

Identification of Some Volatile Descriptors of the Rock-Rose-Like Aroma of Fortified Red Wines from Douro Demarcated Region

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2,2,6-Trimethylcyclohexanone and ethyl dihydrocinnamate, known sensorial descriptors of rock-rose (*Cistus ladaniferus*) aroma, are reported as minor constituents of young fortified wines from the Douro Demarcated region (vintage 1997) for the first time. These compounds were respectively identified by GC–MS in two different fractions (A and B) obtained by preparative GC. Quantitative analysis by GC–MS was performed on 14 fortified wines originating from the different Douro subregions. Ethyl dihydrocinnamate was found to be present at concentrations between 2.3 and 6.7 $\mu\text{g/L}$, while 2,2,6-trimethylcyclohexanone ranged from 0.05 to 0.4 $\mu\text{g/L}$.

Keywords: 2,2,6-Trimethylcyclohexanone; ethyl dihydrocinnamate; gas chromatography–mass spectrometry; preparative GC

INTRODUCTION

Volatile compounds constitute a heterogeneous group of chemical substances widely distributed in the plant kingdom. They have a particular sensorial relevance in associated beverages and food derivatives. They can provide complexity, originality, and a varietal character to the wine's aroma. Some of these volatile metabolites such as the norisoprenoid compounds (C-9, C-11, and C13-carbon) are present in low concentrations in grapes and are usually accumulated in much higher concentrations as glycoconjugates. These conjugates can release their volatile aglycon compounds during fermentation via mild acid or enzymatic hydrolysis, contributing important flavor to the wine (Williams et al., 1989; Sefton et al., 1994). One of the most important operations in Port winemaking is the addition of brandy to the must in order to stop the fermentation. As a result of this shortened fermentation, it is expected that the final Port wine flavor will contain a more important contribution from the primary aroma compounds compared to the secondary aroma compounds released during fermentation. Many of the primary compounds are left intact during winemaking, being extracted from the different parts of grape by alcohol after the addition of brandy to the must.

Among a large number of Portuguese varieties known in this region, Touriga Nacional and Touriga Françesa varieties are distinguished by their superior quality. They are perfectly adapted to the schistous soil that characterizes this region, as well as to the climate, which is cold during the winter and very hot and dry during grape ripening. The high temperatures experienced during final berry maturation are important for the development of aromas, which add complexity and varietal characteristics to the Port wine flavor.

The young Port wines are often characterized by aromas described as "red fruit" (e.g., raspberry, cherry, blackberry), "floral" (e.g., rose, violet, geranium), "nuts", "balsamic", and "resinous" (e.g., rock-rose, pine, eucalyptus). During wine aging, some of the aroma compounds responsible for these aromas disappear or undergo important structural transformations by oxida-

tion (Ribereau-Gayon et al., 1975; Simpson, 1979) and acid-catalyzed reactions (Rapp and Guentert, 1986; Rapp and Mandery, 1986), which lead to changes in sensorial characteristics. These reactions give rise to compounds that impart different kinds of "roasting" aromas (e.g., burnt, caramel, cacao), "fruit" aromas (e.g., plum, fig), as well as nut, honey, tobacco, old wood, etc. To our knowledge, very little is known about the compounds responsible for some of these aromas in Port wines (Williams et al., 1983; Rogerson, 1997; Silva Ferreira, 1998) and how they contribute to the varietal character of the aroma.

The aim of the current work was to study some volatile compounds that contribute to the balsamic-like sweet aroma in young Port wines, described frequently as rock-rose-like flavor (*Cistus ladaniferus*), which has been recently associated with the sensorial quality of Port wines. A simple method using GC–MS was developed to quantify these components, investigating typical concentrations found in both monovarietal Port wines and single varietal grape samples of two Portuguese varieties (Touriga Nacional and Touriga Fancesa) harvested from Douro Valley vineyards.

MATERIALS AND METHODS

Preparative GC. The fortified wine (0.7 L) was extracted with a mixed solvent (2×4 mL) of hexane/diethyl ether (1:1, v/v). The extract's odor was observed to be similar to the odor of the original fortified wine. The organic extract was almost entirely subjected to preparative GC chromatography using a 10% Carbowax 20 M tubular column and FID. Two fractions: A (15–16 min) and B (35–50 min), corresponding to the elution time of the 2,2,6-trimethylcyclohexanone (**1**) and ethyl dihydrocinnamate (**2**) were collected in 0.2 mL of solvent (hexane/diethyl ether); of which 2 μL was directly injected into the GC–MS.

