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Analytical Innovations in the Detection of Phenolics in Wines

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A liquid chromatographic method with online photometric and luminescent detection for the determination of 18 phenolic compounds in wines is reported. Photometric detection is performed at four wavelengths, namely, 256, 280, 320, and 365 nm, using a diode array detection system. The luminescent detection is achieved by means of a postcolumn derivatization reaction of 10 of these compounds with terbium(III) in the presence of synergistic agents, such as ethylenediaminetetraacetic acid (EDTA) and *n*-octyltriphosphine oxide (TOPO). A micellar medium provided by the surfactants sodium dodecyl sulfate and Triton X-100 was used for the determination of the luminescent chelates at λ_{ex} 317 and λ_{em} 545 nm. The long wavelength emission of lanthanide chelates can minimize interferences from background sample matrix, which usually emit at shorter wavelengths. The analytical features of the photometric and fluorometric methods, such as dynamic ranges of the calibration graphs, detection limits, and precision data, have been obtained. The practical usefulness of the developed methods is demonstrated by the analysis of Spanish and Italian wine samples (red, rosé, oloroso, and white), which were diluted and directly injected into the chromatographic system. The accuracy of both methods was checked by assaying a recovery study, which was performed at three different analyte levels for each type of sample.

KEYWORDS: Phenolic compounds; postcolumn derivatization; terbium-sensitized luminescence; wine samples

INTRODUCTION

Phenolics are a wide group of compounds constituted by phenolic aldehydes, hydroxybenzoic and hydroxycinnamic acids, catechins, flavonols, and stilbenes, in their monomeric form or conjugated to some species, such as tartaric acid in the case of cinnamic acids (*1*), among others. These compounds are present in wines because they are secondary metabolites of plants. The composition of phenolics and their concentration depend on grape variety, geographical origin, soil type, collection system, and grape processing. These compounds are responsible of the sensory properties of the wines, and, also, they are anticarcinogenic and have an anti-inflammatory action when they are regularly ingested. A particular example of the importance of monitoring phenolic concentration to control the quality of wines is the presence of aromatic aldehydes, formed by a group of volatile compounds that are extracted from wood lignin during the winemaking process. The presence of these aldehydes in wines is an indicator of fermentation and aging in oak barrels, their absence being indicative of counterfeit aged wines. Vanillin and syringaldehyde are the most abundant aromatic aldehydes in wines.

The occurrence of phenolics has been extensively studied by liquid chromatographic methods (*1–18*). In most of them, conventional reversed-phase columns, constituted by packed microparticulate bonded silica, have been used (*1–5, 7–15, 18*), which generally feature separations of 14–25 compounds in almost 1 h or 32 phenolics in 90 min, which makes routine analysis of these compounds very tedious. The use of other materials such as mesoporous silica has given rise to monolithic columns, which operate at higher flow rates with lower backpressures than conventional columns (*16, 17*). Thus, they allow the analysis of samples using direct injection or low sample dilutions, because the cleaning and regeneration of the column can be done more quickly than in the conventional ones due to the high flow rates afforded. Monolithic columns have been used for the determination of 15 phenolics in red and white wine samples (*16*) with separation times around 30 min and, also, for the direct analysis of cider samples (*19*).

Diode array detection (*1, 3–17*) has been extensively used for the development of liquid chromatography methods, whereas fluorometric (*8, 12*) and mass spectrometry (*2*) detection systems have been used to a lesser extent. Most fluorometric methods proposed are based on measurements of the intrinsic fluorescence of some phenolics. Although these methods feature generally lower detection limits than photometric methods, the

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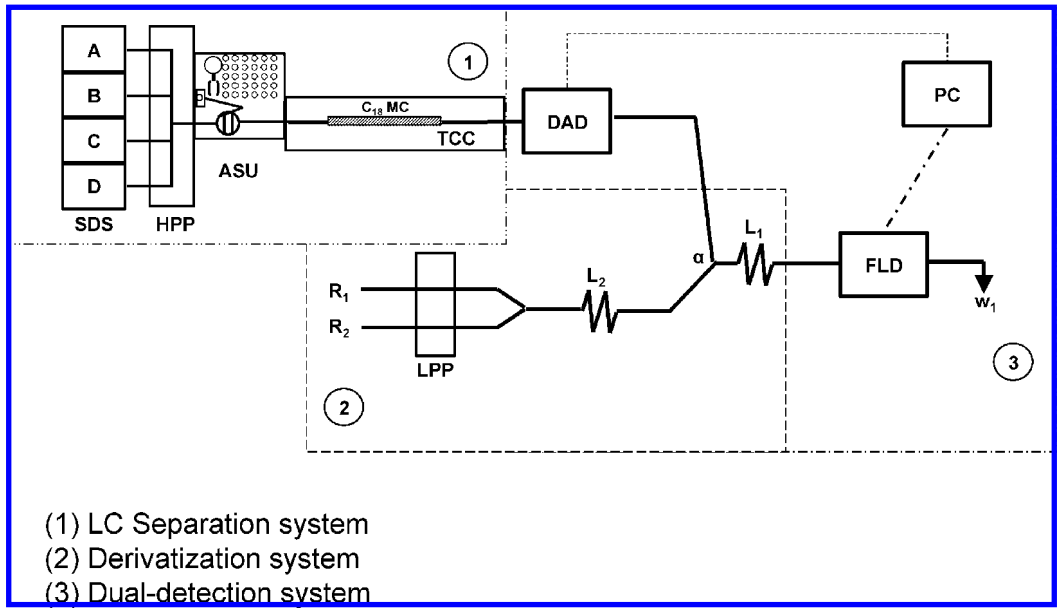

Figure 1. Integrated separation−derivatization and detection approach. A, and B denote 0.02% acetic acid and acetonitrile solutions, respectively. SDS, solvent delivery system; HPP, high-pressure quaternary gradient pump; ASU, autosampler unit; C_{18} MC, C_{18} monolithic column; TCC, thermostated column compartment; DAD, diode array detection system; R_1 and R_2, reagent streams 1 and 2; LPP, low-pressure pump; L_1, mixing reactor; FLD, fluorescence detector; PC, personal computer; w_1, waste.

