1 # New Approach for Differentiating Sessile and Pedunculate Oak: Development of a LC-HRMS Method To Quantitate Triterpenoids in Wood

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15 **Abstract:**
16 Oak aging is a crucial step in winemaking during which the organoleptic properties of wine are
17 modified. Various parameters affect the chemical composition of oak wood including botanical
18 origin, which has been previously shown to be a determinant factor. This study focused on the
19 development of a LC-HRMS method to assay four recently discovered taste-active triterpenes
20 (three sweet and one bitter). The method was applied to evaluate the effect of oak species
21 (*Quercus petraea* and *Quercus robur*) on the concentration of these molecules in wood. The
22 results showed that sessile oak was richer in sweet triterpenes and poorer in the bitter one than
23 pedunculate oak, with high interindividual variations within species. Furthermore, a
24 triterpenoid index was calculated to reveal the triterpenoid composition of oak wood. This index
25 appears to be a promising tool for the unambiguous discrimination of oak species and could
26 offer new insights into oak wood selection by coopers and the monitoring of oak aging by
27 winemakers.
28

29 **Keywords:** wine aging, sweetness, bitterness, taste, Orbitrap, quercotriterpenosides

30 # INTRODUCTION

31

32 The quality of a wine depends both on the quality of the harvested grapes and on how
33 winemaking is conducted. For many wines, this involves an aging period with contact between
34 wine and oak wood.[1] Oak aging is a crucial step, and its effect can be compared with that of an
35 evening dress on the person who wears it. Chosen tastefully and used appropriately, it discretely
36 emphasizes the elegance of the wine. On the contrary, its excessive or inappropriate use
37 produces the opposite effect and borders on caricature. Indeed, the organoleptic properties of
38 wine are modified in contact with oak. These modifications can be due to the moderate oxygen
39 supply[2] during aging or to the release of molecules from wood.[1] For decades, intensive research
40 in this field has led to the identification of the key volatile compounds originating from oak
41 wood: vanillin, β-methyl-γ-octalactone (called oak lactone), eugenol, and 2-
42 furanmethanethiol.[3,4] Moreover, oak wood releases nonvolatile compounds likely to modify the
43 taste of wine. Many works have focused on ellagitannins by establishing their chemical
44 structure,[5,6] their synthesis,[7] their amounts in oak, wine, or spirits,[8,9] and their evolution during
45 the seasoning[10,11] and toasting[12-15] of wood. The sensory properties of isolated ellagitannins has
46 also been investigated,[9,16] and their perception threshold has recently been established thanks
47 to the half-tongue test.[8,17] Apart from ellagitannins, other nonvolatile compounds are released
48 from oak wood such as coumarins,[18] lignans,[19] and triterpenes,[20,21] some of them exhibiting
49 gustatory properties.[22-24]
50 Various parameters such as the origin of oak or cooperage techniques may influence the
51 composition of wood and subsequently its effect on wine taste. Some coopers usually classify
52 the qualities of wood according to ring width (also called "grain") or geographical origin.
53 However, previous studies have demonstrated that species is a better indicator of chemical
54 composition than morphological parameters or provenance.[25,26] In practice, two main species
55 of European oak are used for cooperage and occur together in most French forests: *Quercus*
56 *petraea* Liebl. (sessile oak) and *Quercus robur* L. (pedunculate oak). As reported by various
57 authors, the average levels of oak lactone and ellagitannins are respectively higher and lower
58 in sessile oak wood than in pedunculate oak wood.[27-29] However, high interindividual variations
59 are observed within each species and strongly affect the significance of the species effect.[30-32]
60 For example, Prida et al. showed that some sessile oaks have low levels of oak lactone in the
61 range of those assayed in pedunculate oaks.[31] A similar situation has been described for
62 ellagitannins.[25,33] Therefore, the odorant and taste-active compounds do not seem to be specific

63 of either sessile or pedunculate oak, and the quantitation of oak lactone or ellagitannins in wood
64 samples does not allow oak species to be discriminated. Feuillat et al. carried out a principal
65 component analysis on 18 chemical variables quantified in samples of sessile and pedunculate
66 oak.[25] Even though the results showed that most trees were grouped according to their species,
67 some sessile samples were closer to the pedunculate cluster than to the sessile one.
68 Consequently, the targeted analysis of wood compounds has not allowed the unambiguous
69 discrimination of oak species until now. However, a more recent study by Gougeon et al.
70 applied Fourier transform ion cyclotron resonance (FT-ICR) to the untargeted analysis of oak
71 wood extracts.[34] Thanks to statistical treatment with hierarchical cluster analysis, they clearly
72 distinguished the samples according to their species. These results illustrate the strong influence
73 of botanical origin on oak wood composition.

74 Recently, a taste-guided procedure led to the isolation of two new triterpenoids from
75 oak wood.[21] These compounds, which had never been identified previously, were called
76 quercotriterpenosides (QTTs) I and II and were hypothesized to contribute to the increase in
77 wine sweetness observed during oak aging.[35] Other QTTs have been subsequently identified,[24]
78 and QTTs I, I, and III are the major sweet molecules of this family (Figure 1). Moreover, other
79 triterpenoids carrying a carboxylic acid function on carbon 24 of the genin were identified by
80 Arramon et al.[20] In particular, the glucosyl derivative of bartogenic acid (Glu-BA) is abundant
81 in oak wood and exhibits a bitter taste (Figure 1). Owing to their individual gustatory activities,
82 these triterpenoids could have a significant impact on the organoleptic properties of wine by
83 modulating its taste balance. The aim of the present study was to develop and validate a
84 quantitation method for QTTs I, II, and III and Glu-BA. The method was then applied to assay
85 these taste-active triterpenoids in samples of sessile and pedunculate oak wood whose species
86 has been assigned thanks to a newly developed genetic method.[36,37]
87

88 # MATERIALS AND METHODS

89

90 **Chemicals.** For sample preparation, analytical reagent grade ethanol, tartaric acid, and
91 sodium hydroxide were purchased from Fisher Chemical (Illkirch, France). Ultrapure water
92 (Milli-Q purification system, Millipore, France) was used. Triterpenoids (QTTs I, II, and III
93 and Glu-BA) are not commercial compounds. Their isolation from oak wood by centrifugal
94 partition chromatography and high-performance liquid chromatography (HPLC) and their
95 structural identification by HRMS and NMR have been previously described by Arramon et

96 al.[20] and Marchal et al.[21,24] The standards used in this study have been purified following the
97 same procedures, and their purity as assessed by LC-HRMS was >98%. Acetonitrile and water
98 used for chromatographic separation were of LC-MS grade and purchased from Fisher
99 Chemical.

