegele
Agilent Technologies, Inc.
Waldbronn, Germany

Abstract

This Application Note demonstrates the Agilent 1290 Infinity 2D-LC Solution for resolving complex mixtures by reversed-phase/reversed-phase, two-dimensional chromatography. The method was developed by means of a multi standard mixture and subsequently applied to detect polyphenolic antioxidant compounds in fruit juices and red wine in a qualitative and quantitative manner.

Introduction

Polyphenolic compounds are secondary plant metabolites including, for example, phenolic acids, flavonoides, coumarins and others. Many of these are present in grapes, berries, hop and herbs, as well as in food and beverages such as fruit juices, beer, and wine[1]. This class of compounds has an anti-oxidative effect and potency to protect against free radicals.

The polyphenolic compounds occur in complex matrixes and their analysis requires a high separation capability. Therefore, two-dimensional liquid chromatography is the method of choice. The separation can be done with two reversed-phase separations using columns with different stationary phase chemistries and solvent conditions.

This Application Note demonstrates that the 1290 Infinity 2D-LC Solution is able to resolve complex mixtures of polyphenoles in matrixes by reversed-phase/reversed-phase two-dimensional chromatography. The method was developed using a multi standard mixture and then used to detect polyphenolic antioxidant compounds in fruit juices and red wine in a qualitative and quantitative manner.

Experimental

Equipment

The Agilent 1290 Infinity 2D-LC Solution comprises the following modules:

- Two Agilent 1290 Infinity Pumps (G4220A),
- Agilent 1290 Infinity Autosampler (G4226A) with cooler (G1330A)
- Agilent 1290 Infinity Thermostatted Column Compartment (TCC) (G1316C) with 2-position-4 port-duo valve (G4236A) for 1290 Infinity 2D-LC Solution
- Agilent 1290 Infinity Diode Array Detector (DAD) (G4212A) with 60 mm Max-Light flow cell (G4212-60007)
- Column first dimension: Agilent ZORBAX RRHD Eclipse Plus, C18, 150 × 2.1 mm, 1.8 µm
- Column second dimension: Agilent ZORBAX RRHD Eclipse Plus, Phenyl Hexyl, 50 × 3.0 mm, 1.8 µm

In this configuration, the first and second dimension pumps are identical. Typically, the second dimension pump must be a 1290 Infinity pump to deliver fast gradients to the second dimension column. The first dimension pump is flexible and could also be a 1260/1290 Infinity Quaternary pump, a 1260 Infinity Binary pump, or a 1260 Infinity Capillary pump.

Software

- Open Lab CDS ChemStation Rev. C01.03 with 1290 Infinity 2D-LC Solution add-on software.
- LCxLC Software for 2D-LC data analysis from GC Image LLC., Lincoln, NE, USA.

Method

First dimension pump

Solvent A:	Water + 0.1% formic acid
Solvent B:	Acetonitrile + 0.1% formic acid
Flow rate:	0.1 mL/min
Gradient:	0 min 5% B – 30 min 95% B 40 min – 95% B
Stop time:	40 min
Post time:	15 min

Second dimension pump

Solvent A:	Water + 0.1% formic acid.
Solvent B:	Methanol + 0.1% formic acid.
Flow rate:	3 mL/min.
Initial gradient:	0 min–5% B 0.5 min–15% B 0.51 min–5% B 0.65 min–5% B
Gradient modulation:	0 min 5% B to 30 min 50% B 0.5 min 15% B to 30 min 95% B 0.51 min 5% to 30 min 50% B 0.65 min 5% B to 30 min 50% B

Thermostatted column compartment

- First dimension column on the left side at 25 °C
- Second dimension column on the right side at 60 °C
- Two 80 µL loops, located on the left side, are connected to the 2-position-4-port-duo valve.
- The valve is switched automatically after each second dimension modulation cycle. In this case, the loops are used in a cocurrent manner (the loops are filled and eluted from the same side).