Table 1. Gradient Elution Conditions

time (min)	elution	%[a]	%[b]	flow (mL min^{-1})
0		98.0	2.0	2.0
	isocratic			
7.00		98.0	2.0	2.0
	linear gradient			
9.00		94.0	6.0	2.0
	isocratic			
14.00		94.0	6.0	2.0
	linear gradient			
19.00		84.0	16.0	2.0
	isocratic			
20.00		84.0	16.0	2.0
	linear gradient			
22.00		70.0	30.0	2.0
	isocratic			
24.00		70.0	30.0	2.0
	linear gradient			
25.00		98.0	2.0	2.0
conditioning step				
25.50		20.0	80.0	2.5
	isocratic			
27.00		20.0	80.0	2.5
	linear gradient			
27.10		98.0	2.0	2.0
	isocratic			
30.0		98.0	2.0	2.0

[a] Acetic acid (0.02%, pH 3.85). [b] Acetonitrile.

Table 2. Characteristic Photometric and Fluorometric Wavelengths of the Studied Compounds

	LC-DAD		LC-FL (phenolic−terbium chelate)	
compound	max absorption wavelength (nm)	confirmation wavelength (nm)	max excitation wavelength (nm)	emission wavelength (nm)
gallic acid	280	256	317	545
protocatechuic acid	256	280	315	
p-hydroxy benzoic acid	256	280	245, 299	
salicylic acid	280		295	
vanillic acid	256	280	298	
caffeic acid	324	280		
syringic acid	280	256	293	
catechin	280		302	
vanillin	280	256, 324	324	
p-coumaric acid	280	324		
syringaldehyde	324	280	335	
epicatechin	280	256, 324	299	
ferulic acid	324	280		
rutin	256	365		
trans-resveratrol	324			
quercetin	365			
cis-resveratrol	280			
kaempferol	365			

number of compounds determined by measuring their native fluorescence is low (*8, 12*). An approach that has been described recently is the use of a postcolumn derivatization reaction with terbium(III) for the determination of eight compounds, namely, hydroxybenzoic acids and catechins (*18*). The method involves the formation of luminescent chelates between phenolics and the lanthanide ion in an alkaline medium in the presence of ethylenediaminetetraacetic acid (EDTA) to prevent terbium precipitation. The detection limits achieved were lower than or comparable to those reported by other methods (*1, 3–11, 13–17*).

The work presented here reports the determination of 18 phenolics, which include hydroxybenzoic (gallic, protocatechuic, *p*-hydroxybenzoic, salicylic, vanillic, and syringic) and hydroxycinnamic (caffeic, ferulic, *p*-coumaric) acids, phenolic aldehydes (syringaldehyde, vanillin), catechins (catechin, epicatechin), flavonols (rutin, quercetin, kaempferol), and stilbenes (*cis*- and *trans*-resveratrol). The separation is achieved in <25 min using a monolithic column. Diode array (LC-DAD) and luminescence (LC-FL) detection systems are used simultaneously to detect and quantify these phenolics. The luminescent detection is based on the reaction of terbium(III) with 10 of these phenolics to give rise to luminescent chelates at a slightly basic medium, using tri *n*-octylphosphine oxide (TOPO) and EDTA as synergistic agents. Hydroxybenzoic acids, catechins, and aldehydes are detected by means of this derivatization reaction. Terbium-sensitized luminescence is used for the first

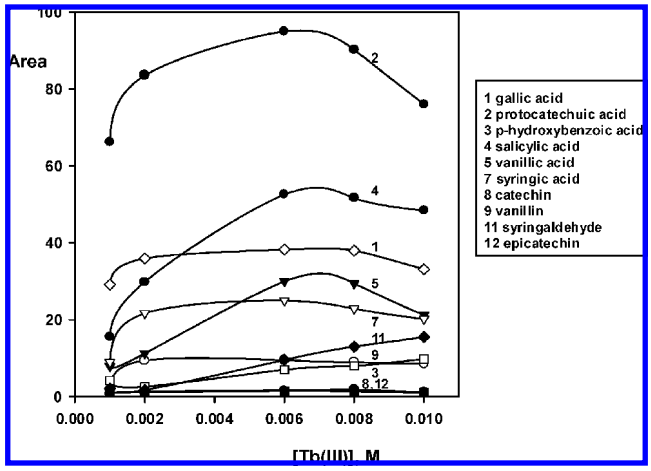

Figure 2. Influence of the terbium(III) concentration for the LC-FL method, at 600 ng mL^{-1} of each phenolic, assayed under the optimum experimental conditions.

time to determine aromatic aldehydes. The luminescence of the chelates is protected from nonradiative processes by a micellar medium provided by Triton X-100 and sodium dodecyl sulfate. The photometric detection is accomplished at four wavelengths: 256, 280, 324, and 365 nm, whereas the luminescent detection is performed using 317 and 545 nm as excitation and emission wavelengths, respectively. Both detection systems are complementary tools to identify and quantify phenolic compounds in different kinds of wine samples, such as red, rosé, oloroso, and white. The only treatment needed is sample dilution prior to the injection onto the chromatographic system. This treatment is simpler than those described elsewhere (*1, 3, 8, 10, 12, 14*), which involve the extraction and/or fractionation of phenolics. The improved selectivity of sensitized luminescence can facilitate the identification of some compounds that cannot be easily identified using UV detection in the presence of complex wine samples. The analytical features of both methods as well as their performance in the analysis of wine samples are compared.

MATERIALS AND METHODS

Apparatus and Instruments. An Agilent 1200 series liquid chromatography system composed of a quaternary pump, a degasser unit, a vial autosampler, a thermostated column compartment, and a diode array detector was used. An SLM Aminco (Urbana, IL) AB2 luminescence spectrometer provided with a 150 W continuous xenon lamp and a 7 W pulsed xenon lamp, furnished with a 176-052-QS Hellma (Hellma Hispania, Barcelona, Spain) flow cell with an inner volume of 18 μL, was used to monitor fluorescence measurements. A Gilson (Villiers-le-Bel, France) Minipuls 3 low-pressure peristaltic pump and Omnifit (Cambridge, U.K.) Teflon tubing of 0.5 mm i.d. were also used for constructing the postcolumn derivatization manifold. Chromatographic separation was performed using an Onyx monolithic C_{18} column (Phenomenex, Torrance, CA), 100 mm × 4.6 mm i.d.; pore sizes were mesopores (13 nm) and macropores (2 μm).

