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Technical Report

Comprehensive Two-dimensional Liquid Chromatography for Determination of Polyphenols in Red Wines

LC×LC-PDA-MS/MS for polyphenol analysis in red wine

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Abstract:

A comprehensive two-dimensional liquid chromatography method was developed and applied to the determination of polyphenols in red wines. To fulfil such a task, a micro cyano and a partially porous octadecylsilane columns were employed in the first and the second dimension, respectively in combination with photodiode array and mass spectrometry (LC×LC-PDA-MSMS) detection. To increase the peak capacity values by using RP modes in both dimensions, a comparison of a conventional full-in-fraction and shifted second dimension gradient was carried out. The separation capabilities of the comprehensive LC approaches tested allowed the analysis of such a complex natural sample without any pre-treatment to effectively reduce the interferences coming from the matrix.

Keywords: comprehensive LC, polyphenols, red wines, mass spectrometry

1. Introduction

Phenolic compounds are secondary metabolites synthesized by plants during normal development and in response to stress conditions. They embrace a considerable range of substances possessing an aromatic ring bearing one or more hydroxyl moieties. Produced and consumed world-wide, wine is an excellent natural source of various polyphenol families that go from phenolic acids (benzoic- or cinnamic-like derivatives) to different classes of flavonoids (flavones, flavan-3-ols, flavonols and anthocyanins).

Sometimes, the polyphenol content in real world-samples can be so complex that they cannot be resolved in a one-dimensional HPLC analysis. In order to overcome this problem, comprehensive two-dimensional liquid chromatography (LC×LC) employing two columns with different selectivity in the two dimensions could be a viable tool. In addition, in order to improve the peak distribution, different gradient elution strategies could be investigated to enhance the orthogonality degree, by means of specific elution gradient approaches to be used in the second dimension.

This technical report describes a novel LC×LC-PDA-MSMS (Fig. 1) instrument, capable of extremely high-resolution power, as well as targeted and untargeted analysis, that was successfully applied to the characterization of the polyphenol content a red wine sample (Fig. 2).

Fig. 1 LC×LC-PDA-MSMS instrumentation

2. Experimental

2-1. Reagents and Materials

LC-MS grade solvents for LC×LC analyses: water (H_2O), acetonitrile (ACN); Acetic acid 99–100%, (glacial). All the solvents and chemicals were purchased from Sigma-Aldrich (Milan, Italy).

Chromatographic separations were carried out using columns provided by Supelco (Bellefonte, PA, USA): Ascentis Cyano (250 mmL. × 1 mmI.D., 5 µm d.p.), and Ascentis Express C_{18} (30 mmL. × 4.6 mmI.D., 2.7 µm d.p.).

The red wine was purchased in a local market. The sample was filtered through a 0.45 µm Acrodisc nylon membrane (Pall Life Sciences, Ann Arbor, MI, USA) before injection.

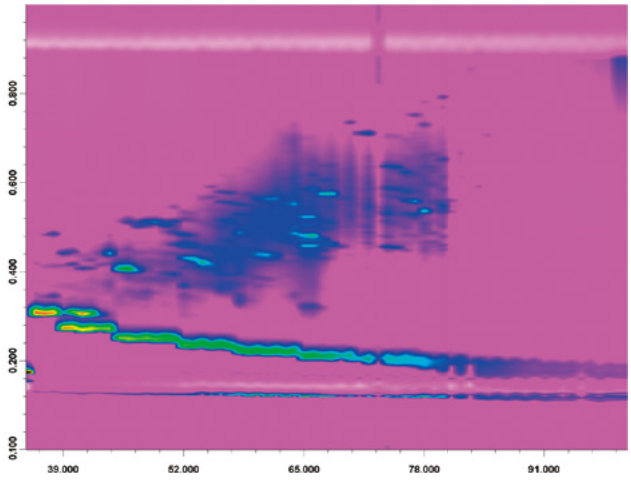

Fig. 2 RP-LC×RP-LC Plot of a red wine sample

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2-2. Instrument

- Shimadzu CBM-20A controller
- Shimadzu LC-30AD dual-plunger parallel-flow pumps (D1-LC)
- Shimadzu DGU-20A5R degassing unit (D1-LC)
- Shimadzu LC-30AD dual-plunger parallel-flow pumps (D2-LC)
- Shimadzu DGU-20A3R degassing unit (D2-LC)
- Shimadzu CTO-20AC column oven
- Shimadzu SIL-30AC autosampler
- Shimadzu SPD-M30A photo diode array detector (1 µL flow cell)
- Shimadzu LCMS-8030 (ESI source)

For connecting the two dimensions: two electronically-controlled 2-position, 6-port high pressure switching valves FCV-32AH (with two 20 µL empty loops).

