Comprehensive two-dimensional liquid chromatography to quantify polyphenols in red wines

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ABSTRACT

A comprehensive two-dimensional liquid chromatography method has been applied for the quantification of polyphenols in red wines and compared to the most commonly employed conventional LC approach. Such methodology comprised the use of a microbore conventional HPLC column packed with totally porous particles in the first dimension and a partially porous column of conventional diameter in the second dimension. Even though a good number of applications in comprehensive LC have been reported, quantification experiments have been rarely described. To this regard, the advantages of comprehensive LC together with the employment of dedicated software capable of detecting and quantifying each peak from the 2D plot, have been taken into account for quantifying the most representative polyphenols in three different commercial Sicilian red wine samples. The optimized method has been validated in terms of linearity, sensitivity, detection and quantification limits. LODs as low as 0.02 ppm were obtained using the one-dimensional HPLC-DAD method, whereas values lower than 0.10 ppm were obtained by comprehensive LC. However, comprehensive LC allowed the quantification of a higher number of compounds with RSD lower than 10% thanks to its improved resolving power. The separation capabilities of comprehensive LC allowed the analysis of complex natural samples without any pre-treatment to effectively reduce the interferences coming from the matrix.

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1. Introduction

In recent years, comprehensive two-dimensional liquid chromatography has been exploited as a powerful analytical technique to study complex samples, particularly in food analysis. Comprehensive LC is characterized by a much greater resolving power compared to one-dimensional LC [1–11]. In a comprehensive system all fractions from the first column are continuously sampled and transferred to the second dimension column for further separation by means of a switching valve [12,13]. Many theoretical and practical aspects of such technique have been recently reviewed [14–20]. Peak capacity is, often, used as a measure of the separation power provided by a particular method. Ideally, the total peak capacity of a comprehensive two-dimensional technique, n_{2D}, is equal to the product of the peak capacities in the first (n_1) and in the second (n_2) dimensions in fully orthogonal 2D systems with non-correlated selectivity in the two dimensions [21,22]. Nevertheless, these conditions are not easily attained in practice, thus being of critical importance the careful selection of each separation mechanism in order to minimize the selectivity correlation in both dimensions [15]. Values of peak capacity up to 2100/h have been achieved in comprehensive LC, practically impossible to obtain by one-dimensional conventional HPLC [9].

Recently, we have demonstrated the possibility of using a microbore totally porous phenyl-silica column in the first dimension combined with a partially porous C_{18} (fused-core technology) column in the second dimension for the separation of polyphenolic antioxidants in wines [10].

Phenolic compounds are secondary metabolites synthesized by plants during normal development and in response to stress conditions [23]. They embrace a considerable range of substances possessing an aromatic ring bearing one or more hydroxy substituents. It has been demonstrated the beneficial, physiological and anti-carcinogenic properties of these compounds for the human health [24–27], which has given rise to an increasing demand in their analysis. Sometimes, the polyphenol content in real world

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samples can be so complex that they cannot be resolved in a one-dimensional HPLC analysis. In order to overcome this problem, different comprehensive RPLC systems comprised of columns of different selectivity in the two dimensions have been developed with the aim to separate such compounds in different real samples [7,8,10,28–30]. However, throughout in literature, only few papers employing a comprehensive LC system provide quantitative results for polyphenols [29,30] or other components [31–33].

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) [34]. To this regard, a challenging task is represented by the possibility of separating and quantifying these compounds in real samples. The advantages of comprehensive LC together with the employment of dedicated software capable of detecting and quantifying each peak from the 2D plot, can be taken into account for quantitative applications [33]. Therefore, the aim of the present work was to evaluate the potential of comprehensive two-dimensional liquid chromatography for the separation and quantification of phenolic antioxidants in red wines, and compare the prediction capability of both two-dimensional and the most commonly used one-dimensional approaches.

