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3-Hydroxy-4,5-dimethyl-2(5*H*)-furanone: A Key Odorant of the Typical Aroma of Oxidative Aged Port Wine

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Application of aroma extract dilution analysis (AEDA) on organic extracts from Port wines barrel-aged over 40 years revealed 5 odor-active compounds corresponding to descriptors used to qualify the characteristic old wine aroma. One of the compounds, described as "nutty" and "spicy-like", and present in at least 9 dilutions above the others, was perceived as particularly important. The compound responsible for this flavor was identified as 3-hydroxy-4,5-dimethyl-2(*5H*)-furanone (sotolon). The levels ranged from 5 to 958 μg/L for wines between 1 and 60 years old. It was also observed that during oxidative aging the concentration of this compound increased with time according to a linear trend ($r > 0.95$). Although the presence of 2-ketobutyric acid was verified, the constant rate of formation of sotolon with aging and its high correlation with sugar derivates (HMF, furfural) suggests other mechanisms, different from those reported for other wines. The flavor threshold of sotolon was evaluated in Port wine at 19 μg/L. Sensorial tests provided valuable information concerning sotolon impact on Port wine aroma. Samples supplemented with this substance were consistently ranked as older. In view of these results it can be expected that sotolon plays a pre-eminent role in the characteristic old Port wine aroma.

KEYWORDS: Port wine flavor; oxidative wine aging; aroma extract dilution analysis; AEDA; sotolon

INTRODUCTION

The duration of the aging process in the production of Port has a fundamental role in determining the quality of the finished product. During this maturation period wine suffers a number of compositional changes, with the levels of some substances decreasing over time while others increase or arise. Changes become more pronounced with extended aging and have significant effects on the color and aroma of the wine.

Because of the route by which they are formed, certain compounds accumulate progressively over time and can be considered genuine age indicators (*1*). The typical aroma developed during barrel storage is the consequence of this chemical behavior, and is usually described as "maderised", "rancio", "burnt", "dry fruit", "tawny", "nutty", and "spicy". It is important to note that the quality of barrel-aged port wine is evaluated solely from these sensorial properties. Furthermore, the age certification of this product is also based on the sensorial analyses effectuated by the "Instituto do Vinho do Porto" (IVP).

Several studies have been published concerning the volatile composition of Port wine (*2*−*7*). The presence of some substances such as aldehydes and methyl ketones were related to the "rancio" odor of oxidative aged Port wine (*8*), as it was also suggested for white wines (*9*). On the other hand, different authors suggested that 3-hydroxy-4,5-dimethyl-2(*5H*)-furanone (sotolon) can contribute to the typical aged aroma of wines. This is the case of Jura wines "vin jaunes" (*10*−*15*), "vins doux naturels" (*16*, *17*), Tokay wines (*13*, *18*), Botrytized wines (*19*, *20*), and Sherry wines (*14*).

Some studies dealing with the descriptive analysis of Port wine aroma have been published (*21*, *22*), as well as some statistical methodology for sensorial analysis (*23*). Nevertheless, the relative contribution of the volatile substances present in Port wine and the respective perceived aroma is still unknown. Several methods using gas chromatography coupled with olfactometry procedures are available in the literature for the purpose of ranking substances by their respective impact on the overall aroma of a foodstuff. They can be divided into the following categories: (i) dilution procedures like CHARM analysis (*24*) or aroma extract dilution analysis (AEDA) (*25*); or (ii) intensity measurement methods such as hyphenated headspace-GC-sniffing (26) or frequency counting with scoring attribution (*27*).

The AEDA technique proved to be very powerful for screening the impact of odor contributors to an aroma and

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Table 1. Sensorial Protocol: Wine Sample Preparation

	sample *J*,1	sample *J*,2	sample *J*,3	sample *J*,4	sample *J*,5
set $J = 1$	R[a]	R + 25 μg/L	R + 50 μg/L	R + 100 μg/L	BS[a]
set $J = 2$	R[a]	BS[a]	BS + 25 μg/L	BS + 50 μg/L	BS + 100 μg/L
set $J = 3$	BS[a]	10Y[a]	10Y + 25 μg/L	10Y + 50 μg/L	10Y + 100 μg/L

[a] Non-supplemented wine samples: 4 year-old Ruby (R), blended sample (BS), and 10-year-old (10Y) with sotolon concentrations of not detected (<2.02 μg/L); 58 μg/L; and 91 μg/L, respectively.

identifying molecules in several foodstuffs (*28*−*32*) and also in wine (*33*, *34*).

Hence, the aim of this study was the chemical characterization of Port, with particular emphasis on the volatile fraction, in order to identify substance(s) with large impact on the characteristic aroma of aged Port wine. AEDA was the chosen technique because it is not time-consuming, it is simple (almost no need to perform "assessors calibration"), and it was appropriate for the screening nature of this work. Validation of the selected substances by sensorial analysis was also attempted.