2-3. Software

- Shimadzu LabSolutions (Version 5.60 SP2)

2-4. 2D Software

- LCxLC-Assist
- ChromSquare (Version 2.0) from Chromaleont, Messina, Italy

3. LC×LC-MS Conditions

First dimension (D1) separations	
Column	: Ascentis Cyano
Flow rate	: 20 µL/min
Mobile phases	: (A) 0.1% acetic acid in water (pH around 3); (B) acetonitrile 0.1% acetic acid.
Gradient elution	: 0.01 min, 2% B; 10 min, 2% B; 60 min, 50% B; 75 min, 100% B; 100 min, 100% B.
Backpressure (at analysis start) = 40 bar	
Injection volume	: 5 mL

Second dimension (D2) separations	
Column	: Ascentis Express C18
Flow rate	: 2.5 mL/min
Mobile phases	: (A) 0.1% acetic acid in water (pH around 3); (B) acetonitrile 0.1% acetic acid.
Gradient elution	:
FIF, full in fraction: 0.01 min, 0% B; 0.10 min, 0% B; 0.75 min, 50% B; 1.00 min, 0% B.	
SG, Shifted gradient: illustrated in Fig. 3	
Backpressure (at analysis start) = 170 bar	
Modulation time of the switching valves : 1 min.	

MS conditions

MS acquisition performed using the ESI interface operating in negative ionization mode:

mass spectral range: 100–800 *m/z*; event time: 0.1 sec; scan speed: 7500 *u/s*; nebulizing gas (N_2) flow: 2 L/min; drying gas (N_2) flow: 15 L/min; Heat block temperature: 250 °C; desolvation line (DL) temperature: 250 °C; Interface voltage: 3.5 kV; detector voltage: 1.80 kV; The flow eluting from the second column was splitted before the MS instrument (approximately 0.4 mL/min to the MS).

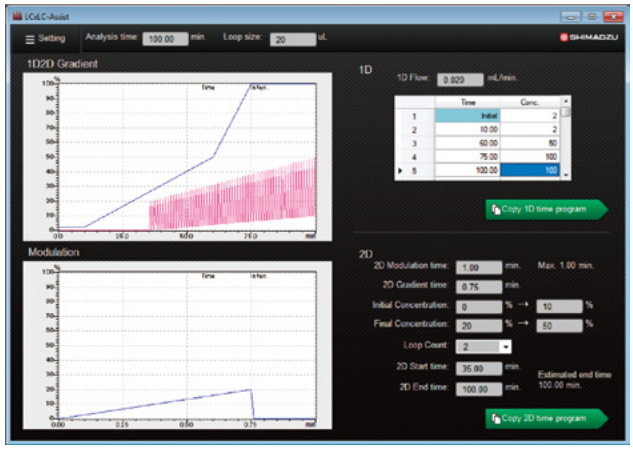

Fig. 3 Scheme of the LCxLC-Assist software

4. Results and Discussion

In an LC×LC system, with two not orthogonal dimensions, an LC×LC separation most likely results in peaks concentrated around the main diagonal line of the separation area. A typical example is the LC×LC analysis of a red wine sample, illustrated in Fig. 4A, by employing a Cyano column in the D1 and a C18 column in the D2 using the conventional full-in-fraction approach.

As a matter of fact, a clear correlation of the D1 and D2 and a small peak-distribution area were observed because the separation mechanisms in the two dimensions were similar. The analytes eluted early in the D1 were only weakly retained in the D2; the analytes eluted in the middle of the D1 were eluted in the middle of the D2 and the analytes eluted late in the D1 were strongly retained in the D2.

To overcome such a limitation, we used a narrower organic solvent span changing the gradient program according to the elution properties. The shifted gradient program, led to a greater coverage of the separation space (Fig. 4B). The blue line is the program of the D1 run and the red line is that of the D2 run. The D2 gradient covered a narrow organic solvent range, which varied continuously during the LC×LC run. The gradient program started with 0% acetonitrile and rose to 20% ACN over 0.75 min; at the end of the analysis, the gradient program in the D2 starts at 10% acetonitrile and rose to 50% acetonitrile. At the end of the analysis, the higher percentage of organic solvent made possible the efficient elution of the strongly retained compounds.

