Development of a quantitation method to assay both lyoniresinol enantiomers in wines, spirits, and oak wood by liquid chromatography-high resolution mass spectrometry

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Abstract:

Wine taste balance evolves during oak aging by the release of volatile and non-volatile compounds from wood. Among them, an enantiomer of lyoniresinol, (+)-lyoniresinol, has been shown to exhibit bitterness. To evaluate the impact of (+)-lyoniresinol on wine taste, a two-step quantitation method was developed and validated. First, (±)-lyoniresinol was assayed in wines, spirits, and oak wood macerates by C-18 liquid chromatography-high resolution mass spectrometry (LC-HRMS). Then, the lyoniresinol enantiomeric ratio was determined by chiral LC-HRMS in order to calculate the (+)-lyoniresinol content. In red and white wines, the average concentrations of (+)-lyoniresinol were 1.9 and 0.8 mg/L, respectively. The enantiomer proportions were not affected by bottle aging, and lyoniresinol appeared to remain stable over time. The sensory study of (+)-lyoniresinol established its perception threshold at 0.46 mg/L in wine. All the commercial wines quantitated were above this perception threshold, demonstrating its impact on wine taste by an increase in bitterness. In spirits, (+)-lyoniresinol ranged from 2.0 to 10.0 mg/L and was found to be released continuously during oak aging. Finally, neither botanical origin nor toasting was found to significantly affect the (+)-lyoniresinol content of oak wood.

Keywords: Lignan, Chiral separation, Bitterness, Orbitrap mass spectrometry, Taste-active compounds, Oak aging

Introduction

The first known racemic mixture, from the Latin word *racemus*, meaning "(wine) grape," was identified in 1848 as racemic acid, which Louis Pasteur found to be a mixture of the two enantiomers of tartaric acid, the most abundant acid in wine [1]. This discovery paved the way for the study of the stereochemistry of molecules from their separation [2] to their synthesis [3, 4] through the comparison of their properties. Indeed, enantiomers exhibit differential behavior when in contact with other chiral compounds. In particular, their biological properties can be very different, as illustrated by the infamous example of thalidomide whose *R*-enantiomer possesses a therapeutic effect while its *S*-enantiomer is teratogenic [5]. The enantioselectivity of interactions between aromas and olfactory receptors has also been widely observed, as with borneol enantiomers ((+)- and (-)-borneol that have unpleasantly peppery and camphor odor, respectively) [6]. Similarly, taste-active compounds such as asparagine or alapyridaine are also subject to distinct properties according to their absolute configuration [7, 8]. Discovering the sensory properties of natural molecules and their interactions with receptors is one of the most exciting challenges for chemists involved in food analysis. The taste of wine is due to thousands of molecules released from grapes that are synthesized by micro-organisms or modified during aging [9]. An important source of active compounds is oak wood, which is used for the traditional step of barrel aging and during which the color, aroma, and taste of wine are modified [10–15]. Within this research, the impact of the stereochemistry of active compounds in wine is a particularly relevant issue. Recent studies have investigated the impact of enantiomers on wine flavor. In the wine aroma field, various studies have focused on the olfactory properties of the enantiomers involved in wine quality [16,17] or off-flavors [18]. Surprisingly, the influence of stereochemistry on the taste properties of wine non-volatiles has received less attention.

Knowledge of the molecular origin of wine taste has increased jointly with the improvement of analytical techniques [19–21]. Even though the study of enantiomers is of particular relevance, it represents a major challenge for analytical chemists. Owing to their identical physical and chemical properties, enantiomers cannot be separated in a symmetrical environment, which complicates their racemic resolution.

Two different approaches are available for the quantitation of enantiomers by LC-MS. First, direct injection can be carried out using a chiral stationary phase (CSP) or a non-chiral column with a chiral mobile phase additive (CMPA). Even though these methods only need simple

preparation of samples and simple chromatographic runs, CSPs are very sensitive to chromatographic conditions and their desorption kinetics are quite poor with low column efficiency. Secondly, the resolution of enantiomers may be assessed by indirect injection after the formation of diastereoisomers by chiral derivatization for separation on a non-chiral column. In the latter case, the enantiomeric purity of the derivatization agent is critical. Moreover, the derivatization procedure must not induce the racemization of the compound or side products, so it is quite complicated to develop a method combining good sensitivity and the efficient separation of enantiomers with easy sample preparation [22].

Among the wine taste-active compounds, lyoniresinol was recently described to be the most abundant and the bitterest lignan extracted from *Quercus petraea* wood [10]. Its perception threshold was evaluated at 1.5 mg/L, whereas its concentrations in most oaked wines were found to be higher, which demonstrates the impact of lyoniresinol on wine taste. It has been isolated from various plants and observed as a mixture of 8*R*,8′*R*,7′*S*-and 8*S*,8′*S*,7′*R*-enantiomers with variable relative abundance [23]. In *Quercus* genus wood, its specific optical rotation measurement indicated that it is present as a racemic mixture [24]. Recently, a racemic resolution based on the natural xylose-derivatives of lyoniresinol was performed in order to purify its enantiomers [25]. Vibrational Circular Dichroism measurements of the enantiomers determined their absolute configurations to be (8*R*,8′*R*,7′*S*)-(+)-lyoniresinol a and (8*S*, 8′*S*,7′*R*)-(−)-lyoniresinol b (Fig. 1). Above all, sensory analysis established that only (+)-lyoniresinol was bitter, whereas (−)-enantiomer was tasteless. This illustrates the crucial influence of stereochemistry on wine taste. In this study, lyoniresinol enantiomers have only been purified and tasted but never quantitated. The determination of their sensory impact requires the comparison between their perception threshold and their concentration in wines and spirits. This highlights the importance to develop powerful and easy-to-use methods to specifically quantitate taste-active enantiomers.

