Estimation of Triterpenes in Wines, Spirits, and Oak Heartwoods by LC–MS

Pedunculate *Quercus robur* L. and sessile *Q. petraea* Liebl oak heartwoods are used commonly in the aging process of wine and spirits. During maturation, the ethanol content of these beverages extracts bitter and astringent compounds such as triterpenes. The authors have extracted, separated, and quantified four major triterpenes from heartwoods using a liquid chromatography–mass spectrometry method in a selected-ion monitoring mode. They used echinocystic acid as an internal standard. Because of their hydroxyl and carboxyl functions, these molecules provided intense deprotonated molecular ions. The authors discuss reproducibility and detection limits and apply the proposed method to the quantification of triterpenes in wines, spirits, and heartwoods of several origins.

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Traditionally, oak woods from *Quercus alba, Q. robur* L., and *Q. petraea* Liebl are used in cooperage to make aging barrels for wine and spirits. Lignins and hydrolyzable tannins are the main extractable compounds that are released by wood during maturation, and they play a role in the flavors of these beverages (1–3). Nevertheless, the role of other compound classes such as triterpenes never has been reported.

Triterpenes commonly are found in the plant kingdom and are of interest because of their health-promoting effects in plant drugs and folk medicine (4). Moreover, the range of the taste properties of these compounds has come to be appreciated (5).

Zepernick and Langhammer (5) focused on the presence of an acidic triterpene (triterpene 1, see Figure 1) named bartogenic acid. Triterpene 1 had been isolated for the first time in an Indian plant, *Barringtonia speciosa,* which was used as a traditional drug in indigenous medicine (6). More recently, Romussi and co-workers (7) discovered the presence of triterpene 1 and its glycoside, triterpene 2, in *Q. robur* L. *var. stenocarpa* oak leaves. The advent of complementary techniques such as liquid chromatography (LC)–electrospray ionization mass spectrometry (MS) in the negative ion mode and two-dimensional nuclear magnetic resonance spectroscopy allowed us to purify and determine structures of triterpenes 1–4 in pedunculate oak heartwood (8). Our main interests are in the acid triterpene content of oak heartwoods used in cooperage, their astringent properties, and their occurrence in wines and spirits.

In this article, we report a highly sensitive and quantitative detection method of four triterpenes in oak heartwoods, wines, and spirits. Because of the lack of UV absorbance and the presence of two acidic groups on the compounds, LC–electrospray ionization MS is well suited for monitoring acidic triterpenes (9). Moreover, LC–electrospray ionization MS is especially powerful for determining high molecular weight compounds such as glycosylated triterpenes, which are polar and cannot be determined by other methods such as gas chromatography (GC)–MS without destruction (9–12).

At the beginning of our study, we established a reliable and fast method for purifying triterpenes in woods. To avoid long extraction and cleanup processes, we performed direct solid–liquid extractions using diethyl ether–powder and ethyl acetate–powder and varying both the extraction strengths of the organic solvents and the mesh of the wood powders. We therefore

intended to develop a LC–MS assay adapted to triterpene 1–4 quantification through calibration plots of triterpenes 1–4 and standard addition in diethyl ether and ethyl acetate samples supplemented with an internal standard, echinocystic acid. Our goal was to establish this methodology as a powerful tool for determining triterpenes in woods and beverages. The liquid–liquid extractions showed good selectivities because diethyl ether extracted first the triterpene aglycones, whereas ethyl acetate extracted the glycosides.

Experimental

Sample calibration curve: To begin, we prepared working standard solutions of pure triterpenes 1 and 2 and their corresponding glycosides, triterpenes 3 and 4. The characterization and the purification of the substances used has been described elsewhere (8). We used the internal standard method to ascertain the linear relationship between quasimolecular ion peak selected-ion recording areas of triterpene 1–4 solutions on selected-ion recording chromatograms and concentrations. Triterpene 2 was present in two isomeric forms, which were integrated together. The retention times for all the compounds were 28.5 min for triterpene 1, 19.5 and 21.0 min for triterpene 2, 15.0 min for triterpene 3, 9.0 min for triterpene 4, and 30.0 min for the internal standard. The molecular weights of the compounds were 517, 533, 679, and 695, respectively, for triterpenes 1–4 and 633 for the internal standard.