Autosampler

Injection volume:	5 µL
Sample temperature:	8 °C
Needle wash:	6 s in methanol

Diode-array Detector

Wavelength:	nm
Slit:	nm
Data rate:	80 Hz
Flow cell:	60 mm Max-Light flow cell

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. Red wine and fruit juice samples were purchased in a local supermarket. A standard solution of all compounds at a concentration of 100 µg/mL was prepared and used for all dilutions.

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.

Results and discussion

To optimize the 1290 Infinity 2D-LC Solution method for the separation of polyphenolic compounds, a standard mixture of polyphenolic and flavonoidic compounds was generated and separated on the 1290 Infinity 2D-LC Solution system (Figure 1A). The inherent compounds were automatically detected by the software peak detection algorithm (Figure 1B). To cover the largest possible space of the two-dimensional separation, a large variety of compounds was used in the standard mixture such as benzoic acids, phenolic compounds, glycosidic compounds, and flavonoidic compounds (Table 1).

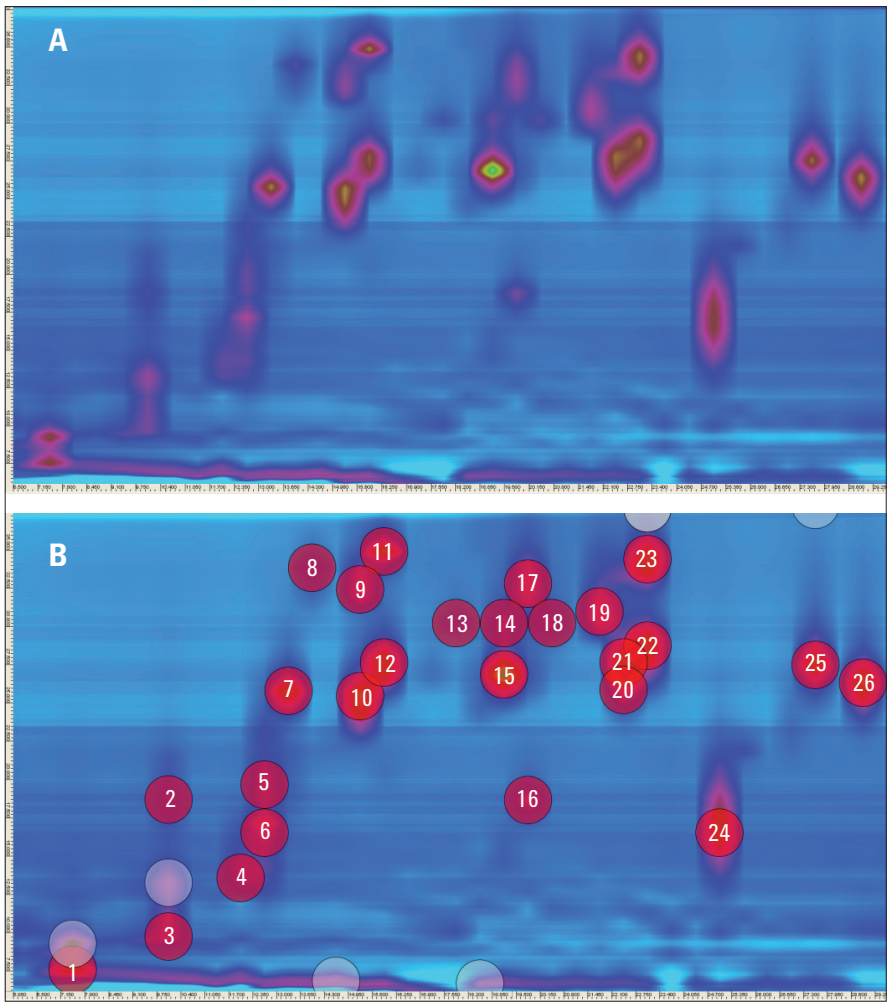

Figure 1
A) Agilent 1290 Infinity 2D-LC Solution plot of the optimized separation of 26 polyphenolic compounds.
B) Agilent 1290 Infinity 2D-LC Solution plot with software detected peak annotation.