As can be seen from Fig. 4B regarding the red wine sample analyzed with LC×LC with a shifted gradient in the D2, a significant improvement in the retention space was attained (Fig. 5). In fact, the use of a shifted gradient with a gradual increase of the proportion of organic solvent gave better separation in the D2 with a less typical diagonal-line distribution. In addition, because of the narrower solvent range, the backpressure was much smoother and steadier.

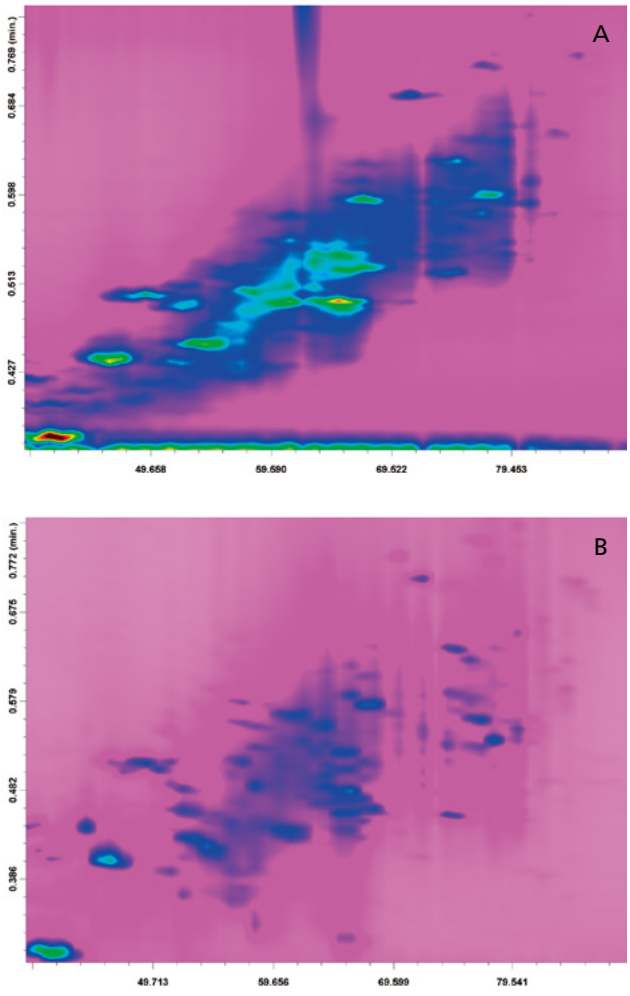

Fig. 4 Separation of a red wine sample by using the FIF (A) and the shifted gradient (B) approaches

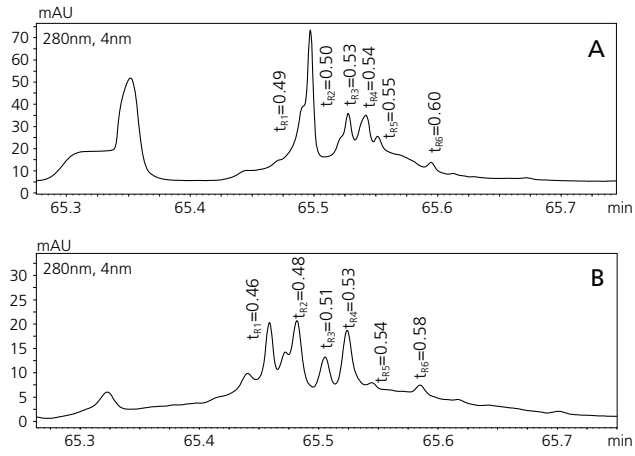

Fig. 5 Analysis of a red wine sample. A comparison of the second-dimension separation of the 65th fraction for both LC×LC plots shown in Fig. 4A–B.

An evaluation of the performance, in terms of peak capacity (n_C), of the two different set-ups tested was carried out, considering both theoretical and practical peak capacity. The theoretical peak capacity values, multiplicative of the individual values obtained for the two dimensions ($^1n_C \times {}^2n_C$), yielded values as high as 690 and 570 for the full in fraction and for the shifted gradients, respectively.

As expected, due to the partial correlation of the two dimensions, "practical" peak capacity values, corrected for both undersampling (number of fractions effectively transferred from the D1 to the D2) and orthogonality (separation space effectively covered by sample compounds), were significantly lower at 75 and 216. The set-up with the use of the shifted second dimension gradients was the most efficient one since it less suffered from the correlation of the two dimensions tested (Table 1).

Selected ion extracted chromatograms for some target compounds occurring in the red wine sample along the relative mass spectra are illustrated in Fig. 6.