Reagents. All chemicals used were of analytical reagent grade. Stock solutions (5000 mg L^{-1}) of phenolics were prepared as follows: phenolic acids, such as vanillic, gallic, protocatechuic, *p*-hydroxybenzoic (Sigma), syringic (Fluka), and salicylic (Aldrich) acids, were prepared by dissolving them in a minimum volume of ethanol (10−25 mL, depending upon the phenolic considered) and bringing them up to the final volume (50 mL) with distilled water. The same procedure was used for hydroxycinnamic acids, such as ferulic, caffeic, and *p*-coumaric (Sigma) acids; the aromatic aldehydes vanillin (Aldrich) and syringaldehyde (Fluka); the catechins catechin and epicatechin, and, also, resveratrol (Sigma), all of which were degassed using nitrogen to prevent their oxidation by dissolved oxygen. Quercetin (Aldrich), rutin (Sigma), and kaempferol (Sigma) were dissolved in absolute ethanol.

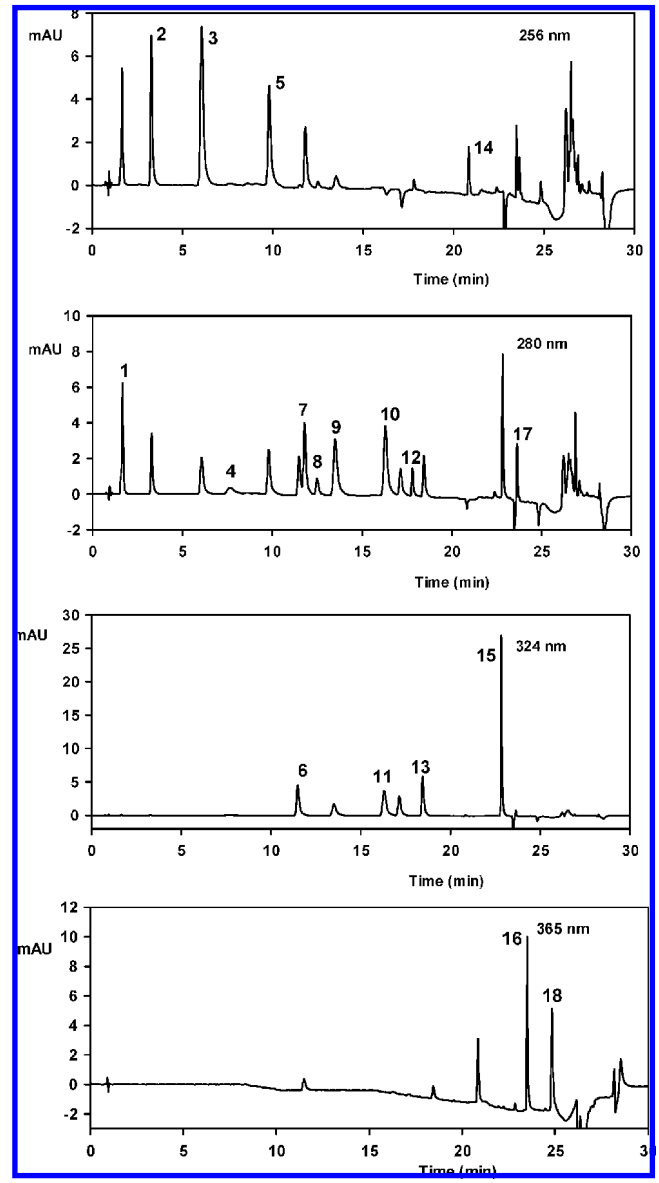

Figure 3. Typical chromatograms achieved using the LC-DAD method at 256, 280, 324, and 365 nm, by injection of a mixture of an aqueous standard solution containing 600 ng mL^{-1} of each analyte (except *cis*- and *trans*-resveratrol, which were 900 and 300 ng mL^{-1}, respectively) and processing under optimum conditions. Peaks: 1, gallic acid; 2, protocatechuic acid; 3, *p*-hydroxybenzoic acid; 4, salicylic acid; 5, vanillic acid; 6, caffeic acid; 7, syringic acid; 8, catechin; 9, vanillin; 10, *p*-coumaric acid; 11, syringaldehyde; 12, epicatechin; 13, ferulic acid; 14, rutin; 15, *trans*-resveratrol; 16, quercetin; 17, *cis*-resveratrol; 18, kaempferol. (The number corresponding to the peak of each analyte appears only at the wavelength chosen for its quantification.)

cis-Resveratrol was obtained by irradiating an aliquot of *trans*-resveratrol stock solution with a UV lamp at 360 nm for 2 h at room temperature. The observed yield for this change was calculated by taking into account the decrease in the area of the peak of *trans*-resveratrol at 324 nm. Fifty percent of *trans*-resveratrol was converted under the mentioned irradiation conditions. Intermediate solutions of 100 mg L^{-1} were prepared by diluting the stock solutions in distilled water, except for quercetin, rutin, resveratrol, and kaempferol, which required absolute ethanol to be stable. Stock and intermediate solutions were stored at 4 °C in the dark and were stable for at least 2 weeks. Working standard solutions were prepared from intermediate solutions by their dilution in distilled water.

The mobile phase used for the separation was constituted by solvent A (acetic acid 0.02%, pH adjusted to 3.85 with sodium hydroxide,

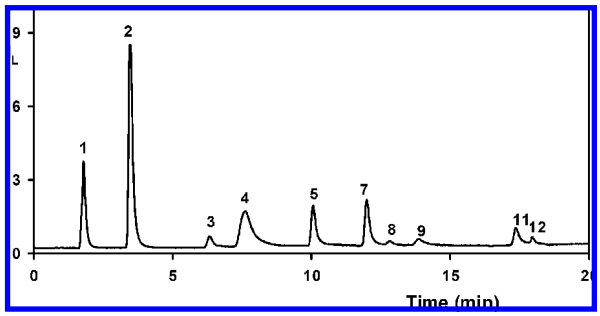

Figure 4. Chromatogram achieved using the LC-FL method by injection of a mixture of aqueous standards at 600 ng mL^{-1} of each analyte and processing under optimum experimental conditions. Peaks: 1, gallic acid; 2, protocatechuic acid; 3, *p*-hydroxybenzoic acid; 4, salicylic acid; 5, vanillic acid; 7, syringic acid; 8, catechin; 9, vanillin; 11, syringaldehyde; 12, epicatechin.

Panreac Quimica, S.A., Barcelona, Spain) and solvent B [HPLC-grade pure acetonitrile (ACN), Panreac Quimica, S.A.], which were mixed by operating in the gradient mode during the chromatographic separation. The derivatization reagent was formed in situ by mixing two streams: the first one contained a solution of terbium(III) (7.5×10^{-3} M), prepared from terbium(III) nitrate pentahydrate (Aldrich), and the second one, a continuously stirred mixture integrated by TOPO (Sigma) (7×10^{-4} M), Triton X-100 (Fluka) (0.8%), EDTA (Fluka) (1.2×10^{-3} M), sodium dodecyl sulfate (Merck) (10^{-3} M), and tris(hydroxymethyl)aminomethane (Merck) buffer (Tris) (0.2 M, pH 9.5).

