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Direct Liquid Chromatography Method for the Simultaneous Quantification of Hydroxytyrosol and Tyrosol in Red Wines

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ABSTRACT: A validated HPLC method with fluorescence detection for the simultaneous quantification of hydroxytyrosol and tyrosol in red wines is described. Detection conditions for both compounds were optimized (excitation at 279 and 278 and emission at 631 and 598 nm for hydroxytyrosol and tyrosol, respectively). The validation of the analytical method was based on selectivity, linearity, robustness, detection and quantification limits, repeatability, and recovery. The detection and quantification limits in red wines were set at 0.023 and 0.076 mg L^{-1} for hydroxytyrosol and at 0.007 and 0.024 mg L^{-1} for tyrosol determination, respectively. Precision values, both within-day and between-day (n = 5), remained below 3% for both compounds. In addition, a fractional factorial experimental design was developed to analyze the influence of six different conditions on analysis. The final optimized HPLC−fluorescence method allowed the analysis of 30 nonpretreated Spanish red wines to evaluate their hydroxytyrosol and tyrosol contents.

KEYWORDS: hydroxytyrosol, tyrosol, HPLC, fluorescence, red wine

INTRODUCTION

Phenolic compounds are responsible for the diverse beneficial effects attributed to a moderate wine consumption,[1] including antioxidant, anticarcinogenic, and antibacterial properties. Among them, tyrosol-related compounds have attracted increasing attention mainly due to their antioxidant role.[2]

Hydroxytyrosol (3,4-dihydroxyphenylethanol) and tyrosol (*p*-hydroxyphenylethanol) are naturally occurring compounds mainly found in olive oil,[3] which are also present in other foods and beverages such as wine. Few studies[4,5] have focused on the appearance and quantification of hydroxytyrosol in wine since they were first described by Di Tommaso et al.[6] in 1998.

Several health-enhancing activities, deriving from their free radical scavenging,[7,8] anticarcinogenic,[9] cardiopreventive,[9−12] and antimicrobial[13] properties, have been attributed to tyrosol and hydroxytyrosol. These specific properties[14] are currently under discussion, as they were settled basically from in vitro assays and cannot be directly correlated with in vivo studies.

Tyrosol and hydroxytyrosol (formed by hydroxylation of the aromatic ring of tyrosol) could be considered as secondary metabolites from the tyrosine formed by yeasts during alcoholic fermentation.[15] Furthermore, the synthesis of tyrosol has been described as directly proportional to the quantity of amino acids present in the must.[16] The formation of both components in wine could be summarized as shown in Figure 1, which is based on the Ehrlich pathway for fusel alcohol production by *Saccharomyces cerevisiae*.[17]

As low concentrations of hydroxytyrosol and tyrosol are found in wine, their beneficial effects (e.g., inhibition of lipid peroxidation) will probably be also correlated to the synergistic effect with other polyphenols present in wine, similar to that proved for olive oils.[14] Anyway, further research involving in vivo tests is necessary to determine the impact of their intake on human health.

The sample preparation methods commonly used for both phenolics prior to chromatographic analysis include liquid−liquid extraction,[26] solid phase extraction,[6,16,20,24,27] and preparative liquid chromatography.[4] Direct injection HPLC analysis has been described[25] only for tyrosol analysis.

Various different techniques have been employed for hydroxytyrosol determination in liquid samples, mainly in olive oils: gas chromatography,[18] capillary electrophoresis,[19] high-performance liquid chromatography,[19,20] nuclear magnetic resonance,[21] and even sequential HPLC-GC.[22] For wine samples, GC-MS after sample derivatization was the most commonly used technique.[4,23] For tyrosol determination, GC-FID,[18] HPLC-DAD,[24] and HPLC-MS[4,25] are the most widely used methodologies.

