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# Synthesis of Deuterium-Labeled 1,1,6-Trimethyl-1,2-dihydronaphthalene (TDN) and Quantitative Determination of TDN and Isomeric Vitispiranes in Riesling Wines by a Stable-Isotope-Dilution Assay

Recep Gök,[†] Pia Bechtloff,[†] Michael Ziegler,[‡] Hans-Georg Schmarr,[‡] Ulrich Fischer,[‡] and Peter Winterhalter*[,†]

[†]Institute of Food Chemistry, Technische Universität Braunschweig, 38106 Braunschweig, Germany

[‡]Institute for Viticulture and Oenology, Dienstleistungszentrum Ländlicher Raum (DLR) Rheinpfalz, 67435 Neustadt an der Weinstraße, Germany

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**ABSTRACT:** The $C_{13}$-norisoprenoid aroma compounds 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) and isomeric 2,10,10-trimethyl-6-methylene-1-oxaspiro[4.5]dec-7-enes, so-called vitispiranes, are considered to be biosynthetically related. They occur at higher concentrations in bottle-aged Riesling wines especially and are important contributors to the varietal aroma of Riesling wines. Because of the variation of the quantitative methods and data reported in the literature, a redetermination of concentration levels for both free and total TDN and isomeric vitispiranes, especially in German Riesling wines, was performed using a stable-isotope-dilution assay (SIDA). For this purpose, a novel six-step synthetic route to TDN and deuterium-labeled TDN was developed. A standardized sample preparation for TDN and vitispiranes and a rapid acid-hydrolysis method at genuine wine-pH conditions for the conversion of the precursors into TDN and vitispiranes were also developed. Automated HS-SPME was applied to 250 wine samples from two wine competitions, and analysis was performed by gas chromatography−mass spectrometry with selected-ion monitoring (GC-SIM-MS) as well as selected-reaction monitoring (GC-SRM-MS).

**KEYWORDS:** *1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), isomeric vitispiranes, synthesis, stable-isotope-dilution assay, petrol note, kerosene off-flavor, Riesling*

## INTRODUCTION

Riesling belongs to the most important grape varieties in Germany with a cultivated area of 23 809 ha, which corresponds to 23.2% of the German wine-growing area. Thus, Germany has 37.2% of the world Riesling cultivation, followed by Romania (6121 ha, 9.6%), the United States (4605 ha, 7.2%), France (4025 ha, 6.3%), Australia (3157 ha, 4.9%), Ukraine (2700 ha, 4.2%), China (2500 ha, 3.9%), and Austria (2068 ha, 3.2%).[1] The varietal character of Riesling wines depends on a number of volatile flavor compounds, including monoterpenes and norisoprenoids. One of the key aroma compounds for Riesling, 1,1,6-trimethyl-1,2-dihydronaphthalene, **1.1** (TDN, Figure 1), is a $C_{13}$-norisoprenoid with a "kerosene" or "petrol-like" note, which occurs in higher concentrations in bottle-aged Riesling wines especially. It was first identified in 1978 by Simpson as being responsible for the "kerosene" off-flavor in aged Riesling wines. The flavor threshold of TDN in wine has been reported by Simpson to be in the range of 20.0 μg/L, applying the method by Meilgaard.[2−4] More recently, Sacks et al. determined a detection threshold of 2.0 μg/L[5] for TDN in wine medium using a trained panel, a value that was later confirmed by Ziegler with 2.3 μg/L.[6] In addition to wine medium, Ziegler also determined the detection threshold of TDN in various other matrices with varying carbonation levels, as well as in sparkling wine (6 bar), the latter threshold amounted to 4.0 μg/L.[6] Despite the low detection threshold of TDN, relatively high consumer-rejection-threshold values for Australian consumers of up to ca. 80.0−160.0 μg/L have been described by Ross et al., depending on the composition of the wine.[7] For German consumers, these values were determined to be in the range of 60.0 μg/L for young wines and 90.0 μg/L for aged wines.[6]

Reasons for the occurrence of TDN at higher concentration levels in Riesling wines especially are still not completely understood. TDN is absent in grapes and young wines and steadily increases in concentration during the fermentation as well as during the aging of wine, predominantly by hydrolysis and rearrangements of acid-labile glycosylated carotenoid metabolites (Figure 1).[8,9]

From a biogenetic point of view, xanthophylls, including violaxanthin and neoxanthin, are the most likely TDN progenitors in grapes, giving rise to the formation of 3-hydroxy-5,6-epoxy-α-ionone, **3**, as primary cleavage product. The subsequent conversion step via compound **4** is plausible but still hypothetical, whereas the reduced form, **5a**, and its allylic rearranged isomer, **5c**, have already been identified in Riesling wine as immediate TDN progenitors (Figure 1).[8,9]

