Contribution of Grape and Oak Wood Barrels to Pyrrole Contents in Chardonnay Wines: Influence of Several Cooperage Parameters

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ABSTRACT

The influence of some enological parameters on pyrrole concentrations in Chardonnay wines was studied. First, a quantitative method to assay five pyrroles was optimized and applied to determine their content in wines produced in different containers. All pyrroles were observed in wines aged in a stainless-steel tank, which indicated that they have a varietal or fermentative origin. However, their concentrations were significantly higher in wines made in new barrels than in older barrels or in a stainless-steel tank, so oak wood may largely contribute during the winemaking process. A quantitative method to assay pyrroles in oak wood extract was also developed to study the influence of several cooperage parameters such as different types of traditional toasting, as well as the temperature and the time of toasting. Significant differences were observed on pyrrole concentrations in oak wood extracts according to these different cooperage parameters. These findings bring new perspectives to the monitoring of winemaking and the aging of Chardonnay wines.

INTRODUCTION

The sensory image of a wine is the result of complex interactions produced by many volatile compounds present in the headspace of a glass of wine. This sensory image, which is conceived in the mind of tasters, contributes to the recognition of a grape variety, a type of wine, or a winemaking process.[1] The characterization of the key aroma compounds is therefore a subject of great importance in enology, in connection with the expectations of producers to optimize and preserve the organoleptic quality of wines. For instance, identification of polyfunctional thiols, responsible for grapefruit or boxwood aromas in Sauvignon blanc wines, has deeply influenced the elaboration process of wines from this variety.[2]

Chardonnay is the second-most planted white grape variety in the world.[3] Its international reputation has given rise to numerous studies to determine its sensory and chemical characteristics. Ballester et al. demonstrated the existence of a Chardonnay wine concept, i.e., experts distinctly recognize these wines among wines made from other grape varieties.[4] Chardonnay wines are characterized by a specific aroma, commonly described as buttery, yellow stone fruit, bready, and woody notes.[5] From a chemical point of view, more than 240 aromatic compounds have been identified to date in these wines. In particular, the contribution of diketones,[6] acetates and ethyl esters,[4,7,8] higher alcohols,[7] volatile phenols,[9,10] and lactones[7,9] has been reported. However, none of them appear specific to the grape variety.[11,12]

The finest Chardonnay wines present a complex bouquet described by experts as having hazelnut, gunflint, white flowers, and verbena nuances.[13] Several varietal compounds have been identified, such as monoterpenes[14,15] and polyfunctional thiols.[16,17] These compounds are commonly associated with the specific notes of Muscat[18] and Gewürztraminer[19] and with the catty and grapefruit-like notes of Sauvignon Blanc wines,[2,20] respectively. The levels measured

in Chardonnay wines suggest their sensory contribution, but they are similar or lower than the values observed in other grape varieties. Therefore, these compounds alone cannot explain the aromatic typicality of these wines.

Benzenemethanethiol, 2-furanmethanthiol, and 2-methyl-3furanthiol are powerful thiols associated with gunflint and smoky notes that can contribute to empyreumatic nuances of wines.[21,22] They have been found in Chardonnay wines at concentrations higher than their detection threshold, which suggests their sensory contribution.[17,23] More recently, two thiol derivatives of pyrroles developing hazelnut aromas were identified in typical Chardonnay wines. Quantitative and sensory analyses revealed that they were present at higher levels in Chardonnay wines, at concentrations above or close to their detection threshold, making these molecules the first key aromatic compounds of Chardonnay pertaining to the hazelnut-like aroma.[13,23] The origin and the formation mechanisms of these molecules have not been elucidated until now. In the same study, five pyrroles reminiscent of hazelnut have also been evidenced. Despite their lack of sensory relevance, they were quantitated at significantly higher concentrations in Chardonnay wines and might appear as chemical markers of the variety or the elaboration process.

Thus, the present work investigated how enological parameters can modulate the concentrations of pyrroles in wine and oak wood. In particular, the origin of five pyrroles was sought: 1-methylpyrrole-2-carboxaldehyde (MPC), 1ethylpyrrole-2-carboxaldehyde (EPC), 2-acetyl-1*H*-pyrrole (AP), 1*H*-pyrrole-2-carboxaldehyde (PC), and 1*H*-pyrrole (P) (Figure 1). Wines were produced in different containers, and pyrrole contents were determined by gas chromatography−mass spectrometry (GC−MS) to determine the relative contribution of grapes and oak wood. The influence of several cooperage parameters such as different types of traditional toasting, as well as the temperature and the time of toasting, was then investigated.

MATERIALS AND METHODS

Chemicals. Dichloromethane (99.9%) and sodium chloride (99.9%) were obtained from Fisher Scientific (Illkirch, France). Pentane (99.8%), absolute ethanol (>99.9%), and methanol (>99.9%) were from Merck (Semoy, France). Ultrapure water (Milli-Q; resistivity, 18.2 MΩ cm; Millipore, Saint-Quentin-en-Yvelines, France) was used. Octan-3-ol (99.9%), 1-methylpyrrole-2-carboxaldehyde (98%), 2-acetyl-1*H*-pyrrole (99%), 1*H*-pyrrole (≥98%), and anhydrous sodium sulfate (99.9%) were obtained from Sigma-Aldrich (Steinheim, Germany). 1*H*-pyrrole-2-carboxaldehyde (99%) was from Acros Organics (Geel, Belgium), and 1-ethylpyrrole-2-carboxaldehyde (97%) was from Fluorochem (Derbyshire, United Kingdom).

Wine Samples and Plant Material. *Wine samples.* This study was carried out with five Chardonnay wines from different wineries in Burgundy and Champagne (France) over four vintages (from 2016 to 2019). For each experiment, a Chardonnay must was sown in duplicate in different containers, a stainless-steel tank (non-oaked control) and barrels of different ages, and vinified in a traditional way to create the modalities “stainless-steel tank”, “new barrels”, and “used barrels” presented in Table 1. Within a series, the barrels were similar concerning the oak wood used and the toasting process. The first experimentation (Beaune 1er cru Clos des Mouches) was performed in 2016 in new oak barrels and barrels of 2, 3, and 4 or more years. A second one was carried out in 2017 (Chassagne Montrachet 1er cru Chenevottes) to compare wines vinified in stainless-steel tanks, new oak barrels, and used barrels (from 1 to 3 years old). Three other experiments (one in Chablis and two in Champagne) were carried out from 2017 to 2019 in stainless-steel tanks, new barrels, and very old barrels (4 or more years old). Pyrrole contents were determined in wines shortly after malolactic fermentation. The method was optimized and validated using a Chardonnay wine (Pays d’Oc, France).

Oak wood samples. Oak wood material originated from France (*Quercus petraea*) and the USA (*Quercus alba*) and was provided by Seguin Moreau cooperage (Table 1). For French oak, the botanical species was confirmed by analyzing the triterpenic composition according to the methodology previously published.[24] The influence of toasting on pyrrole concentrations in oak wood was studied. Staves were submitted to different toasting procedures in real barrel-making conditions (open fire toasting). Three types of oak toasting were tested: "light", "medium", and "heavy". During the toasting process, the internal surface of oak staves was exposed to open fire with a gradual increase in surface temperature from ambient temperature up to 170 °C (light toasting), 180 °C (medium toasting), and 200 °C (high toasting) at the end of toasting, with all temperatures measured by a noncontact infrared thermometer. The total duration of toasting was 25 min for each protocol. Whereas only one side of the staves was heated, both sides were shaved over 3 mm in depth to obtain toasted and untoasted samples. All procedures were performed in replicate with French and American oak wood (Table 1).