Several works have studied hydroxytyrosol and tyrosol quantification through HPLC-DAD or HPLC-MS. To the best of our knowledge, the quantification of both compounds in red wine by fluorescence detection has not been described yet. Indeed, the fluorescence quantification method for hydroxytyrosol has been developed only for plasma or other biological samples.[28]

This study was mainly aimed at the development, optimization, and validation of a sensitive analytical method for the simultaneous hydroxytyrosol and tyrosol determination in red wine by means of high-performance liquid chromatography with fluorescence detection. Quantification limits allowed direct determination in red wine with no sample pretreatment. This method has been successfully applied to the analysis of 30 red wines.

MATERIALS AND METHODS

Reagents. Hydroxytyrosol and tyrosol standards were purchased from Sigma-Aldrich (St. Louis, MO). Analytical grade methanol, formic acid, tartaric acid, and ethanol were supplied by Panreac (Barcelona, Spain). Ultrapure water from a Mili-Q system (Millipore, Bedford, MA) was used throughout this research.

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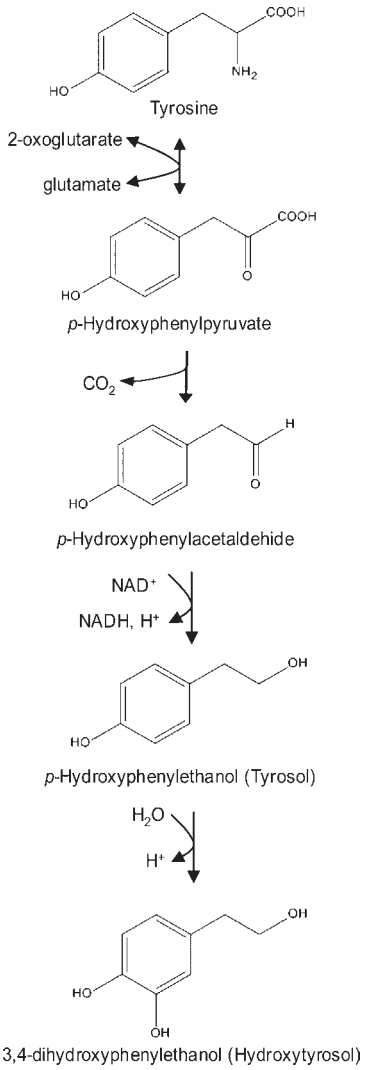

Figure 1. Pathway of formation of tyrosol and hydroxytyrosol in wines by *Saccharomyces cerevisiae* metabolism.

Standard Solutions. A stock standard solution of 100 mg L^{-1} was prepared by dissolving hydroxytyrosol and tyrosol powder (1 mg) in 10 mL of synthetic wine (4 g L^{-1} of tartaric acid, 12% (v/v) of ethanol, pH 3.5, as a wine-like medium). Calibration samples were prepared by dilution in the same matrix.

Wine Samples. The samples included 30 red wines corresponding to 15 varieties of grapes cultivated in Jerez (southern Spain) under different winemaking conditions.

Equipment and Chromatographic Conditions. Chromatographic analysis was carried out in a Jasco high-performance liquid chromatographic system equipped with a diode array detector (model MD-2010), a fluorescence detector (model FP-2020), an HPLC pump module (model PU-2089), a column oven module (model CO-2060), and an autosampler module (AS-2050) and controlled by Chrompass version 1.8 software. The column used was a Mediterranea Sea-C18 column (RP-18, 250 × 4.6 cm; 5 μm particle size) from Teknokroma (Barcelona, Spain) with a guard column made of the same material.

The mobile phase consisted of A (water/formic acid 99.9−0.1%) and B (methanol/formic acid 99.9−0.1%). The elution program involved gradient elution as described by Pereira-Caro et al.,[29] with some modifications. Final elution conditions were as follows: from 100 to 90% A in 5 min; 85% A in 5 min; 65% A in 10 min; 60% A in 20 min; 100% B in 5 min; 100% A in 5 min; followed by 15 min of maintenance and 100% A. The injection volume was 20 μL, and the flow rate was