Table 1 Relative performances, in terms of peak capacity, n_C, of the two set-up investigated

	Full in fraction gradient	Shifted gradient
^1n_C	15	15
^2n_C	46	38
Theoretical D2 n_C	690	570
Practical D2 n_C	75	216

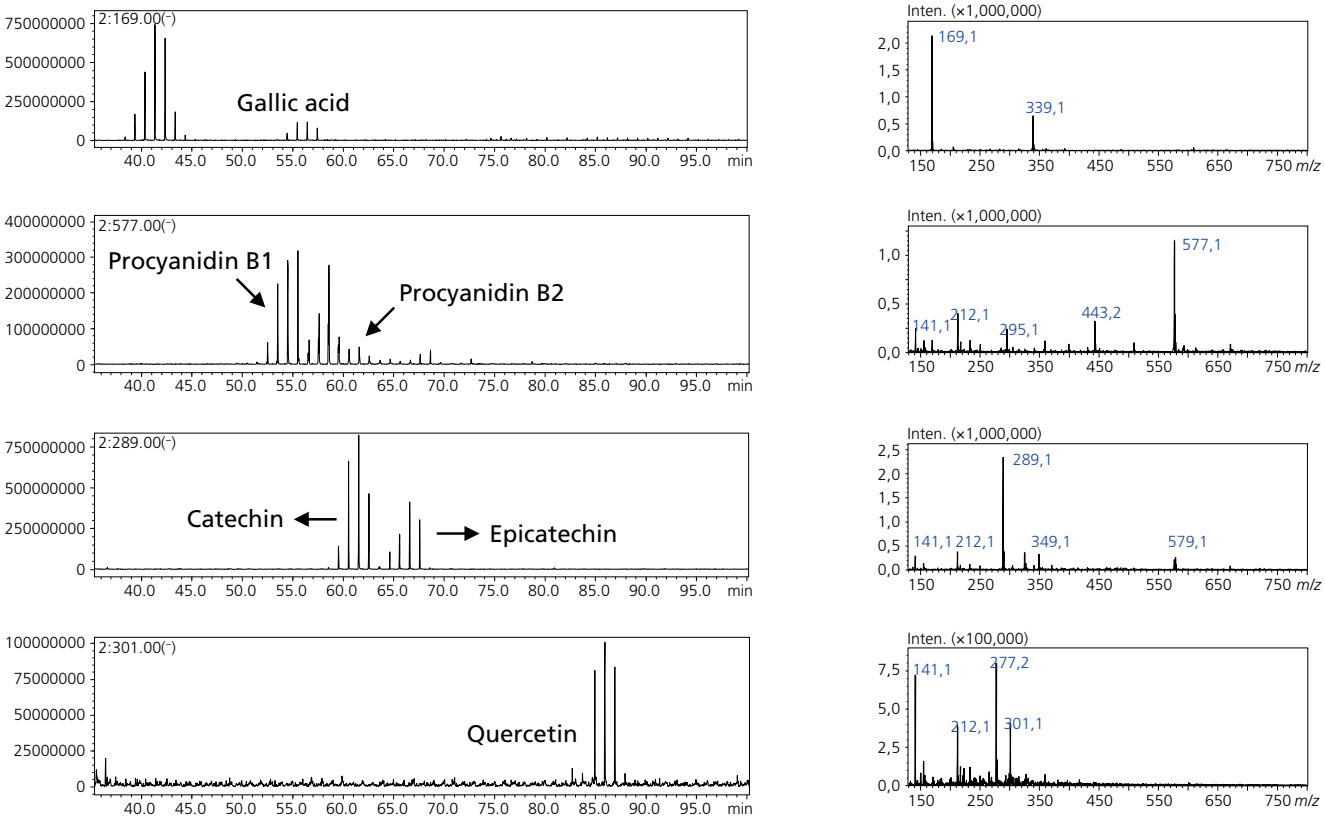

Fig. 6 Ion extracted chromatograms (on the left) along with relative mass spectra (on the right) of the main polyphenolic compounds identified in the red wine sample investigated

5. Conclusions

A comprehensive two-dimensional liquid chromatography system, based on the use of a micro cyano column and a partially porous (C_{18}) column in the first and second dimension, respectively, in combination with photodiode array and mass spectrometry detection, is presented.

Two second dimension gradient approaches, namely full in fraction and shifted were investigated and compared in terms of peak capacity.

The shifted gradient method used increased the effective peak-distribution area in the LC×LC analysis of a red wine sample.

Therefore, the use of a shifted gradient in an LC×LC system brings about a significant improvement in separation power and is a great advantage in the analysis of such complex samples.

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