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Validation of a Mass Spectrometry Method To Quantify Oak Ellagitannins in Wine Samples

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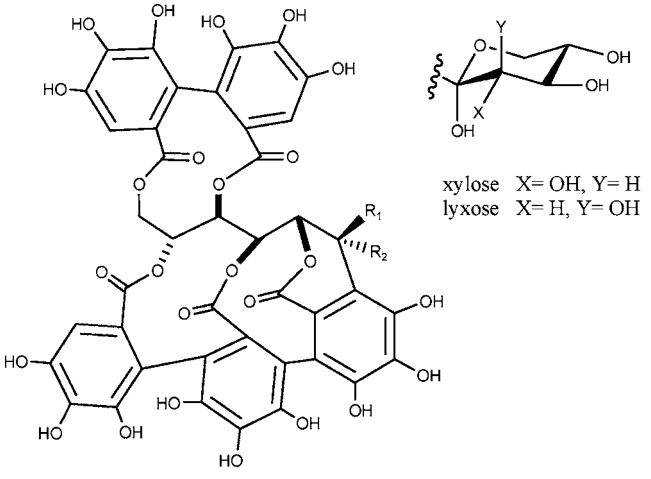
ABSTRACT: Detection and individual quantification of oak wood ellagitannins in oak barrel aged red wine samples are difficult mainly due to their low levels and the similarity between their structures. In this work, a quantification method using mass spectrometry has been developed and validated to quantify wine ellagitannins after sample fractionation with a previously reported method. The use of an internal standard is a requirement to correct mass signal variability. (−)-Gallocatechin, among the different tested compounds, was the only one that proved to be a suitable internal standard making possible the accurate and individual quantification of the main oak wood ellagitannins. The developed methodology has been used to detect and quantify these ellagitannins in different Spanish commercial wines, proving its usefulness.

KEYWORDS: *oak ellagitannins, red wine, mass spectrometry, quantification, internal standard*

INTRODUCTION

Oak wood barrels are commonly used for the aging of red wines to improve their overall quality. During the stay in the barrels, different kinds of compounds are released from the wood to the wine, thus affecting its organoleptic properties such as aroma, color, or astringency. Among these compounds, ellagitannins (Figure 1), with several hydroxyl functions in ortho positions, can take part in oxidation reactions, acting as consumers of oxygen and causing, among other reactions, the transformation of ethanol into acetaldehyde.[1] The acetaldehyde can, in turn, be involved in polymerization reactions between flavanols and between flavanols and anthocyanins,[1,2] affecting wine astringency and color, respectively. Furthermore, ellagitannins could modify wine astringency by themselves because they have the ability to precipitate proteins.[3,4] Recently, the direct involvement of these compounds in wine color by the formation of anthocyanin−ellagitannin hybrids has been postulated by Quideau and co-workers[5] and, in fact, they have been able to successfully achieve the hemisynthesis of malvidin-8-*C*-vescalagin and oenin-8-*C*-vescalagin.[5−7]



(**1**) Grandinin R_1= lyxose, R_2= H (**2**) Vescalagin R_1= OH, R_2= H
(**3**) Roburin E R_1= xylose, R_2= H (**4**) Castalagin R_1= H, R_2= OH

Figure 1. Structures of the main oak ellagitannins.

Ellagitannins can represent up to 10% of oak heartwood,[7,8] but their content depends on several factors such as oak species, age, and processing of wood in cooperage.[7,9−12] From a quantitative point of view, castalagin and vescalagin (Figure 1) are the most abundant ellagitannins in oak wood[12−14] but lyxose/xylose derivatives (grandinin and roburin E) and dimeric forms (roburins A, B, C, and D) are also present.[7,12,15,16] The hydroalcoholic nature of wine allows the extraction of these compounds from wood to wine, but due to their high reactivity, their levels in wine are much lower than could be expected. In addition, the chemical complexity of wines, with a large variety of compounds present in very different amounts, also makes their detection and quantification difficult. As occurs in the analysis of other wine constituents, fractionation of wine samples prior to the analysis can be a useful approach to overcome this problem. Although some fractionation methods have been specifically developed to isolate ellagic acid conjugates from grapes[17] or oak wood ellagitannins from wine,[18,19] they either fail to obtain the targeted compounds in only one fraction or are complex and time-consuming and require large amounts of sample.