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

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

To determine the performance of the system optimized for this method, a subset of the compounds, which covers the entire space of retention times in the first and second dimension was statistically evaluated. The RSD [%] values of the second dimension retention times are typically better than 0.5% and the RSD [%] values for the software calculated peak volumes are typically better than 3% (Table 2). The complete run is displayed in a three-dimensional graphic (Figure 2).

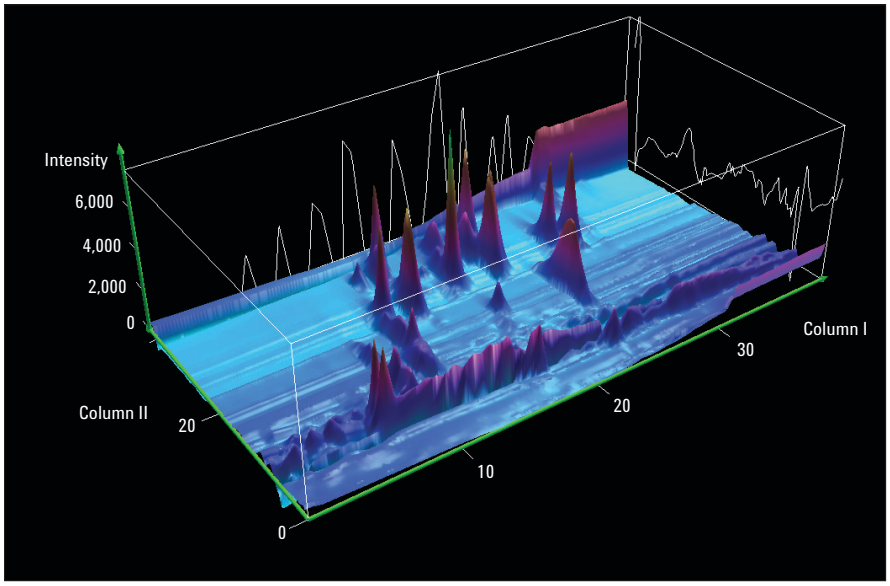

Figure 2
Three-dimensional display of the separation of the 26-compound standard mixture. The first dimension separation takes 40 minutes, and each second dimension separation takes 39 seconds. The back side shows a generated first dimension chromatogram and gives the impression which peaks are coeluting and separated in the second dimensions.

Compound		RT 1st dim. (min)	RT 2nd dim. (sec)	Peak volume	Compound		RT 1st dim. (min)	RT 2nd dim. (sec)	Peak volume
Esculin	Mean	9.75	18.58	177,383	Luteolin	Mean	19.50	32.70	695,601
	s.d.	nd	0.11	4,713		s.d.	nd	0.11	17,592
	RSD (%)	nd	0.57	2.7		RSD (%)	nd	0.33	2.5
Rutin	Mean	13.65	33.68	72,375	7-Hydroxy-flavone	Mean	22.75	34.26	1,388,226
	s.d.	nd	0.07	853		s.d.	nd	0.10	17,195
	RSD (%)	nd	0.22	1.2		RSD (%)	nd	0.30	1.2
Coumaric acid	Mean	13.00	25.57	660,541	Pinoslyvin	Mean	24.70	18,85	1,588,654
	s.d.	nd	0.13	13,037		s.d.	nd	0.23	57,580
	RSD (%)	nd	0.52	2.1		RSD (%)	nd	1.24	3.6
Reservatrol	Mean	18.85	26.65	1,122,219	Chrysin	Mean	27.30	27.42	808,916
	s.d.	nd	0.10	16,089		s.d.	nd	0.11	14,768
	RSD (%)	nd	0.37	1.4		RSD (%)	nd	0.39	1.8
Salicylic acid	Mean	19.50	18.53	211,092	Flavone	Mean	28.60	26.35	1,008,012
	s.d.	nd	0.10	6,895		s.d.	nd	0.13	20,911
	RSD (%)	nd	0.51	3.3		RSD (%)	nd	0.48	2.1

