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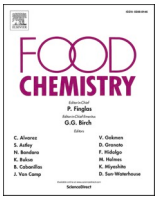

Identification of gallotannins and ellagitannins in aged wine spirits: A new perspective using alternative ageing technology and high-resolution mass spectrometry

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ABSTRACT

This research was focused on identifying gallotannins and ellagitannins degradation pathways to better understand their behavior in complex media such as wine spirits (WS). A WS was aged with chestnut wood staves with three levels of micro-oxygenation, nitrogen, and using wooden barrels. Gallotannins and ellagitannins were identified by LC-ESI-HRMS/MS using a Q-TOF in samples collected at 8, 21, 60, 180, 270, and 365 days of ageing, allowed comparing their relative abundances according to the ageing technology. It was established for the first time, the importance of oxygen in gallotannins and ellagitannins formation/degradation pathways in WS and shading light into the explanation for the steady increase of gallic and ellagic acid contents on WS during ageing. The results also highlighted the presence of penta-*O*-galloyl-β-D-glucose, tetra-*O*-galloyl-β-D-glucose, tri-*O*-galloyl-β-D-glucose, di-*O*-galloyl-β-D-glucose, and mono-*O*-galloyl-β-D-glucose, 2,3-(S)-hexahydroxydiphenoyl-β-D-glucose, pedunculagin, isomers vescalagin/castalagin and two products stemming from ethanol-promoted oxidation of castalagin/vescalagin and vescalin/castalin, in the composition WS aged with chestnut wood.

1. Introduction

The wine spirit (WS) is a distilled beverage that proved along the history to have a relevant socio-economic role in the traditional wine-producing countries across Europe (Grigg, 2004). Besides the iconic French regions of Armagnac and Cognac, Portugal, with its high number of Designations of Origin, is considered a serious producer of this spirit beverage (Belchior et al., 2015). The traditional ageing of WS involves staging the wine distillate in wooden barrels with a continuous innate diffusion of oxygen through the wood and the space between staves (Canas, 2017), under which the beverage undergoes major improvements contributing to the enhancement of its sensory properties

Abbreviations: WS, wine spirits; MOX, micro-oxygenation; LC, liquid chromatography; ESI, electrospray ionization; HR, high-resolution; MS, mass spectrometry; Q-TOF, quadrupole time-of-flight instrument; HHDP, hexahydroxydiphenoyl; PVDF, polyvinylidene difluoride; PCA, principal component analyses; GOH, gallic acid; G, galloyl moieties.

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(Caldeira et al., 2006; Schwarz et al., 2011). Chestnut (*Castanea sativa* Mill.) have been employed for this purpose in Mediterranean countries, since the early Christian era, though scientific investigation on its suitability for cooperage and ageing of wine spirits was recognized only after 1995 (Canas et al., 2018). As a result, for example, the regulation of Lourinhã Designation of Origin, the Portuguese exclusive designation for aged WS, was revised to allow the use of this type of wood, which produces high-quality spirits (Diário da República, 2021).

The ageing process of WS is poorly understood in terms of the compounds involved, mechanisms of reaction, and influence of the affecting factors. The wood, the wine distillate, the environment around the barrel, and the ageing technology (traditional using wooden barrels *vs* alternative using wood fragments in stainless steel tanks without or with micro-oxygenation) have a significant role in the physicochemical phenomena that affect WS' ageing (Caldeira et al., 2021; Caldeira et al., 2006; Canas, 2017; Canas et al., 2020; García-Estévez et al., 2017). These processes include: i) direct extraction of wood constituents; ii) the breakdown of wood biopolymers (lignin, hemicelluloses, and cellulose), followed by the release of derived compounds into the distillate; and iii) chemical reactions involving only the wood extractable compounds, only the distillate compounds, or both (such as oxidation, esterification, polymerization, and Maillard reactions and polycondensation reactions); and iv) the evaporation of volatile compounds and concentration of volatile and non-volatile compounds (Aronson & Ebeler, 2004; Canas et al., 2000; Singleton, 1995).

It is unquestionably accepted by the scientific community that these reactions are the consequence of several phenomena, involving the wood constituents and the compounds of the distillate. Among these phenomena, the release of wood organic extractable compounds into the WS has been the most studied, revealing great influence on the colour, aroma and flavour of the final product (Caldeira et al., 2006; Canas et al., 2000; Schwarz et al., 2009). Some of the most important constituents of the wood, responsible for the occurrence of several phenolic compounds of the wine spirit are lignin, and tannins (Fig. S1) (Canas, 2015).

Phenolic acids are one of the most plentiful phenolic compounds found in oak and chestnut wood, either free or as esters or glycosides (Canas, 2017). These bioactive compounds are also produced stemming from the thermal degradation of gallotannins, ellagitannins and lignin during the heat treatment of wood staves (Newsome et al., 2016). Some of the most relevant phenolic acids identified as chemical markers, and found in high concentration in aged WS, are gallic acid (3,4,5-trihydroxybenzoic acid) and ellagic acid (2,3,7,8-tetrahydroxy-chromeno [5,4,3-*cde*]chromene-5,10-dione). Gallic acid is very reactive due to its three phenolic hydroxyl groups and one carboxyl group, which are responsible for the formation of several ester molecules (Coldea et al., 2020). Phenolic hydroxyl groups of gallate esters can scavenge ROS and break the cycle of the generation of new radicals. Several studies revealed the role of gallic acid, its derivatives, and of ellagic acid in biological processes, with a focus on their antioxidant nature, antimicrobial, anticancer, anti-inflammatory, cardioprotective, gastroprotective, and neuroprotective effects (Sharma et al., 2017; Wang et al., 2020). These qualities are important in spirit beverages since the ingestion of such compounds, under moderate consumption of the drink, may have favorable health effects (Duriez et al., 2001; Kiviniemi et al., 2008; Umar et al., 2003; USDA and HHS, 2020), as opposed to the ethanol-induced damage (WHO, 2018). Gallic acid is the primary and basic building block of gallotannins, while ellagic acid and hexahydroxydiphenoyl (HHDP) moieties are all subunits of ellagitannins. Ellagitannins reach their higher concentration in aged beverages within the first few months of ageing, according to model solution studies (García-Estévez et al., 2017; Vignault et al., 2018), and easily react with oxygen. However, different gallotannins or ellagitannins have different reactivity towards oxygen (García-Estévez et al., 2017 and 2019) due to distinct redox potential or steric effects that are directly related to their chemical structure (Vignault et al., 2018). The redox feature of each molecule may influence their disappearance/degradation, and as a result, it will release gallic or ellagic acid into the WS over time, as previously observed (Canas et al., 2020). Besides, the relationship between these hydrolysable tannins and some sensory features, such as astringency and bitterness, and the overall quality of the aged WS is still unclear (Canas, 2017; Soares et al., 2020).

Few research articles have been published on the processes and molecular reactivity that occur during the ageing of distilled beverages (Awad et al., 2017; Delgado-González et al., 2020; Xiao et al., 2020). In addition, the majority of these studies are focused on a small number of compounds or a few classes that are involved in oxidation reactions during ageing, and there has not been enough information established about the chemical processes of ageing, their relationship with the kind of wood used, and/or the ageing technology. The present research seeks a deeper understanding on gallotannins and ellagitannins, and their derivatives, and to identify their presence on WS, which is associated with the increase of gallic and ellagic acid concentrations during ageing. Towards this end, WS aged by distinct technologies (traditional using chestnut barrels and alternative using micro-oxygenation combined with chestnut wood staves) was collected at different sampling times, and the samples were analyzed by liquid chromatography-electrospray ionization tandem high-resolution mass spectrometry (LC-ESI-HRMS/MS) using a quadrupole time-of-flight instrument (Q-TOF).