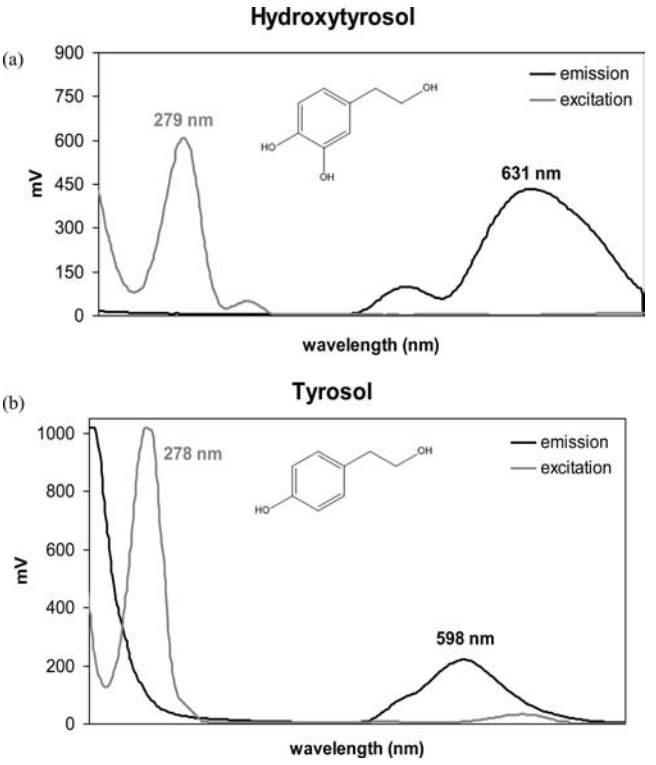

Figure 2. Fluorescence excitation and emission spectra and chemical structures for hydroxytyrosol (a) and tyrosol (b).

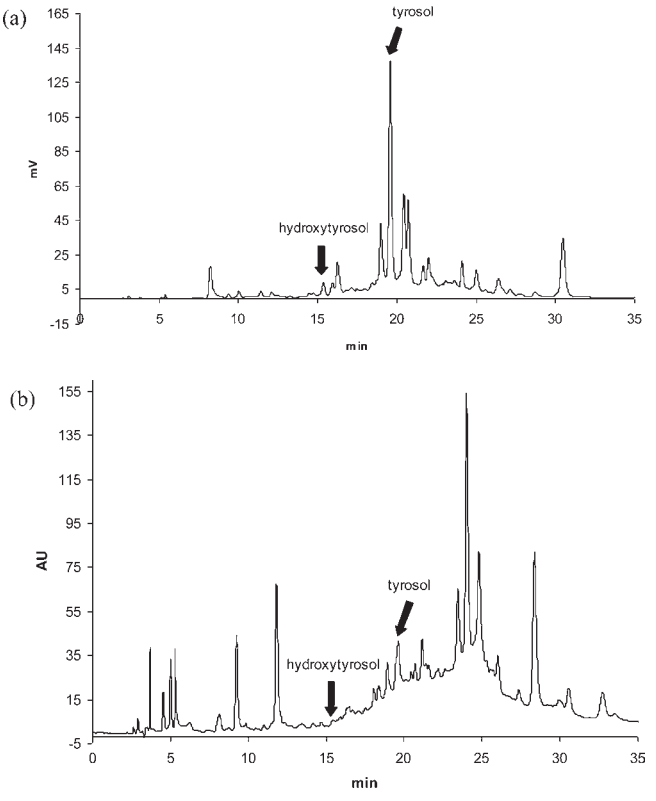

Figure 3. Chromatogram of a Tempranillo red wine: fluorescence (a) and 280 nm (b) channels.

1.0 mL min^{-1}. Chromatography was performed at 37 °C and optimized fluorescence conditions were as follows: excitation at 279 nm, emission at 631 nm for hydroxytyrol, changing from minute 17 to excitation at 278 nm and emission at 598 nm for tyrosol determination. In these chromatographic conditions, retention times were 15.5 and 19.8 min for hydroxytyrosol and tyrosol, respectively.