We added triterpenes 1–4 to 1 mL of the internal standard solution, which was echinocystic acid-3-glucoside at 50 mg/L in methanol, to prepare two sets of calibration solutions — one set of aglycone analytes (triterpenes 1 and 2) was prepared at 80, 40, 20, 10, 5, 2.5, and 1.25 mg/L and a second set of analytes (triterpenes 3 and 4) at 80, 40, 20, 10, 5, 2.5, and 1.25 mg/L. We established one set of calibration curves for triterpenes 1 and 2 and one set for triterpenes 3 and 4. We scanned and recorded intensities of ions *m/z* 633 (internal standard), *m/z* 517 (triterpene 1), *m/z* 533 (triterpene 2), *m/z* 679 (triterpene 3), and *m/z* 695 (triterpene 4) in negative mode using a dwell time of 0.2 s with a capillary voltage of 3500 V. The average highest sensitivity for triterpenes 1–4 corresponded to a cone voltage value of −30 eV. These conditions produced intense traces of the molecular ions $[M - H]^-$ (triterpenes 1–4) without generating fragments, especially for the glycosidic triterpenes 3 and 4.

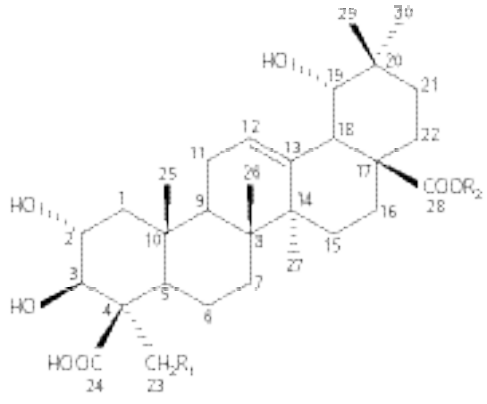
Isolation of triterpenes from woods: We first tried a single-step extraction and purification process for wood sample preparation. To extract selectively aliphatic compounds such as triterpenes, we performed macerations between wood powders (60 g, 60 mesh) and several organic solvents such as water–ethanol solutions, diethyl ether, and ethyl acetate. Despite exhibiting a lower extraction power, the diethyl ether and ethyl acetate extractions showed greater selectivity than the water–ethanol extractions. We chose to perform a first extraction with diethyl ether on wood, and this extraction yielded the triterpene aglycones (triterpenes 1 and 2). A second extraction with ethyl acetate on the remaining solid yielded the glycosylated triterpenes (triterpenes 3 and 4). The corresponding solutions were evaporated, freeze-dried, weighed, and stored in a dessicator at ambient temperature.

Isolation of triterpenes from spirits: We extracted triterpenes 1–4 from six Armagnac brandies (from the Gers region of France) matured in barrels and from different vintages. The extraction procedures were identical for all the samples: We first evaporated the ethanol content (approximately 40%) from these spirits (200 mL initial volume of each spirit). The resulting aqueous solutions were extracted successively with diethyl ether (seven times with 200 mL), which extracted triterpenes 1 and 2, followed by ethyl acetate (seven times with 200 mL), which extracted triterpenes 3 and 4.

LC–MS instrumentation for quantification: We prepared LC–MS samples by dissolving approximately 4 mg of extract in the internal standard solution described above. We injected 40 µL of each sample. LC–electrospray ionization MS was performed on a Micromass Platform II quadrupole spectrometer (Manchester, United Kingdom) equipped with an Agilent series 1100 high performance liquid chromatograph (Agilent Technologies, Palo Alto, California). We used a 250 mm × 4.6 mm, 5-µm d_p Utrasphere C18 ODS reversed-phase column (Beckman Coulter, Fullerton, California).