2. Material and methods

2.1. Experimental design

The experiment was performed on a pilot scale in 50 L glass demijohns with wood staves inside, as previously described (Canas et al., 2020), comprising five ageing modalities: three modalities of micro-oxygenation (MOX) with different concentration of oxygen and one with nitrogen atmosphere as control. In addition, 250 L wooden barrels, representing the traditional ageing technology, were used. Portuguese chestnut wood (*Castanea sativa* Mill.) was used in all modalities. Two replicates of each ageing modality were performed. The barrel and the staves (50 cm length × 5 cm width × 1.8 cm thickness) were manufactured by J. M. Gonçalves cooperage (Palaçoulo, Portugal). The toasting level applied to the staves was medium plus (90 min at an average temperature of 240 °C; 1.8 cm of toasting thickness). The staves were heated in an industrial oven, and the barrels were heated over a fire of wood offcuts, under certain conditions of temperature to ensure similar level of toasting. The quantity of staves inserted into the demijohns was determined in order to replicate the surface area to volume ratio of 250 L barrel (85 cm^2/L), which is the most common barrel size used in the ageing of WS. The wine distillate was produced by the Adega Cooperativa da Lourinhã from Portugal through column distillation (alcohol strength, 78.3 v/v; pH, 5.33; total acidity, as acetic acid, 0.12 g/L of absolute ethanol; volatile acidity, as acetic acid, 0.09 g/L of absolute ethanol), and it was used to fill the several vessels, which were placed in Adega Cooperativa da Lourinhã (winery) in similar environmental conditions.

During the ageing period, five different ageing conditions were applied to the WS:

I) B (barrel) – 250 L chestnut wooden barrel;

II) O15 (micro-oxygenation) – 50 L glass demijohns with chestnut staves and micro-oxygenation (flow rate of 2 mL/L/month during the first 15 days followed by 0.6 mL/L/month until 365 days);

III) O30 (micro-oxygenation) – 50 L demijohns with chestnut staves and micro-oxygenation (flow rate of 2 mL/L/month during the first 30

days followed by 0.6 mL/L/month until 365 days);

IV) O60 (micro-oxygenation) – 50 L glass demijohns with chestnut staves and micro-oxygenation (flow rate of 2 mL/L/month during the first 60 days followed by 0.6 mL/L/month until 365 days);

V) N (nitrogen) – 50 L demijohns with chestnut staves and nitrogen application (flow rate of 20 mL/L/month).

Pure oxygen was supplied using a multiple diffuser micro-oxygenator (VISIO 6, Vivelys, France) with ceramic diffusers. Each ageing condition was performed in two replicates and all samples were analyzed in duplicate by LC-ESI-HRMS/MS following 8, 21, 60, 180, 270 and 365 days of ageing.

2.2. Reagents and instrumentation

Pure oxygen (X50S Food) and nitrogen (X50S Food) were supplied by Gasin, Portugal.

Gallic acid, ellagic acid, vescalagin and castalagin were purchased from Sigma-Aldrich (Steinheim, Germany) or TCI (Zwijndrecht, Belgium). All of them were used as standards (purity > 97%) without further purification. The standard solutions were freshly prepared prior to use with ethanol/water (75:25 v/v). All solvents used in the chromatographic analysis were HPLC gradient grade purchased from Merck (Darmstadt, Germany). LC-MS analyses were performed with LC-MS grade water, acetonitrile and formic acid from Sigma-Aldrich.

LC-ESI-HRMS/MS analyses were conducted on a Bruker Impact II quadrupole time-of-flight mass spectrometer equipped with an ESI source (Bruker Daltoniks, Bremen, Germany). Chromatographic separations were performed in an Ultimate 3000 RSLCnano system (ThermoFisher Scientific) using a Luna C18 column (3.0 μm, 2.0 × 150 mm; Phenomenex) and an elution gradient of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) at a flow rate of 170 μL/min. The elution conditions were as follows: 5–50% B for 6 min; 50–100% B for 4 min; isocratic elution with 100% B for 5 min; 100–5% B for 4 min; and finally, 5% B for 9 min. Before being analyzed WS samples were filtered, using 25 mm diameter sterile syringe filter with a 0.45 μm pore size hydrophilic PVDF membrane, and without any further preparation. The injection volume was 10 μL. The column and the autosampler were kept at 40 °C and 18 °C, respectively. The mass spectrometric parameters were set as follows: end plate offset, 500 V; capillary voltage, −4.5 kV; nebulizer, 40 psi; nitrogen dry gas, 8 L/min; heater temperature, 200 °C. Spectra were acquired in the negative electrospray ionization mode ESI (-). Internal calibration was performed for sodium formate cluster, with a sodium formate solution introduced to the ion source via a 20 μL loop at the beginning of each analysis using a six-port valve. Calibration was then performed using high-precision calibration mode (HPC). Acquisition was performed in the *m/z* 50–1000 range and in a data-dependent MS/MS mode with an isolation window of 0.5, acquisition rate of 3 Hz and a fixed cycle time of 3 s. Precursor ions were selected for auto MS/MS at an absolute threshold of 153, with the active exclusion mode set at three spectra and released after 1 min, nevertheless precursor ions with intensities in the range of 5× the previous intensities were reconsidered.

2.3. Data processing

The acquired data were processed by Data Analysis 4.1 software (Bruker Daltoniks). Extracted ion chromatograms (EIC), with a mass window of ± 5 ppm, were performed for searching characteristic fragment ions of gallotannins and ellagitannins in the tandem scan spectra. All spectra corresponding to metabolites were then manually checked. The mass deviation from the accurate mass of the identified gallotannins and ellagitannins remained below 5 ppm for the precursor and below 10 ppm for product ions. The MS/MS spectra of the products identified are displayed in the Supplementary Material (Figs. S1-S12).

The analytical data analysis was carried out using Statistica software from StatSoft, and IBM® SPSS® Statistics V26.

3. Results and discussion

3.1. Identification and fragmentation patterns

First, gallic and ellagic acids were identified in the samples obtained from the different ageing technologies to understand the increase in their concentration over time (Canas et al., 2020). The presence of gallic acid was confirmed by LC-ESI-HRMS/MS analysis, upon comparison with a standard. This phenolic acid displayed its $[M-H]^{-1}$ ion at *m/z* 169.0329 in the full scan spectrum, and one fragment ion was observed in the MS/MS spectrum: at *m/z* 125.0231, corresponding to the anion obtained upon decarboxylation. The presence of ellagic acid was also attested upon standard comparison. This phenolic compound exhibited the $[M-H]^{-1}$ and $[2\ M-H]^{-1}$ ions at *m/z* 300.9993 and 602.9998, respectively. The tandem mass spectrum of the deprotonated molecule exhibited the expected fragmentation pattern: 283.9965 (loss of hydroxyl radical), 229.0142 (loss of CO_2 and CO), and 185.0242 (loss of two CO_2 and one CO) (Figs. S2, S3).