To study the effect of heat treatment, three randomly selected French oak wood staves were cut in uniform geometric pieces (L =70 mm, l = 50 mm, and w = 18 mm). A calcination oven initially stabilized overnight at the required temperatures was used to apply heat treatments. Heat treatments were done in triplicate, i.e., for a given condition (one temperature and one duration), three pieces (one temperature and one duration), three pieces (one piece per original stave) were simultaneously heated. Treatment conditions were defined according to temperatures observed in industry. Four temperatures were tested: 160, 180, 200, and 250 °C; also, three durations were tested: 10, 30, and 180 min. The modality at 250 °C was heated for only 10 min. Untoasted wood pieces (n =3) were used as a control. All wood samples were ground down to obtain wood powder (<0.5 mm). The temperature during grinding was checked and reported as 30−35 °C. This fact excludes the hypothesis of thermally driven artifact

112 formation of wood compounds, since the minimal temperature required for their formation is
113 120 °C.
114 These powders were soaked in hydro-alcoholic solution (12% ethanol, 5 g/L tartaric acid, and
115 pH adjusted to 3.2 with sodium hydroxide) at a concentration of 50 g/L for 24 h at 25 °C in
116 darkness to simulate wine extraction.[24,25] Samples were filtered on 0.45 µm nitrocellulose filters
117 before analyses. The method was optimized and validated on sessile untoasted stave powder
118 extract.
119 **Sample Preparation for Wine and Oak Wood Extract.** A previous study already
120 demonstrated that solid-phase extraction (SPE) is the best extraction method to quantitate five
121 pyrroles in wines.[13] Nevertheless, parameters such as the SPE cartridge and the elution solvent
122 were tested to optimize the method proposed by Gros et al. (Table 2).
123 Concerning the quantitation of pyrroles in oak wood extract, SPE was compared to
124 liquid−liquid extraction (LLE) and solid-phase micro-extraction (SPME). Optimization was
125 carried out on white wine or oak wood extract spiked at 20 µg/L with a standard solution of five
126 pyrroles prepared at 20 mg/L in ethanol to get significant signals. Octan-3-ol at 17.8 µg/L was
127 used as an internal standard. Three replicated samples were prepared and analyzed for each
128 method.
129 *Solid phase extraction (SPE).* The SPE optimization procedure was carried out
130 similarly for white wine (50 mL) and oak wood extract (25 mL). It was automatized with a
131 Gilson GX-274 ASPEC solid-phase extraction system (Villiers-Le-Bel, France). Four
132 cartridges were tested: HR-X CHROMABOND (500 mg), HLB OASIS (500 mg), LiChrolut
133 EN (500 mg), and LC-18 Supelco (500 mg). The cartridge was first activated with methanol (7
134 mL, 2 mL/min), washed with ultrapure water/ethanol (90/10, v/v; 3 mL, 5 mL/min), and dried
135 by 10 mL air push (6 mL/min). The sample was then loaded onto the SPE cartridge at 3 mL/min.
136 The cartridge was rinsed with water (2 mL, 5 mL/min) and dried by air push (10 mL, 6 mL/

137 min), and then the analytes were recovered by passing 6 mL of solvent (2 mL/min). To optimize
138 the extraction method, several solvents were tested: dichloromethane, pentane/dichloromethane
139 (90/10, 50/50, and 10/90, v/v), and dichloromethane/methanol (95/5, v/v). The organic phase
140 was dried with anhydrous sodium sulfate and concentrated under a gentle stream of nitrogen to
141 reach a final volume around 200 µL.

142 *Liquid-liquid extraction (LLE).* For LLE, 25 mL of oak wood extract was successively
143 extracted with 4, 2, and 2 mL of dichloromethane. The organic phases were collected, dried
144 with anhydrous sodium sulfate, and concentrated under nitrogen flow to obtain 200 µL of
145 extract.

146 *Solid phase micro extraction (SPME).* For SPME, 10 mL of oak wood extract was
147 introduced into a 20 mL standard headspace vial containing 3 g of sodium chloride and sealed
148 with a PTFE-lined cap. The solution was homogenized with a vortex shaker and then analyzed
149 with a Combi PAL sampler (CTC Analytics, Zwingen, Switzerland). The program consisted of
150 swirling the vial at 500 rpm for 5 min at 40 °C, then inserting the fiber into the headspace for
151 30 min at 40 °C as the solution was swirled again, and then transferring the fiber to the injector
152 for desorption at 240 °C for 10 min. Three different fibers were tested: 100 µm
153 polydimethylsiloxane (PDMS), 65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB),
154 and 50/ 30 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/ PDMS) (Supelco,
155 Bellefonte, PA, USA). They were conditioned before use as recommended by the manufacturer.

156 **Validation method.** For both matrices, the linearity was evaluated by the injection of
157 eight calibration levels (Table 2). A correction was applied when needed by subtracting the
158 peak area ratios of the nonspiked sample from the spiked ones. The calibration curves were
159 plotted as the relative peak areas (analyte versus internal standard) as a function of the relative
160 compound concentration (analyte *versus* internal standard). The limit of detection (LOD)
161 (concentration for signal/noise, 5) and the limit of quantitation (LOQ) (concentration for

signal/noise, 10) were calculated using the S/N function of ChemStation software. To evaluate repeatability (intraday precision), five replicates of the same wine and oak wood extract were spiked at two different concentration levels (high and low) with reference standards (Table 3). The reproducibility of the method (interday precision) was determined by analyzing five replicates of the same wine and oak wood extract spiked at two different concentration levels (high and low) over a period of 2 weeks. Finally, accuracy was evaluated by calculating the recoveries with a standard addition technique. Three different samples of wine and oak wood extract were spiked at two different concentrations (Table 3).

Constitution of Grape Juice Extracts for Quantitative Assays. Extraction was performed by SPE according to the method of Gros et al.[13] with the Gilson GX-274 ASPEC solid-phase extraction system (Villiers-Le-Bel, France). An HR-X CHROMABOND (500 mg) was first conditioned (7 mL of MeOH (2 mL/min) and then 3 mL of water (5 mL/min) and dried by 10 mL air push (6 mL/min)). Then, 50 mL of diluted must (50/50, v/v) was spiked with 50 µL of octan-3-ol (17.8 mg/L in EtOH) and poured through the cartridge. The solid phase was rinsed with ultrapure water and dried in air, and elution was performed with 3 mL of pentane/dichloromethane (10/ 90, v/v) and 3 mL of dichloromethane/methanol (95/5, v/v). The eluate was dried with anhydrous sodium sulfate and concentrated under nitrogen flow to obtain 200 µL of extract prior to analysis.