MATERIALS AND METHODS

Wine Material − Port Wine Samples. The wine samples (aged-in-barrel) were supplied by the Instituto do Vinho do Porto (IVP) after certification: 35 samples of a single harvest ranging from 1- to 60-year-old "Colheitas" and thirty-six samples of blended wines ("Tawnys") belonging to the four categories "10 years old" (11 samples), "20 years old" (12 samples), "30 years old" (6 samples), and "40 years old" (7 samples). All samples were matured in oak barrels until analysis.

Sensory Studies. *Sensorial Panel.* The panel employed in all sensorial measurements in this work was composed of 18 persons: university students, Port-wine-makers, and laboratory personnel. The panel is permanent and receives weekly training sessions. Tests were performed in individual booths using tulip glasses containing 30 mL of wine at a controlled room temperature of 20 °C.

Descriptor Selection. The descriptor selection was effectuated by the panel using different barrel-aged wines belonging to the "tawny" category of "40-years-old". The AFNOR NFV-09-021 (*35*) procedure was used to select the most important descriptors related to the typical aroma of aged wines. In a first set of sessions, every member of the panel was asked to freely describe the aroma of the wine. The hedonic and redundant terms, as well as the nonpertinent terms, were then disregarded, and a first group of descriptors was obtained. Then, the panel was asked to determine if the first series of descriptors were present or absent. Those descriptors considered as absent by 50% of the panel were eliminated, and a second group was obtained. The panel was then asked to rank each descriptor belonging to this group on a scale of 0 to 10.

Organic Extract Selection−Aroma Representivity. A 40 year-old port wine was extracted with different organic solvents: hexane, ether, ethyl acetate, and dichloromethane. The same volume of wine (50 mL) was extracted twice with 10 mL and 5 mL, with each of the solvents. Similarity tests were performed between the aroma of the obtained extracts and the wine (*36*). Two 2-mL aliquots of each organic extract were concentrated under nitrogen stream until 0.5 mL. A drop was then put on a perfume sampling paper, and the aroma was compared with the original wine as a pair. The panel was asked to rate the similarity on a discontinuous scale from 0 (no similarity) to 10 (equal) of each sample with the 40-year-old wine. The data obtained were treated according to the ANOVA procedure and Tukey's test was used to establish differences among organic solvents (*37*).

Gas Chromatography/Olfactometry. To identify substances responsible for aromatic notes associated with the selected descriptors of the typical aroma of aged Port, GC−olfactometric analysis was employed. Several dichloromethane extracts from different aged Port wines, from 40 to 60 years old, were submitted to GC−O. Extract aliquots of 2 μL each were injected into the GC which was equipped with an olfactometric detector. Chromatographic conditions were the following: Hewlett-Packard HP 5890 gas chromatograph; column BP-21 (50 m × 0.25 mm × 0.25 μm) fused silica (SGE, France); hydrogen (5.0, Air-liquide, France); flow, 1.2 mL/min; injector temperature, 220 °C; oven temperature, 40 °C for 1 min programmed at a rate of 2 °C/min to 220 °C, maintained during 30 min; splitless time, 0.5 min; split flow, 30 mL/min. The make-up gas employed on the olfactometric device (SGE, France) was air (80% N2; 20% O2) (Air-liquide, France). Two streams were used; one was bubbled in water − nose moistener − the other was applied at the exit of the GC column to lower the temperature of the effluent. This procedure was repeated by a panel of four individuals, using the same operational conditions and on the same chromatograph. The odor zones reported by each panel member were compared for each retention index. The descriptors were selected according to their frequency of citations. Hedonic terms were not considered (good/bad) nor were those considered to be analogues, which were replaced by the most cited.

Aroma Extract Dilution Analysis (AEDA). The dilution factors (FD) of the odorants in Port wine extracts were determined by AEDA as described by (*25*). First, 50 mL of a 60-year-old Port wine was extracted twice with 5 mL of dichloromethane. The organic phases were mixed and dried on sodium sulfate. Two mL of the extract was concentrated to 400 μL under nitrogen stream. Then 2 μL of the concentrated extract was separated by capillary column (BP21). The odor-active regions and the odor qualities were evaluated by each assessor. The extract was stepwise diluted with dichloromethane (1 + 1 by volume), and aliquots of the dilutions were evaluated by the same assessor. The process stopped when no aromas were detected by the assessor.

Ranking Testing. The impact of the sotolon in the typical aroma of aged Port wine was evaluated using ranking tests carried out on wine samples of different ages with or without additions of sotolon. Tests were performed in individual booths, and red light was used to mask visual differences between samples. The results were collected after three tasting sessions with the trained sensorial panel of 18 assessors using the same sample preparation for each session.