Statistical Software. Youden's statistic test is a reliable method to evaluate the robustness of analytical methods by means of an experiment design that involves different variables combined in several tests. A fractional factorial design (2^{6-2}) was used: a total of 16 extractions were carried out in duplicate instead of the 64 possible combinations evaluated (percentage of formic acid in solvent A, percentage of formic acid in solvent B, column temperature, conditioning time, percentage of ethanol in the sample, and solvent flow).

This kind of experimental design has produced good results in the robustness evaluation of chromatographic methods developed for different compounds.[30] Graphic analysis of the main effects and interactions between the variables was used for interpretation of the results.

RESULTS AND DISCUSSION

Development of the HPLC-FD Quantitative Analytical Method. Research on different chromatographic conditions was aimed to achieve the best peak separation and resolution for quantification purposes, as both target compounds appear close to other matrix components. By applying slight modifications to the elution program described by Pereira Caro et al.,[29] no interference peaks appeared at the retention times of the peaks of interest.

With the aim of developing a more selective method of determination by HPLC with a lower detection limit, the fluorescence excitation and the emission spectra of hydroxytyrosol and tyrosol (Figure 2) were analyzed. According to these, wavelengths of 279 and 634 nm, as excitation and emission, respectively, were used for hydroxytyrosol selective detection, changing from minute 17 to excitation at 278 nm and emission at 598 nm for tyrosol determination. These emission conditions widely differ from those previously described in the literature.[28] Those differences may be attributed to the different matrix composition that standards were dissolved in: plasma samples versus wine-like media and, therefore, the different physicochemical characteristics (e.g., pH, viscosity). As an example, a chromatogram of a red wine sample is shown in Figure 3.

Robustness Evaluation by Means of Youden's Statistic Test. The evaluation of the robustness of chromatographic methods can take a long time and can also be very complex when several experimental variables are taken into account. A first option can be introducing variable changes one by one, and the results are subsequently compared by means of Student's *t* test or ANOVA test.[31] A faster and more complete option is the application of a two-level experimental design for simultaneous evaluation of several experimental variables, similarly to a classical Youden's statistic test.[32] A fractional factorial design was used in the present work to determine the robustness of the developed chromatographic method. Fractional factorial design was chosen instead of full factorial design to reduce the total number of necessary experiments. Tables 1 and 2 show the assayed conditions and obtained recoveries.

The effects of six different HPLC conditions (Table 2), including percentage of pH modifier in solvents, flow rate, equilibrating

Table 1. Analytical Parameters and Variations for Robustness Evaluation of the Chromatographic Method for Hydroxytyrosol and Tyrosol Quantification

parameter	nominal condition	variation introduced
% formic acid in solution A	0.10	±0.05
% formic acid in solution B	0.10	±0.05
column temp (°C)	37	±2
equilibrating time (min)	15	±2
% EtOH in sample	12	10 or 15
flow rate (mL min^{-1})	0.10	±0.05

Table 2. Conditions and Results of the Chromatographic Analysis Based on the Fractional Factorial Experimental Design

	conditions assayed						hydroxytyrosol		tyrosol	
expt	% FA[a] on solvent A	% FA on solvent B	column temp (°C)	equilibrating time	% EtOH in sample	flow rate (mL min^{-1})	mean area	RSD[b] (%)	mean area	RSD (%)
1	0.105	0.105	39	13	15	0.95	4.36	0.16	66.89	0.71
2	0.105	0.095	39	13	10	1.05	3.69	2.30	57.66	3.61
3	0.105	0.095	39	17	10	0.95	4.28	0.83	66.65	0.85
4	0.095	0.095	39	13	15	1.05	3.71	0.95	59.30	0.67
5	0.095	0.095	35	13	10	0.95	3.87	0.55	61.79	0.17
6	0.095	0.105	35	17	15	0.95	3.89	1.27	62.28	0.31
7	0.095	0.105	35	13	15	1.05	3.38	0.42	55.85	0.20
8	0.105	0.095	35	17	15	1.05	3.41	0.21	56.17	0.40
9	0.095	0.105	39	17	10	1.05	3.33	3.37	59.72	0.82
10	0.105	0.105	39	17	15	1.05	3.02	0.47	58.37	0.04
11	0.105	0.105	35	17	10	0.95	3.99	0.00	62.90	0.01
12	0.095	0.105	39	13	10	0.95	3.55	0.00	66.37	0.22
13	0.095	0.095	35	17	10	1.05	3.39	1.88	57.02	0.31
14	0.105	0.105	35	13	10	1.05	3.30	1.50	56.47	0.29
15	0.105	0.095	35	13	15	0.95	3.79	0.00	63.77	0.49
16	0.095	0.095	39	17	15	0.95	3.61	1.37	66.47	0.52

[a] FA, formic acid. [b] RSD, relative standard deviation.