GC−MS analyses. Pyrroles were quantitated using an Agilent 6890N gas chromatograph coupled to a mass spectrometer (MSD 5973, Agilent Technologies Inc., Santa Clara, CA). Two µL samples of organic extract were injected in splitless mode (injector temperature, 240 °C; splitless time, 0.75 min) on a BP20 capillary column (50 m × 0.22 mm, 0.25 mm film thickness, SGE, Courtaboeuf, France). The carrier gas was helium N60 (Air Liquide) with a flow rate of 1 mL/min. The oven was programmed at 45 °C for the first minute, heated to 185 °C at 3°C/min, then raised to 240 °C at 10 °C/min, and held at this temperature

for 20 min. The transfer line between GC and MS was set at 250 °C and the ion source at 230 °C. The mass spectrometer was operated in electron ionization mode at 70 eV in selected-ion-monitoring (SIM) mode. Monitored ions are listed in Table 2. Quantitation was performed with calibration curves built using white wine or oak wood extract.

Statistical analyses. Statistical analysis was performed using the Kruskal−Wallis test followed by the post hoc Conover−Iman test and XL-STAT version 2019.1.1.56334 (Addinsoft, Paris, France).

RESULTS AND DISCUSSION

Optimization and validation of pyrrole quantitation method in wine. *Selection of SPE cartridge and elution solvent.* The method described by Gros et al.[13] allowed the determination of five pyrroles by manual SPE on the Lichrolut-EN cartridge and elution of the compounds with 5 mL of dichloromethane. First, the method was optimized by using an automatized Gilson GX-274 ASPEC solid-phase extraction system to gain time and repeatability. Three cartridges were tested (HR-X, HLB, and LC-18) and compared to the LiChrolut EN cartridge (Figure 2A). The analytes were recovered by eluting with 6 mL of dichloromethane. Three replicated measurements of a wine spiked with 20 µg/L of five pyrroles were performed for each cartridge. The LC-18 Supelco cartridge was excluded as its extraction efficiency was lower than that of the other three cartridges. Extraction of AP and PC with the HLB cartridge was not optimal, nor was that of 1*H*-pyrrole with the LiChrolut EN cartridge. The HR-X cartridge presented the best extraction efficiency for all compounds and was therefore chosen to optimize and validate the method. The second criterion evaluated was the solvent used to elute the compounds (Figure 2B). Dichloromethane is a good but not selective solvent for elution. Several solvents and concentrations were tested. Elution with 6 mL of

pentane/dichloromethane (50/50 or 90/10, v/v) or with 6 mL of dichloromethane/methanol (95/5, v/v) was not optimal, so elution with 3 mL of pentane/dichloromethane (50/50, v/v) followed by 3 mL of dichloromethane/methanol (95/5, v/v) was chosen.

Validation of analytical method. Table 3 shows the regression parameters and the critical and detection limits obtained for the five pyrroles in white wine. The linearity was evaluated for a representative range of average pyrrole concentrations usually found in wines (0.02−60 μg/L for P, 0.02−40 μg/L for MPC, 0.02−40 μg/L for EPC, 0.01−25 μg/ L for AP, and 0.07−170 μg/L for PC).[13] The functions are linear (mean correlation coefficients, ≥0.992) over the concentration range generally found in wines. The LOQ values obtained for the five pyrroles were compatible with the analysis of these molecules in wine samples and this method appeared to be much more sensitive than the one previously developed, except for 1*H*-pyrrole,[13] making this new protocol perfectly suitable for the analysis of these compounds in wine (Table 2 and 3). Repeatability and reproducibility were determined using a white wine spiked with two different concentrations (Table 3). The relative standard deviations of the area ratios were lower than 10% for all compounds. Finally, the accuracy was tested by applying recovery calculations from three different white wines spiked at two different concentrations. For both levels, the recovery of each compound ranged from 94 to 108%.

Development and validation of a SPE-GC-MS method to quantitate pyrroles in oak wood extracts. A quantitative method to assay the five pyrroles in oak wood extract was also developed. Since the matrix effect could affect the optimal extraction conditions, it was necessary to compare various SPE cartridges to find a compromise that would best fit the entire set of pyrroles. To reduce sample handling and solvent consumption, SPME could also be an interesting alternative. Consequently, the results obtained from SPE, SPME, and traditional LLE were compared to choose the best preparation protocol.

Extraction mode. To select the best extraction mode, four different SPE cartridges and three different SPME fibers were tested and compared with dichloromethane LLE. For SPE, the analytes were recovered by passing 6 mL of dichloromethane. Three replicated measurements of an oak wood extract spiked with 20 µg/L (corresponding to 400 ng/g of wood) of five pyrroles were performed for each extraction mode. Figure 3A shows that SPE was the best technique for the extraction of pyrroles, with an extraction efficiency 5-fold better than the LLE. In addition, contrary to SPME for which PC was never observed regardless of the fiber used, SPE allowed the extraction of all compounds. Similar results were obtained for the extraction of pyrroles with the LiChrolut EN, HLB, and HR-X cartridges. Since HR-X was already used to quantitate pyrroles in wines, it was also used to analyze these compounds in oak wood extract. Then, several solvent compositions were tested to optimize the elution of pyrroles (Figure 3B). The use of 3 mL of pentane/dichloromethane (10/90, v/v) followed by 3 mL of dichloromethane/methanol (95/5, v/v) appeared to be the best modality to elute these compounds with the best recovery ratios.

Validation of analytical method. Oak wood extract was spiked with the studied compounds at various concentrations to obtain eight calibration levels (P: 2.4−1175 ng/g of wood; MPC: 1.6−783 ng/g of wood; EPC: 1.6−816 ng/g of wood; AP: 1−490 ng/g of wood; PC: 6.9−3432 ng/g of wood). From the data obtained, the developed method showed linear functions throughout the concentration range, with correlation coefficients ranging from 0.990 to 0.998 (Table 3). The LOD and LOQ for pyrroles varied according to their chemical structures, with LOQ values ranging from 0.3 to 29.4 ng/g of wood. The same oak wood extract (n = 5) spiked at two different pyrrole concentrations was analyzed to determine the repeatability of the method. The relative standard deviation of the area ratios was determined between 4 and 10% for the low spiking level and between 2 and 8% for the high spiking level (n = 5). These same extract was spiked and analyzed over 2 weeks to evaluate the

reproducibility. The values obtained varied from 6 to 11% and from 6 to 9% for the low and high levels tested, respectively. Finally, the accuracy was tested by applying recovery calculations from three different oak wood extracts spiked at two different concentrations. For both levels, the recovery of each compound ranged from 85 to 105%. Consequently, these results validated the method and demonstrated its relevance to quantitate the five pyrroles studied in oak wood extracts.

Pyrroles content in white wines elaborated in different containers. Higher levels of pyrroles were observed by Gros et al.[13] in Chardonnay wines than in non-Chardonnay wines. Most of them were made traditionally in barrels, but a few Chardonnay wines were exclusively vinified in a stainless-steel tank without any wood contact. Conversely, small quantities of pyrroles were found in white wines of other grape varieties already made entirely in barrels.