100 **Origin of Oak Wood.** For this study, 46 samples of fresh oak material were collected
101 in 8 different French forests: 3 in the northeast (Saint-Clément, Spincourt, Xures), 2 in the
102 center (Tronçais, Châteauroux), 1 in the northwest (Liffré) and 2 in the southwest (Pierroton,
103 Laveyron). Samples from Pierroton and Laveyron were provided by the French National
104 Institute for Agricultural Research and Dr. Erwan Guichoux. The other samples were supplied
105 by Seguin-Moreau cooperage.

106 The wood material was sampled in the following way. Each sample represented oak
107 shavings from a single living tree drilled at 1 m level. The shavings were collected in a plastic
108 bag filled with silica gel beans. Meanwhile, the green oak leaves from the same tree were
109 collected in a separate plastic bag also filled with silica gel beans. Both samples from one tree
110 were identified by the same code and sent to the laboratory. A genetic analysis was run on the
111 leaves immediately after sample receipt.

112 **Genetic Assignment of Species.** The genetic assignment method was used in this
113 study for the oak samples. It consists in assigning individuals to putative species based on the
114 expected frequencies of their genotypes in those species as described by Guichoux et al.[36,37]
115 The percentage of assignment shows the probability of a tested individual belonging to a given
116 species rather than to another. The 87.5% level is the statistical threshold, which allows
117 individuals to be assigned as pure species. Individuals assigned with a probability >87.5% to
118 one specific species were either *Q. robur* (pedunculate oak) or *Q. petraea* (sessile oak), whereas
119 individuals with a probability <87.5% were considered as hybrids (hybrids F1 and F2,
120 backcross of first generation).

121 **Sample Preparation.** *Preparation of Oak Wood Extracts for LC-HRMS*
122 *Analysis.* Oak wood shavings were dried at 20 °C for 6 h and ground into a homogeneous
123 powder (with linear dimensions of <0.7 mm) with an SM2000 grinder (Reutsch, Germany).

124 Powdered oak wood (2.5 g) was soaked in model solution (50 mL) prepared by
125 dissolving tartaric acid (5 g/L) in an aqueous 12% ethanol solution adjusted to pH 3.5 with
126 aqueous sodium hydroxide (5 M), in glass bottles sealed hermetically with screw caps. Mixtures
127 were agitated (using orbital shakers) at room temperature for 48 h in darkness to avoid any
128 possible photodegradation. Forty-eight hours was chosen on the basis of a preliminary study of

129 duration sufficient for reaching the equilibrium between solid and liquid phase in concentrations
130 of triterpenic compounds.

131 Prior to analysis, each oak wood extract was diluted 25 times with Milli-Q water and
132 filtered through a 0.45 μm PTFE syringe filter. The results were expressed as micrograms per
133 gram (of oak wood) on the basis of the dilution factor. The triterpenes were quantified with
134 external calibration.

135 *Preparation of Stock Solutions and Calibration Samples.* A stock solution of QTT
136 I, QTT II, QTT III, and Glu-BA was prepared in HPLC grade ethanol (1 g/L for each
137 compound). Successive dilutions (by 2, 5, and 10) of this stock solution with Milli-Q water
138 were performed to obtain calibration samples from 100 ng/L to 5 mg/L.

139 **Liquid Chromatography.** The U-HPLC platform consisted of an HTC PAL
140 autosampler (CTC Analytics AG, Zwingen, Switzerland) coupled with an Accela pumping
141 system. For liquid chromatography separation, a C18 column was used as the stationary phase
142 (Hypersil Gold 2.1 mm × 100 mm, 1.9 μm particle size, Thermo Fisher Scientific). The mobile
143 phases were (A) water and (B) acetonitrile. The flow rate was 600 μL/min, and eluent B varied
144 as follows: 0 min, 20%; 0.5 min, 20%; 4 min, 50%; 4.1 min, 98%; 6.1 min, 98%; 6.2 min, 20%;
145 7.5 min, 20%. The injection volume was 5 μL.

146 **High Resolution Mass Spectrometry (HRMS).** U-HPLC was coupled with an
147 Exactive Orbitrap mass spectrometer equipped with a heated electrospray ionization (HESI)
148 probe (both from Thermo Fisher Scientific, Bremen, Germany). Mass acquisitions were carried
149 out for 6 min in negative HRMS ionization mode at 3 kV. The vaporizer temperature of the
150 source was set at 320 °C, the capillary temperature at 350 °C, the nitrogen sheath gas at 75, the
151 auxiliary gas at 18, and the sweep gas at 0 (arbitrary units). The capillary voltage, the tube lens
152 voltage offset, and the skimmer voltage were set at −95, −190, and −46 V, respectively. A mass
153 range of 500–1200 Th was acquired in full scan MS mode with a mass resolution of 25 000
154 (m/Δm, fwhm at m/z 200 Th). The automatic gain control value was 3×10^6 ions, and a source-
155 induced dissociation energy of 20 eV was applied.

156 External mass calibration using Pierce ESI Negative Ion Calibration solution (Thermo
157 Fisher Scientific) was performed before each series of analyses. All data were processed using
158 the Qualbrowser and Quanbrowser applications of Xcalibur version 2.1 (Thermo Fisher
159 Scientific). Detection of the targeted compounds was based on theoretical exact mass and
160 retention time. Peak areas were determined by automatic integration, and final concentrations
161 were expressed on the basis of the dilution factor.