This paper presents the first development of a twostep method for quantitating (+)-lyoniresinol by liquid chromatography-high resolution mass spectrometry (LCHRMS). First, (±)-lyoniresinol was assayed using a non-chiral C18 column. Then, the enantiomeric ratio of each sample was determined by injection onto a CSP column connected to the same Orbitrap analyzer after prepurification by solid phase extraction (SPE). The method was validated and applied to quantitate for the first time (+)-lyoniresinol in three matrices. Its concentrations were measured in commercial red and white wines and compared to its perception threshold in order to establish to what extent it might impact wine taste. Moreover, oaked spirits from Cognac were also analyzed, and the influence of the oak aging period was evaluated. Finally, the

amounts of (+)-lyoniresinol in oak wood were determined to study the influence of cooperage parameters such as oak species and wood toasting.

Materials and methods

Chemicals

Ultrapure water (Milli-Q purification system, Millipore, France) and ethanol (HPLC grade solvent VWR International, Pessac, France) were used for sample maceration. Trifluoroacetic acid (TFA), tartaric acid, and quinine sulfate were purchased from Sigma-Aldrich (France). Acetonitrile (ACN) and water used for chromatographic separation were LC-MS grade and were purchased from Fisher Chemical (Illkirch, France). Lyoniresinol enantiomers were isolated as described previously by Cretin et al. [25].

Oak wood samples, wines, and spirits

Oak wood material originated from France and was provided by Seguin Moreau Cooperage. A part of the study concerned the influence of species on contents of lyoniresinol enantiomers, with 15 samples of *Q. petraea* (sessile oak) and 15 samples of *Quercus robur* (pedunculate oak). The species assignment has been carried out according to the chemical method described by Marchal et al. [26]. The effect of toasting intensity was assayed on one side with oak wood pieces toasted in laboratory oven (air-convection kiln). In this assay, the five different wood staves were cut in five fragments (10 × 5 × 2 cm) and toasted, respectively, at 140, 180, 200, and 250 °C in laboratory kiln for 20 min, while last fragment was left as control (non-toasted). On the other side, other series of samples was collected both on the toasted and nontoasted sides of a stave (13 replicates = different staves) coming from production line of Seguin Moreau cooperage. In this assay, the staves were exposed to open fire bending and toasting qualified as medium toasting. The samples were collected by scraping of internal (exposed to fire) and external part (non-exposed to fire), with a scraping depth of 3 mm. All these samples were ground down to the powder, macerated at 50 g/L for 48 h in a wine-model solution (12% v/v ethanol, 5 g/L tartaric acid solution, pH 3.5) and filtered at 0.45 µm. All concentrations were expressed in mg/g of dry wood. The sample size was 2.5 g in each trial.

Spirits (six commercial Cognac and four "eaux-de-vie" in aging process) were supplied by Rémy Martin. All concentrations were expressed in mg/L of spirits.

Lyoniresinol quantitation was assessed in 59 commercial red wines (29 from various appellations on the Bordeaux left bank, 23 from the Bordeaux right bank, 3 from California, 3 from Australia, and 1 from Italy) and 10 commercial white wines (from Graves). The vintages covered more than one century from 1911 to 2013. All these wines were aged in oak wood. The concentrations were expressed in mg/L of wine.

Wine used for sensory analysis was a non-oaked white Bordeaux from 2013 (12.6% alc. vol.; 5.9 g of glycerol/L; 0.71 g/L of glucose + fructose; pH 3.1).

Quantitation of lyoniresinol enantiomers in wine, spirits, and wood

LC-HRMS analysis

The LC-HRMS apparatus consisted of an HTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland), an Accela U-HPLC system with quaternary pumps and an Exactive Orbitrap mass spectrometer equipped with a heated electrospray ionization (HESI I) probe (both from Thermo Fisher Scientific, Les Ulis, France). LC separations were carried out on two different columns. For (±)-lyoniresinol quantitation, a C18 column (Hypersil Gold 2.1 × 100 mm, 1.9 µm particle size, Thermo Fisher Scientific) was used with water (Eluent A) and ACN (Eluent B) as mobile phases. The flow rate was set at 600 µL/min and the injection volume was 5 µL. Eluent B varied as follows: 0 min, 14%; 0.5 min, 14%; 1.5 min, 19%; 2 min, 19%; 4.5 min, 38%; 4.6 min, 98%; 6.9 min, 98%; 7 min, 14%; 8.6 min, 14%. Enantiomers were separated on a Chiralpak® IB-3 column (2.1 × 150 mm, 3 µm particle size, Chiral Technologies, Illkirch, France) with a flow rate set at 150 µL/min and an isocratic elution mode (80:20; H_2O/ACN).

The mass analyzer was calibrated each week using Pierce® ESI Negative Ion Calibration solution (Thermo Fisher Scientific). The ionization and spectrometric parameters presented in Table 1 were optimized in negative mode for each chromatographic application by continuous injection of a pure solution of lyoniresinol (5 ng/min) with the considered flow rate of solvent. All data were processed using the Qual Browser and Quan Browser applications of Xcalibur version 2.1 (Thermo Fisher Scientific).

Wines and sample preparation

For (±)-lyoniresinol quantitation, wines, spirits, and wood chip macerates were diluted by a factor 3, 10, and 25, respectively. After a 0.45 µm filtration, samples were injected directly in LC-HRMS using the chromatographic and spectrometric parameters described above. To determine the enantiomeric ratio, wines, spirits, and wood chip macerates were pre-purified by SPE. After a dilution with water by a factor 4, 3, and 8, respectively, to reduce the ethanol level and to obtain concentrations suited to further injection in HRMS, aliquots of wine (3 mL), spirits (8 mL), and wood macerates (4 mL) were 0.45 µm-filtered and dropped onto a non-polar column (Bond Elut ENV, PS/DVB polymer, bed weight 200 mg, 125 µm spherical particles, Agilent). Elution was carried out using successively 2 mL of 20% and 2 mL of 40% ACN in water solutions acidified with 0.05% TFA. Aliquots of the 40% ACN fractions containing lyoniresinol were taken, evaporated in vacuo, and suspended in water/methanol solution (95/5 v/v) before analysis by CSP LC using the chromatographic and spectrometric parameters described above.