The origin of pyrroles in wine consequently remained unclear. To explore this issue, five experimentations (three in the Burgundy region and two in Champagne) were performed (Table 1). The objective was to determine the relative contribution of wood to that of grapes. The completed method described above was applied to determine pyrrole content in Chardonnay wines differing in the containers in which they were made. Pyrrole concentrations in wines of each experimentation are presented in Table 4. Mean concentrations of each modality for all experiments were also calculated for the sake of simplicity. Significant differences in the content of four pyrroles were observed in the wines obtained in the five experiments. Overall, MPC was quantitated at 650 ± 872 ng/L, with a minimum value of 20 ng/L and a maximum value of 2915 ng/L. The highest concentrations were found in the "new barrel" modalities for all experiments with an average of 1931 ± 630 ng/L. Intermediate quantities were found in the "1 year-old barrel" modality (335 ± 110 ng/L), whereas the lowest quantities were observed in the "2, 3, or ≥4 year-old barrel" and "stainless-steel tank"

286 modalities. These data are in agreement with the contents observed by Gros et al. in Chardonnay
287 wines.[13] The differences in concentrations observed between wines made in different containers
288 were found to be significant in the nonparametric Kruskal−Wallis test ($p < 0.05$). Similarly, the
289 homologue EPC was more abundant in wines made in new barrels (104 ± 50 ng/L) than in
290 wines made in 1 to ≥4 year old barrels (from 12 to 30 ng/L) or in a stainless-steel tank, where
291 it was detected at the trace level (10 ng/L). The Kruskal−Wallis test showed that these
292 differences were significant in all experiments ($p < 0.05$). AP was quantitated in wines at 358
293 ± 280 ng/L (minimum at 50 ng/L and maximum at 1082 ng/L). Again, significant differences
294 were observed between the "new barrel" modalities and the others for all experiments, with an
295 average content of 718 ± 204 ng/L in the "new barrel" modalities, 280 ± 30 ng/L in 1 year-old
296 barrel modalities, and less than 240 ng/L in the others. PC showed the highest levels of all five
297 pyrroles, with an average concentration of 7.8 ± 12.5 µg/L in wines, a minimum content of 0.26
298 µg/L, and a maximum of 49.2 µg/L, in accordance with previous studies.[12,13] Significantly
299 higher contents ($p < 0.05$) were observed in all wines made in new barrels (24.08 ± 14.06 µg/L)
300 than in wines made in 1 year-old barrels (3.55 ± 0.66 µg/L), 2 year-old barrels (2.21 ± 0.77
301 µg/L), or the oldest or stainless-steel containers (from 1.78 to 0.38 µg/L). Finally, 1*H*-pyrrole
302 was found at 207 ± 136 ng/L in wines and no significant differences could be observed between
303 modalities.

304 For a given container, pyrrole contents could vary from an experimentation to another,
305 probably due to a terroir or vintage effect and to the heterogeneity of the barrels. However,
306 despite such differences between the samples, the same trends could be observed,
307 demonstrating the relevance and robustness of this study. Briefly, the concentrations of MPC,
308 EPC, AP, and PC were higher in wines made in new barrels than in older barrels or in a
309 stainless-steel tank. Therefore, these pyrroles mainly originate from oak wood during the
310 winemaking process. Considering this result, it seems still surprising that low concentrations of

311 pyrroles were found in wines aged in oak barrels but made from other varieties.[13] Moreover,
312 pyrroles were also observed in the "stainless-steel tank" modalities and in a non-oaked control,
313 so these compounds also have, in a lesser extent, a varietal or fermentative origin. Only 1*H*-
314 pyrrole concentrations did not seem to be influenced by the type of container, suggesting that
315 it does not originate from oak wood. The same analysis applied to Chardonnay grape juices
316 prior to any contact with oak wood allowed the detection of all five pyrroles, supporting the
317 hypothesis of a varietal origin (Supporting Information). These observations are in accordance
318 with previous studies highlighting the presence of MPC and PC in Chardonnay and Semillon
319 musts.[13,26] Interestingly, pyrrole concentrations found in musts were below their contents
320 observed in wines, even in modalities made in stainless steel tanks (Supporting Information).
321 This suggested that pyrroles could be revealed or synthesized by fermentative micro-organisms
322 during the winemaking process. PC has already been observed in a hydrolyzed fraction of grape
323 juice and at a higher concentration than in the free volatile fraction.[26–28] These results suggest
324 that PC could be present in musts as a nonvolatile precursor, probably in a glycosidic form, just
325 like terpenoids or norisoprenoids.[29,30] It is well known that glycosidically linked compounds
326 can be released during winemaking due to the mild acid conditions of grape juice and wines[31]
327 or through the action of endogenous or exogenous enzymes with β-glucosidase activity.[29,32,33]
328 Thus, further studies are required to clearly establish the relative contribution of fermentative
329 micro-organisms on pyrrole levels in wine.

330 **Influence of several cooperage parameters on pyrrole concentrations in**
331 **oak wood extract.** *Effect of toasting process.* Oak wood is commonly used during
332 winemaking and aging in most of the world's wine-producing regions because it contributes to
333 the complexity of wine by releasing wood compounds into the wine. Some of these molecules
334 are originally present in significant amounts in fresh wood, but most are revealed during barrel
335 manufacture, especially during toasting.[34,35] During toasting, several hydrothermolysis and

pyrolysis reactions take place, resulting in the degradation of biopolymers such as lignin, polysaccharides, polyphenols, and lipids. The Maillard reaction leads to the formation of several aromatic compounds, including pyrroles, which can be transferred to the wine during the aging process.[36] Previous studies have demonstrated the presence of AP and PC in untoasted and toasted wood,[37–39] but little is known about the other pyrroles. To investigate the influence of toasting on pyrrole contents, the method described above was applied to determine their concentration in different series of wood extracts.

First, five or six French oak staves were heated according to three traditional processes (Table 1): "light toasting" at a maximum temperature of 170 °C, "medium toasting" at a maximum of 180 °C, and "heavy toasting" at a maximum of 200 °C. One of the three toasting levels, "medium toasting", was also applied to four American (*Q. alba*) oak staves. The inner, i.e., toasted, and outer, i.e., untoasted, faces were analyzed, which allowed the effect of toasting to be studied without interference due to intra-individual variability (Table 5). PC was the most abundant pyrrole observed in oak wood extracts. From a quantitative point of view, the contents measured were in accordance with those found in the literature.[38,40] In agreement with previous studies, significantly higher concentrations (Kruskal−Wallis test, $p < 0.001$) were found in toasted face extracts, regardless of the toasting process.[40,41] However, 1*H*-pyrrole was not detected in untoasted or toasted wood extracts. MPC and EPC were found at concentrations lower than their LOQ in untoasted wood extracts, while AP and PC were observed at trace levels (around 2.7 ng/g of wood for AP and 29 ng/g of wood for PC). Their average contents were significantly higher in toasted wood extracts (Kruskal−Wallis test, $p < 0.001$), but no differences between these two compounds were observed regarding the toasting process. On the contrary, EPC and MPC concentrations exhibited significant differences according to the toasting process. Indeed, lower contents of EPC were found in oak wood extract treated with "light toasting" (13.9 ± 2.9 ng/g of wood) than in "medium toasting" or "heavy toasting"

361 modalities (between 21 and 31 ng/g of wood; $p < 0.01$). On the other hand, significant higher
362 contents were observed for MPC in the "heavy toasting" modality (300.3 ± 48.8 ng/g of wood)
363 than in "light toasting" or "medium toasting" samples ($p < 0.05$). Finally, no significant
364 difference in pyrrole contents was found regarding oak species. Similar results were found by
365 Natali et al.[38] and Fernández de Simón et al.[37,42] concerning PC, whereas Cadahía et al.[40] found
366 that the PC content was higher in American wood than in French or Spanish wood. These
367 differences could be due to different methods of wood seasoning and toasting. As has already
368 been pointed out, each cooperage has its own methods and wood composition can vary greatly,
369 despite toasting temperatures that are theoretically the same.[43,44]

