Chemicals with Sweet Aroma Descriptors Found in Portuguese Wines from the Douro Region: 2,6,6-Trimethylcyclohex-2-ene-1,4-dione and Diacetyl

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2,6,6-Trimethylcyclohex-2-ene-1,4-dione (TMCHD), a norisoprenoid with a sweet honey aroma descriptor, is reported for the first time as a minor constituent of single-varietal table and fortified wines from the demarcated Douro region. Olfactory gas chromatography (GC-O) of a volatile wine extract, previously isolated by preparative gas chromatography, indicated the presence of a zone containing an intense honey descriptor. The targeted odor compound was identified by GC-MS, GC-O, and Kovats index. Quantitative analysis using a selected characteristic ion (*m*/*z* 96) indicated that young Douro fortified wines from the 1997 vintage contained up to 4 μg/L TMCHD. The sweet honey sensory threshold limit for TMCHD in a model Port wine solution was found to be 25 μg/L. TMCHD is therefore only likely to contribute as a collective element to Port wine aroma. The wine volatile diacetyl was identified as a strong contributor to the sweet caramel aroma descriptor often associated with Port.

Keywords: *2,6,6-Trimethylcyclohex-2-ene-1,4-dione; diacetyl; GC-MS; preparative GC; olfactory GC; Port wine*

INTRODUCTION

The aroma of wine is exceptionally complex, with contributions from many hundreds, possibly thousands, of volatile compounds. Some of these volatile metabolites such as terpenols and 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 (*1*−*3*).

Among the large number of native grape varieties known in the Douro region, Touriga Nacional and Touriga Francesa are distinguished by their superior quality, being perfectly adapted to the schistous soil that characterizes the region, as well as the climate, which is cold during the winter and very hot and dry during the summer. The high temperatures experienced during final berry maturation are important for the development of aromas, which add complexity and varietal characteristics to the flavor of Douro style wines (*4*).

Young wines are often characterized by distinctive aromas: "sweet" (e.g., caramel, honey), "red fruit" (e.g., red and black currant, cherry), "floral" (e.g., rose, violet), "nutty" (e.g., almond, hazelnut), "balsamic", and "resinous" (e.g., rock-rose, pine, eucalyptus). During wine aging, some of the compounds responsible for these aromas disappear or undergo important structural transformations by oxidations (*5*, *6*) and acid-catalyzed reactions (*1*, *7*), 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), and "nutty", "tobacco", etc.

There is presently little known about the aroma compounds responsible for the characteristic odor descriptors associated with Portuguese wines (*4*, *8*−*11*).

The aim of the current work was to identify volatile compounds that contribute to sweet aroma descriptors often associated with the bouquet of young Portuguese wines from the Douro region of northern Portugal.

MATERIALS AND METHODS

The wines investigated in this study were kindly donated by ADVID (Associação para o Desenvolvimento da Viticultura Duriense). Wines were stored in bottle, without having experienced wood maturation, and were analyzed within 12 months of the 1997 vintage.

Preparative Gas Chromatography (GC). Wine (750 mL) was extracted with dichloromethane (3 × 50 mL), and the organic phase was dried (anhydrous Na_2SO_4) and concentrated to 2 mL under reduced pressure at 20 °C (rotary evaporator). Further concentration to 1 mL was carried out by solvent stripping, using a stream of argon. One hundred microliters of the resulting concentrate was injected and separated by preparative GC, trapping four consecutive volatile fractions, A−D (each 30 min; see Figure 1) in dichloromethane (4 × 4 mL).

Preparative GC−flame ionization detection (FID) used a Varian Aerograph 1740 equipped with a packed column (100 × 1.0 cm i.d.) containing Carbowax 20M in Chromasorb W (60/80 mesh). Oven temperature was programmed from 40 to 210 °C at 2 °C/min. Nitrogen carrier gas flow was 10 mL/min.