Manifold and Procedure. Figure 1 shows the three-step integrated LC separation/derivatization/detection approach. Standards or diluted samples (50 μL), containing the analytes at concentrations within their corresponding dynamic ranges, were injected into the column. The mobile phase was pumped at 2 mL min^{-1}, and the system operated under the gradient conditions included in **Table 1**. The variation of absorbance with time was monitored at 256, 280, 320, and 365 nm. The time necessary to achieve a LC-DAD chromatogram was 25 min. Then, a cleanup and conditioning step were applied to have the chromatographic system ready for the next injection after 5 min.

The eluate of the column after passing through the diode array detection system was merged at point α with the derivatizing solution, which was pumped at a flow rate of 0.8 mL min^{-1}. The mixed solution passed through the reactor L_1, in which the derivatization reaction took place. The fluorescence intensity was monitored at λ_{ex} 317 and λ_{em} 545 nm for 20 min, and the corresponding blank solutions were subtracted. Chromatograms were taken using the original software of the luminescence spectrometer, and the raw data of luminescence intensity and time were exported and treated using adequate software packages for the estimation of the main chromatographic parameters.

Assessment of Precision. Precision was evaluated at two different concentration levels, 200 and 600 ng mL^{-1} (except for *cis*- and *trans*-resveratrol, which were 100 and 300 ng mL^{-1} and 300 and 900 ng mL^{-1}, respectively). Seven solutions were subjected to the chromatographic separation on the same day and 10 solutions made in duplicate on five different days to calculate in-day and interday, respectively, precision for retention times and areas by obtaining the percentage of relative standard deviation of these parameters in each case.

Estimation of LODs. The estimation of LODs was done following IUPAC recommendations (*20*), which involve the use of a signal-to-noise ratio of 3. The signal considered as blank signal was the standard deviation of the *y*-intercept of the calibration curve made using 10 mixtures of aqueous standards of the phenolics determined.

Analysis of Wine Samples. Wine samples were diluted with distilled water to match the linear ranges of calibration for each analyte and were directly injected onto the chromatographic system following the procedure above indicated. Each determination was the mean of three measurements.

RESULTS AND DISCUSSION

Study of Diode Array and Luminescent Detection Systems. Absorbance spectra were recorded in the range of 200–400 nm for selecting the wavelengths of the LC-DAD method. Four wavelengths (256, 280, 324, and 365 nm), which are close to the maximum absorption wavelength of each phenolic, were chosen.

Fluorometric detection was achieved using terbium(III) as derivatizing reagent. The relatively long emission wavelength

Table 3. Analytical Features of LC-DAD and LC-FL Methods

compound	retention time (min)	dynamic range[a] (ng mL^{-1})	slope ± SD	*y*-intercept ± SD	r^2	LOD (ng mL^{-1})
gallic acid	1.66	10–900	0.0642 ± 0.0001	−0.19 ± 0.07	0.9999	3
	1.79	*10–900*	*0.0645 ± 0.0003*	*0.5 ± 0.1*	*0.9999*	*5*
protocatechuic acid	3.26	100–10000	0.0942 ± 0.0004	−3 ± 1	0.9997	36
	3.43	*10–900*	*0.1608 ± 0.0003*	*0.6 ± 0.1*	*0.9999*	*2*
p-hydroxybenzoic acid	6.06	100–15000	0.1652 ± 0.0003	−4 ± 1	0.9999	22
	6.26	*50–2000*	*0.0176 ± 0.0002*	*0.30 ± 0.09*	*0.9990*	*15*
salicylic acid	7.36	50–1000	0.0127 ± 0.0001	0.25 ± 0.06	0.9995	14
	7.51	*50–1000*	*0.0862 ± 0.0009*	*1.5 ± 0.4*	*0.9991*	*14*
vanillic acid	9.80	100–20000	0.0975 ± 0.0002	−3 ± 1	0.9999	30
	10.0	*50–2000*	*0.0538 ± 0.0004*	*0.7 ± 0.3*	*0.9994*	*17*
caffeic acid	11.47	10–900	0.0901 ± 0.0002	0.02 ± 0.08	0.9999	3
syringic acid	11.78	30–15000	0.06116 ± 0.00003	−0.3 ± 0.2	0.9999	10
	11.95	*10–900*	*0.0454 ± 0.0001*	*0.45 ± 0.04*	*0.9999*	*3*
catechin	12.64	20–2000	0.02096 ± 0.00008	−0.22 ± 0.06	0.9999	8
	12.77	*300–5000*	*0.00269 ± 0.00005*	*−0.1 ± 0.1*	*0.9976*	*114*
vanillin	13.64	50–15000	0.0931 ± 0.0001	−1.9 ± 0.6	0.9999	19
	13.82	*100–2000*	*0.0140 ± 0.0002*	*0.4 ± 0.2*	*0.9989*	*40*
p-coumaric acid	16.27	50–15000	0.1141 ± 0.0001	−0.2 ± 0.5	0.9999	14
syringaldehyde	17.22	10–1000	0.0616 ± 0.0002	0.07 ± 0.07	0.9999	3
	17.35	*50–2000*	*0.0221 ± 0.0002*	*0.4 ± 0.1*	*0.9996*	*15*
epicatechin	17.85	20–5000	0.02061 ± 0.00004	−0.25± 0.05	0.9999	8
	17.95	*100–2000*	*0.00361 ± 0.00004*	*0.03 ± 0.03*	*0.9986*	*29*
ferulic acid	18.42	20–15000	0.10498 ± 0.00004	−0.4 ± 0.2	0.9999	5
rutin	20.86	100–20000	0.02364 ± 0.00003	−0.7 ± 0.2	0.9999	24
trans-resveratrol	22.86	10–7500	0.13782 ± 0.00003	0.2 ± 0.08	0.9999	2
quercetin	23.52	200–3500	0.097 ± 0.001	−5 ± 2	0.9995	60
cis-resveratrol	23.67	100–5000	0.0595 ± 0.0003	−1.7 ± 0.7	0.9999	35
kaempferol	24.84	50–2000	0.0892 ± 0.0005	3.1 ± 0.5	0.9999	18

[a] The lower limit of dynamic range for each phenolic is its LOQ. Italics indicate fluorometric method.