370 *Influence of time and temperature.* The formation of flavor compounds by the
371 Maillard reaction depends on the type of sugars and amino acids involved and on the reaction
372 temperature, time, pH, and water content.[45] On the other hand, the aromatic profile of oak wood
373 barrels is considerably modified during the toasting process, depending on the specific
374 combination of toasting time and heating method used by the cooper (not only time and toasting
375 intensity but also variable anoxia and humidity). Thus, the influence of two important
376 parameters, time and toasting temperature, on pyrrole contents in oak wood extract was
377 evaluated (Figure 4). To be more accurate than the real toasting conditions previously used, oak
378 chips were heated at 160, 180, and 200 °C for 10, 30, and 180 min. A heavy toasting modality
379 (250 °C, 10 min) was also tested to accentuate the effect of toasting on volatile compounds. It
380 is well known that heating increases the concentrations of certain compounds up to a certain
381 level of toasting but that if toasting continues beyond this point, the concentrations tend to
382 decrease.[44] However, the toasting level corresponding to the maximum chemical concentration
383 level was shown to depend on the chemical nature of the compounds.[46] In the present study, the
384 influence of toasting appeared to be quite different according to the N-functionalization of the
385 pyrroles (Figure 4). Large confidence intervals were calculated for each modality, which

revealed high inter-individual variability within different oak wood samples. Such heterogeneity has already been highlighted for other compounds.[24,47] However, the same evolution patterns were observed for each sample individually (represented by full lines, Figure 4). At a moderate temperature (160 °C), concentrations of PC and AP significantly increased with toasting time, whereas they tended to decrease with a high heating temperature and a longer time. For instance, at 200 °C, the concentrations after 180′ exposure were lower than that after 10′, with decreases of 45 and 27% for AP and PC, respectively. On the contrary, MPC and EPC were not observed in untoasted wood extracts or in samples heated at a moderate temperature and for a short time. Their concentrations increased with time and temperature, which indicated that they were formed rather during medium or high toasting, whereas the N-non-functionalized pyrroles PC and AP were formed rather during light toasting. Very few data about the influence of toasting time and temperature on pyrrole contents in oak wood can be found in the literature, apart from PC for which similar results were observed.[42] However, the influence of these parameters has been more extensively studied in several food products. Counet et al. highlighted that AP content could increase in dark chocolate even after a low heating treatment (temperature between 70 and 80 °C).[48] In roasted coffee beans, the concentrations of MPC, AP, and PC increase over time at 235 °C, which is not the case of 1*H*-pyrrole, suggesting that this pyrrole might not follow the same reaction pathway.[49]

More results on oak wood are available on other compounds whose genesis is also linked to the Maillard reaction such as furanic derivatives. Furfural and its derivatives are known to be present in untoasted wood at low levels. Their concentrations increase with toasting intensity but decrease at high temperatures (more than 200 °C).[43] According to Chatonnet et al.,[43] volatilization reactions and degradations, such as the opening of the furanic cycle, pigment formation, and condensation with methanol, could explain this phenomenon. Moreover, it is also possible that the functionalization of nitrogen by a methyl or ethyl group increased the

411 thermostability of MPC and EPC. Further research is required to study this hypothesis in more
412 detail and to investigate the chemical mechanisms associated with the synthesis and degradation
413 of pyrroles in both oak wood and wine.

414 The optimized and validated SPE-GC/MS methodologies described in this article
415 allowed five pyrroles to be quantitated in wine and oak wood extracts. They show that the barrel
416 aging process plays the most important role in the modulation of pyrrole contents in the studied
417 wines. However, pyrroles were also observed in musts and wines made in a stainless-steel tank,
418 suggesting in a lesser extent a varietal origin. Further work is required to clarify the relative
419 contribution of grapes and oak wood to their contents, as well as the potential influence of yeast
420 and lactic acid bacteria. Moreover, the present findings throw interesting light on the effect
421 produced by the heating of wood on the quality and quantity of pyrroles. The mechanisms
422 involved require further analysis and highlight the need to continue to study the thermolysis
423 reactions that occur in oak wood.

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588

TABLES

Table 1. Characteristics of (A) Wine and (B) Oak Wood Samples.

A. Wine samples

Origin	Region	Vintage	Type of aging container					
			Stainless-steel tank	New oak barrel	1-year-old barrel	2-year-old barrel	3-year-old barrel	≥4-year-old barrel
Beaune 1er cru Clos des Mouches	Burgundy	2016	0	2	0	2	2	2
Chassagne Montrachet 1er cru Chenevottes	Burgundy	2017	2	2	2	2	2	0
Chablis Grand cru	Burgundy	2019	2	2	0	0	0	2
Cuis 1er cru	Champagne	2017	2	2	0	0	0	2
Cuis 1er cru	Champagne	2018	2	2	0	0	0	2
Total sample			8	10	2	4	4	8

B. Oak wood samples

Type of sample	Origin	Replicates (*n*)	Heat treatment
Staves	France	3	160, 180, 200, 250 °C for 10, 30 or 180 minutes
Staves	France	6	Light toasting with maximal heating temperature of 170°C
Staves	France	5	Medium toasting with maximal heating temperature of 180°C
Staves	USA	4	Medium toasting with maximal heating temperature of 180°C
Staves	France	6	Heavy toasting with maximal heating temperature of 200°C

Table 2. Ions Adopted in Quantitation of Pyrroles, Detection Limits Reported by Gros et al.[13] in White Wine, and Calibration Levels in Wine and Oak Wood Extract for the Present Study.

Compound	abbreviation	*m/z* quantifier (qualifier)	LOD[a] (ng/L)	LOQ[b] (ng/L)	calibration levels (wine; µg/L)	calibration levels (ng/g of wood)
Octan-3-ol	EI	83 (101, 59)				
1H-pyrrole	P	67 (52, 41)	10	25	0.02-60	2.4-1175
1-methylpyrrole-2-carboxaldehyde	MPC	109 (108, 80)	12	25	0.02-40	1.6-783
1-ethylpyrrole-2-carboxaldehyde	EPC	123 (108, 94)	13	32	0.02-40	1.6-783
2-acetyl-1H-pyrrole	AP	94 (109, 66)	8	14	0.01-25	1-490
1H-pyrrole-2-carboxaldehyde	PC	95 (94, 66)	15	37	0.07-170	6.9-3432

[a]LOD, limit of detection; [b]LOQ, limit of quantitation.

