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Role of Glycoconjugates of 3-Methyl-4-hydroxyoctanoic Acid in the Evolution of Oak Lactone in Wine during Oak Maturation

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ABSTRACT: Oak lactone is a natural component of oak wood, but it also exists in glycoconjugate precursor forms. This study concerned the role of glycoconjugates of 3-methyl-4-hydroxyoctanoic acid, specifically a galloylglucoside, glucoside, and rutinoside, in the evolution of oak lactone during cooperage and maturation. The glycoconjugate profiles of 10 French oak samples were obtained by high-performance liquid chromatography−tandem mass spectrometry (HPLC-MS/MS) using stable isotope dilution analysis. The galloylglucoside was found to be the predominant glycoconjugate precursor and ranged in concentration from 110 to 354 μg/g. Maturation trials indicated the galloylglucoside undergoes acid-catalyzed hydrolysis after extraction into wine; after 12 months of maturation, the glucoside was the most abundant precursor, present at between 2- and 11-fold higher concentrations than those observed for powdered oak. Thermal degradation of glycoconjugates was observed only when oak samples were heated at 200 °C for 30 min, demonstrating their thermal stability.

KEYWORDS: *glycoconjugates, maturation, oak wood, oak lactone, toasting, wine*

INTRODUCTION

The (4*S*,5*S*) *cis*- and (4*S*,5*R*) *trans*-isomers of oak lactone (5-*n*-butyl-4-methyl-4,5-dihydro-2(3*H*)-furanone, **1a** and **1b**, Figure 1) are natural components of oak and have been identified in wines and spirits fermented and/or matured in oak barrels, where they impart desirable 'woody', 'coconut', 'vanilla', and 'dark chocolate' sensory attributes.[1,2] *cis*-Oak lactone usually occurs in oak and, therefore, wine, at concentrations equal to or greater than those of *trans*-oak lactone,[3,4] typically at levels between 0 and 1000 μg/L for *cis*-oak lactone and 0 and 400 μg/L for *trans*-oak lactone.[3]

The amount of oak lactone that can be extracted into wine depends on the species and geographical origin of the oak wood[5−8] but is also influenced by the seasoning and toasting processes employed during cooperage,[7,9−11] the duration of maturation, and the volume and previous use of oak barrels.[4,8,12] American oak (*Quercus alba*) generally contains higher concentrations of oak lactone than French oak (*Quercus pedunculata* and *Quercus petraea*),[4−6,11] with sessile oak (*Q. petraea*) usually containing more oak lactone than pedunculate oak (*Q. pedunculata*), although considerable variation has been observed between individual trees of the same species.[7] The effects of seasoning on the concentration of oak lactone remain somewhat unclear, because previous studies have reported both increased[13] and decreased[10] levels of oak lactone following seasoning. In contrast, the dramatic influence of toasting on oak composition is well established.[7,11] Significant increases in the oak lactone content of oak wood have been reported as a function of toasting intensity;[9] however, certain toasting conditions, that is, very high temperatures or prolonged toasting periods, may reduce oak lactone due to volatilization.[12]

Several studies have shown the progressive accumulation of oak lactone in wine and spirits during barrel maturation.[4,13,14] Interestingly, Pérez-Prieto and colleagues found that oak lactone concentrations increased during 270 days of barrel maturation and then continued to increase during the first 180 days of bottle storage, that is, after removal from oak contact.[4] The generation of additional quantities of oak lactone during cooperage, bottle storage, and even in the injector block of a gas chromatograph during analysis of oak extracts,[3] led to the realization that oak lactone occurs in oak wood in both free and precursor forms.