162 **Method Validation.** The quantitation method was validated by studying sensitivity,
163 linearity in working range, intraday repeatability, interday precision, trueness, and specificity.
164 *Sensitivity.* Considering the mass accuracy measurement of the Orbitrap analyzer, the
165 noise level is quite absent in chromatograms of extracted ions (XIC). Consequently, the
166 sensitivity of the method cannot be evaluated by signal-to-noise measurement for LC-HRMS.
167 Thus, instrumental detection limits (IDLs) were determined in accordance with the
168 methodology described by De Paeppe et al.[38] Replicate injections of the lowest levels of the
169 calibration curve (from 100 ng/L to 10 μg/L) made it possible to determine both precision (RSD
170 %) and accuracy (recovering with back-calculated concentrations) for each level. IDLs were
171 defined as the lowest concentration with a precision lower than, for example, 10% and accuracy
172 higher than, for example, 90%.
173 *Linearity.* For all compounds, a calibration curve was obtained by plotting peak area
174 versus the nominal concentration for each concentration level. Linear regressions were chosen
175 with a $1/x$ statistical weight. Linearity was evaluated by correlation coefficient (R^2) and by
176 deviations of each back-calculated standard concentration from the nominal value.
177 *Repeatability and Trueness.* To determine intraday precision, five replicates of two
178 intermediate calibration solutions (5 and 500 μg/L) were injected, and the relative standard
179 deviation (RSD%) was calculated. Interday precision was calculated in the same manner by
180 injecting the same two samples within 5 successive days. Two oak extracts were also fortified
181 with calibration solution corresponding to an addition of 10 or 500 μg/L of each compound.
182 Trueness was evaluated by calculating the recovery ratio (between measured and expected
183 areas).
184 *Specificity.* The specificity of Orbitrap analysis relies considerably on mass accuracy
185 and retention time repeatability. These parameters were assessed concomitantly with the above-
186 described precision and trueness analysis. The isotopic distribution for the quasi-molecular ion
187 $[M - H]^-$ was also observed to confirm the nature of the ions.
188 **Statistical Analyses.** The normal distribution of the data was tested using the
189 Shapiro–Wilk test. The influence of the botanical species on the triterpenoid content was
190 evaluated by the nonparametric Kruskal–Wallis test. Pairwise correlations between variables
191 were studied using Spearman's rank correlations, separately for the two species. All of the
192 statistical analyses were carried out using the software XL-STAT version 2010.5.05 (Addinsoft,
193 Paris, France).
194

195 RESULTS AND DISCUSSION

196

197 **Development of a LC-HRMS Method To Quantitate Triterpenoids in Oak**
198 **Wood Extract.** *Chromatographic and Mass Spectrometry Conditions.* By combining the
199 separation power of U-HPLC and the specificity of Fourier transform mass spectrometry, LC-
200 HRMS appeared to be a powerful technique allowing quantitation of triterpenoids directly in
201 oak wood extract. An acidic hydroethanolic solution was used for solid–liquid extraction to
202 model what occurs during wine aging. A sample dilution factor was chosen to reduce all of the
203 concentrations in the working range. This dilution led to an ethanol level compatible with the
204 requirements of LC (<0.5% here), so samples could be directly injected into the LC-HRMS
205 system after dilution. Chromatographic conditions were determined by using water and
206 acetonitrile to achieve good separation of isomeric compounds in a short run (peaks
207 corresponding to quantified molecules are assigned in Figure 2A). MS parameters were
208 determined by direct injection of target compounds to optimize signal intensity. A low
209 dissociation energy (20 eV) was applied in HESI source to avoid the formation of adducts and
210 to increase repeatability. The mass resolution power (25000 at m/z 200) was chosen as a
211 compromise between the scan rate (4 Hz) and the specificity of ion detection by avoiding mass
212 interferences.

213 *Method Validation.* Peaks were integrated from XICs constructed with 3 ppm accuracy
214 around the theoretical m/z. With such mass accuracy specifications, the classical approach based
215 on signal-to-noise evaluation was not suitable for determining the sensitivity of the LC-HRMS
216 quantitation method.[38,39] In this study, the criteria defined by De Paeppe et al.[38] were applied.
217 For all molecules, IDL was ≤1 μg/L, thus demonstrating the good sensitivity of our method. As
218 in the study by De Paeppe et al.,[38] the instrumental quantitation limit (IQL) was defined as 2
219 times the IDL and limits of detection (LOD) and quantitation (LOQ) were reassessed on the
220 basis of the sample preparation, that is, dilution by a factor of 25. These data are presented in
221 Table 1.

222 Obtaining a good linearity over a wide concentration range is a key issue to quantitate
223 compounds for which the content varies considerably from one sample to another. Preliminary
224 studies showed such strong variations in our samples and provided estimations of the amounts
225 of triterpenoids. On these bases, the working ranges were chosen for each molecule from the
226 IQLs to be 2 or 5 mg/L, depending on the highest concentrations estimated (Table 1). For all
227 compounds, a linear curve was obtained with good correlation coefficients (R^2 > 0.996, Table
228 1). To ensure a good accuracy (>90%) of concentrations back-calculated from the calibration
229 curve at all levels and particularly at low levels, a $1/x$ statistical weight was chosen.

230 Intraday repeatability (RSD) was <8% at 5 μg/L and <5% at 500 μg/L. Interday
231 precision was not as good (up to 16% at 5 μg/L), but this parameter was not so determinant for
232 this study. Indeed, the short U-HPLC runs (7.5 min) enabled the injection of the calibration
233 solutions before each quantitative assay, which prevented interday deviations. Oak wood
234 samples spiked with stock solutions were also injected. Recovery ratios were >89% for all
235 compounds with the addition of 10 μg/L and >96% with 500 μg/L (Table 1). Consequently,
236 these results demonstrated the repeatability and the trueness of the method applied to oak
237 macerates.

238 Analysis of the above samples exhibited very low variations in retention time (<0.04
239 min) and a mass accuracy of <3 ppm for all compounds at various concentrations (Table 1).
240 These results guaranteed the specificity of the method. Moreover, isotopic patterns were
241 recorded for deprotonated ions $[M - H]^-$ of each compound with a mass resolution of 25000 (at
242 *m*/*z* 200). The comparison between these experimental signals and theoretical spectra obtained
243 by simulation showed a high level of similarity (Figure 2B,C), which confirmed the nature of
244 the targeted signals.