Recently, a fractionation method based on that developed by Lee and co-workers[17] and modified according to the nature of the sample (wine samples) and to the nature of the targeted compounds (oak wood ellagitannins) has been developed in our laboratory.[20] This two-step fractionation method allows the obtaining of all the main oak wood ellagitannins in only one

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fraction and requires a low amount of sample (3 mL) and shorter times than those previously existing.[18] The subsequent analysis of the ellagitannin-rich fraction by HPLC-DAD-MS allows the individual identification of the different compounds. However, their individual quantification remains a challenge. First, the standards of oak wood ellagitannins are not commercially available, and some studies on ellagitannins and related compounds quantify any individual compound as ellagic acid.[17] Nevertheless, to obtain more accurate results, it is suitable to quantify each compound with its own standard, thus implying the isolation of these compounds from their sources. Different methods have been employed for this purpose.[4,13,14] Second, because the structures of the main oak wood ellagitannins are very similar (Figure 1) and, consequently, their chromatographic behavior, individual quantification from the chromatogram recorded at a given wavelength lacks accuracy. For this reason, quantification from HPLC-MS^n data, which allows knowledge of the area of the peak for each m/z ratio or for each transition, seems to be more suitable. However, mass data have great variability over the course of several days and, depending on the instrument, even within the same day. For this reason, when quantification is performed from mass data, an internal standard has to be used to correct this variability. A potential internal standard has to fulfill some requirements such as not to be present in the sample and to be structurally related to the compounds that have to be quantified, among others. Despite the structural differences, chlorogenic acid has been employed in previous studies[18,19,21] as internal standard for quantitative analyses of ellagitannins in different kinds of samples, among them, wine[18,19] in which this compound is absent.

Studies carried out in our laboratory following the existing quantitative methods[18,19] did not allow the obtaining of satisfactory results. For this reason, the objective of the present study was to develop and validate a new quantification method by mass spectrometry to be employed in the wine ellagitannin-rich fractions obtained with the previously developed fractionation method.[20] For this purpose, optimization of the mass analysis conditions for each of these compounds, selection of a suitable internal standard, and finally validation of the quantification method have been carried out. The ultimate objective of this study is to achieve an accurate and individual quantification method of the main oak wood ellagitannins in wine samples.

MATERIALS AND METHODS

Chemicals. Oak wood ellagitannin standards (grandinin (**1**), vescalagin (**2**), roburin E (**3**), castalagin (**4**)) were extracted and isolated from medium-toasted chips obtained from *Quercus petraea* (Matt.) Liebl. wood.[20] The identities of the ellagitannins were assigned from the data obtained in the HPLC-DAD-MS and in the ^{1}H and ^{13}C NMR analyses compared to those in the literature.[4,12,22,23] (−)-Gallocatechin, 3,5-dihydroxybenzoic acid, 4-hydroxybenzoic acid, and chlorogenic acid hemihydrate were purchased from Sigma-Aldrich (St. Louis, MO). 1,2,3,4,6-Penta-*O*-galloyl-β-D-glucopyranose (PGG) was purchased from Carbosynth (Berkshire, U.K.).

All of the used solvents were of analytical grade and were purchased from Prolabo (BDH) VWR International (Briare, France). The ultrapure water was obtained from a Direct-Q water purification system equipped with a Millipak 40 (0.22 μm) filter unit (Millipore, Billerica, MA).

HPLC-DAD-MS Analysis. HPLC-DAD analyses were performed in a Hewlett-Packard 1100 series liquid chromatograph (Agilent Technologies, Waldbronn, Germany). An AQUA C-18 reversed-phase, 5 μm, 150 mm × 4.6 mm column (Phenomenex, Torrance, CA) thermostated at 35 °C was used. The HPLC method has been previously developed in our laboratory for the analysis of oak wood ellagitannins.[20] Detection was carried out at 250 nm as the preferred wavelength. Spectra were recorded from 220 to 600 nm.