Two wines certified by IVP were used: 4-year-old ("Ruby") and 10-year-old ("10 anos"). To obtain a "middle sample", these two wines were blended in a mixture of 40% Ruby with 60% 10Anos and designated "blended sample" (BS).

Three sets were studied, as samples were supplemented with three different levels of sotolon: 25 μg/L, 50 μg/L, and 100 μg/L. Each set had five samples, one with no addition, three supplemented samples, and one different wine sample that was common with the next set, as described in **Table 1**.

Samples were randomly codified using four alphanumeric characters. Each set was presented individually, on different days, to each assessor (three per session). The panelists were instructed to smell, but not to taste, the samples and then order them by age using a scale from 1 (youngest) to 5 (oldest) with unit intervals; rank repetition was not allowed. The correlation coefficients between the ranks for each assessor were calculated using the Spearman test (*37*).

The ranks were converted to scores according to the method of Fisher and Yates (*38*). The sample ranked first of five was given a value of −1.16; the second was −0.5; the third was 0; the fourth was +0.5; and the fifth was +1.16. The scores were then subjected to analysis of variance (ANOVA) to determine whether there was a significant difference among samples (at 5% level). To determine which samples were significantly different from another, Tukey's test was used. Samples were arranged according to magnitude, and the honestly significant difference at 95% (HSD) was determined. Any two samples that differed by a value equal to or more than the HSD were regarded as significantly different (*39*).

Threshold Evaluation. Determining the threshold of sotolon constituted a major difficulty in this study. In fact, adding this molecule to a young (<2 years old) Port wine "disrupted" its typical aroma profile (floral, fruity, etc.). In fact, above the recognition threshold the panel rated samples as "not-typical" aged wine. Contrarily, this was not a problem in old wines (>10 years old) because this molecule is one of the typical constituents, being present at concentrations above 50 μg/L. Hence, a compromise solution was taken by selecting a 3-year-old wine to perform this study. In this sample the quantities of sotolon found were above the detection limit of the method and the floral and fruity notes were rated as "weak" by the panel.

The perception threshold of sotolon was established by a triangular test. Wine samples were spiked with this molecule at increasing concentrations. Samples were evaluated by the panel, and the threshold concentration was established when 50% of the panel correctly identified the different sample (*39*).

Chemical Studies. Chemicals were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France): 2-ketobutyric acid (K40-1) (99% purity); 1,2-phenylenediamine (P2,393-8) (99.5%); 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone (W36,340-5) (97%); 3,4-hexanedione (30,693-2) (95%); and 3-octanol (21,840-5) (99%).

2-Ketobutyric Acid Quantification. 2-Ketobutyric acid was quantified after reaction with 1,2-phenylenediamine as previously described (*40*). The quinoxaline derivatives were determined using a Hewlett-Packard 5890 gas chromatograph equipped with a nitrogen phosphorus detector (NPD) and the H. P. Chemstation software. To 50 mL of wine were added 100 μL of 3,4-hexanedione at 543 mg/L as internal standard and 0.5 g of 1,2-phenylenediamine as derivative agent. The pH was adjusted to 2.0 with H_2SO_4 (1 M), and the mixture was reacted at 60 °C during 3 h. After the mixture had returned to room temperature the pH was adjusted to 9.0 with NaOH (1 M), 5 g of Na_2SO_4 was added, and the sample was extracted twice with 5 mL of dichloromethane for five minutes. The extract (2 μg/L) was injected (splitless, 0.3 min) into a BP1-column (Hewlett-Packard, 50 m × 0.22 mm, and 0.20-μm phase thickness). The temperature program was 70 °C (1 min) to 230 °C (20 min) at a rate of 2 °C min^{-1}. Injector and detector temperatures were 220 °C. The carrier gas used was helium at constant pressure and flow of 1.2 mL min^{-1} on initial temperature. The NPD used hydrogen at 50 mL/min and a mixture of nitrogen/oxygen (80/20) at 300 mL/min. The makeup gas was nitrogen at 20 mL/min.

