Determination of Some Phenolic Compounds in Red Wine by RP-HPLC: Method Development and Validation

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Abstract

A methodology employing reversed-phase high-performance liquid chromatography (RP-HPLC) was developed and validated for simultaneous determination of five phenolic compounds in red wine. The chromatographic separation was carried out in a C_{18} column with water acidify with acetic acid (pH 2.6) (solvent A) and 20% solvent A and 80% acetonitrile (solvent B) as the mobile phase. The validation parameters included: selectivity, linearity, range, limits of detection and quantitation, precision and accuracy, using an internal standard. All calibration curves were linear (R^2 > 0.999) within the range, and good precision (RSD < 2.6%) and recovery (80–120%) was obtained for all compounds. This method was applied to quantify phenolics in red wine samples from Santa Catarina State, Brazil, and good separation peaks for phenolic compounds in these wines were observed.

Introduction

Phenolic compounds constitute the most important quality parameters of wine since they contribute to their organoleptic characteristics, particularly color, astringency, and bitterness (1–4). Wine phenolics belong to two main groups of compounds: non-flavonoid and flavonoid. The former group, also called phenolic acids, notably includes hydroxycinnamic acids (e.g., caffeic acid, coumaric acid, and ferulic acid) and the latter group includes anthocyanins, flavonol (quercetin) and flavanol (catechin) (4,5). The types and concentrations of the phenolic compounds in wine have been shown to be influenced mainly by the grape variety, different viticultural practices and enological techniques (6,7). According to several epidemiological studies, phenolic compounds have a positive effect on human health since they decrease the incidence of coronary heart disease, reduce platelet aggregation (8), have antioxidant capacity in vivo (9) and in vitro (10,11) and provide anti-carcinogenic protection (12,13).

High-performance liquid chromatography (HPLC) is a commonly used analytical separation technique that combines high resolution and easy automation with modest sample requirements. Some phenolic compounds found in wine show characteristic absorbances in the UV–vis region, and their chromatograms at 280 nm are widely used to study phenolic compounds because absorption at this wavelength is suitable for the detection of a large number of such compounds, which can easily be detected by HPLC because of its versatility and precision (14). Most frequently, reversed-phase C_{18} columns, a binary solvent system containing acidified water and a polar organic solvent (acetonitrile or methanol) and detection in the UV–vis region are used, and this constitutes a crucial and reliable tool in the analysis of phenolic compounds (15).

Due to the large number, and structural variations, of closely-related food phenolic compounds, analytical procedures for the analysis of individual phenolic compounds are complicated and have been relatively difficult to obtain. Thus, the complexity of the phenolic composition of wine has led to the development of several methods employing high-performance liquid chromatography, in order to determine different groups of phenolics with specific chemical properties in a single chromatographic step, since HPLC can detect different classes of phenolic compounds in a single analysis. Most studies have reported the identification and determination of phenolic compounds in wine using liquid chromatography (4,5,16–19). However, there is a need for a method allowing simultaneous detection of the some main phenolic compounds presents in wine in a single analysis, in order to decrease the analysis time required.

The aim of this study was to develop and validate a method for the identification and quantitation of the phenolic compounds in red wine pertaining two classes, using high-performance liquid chromatography with UV–vis detection. This method was optimized and carefully validated by evaluating the selectivity, linearity, range, accuracy, precision, and limits of detection and quantitation.

Materials and Method

Chemicals and phenolic standard solutions

HPLC-grade acetonitrile and ethanol were supplied by Merck (Darmstadt, Germany), acetic acid, tartaric acid and hydrochloric acid were supplied from Sigma-Aldrich (St. Louis, MO). The water used in the analysis was obtained from a Milli-Q water purification system manufactured by Millipore (Bedford, MA). All solvents used as the mobile phase were previously filtered through 0.45-µm membranes (Millipore) and degassed prior to use.

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Standards of (+)-catechin, *p*-coumaric acid and morin (2',3,4',5,7-penthydroxyflavone) were purchased from Sigma-Aldrich (St. Louis, MO), and quercetin, ferulic acid, and caffeic acid were obtained from Fluka (Steinheim, Germany). All the standars were of purity > 95 %.