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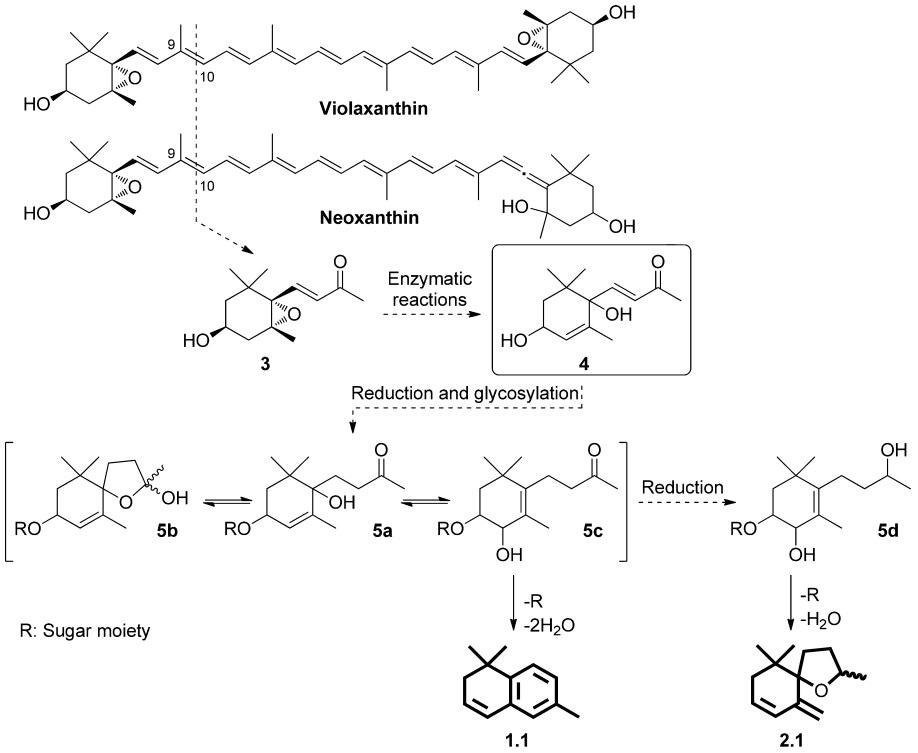


**Figure 1.** Postulated mechanism of formation of TDN and isomeric vitispiranes proposed by Winterhalter and Gök.[9]

The second compound that has also been the focus of the present study is the $C_{13}$-spiroether vitispirane **2.1**, of which two diastereomers can be separated by gas-chromatographic (GC) analysis.[10] The flavor threshold of isomeric vitispiranes was determined by Simpson to be 800.0 $\mu$g/L using the method described by Meilgaard.[3,4] Ziegler[6] determined the detection threshold of vitispiranes to be 101.0 $\mu$g/L in Riesling wine. Biogenetically, TDN and vitispiranes are closely related. Only a reduction step (i.e., reduction of the C9-ketone function in compound **5c**) is required to form the acid-labile progenitor, **5d**, of vitispirane (Figure 1).[9,10]

In the literature, there are various reports on the quantitative analysis of TDN in grapes and wines; however, none have been based on an appropriate isotopically labeled internal standard in a stable-isotope-dilution-assay (SIDA) approach.[5,11−20] Particularly in the flavor-research field, SIDA can be considered as the accepted state-of-the-art method for the most accurate and reliable quantitative determination of volatile compounds.[21] Because of the importance of TDN to the varietal aroma of Riesling wine, we considered it indispensable to develop a reliable SIDA-based quantitative method for the determination of TDN concentrations in Riesling wine. Therefore, a new and convenient synthetic route to TDN and particularly a stable deuterium-labeled isotopologue should be developed. To the best of our knowledge, all of the previously described synthetic routes of TDN[22−25] are not suitable for deuterium labeling as the starting materials used (e.g., $\alpha$- and $\beta$-ionone) may experience possible hydrogen−deuterium (H−D) back-exchange of acidic $\alpha$ C−H positions, as discussed in detail by Gök.[26] Furthermore, in these earlier strategies, chromatographic purification of the target compounds was difficult, possibly because of side reactions that gave rise to the formation of a $C_{13}H_{18}O$-ketone.[23,24] In the case of the vitispiranes, a previously published route for the synthesis was used to prepare deuterium-labeled vitispirane isotopologues.[27−29] The so-obtained deuterium-labeled isotopologues were then used for the development of a SIDA-based quantitation method to determine TDN and vitispirane concentrations in 250 Riesling wine samples.