Once identified the gallic and ellagic acids, a search for their derivatives was carried out by extracting characteristic fragment ions from the tandem mass spectra obtained for all samples included in this study. The isolation of ion at *m/z* 169.0142 allowed the identification of digallate (*m/z* 321.0256) and five gallotannins: mono-*O*-galloyl-β-D-glucose (*m/z* 331.0675), di-*O*-galloyl-β-D-glucose (*m/z* 483.0796), tri-*O*-galloyl-β-D-glucose (*m/z* 635.0919), tetra-*O*-galloyl-β-D-glucose (*m/z* 787.1021) and penta-*O*-galloyl-β-D-glucose (*m/z* 939.1141) (Table 1 and Fig. 1). Some of these compounds had previously been reported in oak and chestnut wood (Canas et al., 2004; Garcia et al., 2012), but only mono-*O*-galloyl-β-D-glucose was found in WSs aged using traditional technology (Canas et al., 2008). Digallate, the esterification product obtained from two gallic acid units, was identified at 6.5 min, showing its deprotonated molecule at *m/z* 321.0256, and the main fragment ions at *m/z* 169.0142, 125.0230 (Fig. S4), which correspond to the deprotonated molecule of gallic acid, $[GOH-H]^{-1}$, and its decarboxylation anion, respectively (Escobar-Avello et al., 2019; Li & Seeram, 2018). For the gallotannins, in addition to the fragment ion at *m/z* 169.0142, two characteristic fragment ions were observed in their tandem mass spectra, which correspond to consecutive losses of gallic acid (-170.0215 u, GOH) and galloyl moieties (-152.0109 u, G). Interestingly, each of these compounds corresponded to a sole chromatographic peak (Fig. 2), thereby suggesting that one sole isomer of mono-*O*-galloyl-β-D-glucose, di-*O*-galloyl-β-D-glucose, tri-*O*-galloyl-β-D-glucose and tetra-*O*-galloyl-β-D-glucose were formed. However, since no comparison with standard compounds was made, the exact identification of the isomer observed was not performed.

The isolation of the fragment ion at *m/z* 300.9979, from the tandem mass chromatogram, allowed identifying four ellagitannins released by the chestnut wood: HHDP-glucose (2,3-(*S*)-hexahydroxydiphenoyl-β-D-glucose, *m/z* 481.0648), pedunculagin (*m/z* 783.0714) and the two isomers vescalagin and castalagin (*m/z* 933.0649 and 933.0653) (Fig. 3) (Singh et al., 2016). Vescalagin and castalagin were previously identified in oak and chestnut woods by Puech *et al.* (1999) and in aged WSs resulting from the traditional technology (Canas et al., 2008). The tandem mass spectra of these four tannins exhibited the three expected diagnostic ions 300.9979/275.0186/249.0394, corresponding to HHDP residue (ellagic acid moiety), 2,2′,3,3′,4,4′-hexahydroxybiphenyl and the decarboxylation of HHDP moiety, respectively (Bowers et al., 2018).

Table 1
Gallic acid derivatives identified by LC-ESI-HRMS/MS.

Identified Compound	RT[a] (min)	Molecular Formula	$[M-H]^-$ (m/z exp)	error (ppm)	Fragment ions m/z (error ppm, molecular formula)
gallic acid	3.5	$C_7H_6O_5$	169.0342	−1.8	125.0303 (9.5, $C_6H_5O_3^-$)
ellagic acid	7.0	$C_{14}H_6O_8$	300.9993	−0.9	283.9951 (5.6, $C_{14}H_5O_7^-$)
			602.9995 $[2M-H]^-$		229.0142 (0.2, $C_{12}H_5O_5^-$)
					185.0242 (-1.1, $C_{11}H_5O_3^-$)
digallate	6.5	$C_{14}H_{10}O_9$	321.0256	4.7	169.0142 (6.5, $C_7H_5O_5^-$)
					125.0230 (-2.4, $C_6H_5O_3^-$)
penta-*O*-galloyl-β-D-glucose	6.7	$C_{41}H_{32}O_{26}$	939.1141	3.4	787.1024 (-3.2, $C_{34}H_{27}O_{22}^-$)
					769.0917 (3.0, $C_{34}H_{25}O_{21}^-$)
					635.0890 (2.5, $C_{27}H_{23}O_{18}^-$)
					617.0803 (3.1, $C_{27}H_{21}O_{17}^-$)
					483.0789 (-7.5, $C_{20}H_{19}O_{14}^-$)
					465.0675 (-3.0, $C_{20}H_{17}O_{13}^-$)
					447.0583 (3.1, $C_{20}H_{15}O_{12}^-$)
					169.0138 (-2.6, $C_7H_5O_5^-$)
tetra-*O*-galloyl-β-D-glucose	6.7	$C_{34}H_{28}O_{22}$	787.1021	−2.8	635.0912 (- 3.4, $C_{27}H_{23}O_{18}^-$)
					617.0806 (- 3.6, $C_{27}H_{21}O_{17}^-$)
					483.0794 (2.8, $C_{20}H_{19}O_{14}^-$)
					465.0689 (3.0, $C_{20}H_{17}O_{13}^-$)
					447.0583 (3.1, $C_{20}H_{15}O_{12}^-$)
					169.0143 (-0.2, $C_7H_5O_5^-$)
tri-*O*-galloyl-β-D-glucose	6.6	$C_{27}H_{24}O_{18}$	635.0919	4.6	483.0798 (- 6.4, $C_{20}H_{19}O_{14}^-$)
					465.0692 (-3.7, $C_{20}H_{17}O_{13}^-$)
					313.0579 (4.4, $C_{13}H_{13}O_9^-$)
					169.0142 (-0.1, $C_7H_5O_5^-$)
di-*O*-galloyl-β-D-glucose	6.2	$C_{20}H_{20}O_{14}$	483.0796	3.3	331.0681 (3.1, $C_{13}H_{10}O_{10}^-$)
					313.0576 (3.4, $C_{13}H_{13}O_9^-$)
					271.0469 (-3.4, $C_{11}H_{11}O_8^-$)
					169.0142 (0.3, $C_7H_5O_5^-$)
mono-*O*-galloyl-β-D-glucose	2.9	$C_{13}H_{16}O_{10}$	331.0672	−0.3	271.0431 (-4.1, $C_{11}H_{11}O_8^-$)
					211.0251 (-1.5, $C_9H_7O_6^-$)
					169.0142 (3.3, $C_7H_5O_5^-$)
HHDP-glucose	3.0	$C_{20}H_{18}O_{14}$	481.0648	5.0	331.0680 (3.1, $C_{13}H_{10}O_{10}^-$)
					301.0000 (-3.2, $C_{14}H_5O_8^-$)
					275.0206 (-3.0, $C_{13}H_7O_7^-$)
					271.0468 (-3.1, $C_{11}H_{11}O_8^-$)
					249.0409 (-1.9, $C_{12}H_9O_6^-$)
vescalagin	3.0	$C_{41}H_{26}O_{26}$	933.0653	−1.4	915.0576 (4.6, $C_{41}H_{23}O_{25}^-$)
					871.0652 (3.2, $C_{40}H_{22}O_{23}^-$)
					631.0597 (4.9, $C_{27}H_{19}O_{18}^-$)
					613.0479 (3.1, $C_{27}H17O_{17}^-$)
					569.0596 (6.0, $C_{26}H_{17}O_{15}^-$)
					300.9996 (-3.9, $C_{14}H_5O_8^-$)
					275.0201 (-1.4, $C_{13}H_7O_7^-$)
					249.0414 (-3.8, $C_{12}H_9O_6^-$)
castalagin	6.1	$C_{41}H_{26}O_{26}$	933.0664	3.8	915.0557 (-2.6, $C_{41}H_{23}O_{25}^-$)
					871.0641 (1.8, $C_{40}H_{22}O_{23}^-$)
					631.0593 (4.3, $C_{27}H_{19}O_{18}^-$)
					613.0481 (3.4, $C_{27}H_{19}O_{18}^-$)
					569.0580 (3.2, $C_{26}H_{17}O_{15}^-$)
					300.9996 (1.9, $C_{14}H_5O_8^-$)
					275.0206 (-3.3, $C_{13}H_7O_7^-$)
					249.0409 (1.7, $C_{12}H_9O_6^-$)
pedunculagin (di-HHDP-glucose)	6.4	$C_{34}H_{24}O_{22}$	783.0714	3.5	481.0644 (-4.2, $C_{20}H_{17}O_{14}^-$)
					301.0000 (-3.2, $C_{14}H_5O_8^-$)
					275.0203 (2.1, $C_{13}H_7O_7^-$)
					249.0210 (-6.4, $C_{12}H_9O_6^-$)
vescalin/castalin oxid. with EtOH	6.3	$C_{29}H_{24}O_{19}$	675.0878	5.7	657.0774 (6.2, $C_{29}H_{21}O_{18}^-$)
					631.0964 (-3.6, $C_{28}H_{23}O_{17}^-$)
					585.0453 (3.6, $C_{26}H_{17}O_{16}$)
					300.9997 (2.5, $C_{14}H_5O_8^-$)
					275.0203 (-2.0, $C_{13}H_7O_7^-$)
					249.0413 (-3.4, $C_{12}H_9O_6^-$)
vescalagin/castalagin oxid. with EtOH	6.5	$C_{43}H_{30}O_{27}$	977.0933	−3.2	959.0819 (2.4, $C_{43}H_{27}O_{26}$)
					933.1051 (5.1, $C_{42}H_{29}O_{25}$)
					887.0617 (-3.4, $C_{40}H_{23}O_{24}$)
					675.0857 (-2.7, $C_{29}H_{23}O_{19}^-$)
					657.0744 (1.6, $C_{29}H_{21}O_{18}$)
					585.0545 (3.9, $C_{26}H_{17}O_{16}$)
					301.0001 (3.6, $C_{14}H_5O_8$)
					275.0197 (3.5, $C_{13}H_7O_7$)
					249.0405 (-0.3, $C_{12}H_9O_6$)