3-Hydroxy-4,5-dimethyl-2(5H)-furanone Quantification. For the extraction procedure, to 50 mL of wine were added 50 μL of 3-octanol in hydro alcoholic solution (1/1, v/v) at 432.9 mg/L as internal standard and 5 g of anhydrous sodium sulfate (increases extractability). The wine was extracted twice with 5 mL of CH_2Cl_2 (SDS, Peypin, France). The two organic phases obtained were blended and dried over anhydrous sodium sulfate. A 2-mL portion of this organic phase was concentrated 5 times under a nitrogen stream with a 1 L/min gas flow. A 2-μL portion of the extract was injected into the GC, which was coupled with an MS detector. Chromatographic conditions were the following: Hewlett-Packard HP 5890 gas chromatograph coupled with a mass spectrometer (HP 5972, electron impact 70 eV, EMV = 2.2 kV, detection mode was selected ion monitoring (SIM) with ion of $m/z = 83$; column BP21 (SGE, France) (50 m × 0.25 mm, 0.25 μm); helium 5.6 Aga pressure, 55 kPa; injector temperature, 220 °C; detector temperature, 280 °C; oven temperature, 40 °C for 1 min programmed at a rate of 2 °C/min to 220 °C, the final step lasting 30 min; splitless time, 30 s; split flow, 30 mL/min.

Optimization and Validation of Analytical Conditions. Studying the stability of compounds with enol-oxo functional groups in gas chromatography, Blanck, et al. (*30*) observed that the degradation of these products became sensitive at low injected quantities (i.e., about 1 ng); this phenomenon is more important since the polarity of the stationary phase is weak. Indeed, for FFAP column (with the highest polarity tested) the losses were minimal: 15% for a quantity close to 1 ng. Thus, we chose to use a FFAP-type capillary column to measure sotolon. Finally, the same dichloromethane extract was injected using a hot injector (classic split/splitless injector; $T = 220$ °C) and using the on-column technique (0.2 μL). It was observed that normalized areas were of similar proportions on both procedures.

The role of the number of successive extractions was studied; the recovery (%) was determined for successive 5-mL dichloromethane extractions. The results for the first and second combined, third, fourth, and fifth extractions were 78.8, 13.3, 4.8, and 2.4% of recovery, respectively.

Although 2× extraction by 5 mL led to a recovery of only 78.83%, it was nevertheless retained. Indeed, the procedure of analysis consisted of the concentration of an aliquot part of the organic phase and not of its totality. Thus, even if the total mass of lactone extracted was higher, because the number of extractions was bigger, it would represent a dilution of sotolon in the injection sample. In fact, between two and three extractions, a dilution of about 50% occurs which was not balanced by the increase of the extracted mass (13.3%).

The reproducibility of the method was calculated from 10 analyses of a wine containing 91.6 μg/L of sotolon. The variation coefficient was found to be 4.98%. The linearity of the method was tested using a young Port wine as matrix; the quantitative analysis of sotolon additions showed that the method was linear for this compound with satisfactory precision. The concentration range tested over seven sotolon additions was between 4.1 and 810 μg/L, and the correlation coefficient between levels added and levels assayed was $r = 0.9998$.

Detection and quantification thresholds were established in a Port wine diluted with 20% (v/v) aqueous-alcoholic solution to bring the sotolon peak to a size as near as possible to the background. Assaying was repeated 10 times; the average value observed was 0.80 μg/L with a standard deviation of 0.122. The detection limit was calculated by adding 3× the standard deviation to this average value, and the quantification limit was calculated by adding 10× the standard deviation to this average value (*34*). Thus, detection and quantification thresholds were found to be, respectively, 1.17 and 2.02 μg/L.

Chemical Ionization Mass Spectrometry (CIMS) and High-Resolution Mass Spectrometry (HRMS). A hybrid Fisons Instruments spectrometer (AutoSpec-EQ with EBEQQ geometry) was used. The mass spectrometer source was kept at 200 °C. Resolution was 5500 and the cycle time for scanning was 1 s for a range of masses between 50 and 650 *m/z*. Acquisition and data processing were carried out using the OPUS system integrated in the apparatus (VAX station 3100 Digital Equipment). For CIMS, the reactive gas was ammonia. For HRMS, calibration was carried out with perfluorokerosene and the measuring accuracy was lower than 50 ppm.

Other Analytical Measurements. Measurements of free SO_2 concentration and chromatic index were performed (*41*). Acetaldehyde, higher alcohols (*42*), acetals (*6*), and furanic aldehydes (*43*) were determined. The Kovat's index was calculated according to the literature (*44*). This determination was performed on polar and apolar phase columns, respectively, such as BP21 (FFAP) (50 m × 0.25 mm × 0.25 μm) and BP1 (50 m × 0.32 mm × 0.4 μm) provided by SGE (France).

RESULTS AND DISCUSSION

Descriptors Selection. Using the AFNOR NFV-09-021 (*35*) procedure, selection of the relevant descriptors of the characterization of the typical aroma of aged Port wine was carried out. Those that obtained the highest ratings were "glue−solvent", "dry fruit", "spicy-like" and "nutty".