An individual stock solution of each standard (1000 mg/L) was prepared, under a N_2 stream, by dissolving the analyte in methanol and storing it in the dark at –18°C. A stock solution was prepared by mixing all standard solutions (150 mg/L) dissolved in synthetic wine (hydroalcoholic solution of 5 g/L tartaric acid, 12% v/v of ethanol, and pH 3.2) (20). The synthetic wine was used in order to avoid interference in the chromatographic separation and in the detection response. The working and calibration solutions were also prepared in synthetic wine by diluting the stock solution with mixed standards in the concentration range of 0.3–150 mg/L. The morin solution was prepared in synthetic wine (16.2 mg/L) and was used as the internal standard. Oxygen was eliminated from all solutions with a nitrogen stream to avoid decomposition of these compounds, and the solutions were stored in the dark at 4°C.

Sample

For the validation of the methodology a sample of commercial red wine from State Santa Catarina, Brazil, was used. For the evaluation of this analytical method, samples of the Cabernet Sauvignon, Syrah and Touriga National wines from Brazil, vintage 2008, were used. The wines were filtered through a 0.45-µm PTFE membrane filter modified with 13 mm of diameter (Millipore) and directly injected into the HPLC.

HPLC analysis

Chromatographic analysis was performed using a Shimadzu (Kyoto, Japan) liquid chromatograph, equipped with a vacuum degasser (DGU-14A), quaternary pump LC-10AT, UV–vis detector (SPD-10AV) and an injector (Rheodyne) with a 20 µL loop. The CLASS-VP software (v. 6.1) was used to control the gradient settings, UV–vis and data acquisition. A C18 reversed-phase column (4.6 mm × 250 mm, 5 µm particle size) (Hichrom, Europe) was used. Preceding the analytical column was a C18 guard column (4.6 mm × 12.5 mm, 5 µm particle size) (Hichrom, Europe), used to prevent any non-soluble residues from the samples from contaminating the column. Peak areas were determined at 280 nm for all phenolic compounds. The ambient temperature was controlled and maintained in 20°C ±1.

Determination of solvent composition, gradient elution, and flow rate

The mobile phase consisted of acetic acid in filtered Milli-Q water adjusted to pH 2.6 as solvent A and 20% of solution A in 80% acetonitrile as solvent B (21).

To determine the gradient conditions, the initial and final compositions of solvents A and B were determined using exploratory gradient elution. Firstly, solvent B was increased linearly from 0% to 100% with a flow rate of 1.2 mL/min in run times of 45 and 60 min. Using this condition 20 µL of the wine sample was injected, and the retention time (t_R) of the first and last peaks in the chromatographic runs, in the two times analyzed, were observed and the values obtained were used to calculate the initial and final percentages of solvent B. The same procedure was carried out with solvent A to determine the best gradient for elution of the compounds. A series of gradients were tested to obtain the best separation of the chromatographic peaks.

Three flow rates were tested (0.8, 1.2, and 1.5 mL/min) with different proportions and run times, for solvents A and B, to determine the best solvent flow rate. Verification was carried out with the injection at 20 µL of the wine sample, to determine the effect on the chromatographic peak resolution.

The identification of the compounds was carried out by separate injection of each standard solution and also with the injection of the stock solution containing all standards. Thus, for each compound the resolution peak and the run time were determined. When all standards were eluted with good resolution, a chromatographic run was performed with the wine sample spiked with the standard solution, to verify the correct identification of all compounds, as also the elution order.

HPLC method validation

After the RP-HPLC gradient conditions were determined, validation tests were performed for selectivity, linearity, range, accuracy, precision, and limits of detection and quantitation (22).

Determination of selectivity

The selectivity of the method was assessed by comparing the chromatograms of the synthetic wine to those of the synthetic wine spiked with the standard solution.

Determination of linearity and range

An intermediate mixed standard solution was prepared by dilution of the stock standard solution with synthetic wine to give a concentration of 150 mg/L for all compounds. The calibration working solutions for each compound were prepared in triplicate and evaluated by injecting 20 µL. A calibration curve for each compound was constructed separately by plotting peak area (y-axis) versus concentration (x-axis). All curves were construction using the internal standard method. The calibration curve was fitted by linear least-squares regression and the value obtained for the correlation coefficient indicated that the method is linear in the range of concentrations studied.