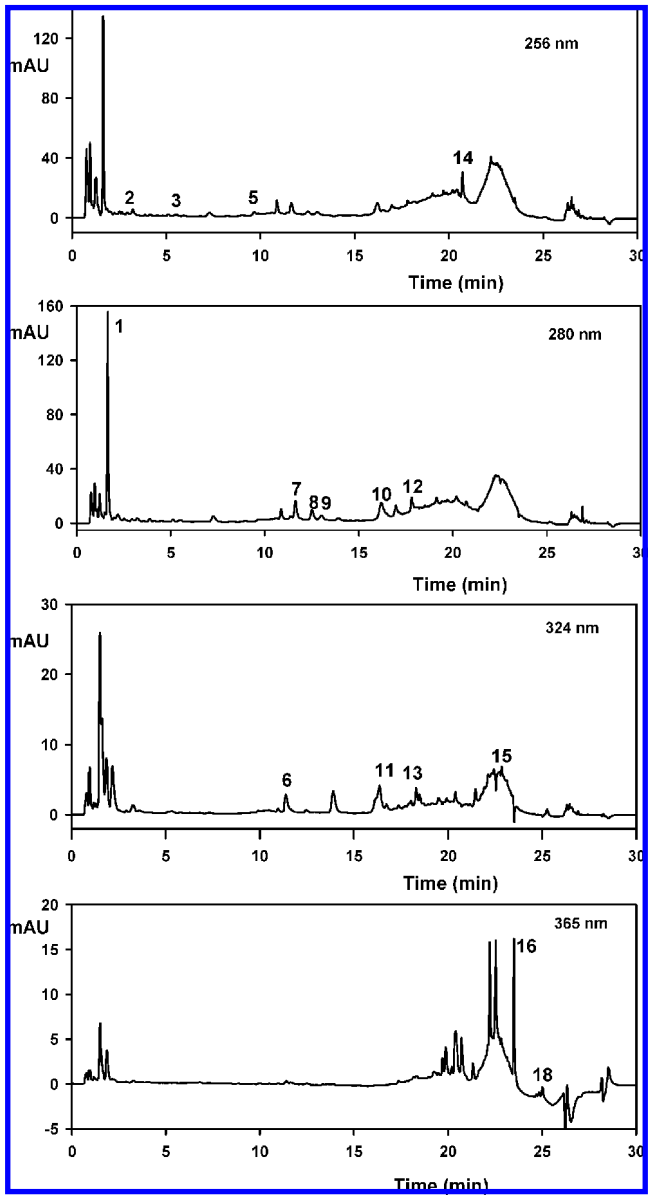

Figure 5. Chromatograms of a red wine sample (sample W2, dilution 1/10) using the LC-DAD method obtained by direct injection of 50 μL of diluted sample. Peaks: 1, gallic acid; 2, protocatechuic acid; 3, *p*-hydroxybenzoic acid; 4, salicylic acid; 5, vanillic acid; 6, caffeic acid; 7, syringic acid; 8, catechin; 9, vanillin; 10, *p*-coumaric acid; 11, syringaldehyde; 12, epicatechin; 13, ferulic acid; 14, rutin; 15, *trans*-resveratrol; 16, quercetin; 17, *cis*-resveratrol; 18, kaempferol.

of terbium(III) chelates can improve the selectivity and sensitivity of the measurements because some fluorescent signals from sample matrix, which could overlap the chromatographic peaks of the phenolics, are minimized or avoided. Postcolumn derivatization with terbium(III), using an alkaline medium and in the presence of EDTA, has been previously reported for the determination of some hydroxybenzoic acids and catechins in white wine samples (*18*). However, aromatic aldehydes do not give any luminescent signal under these experimental conditions. It has been necessary to modify this derivatization reaction to obtain luminescent terbium(III) chelates of aromatic aldehydes. These chelates are formed in a slightly basic medium, provided by a Tris buffer solution, and using TOPO and EDTA as synergistic agents. Hydroxybenzoic acids and catechins also give luminescent signals under these conditions. **Table 2** shows the maximum absorption wavelengths of phenolics, which were

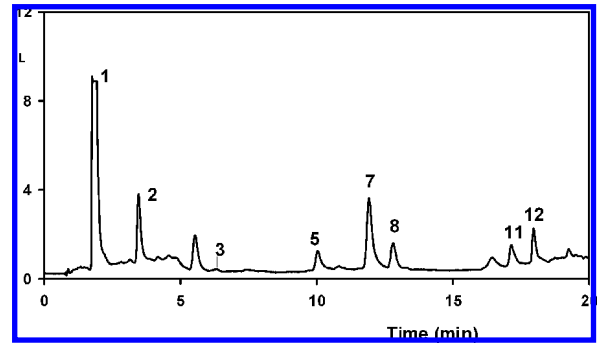

Figure 6. Chromatogram of a red wine sample (sample W2, dilution 1/10) using the LC-FL method obtained by direct injection of 50 μL of the sample. Peaks: 1, gallic acid; 2, protocatechuic acid; 3, *p*-hydroxybenzoic acid; 4, salicylic acid; 5, vanillic acid; 7, syringic acid; 8, catechin; 9, vanillin; 11, syringaldehyde; 12, epicatechin.

Table 4. Precision (%RSD) of LC-DAD and LC-FL Methods

	LC-DAD method				LC-FL method			
	retention time[a]		area[a]		retention time[a]		area[a]	
compound	A	B	A	B	A	B	A	B
gallic acid	2.0	1.8	1.2	3.2	0.5	1.3	3.0	4.7
protocatechuic acid	0.3	3.0	1.3	2.4	0.7	0.6	1.2	2.7
p-hydroxybenzoic acid	0.5	1.4	1.3	2.1	1.3	1.9	4.7	5.3
salicylic acid	1.5	2.3	4.2	5.2	0.8	1.5	3.9	1.5
vanillic acid	0.4	0.7	1.3	3.4	0.4	1.0	2.6	6.4
caffeic acid	0.3	0.8	1.5	3.0				
syringic acid	0.3	0.7	0.8	3.1	0.6	0.4	3.4	5.5
catechin	0.3	0.5	1.6	3.7	0.1	0.6	4.9	5.0
vanillin	0.3	0.6	1.4	3.5	0.5	0.2	5.4	2.0
p-coumaric acid	0.2	0.6	1.3	2.5				
syringaldehyde	0.1	0.3	0.7	3.0	0.2	0.1	4.1	4.7
epicatechin	0.1	0.2	1.7	3.2	0.5	0.08	6.0	2.8
ferulic acid	0.1	0.1	1.2	2.5				
rutin	0.05	0.1	2.0	4.0				
trans-resveratrol	0.01	0.1	1.1	2.4				
quercetin	0.2	0.2	3.3	3.5				
cis-resveratrol	0.1	0.1	1.5	2.3				
kaempferol	0.3	0.09	2.5	9.1				