Table 3. Validation Data for GC-MS Method in Wine (A) and Oak Wood Extract (B)

A. Wine method

					Low spiking level (%)				High spiking level (%)			
Compound	R^2	Slope	LOD[a] (ng/L)	LOQ[b] (ng/L)	Concentration (µg/L)	Repeatability	Reproductibility	Recovery	Concentration (µg/L)	Repeatability	Reproductibility	Recovery
1*H*-pyrrole	0.9966	0.5932	18	37	0.12	6	9	102	29.4	8	10	106
1-methylpyrrole-2-carboxaldehyde	0.9947	2.663	7	16	0.08	9	9	101	19.6	7	9	102
1-ethylpyrrole-2-carboxaldehyde	0.9993	2.938	7.5	15	0.08	7	8	95	20.4	5	9	94
2-acetyl-1*H*-pyrrole	0.9974	3.339	1	2	0.05	7	9	102	12.2	7	8	108
1*H*-pyrrole-2-carboxaldehyde	0.9919	1.048	6	12.9	0.37	8	8	101	93.6	7	9	102

B. Oak wood extract method

					Low spiking level (%)				High spiking level (%)			
Compound	R^2	Slope	LOD (ng/g of wood)	LOQ (ng/g of wood)	Concentration (ng/g of wood)	Repeatability	Reproductibility	Recovery	Concentration (ng/g of wood)	Repeatability	Reproductibility	Recovery
1*H*-pyrrole	0.9912	0.4283	11.8	29.4	29.4	7	7	97	587.5	8	8	85
1-methylpyrrole-2-carboxaldehyde	0.9985	1.474	1.6	7.8	19.6	4	7	104	391.5	2	9	92
1-ethylpyrrole-2-carboxaldehyde	0.9901	1.603	2.7	8.2	20.4	5	8	91	408	2	9	85
2-acetyl-1*H*-pyrrole	0.9974	2.144	0.1	0.3	12.2	10	11	105	244.8	5	9	90
1*H*-pyrrole-2-carboxaldehyde	0.9927	1.436	0.5	6.9	85.8	4	6	104	1716	3	6	87

[a]LOD, limit of detection; [b]LOQ, limit of quantitation.

Table 4. Pyrrole Composition (µg/L) of Wines after Malolactic Fermentation (mean ± standard deviation) Made in Different Experimentations[a,b]

	Stainless-steel tank	New oak barrel	1-year-old barrel	2-year-old barrel	3-year-old barrel	≥4-year-old barrel	Statistical analysis
Beaune 1er cru Clos des Mouches 2016							
1*H*-pyrrole		0.21 ± 0.01		0.21 ± 0.01	0.19 ± 0.00	0.20 ± 0.03	N.S.
1-methylpyrrole-2-carboxaldehyde		1.02 ± 0.04 a		0.25 ± 0.11 b	0.13 ± 0.01 c	0.11 ± 0.01 c	**
1-ethylpyrrole-2-carboxaldehyde		0.07 ± 0.01 a		0.04 ± 0.01 b	0.02 ± 0.00 c	0.02 ± 0.00 c	*
2-acetyl-1*H*-pyrrole		0.49 ± 0.04 a		0.26 ± 0.06 b	0.18 ± 0.01 c	0.15 ± 0.02 c	**
1*H*-pyrrole-2-carboxaldehyde		11.54 ± 1.02 a		2.37 ± 0.75 b	1.73 ± 0.11 c	1.11 ± 0.06 c	**
Chassagne Montrachet 1er cru Chenevottes 2017							
1*H*-pyrrole	0.51 ± 0.04	0.44 ± 0.16	0.45 ± 0.08	0.39 ± 0.22	0.17 ± 0.08		N.S.
1-methylpyrrole-2-carboxaldehyde	0.07 ± 0.04 b	0.98 ± 0.12 a	0.16 ± 0.01 b	0.12 ± 0.09 b	0.09 ± 0.01 b		*
1-ethylpyrrole-2-carboxaldehyde	0.00 ± 0.01 b	0.07 ± 0.00 a	0.03 ± 0.00 ab	0.03 ± 0.01 b	0.01 ± 0.00 b		*
2-acetyl-1*H*-pyrrole	0.08 ± 0.02 b	0.37 ± 0.04 a	0.21 ± 0.03 b	0.18 ± 0.04 b	0.14 ± 0.03 b		*
1*H*-pyrrole-2-carboxaldehyde	0.51 ± 0.01 d	10.71 ± 0.11 a	3.27 ± 0.70 b	2.03 ± 0.93 bc	0.89 ± 0.25 c		***
Chablis Grand cru 2019							
1*H*-pyrrole	< LOQ	< LOQ				< LOQ	-
1-methylpyrrole-2-carboxaldehyde	0.11 ± 0.01 b	2.14 ± 0.16 a				0.29 ± 0.01 b	*
1-ethylpyrrole-2-carboxaldehyde	0.01 ± 0.00 b	0.10 ± 0.01 a				0.03 ± 0.00 b	*
2-acetyl-1*H*-pyrrole	0.47 ± 0.04 b	0.85 ± 0.08 a				0.42 ± 0.05 b	*
1*H*-pyrrole-2-carboxaldehyde	0.26 ± 0.00 b	23.44 ± 9.15 a				0.79 ± 0.36 b	*
Cuis 1er cru 2017							
1*H*-pyrrole	0.12 ± 0.17	0.26 ± 0.06				0.49 ± 0.16	N.S.
1-methylpyrrole-2-carboxaldehyde	0.00 ± 0.00 b	1.63 ± 0.06 a				0.09 ± 0.06 b	*
1-ethylpyrrole-2-carboxaldehyde	0.00 ± 0.00 c	0.11 ± 0.01 a				0.02 ± 0.00 b	*
2-acetyl-1*H*-pyrrole	0.03 ± 0.04 b	0.69 ± 0.11 a				0.04 ± 0.06 b	*
1*H*-pyrrole-2-carboxaldehyde	0.21 ± 0.29 c	32.54 ± 0.22 a				1.02 ± 0.16 b	*
Cuis 1er cru 2018							
1*H*-pyrrole	0.04 ± 0.00	0.04 ± 0.01				0.07 ± 0.03	N.S.

1-methylpyrrole-2-carboxaldehyde	0.02 ± 0.01 b	2.75 ± 0.23 a				0.22 ± 0.14 b	*
1-ethylpyrrole-2-carboxaldehyde	0.02 ± 0.01 b	0.20 ± 0.00 a				0.06 ± 0.02 b	*
2-acetyl-1*H*-pyrrole	0.13 ± 0.03 b	1.07 ± 0.01 a				0.27 ± 0.07 b	*
1*H*-pyrrole-2-carboxaldehyde	0.50 ± 0.22 c	43.36 ± 8.31 a				4.20 ± 2.10 b	*
Mean of experimentations							
1*H*-pyrrole	0.12 ± 0.11	0.20 ± 0.10	0.25 ± 0.26	0.26 ± 0.11	0.22 ± 0.06	0.25 ± 0.21	N.S.
1-methylpyrrole-2-carboxaldehyde	0.07 ± 0.07 c	1.93 ± 0.63 a	0.34 ± 0.11 b	0.20 ± 0.10 c	0.17 ± 0.12 c	0.17 ± 0.11 c	***
1-ethylpyrrole-2-carboxaldehyde	0.01 ± 0.01 c	0.10 ± 0.05 a	0.03 ± 0.01 b	0.02 ± 0.02 bc	0.01 ± 0.01 c	0.03 ± 0.02 bc	***
2-acetyl-1*H*-pyrrole	0.21 ± 0.18 c	0.72 ± 0.24 a	0.28 ± 0.03 bc	0.24 ± 0.05 c	0.19 ± 0.02 c	0.22 ± 0.16 c	***
1*H*-pyrrole-2-carboxaldehyde	0.38 ± 0.22 d	24.08 ± 14.06 a	3.55 ± 0.66 b	2.21 ± 0.77 c	1.28 ± 0.52 c	1.78 ± 1.70 c	***

[a]Note: different letters in row indicate significant differences between samples according to Kruskal−Wallis test followed by post hoc Conover−Iman test. Abbreviations: N.S.: not significant; *$p \leq 0.05$; **$p \leq 0.01$; $p \leq 0.01$. [b]Replicates are indicated in Table 1.