In this contribution, the benefits of comprehensive two-dimensional liquid chromatography compared to conventional HPLC are discussed in terms of linearity, sensitivity, detection and quantification limits and separation performance.

2. Experimental

2.1. Materials and reagents

Standards of phenolic compounds and flavonoids (namely, gallic acid, tyrosol, epicatechin, caffeic acid and rutin) were obtained from Sigma–Aldrich (St. Louis, MO, USA) with the exception of ethylgallate that was supplied by Extrasynthese (Genay, France).

Acetonitrile and water, HPLC grade, were from VWR International S.r.l. (Milan, Italy). Formic acid (HPLC grade) was purchased from Riedel-de Haën (Seelze, Germany). For preparation of calibration curves, stock solutions of each standard with concentration of 1000 mg/L were prepared by dissolving the mixture of pure standards in water/methanol (9:1, v/v). The following dilutions down to 0.78 ppm for one-dimensional analyses and 4.47 ppm for two-dimensional analyses were obtained by diluting each solution with pure water. The Sicilian Nero d'Avola wine samples were obtained by local producers and filtered prior injection through a 0.45 μm nylon membrane (Whatman, Clifton, USA).

2.2. Instruments and software

2.2.1. Conventional LC analyses

One-dimensional analyses were carried out using an Shimadzu HPLC system (Shimadzu, Milan, Italy), including a SCL-10A*vp* controller, two LC-10 AD*vp* pumps, a DGU-14A on-line degasser, a

SPD-M10A*vp* photodiode array detector (10 μL flow cell), a CTO-10A*vp* column oven and a SIL-10AD*vp* autosampler. Data were acquired and processed by using the LCsolution software (Version 1.21 SP1, Shimadzu).

2.2.2. Comprehensive LC analyses

Comprehensive LC analyses were performed on a system earlier described [10]. For data acquisition and method development an LCSolution Software (Version 1.22, Shimadzu), developed by Shimadzu for comprehensive LC instrumentation, has been employed. Integration and thus quantitation of peaks has been performed by using Chrom^{square} Ver. 1.0 software (Chromaleont, Messina, Italy).

2.3. Methods

2.3.1. LC analyses of standard antioxidants and wine samples

One-dimensional analyses were carried out on a conventional octadecyl-silica column (Discovery HS C18, 150 × 2.1 mm; 3 μm, Supelco, Bellefonte, PA, USA). As mobile phases, water (A) and acetonitrile (B), both at pH 3, were used in the following linear gradient conditions: 0 min, 5% B; 20 min, 5% B; 50 min, 40% B; 55 min, 95% B; and 60 min, 95% B. The pH of the mobile phase was adjusted to the appropriate value by adding formic acid. An HPLC oven was used to maintain the column temperature at 30 °C, flow rate was 0.2 mL/min and the 5 μL injections were made by means of an autosampler. The photodiode array detector was operated at 12.5 Hz in the 190–400 nm range.

2.3.2. Comprehensive LC analyses of antioxidant standards and wine samples

Comprehensive LC analyses have been performed under the same experimental conditions reported in our previous work [10]

3. Results and discussion

3.1. Method validation

All phenolic compounds are characterized by a strong chromophore system that allows their UV–Vis detection at detection limits in the low ppm level [35,36], even if they vary for the individual compounds, as a result of the different molar absorptivities.

Calibration curves for six standard compounds were established in the concentration ranges indicated in Tables 1 and 2. These ranges were selected taking into account the normal levels of the compounds in wine, in order to carry out accurate predictions. The curves were constructed using five concentration levels, each one run in triplicate. The responses (peak area *versus* nominal concentration fitted well to the straight line with correlation coefficients values higher than 0.9991 in one-dimensional analyses and 0.9895 in two-dimensional analyses. Parameters of linear regression together with experimental retention time windows ($t_R \pm$ %RSD), LOD and LOQ for the six standards analyzed by RP-LC-DAD and comprehensive RP-LC-DAD, are summarized in Tables 1 and 2, respectively. For quantification, the peak areas were measured in the chromatograms obtained at the wavelengths of

Table 1
Parameters of the linear regression and experimental retention times (t_R)[a], LOD, LOQ for the studied compounds by RP-HPLC-DAD.