[a] Retention times and areas obtained from standard mixtures of 600 ng mL^{-1} of each analyte, except for *trans*-resveratrol (900 ng mL^{-1}) and *cis*-resveratrol (300 ng mL^{-1}). A, experiments carried out on the same day ($n = 7$); B, experiments carried out on different days ($n = 5$).

used to establish the LC-DAD method. Some of these compounds also present other less intense absorption wavelengths, which appear in this table as confirmation wavelengths. These wavelengths can be chosen as an alternative when an improvement of the selectivity is required due to chromatographic overlapping of other peaks at the maximum absorption wavelength, although the signal obtained is lower. **Table 2** also shows the excitation and emission wavelengths corresponding to 10 of the phenolics assayed, which form luminescent chelates with terbium(III). Although they show different maximum excitation wavelengths, their broad excitation bands allow the selection of 317 nm as the working wavelength for the simultaneous excitation of these chelates. This wavelength was chosen to get the maximum sensitivity for catechin, vanillin, syringaldehyde, and epicatechin, which exhibited lower luminescence intensities than hydroxybenzoic acids under the experimental conditions assayed. The luminescence emission is measured at 545 nm, which is the most intense emission wavelength of terbium(III).

Optimization of Variables. The hydrodynamic and chemical variables involved in LC-DAD and LC-FL methods were

Table 5. Phenolic Content of the Spanish Wines Analyzed

	content[b] (mg L^{-1})							
compound	W1	W3	W4	W6	W10	W11	W14	W15
gallic acid[a]	2.8 ± 0.1	30.5 ± 0.8	33 ± 1	3.60 ± 0.3	1.70 ± 0.05	1.9 ± 0.1	2.16 ± 0.08	1.84 ± 0.08
protocatechuic acid[a]	1.268 ± 0.002	1.09 ± 0.01	1.269 ± 0.001	0.58 ± 0.01	0.77 ± 0.02	1.14 ± 0.06	1.524 ± 0.004	0.868 ± 0.002
p-hydroxybenzoic acid[a]	0.8 ± 0.01	0.265 ± 0.005	nd[c]	0.63 ± 0.02	nq	nq	0.34 ± 0.04	0.79 ± 0.02
salicylic acid[a]	nq	nq	0.32 ± 0.02	nd	0.33 ± 0.02	0.215 ± 0.001	0.21 ± 0.01	0.240 ± 0.008
vanillic acid[a]	1.21 ± 0.02	1.020 ± 0.003	0.610 ± 0.003	0.33 ± 0.06	0.30 ± 0.03	1.26 ± 0.04	1.16 ± 0.08	0.208 ± 0.008
caffeic acid	2.90 ± 0.04	9.7 ± 0.1	10.7 ± 0.3	1.71 ± 0.02	0.674 ± 0.003	0.163 ± 0.003	nq	1.68 ± 0.08
syringic acid[a]	4.18 ± 0.02	3.46 ± 0.03	0.37 ± 0.05	1.4 ± 0.1	0.30 ± 0.02	0.36 ± 0.03	0.80 ± 0.03	0.090 ± 0.003
catechin[a]	26.3 ± 0.2	30 ± 2	8.1 ± 0.5	nd	nd	0.95 ± 0.03	1.2 ± 0.1	n.d.
vanillin[a]	nq	nd	nd	nq	nd	nd	0.56 ± 0.04	nd
p-coumaric acid	6.4 ± 0.1	11.8 ± 0.2	11.6 ± 0.3	0.11 ± 0.01	nd	0.51 ± 0.01	nd	0.91 ± 0.05
syringaldehyde[a]	1.3 ± 0.1	1.6 ± 0.1	nd	nd	0.45 ± 0.05	nd	0.43 ± 0.02	nd
epicatechin[a]	14.0 ± 0.1	13.3 ± 0.3	29.1 ± 0.1	1.94 ± 0.80	nd	nq	2.9 ± 0.3	nd
ferulic acid	0.399 ± 0.004	0.445 ± 0.003	0.56 ± 0.02	0.14 ± 0.02	0.42 ± 0.04	nq	0.21 ± 0.02	0.47 ± 0.02
rutin	nd	5.53 ± 0.02	0.75 ± 0.03	0.81 ± 0.02	nq	nd	0.212 ± 0.004	nq
trans-resveratrol	0.42 ± 0.02	0.460 ± 0.004	0.094 ± 0.002	0.83 ± 0.03	nq	0.015 ± 0.007	nq	nq
quercetin	5.3 ± 0.2	6.5 ± 0.2	7.0 ± 0.5	0.47 ± 0.02	0.39 ± 0.01	nd	nd	0.325 ± 0.003
kaempferol	0.32 ± 0.01	0.560 ± 0.009	0.490 ± 0.005	nq	nq	nd	nd	nd

[a] Measured using LC-FL method [b] Mean ± SD ($n = 3$). [c] nd, below detection limit; nq, not quantified, below quantification limit.

