DOI: https://doi.org/10.20870/oeno-one.2025.59.2.8437

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ENOLOGY ORIGINAL RESEARCH ARTICLES

Characterisation of oenological oak tannin extracts by a multi-analytical approach

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Article number: 8437

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Associate editor:
Fernando Zamora

Received:
11 December 2024

Accepted:
30 April 2025

Published:
23 May 2025

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ABSTRACT

Complex ellagitannin extracts from oak are commonly used to improve wine properties, but their compositions are not well known. The composition of eight commercial oak tannin extracts was characterised using a multi-analytical approach. The concentrations of phenolic compounds evaluated by gravimetric analysis of the fraction adsorbed on polyvinylpolypyrrolidone and by UV spectrophotometry varied from 473 to 776 mg/g and from 334 to 576 mg/g, respectively. HPSEC-UV-dRI analysis showed differences in molecular size distribution. The concentration of sugars and polyols (quercitol), determined as alditol acetates by GC-FID, ranged from 59 to 259 mg/g. Nitrogen compounds determined by Kjeldahl analysis were present in very low amounts. Untargeted metabolomics data highlighted 159 features, including ellagitannins and their derivatives (*e.g.*, formed upon toasting) and other molecules such as lignans and triterpenosides, showing large differences between samples. This work demonstrates the variability in the composition of commercial oak tannin extracts, likely to impact their properties.

KEYWORDS: oak, ellagitannins, phenolic compounds, sugars, polyols, size distribution, high-resolution mass spectrometry, metabolomics

INTRODUCTION

Oenological tannins are produced by water and/or alcohol extraction of tannin-rich plants such as grape, quebracho, gallnut, tara, chestnut, and oak. These additives are primarily employed for wine stabilisation and clarification, preventing haze formation thanks to their capacity to precipitate proteins (Haslam & Cai, 1994). They can also have an impact on colour stabilisation (Vignault *et al.*, 2019), contribute to the antioxidant protection of wines and musts (Ugliano *et al.*, 2020; Versari *et al.*, 2013; Vignault *et al.*, 2018), and influence sensorial aspects, such as bitterness (Paissoni *et al.*, 2022) and astringency (Glabasnia & Hofmann, 2006; Hofmann *et al.*, 2006). They have also been reported to affect aroma release and perception of red wine flavour (Pittari *et al.*, 2022).

Oak wood is one of the main sources of oenological tannins (Versari *et al.*, 2013). Oak wood tannins consist of hydrolysable tannins and more specifically, of ellagitannins that release ellagic acid under acidic conditions (Quideau & Feldman, 1996). Ellagitannins are composed of a polyol core and hexahydroxydiphenoyl (HHDP) units formed by oxidative linkage of two galloyl groups (Quideau & Feldman, 1996). Eight major ellagitannins, namely castalagin and its C-1 isomer, vescalagin, grandinin, and roburins A, B, C, D, and E, have been identified in oak wood by LC-MS and/or LC-UV-MS (Cadahía *et al.*, 2001; Fernández de Simón *et al.*, 2006; Viriot *et al.*, 1994).

According to OIV (Office Internationale de la Vigne et du Vin), oenological tannins are required to contain at least 65 % polyphenols to be used in winemaking (OIV, 2022). The official OIV method to determine tannin content in oenological tannin extracts consists of gravimetric analysis of the fraction adsorbed on polyvinylpolypyrrolidone (PVPP), which is a cross-linked synthetic polymer known to specifically bind polyphenols. However, the contents determined in commercial oak wood extracts varied from ≈55 % to ≈73 % (Vignault *et al.*, 2018). This method and other common methods, such as the Total Polyphenol Index or Folin–Ciocalteu index, provided different results, ranging from 16 to 32 % and from 26 to 72 %, respectively (Vignault *et al.*, 2018). The eight oak tannin extracts analysed in this study also showed differences in antioxidant properties measured using different methods (Vignault *et al.*, 2018), and differently affected the colour of a malvidin 3-glucoside solution (Gombau *et al.*, 2019; Vignault *et al.*, 2019).

Moreover, the total polyphenol content of oenological tannins can be analysed using the methyl-cellulose precipitation method, measuring the difference in absorbance (ΔA280) before and after the addition of methyl-cellulose (Sarneckis *et al.*, 2006). However, the response obtained for ellagitanins was significantly lower compared to that of gallo- and condensed tannins for which it was initially developed (Vignault *et al.*, 2018).

Further characterisation of tannin composition can be provided by combining different methods, as shown earlier for gallotannin extracts from gallnut (Watrelot *et al.*, 2020). Liquid chromatography analysis of ellagic acid released from HHDP units after acid hydrolysis (Moutounet *et al.*, 2004) or methanolysis (Lei *et al.*, 2001; Viriot *et al.*, 1994) allows specific determination of ellagitannins but does not provide information on their structure or their mass distribution. Moreover, the low ellagic acid yield (≈15 %) (Moutounet *et al.*, 2004) reflects the low occurrence of HHDP units in the structures. For instance, castalagin and vescalagin contain five gallic acid units, among which two only form a HDDP group, the other three being linked together (30 % ellagic acid yield), and castalin and vescalin contain no HHDP unit and are thus not formally ellagitannins. Castalagin, vescalagin, grandinin, and roburins (A–E) determined by HPLC-UV using calibration with vescalagin accounted together for about 50 % of the material present in a commercial extract (Moutounet *et al.*, 2004). The same eight major ellagitannins were determined in another commercial oak extract using a similar HPLC method developed for their analysis in wine (Navarro *et al.*, 2017), totalling only 144 mg/g (Gombau *et al.*, 2019).

Nuclear magnetic resonance (NMR) analysis provided evidence of heterogeneous mixtures of ellagitannins and carbohydrates in a commercial oak tannin extract. (Melone *et al.*, 2013) Size Exclusion Chromatographic (SEC) analysis suggested that ellagitannins present in this extract consisted mostly of larger molecular weight compounds (> 2000 Da), (Melone *et al.*, 2013) while diffusion ordered spectroscopy (DOSY) NMR analysis detected only lower molecular weight phenolic compounds in other oak tannin samples (Fracassetti *et al.*, 2023).

Besides tannins, oak wood extracts may contain other families of compounds, giving a complex mixture. Hemicelluloses have been detected in water extracts of oak wood (Nonier *et al.*, 2005) and may thus be present in oenological tannins. Quercitol, a polyol, has also been reported as a marker of oak origin (Malacarne *et al.*, 2016; Sanz *et al.*, 2008) and found at concentrations ranging from 5 to 47 mg/g (Malacarne *et al.*, 2016) of dry matter in oenological oak tannins. In addition, lignans (Marchal *et al.*, 2015; Winstel & Marchal, 2019) and coumarins (Winstel *et al.*, 2020), including several bitter compounds, other low molecular weight phenolics, (Martínez-Gil *et al.*, 2020) triterpenoids, some of which contribute sweetness, (Marchal *et al.*, 2011) and various aroma compounds, depending on the botanical species and toasting level, (Jordão *et al.*, 2006) can also be present in oak extracts. Comparison of the compositions of sessile oak (*Quercus petraea Liebl.)* and pedunculate oak (*Quercus robur L.*), which are the major oak species grown in France, showed that they contain different types of triterpenoids, namely quercotriterpenoids in sessile oak and bartogenic acid derivatives in pedunculate oak (*Quercus robur L.)* (Buche *et al.*, 2020). In addition, several oak lactone precursors have also been proposed as sessile oak markers (Buche *et al.*, 2020). However, they were also identified in American oak (*Q. alba*) extracts (Hayasaka *et al.*, 2007).