To date, a number of glycoconjugate precursors have been identified as constituents of oak wood. Masson and co-workers isolated (3*S*,4*S*)-3-methyl-4-*O*-(6′-*O*-galloyl)-β-D-glucopyranosyloctanoic acid (**2**, galloylglucoside) from French oak wood.[15] In a subsequent study, Hayasaka and colleagues identified four isomers of **2**, as well as (3*S*,4*S*)-*cis*- and (3*S*,4*R*)-*trans*-3-methyl-4-*O*-β-D-glucopyranosyloctanoic acid (**3**, glucoside) and (tentatively) 3-methyl-4-*O*-(6′-*O*-α-L-rhamnosyl)-β-D-glucopyranosyloctanoic acid (**4**, rutinoside), in extracts of French and American oak woods.[16] Both **2** and **3** have been shown to produce oak lactone under strong acid hydrolysis and pyrolysis conditions.[15,17]

To date, the majority of investigations concerning the influence of cooperage treatments on oak composition and/or the extraction of oak lactone during wine maturation have relied on compositional comparisons of oak lactone. However, a stable isotope dilution analysis (SIDA) method has recently been developed for the quantitative analysis of the β-D-glucopyranosides of 3-methyl-4-hydroxyoctanoic acid (**3**) using HPLC-MS/MS.[18] We now report the application of this method to investigations into the role of glycoconjugates of 3-

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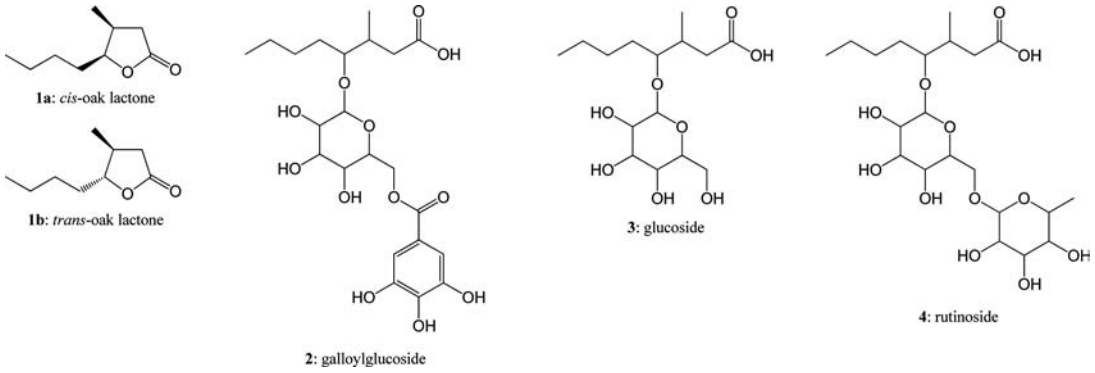

Figure 1. Chemical structures of *cis*- and *trans*-oak lactones **1a** and **1b** and galloylglucoside **2**, glucoside **3**, and rutinoside **4** conjugates of 3-methyl-4-hydroxyoctanoic acid.

methyl-4-hydroxyoctanoic acid (**2**, **3**, and **4**) in the evolution of oak lactone in wine during oak maturation. The effect of toasting on the glycoconjugate content of oak samples was also investigated.

MATERIALS AND METHODS

Oak Samples. Representative samples of oak shavings (approximately 200 g) were obtained from 10 wood stacks, comprising 0.4 m^3 of French oak staves (the equivalent of approximately seven 225 L barrels), sourced according to quality control sampling regimens employed by a French cooperage, Seguin Moreau. The staves from each wood stack were prepared from French oak woods sourced from forests in several departments, Orne, Maine-et-Loire, Yvelines, and Haute-Marne (Table 1), and were naturally seasoned for 24 months prior to sampling. Oak shavings were subsequently ground into a homogeneous powder (with linear dimensions of <0.7 mm) using an SM2000 grinder (Reutsch, Germany).

Table 1. Concentrations of Galloylglucoside (2), Glucoside (3), and Rutinoside (4) Precursors of Oak Lactone Present in Powdered Oak Samples

oak samples		concentration[a] (μg/g)		
stack	department	galloylglucoside	glucoside	rutinoside
504549	Orne	6.7	1.0	0.2
175170	Maine-et-Loire	154.1	8.4	5.0
503544	Yvelines	305.4	26.2	9.1
175171	Maine-et-Loire	137.9	13.1	4.4
503543	Yvelines	110.2	7.0	3.1
180093	Haute-Marne	196.3	20.4	4.5
505502	Maine-et-Loire	209.8	25.9	8.9
175595	Maine-et-Loire	289.6	23.4	20.2
175575	Maine-et-Loire	353.9	23.5	19.4
175168	Maine-et-Loire	245.0	20.6	15.1

[a]Mean values from two replicates.