245 All of these results demonstrated the performance of LC-HRMS to assay triterpenoids
246 in oak wood samples. Use of an internal standard was not necessary to obtain reliable and robust
247 values. Therefore, the development and the validation of this quantitation method were
248 determinant steps allowing the study of the triterpenic composition of oak wood samples after
249 solid/liquid extraction. In particular, the influence of oak botanical species on triterpenoid
250 content was assessed.

251 **Application of the Method To Quantitate Triterpenoids in Sessile and**
252 **Pedunculate Oak Wood.** *Genetic Discrimination of Oak Samples.* The accurate
253 assignment to botanical species by using the morphological features of a tree (wood, bark,
254 leaves, and fruits) is a difficult task. It requires specific skills, and the probability of error is
255 significant. Species may be recognized on cut wood (staves/logs) by using anatomic features,[40]
256 but the rate of success is low. Foliar analysis is a more accurate tool for assessment[41,42] but is
257 cumbersome in the field. The possibility of hybridization and the natural variability of the tree
258 features of oak individuals add even more complexity to the task. To assign botanical species
259 in an unambiguous and accurate way, a genetic study was performed as described
260 previously.[36,37]

261 Fresh leaves were used to facilitate DNA extraction. Table 2 presents the results of the
262 genetic assignment for each oak sample. Among the 46 samples analyzed in this study, 27 were
263 assigned to *Q. petraea* (sessile oak) and 19 to *Q. robur* (pedunculate oak). As is well-known,

264 both species are found in some forests (Tronçais and Liffré), confirming that geographical
265 origin is not in itself a relevant factor to discriminate sessile and pedunculate oak.

266 *Triterpenoid Determination in Oak Wood Samples.* The method developed in this
267 study was applied to quantitate for the first time QTTs I, II, and III in oak wood. The glucosyl
268 derivative of bartogenic acid (Glu-BA) estimated in wine, spirits, and oak wood by Arramon et
269 al.[20] was measured concomitantly. To assay these compounds in oak wood, a solid/liquid
270 extraction was first carried out by soaking wood powder in a hydroalcoholic solution. Previous
271 experiments showed that maximal concentrations were reached after 40 h and remained stable
272 for some days, so the extraction time was 48 h for all samples. All concentrations were
273 expressed as micrograms per gram of dry wood on the basis of the dilution factor (25) and the
274 mass concentration of wood used for soaking (50 g/L).

275 The four triterpenoids were observed in all samples, and all of the concentrations were
276 measured above their LOQ (Table 1; Table S1; Figure 3). For each compound, the results
277 showed a wide range of concentrations with >4 orders of magnitude (0.7–1102.5 μg/g for QTT
278 I; 0.7–1418.5 μg/g for QTT II; 1.2–1408.7 μg/g for QTT III; and 3.2–1952.0 μg/g for Glu-BA).
279 A Kruskal–Wallis test revealed significant differences between the species for each compound
280 (p value < 0.1%). To observe the relationship between triterpenoid composition and botanical
281 species more precisely, the mean concentrations of the quantified compounds were calculated
282 for sessile (n = 27) and pedunculate (n = 19) oak samples (Figure 4). For QTT I, the mean
283 values were 413.5 ± 96.2 μg/g for sessile oak samples and 6.0 ± 2.7 μg/g for pedunculate ones.
284 Similar results were obtained for QTT II and QTT III, demonstrating that sessile oak is richer
285 in QTTs than pedunculate oak (Table 3). On the other hand, mean Glu-BA concentrations were
286 higher in pedunculate oak (795.3 ± 271.3 μg/g) than in sessile oak (24.4 ± 10.7 μg/g, Table 3).
287 This trend was similar for samples of different species originating from the same forests (S-Li-
288 1/P-Li-1–5 and S-Tr-1 and 2/P-Tr-1–3), suggesting that the botanical species had a greater
289 influence on the triterpenoid composition of oak wood than geographical location. Moreover,
290 pairwise Spearman's tests were carried out and revealed significant correlations between
291 concentrations of QTT I, QTT II, and QTT III, for both sessile and pedunculate oak populations
292 (Table S2). Figure 3 (panels a, b, d, and e) shows that within a species samples with a high
293 content in QTT I were also generally rich in QTT II and QTT III. All of these positive
294 correlations might suggest a similar biosynthetic pathway for these regio- and diastereoisomers.
295 In contrast, the relationships between concentrations in QTTs on the one hand and Glu-BA on
296 the other hand seem less clear. Indeed, the scatter plots representing the concentrations in QTT

297 I and Glu-BA in sessile (Figure 3c) and pedunculate (Figure 3f) oak were more widespread and
298 the Spearman correlations between QTTs and Glu-BA were weak or not significant (Table S2).
299 These results could be of particular interest regarding the organoleptic effect of oak
300 aging on wine taste. Indeed, QTTs I, II, and III develop a sweet taste, whereas Glu-BA has been
301 described as bitter. The present findings therefore show that sessile oak contains more sweet
302 triterpenoids, whereas pedunculate oak is richer in bitter triterpene. Apart from triterpenes,
303 previous studies have shown that the chemical composition of oak wood is influenced by
304 botanical species. In particular, sessile oak wood has been shown to contain higher quantities
305 of oak lactone and lower concentrations of ellagitannins than pedunculate oak wood.[27,28]
306 Altogether, the latter findings and these new results concerning taste-active triterpenoids could
307 partly explain why sessile oak is traditionally used for wine aging, whereas pedunculate oak is
308 preferred for maturing spirits.[27]
309 Although statistical tests revealed significant differences for mean concentrations in
310 QTTs and Glu-BA, some extreme values of individual triterpenes were very close for sessile
311 and pedunculate oak, as shown in Table 3. For instance, the minimal concentration of QTT II
312 in sessile oak samples was 23.6 μg/g, whereas the maximal value in pedunculate oak samples
313 was 44.1 μg/g. For Glu-BA, the maximal concentration in sessile samples was 105.5 μg/g, and
314 the minimal concentration was 36.0 μg/g in pedunculate samples. Therefore, even the mean
315 amounts of QTTs and Glu-BA were respectively higher and lower in sessile oak than in
316 pedunculate oak, and high interindividual variations were observed within species for each
317 triterpenoid, as shown by the large confidence intervals. Consequently, the individual
318 quantitation of each triterpenoid did not allow the direct identification of the botanical species.
319 This limitation can be linked with observations concerning other compounds whose
320 concentrations depend on botanical species. For example, Prida et al. showed that a significant
321 number of sessile oak samples contained levels of β-methyl-γ-octalactone similar to or even
322 lower than those in pedunculate oak samples.[31] A similar trend has been observed for
323 ellagitannins.[33] Thus, none of these compounds (oak lactone, ellagitannins, or triterpenoids)
324 allows the unambiguous discrimination of oak species according to their individual
325 concentration in wood.
326 *Differentiation of Sessile and Pedunculate Oak Wood Samples According to a*
327 *Triterpenoids Index.* The scatter diagrams representing Glu-BA concentrations as a function
328 of QTT I levels show a differential distribution according to the species: whereas samples of
329 sessile oak have high levels of QTT I and low levels of Glu-BA (Figure 3c), the inverse situation
330 was observed for samples of pedunculate oak (Figure 3f). Some samples (such as S-La-7 or S-