Thus, oenological tannin extracts from oak show high variability, reflecting differences in the botanical species and process used for their preparation and likely impacting their properties, but their composition is not well known.

The present work aimed to extensively characterise the composition of eight oenological oak tannins available on the market, using a multi-method approach. Phenolic content was analysed by the official gravimetric OIV method and by spectrophotometry measurement at 280 nm. Hydrolysable tannins were assayed by UPLC-DAD analysis of gallic acid, methylgallate, and ellagic acid released after methanolysis. The size distribution of compounds was determined by HPSEC-UV-dRI. Carbohydrates were quantified by GC-MS analysis of their alditol acetate derivatives before and after hydrolysis, and nitrogen content was determined by Kjeldahl analysis. Finally, all samples were compared by a non-targeted metabolomics approach based on HPLC-HRMS profiles.

MATERIALS AND METHODS

1. Materials and chemicals

1.1. Oenological tannins

Eight oenological tannin extracts from oak wood available on the market in the powder form were provided by Biossent (Angeac-Champagne, France), Laffort (Bordeaux, France), ATP group (Larchmont, NY, USA), and IOC (Epernay, France). They were coded T1 to T8. T1–T7 were extracted from French oak (*Q. petraea, Q. robur* or hybrids) and T8 from American oak (*Q. alba*). Samples were stored in the dark in a desiccator until analysis. All sample preparations and analyses were performed in triplicate.

1.2. Chemicals

Acetonitrile, methanol, acetic acid, and formic acid were liquid chromatography−mass spectrometry (LC−MS)-grade purchased from VWR Prolabo (Fontenay-sous-Bois, France). Deionised water was obtained from a Milli-Q purification system (Millipore, Molsheim, France).

Gallic acid, ellagic acid, β-glucogallin, castalagin, vescalagin, castalin, lithium chloride, β-D-allose, myo-inositol and hydrochloric acid (37 %) were provided by Sigma-Aldrich (St. 124 Louis, MO, USA), proto-quercitol was provided by chem-Impex International (Wood Dale, IL, USA) and N,N-dimethylformamide was provided by Carl Roth GmbH (Karlsruhe, Germany). Polyvinylpolypyrrolidone (PVPP) was purchased from BASF SE (Ludwigshafen, Germany).

2. Analysis of oenological tannin extracts

2.1. Gravimetric method

Total polyphenols were quantified according to the official OIV method, (OIV, 2022) *i.e.,* gravimetric analysis of the proportion of material adsorbed on a PVPP column.

2.2. UV spectrophotometry

Tannin extracts were dissolved at 5 g/L in methanol: water (50:50, *v/v*) containing 1 % formic acid and diluted 200-fold. Then, absorbance at 280 nm was measured with a 1 cm optical path cuvette using a Shimadzu-1800 UV-visible spectrophotometer and the total polyphenol content was expressed in mg/g ellagic acid equivalent, after calibration with a commercial standard.

2.3. Gallotannins and ellagitannins by UHPLC-DAD analysis after methanolysis

For acidic methanolysis, 5 mg of tannins were dissolved in 2.5 mL of methanol acidified with 0.6 N HCl. The solution was heated at 120 °C for 260 min and cooled down to room temperature in a water bath. Then, the solution was diluted 2-fold in water and centrifuged at 15000 rpm for 15 min before injection (injection volume 0.5 µL). The UHPLC−DAD equipment was an Acquity UPLC−DAD system (Waters, Milford, MA, USA) equipped with an Amazon X mass spectrometer (Bruker Daltonics, Bremen, Germany), piloted by Hystar software. HPLC analysis was performed using a BEH C18 (1.7 µm × 1 mm × 150 mm) column equipped with a 0.2 µm prefilter (Waters, Milford, MA, USA) placed in an oven at 35 °C, with a 0.08 mL/min flow rate and a 45 min run time. The mobile phase consisted of water:formic acid (99:1, *v/v*) (eluent A) and acetonitrile:formic acid (99:1, *v/v*) (eluent B). The gradient conditions were as follows: 0 min, 1.3 % B; 6.5 min, 10 % B; 12 min, 20 % B; 27 min, 50 % B; 32 min, 99.5 % B; and 40 min, 1.3 % B. The mass spectrometer was equipped with an electrospray source and an ion trap mass analyser. MS analyses were operated in negative ion mode (end plate offset: –500 V; temperature, 200 °C; nebuliser gas: 10 psi and dry gas, 5 L/min; capillary voltage, 4.5 kV in negative mode). Collision energy for fragmentation used for MS2 experiments was set at 1. Compounds were identified according to their UV and MS spectra and comparison with standards and literature data. Gallic acid and its derivatives were quantified at 280 nm, and ellagic acid was quantified at 360 nm, using external calibration with gallic acid and ellagic acid, respectively.

2.4. Molecular size distribution by HPSEC-UV-dRI

High-pressure size exclusion chromatography (HPSEC) was used to determine the molecular size distribution. The HPLC-1260 Infinity II system, (Agilent Technologies) was equipped with 2 Phenogel columns connected in series (500*7.8 mm, 5 µm each, with pore sizes of 50, 1000, and 1 000000 Å), protected by a pre-column (50*7.8 mm) containing the same material, placed in an oven at 60 °C and a diode array detector (DAD). UV absorbance was recorded at 280 nm. Moreover, the system was also coupled to a differential refractometer index (dRI) Optilab T-Rex, Wyatt (Santa Barbara, CA, USA), lamp at λ = 666 nm, with the cell thermostated at 60 °C. This set-up allowed one to detect chromophores like polyphenols with the UV-280 nm detector, whereas dRI is not specific and also detects non-phenolic compounds. The isocratic elution method used a mobile phase (N,N-dimethylformamide with 1 % (*v/v*) acetic acid, 5 % (*v/v*) water and 0.15 M lithium chloride) at 0.8 mL/min (Kennedy & Taylor, 2003). Tannin

extracts were dissolved at 5 g/L in the mobile phase. After centrifugation, 50 µL of the sample was injected. Commercial ellagic acid and castalagin were injected under the same conditions and used as size distribution standards.

2.5. Untargeted metabolomics using UHPLC-HRMS analysis

Untargeted metabolomics analysis was performed on a UHPLC-DAD-HRMS system. Separations were obtained using a Vanquish UHPLC-DAD (Thermo Fischer Scientific, San José, CA, USA) on a (100*1 mm i.d.) Acquity HSST3 column (Waters, Milford, MA, USA; 1.7 µm), operated at 35 °C. The mobile phase consisted of water:formic acid (99:1, *v/v*) (eluent A) and acetonitrile:formic acid (99:1, *v/v*) (eluent B). The flow rate was 0.22 mL/min. The elution program was as follows: isocratic for 1.5 min with 1 % B, 1–10 % B (1.5–4.5 min), isocratic with 10 % B (4.5–7 min), 10–20 % B (7–12 min), 20–40 % B (12–15 min), 40–50 % B (15–16 min), 50–99 % B (16–17 min). ESI-MS/MS analyses were performed with an Orbitrap Exploris 480 from Thermo Fisher Scientific (San José, CA, USA) equipped with an electrospray source and an internal post-source fluoranthene mass calibrant (radical ion at *m/z* 202.0777 in positive ion mode and *m/z* 202.0788 in negative ion mode). The spectrometer was operated in the negative ion mode (ion transfer tube: 280 °C; vaporiser temperature: 300 °C; sheath gas, auxiliary gas, and sweep gas: 40, 10, and 2 (arbitrary units), respectively; voltage set: mode, 2.5 kV in negative mode. The mass range was 150–2000 *m/z*.