Olfactory GC (GC-O). Fractions A−D obtained from preparative GC were each concentrated to 20 μL using a stream of argon. Two microliters of each concentrate was analyzed using a Varian Star 3400 CX gas chromatograph

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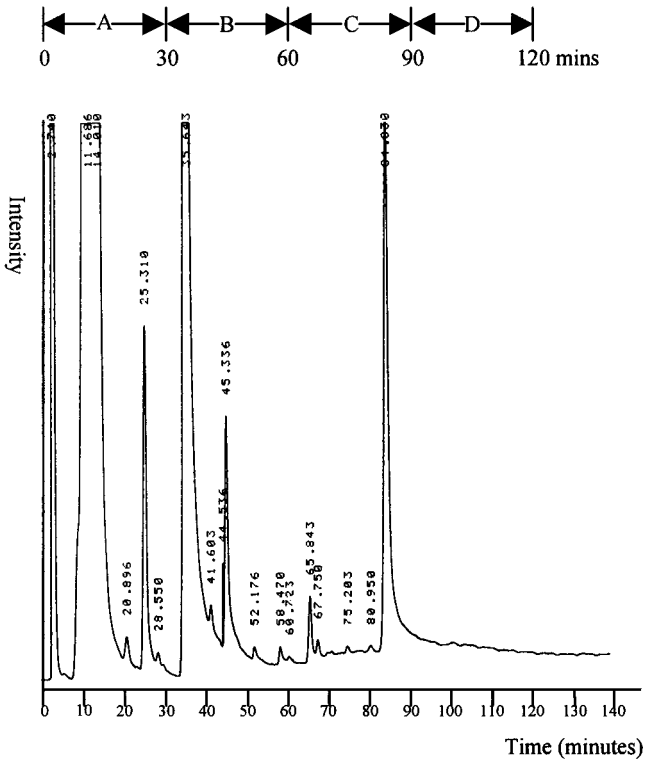

Figure 1. Fractionation of volatiles of a Touriga Francesa table wine extract by preparative GC.

equipped with a Supelcowax 10 column (60 m × 0.25 mm i.d. and 0.25 μm film thickness). Injector (split/splitless, 45 s) and FID temperatures were 250 °C. The carrier gas was helium (1.0 mL/min), with the column effluent being split 1 in 2, to enable both FID and olfactory sniffing port analysis (SGE International). Air, hydrogen, and helium makeup flow rates were, respectively, 300, 30, and 30 mL/min.

Oven temperature was programmed for 20 min at 40 °C, raised at 1.5 °C/min to 200 °C, held for 4 min at 200 °C, followed by a 10 °C/min ramp to 250 °C, and finally held for 120 min at 250 °C.

A second nonpolar Rtx-5MS column (30 m long; 0.32 mm i.d.; 1.0 μm film thickness; DB-5MS equivalent; 5% phenyl-, 95% methylsiloxane) was also employed to verify standard purity and to determine component Kovats indices (KI). Chromatographic conditions were the same as those reported above for the polar Supelcowax column.

GC-O: Isolate Preparation for Qualitative GC—Mass Spectrometry (GC-MS) Analysis. Odorous fractions were collected by the condensation of sniffing port effluent (with sniffing funnel removed) into a 1 mL volume glass chromatography vial (precooled in a freezer at −20 °C). The vial was then closed with a screw-capped septum lid and cooled (2 min at −20 °C), and 10 μL of diethyl ether was added. The closed vial was warmed to allow solvent/component vaporization (homogenization), followed by condensation at −20 °C. The extract was then ready for analysis by GC-MS.

Qualitative GC-MS. Qualitative GC-MS analysis (injection = 2 μL condensate) employed the NIST '98 Mass Spectral Library for the attempted identification of the chemicals responsible for the caramel aroma (GC-O isolate FI; fraction A; Figure 2) and honey aroma (GC-O isolate FII; fraction C; Figure 2). The same chromatographic conditions were used as those reported for the quantitative GC-MS analysis below.