Determination of limits of detection and quantitation

Limits of detection and quantitation (LOD and LOQ, respectively) were calculated based on the standard deviation of the response of the blank and the slope of the calibration curve for each compound. The blank (synthetic wine) was injected 7 times consecutively. The limits were expressed according Equation 1 and 2:

$$LOD = 3.3 \times s/S \quad \text{Eq. 1}$$

$$LOQ = 10 \times s/S \quad \text{Eq. 2}$$

where, s is the standard deviation of the response, and S is the slope of the calibration curve.

Determination of accuracy

The accuracy was determined through the percent recovery with addition of the standard solution to the red wine sample and

synthetic wine sample, at three different concentrations: 1.0, 15.0, and 30.0 mg/L. The analyses were carried out in triplicate, total of 9 injections for each sample.

Determination of precision

The precision was evaluated through the intermediate precision method and within-day repeatability, for which 7 injections of the red wine sample were carried out, and expressed as relative standard deviation (RSD).

Identification and quantitation of phenolic compounds in red wine

Phenolic compounds in Cabernet Sauvignon, Syrah and Touriga Nacional wines from Santa Catarina State, Brazil, were identified through comparison of their retention times and UV–vis spectra with those obtained by injection of the standard solution under the same conditions. Peak area was used for quantitation purposes, using internal standard calibration. The wines were injected at a volume of 20 µL, in triplicate. Values were reported as mg/L.

Statistical analysis

The Statistica v. 6.0 (2001) (StatSoft Inc., Tulsa, OK) program was used for the analysis of variance (ANOVA) of the results obtained, and for the slopes and intercepts of the calibration graphs calculated by least squares regressions and evaluated by the coefficient of determination (R^2) and RSD.

Results and Discussion

Optimization of the chromatographic method

The development of a single liquid gradient for the analysis of several phenolic compounds would represent a useful and quick solution for the study of the presence of the main polyphenols of wine, focusing on their characterization. Table I shows the optimum instrumental parameter values for the chromatographic determination of phenolic compounds in wine. For the optimization of the chromatographic conditions different mobile phases, gradient elution programs, and solvent flow rates were tested.

The mobile phase composition was prepared according to Villaño et al. (21), however, in order to obtain the best separation efficiency two organic solvents were tested: methanol and acetonitrile, as well as the acetic and phosphoric acids used to decrease the pH. Combinations of methanol:water and acetonitrile–water were tested and it was observed that the use of acetonitrile led to the best resolution and separation of the chromatographic peaks, in a shorter run time. With regard to the acids, acetic acid showed the best efficiency separation for the compounds. Based on the results, the optimum solvent combination was acetonitrile–water, acidified with acetic acid (pH 2.6).

Through the injection of the standard solution containing all standards, the total chromatographic run time was determined. Times of 45 and 60 min were tested and it was observed that 60 min was required for all phenolic compounds of interest to appear in the chromatogram. The elution gradient was also optimized to obtain the best resolution of the peaks. Different proportions of the solvents A and B were tested with a run time of 60 min, using 2 and 3 segments, and it was observed that the use of 3 segments is necessary for the gradient elution, to avoid co-elution between the compounds. The first gradient step (0 to 30% in acetonitrile) allowed the elution of catechin and hydroxycinnamic acid (caffeic, coumaric, and ferulic acid). The second gradient was prepared for elution of the morin (SI) and quercetin and both were eluted in the third gradient step, when 100% of acetonitrile reached. All compounds were eluted for 45 min, and the last 15 min was used to recondition the column in preparation for a new chromatographic run (conditioning step). With regard to the phenolic classes analyzed in this method, the elution order was flavanol, followed by hydroxycinnamic acid, and finally the flavonol classes. Figure 1 shows the separation of the 5 phenolic compounds and the internal standard used to identify and quantify phenolic compounds in wine. It was thus verified that this new method is appropriate for the determination of the above-mentioned compounds with distinct peaks and good resolution.