The sample injection sequence was adapted from Arapitsas and Mattivi (Arapitsas & Mattivi, 2018). It included samples, a Quality Control (QC) sample and a blank. Tannin extracts were dissolved in triplicate at 5 g/L in methanol: water (50:50, *v/v*) containing 1 % formic acid. After vortexing and ultrasonication, the solutions were centrifuged for 15 min at 15,000 rpm before injection (0.5 µL) into the UPLC system. The QC was a mix of 100 µL of each sample. Blank was methanol: water (50:50, *v/v*) containing 1 % formic acid. Samples were injected in triplicate following a randomised order. After equilibration, the sequence started with 3 injections of blank, which were followed by 3 injections of QC sample. Then, the QC sample and blank were injected with 6 real samples each. The sequence finished with 1 injection of QC sample and 1 injection of blank. The injection volume was set to 0.5 µl for all samples.

MS data from real samples, QC, and blank samples were processed by Compound Discoverer software (v. 3.2.2.421, Thermo, Waltham, MA, USA), which facilitated peak recognition and dereplication of raw data (retention time alignment, adducts, and isotopic peak clustering). Parameters of the Workflow are described in Table S1. Features corresponding to each retention time associated with a *m/z* value were then filtered based on the variability of the triplicate analysis (CV < 10 %) and the ANOVA results (p-value < 0.05), keeping only features with at least one sample exhibiting low variability and significant differences with another sample. A list of 159 features was thus established. Annotation was performed manually using identification previously done with LC-DAD-MS, completed by mass spectra accuracy with the specific instrumentation mass resolution and in accordance with the four levels of annotation described by Sumner (Sumner *et al.*, 2007).

2.6. Sugar and polyol analysis

Neutral sugar and polyol composition was determined as alditol acetates after reduction and acetylation as previously described (Albersheim *et al.*, 1967). The reaction was performed on 1 mg of tannin extract, before and after hydrolysis with 2 M trifluoroacetic acid. The alditol acetates were quantified by gas chromatography with a flame ionisation detector (GC 2010 Plus Shimadzu chromatograph; carrier gas: Hydrogen 5.6 B50; column: DB225—30 m long/0.25 mm ID/0.25 micron film—JW Scientific). Allose and myo-inositol were used as internal standards for the quantification of neutral sugars and quercitol.

2.6. Quantification of nitrogenous material for estimation of protein content

The total nitrogen concentration (from proteins, peptides, amino acids, and ammonium ions) was determined using the Kjeldahl method (Kjeldahl, 1883) performed on 200 mg of tannin extract. The nitrogen content was converted to amino acid, peptide, and protein content using 6.25 as the conversion factor, assuming that all nitrogen comes from these compounds and that they contain 16 % of nitrogen (Jones, 1931).

3. Statistical Analysis

For all quantitative data except HRMS data, analysis of variance (one-way ANOVA) was performed under R (4.4.0) using the multcompView and agricolae packages. Samples were considered significantly different at p-value < 0.05.

Untargeted HRMS data were processed using Compound Discoverer version 3.1 software for ANOVA, unsupervised hierarchical clustering, and principal component analysis. Hierarchical clustering was generated using complete linkage and Euclidean distance methods and PCA using centred and scaled values.

RESULTS AND DISCUSSION

1. Total polyphenol assays

Table 1 displays the total polyphenol content measured by the official gravimetric OIV protocol and by converting UV-280 nm absorbance values to mg/g ellagic acid equivalent.

The gravimetric analysis method has recently replaced the spectrophotometric method for evaluation of polyphenols in oenological tannin extracts. It relies on the assumption that polyphenols are selectively adsorbed on PVPP while other compounds can contribute absorbance at 280 nm. Moreover, unlike the spectrophotometric method, it does not rely on calibration with a selected standard, which may lead to under- or over-estimation of the concentrations. Both

TABLE 1. Characterisation of the eight oenological oak tannins: concentrations of total polyphenols, sugars and polyols, nitrogen compounds, and residual water and concentrations of gallic acid, methyl gallate, methyl digallate and ellagic acid measured after methanolysis (mg/g).

						HPLC-UV-MS analysis after methanolysis			
Samples	Total polyphenols by gravimetric OIV method	Total phenolic compounds by UV-absorbance at 280 nm[a]	Total sugars and polyols	Total nitrogen compounds[b]	Total polyphenols, sugars, polyols and nitrogen compounds[c]	Gallic acid	Methyl gallate[d]	Methyl digallate[d]	Ellagic acid
T1	473 ± 0^{h}	364.0 ± 5.2^{g}	231 ± 12^{e}	6.7 ± 0.8^{ac}	711 ± 2^{d}	0.6 ± 0.3^{b}	1.3 ± 0.4^{b}	2.4 ± 1.1^{b}	12.2 ± 1.6^{df}
T2	614 ± 0^{a}	490.7 ± 23.7^{a}	190 ± 7^{a}	7.9 ± 0.0^{a}	812 ±7^{a}	0.0 ± 0.0^{a}	8.9 ± 0.0^{a}	0.0 ± 0.0^{a}	26.9 ± 0.0^{a}
T3	755 ± 0^{b}	559.3 ± 9.3^{b}	59 ± 5^{b}	5.9 ± 0.6^{bc}	820 ± 11^{ab}	0.6 ± 0.2^{b}	2.3 ± 0.2^{b}	2.2 ± 0.5^{ab}	32.1 ± 0.3^{a}
T4	758 ± 0^{c}	570.5 ± 14.1^{a}	155 ± 5^{c}	5.4 ± 0.0^{b}	918 ±5^{c}	0.0 ± 0.0^{a}	10.6 ± 0.2^{c}	0.0 ± 0.0^{a}	73.3 ± 2.4^{b}
T5	776 ± 9^{d}	528.7 ± 1.7^{c}	83 ± 2^{b}	5.7 ± 0.1^{bc}	864 ± 12^{bc}	0.6 ± 0.1^{b}	1.6 ± 0.3^{b}	2.3 ± 0.4^{ab}	18.2 ± 3.4^{c}
T6	621 ± 14^{e}	444.9 ± 7.4^{d}	153 ± 10^{c}	6.5 ± 0.6^{a}	780 ± 30^{a}	0.5 ± 0.2^{ab}	1.7 ± 0.2^{b}	3.1 ± 1.7^{bc}	13.8 ± 2.8^{cd}
T7	501 ± 10^{f}	333.7 ± 9.1^{e}	259 ± 7^{e}	32.8 ± 0.6^{a}	793 ± 23^{a}	0.6 ± 0.3^{b}	1.5 ± 0.9^{b}	2.6 ± 1.1^{bc}	7.5 ± 1.0^{ef}
T8	593 ± 0^{g}	396.5 ± 1.4^{f}	216 ± 2^{e}	1.0 ± 0.3^{ab}	811 ± 18^{ab}	0.2 ± 0.0^{ab}	1.5 ± 0.3^{b}	5.0 ± 0.3^{c}	3.7 ± 0.5^{e}

[a] ellagic acid equivalent; [b]converted to amino acids, peptides and proteins from nitrogen content determined using Kjeldahl analysis, using 6.25 as conversion factor; [e]sum of the total sugars and polyols, total polyphenols from OIV method and total nitrogen compounds (converted to amino acids, peptides and proteins); [d]gallic acid equivalent. Different letters indicate significant differences between samples for a given parameter ($p < 0.05$).

assays showed variability between samples, as expected. Polyphenol concentrations ranged from 473 mg/g to 776 mg/g and from 334 to 571 mg/g, using the OIV method and the UV spectrophotometry method, respectively, suggesting the presence of large quantities of non-phenolic material in all extracts, especially in T1, T7, and T8 (Table 1). Absorbance values at 280 nm and polyphenol contents measured by gravimetry were highly correlated ($r = 0.93$), indicating that the proportion of UV-absorbing material retained on the PVPP column was similar for all samples.