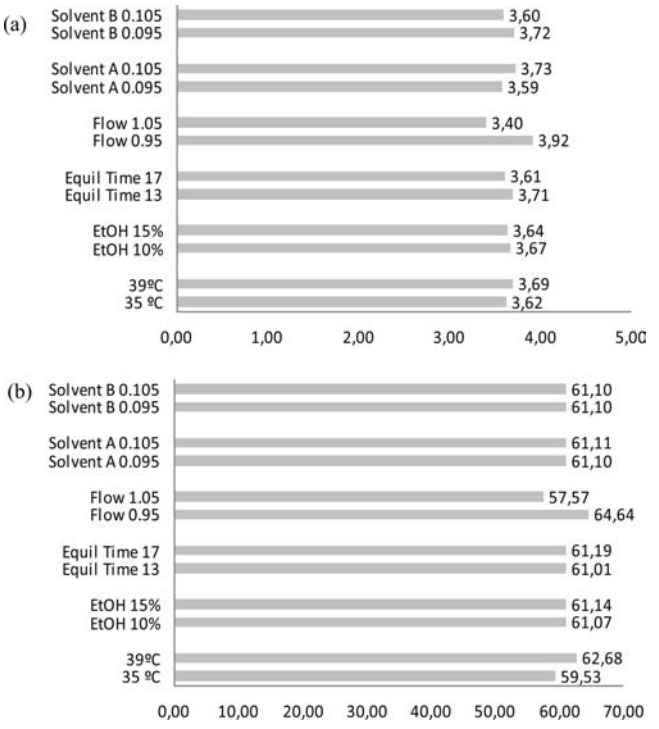

Figure 4. Main effects plot on the mean recovery for hydroxytyrosol (a) and tyrosol (b). Equil. Time, equilibrating time.

time, percentage of ethanol in the sample, and column temperature, on the peak area for hydroxytyrosol and tyrosol were evaluated using fractional factorial design. A total of 16 experiments, instead of the 64 (2^6) available combinations for the tested experimental variables (Table 2), were run. All injections were run in duplicate.

Figure 4 shows the result obtained for the mean peak areas for both hydroxytyrosol and tyrosol under all assayed conditions. Small differences were found for most of the experimental conditions tested, including percentage of acid in solvents, equilibrating time, percentage of ethanol in the sample, and column temperature. The experimental variable flow rate showed the greatest differences for both peak areas. Two levels (0.95 and 1.05 mL min^{-1}) were checked for the flow rate variable: hydroxytyrosol mean areas range from 3.55 to 4.36 and from 3.02 to 3.71 for 0.95 and 1.05 mL min^{-1}, respectively, whereas tyrosol mean areas ranged from 61.79 to 66.89 and from 55.85 to 59.72 for 0.95 and 1.05 mL min^{-1}, respectively (see Table 2). The obtained RSD varied from 0.00 to 3.37 for hydroxytyrosol areas and from 0.01 to 3.61 for tyrosol, the highest deviations being observed for the combination of 39 °C and 1.05 mL min^{-1}. However, even in this case, nonsignificant differences were found in the resulting peak areas for both peaks.

Therefore, the developed method was proven to be robust in the assayed experimental conditions for the six studied variables.

Method Validation. The validation of the quantitative analytical method for simultaneous hydroxytyrosol and tyrosol determination in red wine followed AOAC recommendations.[33] Validation was based on the following parameters: selectivity, linearity, precision of the instrumental system (within- and between-day variability), recovery, robustness, detection and quantification limits, and stability.