Preparation of calibration solution

The method used for (±)-lyoniresinol quantitation in wine was previously described by Marchal et al. [10]. For (±)-lyoniresinol quantitation in oak wood and spirits, two ranges of calibration solutions were prepared by successive dilution of a stock solution of lyoniresinol (1 g/L) in the model solution used for the preparation of oak macerates and in a non-oaked "eau-de-vie" diluted by a factor 10 to provide calibration samples (10 mg/L, 5 mg/L, 2 mg/L, 1 mg/L, 500 µg/L, 200 µg/L, 100 µg/L, 50 µg/L, 20 µ/L, 10 µg/L, 5 µg/L, 2 µg/L). Lyoniresinol was detected according to the theoretical exact mass of its deprotonated ion ([M-H]−) and its retention time. Peaks areas were determined by automatic integration.

Method validation for quantitation of (±)-lyoniresinol on C18 column

The quantitation methods of (±)-lyoniresinol in oak wood macerates and spirits were validated by studying sensitivity, linearity, specificity, intraday repeatability, and trueness.

To determine the sensitivity of the LC-HRMS method, the approach described by De Paepe et al. [27] was used.

A calibration curve was established by plotting the areas for each concentration level versus the nominal concentration. Linear regressions were chosen with a 1/x statistical weight. Linearity

was evaluated by correlation coefficient (R^2) and by deviations of each back-calculated standard concentration from the nominal value.

To evaluate repeatability, the intraday precision was determined by injecting five replicates of two intermediate calibration solutions (50 and 500 µg/L) and the relative standard deviation (RSD%) was calculated. One sample of toasted oak wood, of spirits (S-8), and of red (RW-1) and white wine (WW-2) was chosen among the analyzed samples and was fortified with calibration solution corresponding to the addition of 100, 500, and 1 mg/L of lyoniresinol. Trueness was evaluated by calculating the recovery ratio (between measured and expected areas).

Specificity of the Orbitrap analysis was assessed by evaluating the mass accuracy and retention time repeatability. These parameters were determined concomitantly with the above described precision and trueness analysis.

Method validation for determination of enantiomeric ratio of lyoniresinol on CSP column

The enantiomeric ratio of lyoniresinol (*r*) was defined as a dimensionless number as follows:

r = peak area of (+)−lyoniresinol/peak area of (−)−lyoniresinol

First, the SPE method of lyoniresinol prior to chiral analysis was validated by studying the reproducibility of the SPE by injecting a wine sample onto three cartridges. RSD of the (±)-lyoniresinol peak area obtained for the three cartridges was calculated.

Then, the preservation of the enantiomeric ratio throughout SPE pre-purification was studied. Three replicates of pure (±)-lyoniresinol were compared before and after SPE and the RSD of *r* was calculated. The same experiments were carried out with a wine sample and a Cognac to evaluate the reproducibility between various matrices.

Finally, the method for determining the lyoniresinol enantiomeric ratio was validated by studying the repeatability and specificity of the chiral analysis. Three replicates of an oak wood sample were injected and the RSD of (+)-lyoniresinol and (−)-lyoniresinol peaks areas were calculated. Mass accuracy and retention time repeatability were assessed throughout sample injections.

Sensory analysis

240 Tasting sessions took place in a specific air-conditioned room at 20 °C equipped with individual
241 booths and normalized glasses.
242
243 *Panel training*
244
245 The panel was composed of 30 wine tasters aged from 25 to 65 years. The aim of the first
246 training session was to familiarize the subjects with bitter perception tasted alone or with tartaric
247 acid in order to prepare them to perceive bitterness independently of acidity. Quinine sulfate
248 was presented at 1.5 and 12 mg/L without or with tartaric acid at 3 g/L.
249 During the second training session, the panel was tested for its sensitivity to bitter taste. Two
250 series of increasing quinine sulfate concentrations (1.5, 3, 6, and 12 mg/L) without or with
251 tartaric acid (3 g/L) were presented to the panel. They were asked to sort these modalities into
252 increasing bitterness intensity.
253 Results were interpreted by Page's *L* test described by the International Organization for
254 Standardization [28]. For both series without and with tartaric acid, Page's *L* statistics,
255 respectively 7.40 and 4.06, were considerably above the critical value of 3.09 for an alpha risk
256 of 0.1%, meaning that the tasters sorted the modalities according to the increasing perception
257 of bitterness due to the addition of quinine sulfate. The panel was therefore considered suitably
258 trained for bitterness perception.
259
260 *Determination of (+)-lyoniresinol taste threshold in white wine*
261
262 The taste threshold of (+)-lyoniresinol was evaluated in a white wine (Bordeaux 2013, 12.6%
263 alc. vol.; 5.9 g of glycerol/L; 0.71 g/L of glucose + fructose). Owing to the tiredness of the
264 panelists and the persistence of the bitter taste, two sessions were organized to optimize the (+)-
265 lyoniresinol concentrations for each taster. In the morning session, three concentrations (0.5, 1,
266 and 2 mg/L) were presented in increasing order. Each concentration was displayed according
267 to the triangle test described by the International Organization for Standardization [29].
268 Concentrations presented in the afternoon depended on results from the first session for each
269 taster. They again tasted the lowest concentration at which they had given a correct answer as
270 well as two lower concentrations (0.125 and 0.25 mg/L) following a geometric progression of
271 ratio 2. Tasters who did not give any correct answer during the morning session received two
272 higher concentrations (4 and 8 mg/L) in the afternoon.

Individual thresholds were estimated as the geometrical mean between the lowest concentration of a continuous series of three correct answers and the concentration just below this level. The group threshold was estimated as the geometrical mean between all the individual thresholds.

Statistical analyses

All the statistical analyses were carried out using the R Statistical software (Foundation for Statistical Computing, Vienna, Austria) by a one-way analysis of variance (ANOVA). For each parameter, the homogeneity of the variance was assessed by using the Levene test as well as the distribution of the normality of residues by using the Shapiro-Wilk test.