2. Sugar and polyol composition and nitrogen content

Sugars and polyols were quantified for each tannin before and after hydrolysis (Figure 1), and their total amount is given in Table 1.

T1, T7, and T8 were significantly richer in sugars and polyols, containing over 20 %, whereas T3 and T5 showed contents lower than 10 % (Table 1). Arabinose, glucose, and xylose were detected in all samples along with lower amounts of galactose, rhamnose, and mannose. Much higher concentrations of sugars and especially xylose, but also galactose, arabinose, glucose and, to a lesser extent, rhamnose and mannose were quantified after hydrolysis, meaning that a large proportion was initially present under linked forms (hemicellulose), as reported earlier in oak heartwood. (Nonier *et al.*, 2005) Moreover, large differences in their amounts and proportions were observed between samples. T1 contained over 125 mg/g xylose, including 100 mg/g under linked form, while T3, T4, and T5 were particularly poor in xylose (Figure 1b). Total arabinose and glucose concentrations each ranged between 13 and 42 mg/g, and the proportion of free and linked forms varied between samples, the latter being predominant in T1, T6, T7, and T8. Total galactose was found between 6 and 65 mg/g and mainly in the linked form. Quercitol, which is a known marker of oak origin, (Sanz *et al.*, 2008) was also present in all samples but particularly abundant in samples T2 and T4, representing about 60 mg/g (Figure 1a).

The concentration of amino acids, peptides and proteins was estimated from the analysis of nitrogen content released during the Kjeldahl analysis. Tannin extracts contained less than 8 mg/g of "proteins" except T7 (33 mg/g).

In all the tannin extracts, the sum of polyphenols, sugars, polyols and "proteins" accounted for a minimum of 71.1 % in T1 and a maximum of 91.8 % in T4 (Table 1). Residual water content was also determined, representing 0 to 5.8 % of the sample weight (data not shown). Consequently, part of the sample composition, varying between 8 to 29 % (or 8 to 24 % if we consider the residual water content), remains unexplained. The extracts containing the lowest amount of total sugars and polyols (T3 and T5) showed the highest polyphenol content, whereas the richest (T1, T7, and T8) showed the lowest polyphenol content (Table 1). Total sugars and polyols were negatively correlated with total polyphenol content determined by the gravimetric method ($r = –0.89$).

3. Gallotannins and ellagitannins by UHPLC-DAD after methanolysis

The concentrations of gallic acid and methyl gallate released after methanolysis were low in all extracts. Low amounts of methyl digallate were also detected in most samples (except T2 and T4), indicating that some of the tannin structures contained two gallic acid units linked through a depside bond. The concentrations of ellagic acid measured after methanolysis showed some variability between samples (Table 1), reflecting differences in ellagitannin content and composition. However, they remained quite low, reaching only up to 70 mg/g in T4.

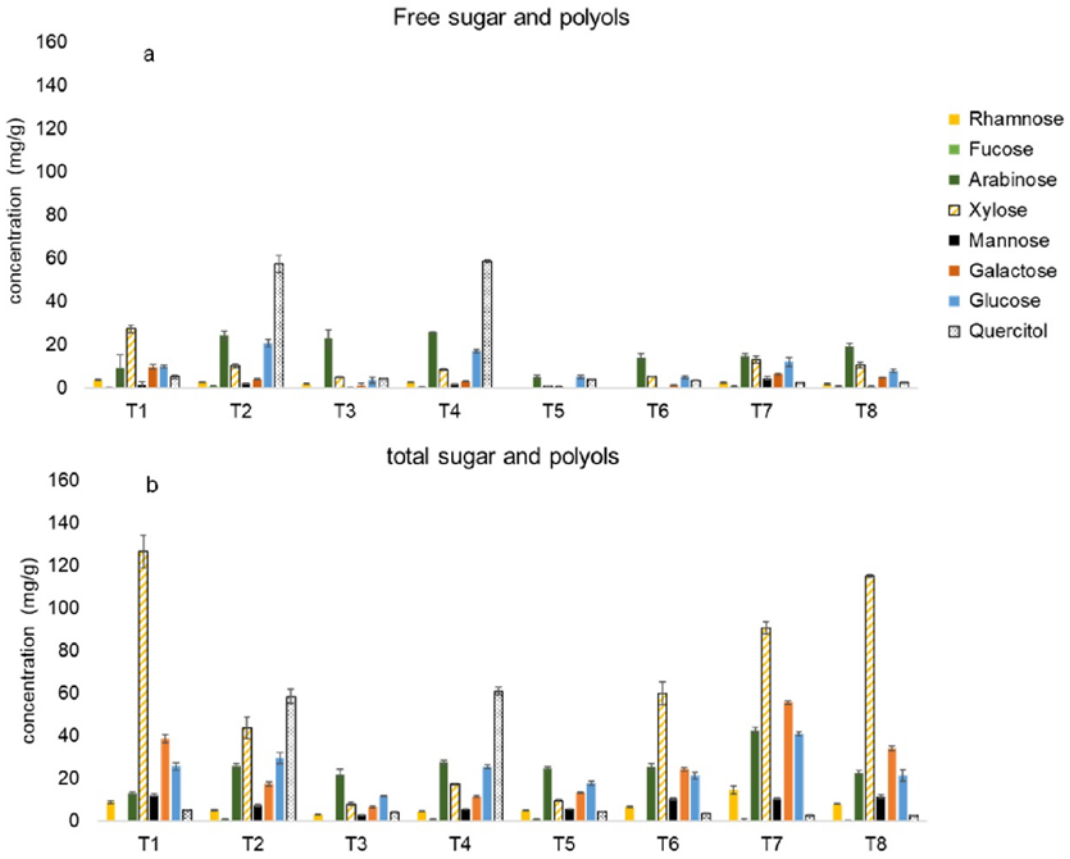


FIGURE 1. Concentrations of sugars and polyols in tannin extracts. (a): free sugars and polyols; (b): total sugars and polyols determined after hydrolysis.

The low conversion rate of ellagitannins to ellagic acid is consistent with their structures containing few HHDP units.

4. Size distribution by HPSEC-UV-dRI analysis

Figure 2 displays the size distribution profiles obtained by HPSEC-UV (280 nm)-dRI for the eight tannin samples along with castalagin and ellagic acid standards.