Sample Preparation for Quantitative GC-MS. Wine volatiles were extracted with a 1:1 mixture of hexane/diethyl ether using the method previously reported (*4*). The internal standard isophorone (100 μL; 130 mg/L) was added to both wine and calibration standards prior to extraction. Quantitative analysis used the GC-MS method described below.

Quantitative GC-MS. Quantitative analysis used a Saturn II (Varian) ion trap mass spectrometer (multiplier voltage, 2550 V; emission current, 10 μA; scan rate, 0.60 s; detector temperature, 170 °C; mass range *m/z*, 30−250) coupled with a Varian 3400 gas chromatograph, equipped with a Supelcowax 10 fused silica capillary column (60 m × 0.25 mm i.d., film thickness = 0.25 μm). Oven temperature was programmed as follows: 40 °C for 20 min; 1.5 °C/min to 200 °C; 200 °C for 4 min; 10 °C/min to 250 °C; 250 °C for 120 min. The helium gas flow was 1 mL/min.

The injector was programmed as follows: 70 °C for 0.1 min; 180 °C/min to 250 °C; 13.9 min at 250 °C; finally isothermal at 70 °C.

2,6,6-Trimethylcyclohex-2-ene-1,4-dione (TMCHD) was quantified by comparing the intensity of its characteristic ion peak (*m/z* 96; KI 1676) with that of the characteristic ion peak for the added internal standard (isophorone; *m/z* 82; KI 1579) in the selected ion chromatograms for both wine extracts and standard solutions.

A linear calibration curve ($r^2 > 0.996$) based on the characteristic ion peak areas was established for standard solutions submitted to the same analysis procedure.

Odor Threshold Determination. The threshold limit for TMCHD was estimated by triangular tests employing a panel of eight experienced tasters; each panelist searched for the lowest concentration that he/she was able to characterize by smell alone. Thresholds were determined in two model ethanolic wine systems (12 and 20% ethanol; pH 3.5; tartaric acid, 5.0 g/L) and were defined by the minimum concentration that 50% of the tasters could sense (*5*). The commercial standard (98%), obtained from Aldrich, was examined by both GC-MS and GC-O to verify purity. The absence of any low threshold odorous impurity (GC-O), examined using both polar (Supelcowax 10) and nonpolar (Rtx-5MS) columns, confirmed both the compound's purity and its sweet honey aroma descriptor.

The threshold limits for both the buttery and caramel aroma descriptors, associated with the fermentation chemical diacetyl, were determined in the synthetic Port wine solution (20% ethanol; pH 3.5; tartaric acid, 5.0 g/L) by an experienced panel of nine tasters. The diacetyl standard (97%) was bought from Aldrich, and its purity was verified by both GC-O and GC-MS.

One odor unit (OU) equals the compound's concentration divided by the compound's sensory threshold limit.

RESULTS AND DISCUSSION

Qualitative Examination of Sweet Aroma Descriptors Found in Red Douro Wines. Four fractions (A−D) were obtained following preparative GC of a CH_2Cl_2 extract of a monovarietal, Touriga Francesa, table wine (Figure 1). This initial separation step enabled the isolation of zones of concentrated volatiles, which were consequently examined by GC-O (Figure 2). Two of the detected odors, which had intense sweet descriptors, namely, caramel (fraction A) and honey (fraction C), were chosen as important targets for identification (Figure 2). These important odor descriptors were common, being present in extracts isolated from both table wines and Port wines made from Touriga Francesa and Touriga Nacional grapes.