Trivial name	Compound type	UV (nm)	t_R (min) ± RSD (%)	Regression equation	R^2	LOD (μg/mL)	LOQ (μg/mL)	Concentration range (μg/mL)
Gallic acid	Benzoic acid-like	270	9.36 ± 1.21	$y = 77099x − 10302$	0.9993	0.03	0.11	0.78–100
Ethylgallate	Benzoic acid ethyl ester-like	270	36.71 ± 0.26	$y = 149614x + 101394$	0.9994	0.02	0.05	0.78–100
Tyrosol	Phenyl-ethyl alcohol-like	278	28.60 ± 0.29	$y = 16150x + 15453$	0.9991	0.13	0.45	0.78–100
Epicatechin	Flavan-3-ol-like	278	34.72 ± 0.18	$y = 20025x + 6789$	0.9991	0.16	0.52	0.78–100
Caffeic acid	Cinnamic acid-like	323	32.66 ± 0.24	$y = 135327x + 72215$	0.9995	0.02	0.05	0.78–100
Rutin	Flavonol-glycoside-like	354	38.58 ± 0.14	$y = 44317x + 36978$	0.9996	0.03	0.10	0.78–100

[a] The retention times (t_R) are the mean of twenty-four replicates.

Table 2
Parameters of the linear regression and experimental retention times (t_R)[a], LOD and LOQ for the studied compounds by RPxRP-HPLC-DAD.

Trivial name	Compound type	UV (nm)	t_R (min) ± RSD (%)	Equation	R^2	LOD (µg/mL)	LOQ (µg/mL)	Concentration range (µg/mL)
Gallic acid	Benzoic acid-like	270	0.25 ± 4.51	$y = 203438x - 669334$	0.9988	0.09	0.30	4.375–70
Ethylgallate	Benzoic acid ethyl ester-like	270	0.89 ± 2.47	$y = 294735x - 337701$	0.9998	0.25	0.85	4.375–50
Tyrosol	Phenyl-ethyl alcohol-like	278	0.64 ± 1.12	$y = 50848x - 35086$	0.9999	0.95	3.17	6.25–50
Epicatechin	Flavan-3-ol-like	278	0.67 ± 0.96	$y = 60965x - 259742$	0.9895	0.79	2.63	6.25–50
Caffeic acid	Cinnamic acid-like	323	0.72 ± 1.27	$y = 594002x - 1350198$	0.9900	0.10	0.33	4.375–50
Rutin	Flavonol-glycoside-like	354	0.99 ± 1.80	$y = 77958x - 172259$	0.9930	0.30	1.01	4.375–50

[a] The retention times (t_R) are the mean of twenty-four replicates corresponding to the second dimension.

maximum absorption for each compound quantified. To carry out this task, the LCsolution software was employed for the mono-dimensional analyses whereas the Chrom^{square} Ver. 1.0 software was used for two-dimensional analyses.

The RSD values for the retention times (mean of 24 replicates) were always lower than 1.3% for conventional LC and 4.51% for comprehensive LC (considering retention times in the second dimension for the richest slice of each standard) confirming the good repeatability of both methods for the determination of polyphenols.