Maturation Trial. Powdered French oak wood (10 g) was soaked in model wine (500 mL), prepared by dissolving tartaric acid (5 g/L) in an aqueous 12% ethanol solution adjusted to pH 3.5 with aqueous sodium hydroxide (5 M), in glass bottles sealed hermetically with screw caps. Mixtures were agitated (using orbital shakers) at room temperature for 72 h before being stored at 16 °C in darkness. Aliquots (10 mL) were sampled after 7 days, 12 months, 16 months, and 27 months of maturation, for quantification of *cis*- and *trans*-oak lactones. Aliquots were also sampled after 12 months of maturation for quantification of glycoconjugate precursors of oak lactone.

Pyrolysis Trial. Powdered French oak wood (2 g) was sealed in 50 mL glass ampules and heated at either 100 or 200 °C for 5 or 30 min (in triplicate). The ampules were then opened, and the toasted oak wood was sampled for quantification of the glycoconjugates of 3-methyl-4-hydroxyoctanoic acid.

Quantification of *cis*- and *trans*-Oak Lactones by Gas Chromatography–Mass Spectrometry (GC-MS). The concentrations of *cis*- and *trans*-oak lactones were determined by headspace-solid phase microextraction–gas chromatography–mass spectrometry according to methodology developed by Carrillo and colleagues.[19] Analysis was carried out using an Agilent 7890A gas chromatograph equipped with a Gerstel MPS 2XL multipurpose autosampler and coupled to an Agilent 5975C mass selective detector. An internal standard solution of 100 μL of 4-heptanolide (100 μg/L in ethanol) was added to each sample (10 mL) in a screw-cap vial, before samples were incubated at 40 °C for 5 min. A DVB-CAR-PDMS fiber (Supelco, Bellefonte, PA, USA) was used to sample the headspace above each model wine sample for 20 min at 40 °C, immediately prior to instrumental analysis. The gas chromatograph was fitted with a 30 m × 0.25 mm × 0.25 μm FactorFour VX-Wax fused silica capillary column. The carrier gas was helium, with a flow rate of 1 mL/min. The oven temperature started at 50 °C and was held at this temperature for 0.3 min, then increased to 200 °C at 12 °C/min and to 240 °C at 20 °C/min, and then held at this temperature for 2 min. The injector temperature was held at 280 °C and the transfer line at 280 °C. For quantification, mass spectra were run in selective ion monitoring (SIM) mode. The ions monitored in SIM runs were m/z 99 for *cis*- and *trans*-oak lactones and m/z 85 for the internal standard. The linear dynamic range was 0 and 3–2000 μg/L, the limit of detection was 1 μg/L, and the precision was <7% relative standard deviation.

Quantification of Glycoconjugates of 3-Methyl-4-hydroxyoctanoic Acid by HPLC-MS/MS. Analysis was carried out using an Agilent 1200 HPLC system coupled to an AB Sciex 4000 Q TRAP hybrid tandem mass spectrometer.

Sample Preparation. For analysis of powdered oak woods, deuterated (3*S*,4*S* and 3*R*,4*R*)-3-methyl-4-*O*-β-D-glucopyranosyloctanoic acid ([$^2H_4$]-**3**, 96 μg/mL in water, 10 μL) was added as an internal standard to each powdered oak sample (0.5 g), together with an aqueous 50% methanol solution (10 mL). The resulting mixture was then sonicated for 60 min and centrifuged (4300*g* for 5 min), and a portion of the supernatant was filtered through a 0.45 μm GHP membrane (Acrodisc, PALL Life Sciences, Cheltenham, Australia) prior to instrumental analysis. For analysis of model wines, the internal standard solution ([$^2H_4$]-**3**, 96 μg/mL in water, 10 μL) was added to each model wine sample (10 mL), and the mixture was shaken briefly and filtered as above, prior to analysis. Preparation of the [$^2H_4$]-**3** internal standard has been reported previously.[18]

HPLC-MS/MS. Analysis was carried out according to a SIDA method developed by Fudge and co-workers[18] with some modifications in mass transitions (two most abundant fragment ions of each compound) monitored for the quantification of glycoconjugates as follows; m/z 487 → 335 and 271 for **2**, m/z 335 → 119 and 89 for **3**, m/z 481 → 205 and 163 for **4**, and m/z 339 → 119 and 89 for [$^2H_4$]-**3** as internal standard with a dwell time of 50 ms.