Caramel Descriptor (KI 0956). The chemical responsible for the intense sweet caramel odor detected in fraction A (KI 0956; Figure 2) was condensed in a vial attached to the GC-O sniffing port exit (isolate FI; 10−12 min). The isolate was examined by GC-MS (see Materials and Methods), and the target compound was tentatively identified, using the NIST '98 spectral data library, to be diacetyl. Confirmation was achieved by comparing mass spectral data for a commercial standard (Figure 3). Further examination of the commercial standard by GC-O, using both the polar Supelcowax and nonpolar Rtx-5MS columns, confirmed its intense caramel aroma, giving respective Kovats indices of 0956 and

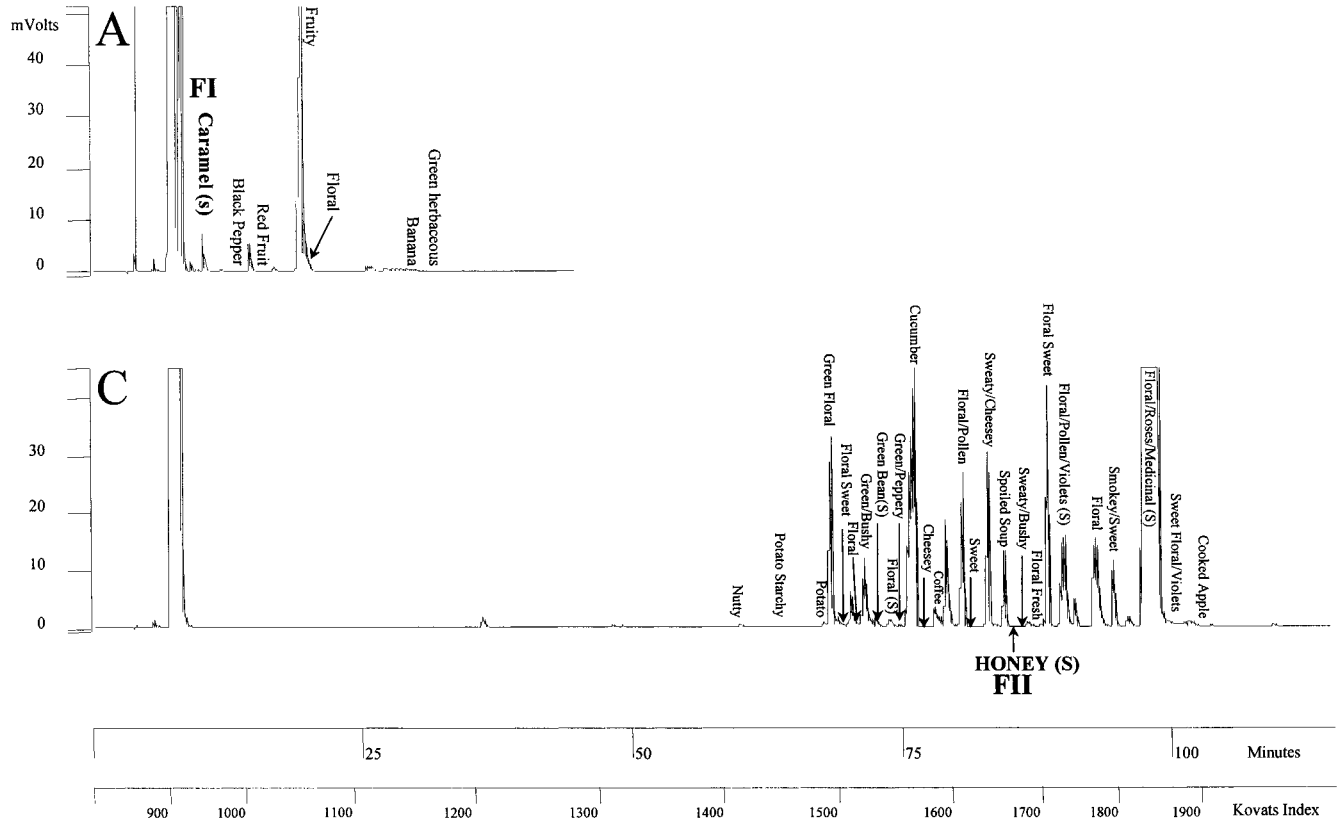

Figure 2. GC-O of volatile fractions A and C isolated from a Touriga Francesa table wine: (s) intense aroma; FI, isolate I "caramel" (10−12 min); FII, isolate II "honey" (85−86 min).