Good linearity was established for both analytes along 15 calibration points. Table 3 lists the analytical characteristics of the calibration curves prepared using detection by absorption at

Table 3. Calibration Curves, HPLC-FD Intra- and Interday Precision ($n = 5$), and Recovery ($n = 3$) of Hydroxytyrosol and Tyrosol in Red Wine Samples Obtained in Accuracy Analysis

compd	detection	interval ($mg\ L^{-1}$)	intercept	slope	regression coeff	LOD ($mg\ L^{-1}$)	LOQ ($mg\ L^{-1}$)	intraday precision ($mg\ L^{-1} \pm$ RSD)[a]	interday precision ($mg\ L^{-1} \pm$ RSD)[b]	mean amount in sample ($mg\ L^{-1}$)[b]	added ($mg\ L^{-1}$)	mean found ($mg\ L^{-1}$)[b]	mean recovery (%)[b]	RSD (%)[b]
hydroxytyrosol	DAD (280 nm)	0.087−105	−0.3133	1.2454	0.9998	0.256	0.853	nd[c]	nd	nd	nd	nd	nd	nd
	fluorescence		0.6466	2.8423	0.9996	0.023	0.076	1.86 ± 1.58	2.34 ± 2.78	1.60	4.00	5.67	101.75	4.90
tyrosol	DAD (280 nm)	0.140−112	0.1285	0.8606	0.9998	0.568	1.894	nd	nd	nd	nd	nd	nd	nd
	fluorescence		1.2086	9.0731	0.9999	0.007	0.024	36.33 ± 1.82	21.12 ± 2.45	14.73	10.00	25.43	106.96	3.26

[a] Tintilla de Rota wine. [b] Tempranillo wine. [c] nd, not determined (because fluorescence detection was selected as the best option).

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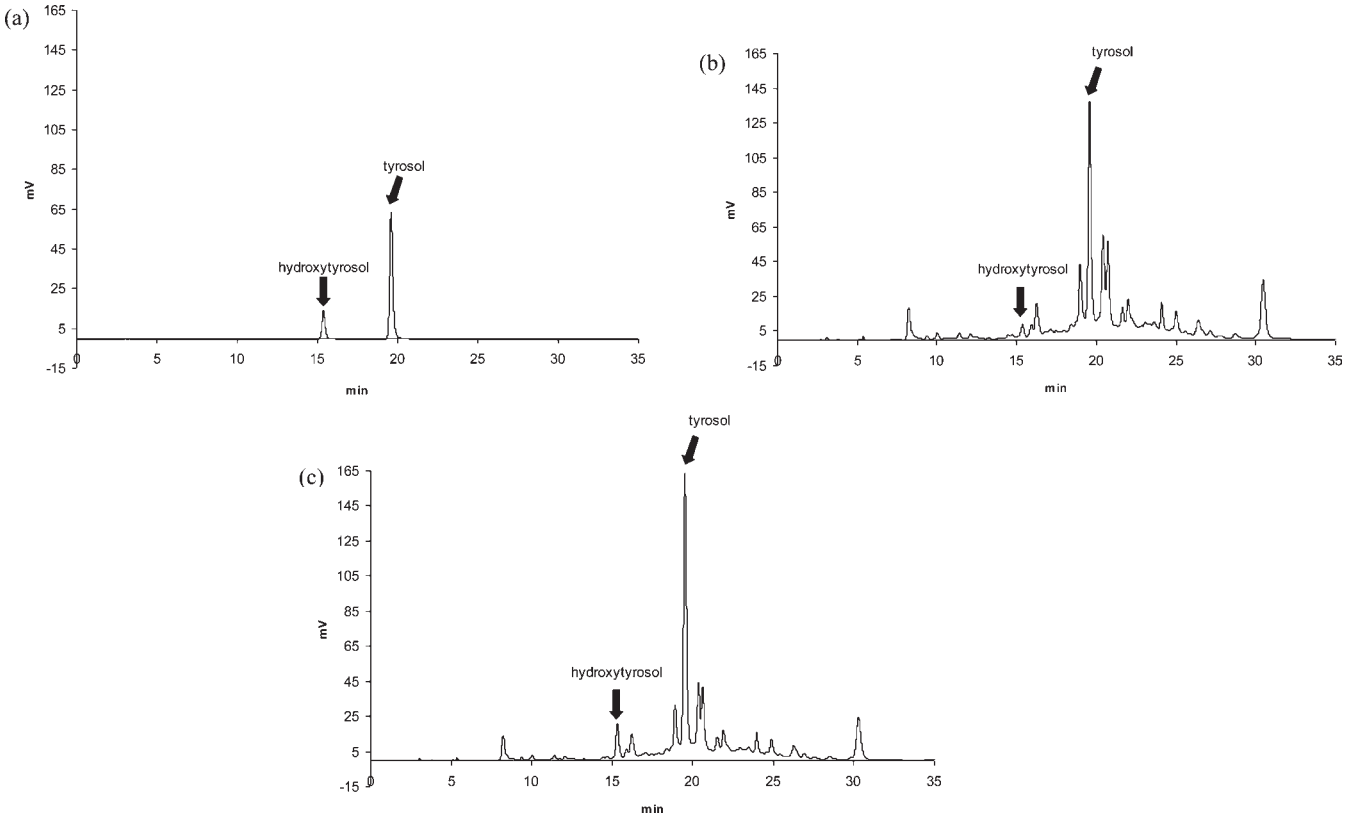