Table I. Chromatographic Conditions for the Determination of Phenolic Compounds in Red Wine

	Chromatographic conditions
Injection volume	20 µL
Guard column	C18 guard column, 4.6 × 12.5 mm, 5 µm particle size
Analytical column	C18 reverse-phase column, 4.6 × 250 mm, 5 µm particle size
Mobile phase	A (acetic acid in water, pH 2.65) B (20 % solution A and 80 % acetonitrile) First segment: 0.01–35 min: 0–30% B Second segment: 35–40 min: 30–50% B Third segment: 40–45 min: 50–100% B Conditioning step: 45–60 min: 100–0% B
Flow rate	1.2 mL/min
Temperature room	20°C ± 1

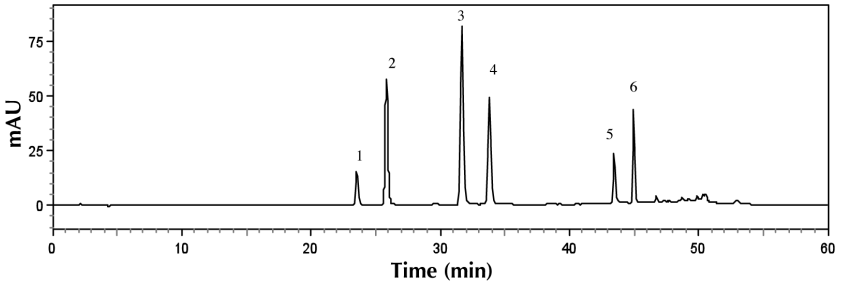

Figure 1. Chromatogram of standard solution containing a mixture of polyphenolic compounds in synthetic wine using optimized chromatographic conditions: 1, catechin; 2, caffeic acid; 3, *p*-coumaric acid; 4, ferulic acid; 5, morin; and 6, quercetin.

HPLC method validation

Determination of selectivity

The selectivity of this method was evaluated considering the peak shape, retention time and chromatographic purity of the spectrum, in order to detect possible interference. No interfering peaks were observed in the blank (synthetic wine) chromatogram at the specific retention time for each compound and in all cases the purity of the peaks, compared with the pure standard solution and wine sample, was higher than 99%.

Determination of linearity and range

The calibration curves were constructed with 6 points, in triplicate, for each phenolic compound, using the internal standard method (morin solution, 16.2 mg/L). The results of the regression analysis and calibration range are shown in Table II. Calibration curves were linear over the concentration range studied. It can be observed that all compounds had a coefficient of determination (R^2) > 0.999, indicating an excellent fit of the phenolic compounds to the model within the range studied (23).

Determination of limits of detection and quantitation

The limits of detection (LOD) and quantitation (LOQ) are shown in Table II. LOD ranged from 0.02 to 0.08 mg/L, while LOQ varied from 0.06 to 0.15 mg/L, and it can be observed that the LOD values were 3 times lower than the LOQ values. These values are within the range obtained by other researches for the determination of phenolics in wine (20,19,24), which verifies that the proposed method is sensitive enough to determine these compounds in red wine.

Determination of accuracy

The accuracy of the method was evaluated by spiking synthetic wine (solution used for calibration curves) and real red wine samples with the standard solution containing the phenolic compounds in known amounts (1.0, 15.0, and 30.0 mg/L) within the calibration range, and obtaining the recovery (%) (Table III). The recovery (%) was calculated using an internal standard. In the synthetic wine, the recovery (%) was calculated as [(concentration found / spiked polyphenol concentration) × 100]; and the recovery (%) for real sample was calculated as [(concentration of the polyphenol found in the sample after spiking minus the concentration of the polyphenol naturally found in the sample) / spiked polyphenol concentration) × 100]. The recovery of phenolic compounds ranged from 86% to 118% for the synthetic wine and 81 to 119% for the red wine, which is in agreement with results reported by other researches for the quantitation of these compounds (18,23,25). These values show that the polyphenol standards in the synthetic wine behaved in a similar manner those in the red wine, which showed a range of recovery between 80% to 120%, indicating that this method is acceptable for the quantitation of these compounds.