331 La-15) contained low amounts of QTTs in comparison with other sessile wood, but their
332 concentration in Glu-BA was even lower. A similar situation was observed for P-Li-4, which
333 contained the minimal concentrations in Glu-BA but also in QTTs among pedunculate oak
334 samples (Table S1 in the Supporting Information). Apart from the absolute concentrations in
335 individual triterpenoids, it seemed that the samples could be grouped into two categories
336 according to their relative amounts of QTTs and Glu-BA. To express this relative composition,
337 a triterpenoids index (TI) was calculated as base 10 logarithm of the ratio between the sum of
338 concentrations in QTTs and the concentration in Glu-BA (all expressed as μg/g).
339

340 $$\mathrm{TI} = \log \frac{[\mathrm{QTT\ I}] + [\mathrm{QTT\ II}] + [\mathrm{QTT\ III}]}{[\mathrm{GluBA}]}$$

341

342 The average values of this index were calculated for sessile and pedunculate oak wood
343 samples (Figure 5 and Table 3). The mean TI was positive for sessile samples and negative for
344 pedunculate samples (1.9 and −1.5, respectively). A Kruskal–Wallis test showed that these
345 differences were statistically significant (p value < 0.1%). More interestingly, confidence
346 intervals were smaller than for absolute concentrations in triterpenes expressing a less extended
347 range of values. Indeed, all sessile oak samples had positive TI values (from 1.2 to 2.4), whereas
348 all pedunculate oak samples exhibited negative TI values (from −2.2 to −0.8). Contrary to the
349 absolute concentrations in individual triterpenoids, there was a huge gap (2 log points) between
350 the closest values in both species, that is, between the lowest value in sessile oak (1.2) and the
351 highest value in pedunculate oak (−0.8).

352 Consequently, the calculation of a TI reflecting the relative composition in triterpenoids
353 of wood appeared to avoid any ambiguity in the assignment of the botanical species. In practice,
354 a positive TI value might indicate that the sample came from sessile oak, whereas a negative TI
355 value might correspond to a pedunculate oak sample. These results highlight probable
356 differences between species in terms of triterpenoid biosynthesis, with variations of the
357 enzymes involved in the decoration of the genin. Such differences have already been observed
358 and are related to the wide diversity of triterpenoids occurring in the plant kingdom.[43,44]

359 This study presents the development of an LC-HRMS method to quantitate sweet
360 (QTTs) and bitter (Glu-BA) triterpenoids in oak wood. The results provide new insights into
361 the chemical composition of oak wood and the interpretation of its organoleptic effect on wine
362 and spirits. Sessile oak was found to be rich in sweet QTTs and poor in bitter Glu-BA, whereas
363 pedunculate oak samples exhibited high levels of Glu-BA and low levels of QTTs. This may

364 explain why sessile oak is preferred for wine aging. Apart from this significant trend, the high
365 interindividual variations in concentrations observed for these compounds within each species
366 are similar to previous observations concerning oak lactone and ellagitannins. However, a TI
367 reflecting the triterpenoid profile of wood samples was calculated and showed a clear-cut
368 differentiation between species without any equivocal sample or recovery between species. This
369 TI therefore seems to be a promising tool for identifying oak species in a chemical manner in
370 addition to genetic assignment. Until now, unambiguous chemical differentiation of species had
371 only been reached by nontargeted analysis with FT-ICR followed by statistical treatment.[34]
372 Such an approach gives an accurate fingerprint of the studied sample, and its species is assigned
373 by comparing the fingerprint to a database collection of previously analyzed samples. Although
374 FT-ICR is a very powerful technique, it is also very costly, so its use is reserved for the few
375 laboratories specializing in mass spectrometry. This constitutes a limitation for its routine
376 application by the cooperage industry. On the other hand, the targeted method presented here
377 involves the quantitation of only four compounds with straightforward sample preparation and
378 a short analysis time. Various LC-MS instruments could be used to perform this targeted
379 analysis. The species assignment can be obtained directly, without any statistical treatment or
380 comparison with a database. Moreover, it is based on taste-active molecules that are likely to
381 modify the organoleptic properties of wine. It thus appears promising for various applications,
382 in particular for better selecting the oak wood used in cooperage and allowing a more
383 harmonious marriage with wine. Further work will focus on the evolution of the TI during barrel
384 production and oak aging. Indeed, the direct evaluation of the TI in wine and spirits aged in
385 wood might provide a reflection of the aging conditions, a factor that could be useful for
386 traceability.
387

388 **Supporting Information**

389 Individual concentrations of QTT I, QTT II, QTT III, and Glu-BA measured and TI calculated
390 for the 46 oak wood samples (Table S1); correlation coefficient (ρ) and *p* values corresponding
391 to Spearman correlation test between concentrations of QTT I, QTT II, QTT III, and Glu-BA
392 in sessile and pedunculate oak samples (Table S2).
393