T2, T3, T4, and T5 present the largest peak areas while T1, T6, T7, and T8 show lower intensities in UV and dRI chromatograms. UV (280 nm) chromatogram areas (data not shown) correlated well with the UV (280 nm) absorbance measured by spectrophotometry provided in Table 1 ($r = 0.91$). Column yields varied between 95 and 105 %, suggesting that the phenolic material detected by HPSEC-UV (280 nm) was well representative of the UV (280 nm) absorbing compounds. The slightly lower correlation with total polyphenol values obtained by the gravimetric method suggests that some of the lower molecular weight phenolic compounds were not retained on the PVPP column. Indeed, r increases (from 0.84 to 0.91) when only the chromatogram areas of P2 and P3 are considered.

Few studies discuss the specific affinity of PVPP towards ellagitannins. Consequently, it is difficult to identify at this stage whether one of the fractions detected by HPSEC-UV (280 nm) was better retained on PVPP, compared to another. On the other hand, it was demonstrated that polyphenol affinity towards PVPP increases with the number of OH groups (Durán-Lara *et al.*, 2015) and aromatic rings (Magalhães *et al.*, 2010), so that higher molecular weight ellagitannins would be better retained, which is consistent with our observation.

Moreover, dRI and UV-chromatograms show similar size distribution profiles, meaning that dRI chromatograms mainly correspond to UV (280 nm) absorbing material (Figure 2a–b).

Chromatograms were divided into five populations, coded P1 to P5 (Figure 2). P1, eluted between 19 and 22.5 min, contains large polymers detected by dRI, only in T7 (Figure 2b), but showing no or very little UV absorbance, indicating that they are not phenolic material. This may correspond to proteins, glycoproteins, or polysaccharides, since T7 contains the highest concentration of nitrogen compounds as well as large amounts of polysaccharides. P2 and P3, eluted at 22.5–26 min and 26–27.5 min, respectively, represent the major part of UV- and dRI-chromatrogram areas. P2 probably corresponds to higher molecular weight ellagitannins, whereas P3 has the same hydrodynamic volume as castalagin (Figure 2a–b). P4 UV-chromatogram perfectly overlays with ellagic acid UV-chromatogram (Figure 2a). However, the dRI detector shows the presence of other compounds, eluting slightly later, which are not phenolic compounds as they are not detected at 280 nm (Figure 2a–b). Finally, P5 contains smaller molecules absorbing at 280 nm, which may correspond to phenolic acids and aldehydes or HMF (hydroxymethylfurfural).

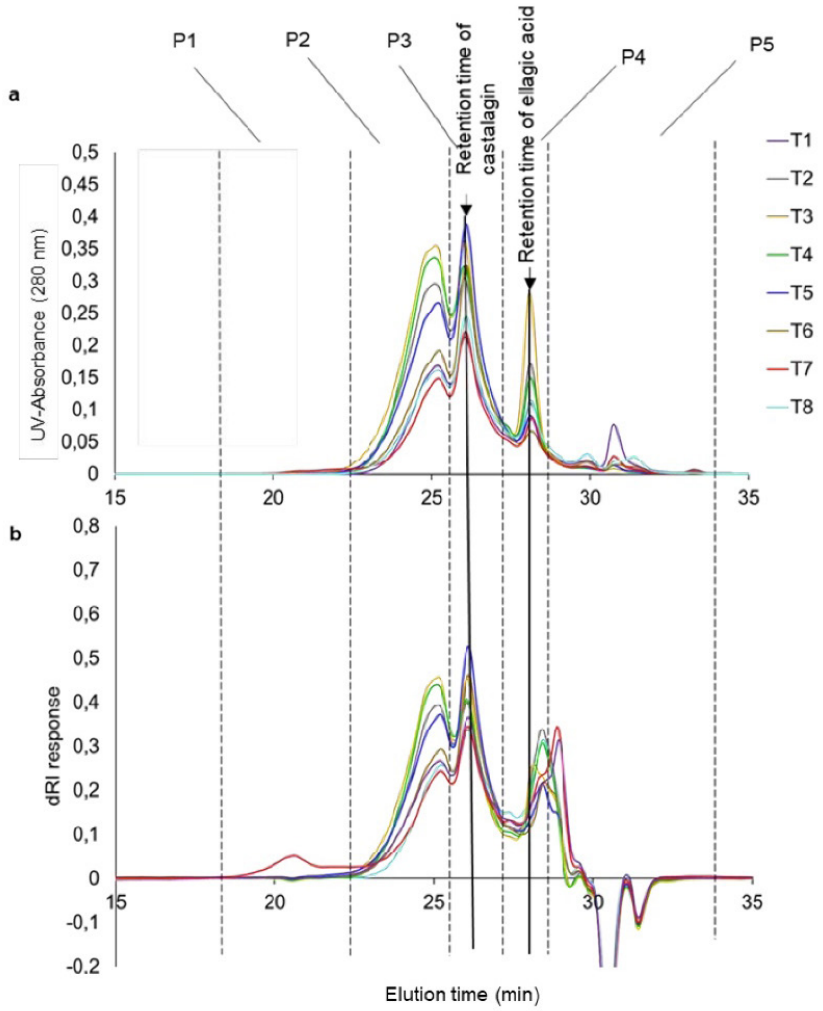


FIGURE 2. HPSEC analysis of oak tannin extracts. (a) UV detection at 280 nm; (b) dRI detection. Retention times of ellagic acid and castalagin and populations P1 to P5 are also indicated.

5. Untargeted metabolomics using UHPLC-HRMS analysis

The chemical composition of the 8 samples was investigated by untargeted metabolomics using UHPLC-HRMS analysis in the negative ion mode. Features corresponding to each retention time associated with a *m/z* value were selected as described in Materials and Methods. The 159-feature dataset thus obtained was first analysed by PCA. Its reliability is shown by the proximity of projections of triplicates of each tannin sample and of all QC samples on the first two PCs (Figure 3). Components 1 and 2 of the PCA represented 58.1 % of the total variance. The first axis (explaining 29.0 % of the dataset) contrasted T2, T3, and T4 with T1 and T7, while T5 and T6 were close together and separated from all other samples on the second axis (26.9 %).

Annotation of the compounds was performed manually by comparison of retention times and mass spectra, and, when available, fragmentation spectra and UV-visible spectra, with an internal database and with data published in the literature. A given compound could be associated with several features with identical retention times but different *m/z* values, corresponding to molecular or pseudo-molecular ions, adduct and fragment ions, and some isotopic peaks. Data and annotation of the features following the four levels of annotation described by Sumner (Sumner *et al.*, 2007) are presented in the Table S1, along with the results of ANOVA performed for each feature to determine whether intensity differences between the two samples were significant.