Concentrations of **2**, **3**, and **4** were obtained by measuring the peak area (sum of the two mass transitions) of **2**, **3**, or **4** relative to that of the known amount of [$^2\mathbf{H}_4$]-**3** and were therefore expressed as [$^2\mathbf{H}_4$]-**3** equivalents. The *cis*- and *trans*-isomers of **3** share close retention times and product ion spectra,[16] which does not permit their individual quantification; the glucoside concentrations reported therefore represent a sum of both isomeric forms. Of the multiple galloylglucoside isomers, the most dominant isomer, (3*S*,4*S*) 3-methyl-4-*O*-(6′-*O*-galloyl)-*β*-D-glucopyranosyloctanoic acid (**3**), was measured for this study.

Data Analysis. Chemical data were analyzed by ANOVA using GenStat (14th ed., VSN International Limited, Herts, UK). Mean comparisons were performed by least significant difference (LSD) multiple-comparison test at $P < 0.05$.

RESULTS AND DISCUSSION

The French oak woods sourced for this study were not randomly selected, but were instead deliberately chosen to reflect the natural variation in free oak lactones that can occur. It should also be noted that in the absence of isotopically labeled analogues of galloylglucoside **2** and rutinoside **4**, their relative concentrations were determined using deuterated glucoside [$^2\mathbf{H}_4$]-**3** as an internal standard, that is, as glucoside equivalents. That said, a high degree of reproducibility of glycoconjugate measurements was observed;, that is, within ca. 10% standard error (Tables 1, 2, and 3). As such, it was assumed that relative changes in concentrations of **2** and **4** could be accurately determined, but direct comparisons with glucoside levels are at best approximations.

Table 2. Concentrations of Galloylglucoside (2), Glucoside (3), and Rutinoside (4) Precursors of Oak Lactone Present in Model Wine Following 12 Months of Maturation with Powdered Oak

	concentration[a] (μg/L)		
oak sample	galloylglucoside	glucoside	rutinoside
504549	2.1	12.4	2.3
175170	1234.3	1387.8	82.9
503544	1171.5	3834.6	174.7
175171	100.4	1516.5	34.0
503543	71.3	1139.8	43.1
180093	3.1	1174.5	51.3
505502	3.6	1009.8	140.2
175595	40.8	4159.4	313.5
175575	277.6	5209.1	355.2
175168	1152.7	2615.5	233.1

[a]Mean values from two replicates.

Glycoconjugate Content of French Oak Woods. Similar glycoconjugate profiles were observed for almost all of the different French oak woods (Table 1). With the exception of the oak sourced from Orne (stack 504549), **2** was the most abundant glycoconjugate precursor and ranged in concentration from 110 to 354 μg/g. The concentration of **3** ranged from 7 to 26 μg/g, whereas smaller quantities of **4**, 3−20 μg/g, were also observed. Glycoconjugate levels were considerably lower for the Orne sample, being 7, 1, and 0.2 μg/g for **2**, **3**, and **4**, respectively. The influence of geographic origin on the composition of oak wood has been reported previously,[7] albeit not with respect to oak lactone glycoconjugates. The concentrations of **2** observed in the current study were comparable to the levels reported by Masson and colleagues for fresh sessile oak woods, being 0−495 μg/g;[20] whereas the observed levels of **3** were within the 0−50 μg/g concentration ranges previously reported for French and American oak samples.[18]

Evolution of Oak Lactone in Wine during Oak Maturation. The evolution of *cis*- and *trans*-oak lactones in model wines treated with powdered French oak wood is shown in Figure 2. As intended, the different oak samples showed considerable variation in oak lactone levels, but similar trends were apparent. In most cases, the concentration of oak lactone increased linearly during the first 16 months of maturation and then plateaued. However, the *cis*-oak lactone concentrations of two samples (505502 and 503544) and the *trans*-oak lactone concentration of one sample (175171) increased throughout the 27 month maturation period, whereas the *trans*-oak lactone content of two samples (175168 and 503544) initially increased but then decreased slightly between the 16 and 27 month sampling points. There was no apparent relationship between the concentrations of *cis*- and *trans*-oak lactones. With the exception of one sample (175171), *cis*-oak lactone was the predominant isomer, being 57−90% of total oak lactone content, in agreement with the literature (see, e.g., refs 2−5, 7, 20, and 22).