Results and discussion

Development of an LC-HRMS method to quantitate lyoniresinol enantiomers in oak wood macerates, wines, and spirits

Quantitation of lyoniresinol enantiomers requires the chromatographic separation of (±)-lyoniresinol enantiomers, which is not achievable in a symmetrical environment. The classical chromatographic conditions of the LC-HRMS analysis, i.e., C18 column with non-chiral solvents (water and ACN), do not allow this separation. To achieve this racemic resolution, we used an analytical CSP column. Recently, the efficiency of the Chiralpak® IB-3 column to separate lyoniresinol enantiomers has been demonstrated by Cretin et al. [25]. However, in this study, only pure compounds have been injected on the CSP column. Indeed, the nature of the polymeric stationary phase did not allow the column to be washed with high levels of ACN after each sample or to withstand the resulting wide variations of pressure and mobile phase polarity. Since wine, spirits, and wood macerates are complex matrices containing thousands of molecules, only pre-purified fractions could be injected onto this column in order to avoid its clogging. For this reason, we decided to implement in this work a pre-purification step by SPE prior to CSP analysis. Nevertheless, pre-treatment could have decreased the accuracy and robustness of quantitation. Therefore, C18 LC-HRMS analysis was used first to quantitate (±)-lyoniresinol, according to the quantitation method developed by Marchal et al. in wine [10] and adapted here to spirits and oak wood macerates. Then, CSP LC-HRMS analysis was used to obtain the racemic resolution of (±)-lyoniresinol and calculate the lyoniresinol enantiomeric

ratio after development of a SPE pre-purification. This two-step method aimed at determining for the first time the content of lyoniresinol enantiomers in wines, spirits, and oak wood samples.

Chromatographic and mass spectrometry conditions for quantitation of (±)-lyoniresinol on C18 column

The separation efficiency of U-HPLC and the specificity of FTMS have already been shown to be a powerful technique for (±)-lyoniresinol quantitation in wine [10]. The same chromatographic and spectrometric methods were used for (±)-lyoniresinol quantitation in oak wood extracts and spirits. However, while the dilution factor of the wine samples before injection was about 3, oak wood samples and spirits required greater dilution (by a factor 25 and 8, respectively). Indeed, decreasing the ethanol level avoids deterioration of the chromatographic separation and reduces all the concentrations to within the working range.
MS parameters were adjusted by direct injection of lyoniresinol in order to increase the detection sensitivity. Since no significant adducts were observed, the analysis was carried out in negative mode with no application of dissociation energy in source. The mass accuracy measurement of the Orbitrap analyzer (<3 ppm) confers high selectivity to the detection, thus allowing the analysis to be conducted in full scan mode.

Chromatographic and mass spectrometry conditions for determination of lyoniresinol enantiomeric ratio on CSP column

SPE was performed to pre-purify the samples prior to chiral analysis. Preliminary tests had shown that the PS/DVB stationary phase (Bond Elut ENV) was well suited to this application since it has high lyoniresinol retention capacities. Injections of the SPE fractions onto the C18 column showed that most of the lyoniresinol was eluted in the 40% ACN fraction (from 50 to 80% of recovery), while a lot of polar compounds were eluted in the 20% ACN fraction. As the enantiomeric ratio was the same in all SPE fractions containing lyoniresinol, only the 40% ACN fraction was submitted to CSP chromatography in order to determine the lyoniresinol enantiomeric ratio. Chromatographic conditions were determined to achieve a good separation of the lyoniresinol enantiomers. The isocratic mode was used to obtain an optimized equilibrium between the polysaccharide-derived CSP and the mobile phase in order to obtain efficient and resolved lyoniresinol enantiomers separation (Fig. 2).

Spectrometric parameters such as HESI probe temperature (250 °C), capillary temperature (320 °C), and sheath gas flow were chosen on the basis of the flow rate (150 µL/min) and the composition of the mobile phase (80:20 H_2O/ACN) in order to obtain the optimal ionization of the targeted compound. Since the elution conditions were stable and as enantiomers have the same chemical properties in a symmetrical environment, (+)-lyoniresinol and (−)-lyoniresinol must have a similar ionization yield in the probe and the same behavior in the spectrometer. Consequently, the enantiomeric ratio (*r*) can be calculated directly by integrating the chromatographic peaks corresponding to both enantiomers.

Validation of (±)-lyoniresinol quantitation on C18 column

The method for quantitating (±)-lyoniresinol has already been published in wine [10] but its application to spirits and oak wood macerates needed validation. The construction of an extracted ion chromatogram (XIC) with a 3 ppm accuracy around the theoretical *m/z* of $C_{22}H_{27}O_8^-$ allowed the detection of the (±)-lyoniresinol deprotonated ion. Noise was almost absent and the corresponding peak at 2.16 min (Electronic Supplementary Material, ESM) was automatically integrated. With LC-HRMS, the classical approach of sensitivity determination based on signal-to-noise evaluation is no longer pertinent. The lowest levels of the calibration curve (from 2 to 50 µg/L) were injected into five replicates and both precision (RSD%) and accuracy (recovering with back-calculated concentrations) were determined for each level. The lowest concentration with a precision lower than, e.g., 10 % and accuracy higher than, e.g., 90% was defined as IDL (5 µg/L in this study, both in oak wood extracts and in spirits). De Paepe et al. [27] defined the instrumental quantitation limit (IQL) as two times the IDL. Limits of detection (LOD) and quantitation (LOQ) were further reassessed by considering the wood concentration of macerates (50 g/L of wood chips) and the dilution factor used for the sample preparation for each matrix (dilution factor of 10 and 25 for spirits and oak wood samples, respectively). These data are presented in Table 2.

In this study, samples of three different origins were analyzed because it is essential to obtain a good linearity over a wide range of concentrations. On the basis of previous studies, the working ranges were chosen to range from the IQLs to 5 mg/L. A linear curve was obtained with good correlation coefficients both in oak extracts and in spirits (R^2 of 0.997 and 0.996, respectively). To ensure good accuracy (>90%) of concentrations back-calculated from the calibration curve at all levels and particularly at low levels, a 1/x statistical weight was chosen.

Intraday repeatability (RSD%) was lower than 4% for 5 and 500 µg/L. Oak wood samples and spirits spiked with stock solutions were also injected. Recovery ratios were higher than 94% for additions of 100 µg/L, 500 µg/L, and 1 mg/L suggesting that matrix effects were negligible. Consequently, these results demonstrated the repeatability and trueness of the method.

Analysis of the above samples exhibited very low variations in retention time (<0.02 min) and a mass accuracy lower than 1.5 ppm for all compounds at various concentrations. These results guaranteed the specificity of the method.