Most of the signals showing high intensity could be attributed to phenolic compounds. Ellagic acid, gallic acid, castalagin, vescalagin, roburins A/D (two isomers), roburins B/C (one signal), and three isomers of grandinin and roburin E were among the major signals detected (Table S1). Another major signal detected at *m/z* 608.02631 corresponding to the doubly charged ion of a molecule with a M_w of 1218.06691 Da ($C_{55}H_{30}O_{33}$) was tentatively attributed to castacrenin F, a vescalagin coupled to an ellagic acid. This structure has been described earlier in Oakwood and proposed to result from partial hydrolysis of higher molecular weight tannins such as roburins or castaneins (Tanaka *et al.*, 1997). Other important signals differed from vescalagin/castalagin and roburin A/D by the loss of 18.01026 a.m.u (H_2O). The signal at *m/z* = 931.04610 was attributed to dehydrocastalagin, the major thermal degradation product of castalagin (Glabasnia

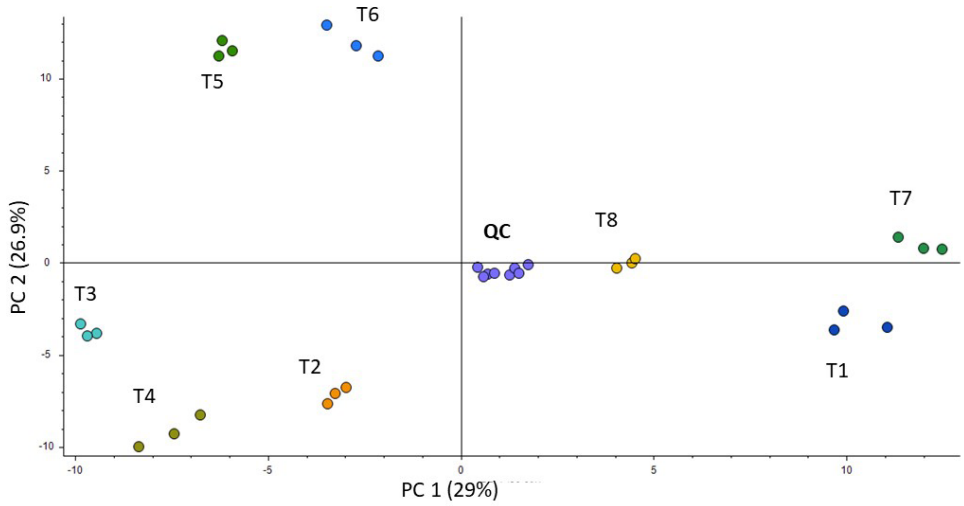


FIGURE 3. PCA plot of untargeted UHPLC-HRMS analysis data of oak tannin samples.

& Hofmann, 2007), while the deoxy products formed from vescalagin and roburin A in toasting experiments (Glabasnia & Hofmann, 2007) were not detected. The signal at *m/z* 527.02826 was attributed to castacrenin E, detected as the doubly charged ion $[M\text{-}2H]^{2-}$. Castacrenin E, resulting from oxidative decarboxylation of castacrenin D (vescalagin substituted by a gallic acid through a C-C bond), has been detected in oakwood (Tanaka *et al.*, 1997). It was also found among products formed in toasted oakwood (Chira *et al.*, 2020), as well as another compound detected at *m/z* 1011.07477, not yet identified. Signals at *m/z* 961.05859, 1085.07507, and 593.02087 (doubly charged ion) were tentatively attributed to 33-carboxy-33-deoxyvescalagin, (Glabasnia & Hofmann, 2007) castacrenin F, which is a vescalagin substituted by ellagic acid through a C-C bond, and castacrenin G, an oxidative metabolite of castacrenin F (Tanaka *et al.*, 1997), respectively.

Other signals were attributed to carbohydrates, triterpene saponins (Arramon *et al.*, 2002), and lignans (Marchal *et al.*, 2015) reported earlier in oak as detailed below.

The heatmap obtained by unsupervised hierarchical cluster analysis performed on the 159-feature dataset (Figure 4) confirmed that injections of triplicate samples are grouped and separated into three clusters: T1, T8, and T7; T2, T3, and T4, and T5 and T6. This analysis also highlighted clusters of features that were over- or under-represented in some samples.

In addition, the features that were attributed molecular formula were plotted on a van Krevelen diagram (Figure 5).

The first cluster (A) contains eleven signals showing significantly lower intensities in T7. Eight of them were attributed to ellagic acid and its adducts, and one to quinic acid. The other three are unknown. However, three of them were attributed a molecular formula that suggests that they are lower molecular weight phenolic compounds and are in the ellagitannin region on the van Krevelen diagram (Figure 5).

The next cluster (B) contains 37 features that were more abundant in T2, T3, and T4 than in other samples. The major compounds identified in this cluster were ellagitannin derivatives, showing high molecular weight and intermediate polarity, such as castacrenin F. Two signals detected at *m/z* = 915.05334 and *m/z* = 915.05328 were tentatively attributed to dehydrated castalagin/vescalagin ($C_{41}H_{24}O_{25}$ detected as $[M\text{-}H]^-$) and dehydrated roburin A/D ($C_{82}H_{48}O_{50}$ detected as $[M\text{-}2H]^{2-}$ at *m/z* = 915.55542). The signal at 1041.08557 was attributed to the formula $C_{47}H_{30}O_{28}$, which may correspond to a trihydroxybenzene substituted vescalagin/castalagin structure, described in chestnut (Venter *et al.*, 2019). A signal detected at *m/z* = 1011.07477 presumably corresponds to a C-glycosidic ellagitannin, detected earlier and formed after oakwood toasting, along with castacrenin F. (Chira *et al.*, 2020) This cluster thus seems characteristic of toasted oakwood extracts, It also contained a series of highly polar compounds, including several gallic acid hexosides, gallic acid deoxyhexoside, ellagic acid hexoside, some ellagitannins, and unknown compounds with a high O/C ratio in their molecular formula (Figure 5), suggesting that they are acidic sugars or organic acids, as well as a series of unknown compounds, eluting between 11 and 17 min and, thus, rather apolar.

The next cluster (C) appeared overrepresented in T1, present in T2, T6, T7, and T8, and almost absent in T3, T4, and T5. Among its 23 features, 17 were eluted very early (Rt < 1 min) and attributed molecular formula with O/C and H/C ratios suggesting that they were carbohydrates (Figure 5). Some