The mass spectrometer was connected to the HPLC system via the DAD cell outlet. MS detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple-quadrupole linear ion trap mass analyzer that was controlled by Analyst 5.1 software. Zero grade air served as nebulizer gas (50 psi) and turbo gas for solvent drying (400 °C, 60 psi). Nitrogen served as curtain (20 psi) and collision gas (high). Both quadrupoles were set at unit resolution. The ion spray voltage was set at −4500 V in the negative mode. The mass spectral method consisted of two mass experiments: Full mass analysis (EMS mode) was employed to obtain the signals at the m/z ratios corresponding to the ellagitannins and to the internal standard. Optimization of the conditions was carried out automatically by direct infusion of the ellagitannins. Full mass settings were tuned by direct infusion of a solution of castalagin. Settings used were as follows: declustering potential (DP), −95 V; entrance potential (EP), −7 V; collision energy (CE), −10 V. Multiple reaction monitoring analysis (MRM mode) was employed to detect the transitions (each parent ion−daughter ion pair) corresponding to the analyzed ellagitannins (castalagin (933/631), vescalagin (933/301), and grandinin and roburin E (1065/249)) and to the internal standard (gallocatechin (305/125)). The settings employed in the MRM analysis were tuned for each transition by direct infusion of a solution of the corresponding compound. Settings used for the selected transitions are shown in Table 1.

Table 1. Settings Used in MRM Analysis for Each Selected Transition

		parameter			
compound	transition	DP (V)	CE (V)	CXP[a] (V)	EP (V)
grandinin/roburin E	1065/249	−70	−68	0	−10
vescalagin	933/301	−100	−38	−28	−10
castalagin	933/631	−100	−38	−28	−10
gallocatechin	305/125	−100	−38	−28	−10

[a]CXP, collision cell exit potential.

Internal Standard Selection. Different compounds were tested as internal standards Five different solutions of castalagin in different concentrations (in a range from 1.5 to 12.5 mg L^{-1}) were prepared every day from the same stock solution of castalagin (concentration 100 mg L^{-1}) and were analyzed in triplicate on four nonconsecutive days, after the addition of the tested internal standard (each internal standard was tested separately): chlorogenic acid (5 mg L^{-1}), pentagalloylglucose (10 mg L^{-1}), 4-hydroxybenzoic acid (5 mg L^{-1}), 3,5-dihydroxybenzoic acid (5 mg L^{-1}), or gallocatechin (15 mg L^{-1}). The relationship between the castalagin and the internal standard (IS) signals in both mass modes employed (EMS and MRM modes) was determined by the following expression:

$$f = \frac{\text{ellagitannin signal/ellagitannin concn}}{\text{IS signal/IS concn}}$$

From these data, the intra- and interday coefficients of variation of the f ratios were calculated. In the same way, the intraday and interday coefficients of variation were also calculated for the internal standard signal and for the castalagin signal/castalagin concentration ratio.

Model Calibration Development. Calibration curves were built from the data resulting from triplicate HPLC-DAD-MS analyses of solutions of each ellagitannin at different concentrations. Gallocatechin was added as internal standard to all of the solutions in a concentration of 15 mg L^{-1}. To build grandinin, vescalagin, and roburin E calibration curves, seven levels of concentration (in a range from 7.5 × 10^{-2} to 5 mg L^{-1}) were analyzed. The calibration curve of castalagin has been

built in a wider range of concentrations (from 7.5×10^{-2} to 50 mg L^{-1}), including two higher concentration levels.