GC−O Results. The similarity test effectuated by the panel (between the wine and the respective organic extracts) showed that the typical aroma of the aged Port was better represented in the dichloromethane extract. Hence, this solvent was chosen to perform the GC−Olfactometry. Sixteen odor-active zones were selected as the most frequently cited by the four members of the GC−O panel. Among them, five showed aromas close to those descriptors selected as characteristic of oxidative aged Port wine. They were described as "ethyl acetate−glue" for a retention index (RI) of 1122, "caramel−candy" (RI = 1138), "roasted-peanut" (RI = 1312), "burnt-sugar" (RI = 2030) and "nut", "spicy", "old Port wine", and "burnt sugar" (RI = 2172). The latter olfactory zone was considered as particularly intense

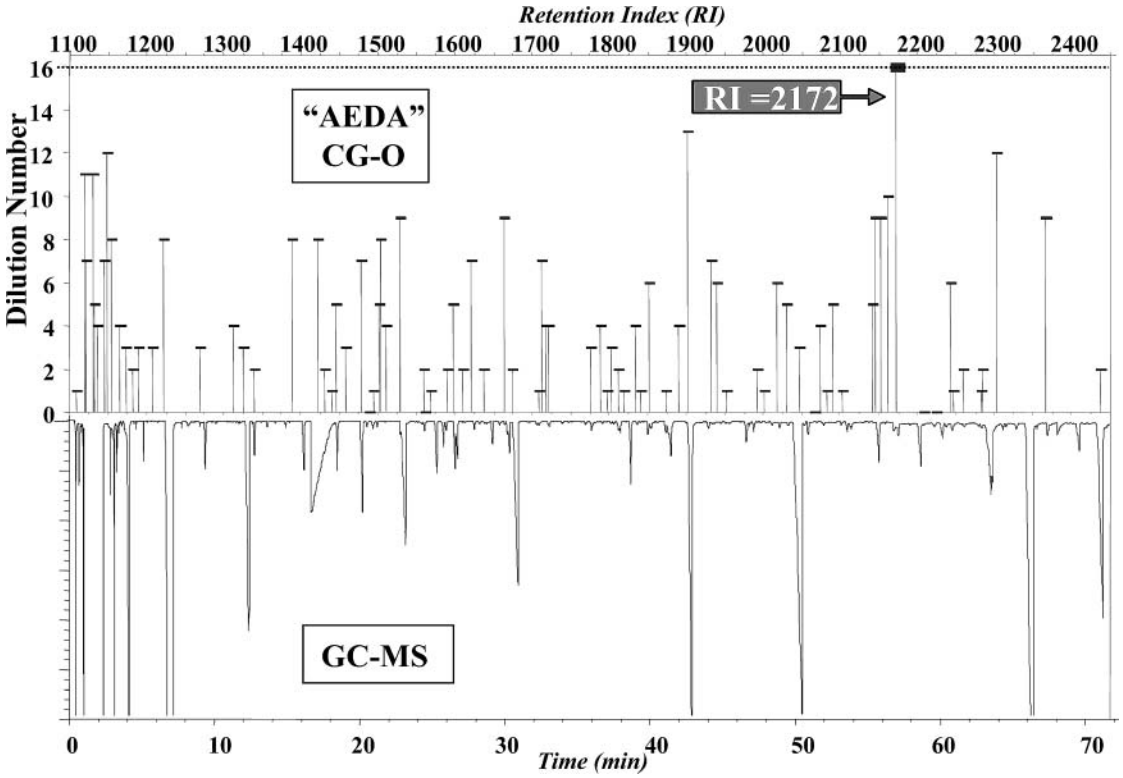

Figure 1. FD and GC-MS chromatograms of a dichloromethane extract of a 60-year-old wine.

and persistent, and most important, the only one that matched closely with one of the descriptors.

AEDA Results. AEDA was used as a screening methodology, not as a quantification measure but to evaluate among the aromatic zones the relative importance of the volatiles for further identification. The odor importance of each of the sixteen zones reported by the GC−O analysis was ranked by AEDA, comparing the FD values obtained. The resulting FD chromatogram and the GC−MS chromatogram of a dichoromethane extract of an aged wine (60 years old) are shown in **Figure 1**.

The dilution factors observed for the five odor zones selected were 128, 32, 16, 32, and 65536, respectively, for the retention indexes 1122 ("caramel-candy"), 1138 ("roasted peanut"), 1312 ("roasted peanut"), 2030 ("roasted peanut"), and 2172 ("nut", "spicy"). The last odor-active compound clearly prevailed over all the others being reported with a particularly high dilution factor. Repetitions of AEDA analysis of extracts obtained from other wines older than 40 years were in agreement with this last observation, i.e. they all presented a higher FD for the RI = 2172 odor zone. Hence, on the basis of these observations the efforts of identification were concentrated on the molecule(s) present in this zone.