[a] RT – Retention time.

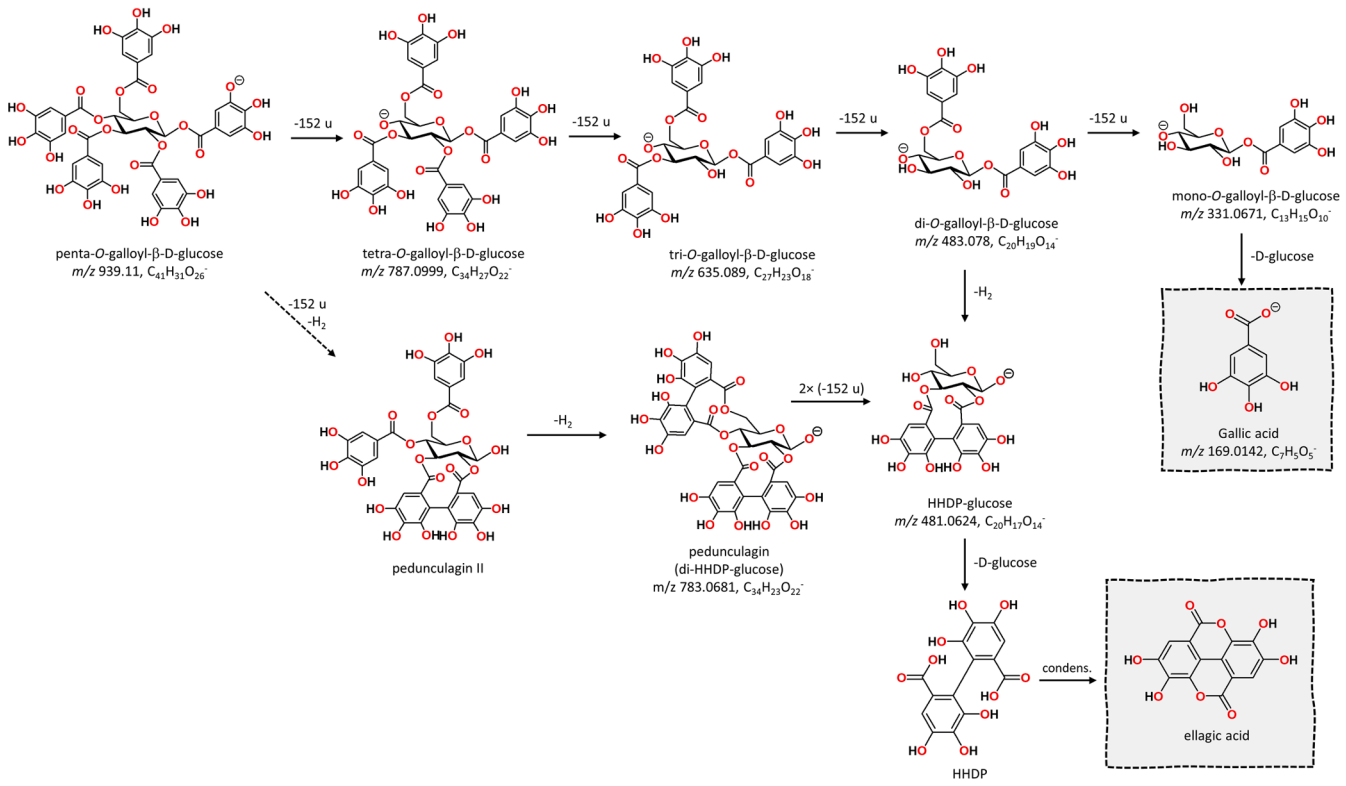

Fig. 1. Proposed fragmentation patterns and degradation pathways of gallotannins.

In addition to these diagnostic ions, the tandem mass spectrum of the isomer of HHDP-glucose showed the fragment ion at *m/z* 271.0469 that corresponds to the cross-ring fragment ion of glucose (Delgado-González et al., 2020). The two isobaric ions detected at *m/z* 933.0653 (RT 3.0 min) and 933.0664 (RT 6.1 min) were unequivocally assigned to vescalagin and castalagin, respectively, based on identical retention time and fragmentation pattern, when compared with their corresponding commercial standards. Coherently, these two ions exhibited very similar fragmentation patterns: 915.0523 (loss of water), 871.0625 (loss of water and CO_2), 631.0566 (loss of HHDP moiety), 613.0460 (loss