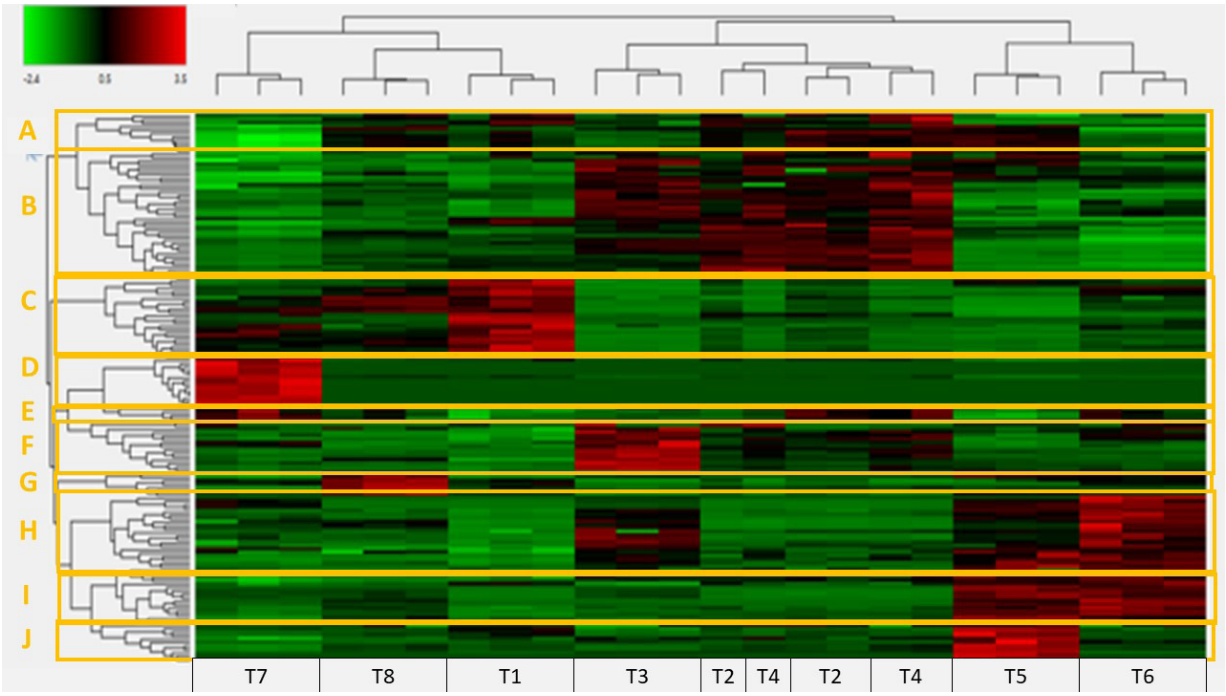


FIGURE 4. Unsupervised hierarchical cluster analysis of metabolites and samples, showing the letter codes for metabolite clusters.

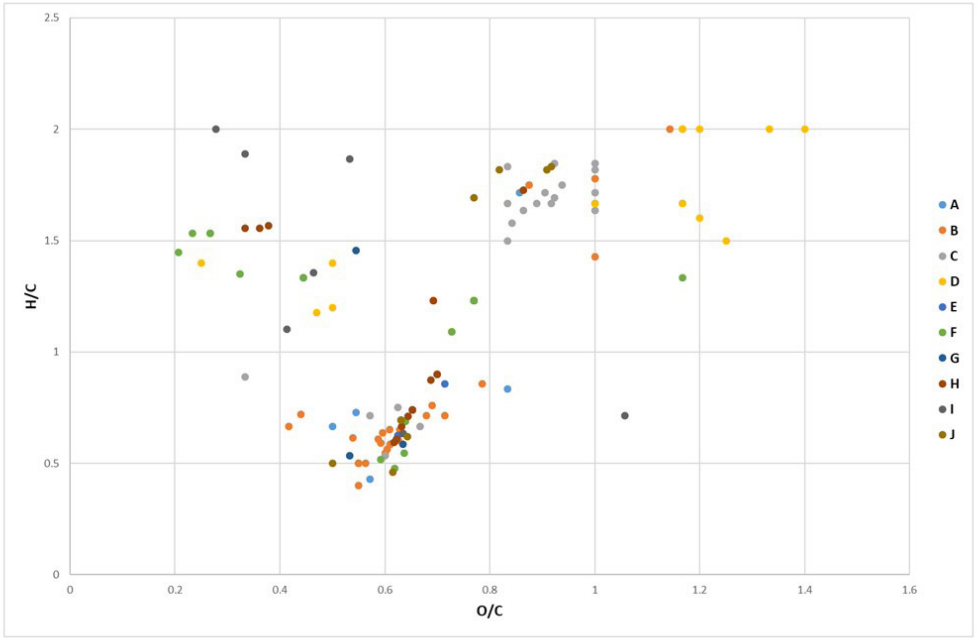


FIGURE 5. Van Krevelen diagram based on the H/C and O/C atomic ratios performed on features attributed to a molecular formula. Colour codes indicate the clusters.

of these features could be tentatively attributed to a uronic acid (glucuronic or galacturonic acid), a methyluronic acid (probably 4-O-methylglucuronic acid), along with pentoside and acetylpentoside derivatives of these structures. They likely correspond to 4-O-methylglucuronoxylan and O-acetyl-(4-O-methylglucurono)xylan structures that are the most abundant hemicellulose structures in heartwood (Teleman *et al.*, 2002). Five other features were attributed molecular formula that may correspond to phenolic compounds.

Cluster D contained 13 features that were characteristic of T7 and present in very low amounts in all other samples, none of which could be identified. Among them, 11 contained sulfur atoms and may correspond to sulfonated compounds. In particular, the compounds detected at m/z = 245.0489

and *m/z* = 325.0057 were attributed the molecular formula $C_8H_{14}O_5S$ and $C_8H_{14}O_8S_2$, differing by a sulfonate (SO_3) group. As sulfonates are known to be formed by reaction of bisulfite ions with various molecules, including phenolic compounds, sugars, keto-acids, and aldehydes (Nikolantonaki *et al.*, 2022), their presence suggests that T7 has been exposed to bisulfite ions during its extraction process.

Cluster E contained only 4 features that were present in all samples, although more abundant in some of them (especially T2, T4, and T7). Three of these signals corresponded to gallic acid, while the last one was tentatively attributed to a gallic acid adduct of vescalagin or castalagin, possibly castacrenin D identified in chestnut (Tanaka *et al.*, 1997).

Cluster F contained 15 features that were present in higher amounts in T3 than in the other extracts. Half of these features were tentatively attributed to hydrolysable tannin derivatives. Signals at *m/z* = 331.06708 and *m/z* = 469.00458 were tentatively attributed to gallic acid hexoside and valoneic acid dilactone, respectively. The signal at 698.05792 corresponds to the doubly charged ion of a $C_{61}H_{42}O_{39}$ structure that can be attributed to a vescalagin /castalagin substituted with two galloyl groups and one hexose and oxidised (-2H). That detected at *m/z* = 593.02087 may correspond to castacrenin G, identified earlier in chestnut and resulting from oxidation of castacrenin F (Tanaka *et al.*, 1997). Other major signals of this cluster correspond to apolar compounds eluted around 16 min that were tentatively attributed to triterpene saponins, namely bartogenic acid, *i.e.*, 2α,3β,19α-trihydroxyolean-12-ene-24,28-dioic acid (*m/z* = 517.31689, $C_{30}H_{46}O_7$), and two related compounds containing an additional OH group (*m/z* = 533.31189, $C_{30}H_{46}O_8$) that have been identified in oak heartwood (Arramon *et al.*, 2002). In a recent study comparing the composition of sessile oak (*Quercus petraea Liebl.*) and pedunculate oak (*Quercus robur L.*), bartogenic acid derivatives were shown to be specific to the latter (Buche *et al.*, 2020). The last two compounds of intermediate polarity were tentatively attributed to lignans, as their *m/z* values at 551.21326 correspond to the $[M-H]^-$ ion of pentosyl lyoniresinol ($C_{27}H_{36}O_{12}$). Two pentosyl derivatives of lyoniresinol, namely lyoniside and nudiposide, which are 9'-O-β-xylopyranosides of (+)-lyoniresinol and (-)-lyoniresinol, respectively, have been isolated from oakwood (Marchal *et al.*, 2015).

Cluster G contained 7 features showing higher intensity in T8. Two signals, detected at *m/z* = 931.0481 and at *m/z* = 315.01468, were tentatively attributed to dehydrocastalagin and methylellagic acid. The former has been reported to be an oxidation product of castalagin formed upon toasting. (Glabasnia & Hofmann, 2007) The signal at *m/z* = 487.18167 was attributed the formula $C_{22}H_{32}O_{12}$. This may correspond to a 6′-O-gallate derivative of (3S,4S)-4-β-D-glucopyranosyloxy-3-methyloctanoic acid that has been identified as a precursor of *cis*-β-methyl-γ-octalactone (Masson *et al.*, 2000) and reported as a marker of sessile oak (Buche *et al.*, 2020). However, this compound was also detected in *Q. alba*, along with three isomers (Hayasaka *et al.*, 2007). The other compounds detected in this fraction remain unidentified.