Figure 5. HPLC-FD chromatograms corresponding to (a) spiking solution sample of 4 and 10 mg L^{-1} for hydroxytyrosol and tyrosol, respectively; (b) Tempranillo wine sample before spiking; and (c) Tempranillo wine sample fortified with the spiking solution.

280 nm against detection by fluorescence. As seen in Figure 3 and Table 3, the analytical sensitivity of the fluorescence method is much higher (almost 3 and 10 times higher for hydroxytyrosol and tyrosol, respectively) than that recorded with detection by absorption at 280 nm. Fluorescence was also more selective than DAD, as previously described for other phenolics.[34] Table 3 also shows the quantification (LOQ) and detection (LOD) limits determined by replicate analyses and considered to be 10 and 3 times the standard deviation of baseline noise from 12 standard samples, respectively.

Method precision was studied as intra- and interday assay ($n = 5$) for each compound. Within-day variation was assessed by analyzing replicates of the same wine sample (Tintilla de Rota wine, Table 3). The method was found to be precise with RSD values of 1.58% for hydroxytyrosol and 1.82% for tyrosol (intraday assay). Interday RSDs were below 3% for both analytes for the same wine sample (Tempranillo variety, Table 3) injected on five separate days.

Method accuracy was established by determining the recovery of hydroxytyrosol and tyrosol spiked to the sample (4 and 10 mg L^{-1}), running in triplicate, according to the proposed method. Figure 5 shows HPLC-FD chromatograms corresponding to the spiking solution (Figure 5a), as well as nonspiked (Figure 5b) and spiked red wine samples (Figure 5c).

The results obtained are shown in Table 3. In all analyzed wine samples, mean recovery for each concentration ranged from 96.40 to 106.27% ($n = 3$) for hydroxytyrosol and from 104.74 to 110.99% ($n = 3$) for tyrosol. The RSD of the results for each compound was below 5% (Table 3).

Application to Real Samples. The optimized procedure was successfully applied to the determination of hydroxytyrosol and tyrosol levels in 30 red wine samples. Results are shown in Table 4. Almost all analyzed samples presented values above the LOD and LOQ for fluorescence detection in both analytes.

Among the red wines analyzed, the Cabernet Sauvignon B and Tempranillo F varieties for vintages 2009 and 2010, respectively, contained the highest amounts of hydroxytyrosol. On the other hand, Merlot variety showed the highest tyrosol content. In all red wines analyzed, tyrosol concentration ranged from 20.51 to 44.46 mg L^{-1}, whereas hydroxytyrosol ranged from not determined to 5.02 mg L^{-1}.