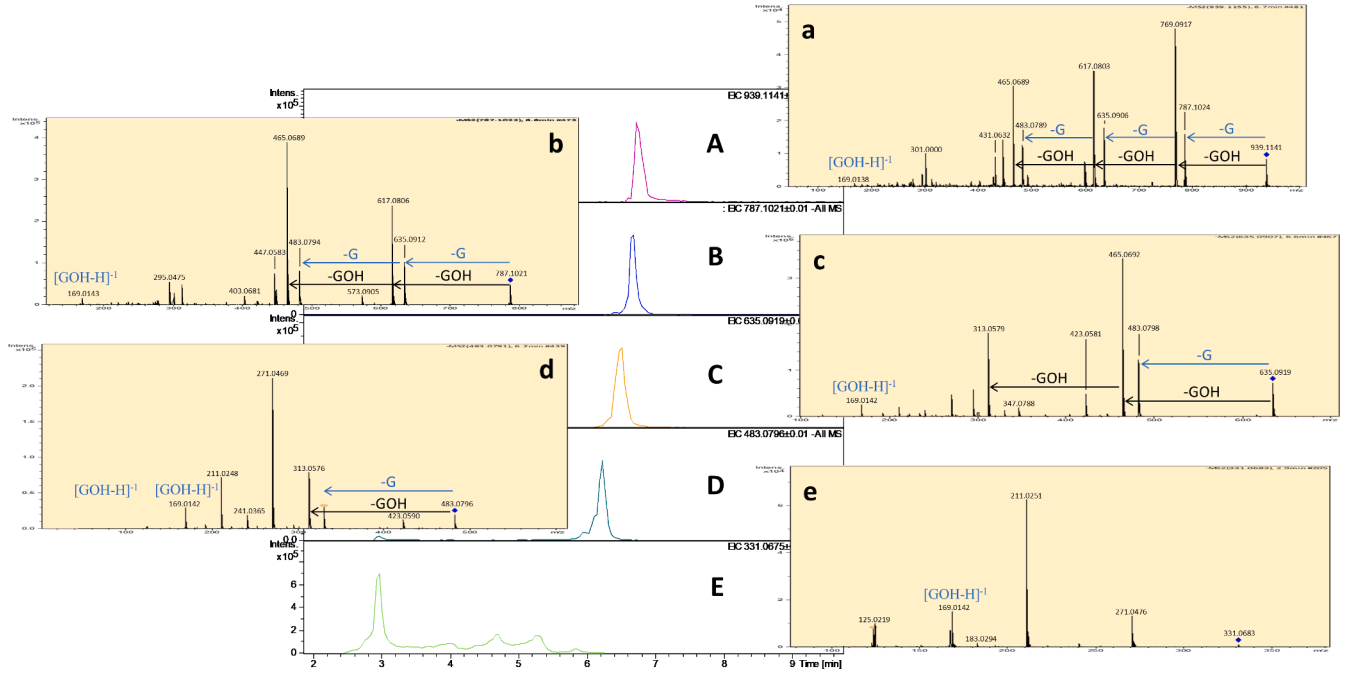

Fig. 2. Extracted ion chromatogram and respective tandem mass spectrum of: penta-*O*-galloyl-β-D-glucose (*m/z* 939.1141) (A,a); tetra-*O*-galloyl-β-D-glucose (*m/z* 787.1021) (B, b); tri-*O*-galloyl-β-D-glucose (*m/z* 635.0919) (C, c); di-*O*-galloyl-β-D-glucose (*m/z* 483.0796) (D, d); and mono-*O*-galloyl-β-D-glucose (*m/z* 331.0675) (E, e). GOH (Gallic Acid) and G (Galloyl).

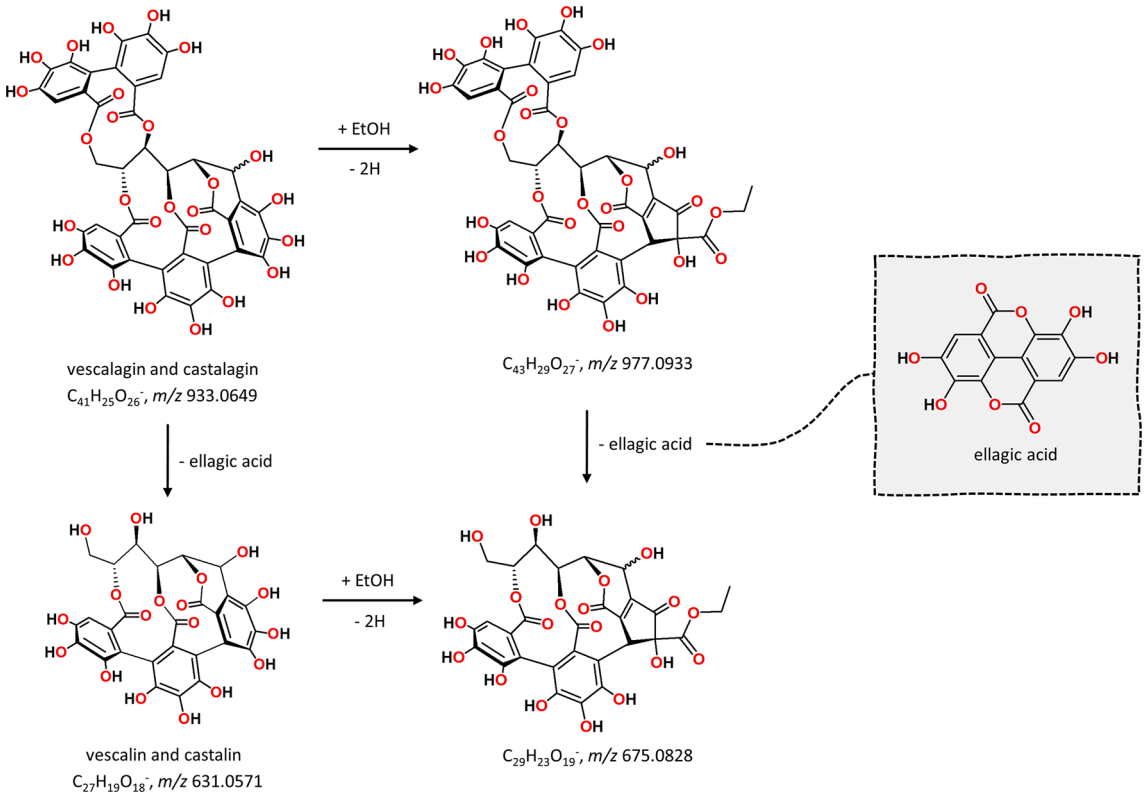

Fig. 3. Structures of vescalagin/castalagin isomers and their degradation pathway for the two derivatives that were tentatively assigned to the ethanol-promoted oxidation products of vescalagin/ castalagin and vescalin/castalin.

of water and of HHDP), 559.0562 (loss of HHDP, water and CO_2) (Figs. S5-S10).

3.2. Ethanol-promoted oxidation products

Two additional products were tentatively assigned to products stemming from ethanol-promoted oxidation of vescalagin/castalagin and vescalin/castalin (Fridrich et al., 2008), at *m/z* 977.0933 and 675.0828, respectively (Fig. 3). These derivatives not only displayed in the full scan spectra a *m/z* compatible with the deprotonated molecules of ethanol-promoted degradation products reported by Fujieda et al. (2008), but also displayed in the tandem mass spectra the three diagnostic ions 300.9979/275.0186/249.0394, characteristic of ellagitannins. The observation of specific losses in the fragmentation pattern of these derivatives, further corroborate the assigned structures: loss of water (18.0100 u), loss of CO_2 (43.9893 u), loss of water and of $CO_2CH_2CH_2$ moiety (90.0311 u) from the deprotonated molecule. The tandem mass spectrum of the product obtained upon ethanol-mediated oxidation of vescalagin/castalagin also showed similar losses from the fragment ion $[M-HHPD]^{-1}$ observed at *m/z* 675.0857 (Figs. S10-S13).

3.3. Relative abundances of hydrolysable tannins during the ageing process

3.3.1. Gallotannins

The relative abundance of each gallic acid derivative, using the ratio between the peak area of each derivative over the total peak area of all compounds found in each sample, was determined for each sample analyzed to understand the impact of oxygen on gallotannins degradation during WS's ageing (Tables S1-S4). This allowed us to compare the relative content of each derivative identified by LC-ESI-HRMS/MS between the distinct ageing modalities and in several sampling times for the two replicates and their duplicated analysis.

The alternative ageing technology based on different MOX flow rates, provided information on whether, or not, gallotannins and ellagitannins released from the wood and their subsequently degradation depend on the concentration of dissolved oxygen. Both traditional and alternative ageing technologies are complex, where different organic species are always being generated. Some of these species result directly from the wood extraction, and others from reaction processes that occur in the reaction medium. Nevertheless, alternative ageing technology with different MOX flow rates already provided information about the role of oxygen in the chemical phenomena underlying ageing. Indeed, it was previously reported that higher gallic acid levels (as well on syringic acid and vanillin) were associated with lower levels of micro-oxygenation, while higher levels of ellagic acid (also of syringaldehyde, coniferaldehyde, and sinapaldehyde) were associated with higher levels of micro-oxygenation (Canas et al., 2020). Nitrogen application was used as a way of preventing oxygen uptake. This modality gave rise to the lowest concentrations of these compounds in comparison with micro-oxygenation effect (Canas et al., 2020). In our previous work, we refer that the WS in which the lowest amount of oxygen was supplied had a larger amount of gallic acid (45–123 mg/L) at 21, 60, 180, 270, and 365 days of ageing (Canas et al., 2020). Herein we have identified the same trend, although for traditional ageing methodology (barrel, B), on the other hand, the relative percentage of gallic acid is somehow lower in modalities with higher levels of oxygen, indicating that gallic acid is being consumed (Fig. 4a and S14). Interestingly, and reinforcing the previous idea, the nitrogen trial (N) showed reduced gallic acid levels, but with higher relative percentage (Fig. 4a), which demonstrates that the kinetics are slower, and direct extraction of wood components or the breakdown of wood biopolymers is also affected by the availability of oxygen in the medium.