Sotolon Identification. The mass spectrum of the old-Port-like flavor compound naturally present in wine, obtained for a retention index 2172 using low-resolution mass spectrometry (GC/LRMS), presented the same peaks, an identical fragmentation, and relative abundances comparable to those of 3-hydroxy-3,4-dimethyl-2(*5H*)-furanone indexed in the NBS 75000 library. The spectrum interpretation has been provided by Martin et al (*14*). Analysis carried out with chemical ionization mass spectrometry (GC/CIMS) showed a basic peak with $m/z = 146$, which corresponded to $[M + NH_4]^+$. This was in agreement with the relative acidity of this compound. High-resolution mass spectrometry confirmed our previous results. Indeed, in view of the accuracy of the determinations, the fragment masses are completely in agreement with the structure suggested (*45*). Moreover, the retention index of the reference compound was respectively 2172 and 1066 for the FFAP and BP1 columns. These values were identical to those obtained for the wine extract and are in agreement with those reported in the literature (*46*). Finally, by GC−O using the same operation conditions, it was verified that the "aromatic quality" of the pure product matched that present in the wine extract.

Sotolon Levels in Wines. The concentration of 3-hydroxy-4,5-dimethyl-2(*5H*)-furanone increases with storage time from a few dozen μg/L in young wines, to about 100 μg/L in 10-year-old wines, to200 μg/L after 10 additional oxidative aging years. The highest contents were observed for wines older than 50 years, i.e., almost 1 mg/L **Figure 2**.

The high correlation coefficient calculated ($r > 0.95$) clearly demonstrated the dependence of sotolon contents on the time of barrel aging under oxidative conditions which makes this molecule an "age indicator". The predictive precision for age estimation (age*) was calculated by construction of the simple linear model (age* = 5.67[sotolon] + 0.067) for 95% of confidence interval as being equal to 11 years. The rate of formation roughly estimated (slope of linear model) as 6 μg/L/year was also observed for the "Tawnys" categories of 10-, 20-, 30-, and 40-year-old blended wines as shown in **Figure 3**.

Formation Mechanisms. Among the mechanisms possibly explaining the formation of 3-hydroxy-4,5-dimethyl-2(*5H*)-furanone in various foodstuffs, two seem to be of particular interest in Port wines. They involve a step of condensation between two carbonyl compounds followed by cyclization. In French "flor sherry" type wine "vin jaune", sotolon could be formed during aging by the aldolic condensation between acetaldehyde and 2-ketobutyric acid produced from threonine due an enzymatic reaction, which is possible only because of the presence of a yeast "flor" (*15*). In Port wine, this "flor" is not present and there is no microbial intervention after the end of alcoholic fermentation, with the exception of some contamination due to an alcohol-resistant lactic bacteria (*47*). A strictly

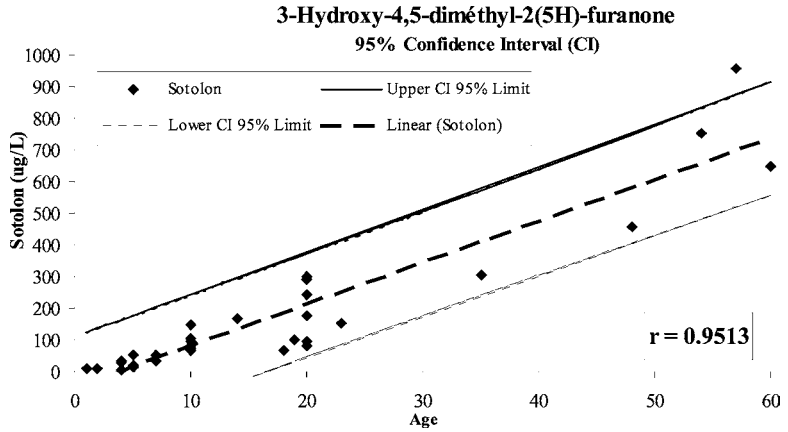

Figure 2. Concentration of sotolon observed in "Colheita" category Port wine (μg/L).