We developed a gradient in which solvent A was 95:5 (v/v) water–acetic acid and solvent B was 95:5 (v/v) methanol–acetic acid at a flow rate of 1 mL/min and an injection volume of 40 µL. The time program for analytic gradient elution was 0 min at 50% solvent B, 5 min at 50% solvent B, 12 min at 65% solvent B, 29 min at 68% solvent B, 31 min at 100% solvent B, and 36 min at 100% solvent B, successively.

Concerning the MS procedure, we performed full scans from *m/z* 200 to *m/z* 1500. The capillary temperature and voltage were maintained at 120 °C and 3.5 kV, respectively. The nitrogen drying gas flow was 220 L/h, and electrospray ionization nebulizing gas (also nitrogen) was 9 L/h. The pressure in the analyzer was 500 bar. Concerning identi-



Triterpene		R_1	R_2
1	2α,3β,19α-Trihydroxyolean-12-ene-24,28-dioic acid	H	H
2	2α,3β,19α,23-Tetrahydroxyolean-12-ene-24,28-dioic acid	OH	H
3	28-β-D-Glucopyranosyl-2α,3β,19α-trihydroxyolean-12-ene-24,28-dioic acid	H	β,D-Glucopyranosyl
4	28-β-D-Glucopyranosyl-2α,3β,19α,23-tetrahydroxyolean-12-ene-24,28-dioic acid	OH	β,D-Glucopyranosyl

Figure 1: Molecular structures of the triterpenes studied.

fication and quantification, we performed both total-ion chromatograms and selected-ion recording as follows: we scanned mass spectra in the negative ionization mode with a cone voltage of either 30 eV or 75 eV to induce fragmentation of the parent ions. For quantification, we used the same conditions that were used for the calibration curves (confer 1.1); that is, selected-ion recording mode with −30 V for the cone voltage. Analytical data were acquired by MassLynx 3.4 software (Micromass).

Linearity of calibration diagram: We established linear regression calibration curves by plotting the peak area ratios of triterpenes 1–4 to echinocystic acid versus standard concentration. The response coefficients (K) between triterpenes (X) and internal standard (EI) was calculated using the following equation:

$$K_{X/EI} = \frac{[X] \cdot A_{EI}}{[EI] \cdot A_X} \quad [1]$$

where [X] is the concentration of triterpene X, [EI] is the concentration of the internal standard or 50 mg/L, A_X is the peak area of triterpene X as determined by selected-ion recording, and A_{EI} is the peak area of the internal standard as determined by selected-ion recording. The peak areas were integrated manually.

We obtained mean relative error for response coefficients K by making five measurements of the four concentrations used to build the calibration curve (Table I).

The second step was to analyze the samples (wines and spirits) to quantify their triterpene content. We analyzed these samples using the same internal standard concentration of 50 mg/L. The concentration of X in sample (vial) becomes [X]′ and is calculated with the following formula:

$$[X]' = [EI] \cdot K_{X/EI} \left(\frac{A'_X}{A'_{EI}} \right) \quad [2]$$

where [X]′ is the concentration of X in the sample vial, [EI] is the concentration of the internal standard or 50 mg/L, $K_{X/EI}$ is the response coefficient between X and EI, A'_X is the peak area of triterpene X in the sample as determined by selected-ion recording, and A'_{EI} is the peak area of the internal standard in the sample as determined by selected-ion recording.

The concentration of X in bottles of wines and spirits $[X]_{arm}$ was deduced by

$$[X]_{arm} = M_r^{ES} \left(\frac{[C^{X'}_{vial}]}{M^{ES}_{vial}} \right) \quad [3]$$

where $[X]_{arm}$ is the concentration of X in the bottles measured in grams per liter, M_F^{ES} is the mass of ethyl acetate or diethyl ether obtained from 200 mL of wine or brandy measured in milligrams, $C^{X'}_{vial}$ is the concentration of X in the vial, and M_F^{ES} is the mass of powder in the vial measured in milligrams.

We calculated the triterpenes in heartwood powders by taking into account the mass of initial wood used (60 g).