The galloyl-β-D-glucose degradation contents, which all produced

free gallic acid and, later, digallate, are depicted in Fig. 4a, that was created using relative abundance graphs calculated by the ratio between each signal area and total areas (Table S1-S4). It is evident that all kinetics followed the same pattern: a sudden and slow increase at the beginning of the aging process, and their relative content of gallotannins is similarly comparable: there is a higher percentage of mono-*O*-galloyl-β-D-glucose in all modalities (8–10%), followed by tri-*O*-galloyl-β-D-glucose (0.8–1.5%). In all modalities, the most substituted gallotannin, penta-*O*-galloyl-β-D-glucose, is the least abundant. Regardless of the ageing modality, it seems that after 270 days of ageing, all systems tend to balance out in terms of their gallotanins relative composition. Even though their areas increase with time, which corresponds to the fact that the amounts of these substrates increase. (Table S1-S4). More precisely, it can be seen that penta-*O*-galloyl-β-D-glucose relative percentage expression in all samples was very low, 0.8–1.5%. Its kinetic pattern is also similar for all ageing modalities, with an increase in penta-*O*-galloyl-β-D-glucose levels for early ageing days followed by stabilization after 180 days. The mono-*O*-galloyl-β-D-glucose stood out from the other penta-*O*-galloyl-β-D-glucose metabolic by-products in all WSs. However, it should be highlighted that after 365 days, its relative percentage is greater in WSs from modalities with lower oxygen loadings (B, N), 9.6 and 8.8%, respectively, than that for micro-oxygenation modalities (7.0–7.7%). The degradation product from penta-*O*-galloyl-β-D-glucose with the second highest relative percentage in all WSs was identified as tri-*O*-galloyl-β-D-glucose (0.8–1.5%), suggesting that the more substituted metabolites are more prone of losing their gallic acid units than of undergoing other degradation pathways, and thus no substantial amounts will remain in the WS complex media. A relationship with the quantity of oxygen present in the media can also be observed: the WS aged with less dissolved oxygen have more galloyl-β-D-glucose substrates (e.g. 13.4% (B) and 10.0% (O60) after 365 days), which is related to the equilibrium that is generated in the hydrolysis reaction - the more gallic acid consumed, the more displaced the hydrolysis is, and less galloyl-glucose esters are produced.

Higher amounts of mono- and di-*O*-galloyl-β-D-glucose were found in traditional ageing modality (B), 10.5 and 1.2% respectively, showing the role of oxygen in the promotion of gallic acid unit loss in metabolites with galloyl groups. In this regard it is important to note that there are two competing mechanisms: the hydrolysis reaction that produces gallic acid, and the oxidation of gallic acid and of galloyl-glucose moieties. The more gallic acid consumed, the more displaced the hydrolysis equilibrium is, thereby leading to less production of galloyl-glucose esters.

3.3.2. *Ellagitannins*

Regardless the ageing modality, ellagitannins were identified as minor compounds with modest relative abundances. Nonetheless, similarly to the previous analysis, and taking into account the results of our previous study, which revealed a higher concentration of ellagic acid (23 mg/L − it is worth noting that it is approximately 20 times lower than the concentration of gallic acid), after 365 days of aging with micro-oxygenation modality (Canas et al., 2020), we attempted to understand the relationship between the identified ellagitannins and their relationship with the amount of ellagic acid present in aged WSs. Regarding the identified ellagitannins (Fig. 3, Table S1), pedunculagin was the least abundant (<0.12%), which is understandable given that it is derived from the penta-*O*-galloyl-β-D-glucose metabolite that shows a low relative abundance, as previously demonstrated. Its degradation is related to HHDP-glucose formation, whose amounts were three times higher when higher levels of micro-oxygenation were used (Fig. 4b).

Concerning the two isomers vescalagin and castalagin, both exhibit comparable behavior over time; the first generally having lower levels in WSs analyzed after 180 days. At 365 days of ageing, both vescalagin and castalagin had a relative abundance very low for all modalities (maximum of 0.55%), thereby highlighting their prompt reactivity under these conditions (García-Estévez et al., 2019). Therefore, it is not surprising to identify vescalagin and castalagin oxidation product with ethanol in very low percentage, up to 0.42%, for O30 and O60 conditions. WSs exposed to a higher level of oxygen (MOX) presented not only an increased amount of this oxidation product, but also of pedunculagin (di-HHDP-glucose) and HHDP-glucose, indicating the degradation of both vescalagin and castalagin isomers. It is also noteworthy that the esterification product of two gallic acid units was a product with very low relative expression in the group of WS's investigated substrates. Its relative percentage showed a slow kinetics evolution reaching the level of 0.90% for B, 0.65% for O15, 0.81% for O30, 0.55% for O60 and 0.62% for N, after 365 days of ageing.

Vescalin/castalin ethanol-promoted oxidation products exhibited a relative expressiveness in the same range for WSs exposed to oxygen, ~0.2% for all modalities. Other two substrates with low relative expressiveness trend were HHDP-glucose and digallate (Fig. S14). HHDP-glucose which had an estimated content of only 0.5–0.8% in all modalities, except for the WSs exposed to micro-oxygenation, in which a content of 0.8% was observed for this derivative (Fig. 4b) and during a longer ageing period. Conversely, in WSs exposed to higher oxygen level, the relative percentage of digallate is reduced: 0.55% (O60) *versus* 0.90% (B), 0.80% (O30) or 0.65% (O15) (Table S1).

The MOX strategy used in this study allowed highlighting that gallotannins and ellagitannins have distinct oxygen reactivities, and their disappearance/degradation over time is linked to the increase of gallic or ellagic acid in the WS. This finding is consistent with prior ones that revealed different contents of gallic acid according to the MOX level (Canas et al., 2020), as it is consumed when intervening in reactions like esterification or oxidation.

The nitrogen modality (N) profile for the total contents of vescalagin/castalagin and their oxidation derivatives evolved in a similar way to micro-oxygenated modalities (~1.4% at 365 days). This tendency has already been seen, but not as evident, for the level of galloyl-β-D-glucose substrates, as these substrates are more dependent on dissolved oxygen. The nitrogen modality results confirm what we previously showed: the wood releases oxygen into the WS, which can then react with substrates (Canas et al., 2020). The outcomes also indicate that wood play an essential role for understanding ageing chemistry and, as a result, for further exploring ageing technologies, toward the enhancement of ageing process and of the final WSs, similarly to what has been done for wines. Future studies should consider different types of wood and how oxygen is taken from their matrices.

3.4. *Global analysis*

To enhance the kinetics of hydrolysable tannins contents, a global analysis was done using component variance analysis and two principal component analyses (PCA) to understand the behavior of the analytes as a whole in relationship to compounds, the ageing time and ageing modalities, as well as to identify coherent patterns between them.