Two calibration curves were built for each ellagitannin from data supplied by mass spectrometry. The first of them was built using the signal obtained for each ellagitannin m/z ratio corrected with the signal obtained for the internal standard m/z ratio (EMS mode). The other was built using the signal obtained for the selected transition for each ellagitannin corrected with the signal obtained for the selected transition of the internal standard (MRM mode). In both cases, correction was done by calculating the s ratio as follows:

$$s = \frac{\text{ellagitannin signal}}{\text{IS signal/IS concn}}$$

The s ratio was plotted versus the ellagitannin concentration to build the calibration curves.

Validation of the HPLC-MS Method. To validate the HPLC-MS calibration methods, the following parameters were determined following the FDA guidance for the validation of analytical methods.[24]

Curve Adjustment. Coefficients of correlation were calculated, and an analysis of the variance of adjustments was carried out.

Accuracy. Recovery rates were calculated for all of the calibration models. A known concentration solution of each ellagitannin to which the internal standard was added was analyzed in triplicate. The concentration determined by means of the calibration model was compared to the real concentration of the standard through the calculation of the recovery rate ((determined concentration/real concentration) × 100).

Intra- and Interday Precision. To determine the intraday precision a series of castalagin solutions at nine concentration levels (from 0.4 to 25 mg L^{-1}) were analyzed in duplicate, and the values of the recovery rates were calculated using the corresponding calibration model. The coefficient of variation of these recovery rates was used to determine the intraday precision. The interday precision was determined in the same way, but in this case, the coefficient of variation of the recovery rates were calculated from the triplicate analysis at five concentration levels (from 1.5 to 12 mg L^{-1}) on three nonconsecutive days.

Detection (LOD) and Quantification (LOQ) Limits. To determine the LOD, a solution of each ellagitannin in a low concentration (signal-to-noise ratio between 2.5 and 5) was analyzed. The internal standard was added to each solution, which was then analyzed at least seven times. The s ratio was calculated for each analysis as previously described under Model Calibration Development. To calculate the LOD, the standard deviations of the s ratios were multiplied by a statistical factor based on the number of analyses as

$$\text{LOD} = t_{n-1;0,01} \times \text{SD}$$

where t_{n-1} is the statistical factor for $n-1$ degrees of freedom, n is the number of analyses performed, and SD is the s ratio standard deviation. LOD concentration was calculated from this value using the corresponding calibration model. The LOQ was calculated as 3 times the LOD.

Other Parameters. To determine the compound stability in the processed samples, the concentration of castalagin in a 2.5% acetic acid solution was determined both before and after 4 °C storage for 2 months. Moreover, the compound stability in the processed samples after the internal standard addition was also determined by quantifying castalagin in the same 2.5% acetic acid solution in presence of gallocatechin (15 mg L^{-1}) before and after 2 weeks of 4 °C storage.

Statistical Analysis. The SPSS 13.0 for Windows software package (SPSS, Inc.,Chicago, IL) was used for data processing.

Sample Analysis. Twelve Spanish commercial wines were analyzed, including red, white, and rosé wines, which were made from grapes of different varieties and aged for different periods using oak wood from different geographical origins.

Wines were fractionated with the method previously developed.[20] Gallocatechin was added as internal standard in a final concentration of 15 mg L^{-1} to the fraction containing the ellagitannins, which was then analyzed by HPLC-DAD-MS. Ellagitannin concentration was individually determined using the quantification models built on MRM mode analysis.