All these results demonstrated the ability of LC-HRMS to assay lyoniresinol in oak wood samples and spirits. Moreover, recovery ratios were also measured above 92% in red and white wines.

Validation of method for determining lyoniresinol enantiomeric ratio on CSP column

The reproducibility (RSD%) of the SPE method was calculated to be less than 4% between three distinct cartridges, thereby demonstrating the high reproducibility of this step. Then, pure (±)-lyoniresinol was submitted to SPE. The enantiomeric ratio was measured for the 40% ACN fraction and for (±)-lyoniresinol directly injected onto the CSP column. These two ratios were compared in order to study the stability of the ratio during SPE treatment. A similar comparison was carried out with a sample of wine to evaluate a potential matrix effect. The results showed that the same enantiomeric ratios were observed with and without SPE pre-purification for both matrices (RSD of 1.4% in water and 2% in wine), meaning that SPE treatment did not affect the lyoniresinol enantiomeric ratio. This validated the trueness and reproducibility of the SPE method. Successive injections of the same sample exhibited RSD% lower than 2% for the enantiomeric ratio both in wine and in Cognac. The sample analyses revealed very low variations in retention time for both enantiomers (<0.12 min) and the mass accuracy of lyoniresinol enantiomers was lower than 3 ppm. These results demonstrated the good repeatability and specificity of the CSP LC-HRMS method.

Application of the method to quantitate lyoniresinol enantiomers in wines and spirits

The quantitation of (±)-lyoniresinol followed by the determination of its enantiomeric ratio made it possible to calculate the content of both lyoniresinol enantiomers in wines and spirits.

First, the release of (+)-lyoniresinol was evaluated by analyzing oaked wines. Then, its perception threshold was evaluated in wine in order to establish its sensory impact. The method was also applied to another matrix of oenological interest by analyzing spirits.

Quantitation of (+)-lyoniresinol in wines

To determine the importance of (+)-lyoniresinol in wine, we used our two-step method to analyze 69 wines with vintages from over more than a century (from 1911 to 2013) (ESM). All these wines were commercial and were indicated as being aged in oak wood but the details of the aging conditions were not known for each sample. Average (±)-lyoniresinol concentrations in wine were measured at 3.3 and 1.4 mg/L for red and white oaked wines, respectively. These contents confirm a previous study in which concentrations of total lyoniresinol varied from 1.3 to 2.4 mg/L in various vintages of one commercial white wine [10]. Red wines are commonly aged longer and with a higher percentage of new oak barrels than white wines, which could explain the difference between the mean levels. Moreover, we observed a high variability of lyoniresinol content in red wines (from 0.6 to 9.9 mg/L). Since old vintages of the same estate contained a (±)-lyoniresinol content similar to that of recent vintages (Fig. 3), these variations did not appear to be related to the duration of bottle aging but more likely to winemaking practices.

Mean lyoniresinol enantiomeric ratios were in the same range for red and white wines, respectively, 1.27 and 1.19. Moreover, Fig. 3suggests that bottle aging did not affect the relative proportions of either enantiomer.

Concentrations of lyoniresinol enantiomers in red and whitewinesarepresentedinTable3. The average concentrations were 1.9 and 0.8 mg/L in red and white wines, respectively.

Sensory analysis and determination of (+)-lyoniresinol taste threshold

The gustatory impact of lyoniresinol enantiomers was previously established by Cretin et al. [25]. Experts described for the first time that (+)-lyoniresinol exhibited a strong bitterness while (−)-lyoniresinol exhibited no taste. To determine the sensory impact of (+)-lyoniresinol in wine, its concentrations have to be compared with its perception threshold.

For this purpose, various (+)-lyoniresinol concentrations were prepared in a non-oaked white wine. Given the enantiomeric ratio of lyoniresinol in wine, concentrations of (+)-lyoniresinol were prepared on the basis of half (±)-lyoniresinol taste threshold previously established at 1.5

mg/L [10]. To avoid sensory tiredness and weariness among the panelists, tastings were conducted in two sessions. (+)-Lyoniresinol concentrations were distributed according to a geometric progression with a common ratio of 2, and the samples were assessed using a triangulation test. The group taste threshold was calculated to be 0.46 mg/L with a high range of individual detection thresholds from 43 µg/L to 4mg/L.

Table 3 shows that mean concentrations of (+)-lyoniresinol were 4.1- and 1.7-fold higher than its threshold in red and white wines, respectively. The (+)-lyoniresinol content was found to be higher than 0.46 mg/L in most of the commercial wines used in this study (Table 3 and Table S1), except for three of them in which the concentrations were very similar.

These analytical and sensory results clearly demonstrated that (+)-lyoniresinol has a significant impact on white wine taste by increasing its bitterness. The perception threshold had not been determined in red wine and matrix effects could slightly affect its value. However, the high differences between the levels assayed in red wines and the threshold measured in white wine suggest that (+)-lyoniresinol must also impact the taste of oaked red wines.

Quantitation of (+)-lyoniresinol in spirits

To evaluate the presence of lyoniresinol in spirits, six commercial Cognacs were first analyzed (ESM). Total lyoniresinol content was 8.8 mg/L on average with large variations (from 3.4 to 17.5 mg/L). The mean lyoniresinol enantiomeric ratio was very similar to that measured in wine (1.18) and (+)-lyoniresinol concentrations varied from 2.0 to 10.0 mg/L. The higher levels of lyoniresinol observed in these samples in comparison with values measured in wines could be due to various factors. Indeed, Cognac spirits contain higher percentage of ethanol and are aged during a longer period than wine. Moreover, a part of Cognac, called “angel share” is evaporated during barrel aging, causing a concentration of non-volatiles compounds. But this phenomenon is estimated between 2 and 4% per year, so its influence on lyoniresinol content seems to be limited and not sufficient to explain the values showed in Fig. 4 [30].

Furthermore, as Cognacs contain a higher percentage of ethanol and are aged for a longer period than wine, which could explain the higher levels of lyoniresinol observed in these samples. As a Cognac is a result of a blend of several “eaux-de-vie” from different vintages, the inter-sample variations could partly be related to the mean age of the sample. To examine the influence of aging time on lyoniresinol release, four “eaux-de-vie” that had been aged for different periods (from 4 to 20 years) were taken directly from barrels without any blending (Fig. 4).