Moreover, limits of detection (LOD) and quantification (LOQ) were determined by using the untransformed comprehensive LC chromatogram at signal-to-noise ratio (S/N) levels of 3 and 10, respectively. In this concern, it has to be taken into account that whereas in conventional LC the LOD and LOQ calculations are straightforward, in comprehensive LC each constituent in the sample must be eluted in more than one fraction (at least three on average) in the second dimension [37]. This phenomenon increases the LOD and LOQ values, given that this small amount is divided in several second dimension peaks. Even though this factor is purely related to experimental conditions, to find more precise LOD and LOQ calculations, it has been considered that at the concentration levels used (Table 2), the compound is eluted in a single slice, that is, all the compound is formed only by a second dimension peak. LOD and LOQ values ranged from 0.02 to 0.16 ppm and from 0.05 to 0.52 ppm, respectively, in conventional LC. On the other hand, in Comprehensive LC, LOD values ranged from 0.09 to 0.95 ppm and from 0.30 to 3.17 ppm for LOQ. These values completely agreed with those reported in the literature [30]. The differences appreciated between these two techniques are due to the sample dilution produced in the second dimension of the comprehensive LC separation because of the high flow rates employed.

Once the analytical conditions were successfully tested with standard compounds and the calibration curves correctly calculated, the present methods were used for the polyphenols quantification in wine samples both by conventional and comprehensive LC.

3.2. Quantification of polyphenols in wines. Comparison between LC and LC × LC

In Fig. 1, the chromatograms obtained for the analysis of polyphenols from a red wine sample without any pre-treatment using conventional LC are shown at 278 nm (Fig. 1A) and 354 nm (Fig. 1B), being these two wavelengths the extreme values employed for the quantification, so that it is possible to appreciate the different groups of compounds separated. Using this LC method, it was possible to identify 13 phenolic compounds by combining the information coming from UV–Vis spectra, chromatographic retention and the data found in the literature. However, it is possible to see how the chromatogram is characterized by a "mountain" formed by unresolved interferences generated from the matrix. This behaviour was previously described in the literature [38]. Therefore, the quantification of these samples by using this methodology could be difficult or inaccurate. Nevertheless, this method was

employed to analyze three different wine samples, whose profile was very similar to that shown in Fig. 1. The quantitative data generated from these analyses at least in triplicate using the calibration curves previously developed are shown in Table 3. As it can be observed, the compounds found in higher amount in all wines were gallic acid followed by a procyanidin, epicatechin and catechin. These results are in good agreement with those reported in literature for red wines [38–43], although the precise contribution of each phenolic compound will depend in great extent of different factors such as the grape variety or the geographical origin. Some other minor polyphenolics could be also quantified.

In order to improve the separation as well to reduce the interferences from the matrix that hamper quantitation data, a comprehensive LC method previously developed at our lab [10] to separate and identify polyphenolic compounds from wines, was tested in this contribution. In Fig. 2, the two-dimensional plots corresponding to the separation of the same wine sample of Fig. 1 are shown, at 278 nm (Fig. 2A) and 354 nm (Fig. 2B). As it can be clearly appreciated from this figure, compounds were well separated although a low degree of orthogonality considering that two RP separations are coupled. To carry out the peaks quantification a dedicated software [33] was employed. The results obtained from the quantitative analysis of the three wine samples using the previously developed calibration curves are also shown in Table 3.

As it can be seen from the comparison of these figures, at first sight some clear advantages related to the use of comprehensive LC can be found. Among them, it is possible to see how compounds 9 (rutin) and 10 (isoquercitrin) could be now perfectly separated. Besides, it is possible to see how the interferences produced by the matrix are significantly reduced, allowing the more accurate
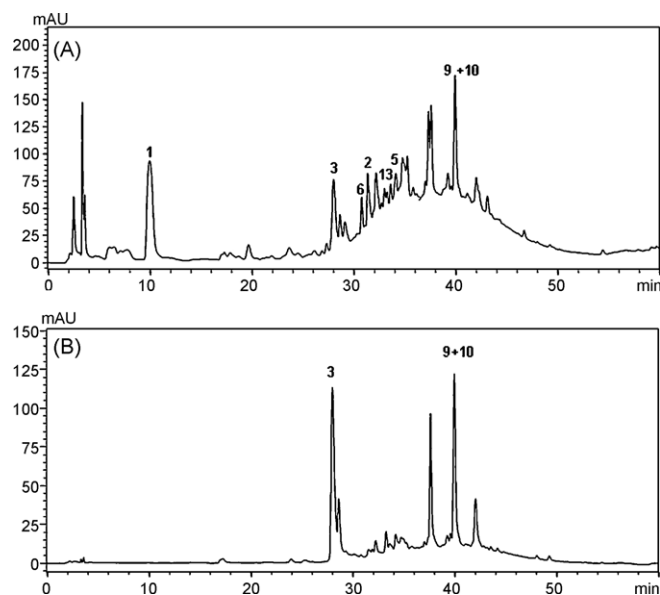