RESULTS AND DISCUSSION

Mass Parameter Optimization. Mass parameters were tuned to obtain the highest signal for the pseudomolecular ions or for the selected transitions of the analyzed compounds. The transitions (Table 1) were selected on the basis of the fragmentation pattern obtained in the MS^2 analysis for each compound. The most abundant fragment ion obtained in this analysis was selected as daughter ion. Because castalagin and vescalagin have similar structures and identical m/z ratios, their fragmentation patterns in mass spectrometry were, as expected, very similar. Both compounds showed main fragment ions at m/z 631 $[M - H - 302]^-$ and m/z 301 $[M - H - 632]^-$. The first one resulted from the loss of an ellagic acid moiety, whereas the second one corresponded to ellagic acid formed after the simultaneous loss of a glucose moiety and the nonahydroxytriphenoyl (NHTP) unit. Nevertheless, optimization of the conditions has allowed the obtaining of a specific fragmentation pattern for each compound where the most abundant fragment ion is different and with also different minor fragment ions. In the case of castalagin the main MS^2 ion was that at m/z 631 (100%), followed by that at m/z 301 (50%) and by that originated from the loss of a water molecule, $[M - H - H_2O]^-$ (m/z 915, 31%). On the contrary, in the case of vescalagin the main MS^2 ion obtained was that at m/z 301 (100%), followed by that at m/z 631 (55%) and by that at m/z 249, $[M - H - 684]^-$ (52%). Thus, it was possible to monitor a different transition for each of these two compounds, making the quantification method more selective. This differentiation between isomers was not possible for grandinin and roburin E. In this case, the modification of the conditions did not yield differences in their fragmentation patterns, obtaining in both cases a major fragment ion at m/z 249. However, the previous separation by HPLC allows the individual quantification of these compounds from the signals obtained for the same transition.

Table 2. Coefficients of Variation of Castalagin Signal/Concentration Ratio, the Internal Standard Signal, and the f Ratio Intra- and Inter-Day in Both Mass Modes

compound	day 1	day 2	day 3	day 4	interday
			EMS Mode[a]		
castalagin	8.8	5.3	18.1	25.0	71.9
chlorogenic acid	12.1	7.7	12.0	20.7	54.7
f ratio	9.3	10.1	17.4	21.6	47.3
PGG	5.0	6.0	6.6	12.6	62.5
f ratio	6.9	8.9	12.4	15.3	29.8
			MRM Mode[b]		
castalagin	12.1	5.1	23.7	24.0	76.3
chlorogenic acid	5.8	1.7	17.3	20.5	30.2
f ratio	15.9	5.5	21.5	23.2	58.8
PGG	6.2	13.2	7.7	15.8	46.7
f ratio	5.8	4.3	11.8	13.1	19.8

[a]Full mass analysis. [b]Multiple reaction monitoring.

Internal Standard Selection. Mass signals have great variability, making necessary the use of an internal standard to allow the obtaining of reproducible results over time. In this work, different compounds were tested as internal standard to correct the variability in the ellagitannin signal and to use mass

Table 3. Calibration Curves Built for Grandinin, Vescalagin, Roburin E, and Castalagin in both EMS and MRM Modes

ellagitannin	concentration range analyzed (mg L^{-1})	mass mode analysis	model calibration equation	correlation coefficient
grandinin	$7.5 \times 10^{-2} - 5$	EMS	$y = 1.8072x^2 + 0.0291x + 3 \times 10^{-6}$	0.9990
		MRM	$y = 9.8641x^2 + 0.1189x + 6 \times 10^{-6}$	0.9993
vescalagin	$7.5 \times 10^{-2} - 5$	EMS	$y = 0.341x^2 + 0.0185x$	0.9990
		MRM	$y = 0.2544x^2 + 0.0032x + 4 \times 10^{-7}$	0.9993
roburin E	$7.5 \times 10^{-2} - 5$	EMS	$y = 1.7758x^2 + 0.0638x + 4 \times 10^{-6}$	0.9991
		MRM	$y = 10.323x^2 + 0.2616x + 7 \times 10^{-6}$	0.9993
castalagin	$7.5 \times 10^{-2} - 50$	EMS	$y = 0.2941x^2 + 0.2559x$	0.9997
		MRM	$y = 5.6273x^2 + 0.249x$	0.9997

Table 4. Recovery Rates Calculated for Each Ellagitannin by Means of Both Quantification Modes

	recovery rate (%)	
ellagitannin	EMS mode	MRM mode
castalagin	100.7 ± 1.2	100.6 ± 1.0
vescalagin	100.1 ± 1.2	100.7 ± 1.1
grandinin	99.6 ± 1.2	99.3 ± 0.9
roburin E	99.3 ± 1.0	99.5 ± 0.7