Method validation: We performed a series of diethyl ether and ethyl acetate fractions of wine and Armagnac sample analyses to quantify their triterpene contents. Triterpenes 1 and 2 were extracted and quantified in the diethyl ether fractions, whereas triterpenes 3 and 4 were extracted and quantified in the ethyl acetate fraction. The coefficient responses (average values, n = 5, the same day) were 0.9 for triterpene 1, 2.73 for triterpene 2, 6.78 for triterpene 3, and 4.31 for triterpene 4.

To assess the repeatability, we quantified the same solution of triterpenes 1–4 eight times during the same day. The standard deviations obtained were 1.5% for triterpene 1, 1.1% for triterpene 2, 1.6% for triterpene 3, and 1.1% for triterpene 4. These results showed that the method is repeatable within the same day.

The reproducibility assessment was performed by quantifying the same solution each day during three days. The standard deviations obtained were 4% for triterpene 1, 3.9% for triterpene 2, 2.3% for triterpene 3, and 0.5% for triterpene 4, which displayed low variations between days.

Limits of detection and quantification: We calculated the limits of detection and quantification by analyzing control solutions (free of triterpene 1) and solutions spiked with concentrations of triterpenes 1–4. For method validation, the limits of quantification and detection should correspond to the concentration for which the signal-to-noise ratios are less than 3 and 10, respectively. The detection limits we obtained were 5.5 mg/L for triterpene 1, 6.0 mg/L for triterpene 2, 3.0 mg/L for triterpene 3, and 1.5 mg/L for triterpene. The limits of quantification were 7.0 mg/L for triterpene 1, 8.0 mg/L for triterpene 2, 6.0 mg/L for triterpene 3, and 2.5 mg/L for triterpene 4.

Table I: Response coefficient K for triterpenes 1–4*

Triterpene	K (Mean)	Standard Deviation	Relative Standard Deviation (%)	R^2
1	0.9	0.07	7.7	0.9988
2	2.73	0.1	3.8	0.9984
3	6.78	0.06	0.9	0.9998
4	4.31	0.09	2.1	0.9996

*n = 5. Initial concentrations measured in milligrams per liter.

Results and Discussion

Optimization of LC–MS quantification method: The LC–electrospray ionization MS experiments first were performed in both positive and negative mode under acidic conditions in the mobile phase. In terms of sensitivity, acidic triterpenes displayed stronger signals in the negative mode. We chose the final mobile phases — 95:5 (v/v) water–acetic acid and 95:5 (v/v) methanol–acetic acid — as compromises between good chromatographic separation and good electrospray negative ionization. Our major problem was finding a suitable internal standard; among the many tested, we found that echinocystic acid-3-glucoside, an acidic triterpene glucoside, was the most appropriate.

LC–MS identification in wines, spirits, and heartwoods: Using this method, we identified triterpenes 1–4 in a selection of commercial wines, spirits, and heartwood powders. Figure 2 shows a quantitative LC–electrospray ionization MS chromatogram (selected-ion recording mode) corresponding to a diethyl ether extract of an Armagnac brandy (V.S.O.P.). The internal standard was the 50 mg/L, m/z 633 echinocystic acid-3-glucoside. The aglycone triterpenes 1 and 2, which are extracted by diethyl ether, displayed weak signals in red and white wines, whereas those signals were stronger in spirits and heartwood extracts. Indeed, because of the higher percentage of ethanol in spirits compared with wines and because of the maturation time in barrels (two years for spirits, six months for wines), triterpenes 1 and 2 were extracted from spirits much more efficiently.

Figure 3 shows the mass spectra for triterpenes 1–4 in the diethyl ether and ethyl acetate fractions of the V.S.O.P. Armagnac

brandy. These spectra were determined in the total-ion current mode. The highest selected-ion recording peak areas were obtained at a cone voltage of 30 V, which we chose for quantification experiments in selected-ion recording mode. Triterpenes 1–4 provided very intense deprotonated molecular ions $[M - H]^-$, and thanks to the intense responses of the base peaks, we easily could determine the molecular masses of triterpenes 1–4. The aglycone triterpenes 1 and 2 also yielded intense dimeric quasi-molecular ions at *m/z* 1035 and *m/z* 1067, representing 80% and 50% of their base peak signals *m/z* 517 and *m/z* 533, respectively. They corresponded to the weak association of two molecules because their signals corresponded to $[2M - H]^-$ species. When the cone voltage was set at 75 V, the deprotonated ions of triterpenes 3 and 4 generated daughter ions (*m/z* 517 and *m/z* 533, respectively), which corresponded to losses of the glycosidic linkages (loss of 162 amu). To collect more structural information, we tried to favor fragmentations with increasing cone voltage (as high as 110 V), but the fragmentation of the glycosidic link was the only one we observed.