An increase in total lyoniresinol was observed over time while the enantiomeric ratio remained stable. Thus, (+)-lyoniresinol varied from 3.2 to 8.2 mg/L and appeared to be continuously released during the oak aging of spirits. Despite the release of this bitter compound, spirits are known to improve during oak aging. Other taste-active molecules such as quercotriterpenosides [31,32] could therefore be released concomitantly and might modulate the effect of (+)-lyoniresinol on taste balance in spirits. The determination of (+)-lyoniresinol perception threshold in spirits would clarify its gustatory effect in this matrix.

Quantitation of (+)-lyoniresinol in oak wood extracts

Winemakers generally aim at producing wines with low levels of bitterness so as not to depreciate the taste balance of wine. This issue is of particular economic interest for the wine industry. Previous studies have demonstrated that the concentration of oak molecules released in wine vary considerably according to cooperage parameters such as differences in the botanical origin of the oak or the degree of toasting [33–35]. Given the sensory impact of (+)-lyoniresinol on bitter taste in wine, we investigated whether these technological features could have a significant influence on its content in oak wood.

Influence of oak wood species on (+)-lyoniresinol content in oak wood

(±)-Lyoniresinol concentration in oak wood was measured at 0.79 and 0.67 mg/g on average for sessile and pedunculate oak samples, respectively. A one-way ANOVA showed that there was no significant effect of oak species on total lyoniresinol content (p value = 0.16).
Mean enantiomeric ratios were estimated at 1.09 for sessile and at 0.99 for pedunculate samples, suggesting that (+)-lyoniresinol and (−)-lyoniresinol were present as a racemic mixture in oak wood, as shown in Fig. 5. Similar results were reported previously for *Q. robur* [24] and for *Q. petraea* [25]. No significant difference in (+)-lyoniresinol content was observed between species by an ANOVA analysis (p value = 0.07). Indeed, lyoniresinol enantiomeric ratios displayed very high inter-individual variations within species, varying from 0.56 to 1.43 and from 0.36 to 1.45 for sessile and pedunculate oak, respectively. As a result, (+)-lyoniresinol was observed from 0.14 to 0.60 mg/g and from 0.12 to 0.52 mg/g in sessile and pedunculate oak, respectively. Such inter-individual variations have been previously observed in both species for other compounds such as β-methyl-γ-octalactone [36], ellagitannins [37], or

triterpenoids [26]. In a next study, investigations could focus on the influence of geographical origin on the lyoniresinol enantiomeric composition of oak wood.

Influence of wood toasting temperature on (+)-lyoniresinol content

The analysis of oak wood chips toasted at different temperatures showed that (±)-lyoniresinol remained stable up to 200 °C, but might be slightly degraded around 250 °C. This result was in agreement with a previous study that described a decrease in (±)-lyoniresinol concentration at 250 °C [35]. However, statistical analysis did not reveal any significant changes in (+)-lyoniresinol content between the control oak wood modality and those toasted from 140 to 250 °C (Fig. 6). This lack of significance could be partly due to the high interreplicate variability at each temperature attributable to the heterogeneity of the wood chips used for each soaking modality.

To offset this heterogeneity, some staves were selected for their similar (±)-lyoniresinol content and were toasted only on their inner side, as is the case in the cooperage industries for barrel making. Samples were collected on both the inner and outer sides of these staves and (+)-lyoniresinol was quantitated. The results showed a very low standard deviation allowing a more advanced investigation of the toasting effect. Statistical analysis did not show any significant difference between the inner and the outer side of the staves either for total lyoniresinol (p value = 0.62) or for (+)-lyoniresinol mean concentrations (p value = 0.83) (0.79 mg/g for inner side and 0.80 mg/g for outer side) as shown in Fig. 7.

Thus, the cooperage parameters used here did not have any significant impact on (+)-lyoniresinol concentration.

Conclusion

Lyoniresinol is a bitter lignan released from oak wood during wine aging. While previous works have shown that it is racemic in oak wood, only its dextrorotatory enantiomer exhibits bitterness but its concentrations in wines and spirits remained unknown. To assay this taste-active enantiomer, a two-step quantitation method using LC-HRMS was designed. First, the total lyoniresinol content was measured on a classical C18 UHPLC-HRMS set-up. Then, the precise enantiomeric ratio of lyoniresinol was determined by means of sample purification on SPE followed by CSP LC-HRMS analysis. The key parameters were investigated and, after

538 validation of the method, (+)-lyoniresinol was quantitated with precision in three distinct
539 matrices: wines, spirits, and oak wood. An overview of (+)-lyoniresinol in wine was obtained
540 for the first time by the analyzing 69 commercial red and white wines from various vintages
541 from over more than one century and from distinct areas. Almost all the values were above the
542 perception threshold of (+)-lyoniresinol, which was determined to be 0.46 mg/L. Consequently,
543 this work demonstrates that (+)-lyoniresinol has a significant impact on wine taste balance by
544 increasing its bitterness. Furthermore, high concentrations of (+)-lyoniresinol have been
545 observed in commercial Cognac (up to 10 mg/L), and the analysis of oaked "eaux-de-vie" from
546 different aging periods suggests that it is released continuously during barrel aging. Finally, the
547 study of cooperage parameters showed that (+)-lyoniresinol concentrations are not influenced
548 by the botanical origin of oak or by toasting. (+)-lyoniresinol concentrations cannot therefore
549 be adjusted by modulating these technical parameters.
550 The methodological development presented here offers new insights into the finer
551 understanding of the molecular origin of wine taste by unraveling the differential contribution
552 of lyoniresinol enantiomers. A similar analytical strategy could be applied to other wine and
553 food active compounds with stereochemical diversity.

Acknowledgments

Seguin-Moreau, Remy-Martin, France Agrimer, and CIVB are kindly thanked for financial support. BC's grant was provided by Fondation Jean Poupelain. The authors acknowledge Ray Cooke for copyediting the manuscript. The authors are also very grateful to Dr. A. Prida and Dr. J.-C. Mathurin for providing samples.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no competing interest.