The concentrations of oak lactone measured after 7 days of maturation were considered to reflect the free oak lactone content of the different powdered oak samples, which should have been exhaustively extracted in this time.[23] As such, the formation of additional quantities of oak lactone was attributed to the extraction and hydrolysis of glycoconjugates of 3-methyl-4-hydroxyoctanoic acid, as oak lactone precursors. The glycoconjugate content of oak wood (Table 1) generally reflected the total oak lactone content of model wine after 27 months of maturation (Figure 2). The Orne oak sample, which contained the lowest glycoconjugate levels, yielded the model wine with the lowest oak lactone concentrations. The four oak samples with the highest levels of **2** (which ranged from 245 to 354 μg/g), corresponded to model wines containing high oak lactone levels (980−1170 μg/L). Similarly, two samples with low levels of **2** (110 and 154 μg/g) corresponded to model wines with low levels of oak lactone (330 and 390 μg/L, respectively). However, three samples with moderate galloylglucoside levels (138−210 μg/g) yielded model wines with comparatively high oak lactones levels (1190−1850 μg/L).

Glycoconjugate Content of Model Wines during Oak Maturation. The concentrations of glycoconjugates in model wines were determined after 12 months of maturation to investigate the extent to which they had been hydrolyzed (Table 2). For three oak samples (175170, 503544, and 175168), a significant proportion of galloylglucoside remained after 12 months of maturation (i.e., 20−40%), but for the remaining samples, >95% of **2** had been consumed. Considerable variation was observed in the hydrolytic behavior of **4**. In some cases (503544 and 175575) there was little change in rutinoside levels, whereas for one sample (175171) >60% of **4** had been hydrolyzed, but moderate losses of between 20 and 40% were observed for most samples. Perhaps the most notable finding was the significant change in glucoside concentrations. With one exception (being the Orne oak sample), model wines showed between 2- and 11-fold increases in concentrations of **3**, relative to the levels corresponding to quantitative extraction from powdered oak. This finding can be explained only by sequential hydrolysis of **2** and, to a lesser extent, **4**, that is, cleavage of gallate and pentose moieties, respectively, to generate additional quantities of **3**. Certainly the

Table 3. Concentrations of Galloylglucoside (2), Glucoside (3), and Rutinoside (4) Precursors of Oak Lactone in Powdered Oak Following Heating at 100 or 200 °C for 5 or 30 min

oak sample		concentration[a] (μg/g)		
		galloylglucoside	glucoside	rutinoside
503543	toasted for 5 min at 100 °C	139.4.0 a	7.9	2.5 a
	toasted for 30 min at 100 °C	133.4 a	8.4	2.3 a
	toasted for 5 min at 200 °C	130.3 a	8.7	2.3 a
	toasted for 30 min at 200 °C	100.6 b	8.2	0.7 b
		$P < 0.05$	ns	$P < 0.001$
505502	toasted for 5 min at 100 °C	220.9 a	28.2 a	7.3 a
	toasted for 30 min at 100 °C	223.0 a	29.1 a	7.8 a
	toasted for 5 min at 200 °C	209.6 a	29.7 a	6.9 a
	toasted for 30 min at 200 °C	137.9 b	32.2 b	3.0 b
		$P < 0.005$	$P < 0.005$	$P < 0.001$
175575	toasted for 5 min at 100 °C	270.3	24.0	12.8 a
	toasted for 30 min at 100 °C	280.9	24.5	14.3 a
	toasted for 5 min at 200 °C	277.0	26.9	13.1 a
	toasted for 30 min at 200 °C	231.6	30.6	6.5 b
		ns	ns	$P < 0.001$

[a]Mean values from three replicates.