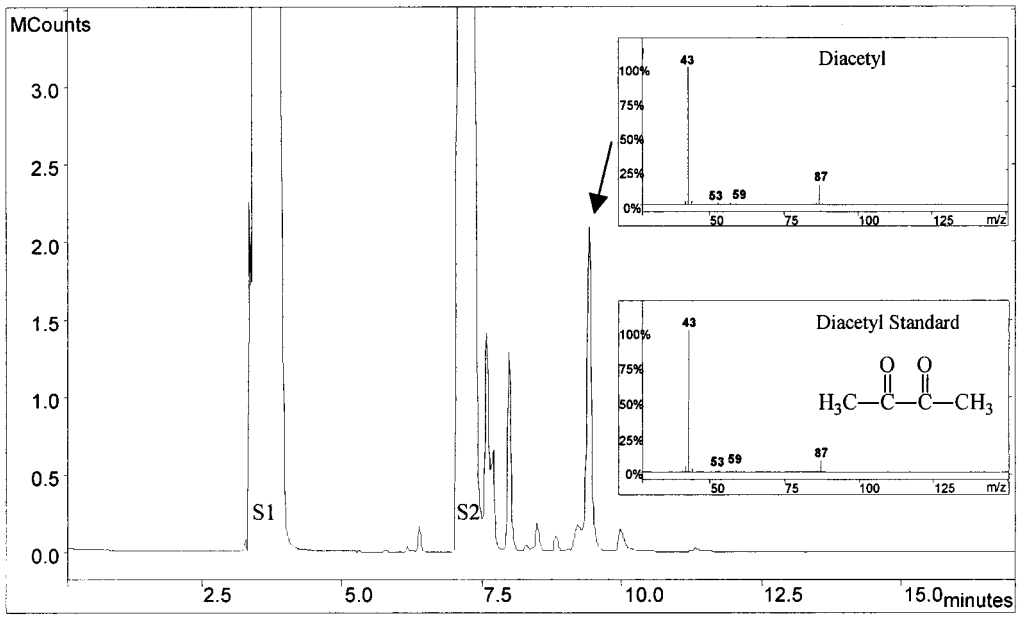

Figure 3. GC-MS identification of the odorous caramel peak in isolate FI. S1, diethyl ether; S2, dichloromethane.

0590. This chemical is more commonly described as having a buttery aroma; however, we found that at elevated concentrations, it contributes a sweet caramel-like odor. This finding is in agreement with the caramel/fatty descriptor given to diacetyl, an aroma active component found in rehydrated French beans, bell peppers, and leeks (*12*).

Honey Descriptor (KI 1676). The condensation method employed for the detection of the caramel aroma found in fraction A failed to identify the chemical responsible for the honey descriptor present in fraction C. The isolate (85−86 min; isolate FII, Figure 2) collected from the GC-O sniffing port contained excessive quantities of masking components. Following GC-MS structural determination of compounds present in the odorous zone containing the isolate FII, various aromatic chemicals including furans, lactones, and diones emerged as probable targets. The standard 2,6,6-trimethylcyclohex-2-ene-1,4-dione was consequently identified by GC-MS as a likely candidate having the same Kovats index (1676) as the detected honey descriptor. Further GC-MS analysis, investigating relatively dilute