Table 5. LOD and LOQ Calculated for Each Ellagitannin by Means of Both Quantification Modes

ellagitannin	no. of analyses, *n*	mass mode analysis	LOD (mgL^{-1})	LOQ (mgL^{-1})
castalagin	10	EMS	6.6×10^{-3}	2.0×10^{-3}
		MRM	4.1×10^{-4}	1.2×10^{-4}
vescalagin	9	EMS	1.8×10^{-3}	5.3×10^{-3}
		MRM	1.5×10^{-3}	4.6×10^{-3}
grandinin	9	EMS	1.8×10^{-3}	5.3×10^{-3}
		MRM	1.7×10^{-3}	5.0×10^{-3}
roburin E	9	EMS	1.8×10^{-3}	5.4×10^{-3}
		MRM	1.7×10^{-3}	5.1×10^{-3}

spectrometry to quantify these compounds accurately and precisely in low concentrations. Chlorogenic acid has been employed previously as internal standard in quantitative analysis of oak ellagitannins in wine samples,[18,19,21] and for this reason it was the first compound to be tested. The tests conducted were designed to find a stable relationship between the ellagitannin and the internal standard mass signals. The results obtained (Table 2) showed that there was a high variability in the signal corresponding to castalagin and also in those corresponding to the internal standard. The calculated *f* ratio also showed a high variability both intra- and interday and, in some cases, higher than the ellagitannin signal variability. Although in both mass methodologies employed there was a great variability, it could be seen that the variability found in MRM mode was in most cases lower than that found in EMS mode, pointing out that full mass signal seems to be less stable. The results of these first experiments with chlorogenic acid indicated that the use of this compound did not allow the correction of the variability of the ellagitannin signal. This is probably due to the difference between its structure and that of the ellagitannins, but it might also be related to differences in the composition of the mobile phase at the moment of the mass analysis because, with the chromatographic method employed in this study,[20] chlorogenic acid elutes much later and with higher percentages of methanol than ellagitannins. Furthermore, during these experiments a decrease with time in the levels of castalagin in the solutions that contained chlorogenic acid was observed, whereas the same solutions in the absence of chlorogenic acid were stable, as can see below

Table 6. Ellagitannin Concentrations Quantified in Different Spanish Commercial Wines

wine	grape variety	aging time (months) barrel/bottle[a]	[grandinin] (mg L^{-1})	[vescalagin] (mg L^{-1})	[roburin E] (mg L^{-1})	[castalagin] (mg L^{-1})	total ellagitannin content (mg L^{-1})
1 (red)	Tempranillo	4 (A)/12	3.4 ± 0.5	4.45 ± 0.03	1.6 ± 0.1	10.5 ± 0.5	20 ± 1
2 (red)	Tempranillo	6 (A)/12	0.36 ± 0.03	0.90 ± 0.07	0.18 ± 0.01	2.24 ± 0.09	3.7 ± 0.2
3 (red)	Graciano	12 (F)/8	3.55 ± 0.03	6.4 ± 0.2[b]	2.08 ± 0.07	11.8 ± 0.2	23.8 ± 0.5
4 (red)	Tempranillo	12 (F)/8	3.4 ± 0.1	3.7 ± 0.3	1.73 ± 0.04	10.5 ± 0.2	19.4 ± 0.6
5 (red)	Tempranillo	12 (A)/6	2.2 ± 0.1	1.73 ± 0.05	0.96 ± 0.01	4.43 ± 0.08	9.3 ± 0.2
6 (red)	98% Tempranillo, 2% Cabernet Sauvginon	4 (A)/24	1.37 ± 0.08	0.63 ± 0.03	0.490 ± 0.008	2.83 ± 0.03	5.3 ± 0.2
7 (red)	Tempranillo	6 (F,A)/24	0.88 ± 0.02	0.39 ± 0.03	0.133 ± 0.008	0.92 ± 0.03	2.33 ± 0.08
8 (red)	Tempranillo	12 (A)/12	0.73 ± 0.02	0.194 ± 0.002	0.158 ± 0.005	1.07 ± 0.04	2.15 ± 0.06
9 (red)	Tempranillo	18 (F)/12	0.58 ± 0.05	0.40 ± 0.02	0.115 ± 0.008	0.61 ± 0.01	1.71 ± 0.09
10 (red)	Tempranillo	12 (A)/24	1.21 ± 0.06	1.91 ± 0.06	0.39 ± 0.01	4.0 ± 0.3	7.5 ± 0.4
11 (white)	Chardonnay	3 (A)/12	0.061 ± 0.001[b]	0.14 ± 0.02	<LOQ	0.328 ± 0.008	0.53 ± 0.03
12 (rosé)	50% Tempranillo, 50% Merlot	3 (C)/12	3.5 ± 0.1	2.3 ± 0.1	1.5 ± 0.1	4.69 ± 0.08	12.0 ± 0.4