For preparative GC–FID, a Varian Aerograph 1740 was equipped with a packed column (100×1.0 cm i.d.) containing a chemical bonded phase of Carbowax 20 M in Chromasorb W (60/80 mesh). Oven temperature was programmed 40–200 °C at 1 °C/min. Nitrogen carrier gas flow was 10 mL/min. Small glass flasks with a mixture of hexane and diethyl ether (1:1, v/v) were used for trapping separated fractions for further analysis by GC–MS. For GC-sniffing, the chromatograph was

equipped with a special glass nose funnel directly at the end of the preparative column and the respective odor was sniffed by two trained members.

Preparation of Fortified Wine Extracts for GC-MS Determination. The wines were extracted with hexane/diethyl ether (1:1, v/v), based on the procedure described by Bertrand (1975). A 100 μ L portion of a solution of the internal standard isophorone (200 μ g/L) and sodium sulfate (~5 g) to promote extraction by "salting out" of the volatile compounds was added to 50 mL of a fortified wine. Isophorone was selected as an internal standard because it is unknown in wine and has a structure similar to that of 2,2,6-trimethylcyclohexanone. This slurry was stirred for 5 min with 4 mL of hexane/diethyl ether (1:1, v/v). The organic layer was separated, and the extraction was repeated twice using a further 4 mL and 2 mL of the same solvent. The organic layers were combined and injected (2 μ L), without any preconcentration, into the GC-MS. Extractions and analyses were carried out in duplicate.

Preparation of Grape Extracts. Grapes of the Touriga Nacional and Touriga Francesa varieties from 1997 vintage were obtained from several vineyards located in different subregions within the Douro Demarcated region (Cima Corgo and Douro Superior). One hundred berries were harvested from several parts of grapevines selected randomly along the vineyards studied. Grape seeds were removed carefully from the frozen berries, and volatiles present in the pulp and the skins were extracted (Ultra-Turrax, for 5 min) with 25 mL of a solution of only deionized water/pure ethanol (12%, v/v). The composition of this solution was chosen as being representative of the extraction conditions encountered during vinification. The resulting slurry was centrifuged for 5 min (5000g). The hydro alcoholic extract was collected, and the residue was then re-extracted using the same conditions. The hydro alcoholic phases were combined and diluted with the same solution to the volume of 100 mL. The volatile compounds were then extracted with hexane/diethyl ether (1:1, v/v) and analyzed as previously described for the fortified wines.

Quantitative Analysis. This was performed by GC-MS. The concentrations of volatile compounds were determined by comparing the intensity of the component peaks with that of the added isophorone standard in the selected ion chromatograms of both the wine extract and the standard solution of available reference compounds. 2,2,6-Trimethylcyclohexanone (**1**) and ethyl dihydrocinnamate (**2**) compounds were quantified by the relative abundance of their respective characteristic ion peaks at m/z 82 and 104. The volatile compounds *trans*-anethole, 2-phenylethanol, linalool, eugenol, α -terpineol, geraniol, ethyl cinnamate, DL-citronellol and benzaldehyde were determined by the procedure described previously in the paper for the 2,2,6-trimethylcyclohexanone compound, using the relative abundance of the respective characteristic ion peaks at m/z 148, 122, 93, 164, 93, 93, 131, 95, and 77. Calibration curves (based on characteristic ion peak area) were established using a standard solution submitted to the same procedure. Linear calibration curves ($r^2 > 0.98$) in the ranges of 0.05 mg/L (limit of detection) to 0.5 mg/L were obtained for compounds **1** and **2**. The repeatability of this method, from extraction to GC-MS analysis, for five samples gave a coefficient of variation less than 7%.

GC-MS analyses were carried out with a Saturn II (Varian) ion trap mass spectrometer (multiplier voltage, 2550 V; emission current, 10 mA; scan rate, 0.600 s; detector temperature, 170 °C; mass range m/z 30–250) coupled with a Varian 3700 gas chromatograph, equipped with a Supelcowax 10 fused silica capillary column (60 m \times 0.25 mm i.d., film thickness 1 μ m). Oven temperature was held at 50 °C for 5 min and then programmed to 200 °C at 3 °C/min, set at 200 °C for 4 min, and then programmed to 250 °C at 3 °C/min. The helium carrier gas flow rate was 1 mL/min.