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Tables

Table 1 Ionization and spectrometric conditions for HRMS analyses

Column	Hypersil Gold	Chiralpak® IB-3
Chromatographic flow rate	600 µL/min	150 µL/min
Sheath gas flow[a]	75	50
Auxiliary gas flow[a]	18	9
HESI probe temperature	320 °C	250 °C
Capillary temperature	350 °C	320 °C
Electrospray voltage	−3 kV	
Capillary voltage	−60 V	
Tube lens voltage offset	−135 V	
Skimmer voltage	−26 V	
Mass range (in Th)	200–800	
Resolution [b]	25,000	100,000
AGC value	5.10^5	10^6

[a] Sheath gas and auxiliary gas flows (both nitrogen) are expressed in arbitrary units

[b] Resolution $m/\Delta m$, fwhmat m/z 200 Th

Table 2 Validation parameters for HRMS quantitation of (±)-lyoniresinol in oak wood and spirits

Matrices	Sensitivity[a]				Linearity		Specificity		Repeatability and trueness				
	IDL (µg/L)	IQL (µg/L)	LOD	LOQ	Working range	R^2	t_R variation	Mass accuracy[b]	Intraday repeatability[c]		Recovery		
									50 µg/L	500 µg/L	100 µg/L	500 µg/L	1 mg/L
Oak wood	5	10	2.5 µg/g	5 µg/g	10 µg/L–5 mg/L	0.997	0.02 min	1.5 ppm	2.6 %	3.6 %	97 %	98 %	96 %
Spirits	5	10	40 µg/L	80 µg/L	10 µg/L–5 mg/L	0.996	0.01 min	1.5 ppm	4 %	1.5 %	94 %	97 %	95 %

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

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

[c] Injections of five replicates at two concentrations (50 and 500 µg/L)

Table 3 Presence of (+)-lyoniresinol and (−)-lyoniresinol in 59 red and 10 white wines

		Mean (mg/L)	First quartile (mg/L)	Median (mg/L)	Third quartile (mg/L)
Red wines	(+)-Lyoniresinol	1.9	1.0	1.6	2.5
	(−)-Lyoniresinol	1.5	0.7	1.1	2.1
White wines	(+)-Lyoniresinol	0.8	0.7	0.8	0.8
	(−)-Lyoniresinol	0.7	0.6	0.6	0.7

Figures

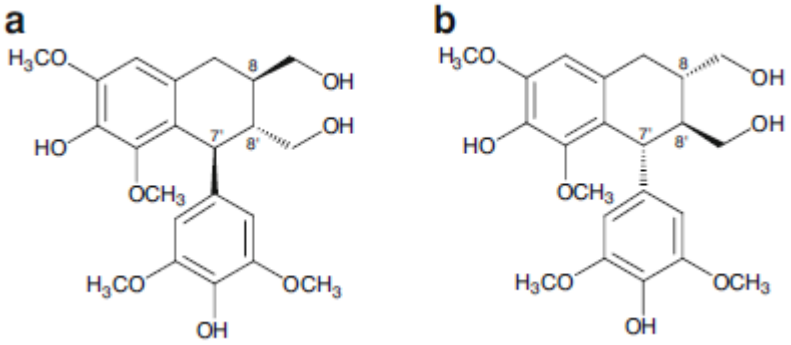

Fig. 1 Chemical structures of (8*R*, 8′*R*, 7′*S*)-(+)-lyoniresinol (a) and (8*S*, 8′*S*, 7′*R*)-(−)-lyoniresinol (b) (absolute configuration)

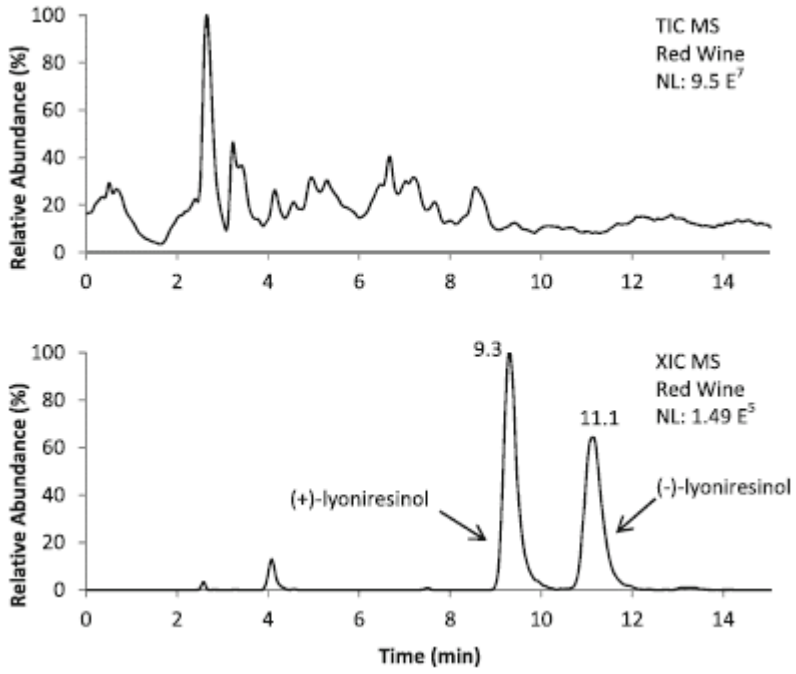

Fig. 2 Negative LC-HRMS XIC of an oak wood macerate corresponding to $C_{22}H_{27}O_8^-$ ion (*m/z* 419.1712 with a 3 ppm accuracy)