Table II. Compound Identification, Regression Equation Including the Coefficient of Determination (R^2) and Range of Calibration (n = 6), LOD, and LOQ

Compound (Name)	Regression equation	R^2	Calibration range*	LOD*	LOQ*
1 (Catechin)	$y = 0.7488x + 0.0502$	0.9992	0.3–150	0.04	0.12
2 (Caffeic acid)	$y = 3.3608x + 0.2287$	0.9991	0.3–75	0.02	0.06
3 (*p*-Coumaric acid)	$y = 4.9013x + 0.3672$	0.9998	0.3–30	0.08	0.24
4 (Ferulic acid)	$y = 2.9768x + 0.2145$	0.9998	0.3–30	0.04	0.13
5 (Quercetin)	$y = 1.5904x + 0.0477$	0.9991	0.3–75	0.05	0.15

* expressed in mg/L

Table III. Determination of the Method Accuracy Expressed as Recovery (%) from the Synthetic Wine and Real Red Wine Samples with 3 Different Concentrations of the Standard Solution

	Synthetic wine†			Real red wine†		
Compounds*	1.0 mg/L	15 mg/L	30.0 mg/L	1.0 mg/L	15 mg/L	30.0 mg/L
1	108.94 ± 10.93	88.20 ± 0.33	97.54 ± 0,82	93.09 ± 2.44	119.88 ± 1.26	95.76 ± 1.06
2	117.61 ± 0.03	104.51 ± 0.53	96.63 ± 0,62	98.88 ± 2.30	118.35 ± 2.42	109.29 ± 0.97
3	118.22 ± 0.54	110.84 ± 0.39	96.17 ± 0.21	108.11 ± 2.02	117.61 ± 4.06	93.93 ± 0.90
4	116.22 ± 0.89	106.87 ± 0.36	96.56 ± 0.44	119.20 ± 2.30	115.78 ± 1.18	100.19 ± 1.03
5	100.15 ± 0.07	86.70 ± 0.59	101.60 ± 0.35	93.15 ± 2.10	84.17 ± 2.09	81.34 ± 0.72

* Compounds identified: 1, catechin; 2, caffeic acid; 3, p-coumaric acid; 4, ferulic acid; 5, quercetin.
† Results: recovery (%) ± RSD (%)

Table IV. Precision of the Method According to Retention Time (t_R) and Average Concentration of the Phenolic Compounds (mg/L) in Red Wine Sample

Compounds*	t_R min. (SD)	RSD %	Average Conc. (SD)	RSD %
1	23.20 (0.13)	0.59	30.1 (0.41)	1.31
2	25.59 (0.10)	0.43	10.2 (0.2)	1.70
3	31.52 (0.15)	0.50	4.32 (0.09)	2.54
4	34.74 (0.26)	0.79	0.39 (0.008)	2.68
5	44.99 (0.12)	0.26	7.09 (0.18)	2.59

* 1, catechin; 2, caffeic acid; 3, p-coumaric acid; 4, ferulic acid; 5, quercetin.

Table V. Mean Concentration (mg/L) for Phenolic Compounds in Commercial Red Wines*

Compounds	Cabernet Sauvignon	Syrah	Touriga Nacional
Catechin	50.42 ± 0.62[a]	31.27 ± 0.40[b]	22.34 ± 0.29[c]
Caffeic acid	7.16 ± 0.38[a]	11.76 ± 0.20[b]	19.88 ± 0.81[c]
p-Coumaric acid	1.50 ± 0.05[a]	3.51 ± 0.09[b]	5.08 ± 0.12[c]
Ferulic acid	0.11 ± 0.02[a]	0.31 ± 0.01[b]	1.99 ± 0.06[c]
Quercetin	15.35 ± 0.96[a]	6.95 ± 0.18[b]	9.06 ± 0.21[c]

* Average ± standard deviation. The analyses were carried out in triplicate. Different letters on the same line represent a significant difference ($p < 0.05$) between the samples.

Determination of precision

The precision of this methodology was based on the retention time and the average polyphenol concentration, expressed as relative standard deviation (RSD%) (Table IV). The values obtained in this study, that is, 0.43% to 0.79% and 1.31% to 2.68% for retention time and average concentration, respectively, were considered to be sufficiently low to quantify phenolic compounds in wine, given that it is considered to be a matrix of high complexity. These ranges of RSD values are in agreement with those of other studies which validated the analytical methodology for simultaneous detection of macro-elements (20,22,26,27).