Determination of Aromatic Thresholds. The threshold limits of 2,2,6-trimethylcyclohexanone and ethyl dihydrocinnamate were determined by a panel of eight enologist experts, using a triangular test; each taster looked for the lowest concentration he/she was able to characterize by smell alone. The thresholds were determined in both deionized water and

in a model ethanolic wine system (12% ethanol; pH 3.2; tartaric acid, 5.0 g/L) at 20 °C. Tasters were informed which compound was being determined. The chemicals used as standard were the purest commercially available (over 98%), and they were examined by GC-MS in order to control their purity.

Chemicals and Solvents. The following reference compounds were used: 2,2,6-trimethylcyclohexanone 98% (Aldrich, cat. no. T75736), ethyl hydrocinnamate 99% (Aldrich, cat. no. 28,441-6), *trans*-anethole (Sigma, cat. no. A8639), 2-phenylethanol (Sigma cat. no. P6134), linalool 95–97% (Sigma, cat. no. L-5255), α -terpineol 95% (Sigma cat. no. T3407), geraniol 98% (Sigma cat. no. G5137), DL-citronellol 95% (Sigma cat. no. C5904), benzaldehyde >99% (Merck, cat. no. 801756), *n*-hexane (Merck, Lichrosolv), and diethyl ether (Pronalab, cat. no. 12-19).

RESULTS

Identification of 2,2,6-trimethylcyclohexanone and ethyl dihydrocinnamate, which were two of the main volatile compounds found to be responsible for the odor in the leaves of the rock-rose bush, was important in order to attempt to describe this aroma descriptor (Ramalho et al., 1999; Simon-Fuentes et al., 1987; Ohno, 1981), often associated with young Port wines. The presence of these compounds was then investigated in monovarietal fortified wines made from either Touriga Nacional or Touriga Francesa grapes (1997 vintage) chosen for their aromatic richness and complex aroma.

Identification of Volatile Descriptors of Rock-Rose Aroma in Fortified Wines. Recent work in our laboratory investigating the aroma characterization of this *Cistus* plant (rock-rose) allowed us to identify several compounds, namely 2,2,6-trimethylcyclohexanone (**1**) as the main aroma and 2,6,6-trimethyl-2-cyclohexenone in a lower quantity (Ramalho et al., 1999). These compounds, apparently derived from carotenoid degradation, are referred to in the literature as having a woody and haylike odor (Shimoda et al., 1995). Another potent flavorant described in this bush is ethyl dihydrocinnamate (**2**) (shikimate-derived metabolite), which has been confirmed by preparative GC and sniffing to have a fruity and balsamic-like sweet odor (Ohno, 1981). This latter constituent has recently been identified as a minor constituent of Pinot noir wines of the Burgundy region in France (Moio and Etievant, 1995; Aubry et al., 1996), while 2,2,6-trimethylcyclohexanone (**1**) has already been cited as constituent of white wines produced from the Muscat de Frontignan and Sauvignon Blanc grapes (Etievant et al., 1983; Sefton et al., 1994). These compounds may be present at extremely low levels and thus require special concentration techniques for analytical detection. This was emphasized by the GC-MS analysis performed directly on the total wine extract, which resulted in inadequate identification of these compounds.

For the initial characterization of the rock-rose sensorial descriptor, a fortified wine from the Douro region with particular intense fruity-, floral-, nut-, and wood-like aromas was examined. Wine volatiles were isolated and concentrated by extraction with diethyl ether/hexane (1:1, v/v), followed by purification by preparative GC. The eluted compounds were collected in a small volume of solvent and analyzed after direct injection into a GC-MS. These purification and preconcentration techniques do not require extensive sample preparation, involving sample heating or solvent evaporation before GC-MS analysis. Figure 1 shows the preparative GC gas chromatogram of a fortified wine extract and the