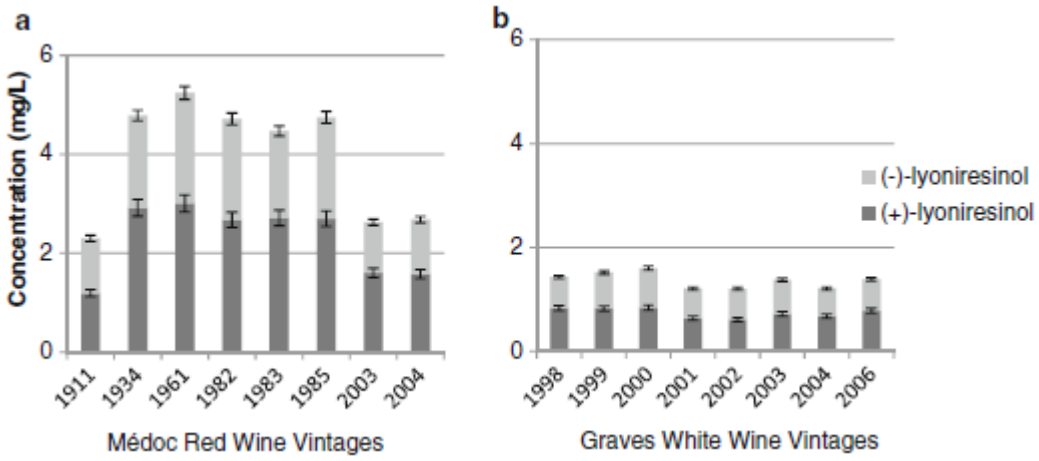

Fig. 3 Variations in (+)-lyoniresinol and (−)-lyoniresinol content in different vintages of a Médoc red estate from 1911 to 2004 (a) and different vintages of a Graves white estate from 1998 to 2006 (b). Error bars indicate 95% confidence intervals

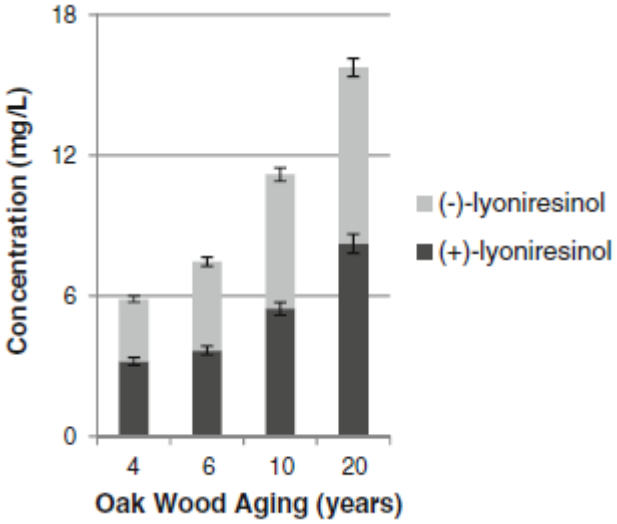

Fig. 4 Concentrations of lyoniresinol enantiomers in "eaux-de-vie" aged for 4, 6, 10, and 20 years. Error bars indicate 95% confidence intervals

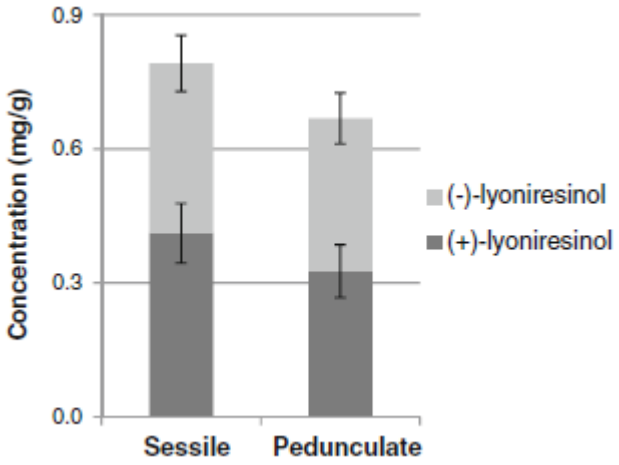

Fig. 5 (+)-Lyoniresinol and (−)-lyoniresinol concentrations in sessile and pedunculate oak wood samples. Error bars indicate 95% confidence intervals

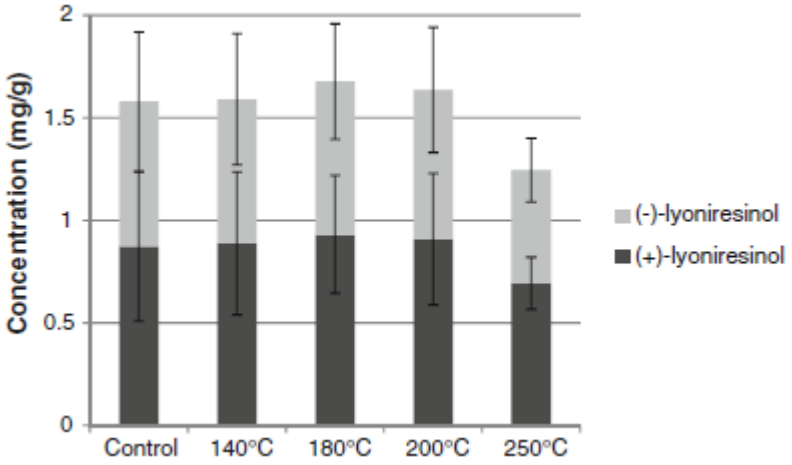

Fig. 6 Evolution of (+)-lyoniresinol and (−)-lyoniresinol concentrations at various oak wood toasting temperatures (from 140 to 250 °C). Error bars indicate 95% confidence intervals

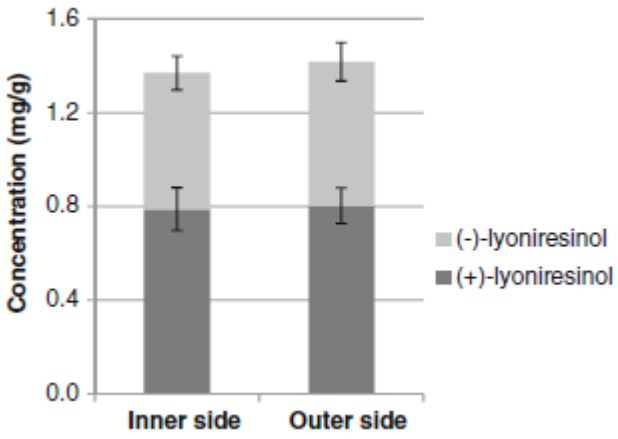

Fig. 7 (+)-Lyoniresinol and (−)-lyoniresinol concentrations on the inner and outer sides of oak wood staves. Error bars indicate 95% confidence intervals