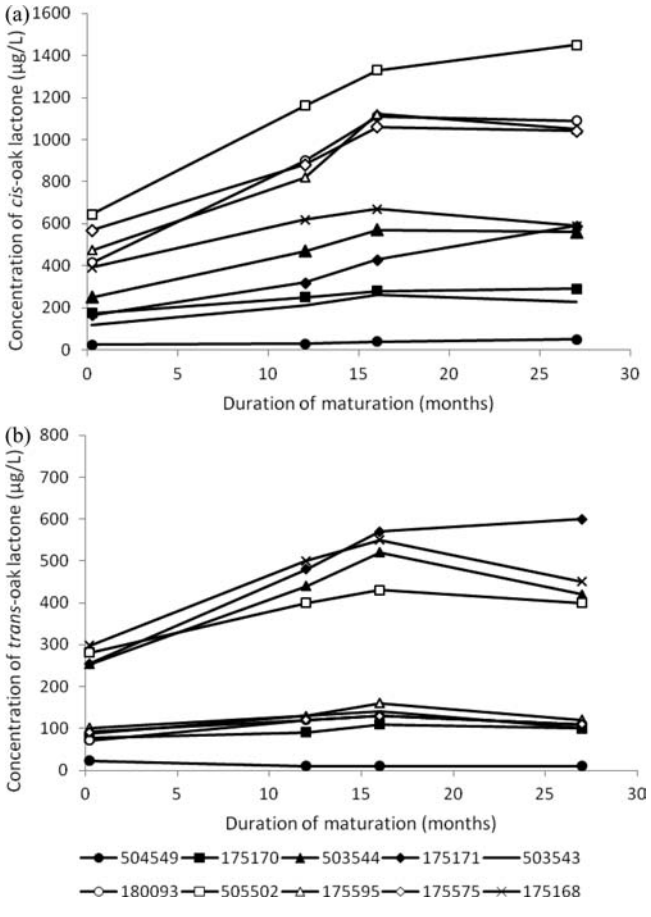

Figure 2. Evolution of (a) *cis*- and (b) *trans*-oak lactones in model wine during oak maturation.

model wines containing the highest levels of **3** after 12 months of maturation corresponded to the powdered oak woods that contained the highest galloylglucoside levels, that is, 503544, 175595, 175575, and 175168 (Tables 1 and 2). Subsequent hydrolysis of **3** may occur slowly, given the stability of the glucoside to mild acid conditions,[17] but would release 3-methyl-4-hydroxyoctanoic acid, which can then lactonize to yield oak lactone, explaining the evolution of *cis*- and *trans*-oak lactones during maturation (Figure 2). The different rates of evolution observed for each isomer can be attributed to differences in their thermodynamic stability, as shown previously.[21]

For the glycoconjugate content of oak wood to be used as a diagnostic tool for predicting the concentration of oak lactone in wine after oak maturation, a larger number of samples would need to be analyzed to better establish the variability within oak samples. As with free oak lactone, natural variation of glycoconjugates may also be high. Interestingly, in the current study, a high level of correlation was found between the concentrations of *cis*- and *trans*-oak lactones measured after 7 days and 12 months of maturation (Figure 3).

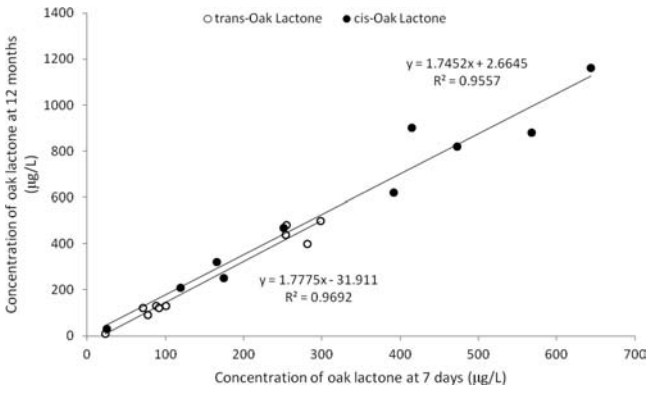

Figure 3. Linear regression of the oak lactone concentrations of model wines after maturation for 7 days and 12 months.