Method application and quantitation of phenolic compounds in red wine

The proposed method was applied to the determination of the phenolic compounds (flavonoids and phenolic acids) in commercial samples of Brazilian red wines: Cabernet Sauvignon, Syrah, and Touriga Nacional (Table V). The three varieties were significantly different ($p < 0.05$) in terms of the composition of the compounds determined. Catechin was the predominant phenolic in both wines, which is an excellent quality for these Brazilian wines since according to De Quirós et al. (19) among the phenolics present in wine, catechin has an important antioxidant activity. The Cabernet Sauvignon wine contained a higher content of flavonoids (catechin and quercetin) than Syrah and Ttouriga Nacional. Overall, the results for the phenolic compounds in these Brazilian wines quantified by RP-HPLC are similar to, and often higher than, those found by other researchers which could be due to the different geographical origins of the wine, since the content of these compounds depends on the climate and temperature (4,28,29).

Conclusion

A methodology for the simultaneous determination of 5 representative phenolic compounds in red wine by RP-HPLC was developed and validated in this study. The proposed method offers the following advantages: the sample can be directly injected without time-consuming sample preparation or previous fractionation; and it exhibits excellent precision, accuracy and detection limits, with good recoveries (80–120%), for the determination of phenolics in wine. The chromatographic method yielded a very satisfactory separation of the standards and under the same conditions the separation of phenolics in real samples. Thus, this procedure can be used to determine the phenolic compounds in various types of wine, as well as to characterize and differentiate wine samples. Finally, the results indicate that this method could be used for application in quality control and industrial laboratories interested in obtaining information on the main some phenolic compounds present in wine.