s.d. = standard deviation
nd = not determined

Table 2
Performance data of selected compounds of the used standard mixture. Retention time RSD [%] are typically better than 0.5% and the peak volume RSD [%] are typically better than 3%.

The second part of this Application Note demonstrates the application of the developed separation method to real samples that contain polyphenolic and flavonoidic compounds, such as fruit juices and red wine. For example, fruit juice from red grapes was analyzed with the 1290 Infinity 2D-LC Solution using the developed method (Figure 3). In this sample, simple hydroxyl benzoic acid derivatives could be identified. They were identified by their retention time in the first and second dimension and a comparison of UV spectra to standard compounds. Typical compound ingredients in grape juice are gallic acid and syringic acid[1]. A more complex sample was obtained by a multi antioxidant fruit juice mixture bought in a local supermarket (Figure 4). This mixture contained juice and fruit pulp, and was made of a selection of fruits, known for simple and complex antioxidant ingredients. It was possible to identify simple compounds like hydroxyl benzoic acid derivatives as well as more complex glycosidic natural compounds such as rutin and esculin. The compounds were identified by their retention times in the first and second dimension as well as UV spectra compared to standards.

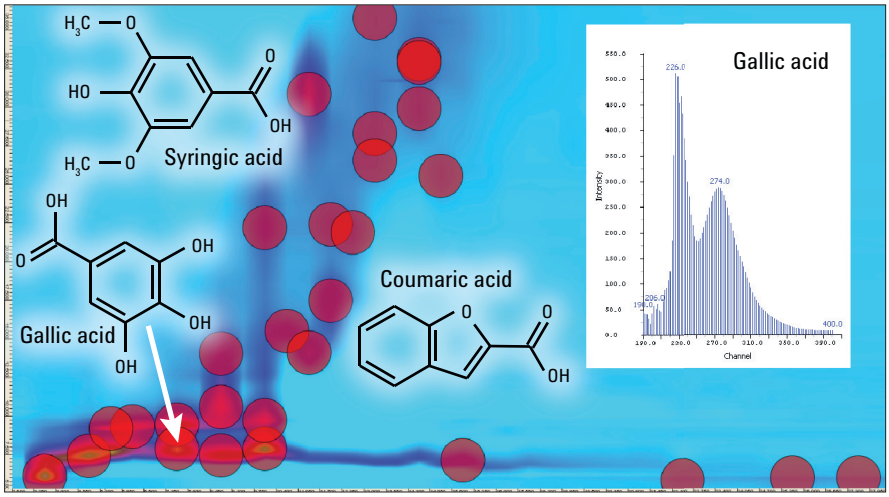

Figure 3
Sample of red grape juice separated by the Agilent 1290 Infinity 2D-LC Solution. The main components are typically hydroxyllic benzoic acid compounds such as gallic acid (insert: UV spectrum of gallic acid).