Influence of Toasting on Glycoconjugate Content of French Oak Woods. The formation of *cis*- and *trans*-oak lactones from galloylglucoside and glucoside conjugates under acid hydrolysis and pyrolysis conditions was reported in an earlier study.[17] A 90% conversion of **3** to oak lactone was reported after hydrolysis for 48 days at pH 3.0 and 100 °C, but

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only trace levels of oak lactone were detected when hydrolysis was performed at 45 °C. A 20% conversion of **2** to oak lactone was reported after hydrolysis for 35 days at pH 3.0 and 100 °C. These results demonstrated the relative stability of **3** in mild acid and at moderate temperatures. Pyrolysis of **2** and **3** for 30 min at 235 °C yielded 15 and 25−30% oak lactone conversions, respectively. However, the extent to which these precursors were thermally degraded could not be determined, due to the lack of an appropriate quantitative method.

The current study therefore included an investigation into the influence of toasting temperature and duration on the thermal degradation of glycoconjugates of 3-methyl-4-hydroxyoctanoic acid. Three French oak samples comprising different glycoconjugate profiles were subjected to pyrolysis at 100 or 200 °C for 5 or 30 min (Table 3). These conditions were chosen to allow compositional comparisons with acid hydrolysate data from previous studies[17] and to simulate the toasting conditions of barrel cooperage. The pyrolytic behavior of glycoconjugates was similar for each of the French oak samples. At 100 °C, irrespective of the duration of toasting, no significant changes in glycoconjugate concentrations were observed. However, thermal degradation of **2** and **4** occurred after 30 min of heating at 200 °C, with concentrations reduced by approximately 20−40 and 50−70%, respectively. In contrast, no meaningful differences were observed in glucoside concentrations.

These results further demonstrate the thermal stability of glycoconjugates of 3-methyl-4-hydroxyoctanoic acid. The uppermost conditions employed in the current study, that is, 200 °C for 30 min, would not be expected to generate significant quantities of oak lactone, in agreement with the findings of Campbell and co-workers that heating French and American oaks to 200 °C for 1 h had little effect on *cis*- and *trans*-oak lactone concentrations.[22] Instead, higher temperatures and/or extended durations of toasting would be needed to pyrolyze a greater proportion of the oak lactone glycoconjugate pool. During barrel toasting, temperatures of about 200 °C are typical, but can reach as much as 250 °C for short periods of time. Higher temperature conditions are more likely to favor oak lactone formation due to the thermal degradation of glycoconjugate precursors. However, it should be noted that heavily toasting oak can result in both the generation and loss of oak lactone; when French and American oaks were heated at 235 °C for 1 h, significantly reduced oak lactone concentrations resulted,[22] presumably due to volatilization and/or thermal degradation.[12]

Whereas hydrolysis of **2** during maturation gave additional quantities of **3** (Table 2), pyrolysis did not (Table 3), which suggests a different degradative pathway was involved. Pyrolytic (thermal) degradation likely involves cleavage of the glycosidic linkage to give 3-methyl-4-hydroxyoctanoic acid, which yields oak lactone upon lactonization.[21] The formation of oak lactone from galloylglucoside and glucoside precursors at pH 3.0 and 100 °C described previously[17] likely involves acid-catalyzed hydrolysis, rather than thermal degradation, because, in the current study, thermal degradation of glycoconjugates was not observed at 100 °C.

In summary, the role of galloylglucoside, glucoside, and rutinoside conjugates of 3-methyl-4-hydroxyoctanoic acid as precursors to *cis*- and *trans*-oak lactones has been demonstrated. Oak lactone can be generated from these glycoconjugates by (i) thermal degradation during toasting, where both temperature and the duration of toasting can influence yield; and (ii) slow acid-catalyzed hydrolysis, after extraction from oak wood into wine during maturation. As with free oak lactone, the glycoconjugate content of oak wood showed considerable variability. Future work could therefore investigate the influence of factors such as geographic origin, species, and seasoning on the glycoconjugate profiles of both French and American oaks.

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Notes

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