Table 5. Pyrrole Concentrations (Mean ± Standard Deviation in ng/g of Wood) in Oak Wood Extract Made with Untoasted or Toasted Face of Staves According to Several Heat Treatments

Origin	Heat process	Stave side	MPC	EPC	AP	PC
France	Light	untoasted	< LOQ	< LOQ	2.0 ± 0.4	23.5 ± 2.6
		toasted	185.3 ± 27.2	13.9 ± 2.9	70 ± 13,0	2272.7 ± 498.3
France	Heavy	untoasted	< LOQ	< LOQ	1.1 ± 0.9	16.3 ± 4.3
		toasted	300.3 ± 48.8	21.5 ± 3.6	63.7 ± 15,0	2421.8 ± 792
France	Medium	untoasted	< LOQ	< LOQ	5.0 ± 1.6	40.0 ± 13.1
		toasted	180.8 ± 60.3	31.2 ± 12.7	73.3 ± 45.8	1938.9 ± 980.3
USA	Medium	untoasted	< LOQ	< LOQ	3.0 ± 0.5	37.3 ± 1.6
		toasted	175.1 ± 30.5	27.5 ± 2.5	76.8 ± 25.1	2262.2 ± 248.9

FIGURE GRAPHICS

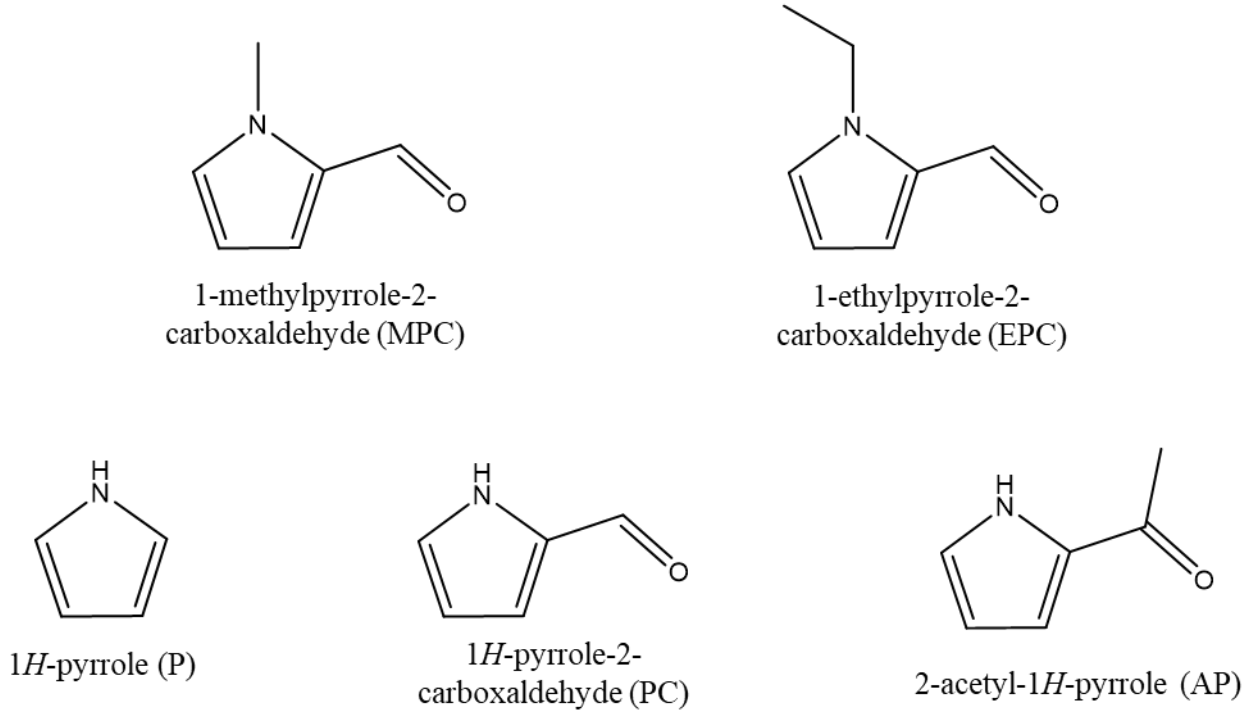


Figure 1. Chemical structures of the five pyrroles studied.

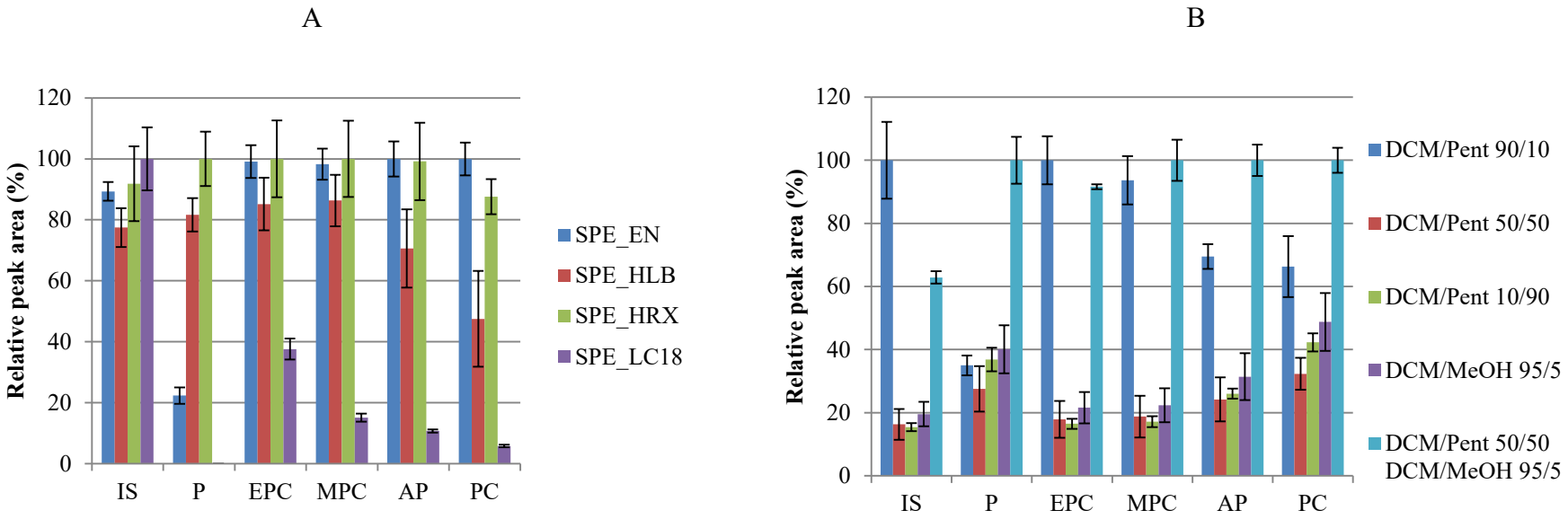


Figure 2. Relative peak area ± standard deviation (n = 3) of pyrroles extracted by SPE (A) regarding different cartridges used and (B) regarding different solvents used (v/v) for SPE with HR-X cartridge in white wine. IS, internal standard; P, 1*H*-pyrrole; EPC, 1-ethylpyrrole-2-carboxaldehyde; MPC, 1-methylpyrrole-2-carboxaldehyde; AP, 2-acetyl-1*H*-pyrrole; PC, 1*H*-pyrrole-2-carboxaldehyde; DCM, dichloromethane; Pent, pentane; MeOH, methanol.