Estimation in wines, spirits, and heartwoods: We analyzed 10 red wines, 5 white wines, and 6 Armagnac brandies in duplicate for triterpenes 1–4. Table II lists the results from quantitation of triterpenes 1–4 in wines and spirits. The presented data are average values of duplicate samples of wines or Armagnac brandies. They show a large degree of quantitative variation between wines and, logically, between wines and spirits. Triterpenes are quite hydrophobic compounds that are efficiently dissolved and extracted by high alcohol content solutions. Because Armagnac brandies are matured at high ethanol content (55–60%) in new oak barrels for two years, triterpenes will be extracted much more efficiently in these spirits than in wines (12% ethanol). Indeed, triterpenes 1 and 2 average contents (concentration ± standard deviation) vary between 11 ± 0.4 and 69.2 ± 2.6 mg/L, 7.1 ± 0.7, and 34.9 ± 3.4 mg/L, respectively in spirits, whereas the maximum level in wines hardly reaches 5.4 ± 0.5 mg/L; their levels in spirits are 100-fold greater than those found in white and red wines.

In terms of content, the aglycone triterpenes (triterpenes 1 and 2) are the most extracted in wines and spirits, and they are accompanied by minor amounts of glycosidic triterpenes (triterpenes 3 and 4) (trace amounts in wines). In one of the red wines (Burgundy 1996), we found a small amount

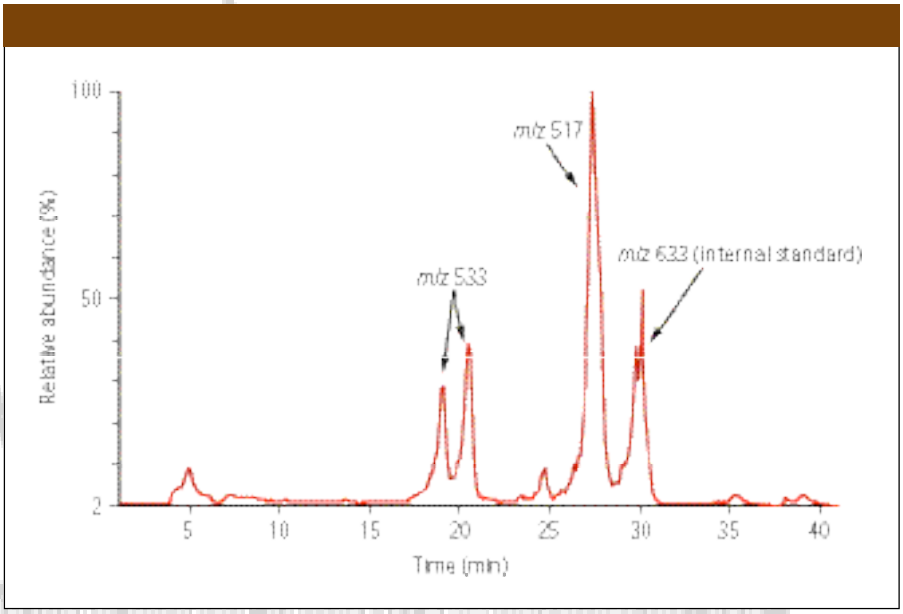


Figure 2: LC–electrospray ionization MS separation of triterpene aglycones of V.S.O.P. Armagnac brandy (diethyl ether fraction) in selected-ion recording mode on quasimolecular ions $[M - H]^-$ of *m/z* 517, 533, and 633.