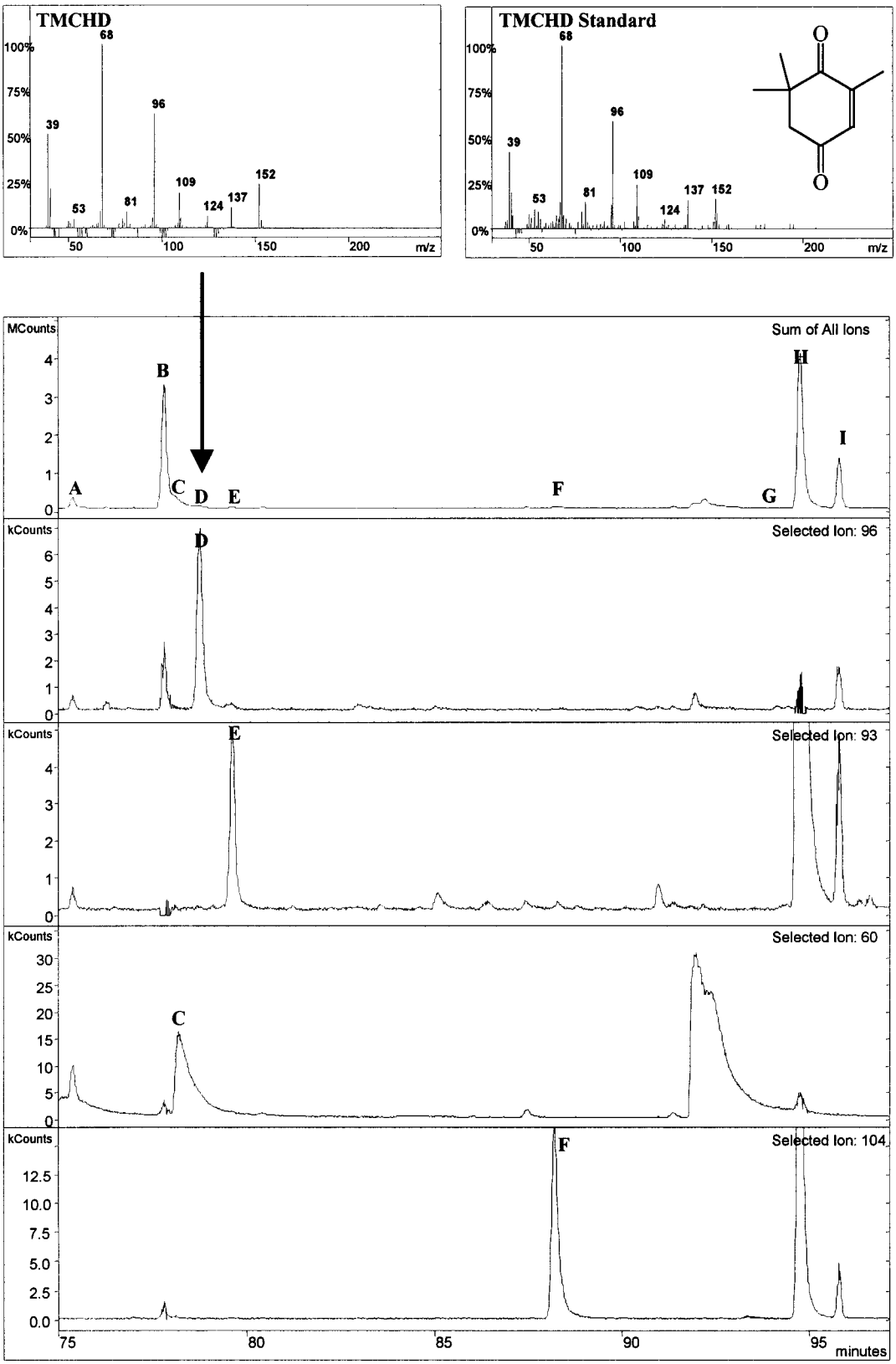

Figure 4. GC-MS identification of the honey aroma peak present in a hexane/diethyl ether extract of a Touriga Francesa Port wine: (A) ethyl decanoate (KI 1639); (B) diethyl succinate (KI 1668); (C) 3-methylbutanoic acid (KI 1671); (D) TMCHD (KI 1676); (E) α-terpineol (KI 1694); (F) 2-phenylethyl acetate (KI 1783); (G) benzyl alcohol (KI 1882); (H) 2-phenylethanol (KI 1887); (I) BHT.