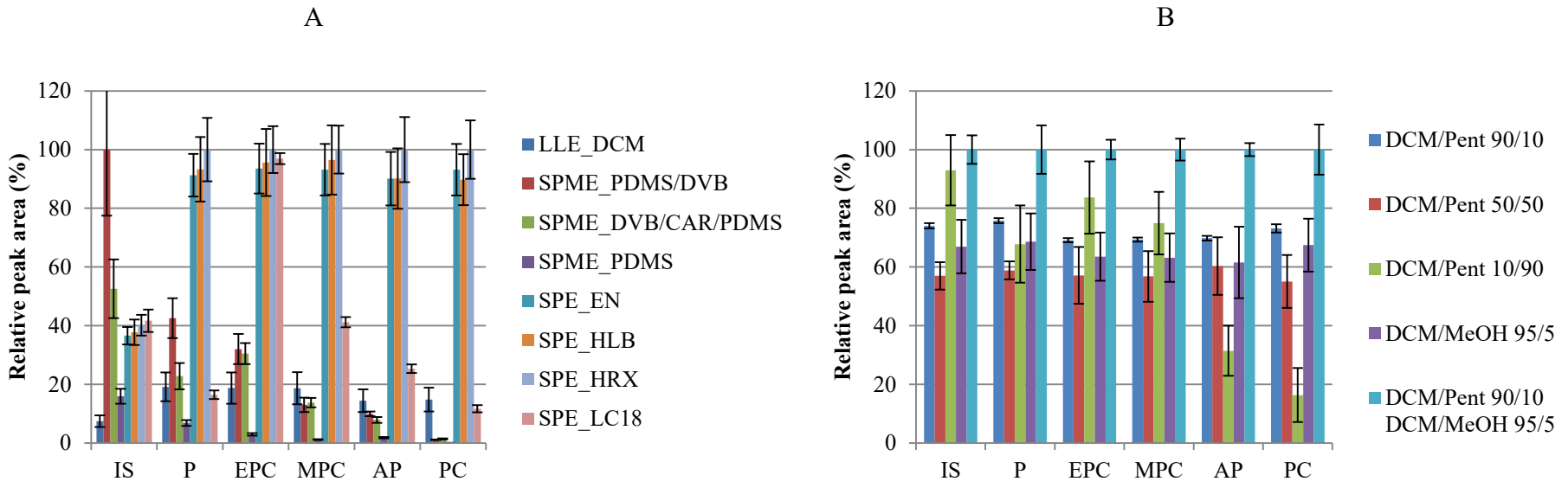
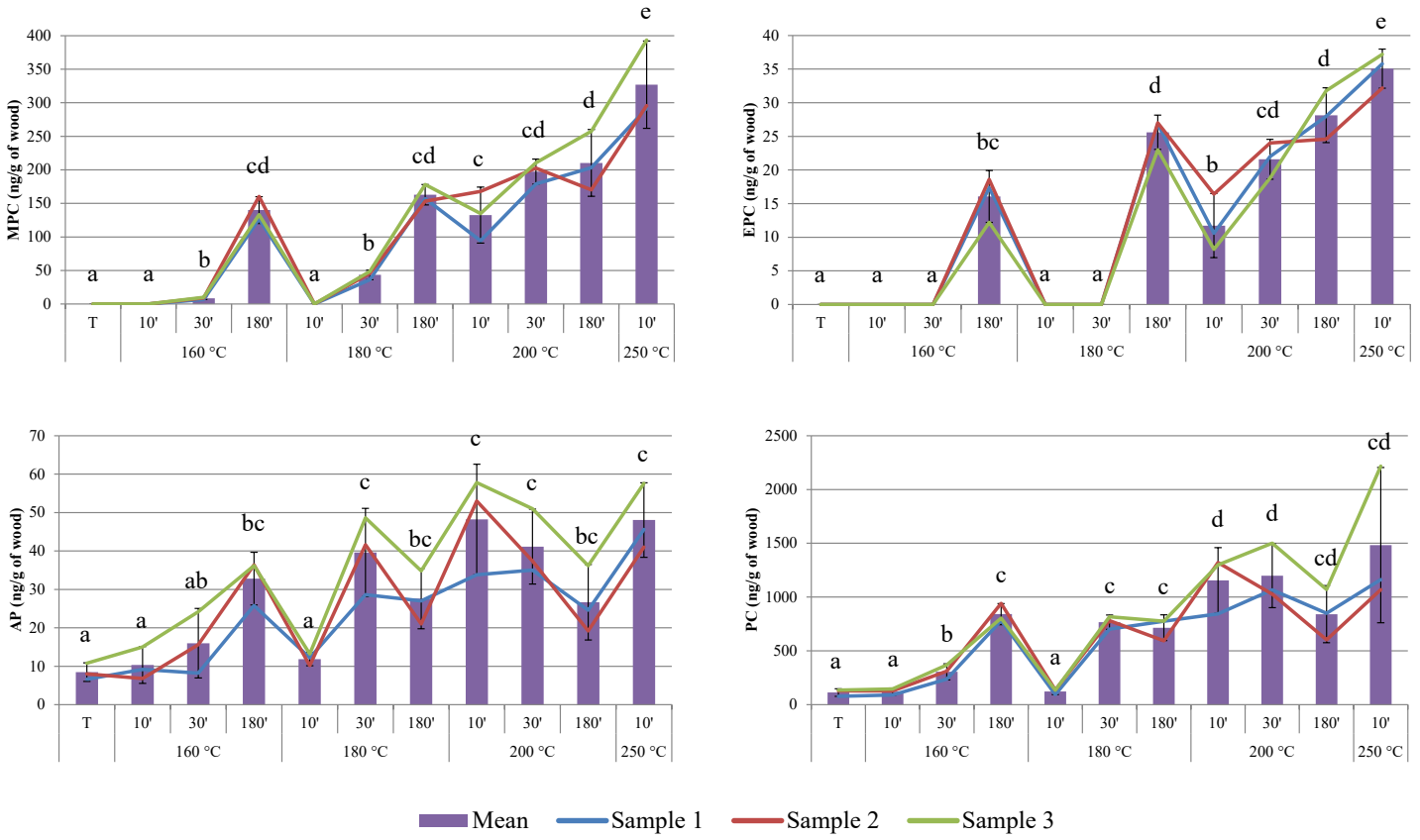


Figure 3. Relative peak area ± standard deviation (n = 3) of pyrroles (A) regarding different extraction mode (nature of solvent, fiber, and cartridge) and (B) regarding different solvents used (v/v) for SPE with HR-X cartridge in oak wood extract. IS, internal standard; P, 1*H*-pyrrole; EPC, 1-ethylpyrrole-2-carboxaldehyde; MPC, 1-methylpyrrole-2-carboxaldehyde; AP, 2-acetyl-1*H*-pyrrole; PC, 1*H*-pyrrole-2-carboxaldehyde; DCM, dichloromethane; Pent, pentane; MeOH: methanol.



589 **Figure 4.** Mean concentration ± standard deviation (in ng/g of wood) of pyrroles in oak wood chip extract regarding toasting treatment. Different
590 alphabetical letters indicate significant differences.

FOR TABLE OF CONTENTS ONLY