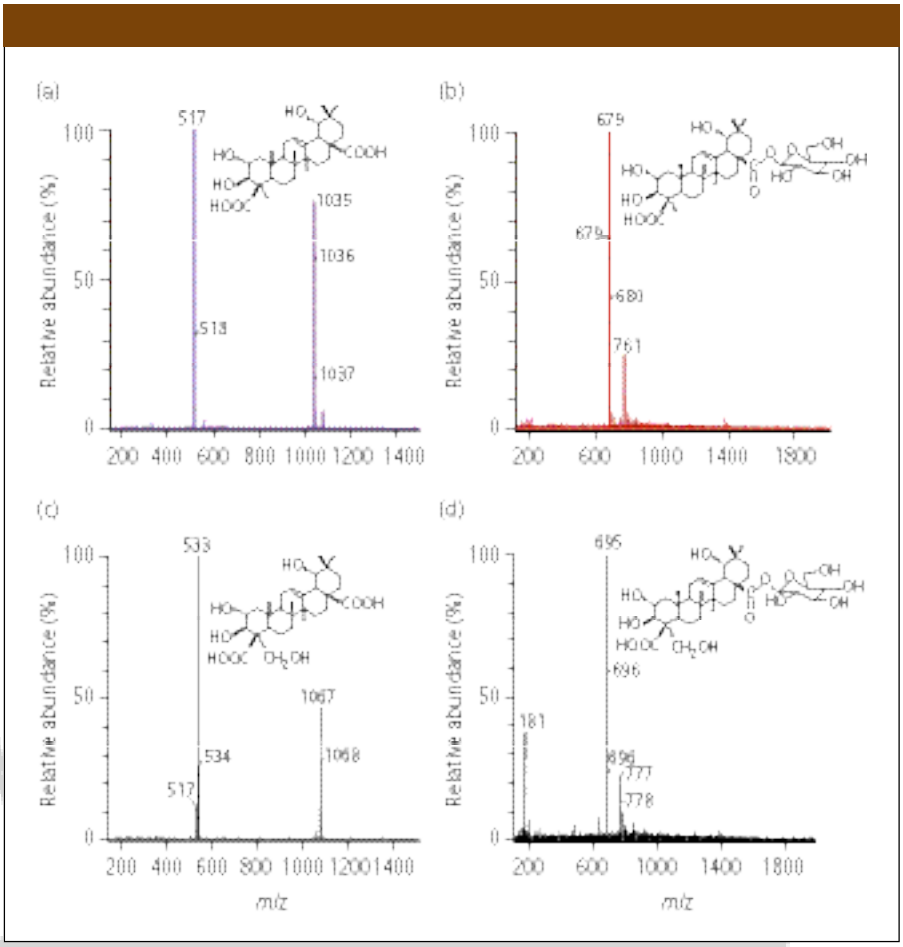


Figure 3: Mass spectra of the deprotonated (a) 2α,3β,19α-trihydroxyolean-12-ene-24,28-dioic acid (triterpene 1, *m/z* 517), (b) 28-β-D-glucopyranosyl-2α,3β,19α-trihydroxyolean-12-ene-24,28-dioic acid (triterpene 3, *m/z* 679), (c) 2α,3β,19α,23-tetrahydroxyolean-12-ene-24,28-dioic (triterpene 2, *m/z* 533), and (d) 28-β-D-glucopyranosyl 2α,3β,19α,23-tetrahydroxyolean-12-ene-24,28-dioic (triterpene 4, *m/z* 695).

Table II: Concentrations of triterpenes 1–4 in red and white wines, Armagnac brandies, and heartwoods*