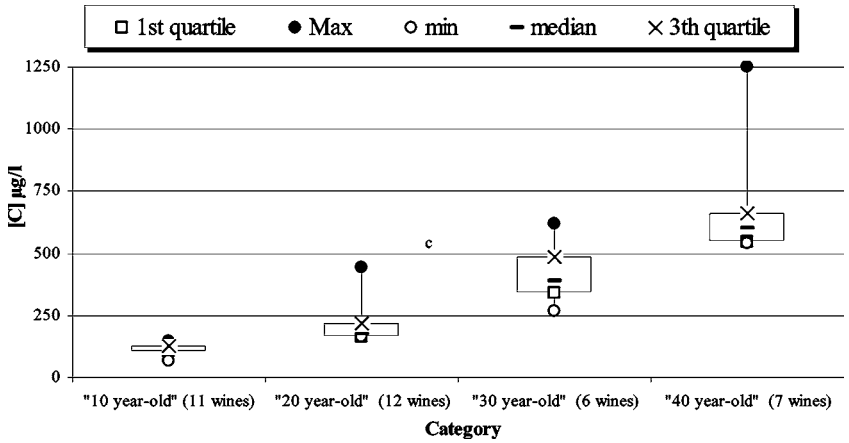

Figure 3. Concentration of sotolon observed in "Tawnys" categories of 10-, 20-, 30-, and 40-year-old wines (μg/L).

chemical degradation of threonine into 2-ketobutyric acid in acidic conditions has also been suggested (*48*).

The quantities of 2-ketobutyric acid found in the samples ranged from 188 to 1722 μg/L. They were always inferior to 500 μg/L for wines older than 10 years, being relatively lower than expected, when compared to the normal levels of threonine present in port wines (e.g., close to 10 mg/L) and sotolon.

Furthermore, the levels of keto-acid do not show any correlation with age nor with the quantities of sotolon found ($r < 0.4$) contrary to the good correlation observed between the furanone and acetaldehyde ($r = 0.8906$). Although the presence of 2-ketobutyric acid can contribute to sotolon formation, due to the linear trend levels observed with time for the furanone, it seems unlikely that this keto-acid constitutes the only source of sotolon in Port wine.

In addition, other studies have shown that 3-hydroxy-4,5-dimethyl-2(*5H*)-furanone can be formed by the Maillard reaction as a result of condensation of molecules such as butane-2,3-dione (diacetyle) and hydroxyacetaldehyde which can arise from this mechanism (*28, 29, 49*). These findings seem relevant considering the high sugar content of Port wine (100 g/L) and the high correlation coefficient observed between sotolon and 5-hydroxymethylfurfural ($r = 0.9015$) in this work. More studies are needed to explain which mechanisms are responsible for sotolon formation during Port wine aging particularly using labeled isotopic precursors in a manner similar to recent works (*50*) where it has been demonstrated that sotolon also can be formed from ascorbic acid degradation products.

Sensorial Impact. The flavor threshold of sotolon was determined to be 19 μg/L in Port wine. This result is totally in agreement with those previously obtained in 12% (v/v) hydro

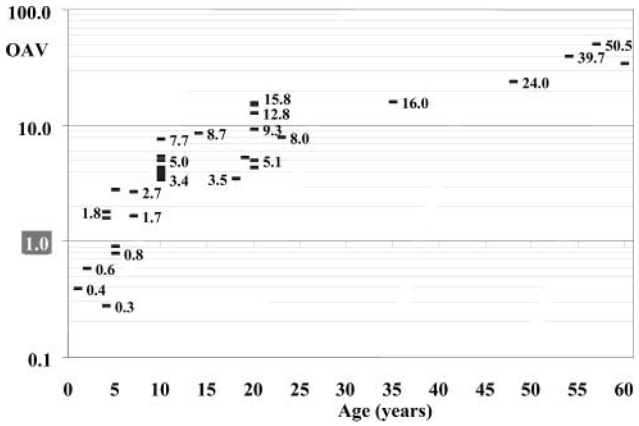

Figure 4. Plot of the logarithm of OAV as a function of age (Fecher's law).

alcoholic solution and "flor" sherry type wine "vin jaune", respectively, by (*19*) and (*14*). Considering uniquely the single effect of sotolon in Port wine expressed by the "odor aroma value" (OAV) it can be seen that sotolon has a positive value in wines close to 10 years old as shown in **Figure 4**.

The behavior shown in the figure is in agreement with the empirical observation in the Port wine industry upon which the "rancio" aroma constitutes a "quality factor" for wines aged in barrels for more than 10 years.

Ranking Results. The estimation of the importance of sotolon in the typicality and persistence of aroma for "flor sherry" has already been published (*14*). Nevertheless, Port wine is submitted to long aging periods (up to 60 years and more) and the value of the product is related to the characteristic aroma