The component variance analysis for the studied WSs was based on the ageing modalities (B, O15, O30, O60, and N), ageing time (8, 21, 60, 180, 270 and 365 days), and hydrolyzable tannins content (Fig. 5a and Table S5). It clearly reveals that time and technology had a highly significant ($p < 0.001$) impact on the chemical composition. This conclusion appears to be true for most of the studied substrates, except for vescalagin and pedunculin, whose high variations cannot be explained by the ageing time or the ageing technology. It is worth mentioning that ellagic acid and vescalagin/castalagin oxidation products with ethanol also exhibit a higher correlation with MOX modality (Fig. 5a and S17). In addition, for most of the compounds, the interaction MOX × t (Fig. 5a, Table S5) is substantial, revealing that the variation over time depends significantly on the MOX modality used. Given the diverse ageing modalities employed as variability sources, PCA analysis revealed that the analyzed substrates contents seemed to have a wide range of values and significant variability, indicating good data scattering (Fig. 5b). Principal component 1 (PC1) distinguishes the WSs according to the ageing time and reveals the differences between gallic acid and other

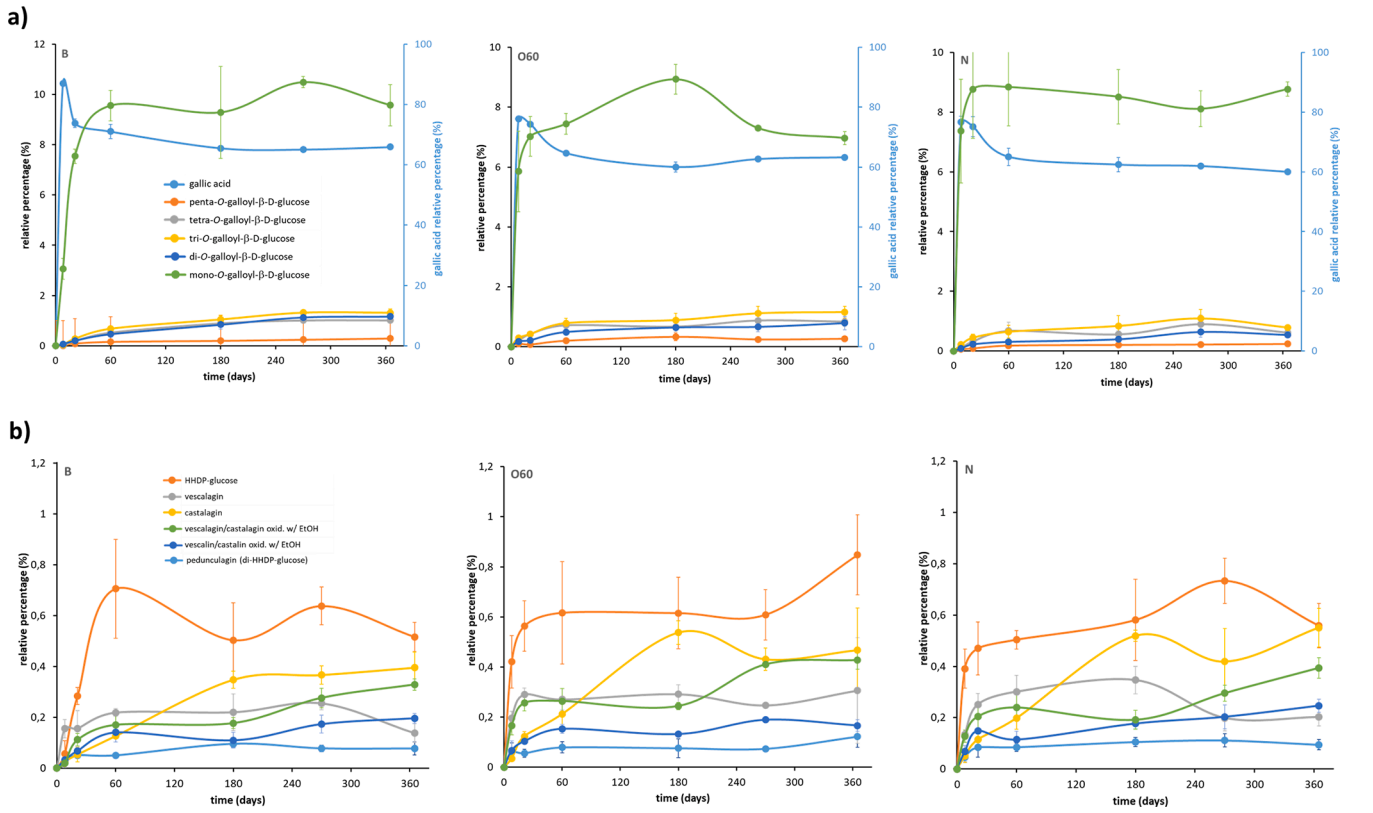

Fig. 4. Contents of a) gallic acid and galloyl-β-D-glucose derivatives (gallic acid relative percentage is given on the blue axis) and b) HHDP-glucose, pedunculagin, vescalagin, castalagin and their derivatives in the aged WSs according to the ageing modalities (B, O60, and N), and the ageing time (8, 21, 60, 180, 270 and 365 days); B – Chestnut barrel, O15, O30, O60 – MOX modalities combined with chestnut staves, N – nitrogen (control). The lines are used to illustrate a trend to make the reader's perception easier. See also Figures S13-S15 for more details and graphics of all ageing modalities. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

compounds (Fig. 5b). The difference in N modality and the other modalities is explained by principal component 2 (PC2) (Fig. 5b). Furthermore, the two PCA graphs reveals the stabilization of the chemical composition of the WSs, as it is shown by the correlation between samplings at 270 and 365 days. A positive correlation for studied substrates can be described in two main clusters: i) penta-*O*-galloyl-β-D-glucose, tetra-*O*-galloyl-β-D-glucose, tri-*O*-galloyl-β-D-glucose, di-*O*-galloyl-β-D-glucose and digallate; and ii) pedunculagin, HHDP-glucose, mono-*O*-galloyl-β-D-glucose, vescalagin/castalagin and two products stemming from ethanol-promoted oxidation of castalagin/vescalagin and vescalin/castalin. This observation supports the suggested degradation pathways for gallotannins and ellagittanins (Figs. 1 and 3). The negative correlation found between gallic acid and other substrates indicates that gallic acid is derived from the breakdown of most of those substrates. Ellagic acid and the ethanol-promoted oxidation products of vescalagin/castalagin show a weak correlation, indicating that the degradation of these compounds is associated with ellagic acid formation (Fig. 3).

Principal component 3 (PC3) distinguishes MOX modalities from N and B modality (Fig. 5b). PC3 further reveals that WSs aged using traditional technology (B) had more mono-*O*-galloyl-β-D-glucose, as well as MOX modalities are related to ethanol-promoted oxidation products of vescalagin/castalagin and vescalin/castalin in WSs. However, PC2 and PC3, analysis only account for 9% and 7% of the total variance, respectively, whereas PC1 accounts for 40% (time factor), which had already been revealed by the component variance analysis. The positive correlation of MOX modalities with the studied substrates highlights and supports the remarkable effect of oxygen on the chemical composition of aged WS.

4. Conclusions

The findings herein described contribute to a better knowledge of the WS ageing chemistry by providing novel information on the influence of oxygen/MOX level and chestnut wood on the hydrolysable tannins composition during 365 days of WS ageing. Additionally, the results attest and explain previous studies on the release of gallic or ellagic acid in the WS over time.

LC-ESI-HRMS/MS analysis of WS produced under distinct ageing technologies enabled not only the tentatively identification of multiple gallic and ellagic acid derivatives but also the evaluation of their relative content according to the technology used. Among the wood-related derivatives identified are digallate (*m/z* 321.0256) and five gallotannins: mono-*O*-galloyl-β-D-glucose (*m/z* 331.0675), di-*O*-galloyl-β-D-glucose (*m/z* 483.0796), tri-*O*-galloyl-β-D-glucose (*m/z* 635.0919), tetra-*O*-galloyl-β-D-glucose (*m/z* 787.1021) and penta-*O*-galloyl-β-D-glucose (*m/z* 939.1141). Additionally, two ellagitannins were identified as HHDP-glucose (*m/z* 481.0648) and pedunculagin (*m/z* 783.0714), and two were unequivocally assigned to vescalagin (*m/z* 933.0649) and castalagin (*m/z* 933.0653), based on comparison with standards. The identification of products presumably stemming from the ethanol-promoted oxidation of the ellagitannins vescalagin/castalagin (*m/z* 977.0933) and vescalin/castalin (*m/z* 675.0878) should also be stressed.