The hydroxytyrosol levels observed agree with those reported by other authors.[5,6] However, tyrosol concentrations were quite higher than reported by Di Tommaso et al.[6] (3.61−4.80 mg L^{-1}), although they agree with those contributed by other authors for different red wine varieties such as Pinot noir Champagnes, 18 mg L^{-1};[35] Mazuelo, 20−30 mg L^{-1};[16] Graciano, Tempranillo, or Cabernet Sauvignon, 7−26 mg L^{-1};[36] autochthonous Italian wines, 17−62 mg L^{-1};[25] or autochthonous Hungarian and Canadian wines, 38−82 mg L^{-1}.[24] On the basis of these observations, our findings were not unexpected, because a number of factors such as temperature, nitrogen source availability, and sugar content are widely known to affect yeast activity during wine fermentation.[16]

In conclusion, a simple, rapid, and reliable RP-HPLC-FD method was validated for routine analysis of hydroxytyrosol and tyrosol in red wine samples. The analytical method was proven to be robust, selective, linear, precise, and accurate in the intervals studied for both phenolics. The obtained detection and quantification limits were lower than those found so far in the literature for HPLC determination in both components.

The proposed method enables the determination of hydroxytyrosol and tyrosol contents in wine by direct injection with no

Table 4. Hydroxytyrosol and Tyrosol Concentrations in 30 Spanish Wines (n = 3)

wine	vintage	hydroxytyrosol[a]	tyrosol[a]
Tempranillo A	2009	1.84 ± 1.56	22.76 ± 1.00
Tempranillo B		1.46 ± 1.03	20.51 ± 0.74
Tempranillo C		0.82 ± 0.71	21.06 ± 1.69
Blasco		1.55 ± 0.98	27.38 ± 0.66
Cabernet Sauvignon A		3.63 ± 0.77	31.95 ± 0.57
Cabernet Sauvignon B		5.02 ± 1.28	32.57 ± 1.38
Petit Verdot A		4.12 ± 0.63	38.50 ± 0.25
Petit Verdot B		2.33 ± 2.43	40.59 ± 2.82
Syrah A	2010	1.11 ± 2.43	34.11 ± 2.66
Syrah B		0.82 ± 0.71	40.98 ± 0.74
Merlot		1.77 ± 1.49	44.46 ± 2.13
Tintilla de Rota A		2.66 ± 0.17	28.91 ± 0.09
Tintilla de Rota B		1.65 ± 3.58	30.97 ± 2.59
Melonera I		0.45 ± 1.47	35.31 ± 1.18
Melonera B		0.53 ± 1.64	36.86 ± 0.16
Tempranillo A		nd[b]	43.21 ± 3.11
Tempranillo B		nd	43.13 ± 0.21
Tempranillo C		nd	44.26 ± 0.61
Tempranillo D		1.78 ± 0.25	30.60 ± 0.58
Tempranillo E		3.87 ± 1.34	25.03 ± 0.62
Tempranillo F		4.65 ± 0.56	29.93 ± 0.27
Vitis silvestris A		3.09 ± 0.90	40.20 ± 0.30
Vitis silvestris B		0.28 ± 0.49	23.71 ± 1.78
Vitis silvestris C		0.62 ± 2.92	32.12 ± 3.11
Vitis silvestris D		0.77 ± 1.49	20.38 ± 1.91
Palomino negro		1.10 ± 0.37	29.57 ± 0.33
Rome A		1.67 ± 0.39	35.57 ± 0.46
Rome B		1.86 ± 0.83	35.41 ± 0.26
Garnacha A		1.02 ± 0.80	30.15 ± 0.47
Garnacha B		1.28 ± 0.83	26.73 ± 0.63

[a] mg L^{-1} ± RSD (%). [b] nd, not detected.

sample pretreatment. On the other hand, the findings of this study provide deeper knowledge regarding the content of these biologically active phenolic compounds in Spanish red wines.

This content provides useful information for exploring the polyphenol content of different wines as well as characterizing them on the basis of the abundance of these potentially beneficial compounds.

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