Sample	Triterpene 1	Triterpene 2	Triterpene 3	Triterpene 4
Red wines (mg/L)				
Bordeaux Graves 2000	1.02 ± 0.07	0.4 ± 0.04	—	—
Bordeaux Haut médoc 1998	0.9 ± 0.06	1.2 ± 0.1	0.9 ± 0.05	—
Bordeaux Pessac Léognan 1999	1.2 ± 0.08	2.5 ± 0.25	1.1 ± 0.1	—
Burgundy 2000	4.4 ± 0.31	1.1 ± 0.1	—	—
Burgundy 1996	—	—	—	7.9 ± 0.17
White wines (mg/L)				
Bordeaux Graves 2000	0.72 ± 0.05	5.42 ± 0.5	5.08 ± 0.3	0.26 ± 0.01
Bordeaux Graves 1999	0.45 ± 0.03	0.4 ± 0.04	—	—
Armagnac brandies (mg/L)				
V.S.O.P. (4–6 years old)	11 ± 0.4	44 ± 4.4	1 ± 0.1	—
X.O. (8–9 years old)	69.2 ± 2.6	34.9 ± 3.4	4.5 ± 0.3	1.9
H.A. (10–12 years old)	43.4 ± 1.7	21.7 ± 2.2	7.7 ± 0.5	3.5
1980 (Millésime)	29.3 ± 1.1	9.9 ± 0.9	5.9 ± 0.4	3.6
1974 (Millésime)	16.1 ± 0.6	7.1 ± 0.7	9.1 ± 0.5	4.5
1968 (Millésime)	24 ± 0.8	16.7 ± 2.1	15.8 ± 1.0	4.2
Heartwoods (mg/kg)				
Q. robur L. (Limousin, France)	81.5 ± 3.0	271 ± 10.5	92.5 ± 5.2	—
Q. robur L. (Tronçais, France)	191 ± 15.2	538.5 ± 21.5	—	—
Q. robur L. (France, Gers, France)	254.5 ± 19.1	427 ± 17.1	96 ± 5.7	—
Q. petraea Liebl (Tronçais, France)	124 ± 9.8	158 ± 6.2	—	—
Q. petraea Liebl (Limousin, France)	244.5 ± 19.6	499 ± 20.4	14 ± 0.9	—
Q. petraea Liebl (Allier, France)	64 ± 4.8	1.5 ± 0.05	—	—
Q. alba (Oregon)	10 ± 0.7	—	—	—

*n = 3.

of aglycone triterpenes (trace), whereas the wine contained large amounts of glycosidic triterpene 4 (7.9 ± 0.17 mg/L). Taking into account differences between triterpene concentrations in wines and spirits, we deduced that their extraction efficiency is dependent upon alcohol percentage, origin, and oakwood species used during maturation. Indeed, heartwoods from pedunculate oaks *Q. robur* L. are used especially for spirit aging, and sessile oaks *Q. petraea* Liebl. more often are used for white and red wines.

In heartwoods, the determinations displayed strong differences between different origins and species. The pedunculate oakwoods *Q. robur* L. showed the higher aglycone triterpene content of 191 ± 15.2 mg/L and 538.5 ± 21.5 mg/L for triterpenes 1 and 2, respectively, in *Q. robur* L. from Tronçais (France), and 254.5 ± 19.1 and 427 ± 17.1 mg/kg for triterpenes 1 and 2, respectively, in *Q. robur* L. from the Gers region (France). Nevertheless, the sessile oakwood *Q. petraea* Liebl. from Limousin (France) also displayed high concentrations of 244.5 ± 19.6 and 499 ± 20.4 mg/kg (triterpenes 1 and 2) as opposed to *Q. petraea* Liebl. from Allier, which provided 64 ± 4.8 and 1.5 ± 0.05 mg/kg. On the basis of these results, we cannot assign the stronger triterpene content to the pedunculate heartwoods (as opposed to the sessile ones).

On the other hand, the American oak *Q. alba* (Oregon) seems to contain the weakest concentrations of triterpenes — 10 ± 0.7 mg/kg of triterpene 1.

In conclusion, the concentrations of triterpenes 1–4 in seven trees reported in this article are likely to show individual differences. As a consequence, a detailed study of the four triterpenes with a broader sampling in the three previous oak species is necessary to ascertain the influence of species on the triterpene content of wines and spirits.

Conclusion

We developed and validated a sensitive, selective HPLC–electrospray ionization MS method in selected-ion recording mode for the quantitative determination of acidic triterpenes in wines and spirits. This method was applied successfully to quantify these compounds for the first time in a wide range of wines and Armagnac brandies. Preliminary studies indicated that these molecules could play an important role in the flavor of these beverages.

Acknowledgments

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