Regarding the relative proportion of examined hydrolysable tannins, mono-*O*-galloyl-β-D-glucose had higher relative percentage expression among the gallotannins identified, which had direct relationship with the amount of oxygen available in the medium. Its relative proportion is higher in WSs resulting from the traditional technology using barrels than in those resulting from MOX combined with staves. On the other hand, the overall levels of the other galloyl-β-D-glucose moieties were

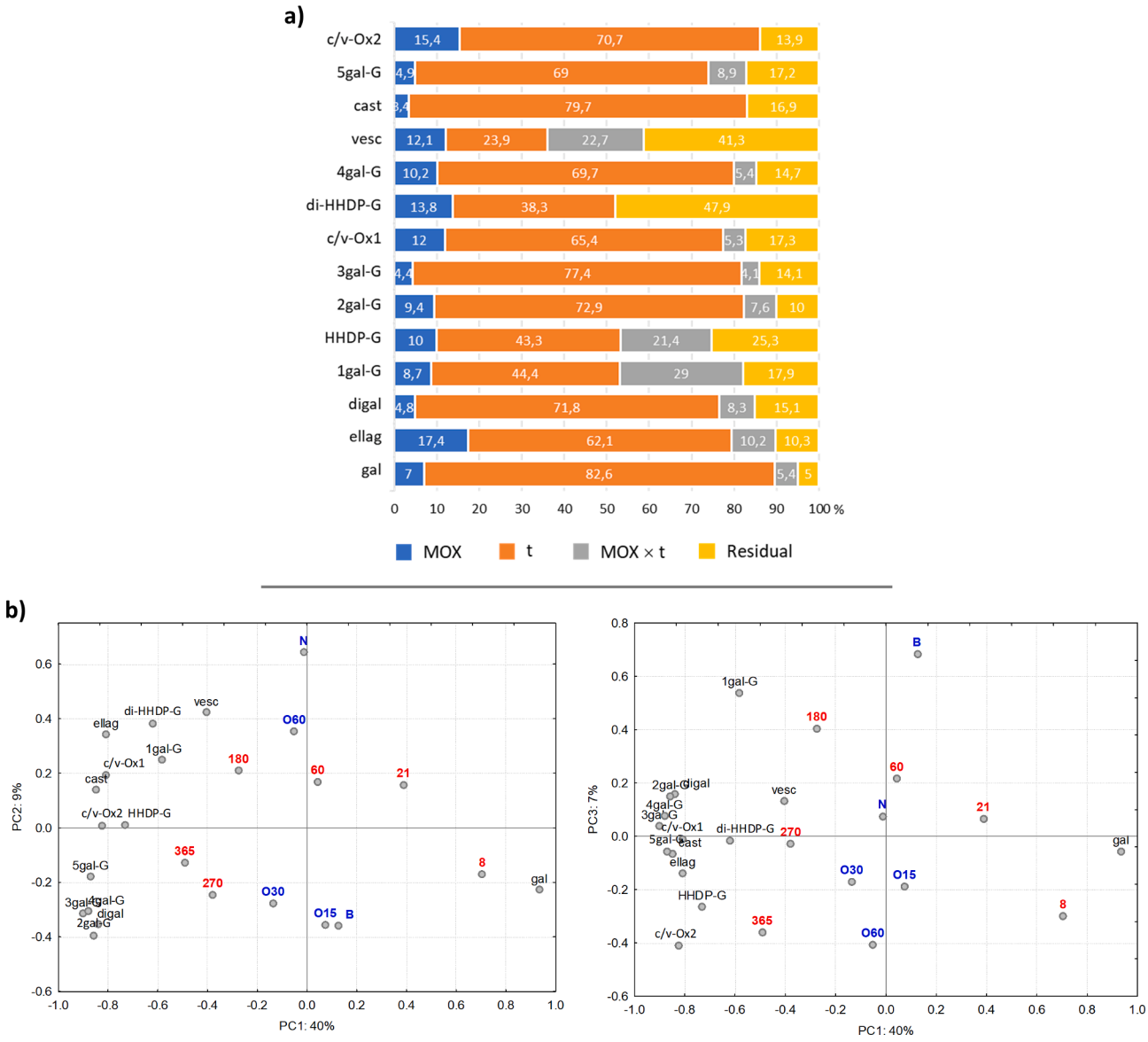

Fig. 5. a) Component variance analysis (in percentage) for the WSs according to the analyzed factors (MOX – oxygenation modalities; t – ageing time; MOX × t – interaction between the two factors; b) Projection of WSs samples, gallotannins and ellagitannins compounds in the space defined by the firsts three components obtained with Principal component analysis. Legend: gal – gallic acid, ellag – ellagic acid, digal – digallate, 5 Gal-G – penta-*O*-galloyl-β-D-glucose, 4 Gal-G – tetra-*O*-galloyl-β-D-glucose, 3 Gal-G – tri-*O*-galloyl-β-D-glucose, 2 Gal-G – di-*O*-galloyl-β-D-glucose, 1 Gal-G – mono-*O*-galloyl-β-D-glucose, di-HHDP-G – pedunculagin (di-HHDP-glucose), HHDP-G – HHDP-glucose, vesc – vescalagin, cast – castalagin, c/v-Ox1 – castalin/vescalin oxidation product with ethanol, c/v-Ox2 – vescalagin/castalagin oxidation product with ethanol; B – 250 L chestnut wooden barrel; N –50 L demijohns with chestnut staves and nitrogen application (control) (flow rate of 20 mL/L/month); O15 – 50 L glass demijohns with chestnut staves and MOX (flow rate of 2 mL/L/month during the first 15 days followed by 0.6 mL/L/month until 365 days); O30 – 50 L demijohns with chestnut staves and micro-oxygenation (flow rate of 2 mL/L/month during the first 30 days followed by 0.6 mL/L/month until 365 days); O60 –50 L glass demijohns with chestnut staves and micro-oxygenation (flow rate of 2 mL/L/month during the first 60 days followed by 0.6 mL/L/month until 365 days); 8, 21, 60, 180, 270, 365 – days of ageing.

very low.

The two isomers vescalagin and castalagin were also examined and it was not surprising their low relative contents in the studied WSs. Both showed less expression in the presence of oxygen, which highlights their prompt reactivity under this condition. Indeed, the presence of products stemming from their degradation (hydrolysis), which exhibit more than two-fold the relative percentage of vescalagin and castalagin isomers, demonstrates their importance in contributing to the increased complexity of wooden-derived substrates in aged WSs.

The experimental approach used, combining the identification ability of LC-ESI-HRMS/MS with distinct ageing technologies, proved to have great potential to unravel new molecules generated on WS, and can be replicated to brandies, whiskeys, wine and other aged beverages, contributing to a better understanding of the chemistry of these complex matrices.

CRediT authorship contribution statement

Tiago A. Fernandes: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision. **Alexandra M.M. Antunes:** Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Ilda Caldeira:** Investigation, Data curation, Writing – review & editing. **Ofélia Anjos:** Data curation, Writing – review & editing, Visualization. **Victor de Freitas:** Writing – review & editing. **Laurent**

Fargeton: Writing – review & editing. **Benjamin Boissier:** Writing – review & editing. **Sofia Catarino:** Writing – review & editing, Funding acquisition. **Sara Canas:** Formal analysis, Investigation, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.132322.

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