Table 2. Sample Evaluation Results of the Multiple Comparison Test (Significance Level = 95%)

samples evaluation	sample *J*,1	sample *J*,2	sample *J*,3	sample *J*,4	sample *J*,5
set $J = 1$; 4 years old ("Ruby")	R[a]	R + 25 μg/L	R + 50 μg/L	R + 100 μg/L	BS[a]
samples average ranks ($n = 17$)	1.3	2.2	2.9	3.3	4.4
mean rating scores (HSD, 95% = 0.46)[b]	0.89 (a)	0.42 (b)	–0.03 (bc)	–0.26 (c)	–1.02 (d)
set $J = 2$; "blended sample"	R[a]	BS[a]	BS + 25 μg/L	BS + 50 μg/L	BS + 100 μg/L
samples average ranks ($n = 15$)	*1.1*	*2.6*	*2.8*	*3.8*	*4.7*
mean rating scores (HSD, 95% = 0.45)[b]	0.93 (a)	0.18 (b)	0.08 (bc)	–0.36 (c)	–0.83 (d)
set $J = 3$; 10 years old (10Y)	BS[a]	10Y[a]	10Y + 25 μg/L	10Y + 50 μg/L	10Y + 100 μg/L
samples average ranks ($n = 15$)	1.1	2.7	3.1	3.9	4.2
mean rating scores (HSD, 95% = 0.57)[b]	0.93 (a)	0.15 (b)	–0.07 (bc)	–0.45 (c)	–0.55 (c)

[a] Non-supplemented wine samples 4-year-old "Ruby" (R), "blended sample" (BS), and 10-year-old ("10Y") with a concentration of sotolon, respectively, of not detected (<2.02 μg/L), 58 μg/L, and 91 μg/L. [b] The results are shown using letters to indicate differences; any means not followed by the same letter are significantly different at the 5% level.

developed during this long maturation. To gather more information concerning the impact of sotolon on the typical aroma associated with the age of Port wine by the consumer, a simple ranking experiment was carried out.

A trained panel was given three sets of wines supplemented with three levels of lactone as described in the Materials and Methods Section. The panel was asked to rank the samples from youngest (1) to oldest (5) based only on the perceived aroma.

The correlation between the rank order for age attributed by each assessor and the rank order for sotolon concentration in samples (or real age) was calculated by the Spearman method (*37*). Assessors were eliminated when their correlation coefficients were lower than −0.5. From a maximum of 18, the total number of answers taken into account for the first, second, and third sets were, respectively, 15, 15, and 17. The ranks were converted into scores, and the ANOVA treatments, for 95% significant level, showed for each set no differences between assessors and differences among samples with p values for sets $J = 1$ (4 years old), $J = 2$ (blended sample), and $J = 3$ (10 years old) of 1.233×10^{-17}, 1.489×10^{-17}, and 1.252×10^{-11}, respectively. Obtained results for the simple ranking test are shown in **Table 2**.

The panel ranked the three sets of samples on an increasing manner according to the real age and the increasing levels of sotolon additions (samples average ranks, **Table 2**). To determine which samples differ significantly in "average ranked age" after scoring translation, the comparison of the samples mean scores using HSD was calculated. For the first set ($J = 1$; 4 years old) and second set ($J = 2$; blended sample) samples 1, 2, and 5 were significantly different from each other; the panel recognized the odd samples, sample 1,5 and sample 2,1, respectively, for the first and second sets. The lower level of sotolon addition (25 μg/L) in the first set was considered different from the sample not supplemented (sample 1,1/sample 1,2). This was not verified in the second set (sample 2,2/sample 2,3), and it could be related to the concentrations of sotolon present in samples not supplemented under and above threshold values in the case of "Ruby" and "blended sample", respectively. It is important to note that in the second set sample 2,5 clearly rated as the oldest. Among the samples of the third set (10-years-old) the differences are not so important, with only samples 3,1 and 3,5 being significantly different from all the others. Nevertheless, samples supplemented with 50 μg/L and 100 μg/L differed from the nonsupplemented samples.

CONCLUSION

The GC−olfactometry and AEDA analysis of an aged Port wine led to the identification of a substance, 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone (sotolon), which could be related to the descriptors "nutty", "spicy". The quantification of this molecule both in "Colheita" and "Tawnys" categories clearly showed a high dependence between sotolon levels and maturation time. 2-Ketobutyric acid was found in samples which could contribute to explaining sotolon formation in Port wines. Nevertheless, the high correlation coefficient observed between 5-hydroxymethylfurfural and sotolon suggests that different mechanisms, related to sugar degradation, could be responsible for the constant rate of formation observed. Further work needs to be done, preferably using more powerful techniques such as isotopic labeled compounds, to clarify this point.

The threshold value determined as 19 μg/L was always above the quantities present in wines older than 10 years. Finally, it was attempted to correlate the levels of sotolon found with the typical aroma of aged Port wine. A ranking test was implemented in which increasing quantities of sotolon were added to different sets of wines. These tests provided some valuable information concerning sotolon impact on aged port wine aroma. In fact, samples supplemented with this substance were consistently ranked as older. However, these results need further sensorial experimental approaches, such as age scoring attribution, in order to establish the extent to which sotolon plays a role in aged Port wine.

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