[a](A) American oak; (F) French oak; (C) Caucasian oak. [b]Values calculated by extrapolation.

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(see Other Parameters under Model Validation). Thus, chlorogenic acid was not a suitable internal standard for the ellagitannin quantification by mass spectrometry.

Pentagallolylglucose (PGG) was then selected as a possible internal standard, because its structure is more similar to those of oak ellagitannins than chlorogenic acid. PGG possesses a molecule of glucose esterified in its hydroxyl groups by gallic acid moieties as oak ellagitannins do. To test its usefulness as internal standard, the same experiments as those performed in the case of chlorogenic acid were carried out. The values obtained (Table 2) for the coefficient of variation of the f ratio were lower than those obtained in the case of chlorogenic acid, pointing out a better correction of the castalagin signal variability by using the PGG signal. However, the obtained values were still too high to allow the quantification.[24] In this case, the difference between the castalagin and the PGG chromatographic retention times might be the reason for the great variability found in the mass signal relationship. Due to the lower polarity of PGG in relation to ellagitannins, the methanol percentage in the mobile phase with which PGG eluted is higher than in the case of ellagitannins, which eluted with 100% aqueous mobile phase. As a consequence, there is a great difference in the ionization process of these compounds despite their similar structures.

For this reason, different phenolic compounds that could elute with a predominantly aqueous mobile phase were tested as internal standards. 4-Hydroxybenzoic acid, 3,5-dihydroxybenzoic acid, and gallocatechin showed low retention times, eluting with mobile phases with methanol percentages lower than 5%. The same experiments as in the previous cases were carried out. Among the compounds assayed, only gallocatechin was able to correct the high variability of the ellagitannin mass signal. For this reason the methods built using gallocatechin as internal standard were validated for all oak ellagitannins that were going to be quantified, providing low values of the coefficient of variation, both intra- and interday, as can be seen below underModel Validation. Although gallocatechin can be found in wine samples, with the present methodology ellagitannin quantification is performed after submitting wine samples to the fractionation method previously developed.[20] Thus, after sample fractionation, ellagitannins and wine native gallocatechin are present in different fractions. Owing to this, gallocatechin can be used as internal standard in the ellagitannin quantification by mass spectrometry, being added to the ellagitannin-rich fraction after wine fractionation.

Model Calibration Development. Table 3 shows the obtained results, that is, the calibration curves built for grandinin, vescalagin, roburin E, and castalagin in both EMS and MRM modes, as well as the correlation coefficients and the range of concentration for each ellagitannin. The calibration curve of castalagin has been built in a wider range of concentrations including two higher levels because of the concentrations usually reported in wines for castalagin,[18] which are higher than those found in the cases of grandinin, vescalagin, and roburin E.

MRM analysis, due to its high selectivity and sensitivity, is the most appropriate mass methodology to perform the quantification. Nevertheless, some mass spectrometers do not allow the simultaneous monitoring of more than one mass transition. For this reason, the possibility of carrying out the quantification using the mass signal obtained in the full mass analysis for each m/z ratio was also assayed.