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397 **Notes**

398 The authors declare no competing financial interest.

399

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Figures

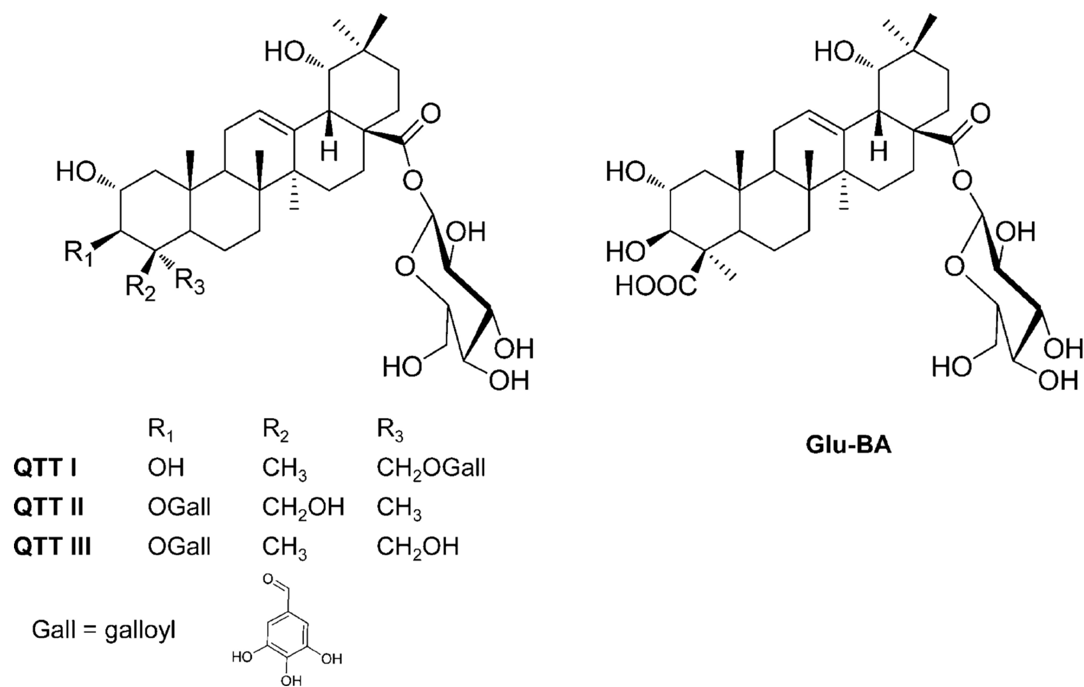

Figure 1. Chemical structures of QTT isomers and Glu-BA.

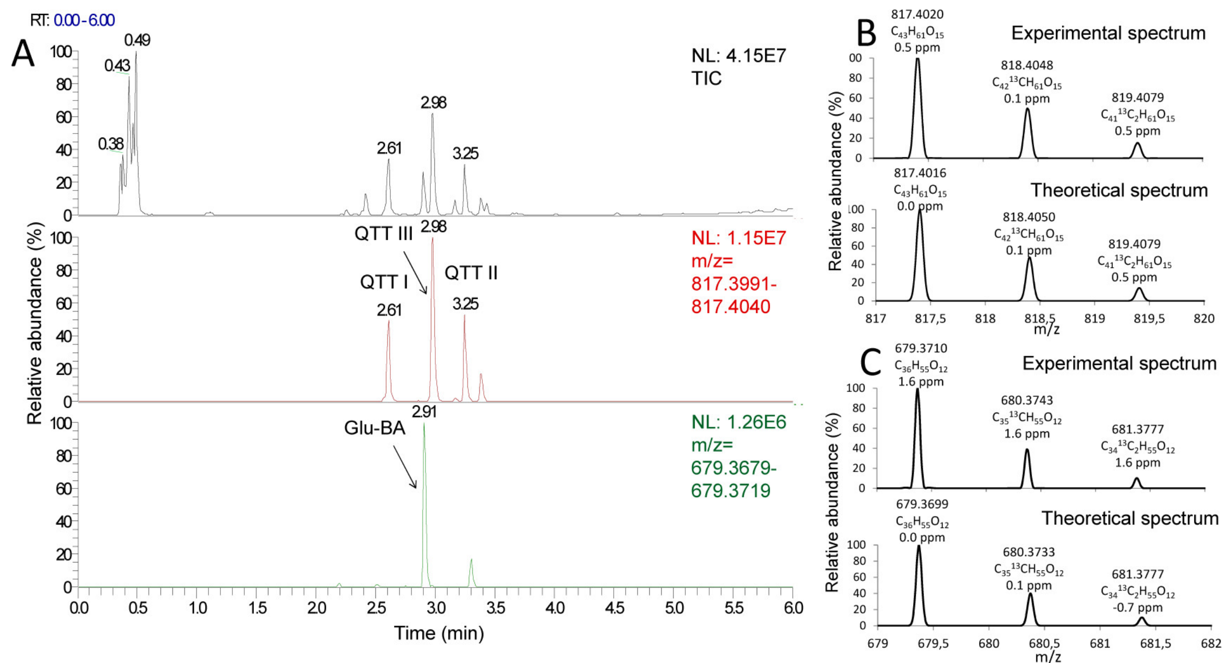

Figure 2. (A) LC-HRMS chromatograms of a sample of sessile oak. From top to bottom: TIC, XIC for QTT, and XIC for Glu-BA. XICs were recorded in a 3 ppm window around the molecular mass corresponding to $C_{43}H_{61}O_{15}^{-}$ (*m*/*z* 817.4016) and $C_{36}H_{55}O_{12}^{-}$ (*m*/*z* 679.3699). (B, C) Detail of the experimental and theoretical HRMS spectra corresponding to the isotopic pattern of QTT III and Glu-BA, respectively (at a resolution of 25000 at *m*/*z* 200).