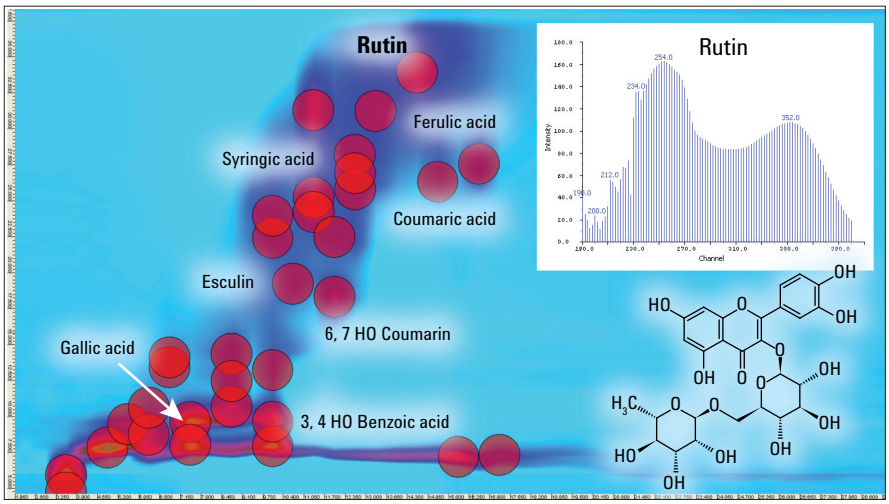

Figure 4
Agilent 1290 Infinity 2D-LC Solution separation of a mixed antioxidant juice containing red and green grape, apple, black current, cherry, cranberry, pomegranate, bilberry. In addition to the simple hydroxy benzoic acid derivatives, more complex glycosidic compounds like rutin and esculin were identified.

Finally, a Merlot red wine sample was analyzed by means of the 1290 Infinity 2D-LC Solution with the developed method (Figure 5). The two-dimensional-plot shows the typical early eluting compounds such as the derivatives of hydroxyl benzoic acid, which are known to be inherent in red wine[1] and also a very potent antioxidant compound typical for red wine, resveratrol. This compound was first indentified qualitatively by its retention times and its UV spectrum. In addition to the qualitative identification, it is also possible to make quantitative statements from a sample analyzed by 1290 Infinity 2D-LC Solution.