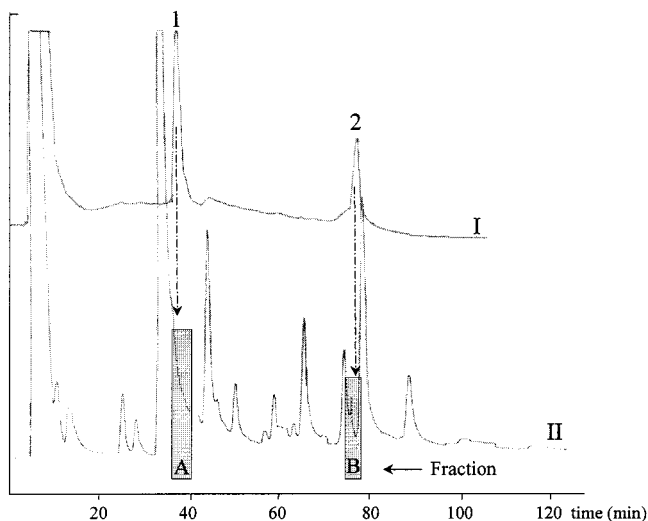


Figure 1. Preparative GC gas chromatograms of (I) a mixture of authentic 2,2,6-trimethylcyclohexanone (**1**) and ethyl dihydrocinnamate (**2**) and (II) Et₂O/hexane (1:1, v/v) extract of a fortified wine.

collected A and B fractions, respectively, corresponding to the elution times of 2,2,6-trimethylcyclohexanone and ethyl dihydrocinnamate compounds. The chromatograms of the two fractions A and B (Figure 2), isolated by preparative GC, show that other important volatile compounds are coeluted with both compounds, 2,2,6-

trimethylcyclohexanone and ethyl dihydrocinnamate. Identification of the aroma of compounds **1** and **2**, when sensed directly at the end of the preparative column (GC sniffing) was difficult, due to the high aroma intensity of coeluting compounds, which masked their odors. The two compounds were identified by GC-MS using two columns of different polarity (Supelcowax 10 fused silica capillary and DB-1 columns), from which the resulting chromatographic peaks give clear EI mass spectra (Figure 2) and identical retention indices compared with authentic compounds.

Although chromatographic peaks of these compounds were directly visible in total ion chromatography (full scan), their relative intensity was very low. Quantification of both, 2,2,6-trimethylcyclohexanone and ethyl dihydrocinnamate found in the fortified wines demanded a special GC-MS detection selecting the most characteristic ion peaks given by their EI mass spectrums.

Quantitative Determination and Sensory Evaluation. Figure 3 shows the GC-MS total ion chromatogram of fortified wine volatiles extracted using Et₂O/hexane, as well as part of the selected ion chromatograms corresponding to the elution zones of 2,2,6-trimethylcyclohexanone (40–50 min) and ethyl dihydrocinnamate (90–98 min). Their content in wine was determined by the relative abundance of the respective characteristic ion peaks at *m/z* 82 and 104 (Table 1).

For this study, 14 fortified wines from different subregions (Douro Superior e Cima Corgo) were tasted