## MATERIALS AND METHODS

**Chemicals.** L-(+)-Tartaric acid (>99.5%), succinic anhydride (>99%), anhydrous aluminum chloride (>99.5%), [$^2H_8$]-toluene (>99.6 atom % D), toluene (>99.8%), hydrogen chloride−ethanol solution (1.25 M HCl), trimethylaluminum (2 M in *n*-hexane), trimethylsilyl trifluoromethanesulfonate (>99%), trifluoroacetic acid (>99%), trifluoroacetic anhydride (>99%), cerium(III) chloride heptahydrate (>98%), sodium borohydride (>96%), *p*-toluenesulfonic acid (>98.5), hydrochloric acid (37%), sodium bicarbonate (>99.7%), and magnesium sulfate (>99.5%) were purchased from Sigma-Aldrich (Steinheim, Germany). Calcium chloride (>98%), sodium chloride (>99.8%), sodium hydroxide (>99%), and sodium sulfate (>99%) were from Carl Roth (Karlsruhe, Germany). The solvents dichloromethane, diethyl ether, ethyl acetate, and ethanol were analytical-grade; methanol and *n*-hexane were HPLC-grade (VWR Chemicals, Fontenay-sous-Bois, France). Cyclohexane was obtained from Riedel-de Haën (Seelze, Germany). For all experiments, deionized water (Nanopure, Werner, Leverkusen, Germany) was used.

**General Experimental Methods.** Moisture-sensitive reactions were carried out under a static atmosphere of argon gas. All reagents were commercially obtained at the highest commercial quality and used without further purification. Synthesized reference substances were purified by means of silica-gel chromatography, and individual purities were checked by GC and spectroscopic methods, ensuring sufficient purities for their intended uses in quantitative analysis.

Thin-layer chromatography (TLC) was done on silica-gel-60 $F_{254}$ plates (Merck, Darmstadt, Germany), and column chromatography (CC) and flash chromatography (FC) were done on silica gel 0.04−0.063 mm, 230−400 mesh ASTM (Merck).

**Instrumental Analysis for Synthesis Control.** For GC-MS analyses, a Hewlett-Packard HP-5890 gas chromatograph was used and coupled to an HP 5972 mass-selective detector (MS, Agilent,


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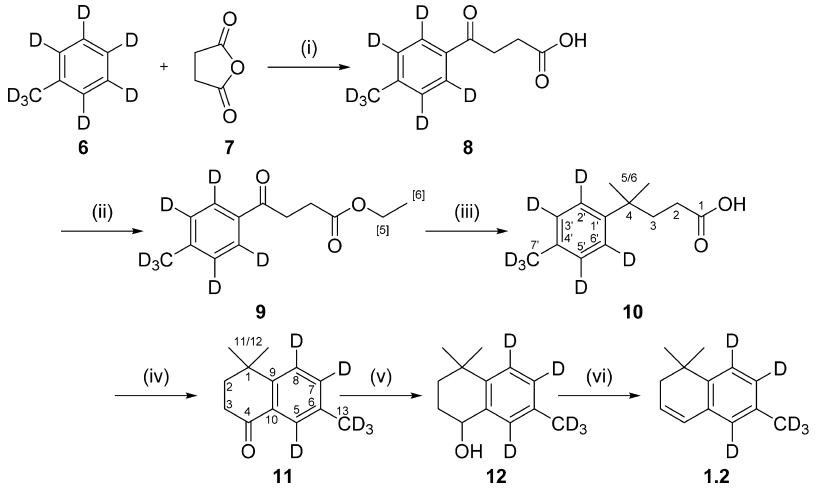


**Figure 2.** Novel synthetic route to TDN-$d_6$ **1.2** and the numbering system for the synthesized compounds (cf. instrumental analysis for synthesis control).

Waldbronn, Germany). Chromatographic separation was achieved with a 30 m × 0.25 mm i.d. fused silica capillary column coated with 0.25 $\mu$m of a polyethylene glycol stationary phase (VF-WAXms, Agilent). The following temperature program was used: the temperature started at 50 °C (1 min isothermal), then was increased at 10 °C/min to 240 °C, and was held at this temperature for 10 min. The carrier gas used was helium with a constant flow of 1 mL/min. The split−splitless-injection port was heated to 240 °C, and the split ratio was set to 1:20. The MS-transfer-line temperature was set to 240 °C, ionization was done in electron-impact mode (EI+), and the ionization energy was set to 70 eV.