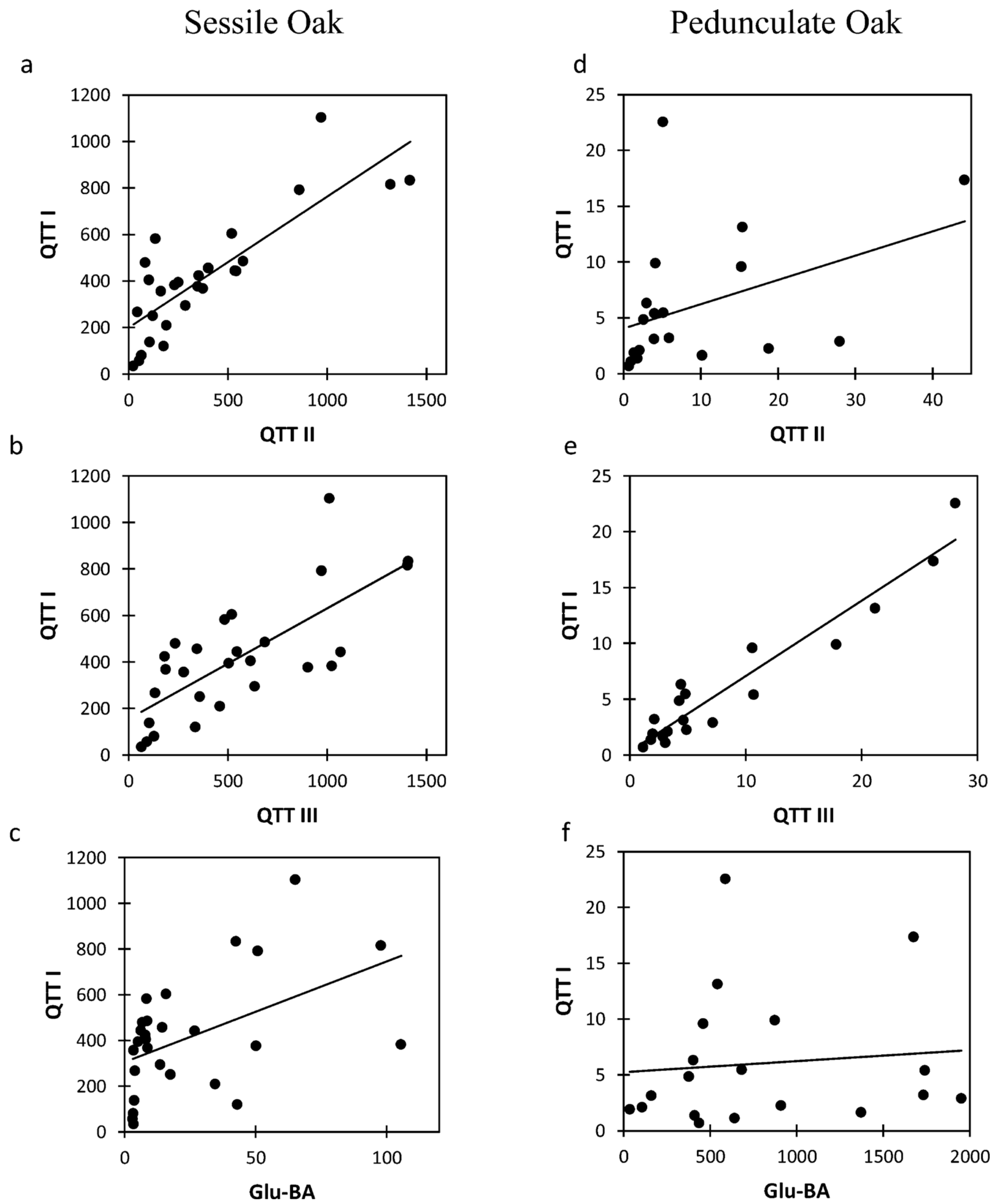

Figure 3. Scatter diagrams representing the relationships between concentrations of QTTs I, II, and III and Glu-BA in sessile and pedunculate oak wood samples. All concentrations are expressed in μg/g of wood.

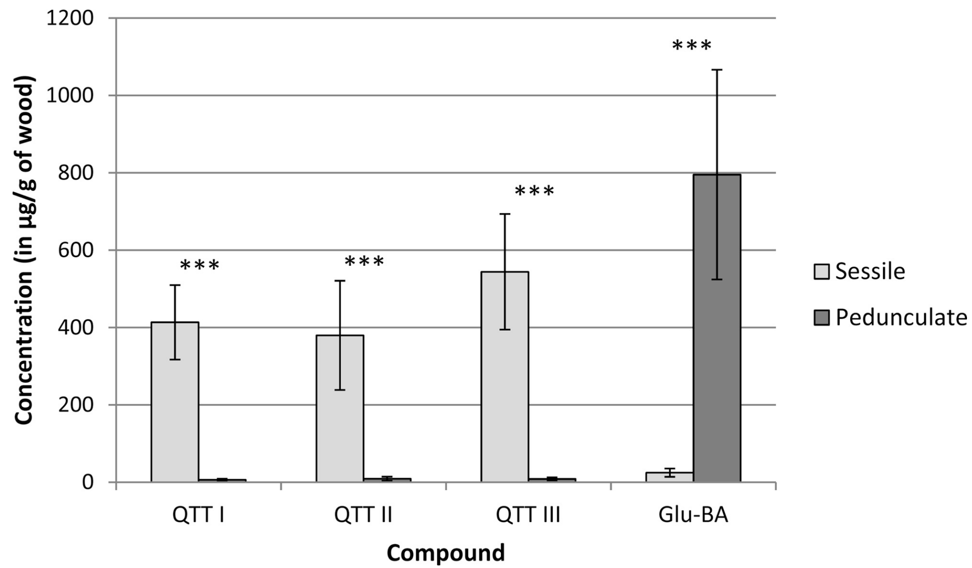

Figure 4. Mean concentrations (in µg/g) of QTTs I, II, and III and Glu-BA in sessile (27 samples) and pedunculate (19 samples) oak wood. Error bars indicate 95% confidence intervals. (∗∗∗) significant for Kruskal–Wallis test with $p < 0.001$.