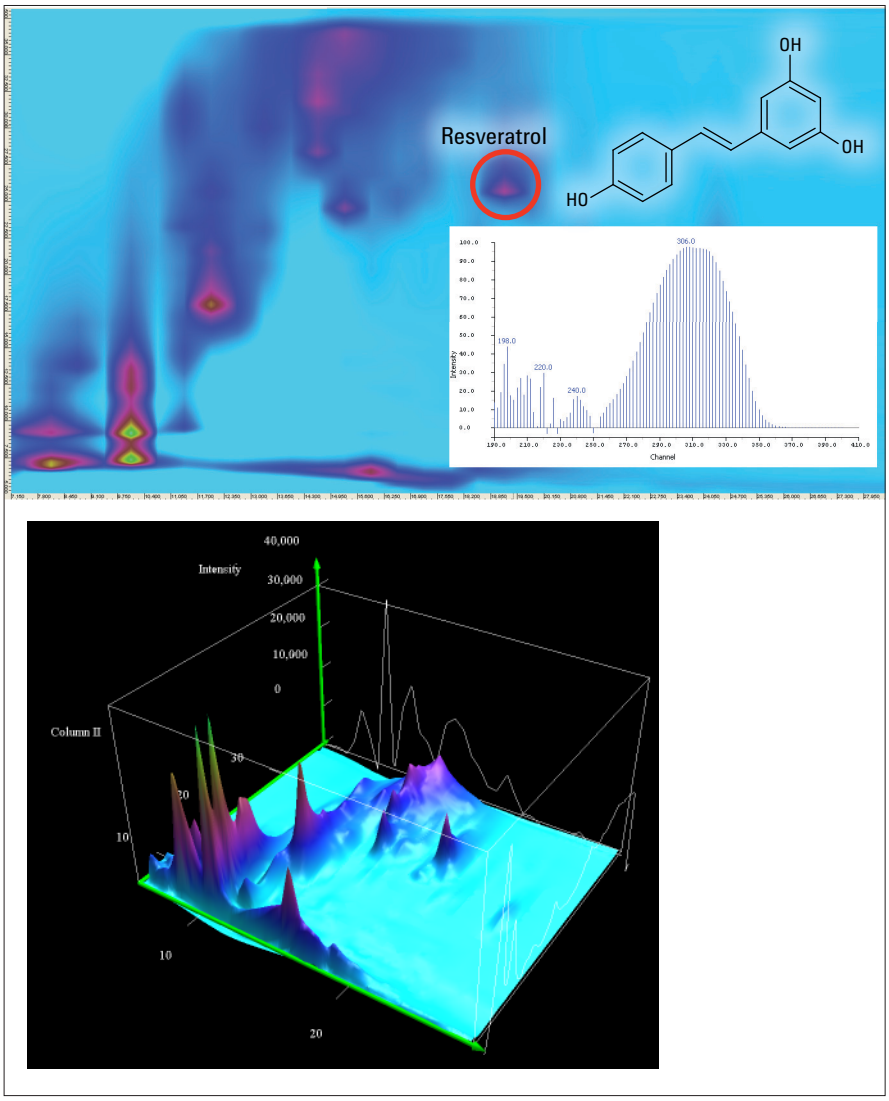

Figure 5
2D-LC analysis of red wine visualized with LCXLC software. The upper picture shows the compounds separated from the red wine sample in a 2-dimensional map including the UV spectrum of resveratrol. The lower part shows a 3-dimensional plot of the separation.

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Therefore, a dilution series of standard resveratrol was measured and combined with the samples in a project for quantification within the LCxLC software (Figure 6). The calibration curve was measured between the obtained limit of quantification (LOQ) at 1 µg/mL up to 50 µg/mL with a linearity of 0.9987, which is excellent for a 2D-LC analysis quantification. From the samples a quantity of about 4.5 mg/L resveratrol in the measured Merlot red wine was determined.

Conclusion

This Application Note demonstrates that the 1290 Infinity 2D-LC Solution can be used for the separation of complex natural product samples. The separation was optimized with a multicomponent standard solution. The separation performance was excellent with retention time RSD values typically below 0.5 % and peak volume RSD values typically below 3%.

The method was used for quantification of ingredients in red wine. The linearity and LOQ achieved with the Agilent 1290 Infinity 2D-LC Solution were excellent for a 2D-LC separation and quantitative determination of the compounds in sample matrix.