Fig. 1. Conventional LC chromatograms at 278 nm (A) and 354 nm (B) of polyphenols of wine 3. For peak identification see Table 3.

Table 3
Quantification of polyphenolic compounds by one-dimensional and comprehensive LC.

ID	Compounds	Chemical class	Wine 1		Wine 2		Wine 3	
			1D (μg/mL)	2D (μg/mL)	1D (μg/mL)	2D (μg/mL)	1D (μg/mL)	2D (μg/mL)
1	Gallic acid	Benzoic acid-like	67.8 ± 2.2	67.0 ± 1.6	41.1 ± 0.5	40.0 ± 4.3	45.9 ± 0.1	46.0 ± 4.0
2	Procyanidin	Flavan-3-ol-like	–	38.4 ± 3.3	26.5 ± 0.7	27.3 ± 2.9	34.8 ± 0.4	36.1 ± 1.5
3	Caftaric acid	Cinnamic acid-like	–	19.2 ± 0.9	16.7 ± 0.3	18.2 ± 1.7	–	30.3 ± 1.9
4	Tyrosol	Phenyl-ethyl alcohol-like	–	28.8 ± 0.1	–	26.0 ± 4.0	–	24.8 ± 2.1
5	Epicatechin	Flavan-3-ol-like	28.4 ± 0.1	28.3 ± 4.1	23.0 ± 0.2	23.2 ± 3.5	18.9 ± 0.3	19.6 ± 2.6
6	Catechin	Flavan-3-ol-like	22.0 ± 0.4	21.3 ± 2.6	18.4 ± 0.2	17.4 ± 1.1	23.8 ± 1.4	21.0 ± 0.3
7	Caffeic acid	Cinnamic acid-like	–	16.0 ± 1.6	–	5.1 ± 0.6	–	5.9 ± 0.5
8	Ethylgallate	Benzoic acid ethyl ester-like	–	13.2 ± 1.1	–	12.6 ± 0.2	–	12.6 ± 1.4
9	Rutin	Flavonol-glycoside-like	–	5.4 ± 0.6	–	7.6 ± 1.3	–	–
10	Isoquercitrin	Flavonol-glycoside-like	–	9.9 ± 1.2	–	11.8 ± 1.8	–	14.2 ± 1.5
11	p-Coumaric acid	Cinnamic acid-like	–	6.9 ± 0.1	–	4.2 ± 0.2	–	4.9 ± 0.3
12	Syringic acid	Flavan-3-ol-like	3.2 ± 0.2	–	–	–	–	–
13	Syringaldehyde	Flavan-3-ol-like	0.5 ± 0.2	–	–	–	2.39 ± 0.6	–

quantification of the isolated peaks. Moreover, it can be also noted that in addition to the improved separation power of this technique compared to the one-dimensional, comprehensive LC provides an increased identification power. This property is based on the possibility to correlate the position of each compound in the 2D plot as function of the retention times of the two dimensions (and not only a retention time, as in conventional LC), and the possibility to obtain clearer spectra due to the better separation between compounds from the matrix interferences.