Data were fitted to different calibration models, and the best results were obtained for a quadratic model (Table 3). The quadratic trend is more evident at the lowest concentrations, and because ellagitannins are normally found in wines in low concentrations, this type of adjustment seems to be the most appropriate to build the calibration curves.

Model Validation. Both developed methods, those built from the signal obtained in EMS analyses and those built using mass signals obtained in the MRM analyses, were validated. Validation was carried out following FDA guidelines to validate analytical methods.[24] The following parameters were determined.

Calibration Curve Adjustment. The correlation coefficients were >0.999 in all cases. Moreover, the analysis of the variance of adjustments showed that all of them were statistically significant ($p < 0.01$).

Accuracy. All of the recovery rates obtained did not show statistically significant differences with a 100% recovery value (Table 4).

Intra- and Interday Precision. In the case of intraday precision, the value of the coefficient of variation when the signal used was that obtained in the full mass analysis was 2.1%, whereas when the signal used was that obtained in the MRM analysis, it was 1.5%.

The values obtained for the interday coefficients of variation were 5.8 and 5.2% when the signal employed was that obtained in the full mass analysis or in the MRM analysis, respectively.

The obtained values were within FDA criteria acceptance.[24] Furthermore, it can be observed that in models built from the MRM analysis there was a lower variability than those built from full mass analysis, as a result of the greater selectivity of the former mass methodology.

LOD and LOQ. Table 5 shows the values of the LOD and LOQ obtained in both models for each ellagitannin. The obtained values of LOD and LOQ were on the order of the LOD and LOQ values existing in the literature.[19] It can be observed that models built from the signal obtained on the MRM analysis provided the lowest detection and quantification limits, as a result of the higher sensitivity of this mass methodology. In both cases they were lower than the usual concentration levels of ellagitannin in wine samples.[19,25]

Other Parameters. To achieve an accurate quantification of oak ellagitannins in wine samples, they have to be isolated from other wine components by the fractionation method previously developed.[20] As a result, ellagitannins are isolated in a 2.5% acetic acid solution. Often, the samples are stored because they are processed one day and analyzed by HPLC-MS on a different day. The compound stability in the processed samples was determined, and the results showed that there were no statistically significant differences between the concentration determined before and after the storage, pointing out the processed sample stability (data not shown).

Moreover, as previously mentioned, the internal standard can affect the ellagitannin stability. The compound stability in the processed samples after internal standard addition was also determined, and the results showed that gallocatechin did not affect castalagin stability because there were no statistically significant differences in its concentration before and after storage (data not shown).

Ellagitannin Determination in Wine Samples. To prove the versatility of the developed analysis method, the ellagitannin content of 12 Spanish commercial wines was determined (Table 6). Selected wines were made from grapes

of different varieties and aged during different periods using oak wood from different geographical origins (French oak (F), American oak (A), or Caucasian oak (C)). Ten red wines, one white wine, and one rosé wine were fractionated with the method previously developed,[20] and their ellagitannin contents were determined by the calibration model developed in the present work. Individual quantification of ellagitannins was carried out by the corresponding MRM calibration models as they are the most accurate and precise and allow the quantification of low ellagitannin amounts. Regardless of the variety of grape employed in wine elaboration or the geographical origins of the oak employed for wine aging, it was possible to quantify ellagitannins in all cases except one, for which roburin E could be detected but in amounts below the LOQ. Despite the high variability in the observed values, the determined amounts of oak ellagitannins in wines were similar to those previously reported in other published studies.[4,19,25]

In conclusion, the mass spectrometry method developed has been validated for the individual quantification of oak ellagitannins in wine after sample fractionation. Different compounds have been tested as internal standards, and among them, only gallocatechin has provided good results. The present method allows the accurate and precise quantification of oak ellagitannins with low detection and quantification limits. The best results in the validation process have been achieved with MRM analysis, showing the highest precision and the lowest detection and quantification limits. This validated methodology has been successfully applied to the individual detection and quantification of the main oak ellagitannin in different commercial wines.

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