diethyl ether/hexane wine extracts (50 mL of wine extracted into 10 mL of solvent), revealed a target compound (KI 1676) with a mass spectrum identical to that of the TMCHD standard. GC-O analysis of the standard using the polar column confirmed both the compound's Kovats index and its intense honey aroma descriptor. TMCHD (KI 1676) was well separated from other peaks (Figure 4), eluting after ethyl decanoate (KI 1639) and diethyl succinate (KI 1668) and before α-terpineol (KI 1684). Diethyl succinate and α-terpineol serve

Table 1. Levels of TMCHD Present in Single-Varietal Port Wines from the 1997 Vintage[a]

cultivar	wine	TMCHD, KI 1676 (μg/L)
Touriga Francesa	TF1	2.3
	TF2	2.1
	TF3	2.2
	TF4	2.6
	TF5	3.0
	TF6	2.6
	TF7	3.3
Touriga Nacional	TN1	3.1
	TN2	2.4
	TN3	2.6
	TN4	2.9
	TN5	2.6
	TN6	2.3
	TN7	2.3
	TN8	3.3
Tinta Roriz	TR1	2.4
	TR2	2.9
Tinta Barroca	TB1	2.5
Tinto Cão	TC1	2.9

[a] All data are averages obtained from duplicate extractions.

Table 2. Sensory Threshold Determinations for the TMCHD Sweet/Honey Descriptor in Model Wine Solutions[a]

	concn (μg/L)					
model wine	3.1	6.3	12.5	25.0	50.0	100.0
12% EtOH	0	0	37.5	50.0	62.5	87.5
20% EtOH	0	25.0	37.5	75.0	62.5	75.0

[a] 12 and 20% ethanol; pH 3.5; tartaric acid, 5.0 g/L. Data represent the percent of tasters able to sense the sweet/honey descriptor.

as useful markers for the Supelcowax 10 column. Examination of the standard by GC-O using the nonpolar column confirmed its distinctive sweet honey aroma, with Kovats index 1153. TMCHD is most likely derived from carotenoid degradation and is a likely contributor to the sweet, honey aroma often associated with Douro wines.

Quantitative Analysis of TMCHD in Douro Fortified Wines. The analysis of 19 monovarietal fortified wines from the Douro (1997 vintage) indicated typical levels of TMCHD to be between 2 and 4 μg/L (Table 1). The observed levels were similar for each of the five cultivars examined. Table wines made from both Touriga Nacional and Touriga Francesa grapes were also observed to contain this odorous compound (GC-MS); however, levels were not quantified.

Method reproducibility, examining extraction through GC-MS analysis, for six extracts of the same wine gave a coefficient of variation of <6%.

The present literature suggests that compounds such as TMCHD are formed from grape precursor(s), being released by either acid or enzymatic hydrolysis. Indeed, TMCHD has been shown to be a minor product resulting from the acid hydrolysis of the norisoprenoids 3-oxo-α-ionol and vomifoliol (*13*). It has been identified as a precursor constituent of white grapes (*14*, *15*), with up to 20 μg/L being released following acid precursor hydrolysis of a 1988 Chardonnay juice (*15*). This level is particularly interesting, being close to the sweet honey threshold limit of 25 μg/L estimated in the present study.

More recently, TMCHD was released by enzymatic precursor hydrolysis from volatile precursors extracted from red Merlot grape juice (*16*). The compound has also been shown to be a component of oak (*17*), but to our knowledge it has not been identified as a free constituent of wines made from either red or white grapes and it has not been characterized by olfactory techniques.

The compound has also been identified in honey (*18*, *19*), tea (*20*−*23*), saffron (*24*), and tobacco (*25*−*28*).

Sensory Analysis of TMCHD. An experienced panel of eight tasters estimated the sweet honey threshold limit (by smell alone) for TMCHD in a model Port wine solution to be 25 μg/L. Similar results were obtained for both the 12 and 20% synthetic wine solutions (Table 2). Although TMCHD levels in young wines were found to be only between 2 and 4 μg/L (Table 1), it is likely that the norisoprenoid will contribute as a collective element to the sweet descriptors associated with Douro wines.