Table 6. Phenolic Content of the Italian Wines Analyzed

	content[b] (mg L^{-1})						
compound	W2	W5	W7	W8	W9	W12	W13
gallic acid[a]	98.0 ± 4.0	87.2 ± 0.8	9.45 ± 0.07	10.9 ± 0.2	15.3 ± 0.9	2.0 ± 0.1	2.6 ± 0.1
protocatechuic acid[a]	1.6 ± 0.1	2.2 ± 0.2	1.615 ± 0.001	1.50 ± 0.080	1.642 ± 0.003	0.65 ± 0.01	0.41 ± 0.04
p-hydroxy benzoic acid[a]	nq[c]	nq	0.344 ± 0.004	0.48 ± 0.04	0.175 ± 0.005	0.252 ± 0.004	0.11 ± 0.04
salicylic acid[a]	nd	nd	0.20 ± 0.01	0.886 ± 0.002	0.32 ± 0.02	0.090 ± 0.004	nq
vanillic acid[a]	1.25 ± 0.05	2.2 ± 0.1	0.27 ± 0.01	0.34 ± 0.02	0.29 ± 0.02	0.114 ± 0.008	0.106 ± 0.008
caffeic acid		7.27 ± 0.07	1.81 ± 0.01	1.574 ± 0.006	3.20 ± 0.01	1.312 ± 0.006	1.43 ± 0.01
syringic acid[a]	6.3 ± 0.4	5.1 ± 0.3	0.200 ± 0.008	0.46 ± 0.01	0.139 ± 0.002	0.30 ± 0.01	0.128 ± 0.004
catechin[a]	3.8 ± 0.2	33 ± 2	nd	1.00 ± 0.02	nd	3.2 ± 0.1	1.30 ± 0.06
vanillin[a]	nd	nd	nd	nq	nq	nd	0.20 ± 0.02
p-coumaric acid	16.3 ± 0.2	24.6 ± 0.2	2.04 ± 0.03	1.296 ± 0.006	1.68 ± 0.05	0.590 ± 0.004	0.60 ± 0.04
syringaldehyde[a]	3.0 ± 0.2	2.12 ± 0.03	nq	nd	nd	nq	0.14 ± 0.01
epicatechin[a]	32.0 ± 0.3	26.5 ± 0.5	0.332 ± 0.006	0.52 ± 0.02	0.50 ± 0.04	1.32 ± 0.1	0.43 ± 0.02
ferulic acid	0.46 ± 0.02	0.36 ± 0.01	1.00 ± 0.05	0.34 ± 0.02	0.540 ± 0.005	0.486 ± 0.006	0.344 ± 0.002
rutin	19.0 ± 1.0	3.5 ± 0.2	0.961 ± 0.001	0.46 ± 0.02	0.65 ± 0.03	0.73 ± 0.02	0.20 ± 0.02
trans-resveratrol	0.34 ± 0.01	0.72 ± 0.03	nd	0.050 ± 0.002	nq	0.040 ± 0.004	0.008.4 ± 0.0002
quercetin	11.24 ± 0.05	13.0 ± 0.3	nd	nd	nd	nd	nd
kaempferol	0.216 ± 0.002	0.47 ± 0.01	nd	nd	nd	nd	nd

[a] Measured using LC-FL method. [b] Mean ± SD ($n = 3$). [c] nd, below detection limit; nq, not quantified, below quantification limit.

optimized using the univariate methodology. Values chosen were those yielding the maximum absorbance and luminescence signals with a minimum standard deviation.

Chromatographic Variables. The luminescence of lanthanide chelates can be quenched by the vibration of hydroxyl groups of water molecules (*21*), which is really notable when RP-LC is used due to the relatively high content of water in the mobile phases used. The addition of a synergistic agent or the introduction of an acid in the mobile phase can help to achieve the required chromatographic sensitivity. It has been previously described that an adequate luminescent signal can be obtained in the presence of acetate ions (*22, 23*), which can be ascribed to their capability to displace water from the first coordination sphere of terbium(III) (*24*).

Chromatographic variables were optimized to separate the analytes in the shortest separation time. The study of the effect of the apparent pH of the mobile phase showed that it is a critical variable to achieve the required chromatographic selectivity. The shape of peaks was more definite at pH 3.85, using 0.02% acetic acid. The pH of the mobile phase was critical for the separation of the peaks of caffeic and syringic acids and the peaks of syringaldehyde and epicatechin. Binary mixtures of acetic acid (0.02%, pH 3.85) and ACN using different gradient profiles were tried at the optimum flow rate (2 mL min^{-1}), finding that the use of increasing percentages of ACN in two subsequent steps (**Table 1**), from 14 to 19 min and from 20 to 22 min, was useful for the resolution of *p*-coumaric, syringaldehyde, epicatechin, and ferulic acid. Initial percentages of ACN >2% allowed lower retention times for all of the compounds, but they were overlaped. The optimum conditions found were those appearing in **Table 1**, which were used for the chromatographic separation. As can be also seen from this table, cleaning and conditioning steps, which take only 5 min, are included in the gradient routine to ensure reproducibility of retention times between two repeated injections.

Postcolumn Derivatization Variables. The study of the flow rate of the derivatizing reagent solution showed that 0.8 mL min^{-1} provided the optimum derivatizing solution column effluent ratio when 2 mL min^{-1} was used for the chromatographic separation. The length of the reactor L_1 used was 50 cm, which is enough for the development of the derivatization reactions due to the relatively fast rate for the terbium chelate formation. A Tris buffer solution was chosen to increase the pH of the chromatographic eluent, which is required to achieve the optimum luminescence signal without terbium hydroxide precipitation. The concentration of this buffer was studied in the range of 0.05–0.2 M, the pH being adjusted to 8.7 using the highest concentration. The influence of terbium(III) con-

Table 7. Mean Recoveries Obtained by the Analysis of Different Wine Samples

compound	recovery (%)			
	W1	W2	W7	W11
gallic acid[a]	102.3	95.8	102.9	94
protocatechuic acid[a]	92.6	90.6	94.0	81.3
p-hydroxy benzoic acid[a]	99.8	92.7	98.4	92.1
salicylic acid[a]	108.9	88.6	104.8	94.2
vanillic acid[a]	87.8	94.2	99.7	88.5
caffeic acid	108.9	112.3	103.3	115.0
syringic acid[a]	99.3	101.9	89.3	95.6
catechin[a]	93.6	110.7	115.0	103.5
vanillin[a]	97.8	103.4	101.6	92.7
p-coumaric acid	96.8	105.8	101.1	85
syringaldehyde[a]	83.8	103.5	94.9	68.0
epicatechin[a]	98.0	108.4	76.0	89.2
ferulic acid	90.5	93.6	86.0	102.9
rutin	68.0	114.2	99.2	100.5
trans-resveratrol	93.6	105.7	95.7	96.0
quercetin	116.9	108.8	109.7	115.1
kaempferol	88.0	100.2	88.1	88.6

[a] Measured using LC-FL method.

centration was evaluated for each phenolic−terbium chelate in the range of 3×10^{-4}−1.0×10^{-2} M (**Figure 2**). The concentration of TOPO was studied in the range of 1.5×10^{-4}−1.6×10^{-3} M. This synergistic agent notably enhances the sensitized luminescence of salicylic acid and catechin when it is used at (2–7.5) $\times 10^{-4}$ M. This behavior was less significant for the other phenolics assayed. EDTA was mainly used to prevent the terbium precipitation, and its influence was studied in the range of 2×10^{-4}−1.0×10^{-2} M. It was found that this compound improves the signal of gallic acid, but decreases the signal from the other compounds. Thus, a compromise solution was taken by using 1.2×10^{-3} M EDTA. The surfactants Triton X-100 and sodium dodecyl sulfate were used to protect the luminescence of the chelates from nonradiative processes. Triton X-100 had a positive influence on the signal of gallic acid, although the luminescence from other phenolic chelates was unaltered until 0.8%, from which a decrease was observed, this concentration being chosen as optimum. The other surfactant increases the signal of syringaldehyde, which was practically negligible in its absence. **Figures 3** and **4** depict the chromatograms obtained for a standard mixture of phenolics, carried out under optimum conditions using LC-DAD and LC-FL methods, respectively. As can be seen from **Figure 3**, the highest number of analytes appears in the chromatogram obtained at 280 nm.