From the results presented in Table 3, it can be observed that a higher number of compounds could be correctly identified and quantified in comprehensive LC. Each compound was quantified with the previously constructed calibration curves corresponding to the standard, if available, or to the most similar compound included. In general, good agreement was found among the data obtained using the two techniques. Besides, several compounds like caftaric acid, tyrosol, p-coumaric acid and ethylgallate were identified and quantified using comprehensive LC that were not correctly separated in conventional LC. The quantification of other phenols, like syringaldehyde or syringic acid was more favourable by conventional LC. This situation was due to fact the amount of these

compounds was very close to the quantification limits. As it was mentioned before, the use of very high flow rates in the second dimension in the comprehensive LC set-up produces a very well known dilution effect. This effect limits in some extent the sensitivity of this technique, when used in the configuration used in this work, avoiding to reach detection and quantification limits as low as in conventional LC.

To further demonstrate the practical applicability and the quantification performance of the employed comprehensive LC methodology, a statistical comparison of the concentrations calculated by both methodologies was carried out for the compounds properly separated and quantified by both of them. Prior to the means comparison of the two sets of data, it is necessary to test whether the difference between the variances of both sets is significant. With this aim, in a first stage, the variances of the predictions made by both techniques were compared by means of the "*F* test". Statistically similar variances were not found in all cases. Then, mean values of concentration obtained by one- and two-dimensional methodologies were compared. Thus, the *t*-test was applied when the variances resulted similar and a slightly modified method was applied when the variances were statistically distinguishable, as in these cases it was not possible to calculate a pooled standard deviation. All comparison tests were carried out at a 95% confidence level. No statistical differences were found in any case between the predictions made by the one- and two-dimensional approaches.

Thus, in this work, the ability of comprehensive LC to effectively separate and quantify phenolic compounds from wines has been correctly validated and compared to conventional LC for the first time.

4. Conclusions

In the present contribution, the possibility of carrying out quantitative studies from polyphenolic compounds from wines has been demonstrated for the first time, in comparison with the most extensively used conventional LC. A good accordance between quantitative results in conventional and comprehensive LC was attained. Moreover, a high degree of separation could be obtained by comprehensive LC making it possible to quantify some peaks coeluted in one-dimensional LC. Besides, although the sensitivity of comprehensive LC was lower, due to the dilution obtained caused by the high second dimension flow rates employed, the higher resolution power of the comprehensive LC system allowed to obtain clearer UV spectra from the matrix interferences to be used for peak identification. The methodology could be used to study the polyphenolic quantitative pattern in other food related products other than wine.
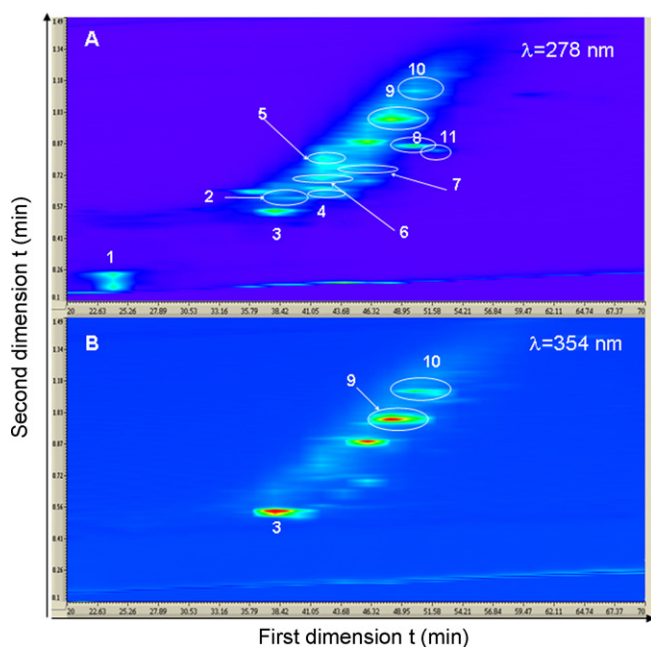

Fig. 2. Two-dimensional plots of wine 3 at 278 nm (A) and 354 nm (B). For identification see Table 3.

Acknowledgements

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