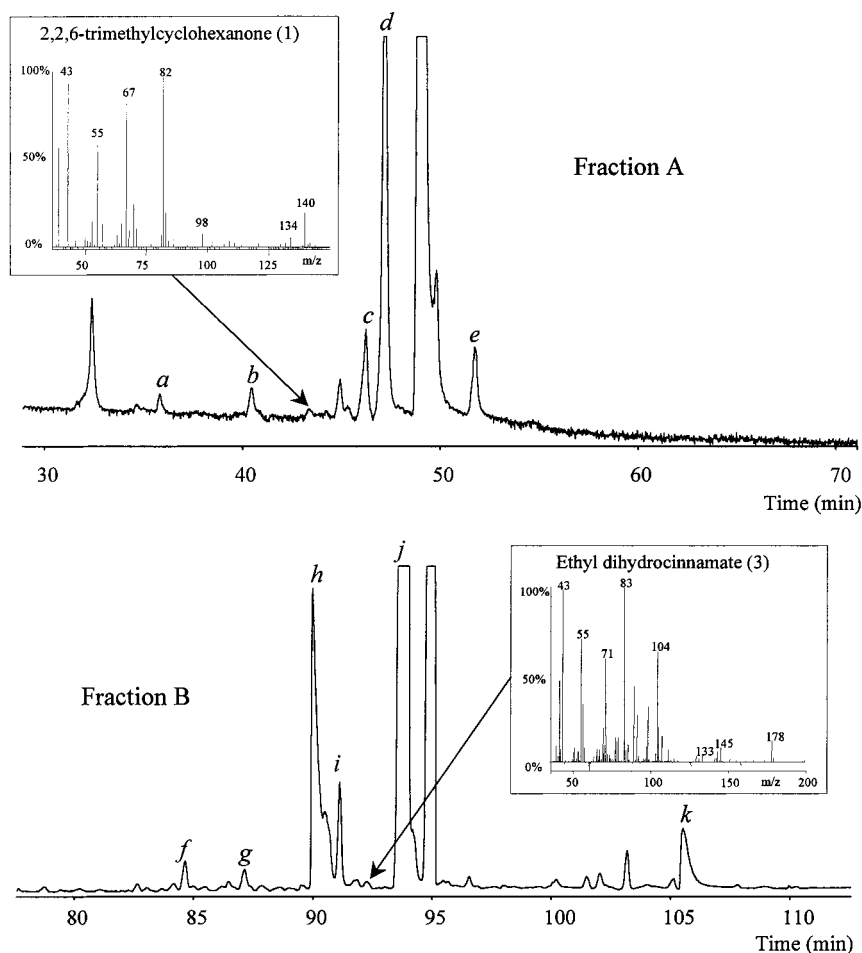


Figure 2. GC-MS total ion chromatograms of fractions A and B isolated by preparative GC (Figure 1) corresponding to the elution zones containing the 2,2,6-trimethylcyclohexanone (**1**) and ethyl dihydrocinnamate (**2**): (a) ethyl hexanoate; (b) hexyl acetate; (c) 1-hexanol; (d) ethyl lactate; (e) (*E*)-3-hexen-1-ol; (f) 2-phenylethyl formate; (g) 2-phenylethyl acetate; (h) butyl hexanoate; (i) benzyl alcohol; (j) phenylethyl alcohol; (k) octanoic acid.

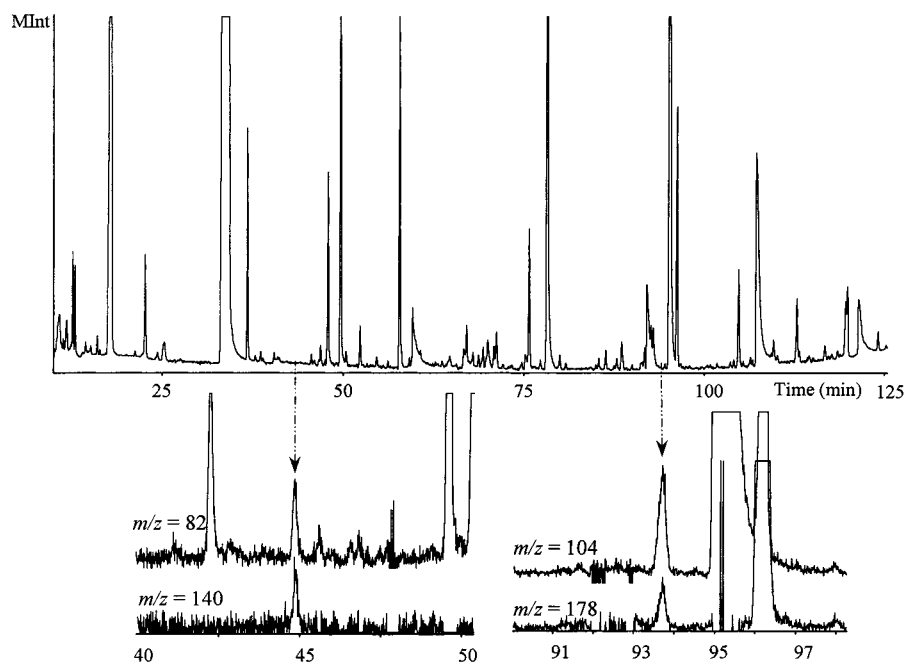


Figure 3. GC-MS total ion chromatogram of Et₂O/hexane extract of a fortified wine. Chromatogram profiles of the selected ion peaks at *m/z* 140 and 82 corresponding to 2,2,6-trimethylcyclohexanone and the ion peaks at *m/z* 178 and 104 characteristic of ethyl 2,3-dihydrocinnamate EI mass spectra.