Analytical Features. Calibration graphs were run under the optimum experimental conditions by using an external calibration method. **Table 3** shows the retention time for each phenolic, the calibration parameters, and the detection limits (LODs) obtained for both methods, which were calculated according IUPAC recommendations (*20*). These detection limits are lower than or comparable to those of other methods previously reported (*1–17, 19*). For LC-DAD, this fact can be explained by two reasons: (1) the use of a monolithic column, which provides narrower peaks than the particulate columns, enhancing the peak height and improving the signal-to-noise ratio; (2) a higher injection volume (50 μL) than those used in the other methods, which are typically 10–20 μL. This injection volume does not provide notable peak broadening due to the relatively high flow rate used in the present method. The r^2 values obtained indicate a very good correlation of experimental data to calibration curves. The precision of both methods has been studied for retention times and areas at two different analyte concentration levels, and the results obtained at 600 ng mL^{-1} for most analytes, expressed as the percentage of relative standard deviation, are summarized in **Table 4**. It can be seen that the repeatability of peak areas for both methods was equal to or lower than 6%, the results for the LC-FL method being slightly higher than those obtained by applying the LC-DAD method. The precision was evaluated also at 200 ng mL^{-1}, finding that the results obtained ranged from 0.1 to 1.3% and from 0.3 to 4.3% for the in-day and interday, respectively, precisions of retention times and from 1.2 to 7.4% and from 3.2 to 9.7% for the in-day and interday, respectively, precisions of areas obtained using the FL method. For the DAD method, the results ranged from 0.07 to 1.5% and from 0.15 to 3% for the in-day and interday, respectively, precisions of retention times and from 0.5 to 7.5% and from 1.3 to 10.7% for the in-day and interday, respectively, precisions of areas. This difference could be explained by bearing in mind that LC-FL can be affected by more sources of variability of the results, such as the use of a second pump and the derivatization reaction.

Applications. LC-DAD and LC-FL methods were applied to the analysis of 15 wine samples belonging to different wine varieties (red, oloroso, rosé, and white). Samples W1−W5 were red wines, W6 was rosé wine, W7–W10 included oloroso wines, and W11–W15 were white wine samples. These wine samples belonged to Córdoba (Spain) (samples W1, W3, W4, W6, W10, W11, W14, and W15) and Sicily (Italy) (samples W2, W5, W7–W9, W12, and W13) geographical areas. Wine samples were diluted with distilled water to match the linear range of the calibration graph for each analyte. **Figures 5** and **6** correspond to the typical chromatograms achieved for a red wine sample using LC-DAD and LC-FL methods, respectively. As can be seen from **Figure 5**, there is an increase in the baseline of the chromatograms in the range of 22–24 min. It has been reported that this baseline drift can be ascribed to the presence of polymeric compounds, which have not been separated in the experimental conditions (*1*). However, the chromatogram corresponding to the LC-FL method (**Figure 6**) is cleaner than that obtained for the LC-DAD method. This demonstrates the higher selectivity level obtained using terbium-sensitized luminescence, which is less prone to baseline drifts and to the interference from other sample components. An example of the complementarity of the information supplied by both LC-DAD and LC-FL methods in the analysis of real samples is the determination of salicylic acid, which cannot be determined with accuracy in some wine samples using photometric measurements, due to the presence of an overlapping peak at the same retention time. However, this interference is not observed for the LC-FL method, which also gives satisfactory results when standards of salicylic acid are added. Thus, the LC-FL method is suitable for the identification and confirmation of some of the peaks found by LC-DAD. **Tables 5** and **6** summarize the phenolic content found for the Spanish and Italian wines analyzed, respectively; it can be seen that the highest values were found in red wine samples. The phenolics that form luminescent chelates with terbium(III) were determined using both LC-DAD and LC-FL methods, and the results obtained by both methods were statistically compared using a regression test. The regression study carried out with the concentration values obtained by both methods showed that they did not differ significantly taking into account the correlation parameters obtained ($Y = 0.185 + 0.979X$, $r = 0.954$, Y and X being the results for LC-FL and LC-DAD, respectively). *cis*-Resveratrol was not found in any of the samples. Sicilian red wines contain high concentrations of gallic acid, which agree with the values found in the literature (*2*). Protocatechuic and syringic acids are the most abundant phenolics found in white wine samples.

The recovery study was carried out by adding three different amounts of each analyte to four of the samples analyzed at concentrations in the range of 0.5–20 mg L^{-1} and subtracting the results obtained from similarly unspiked samples. **Table 7** shows the mean recovery values obtained, which were in the range of 68.0–116.9%, most of them being >85%, except those for rutin in sample W1 and syringaldehyde in sample W11, which gave values of 68%, although the values obtained for the other analyzed samples were closer to 100%. Also, the LC-DAD method gave results similar to those of the LC-FL method. The recoveries obtained for the rest of compounds were in agreement with those provided by other methods (*3, 6, 13, 14, 16*). In some of these methods, the accuracy was determined using standards or synthetic wine samples (*6, 13, 14*) and, therefore, the results cannot be directly compared with those obtained with the proposed method, which were obtained in the presence of commercial wine samples. Despite this fact, the results obtained were closer to 100% than those provided by other methods involving several extraction steps (*3*).

This study shows the usefulness of terbium-sensitized luminescence as a complementary tool for the identification and quantification of phenolic compounds in complex wine samples. This is the first time that aromatic aldehydes have been determined using terbium-sensitized luminescence. The spectral selectivity achieved using this approach can be profited to avoid or minimize the potential interferences from the sample matrix. The sensitivity levels achieved with both LC-DAD and LC-FL methods allow higher dilution of samples to be done, which is useful to improve the selectivity. The practical application of both methods has been shown by the analysis of 15 wine samples with satisfactory results.

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