Quantitative and Sensorial Data for Diacetyl. Levels of diacetyl were not quantified in the present study. However, the maximum level found in Port wine in a recent study (*10*) was 7.8 mg/L. The sensory detection level for diacetyl has been reported as being 4 and 12 mg/L, respectively, for white and red table wines (*29*). Martineau et al. (*30*), however, determined significantly lower detection limits for the varietal wines Chardonnay (0.2 mg/L), Pinot noir (0.9 mg/L), and Cabernet Sauvignon (2.8 mg/L), Silva Ferreira (*10*) concluded that the role of diacetyl in the aroma of Port wine is limited due to its being present at levels below its perception threshold. However, in the current study, diacetyl descriptors were observed to have considerably lower sensory threshold limits in a model Port solution [20% EtOH; 5 g/L tartaric acid; pH 3.5): buttery (19,5μg/L); caramel (1.25 mg/L]. It was also clear that a transition occurred, with the buttery aroma intensifying with increasing concentration to give a much sweeter, caramel-like odor (Table 3). As previously stated, the maximum level of diacetyl, which has been detected in Port wine, is reported to be 7.8 mg/L (*10*). The calculation of odor units, applying the caramel/buttery threshold limit of 1.25 mg/L (Table 3), gives an estimated maximum for the Ports of Silva Ferreira of 6.24 OU. This level surpasses the detected aroma threshold found for a synthetic Port wine by >6 times. On the basis of the threshold levels determined in this paper, which differentiate between buttery and buttery/caramel notes for model fortified wines, diacetyl is considered for the

Table 3. Sensory Threshold Determinations for Diacetyl Descriptors in a Model Port Wine Solution[a]

	concn (μg/L)										
descriptor	4.9	9.8	19.5	39.0	78.0	156	312.5	625	1250	2500	5000
buttery	0	33.3	55.6	66.7	100	100					
sweet/buttery	0	0	0	11.1	33.3	44.4	55.6	77.8	100	100	100
caramel/buttery	0	0	0	11.1	33.3	33.3	33.3	44.4	77.8	77.8	88.9

[a] 20% ethanol; pH 3.5; tartaric acid, 5.0 g/L. Data represent the percent of tasters able to sense each descriptor.

first time as an important contributor to the sweet caramel aroma descriptor often associated with Port.

Diacetyl accumulates in wine during alcoholic fermentation (*31*); however, the associated mechanism has not been studied (*32*). In the case of beer, diacetyl accumulates as a result of the oxidative decarboxylation of α-acetolactate, which is synthesized by *Saccharomyces cerevisiae* and excreted from the cell (*33*). Yeasts also have the ability to remetabolize diacetyl (*31*). The factors that influence final wine diacetyl concentration have been summarized by Martineau et al. (*32*).

In the case of Port production, the autovinification tanks and traditional open lagars permit a limited oxygenation of the fermenting must, which should favor increased diacetyl production. It is likely that maximum diacetyl biosynthesis will coincide with the highest oxygen concentrations, which occur during the initial phase of fermentation. As a result of fermentation being cut short (between 24 and 48 h) by the addition of wine alcohol, it is probable that diacetyl remetabolism by yeast will be less significant, resulting in a relatively high concentration in wine. These hypotheses require further investigations.

Diacetyl levels are also known to increase as a result of the malolactic fermentation (*32*); however, this is less likely to occur in fortified wines, unless favorable conditions prevail for bacterial spoilage.

Further research is planned to investigate levels of TMCHD in wood-aged Ports, which may include contributions extracted from oak (*17*). Bottle-aged white wines will also be investigated, because they commonly develop resinous, waxy, and honey odors (*34*).

ABBREVIATIONS USED

TMCHD, 2,6,6-trimethlcyclohex-2-ene-1,4-dione; GC-O, olfactory gas chromatography; FID, flame ionization detection.

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