Table 1. Concentration of Some Volatile Components of Monovarietal Fortified Wine (1–10) Made from Touriga Nacional and Touriga Francesa Grapes from the Douro Demarcated Region (Vintage 1997), Including the Main Sensory Descriptive Odors Determined by a Taste Panel of 12 Experts (Concentrations Are the Mean of Two Replicate Determinations)

| compounds ($\mu\text{g/L}$): | Touriga Nacional | | | | | | | | Touriga Francesa | | | | | |
|---|------------------|-----------------|-----------------|------|---------|-----------------|-----------------|-------|------------------|-----------------|------|-----------------|-----------------|-----------------|
| | Douro Superior | | | | | | Cima Corgo | | Douro Superior | | | Cima Corgo | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| 2,2,6-trimethylcyclohexanone (1) | 0.39 | nd ^a | nd ^a | 0.39 | 0.20 | 0.21 | nd ^a | 0.30 | tr ^b | nd ^a | 0.33 | tr ^b | 0.06 | tr ^b |
| ethyl 2,3-dihydrocinnamate (2) | 2.30 | 4.40 | 3.10 | 4.10 | 4.20 | 6.70 | 6.10 | 6.20 | 2.90 | 4.00 | 3.60 | 3.60 | 3.50 | 4.10 |
| ethyl cinnamate | 7.6 | 4.6 | 3.2 | 7.0 | 5.7 | 8.2 | 7.6 | 6.7 | 5.6 | 11.0 | 5.0 | 3.6 | 5.3 | 4.4 |
| eugenol | 6.0 | 6.0 | 7.8 | 7.2 | 9.3 | 9.8 | 10.2 | 6.0 | 8.3 | 10.0 | 7.8 | 7.5 | 7.4 | 8.9 |
| benzaldehyde | 217.6 | 703.0 | 100.2 | 71.1 | 64.2 | 187.3 | 113.9 | 267.2 | 136.1 | 119.3 | 81.0 | 119.8 | 26.0 | 60.6 |
| linalool | 55.9 | 55.1 | 32.0 | 32.7 | 23.0 | 61.0 | 44.4 | 54.6 | 19.8 | 43.7 | 31.7 | 19.1 | 20.8 | 19.2 |
| α -terpineol | 53.2 | 52.8 | 31.1 | 29.4 | 21.1 | 58.2 | 38.0 | 46.9 | 18.2 | 44.8 | 28.6 | 26.2 | 20.0 | 20.3 |
| DL-citronellol | 17.1 | 16.9 | 9.0 | 13.4 | 15.8 | 18.0 | 14.7 | 16.2 | 11.4 | 16.5 | 10.1 | 7.0 | 9.9 | 9.0 |
| trans-anethole | nd ^a | nd ^a | nd ^a | 14.6 | 16.3 | nd ^a | 13.7 | 11.1 | nd ^a | nd ^a | 10.4 | nd ^a | nd ^a | 12.4 |
| geraniol | 21.5 | 21.5 | 11.6 | 15.3 | 13.9 | 24.3 | 17.9 | 20.8 | 12.6 | 17.9 | 12.8 | 10.5 | 12.4 | 10.6 |
| 2-phenylethyl acetate | 1173 | 938 | 596 | 1179 | 1147 | 999 | 626 | 662 | 420 | 1044 | 1037 | 675 | 247 | 347 |
| 2-phenylethanol (mg/L) | 12.5 | 16.6 | 11.8 | 16.6 | 18.3 | 10.7 | 10.7 | 12.4 | 10.2 | 12.0 | 11.0 | 18.6 | 10.7 | 11.8 |
| main sensory descriptive odors ^c | RF, Fl, Res | Sp, Rt | RF, Fl | Fl | RF, Res | RF, Nt, Res | RF, Nt | RF | Fl, Sp | Fl | Res | Nt, Rt | oxidative odors | RF, Res |

^a nd, not detected. ^b tr, represents concentration less than 0.05 $\mu\text{g/L}$. ^c RF, red fruity; Fl, floral; Res, resinous; Sp, spice; Rt, roasting.

by 12 expert enologists, evaluating the different kinds of aromas associated with Port wines such as red fruit-, nut-, floral-, resinous-, roasting-, and spice-like odors (Table 1). Although the tasters were particularly attentive to the rock-rose descriptors, it was difficult to detect a wine with a typical woody and haylike aroma, ascribed by 2,2,6-trimethylcyclohexanone associated with the rock-rose plant. In general, the vintage of 1997 did not produce wines typified by this kind of aroma. However, the concentration of ethyl dihydrocinnamate was much higher than that of 2,2,6-trimethylcyclohexanone (1). The odor thresholds of the pure compounds in water and in a model ethanolic wine system were determined (Table 2). To our knowledge, these perception limits have not been previously investigated. Ethyl dihydrocinnamate was easily detected at low concentration (1.9 $\mu\text{g/L}$) by the majority of the panel in both model systems