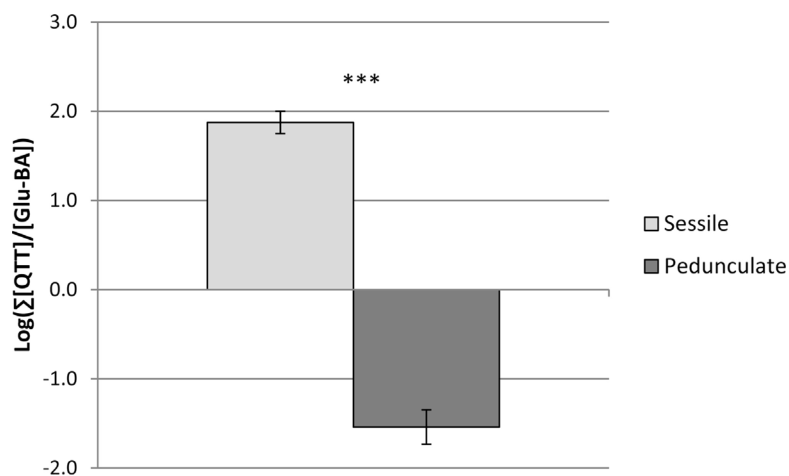

Figure 5. Mean base 10 logarithm of the ratio between the sum of concentrations in QTTs I, II, and III (∑QTT) and concentration in Glu-BA for sessile and pedunculate oak wood. Error bars indicate 95% confidence intervals. (∗∗∗) significant for Kruskal–Wallis test with $p < 0.001$.

Tables

Table 1. Validation Parameters for HRMS Quantitation Method of Oak Wood Triterpenoids Including Sensitivity, Linearity, Specificity, Repeatability, and Trueness

	sensitivity[a]			linearity		specificity		repeatability and trueness					
								intraday repeatability[b] (%)		interday repeatability[c] (%)		recovery (%)	
	IDL (µg/L)	LOD (µg/g)	LOQ (µg/g)	working range	R^2	t_r variation (min)	mass accuracy[d] (ppm)	5 µg/L	500 µg/L	5 µg/L	500 µg/L	10 µg/L	500 µg/L
QTT I	0.5	0.25	0.5	1 µg/L–2 mg/L	0.999	0.04	0.9	7	4	11	11	91	96
QTT II	0.5	0.25	0.5	1 µg/L–2 mg/L	0.996	0.03	1.8	6	5	14	10	93	96
QTT III	1	0.5	1	2 µg/L–2 mg/L	0.998	0.03	2.8	8	3	16	12	89	97
Glu-BA	0.5	0.25	0.5	2 µg/L–5 mg/L	0.999	0.04	2.2	7	4	13	11	94	98

[a]Instrumental detection limit (IDL) was determined as the lowest concentration with precision lower than, e.g., 10% and accuracy higher than, e.g., 90%. Limit of detection (LOD) was calculated from IDL by considering the wood concentration of macerates and the dilution factor used for sample preparation. Limit of quantitation (LOQ) was defined as twice the LOD.

[b]Injections of five replicates at two concentrations (5 and 500 µg/L).

[c]Injections during 5 consecutive days at two concentrations (5 and 500 µg/L).

[d]The experimental mass used for the determination of mass accuracy was the main mass measured for the target compound across the chromatographic peak. The value given in the table is the maximum deviation observed within all calibration solutions.

Table 2. Results of Genetic Assignment of Species for the 46 Oak Samples

sample code	species	forest	sample code	species	forest
S-Ch-1	sessile	Chateauroux	P-Xu-1	pedunculate	Xures
S-Ch-2	sessile	Chateauroux	P-Xu-2	pedunculate	Xures
S-Ch-3	sessile	Chateauroux	P-Li-1	pedunculate	Liffré
S-Ch-4	sessile	Chateauroux	P-Li-2	pedunculate	Liffré
S-Li-1	sessile	Liffré	P-Li-3	pedunculate	Liffré
S-Tr-1	sessile	Tronçais	P-Li-4	pedunculate	Liffré
S-Tr-2	sessile	Tronçais	P-Li-5	pedunculate	Liffré
S-La-1	sessile	Laveyron	P-SC-1	pedunculate	St Clément
S-La-2	sessile	Laveyron	P-SC-2	pedunculate	St Clément
S-La-3	sessile	Laveyron	P-Sp-1	pedunculate	Spincourt
S-La-4	sessile	Laveyron	P-Sp-2	pedunculate	Spincourt
S-La-5	sessile	Laveyron	P-Tr-1	pedunculate	Tronçais
S-La-6	sessile	Laveyron	P-Tr-2	pedunculate	Tronçais
S-La-7	sessile	Laveyron	P-Tr-3	pedunculate	Tronçais
S-La-8	sessile	Laveyron	P-Pi-1	pedunculate	Pierroton
S-La-9	sessile	Laveyron	P-Pi-2	pedunculate	Pierroton
S-La-10	sessile	Laveyron	P-Pi-3	pedunculate	Pierroton
S-La-11	sessile	Laveyron	P-Pi-4	pedunculate	Pierroton
S-La-12	sessile	Laveyron	P-Pi-5	pedunculate	Pierroton
S-La-13	sessile	Laveyron			
S-La-14	sessile	Laveyron			
S-La-15	sessile	Laveyron			
S-La-16	sessile	Laveyron			
S-La-17	sessile	Laveyron			
S-La-18	sessile	Laveyron			
S-La-19	sessile	Laveyron			
S-La-20	sessile	Laveyron			

Table 3. Mean, Minimal, and Maximal Values of QTTs I, II, and III Glu-BA and TI Observed in Sessile and Pedunculate Oak Wood Samples

	sessile oak (*n* = 27)			**pedunculate oak (*n* = 19)**		
	mean	**min**	**max**	**mean**	**min**	**max**
QTT I[a]	413.5 ± 255.1	33.2	1102.5	6.0 ± 2.7	0.7	22.5
QTT II[a]	379.5 ± 141.1	23.6	1418	9.1 ± 5.0	0.7	44.1
QTT II[a]	543.8 ± 149.5	64.1	1408.7	8.5 ± 3.8	1.2	28.1
Glu-BA[a]	24.4 ± 10.7	3.2	105.5	795.3 ± 271.3	36	1952
TI[b]	1.9 ± 0.1	1.2	2.4	–1.5 ± 0.2	–2.2	–0.8

[a]Concentrations in QTTs I, II, and III and Glu-BA were expressed in µg/g of dry wood.

[b]TI, triterpenoids index (adimensional).

$$\mathrm{TI} = \log \frac{[\mathrm{QTT\ I}]+[\mathrm{QTT\ II}]+[\mathrm{QTT\ III}]}{[\mathrm{GluBA}]}$$