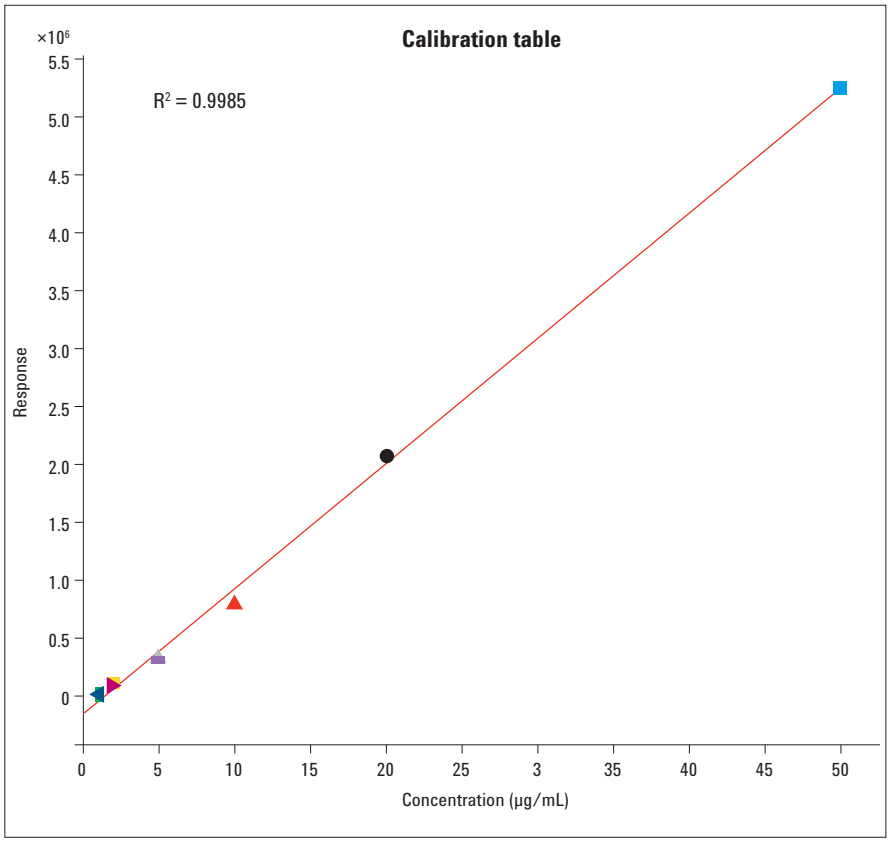

Figure 6
Calibration of 1290 Infinity 2D-LC Solution for resveratrol quantification, 1–50 µg/mL found in merlot: 4.5 µg/mL, 4.5 mg/L.

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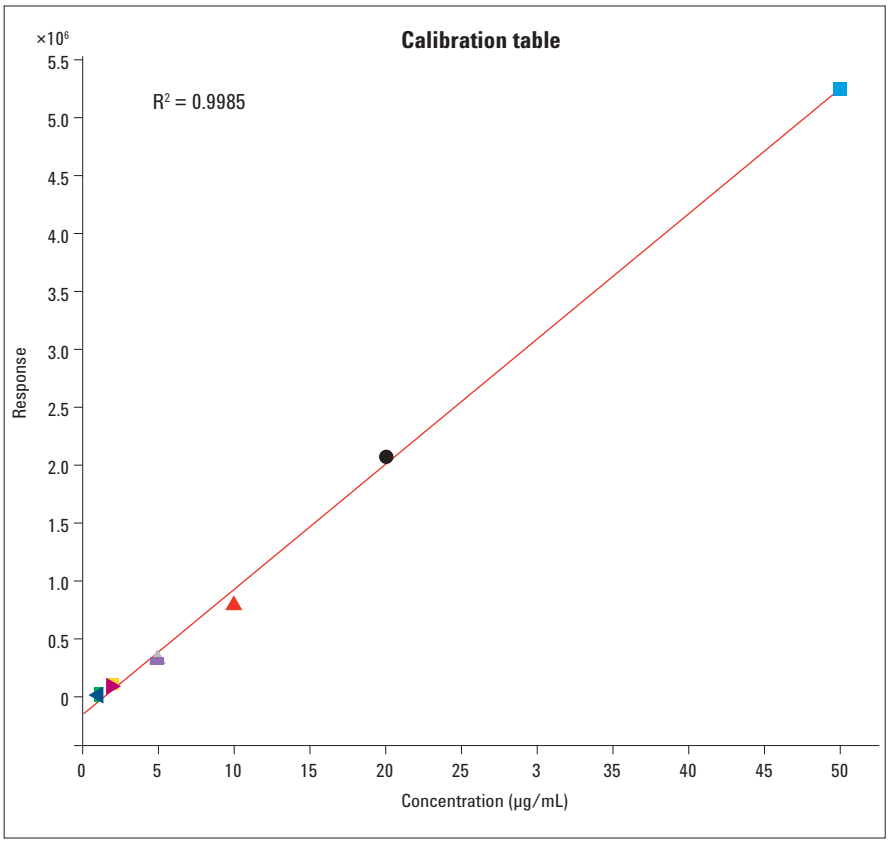

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Reference

1.
Kivilompolo M., Oburka V., Hyötyläninen T., Comprehensive two-dimensional liquid chromatography in the analysis of antioxidant compounds in wines and juices. Anal. Bioanal. Chem. **2008**, 391, 373-380.

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ZOEX | EUROPE
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
www.go-jsb.com

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