References

1. F. Shahidi and M. Naczk. *Wine in food phenolics: Sources, chemistry, effects, applications*. Technomic Publishing Co, PA, 1995, pp.. 136-148.
2. R. Boulton. The copigmentation of anthocyanins and its role in the color of red wine: A critical review. *Am. J. Enol. Vitic.* **52:** 67-72 (2001).
3. I. Lesschaeve and A.C. Noble. Polyphenols: Factors influencing their sensory properties and their effects on food and beverage preferences. *Am. J. Clinic. Nutr.* **81:** 3305-3355 (2005).
4. S. Gómez-Alonso, E. García-Romero, and I. Hermosín-Gutiérrez. HPLC analysis of diverse grape and wine phenolics using direct injection and multidetection by DAD and fluorescence. *J. Food Compos. Anal.* **20:** 618-626 (2007).
5. Z. Spacil, L. Nováková, and P. Solich. Analysis of phenolic compounds by high performance liquid chromatography and ultra performance liquid chromatography. *Talanta* **76:** 189-199 (2008).
6. M.A. Rodriguez-Delgado, G. González-Hernández, J.E. Conde-González, and J.P. Pérez-Trujillo. Principal component analysis of the polyphenol content in young red wines. *Food Chem.* **78:** 523-532 (2002).
7. D. Matejícek, O. Mikes, B. Klejdus, D. Sterbová, and V. Kubán. Changes in contents of phenolic compounds during maturing of barrique red wines. *Food Chem.* **90:** 791-800 (2005).
8. J. Burns, P.T. Gardner, J. O'Neil, S. Crawford, I. Morecroft, D.B. McPhail, C. Lister, D. Matthews, M.R. Maclean, M.E.J. Lean, G.G. Duthie, and A. Crozier. Relationship among antioxidant activity, vasodilation capacity and phenolic content of red wines. *J. Agric. Food Chem.* **48:** 220-230 (2000).
9. J. Wang, L. Ho, Z. Zhao, I. Seror, N. Humala, D.L. Dickstein, M. Thiyagarajan, S.S. Percival, S.T. Talcott, and G.M. Pasinetti. Moderate consumption of Cabernet sauvignon attenuates A neuropathology in a mouse model of Alzheimer's disease. *FASEB J.* **20:** 2315-2320 (2006).
10. M.S. Fernández-Pachón, D. Vollaño, M.C. García-Parrilla, and A.M. Troncoso. Antioxidant activity of wines and relation with their polyphenolic composition. *Anal. Chim. Acta* **513:** 113-118 (2004).
11. F. Cimino, V. Sulfaro, D. Trombetta, A. Saija, and A. Tomaino. Radical-scavenging capacity of several Italina red wines. *Food Chem.* **103:** 75-81 (2007).
12. E.N. Frankel, C.A. Bosanek, A.S. Meyer, K. Silliman, and L.L. Kirk. Commercial Grape Juices Inhibit the in Vitro Oxidation of Human Low-Density Lipoproteins. *J. Agric. Food Chem.* **46:** 834-838 (1998).
13. K.W. Singletary, M.J. Tansbury, M. Giusti, R.B. Van Breemen, M. Wallig, and A. Rimando. Inhibition of Mammary Tumorigenesis by Concord Grape Juice Constituents. *J. Agric. Food Chem.* **51:** 7280-7286 (2003).
14. M.N. Bravo, S. Silva, A.V. Coelho, L. Vilas Boas, and M.R. Bronze. Analysis of phenolic compounds in Muscatel wines produced in Portugal. *Anal Chim. Acta* **563:** 84-92 (2006).
15. H.S. Lee. HPLC analysis of phenolic compounds. L.M.L. Nollet. *Food analysis by HPLC*, 2nd ed., Marcel Dekler Inc., New York, NY, 2000.
16. E. Revilla, and J.M. Ryan. Analysis of several phenolic compounds with potential antioxidant properties in grape extracts and wines by high-performance liquid chromatography-photodiode array detection without sample preparation. *J. Chromatogr. A* **881:** 461-469 (2000).
17. M. López, F. Martinez, C. Del Valle, C. Orte, and M. Miró. Analysis of phenolic constituents of biological interest in red wines by high-performance liquid chromatography. *J. Chromatogr. A* **922:** 359-363 (2001).
18. S. Muñzoz, M. Mestres, O. Busto, and J. Guash. Determination of some flavan-3-ols and anthocyanins in red grape seed and skin extracts by HPLC-DAD: Validation study and response comparison of different standards. *Anal. Chim. Acta* **628:** 104-110 (2008).
19. A.R.B. De Quirós, M.A. Lange-Yusty, and J. López-Hernández. HPLC-analysis of polyphenolic compounds in Spanish white wines and determination of their antioxidant activity by radical scavenging assay. *Food Res Int.* **42:** 1018-1022 (2009).
20. M.S. García-Falcón, C. Pérez-Lamela, E. Martínez-Carbalho, and J. Simal-Gándara. Determination of phenolic compounds in wines: Influence of bottle storage of young red wines on their evolution. *Food Chem.* **105:** 248-259 (2007).
21. D. Villaño, M.S. Fernández-Pachón, A.M. Troncoso, and M.C. García-Parrilla. Influence of enological practices on the antioxidant activity of wines. *Food Chem.* **95:** 394-404 (2006).
22. ICH. Harmonised Tripartite Guideline, Q2(RI), Validation of Analytical Procedures: Text and Methodology, 1994.
23. L.R. Snyder, J.J. Kirkland, and J.L. Glajch. *Practical HPLC method development,* 2nd ed., John Wiley & Sons Inc., New York, NY , 1997.
24. F. Nave, M.J. Cabrita, and C.T. Costa. Use of solid-supported liquid-liquid extraction in the analysis of polyphenols in wine. *J. Chromatogr. A* **1169:** 23-30 (2007).
25. R. Sladkovský, P. Solich, and M. Urbánek. High-performance liquid chromatography determination of phenolic components in wine off-line isotachophoretic pretreatment. *J. Chromatogr. A* **1040:** 179-184 (2004).
26. A.C. Muñoz-Muñoz, A.C. Grenier, H. Gutiérrez-Pulido, and J. Cervantes-Matínez. Development and validation of High Performance liquid Chromatography-Diode Array detection method for the determination of aging markers in tequila. *J. Chromatogr. A* **1213:** 218-223 (2008).
27. M.L. Francisco, and A.V.A. Resurreccion. Development of a reversed-phase high performance liquid chromatography (RP-HPLC) procedure for the simultaneous determination of phenolic compounds in peanut extracts. *Food Chem.* **117:** 356-363 (2009).
28. D.M. Goldberg, E. Tsang, A. Karumanchiri, E.P. Diamandis, and N.G. Soleas. Method to Assay the Concentrations of Phenolic Constituents of Biological Interest in Wines. *Anal Chem.* **68:** 1688-1694 (1996).
29. V. Rastija, G. Srecnik, and M.M. Saric. Polyphenolic composition of Croatin wines with different geographical origins. *Food Chem.* **115:** 54-60 (2009).

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