Cluster H (21 features) corresponded to molecules that were present in higher amounts in T3, T5, and T6, and in lower amounts in T1, T2, and T4. The most abundant features corresponded to vescalagin and other known oak ellagitannins. The signals at *m/z* = 924.05865 (two isomers) could be tentatively attributed to roburins A and D, detected as the doubly charged ions, and the signal at *m/z* = 990.07971 to roburin B or roburin C. The three signals at *m/z* = 1065.10632 likely correspond to grandinin, roburin E and a pentoside of vescalagin or castalagin. The signal at *m/z* = 527.02826 was tentatively attributed to castacrenin E, identified earlier (Tanaka *et al.*, 1997). Three other signals were also attributed to hydrolysable tannins, namely gallic acid deoxyhexoside and HHDP hexose with a closed hexose core (*m/z* = 481.06204, $C_{20}H_{18}O_{14}$) (Venter *et al.*, 2019). This cluster also contained three polar features, including one signal attributed to a tetrasaccharide (two hexose and two pentose) and four signals eluted at higher retention times that were tentatively attributed to triterpene saponin derivatives. Thus, signals at 695.36487 and 679.36975 likely correspond to hexosides of the triterpene saponins present in cluster E, probably their glucosides that have been identified in oakwood. (Arramon *et al.*, 2002) The signal at *m/z* = 725.37543 can be interpreted as a methoxyl derivative of the triterpene saponin detected at *m/z* = 533.31183.

The last two clusters are cluster I (16 features, more abundant in T5 and T6) and J (11 features, overexpressed only in T5). The most abundant compound in cluster I was attributed to castalagin. Two signals at *m/z* = 571.18225 and *m/z* = 581.22388 were tentatively attributed to a galloyl and a hexoside derivative of lyoniresinol, likely corresponding to quercoresinol, *i.e.*, lyoniresinol 9-*O*-gallate, and lyoniresinol 9'-*O*-β-glucopyranoside identified earlier in oak heartwood of *Q. petraea* and reported to taste slightly sweet and bitter, respectively (Marchal *et al.*, 2015). This cluster also contained two apolar compounds that may be lipid derivatives and several unknown compounds. Three ellagitannin derivatives were tentatively identified in cluster J. The signal at *m/z* = 961.05859 likely corresponds to 33-deoxy-33-carboxyvescalagin reported earlier in oakwood extracts (Glabasnia & Hofmann, 2006) and those at *m/z* = 1047.09607 to dehydrated roburin E and grandinin. Cluster J also contained four polar compounds that were tentatively identified as disaccharides and several unknown compounds.

Non-targeted metabolomics data separated oak tannin samples into three major groups, providing further insight into their composition differences. In the first group (T1, T7, T8), HRMS features attributed to ellagitannin derivatives (clusters B, F, G, H, I, J) were under-represented and those attributed to carbohydrates corresponding to hemicellulose structures (cluster C) over-represented, consistent with the low phenol content and high concentrations of sugars in the bound form in these samples. T7 showed a particular composition characterised by the presence of molecular markers containing sulfur atoms (cluster D), possibly reflecting the use

of sulfites in the extraction process. It also contained higher molecular weight compounds detected by dRI but showing no UV absorbance, along with higher amounts of nitrogen, suggesting that these compounds correspond to proteins or glycoproteins. Features (cluster G) overrepresented in T8 included signals attributed to dehydrocastalagin formed from castalagin upon thermal treatment (Glabasnia & Hofmann, 2007) and to a lactone precursor reported in American oak (T8) (Hayasaka *et al.*, 2007). Samples in the second group (T2, T3, and T4) were characterised by higher levels of HRMS features attributed to ellagitannins (clusters B, F, and H in T3), including some that are formed upon toasting, in relation with their high concentrations of phenolic compounds and especially of the higher molecular weight (P2) population. T2 and T4 also contain much higher quercitol contents (60 mg/g) than all other samples. T3 is particularly rich in bartogenic acid derivatives that have been detected in pedunculate oak but not in sessile oak (Buche *et al.*, 2020) and can thus be considered as markers of the oak species. It also contains lyoniresinol derivatives, some of which may contribute bitterness or sweetness (Marchal *et al.*, 2015). The last group (T5 and T6) was characterised by features corresponding to ellagitannins, including roburins, castalagin, grandinin (clusters H and I), in agreement with their SEC profile showing a high proportion of lower molecular weight ellagitannins (P3).

CONCLUSION

In this study, eight commercial tannin extracts from oak were characterised by a combination of analytical approaches. Our results confirm that the studied samples show very complex compositions with large variability between them. Total polyphenol contents determined by the official OIV method ranged from 47.3 to 77.6 %, and those evaluated from the UV 280 absorbance were slightly lower (334 to 570 mg/g ellagic acid equivalent). Nitrogen contents were very low in all samples (less than 1 %, when expressed as amino acids and proteins, except in T7) while carbohydrates, including quercitol and sugars in both free and linked forms, represented 6 to 26 % of the sample weights. Carbohydrates, polyphenols, and nitrogen compounds accounted for ≈71 to 92 % of the samples, meaning that 8 to 29 % (8 to 24 % when considering the residual water content) remains unexplained.

The concentrations of ellagic acid after acidic methanolysis revealed some variability between samples but remained very low (0.4 to 7 %), as expected from ellagitannin structures.

HPLC-SEC-UV-dRI analysis provided information on the molecular weight distribution, showing large variations between samples. The chromatograms of oak tannin extracts were divided into five populations (from P1 to P5). The extracts containing higher polyphenol concentrations present the largest areas of P2, corresponding to larger molecular weight ellagitannins, whereas P3, corresponding to the molecular size of castalagin/vescalagin, is the greatest population in the poorest samples. Larger polymers showing no UV absorbance were also detected by refraction index in T7.

Finally, a non-targeted metabolomic approach based on UHPLC–HRMS/MS analysis provided fingerprints of oak oenological tannins. Most of the intense signals were attributed to phenolic compounds, namely ellagic acid, gallic acid, and ellagitannins, while other signals were attributed to carbohydrates, terpene saponins, and lignans reported earlier in oak. This analysis highlighted clusters of features that were over- or under-expressed in some samples.

In summary, this work confirms that commercial oak tannin extracts used in the wine industry show large differences in their ellagitannin and carbohydrate contents and their composition, related to the oak species and process used, and potentially impacting their characteristics. Future studies will aim at relating analytical data with some variables linked to tannin functionality and sensory properties, to bring a better understanding of compounds of interest for particular applications. Links with botanical origin and process will also be analysed.

ACKNOWLEDGEMENTS

People involved in the experiments are gratefully acknowledged, in particular Christine Guilloton for the total polyphenols assay with the OIV-method.

This research was funded by FEDER and Region Grand-Est, in the frame of "Programme Operationnel FEDER/FSE/IEJ 2014-2020". Funding from FEDER and Region Occitanie in the frame of PRRI Phenoval-LR00210582014-2020 for the HPLC-HRMS equipment is also gratefully acknowledged.

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