Table 2. Determination of Aromatic Thresholds of 2,2,6-Trimethylcyclohexanone and Ethyl Dihydrocinnamate in Water and in Ethanol Model Wine System (12% Ethanol; pH 3.2; Tartaric Acid, 5.0 g/L)

| | thresholds ^a ($\mu\text{g/L}$) | | |
|------------------------------|---|------------|-------------|
| water | | | |
| ethyl dihydrocinnamate | 1.9 (75%) | 18.8 (87%) | 47.0 (100%) |
| 2,2,6-trimethylcyclohexanone | 44.3 (25%) | 88.6 (87%) | |
| model wine system | | | |
| ethyl dihydrocinnamate | 1.9 (62%) | 18.8 (75%) | |
| 2,2,6-trimethylcyclohexanone | 443.0 (25%) | | |

^a The number in parentheses indicates the percentage of tasters able to appreciate the amounts checked.

tasted, indicating an intensive odor. 2,2,6-Trimethylcyclohexanone gave a much higher odor threshold in both water and model ethanolic wine system studied. The presence of ethanol made it much more difficult for the panel to detect the two compounds, especially 2,2,6-

trimethylcyclohexanone, with only 25% of the panel detecting the concentration 443 $\mu\text{g/L}$. Ethyl dihydrocinnamate has therefore comparatively a much more intense odor than the 2,2,6-trimethylcyclohexanone, and its contribution to wine aroma is potentially more important. This is emphasized by the relatively high concentrations found in fortified wines, which exceed the odor threshold implying than ethyl dihydrocinnamate contributes individually to wine aroma responsible for a fruity and balsamic-like sweet odor. This contribution seems to be more important in wines made from Touriga Nacional compared to Touriga Francesa wines.

It is likely that Port wine contains other volatile compounds, which impart similar aroma to compounds **1** and **2**. For example, ethyl cinnamate (quantified in Table 1) is characterized by a fruity and balsamic-like sweet aroma similar to ethyl dihydrocinnamate, which may also contribute to the rock-rose-like aroma of wines (Moio and Etievant, 1995; Arctander, 1969). The quantitative balance of the complex mixture of volatile flavors appears to give an important contribution to the wine's aroma described by the panel. Table 1 shows some of these compounds, which could contribute collectively to the overall aroma. The majority of compounds listed in Table 1, namely linalool, α -terpineol, citronellol, geraniol, 2-phenylethyl acetate, and 2-phenylethanol, are extremely odorous and either individually or collectively could give enhanced floral aromas to wines. The individual contribution of linalool and citronellol seems to be important for some wine aroma in which their concentration was close to known sensorial threshold levels as described in the literature (50 and 18 $\mu\text{g/L}$, respectively) (Darriet, 1993). The remaining compounds eugenol, benzaldehyde, and anethole are, respectively, associated with strong clove-, bitter almond-, and aniseed-like odors and are likely to have a particular sensorial relevance to Port wines.

Similar levels of 2,2,6-trimethylcyclohexanone and ethyl dihydrocinnamate were found present in grapes of Touriga Nacional and Touriga Francesa varieties, harvested in different vineyards from the Douro valley. 2,2,6-Trimethylcyclohexanone was found at concentrations between 10 and 45 ng/berry, while ethyl dihydrocinnamate was higher, ranging from 48 to 85 ng/berry. The berry levels suggest that the corresponding concentrations in wines ought to be greater than those found in Table 1, especially for 2,2,6-trimethylcyclohexanone. This decrease may not only result from chemical reactions and losses from the winemaking process but also from the partial transformation into other volatile compounds by yeast during the short fermentation. This latter hypothesis is being studied in our laboratory, as well as the identification of some of these yeast metabolites, which may also have an important impact on the wine flavor.

Further research will study 2,2,6-trimethylcyclohexanone and ethyl dihydrocinnamate compounds in wines and grapes of different Portuguese varieties from the Douro Demarcated Region, as well as the seasonal influence on their composition, which seems to be associated with the degree of berry maturation. The sensorial descriptors of the rock-rose odor may provide important information about this aroma often referred to in young Port wines.

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