NMR experiments were performed on a Bruker FT 300 (300 MHz NMR spectrometer, Rheinstetten, Germany). Samples were recorded at room temperature in $CDCl_3$ (>99.96% D, Deutero GmbH, Kastellaun, Germany) and referenced to tetramethylsilane (TMS, $\delta$ = 0 ppm, Sigma-Aldrich, Deisenhofen, Germany) as the internal standard. Chemical shifts ($\delta$) are reported in parts per million (ppm); coupling constants ($J$) are reported in in hertz (Hz). The numbering system of the synthesized compounds is that of IUPAC for open-chain molecules (**8**−**10**) and that of Miginic[23] for TDN-like molecules (**11**, **12**, and **1.2**; cf. Figure 2).

Infrared spectra (IR) were recorded with a Bruker Tensor 27 attenuated-total-reflectance infrared instrument (ATR-IR, Rheinstetten, Germany).

**Synthesis of TDN and TDN-$d_6$.** The synthesis of TDN was carried out in an analogous manner to the synthesis of TDN-$d_6$, starting with succinic anhydride and toluene (cf. Figure 2).

*4-(4′-[$^2H_7$]-Methylphenyl)-4-oxobutanoic Acid (**8**).* Succinic anhydride, **7** (2 g, 20.00 mmol), and powdered anhydrous aluminum chloride (5.34 g, 40.00 mmol) were ground in a mortar for 1 min under a hood. The mixture was ground vigorously for an additional 10 min after the addition of [$^2H_8$]-toluene, **6** (1.9 mL, 20.00 mmol). The progress of the reaction was monitored by means of TLC. After completion of the reaction, the mixture was treated with crushed ice−HCl (40 mL; 3:1, v/v). The resulting solid was filtered, washed several times with water, and dried under reduced vacuum. The product, **8**, was obtained as a white powder (2.09 g, yield 53%). IR ($\tilde{\nu}$, $cm^{-1}$): 2926 (w), 1660 (s), 1606 (m), 1398−1360 (m), 1226 (s), 1173 (m), 1064 (w), 927 (m), 907 (s), 522 (m), 451 (m). $^1H$ NMR (300 MHz, $CDCl_3$, $\delta$, ppm): 3.30 (t, 2H, $J$ = 6.5 Hz, H-3), 2.81 (t, 2H, $J$ = 6.5 Hz, H-2). $^{13}C$ NMR (75 MHz, $CDCl_3$, $\delta$, ppm): 197.6 (Cq, C-4), 178.7 (Cq, C-1), 144.1 (Cq, C-4′), 133.9 (Cq, C-1′), 33.2 ($CH_2$, C-3), 28.2 ($CH_2$, C-2).

*4-(4′-[$^2H_7$]-Methylphenyl)-4-oxobutanoic Acid Ethyl Ester (**9**).* A mixture of compound **8** (4.52 g, 22.71 mmol) and a hydrogen chloride−ethanol solution (1.25 M, 25 mL) was stirred at room temperature for 4 h. The excess of ethanol was evaporated under reduced pressure. The residue was taken up in ethyl acetate (200 mL) and extracted with water (50 mL). The organic phase was separated, and the aqueous phase was re-extracted with ethyl acetate (3 × 50 mL). The combined organic phases were washed with saturated sodium bicarbonate and brine, dried over sodium sulfate, and evaporated. After concentration, the product, **9** (5.10 g, yield 98%, 94% by GC), was obtained as a yellowish oil. IR ($\tilde{\nu}$, $cm^{-1}$): 2986 (w), 1720 (s), 1675 (m), 1580 (m), 1476 (w), 1414 (w), 1374 (m), 1347 (m), 1220 (s), 1148 (s), 1063 (m), 1028 (m), 984 (m), 940 (m), 860 (m), 658 (w), 523 (m), 484 (m). $^1H$ NMR (300 MHz, $CDCl_3$, $\delta$, ppm): 4.16 (q, 2H, $J$ = 7.1 Hz, H-[5]), 3.29 (t, 2H, $J$ = 6.6 Hz, H-3), 2.75 (t, 2H, $J$ = 6.6 Hz, H-2), 1.27 (t, 3H, $J$ = 7.1 Hz, H-[6]). $^{13}C$ NMR (75 MHz, $CDCl_3$, $\delta$, ppm): 197.9 (Cq, C-4), 173.1 (Cq, C-1), 144.8 (Cq, C-4′), 134.0 (Cq, C-1′), 60.7 ($CH_2$, C-[5]), 33.3 ($CH_2$, C-3), 28.4 ($CH_2$, C-2), 14.3 ($CH_3$, C-[6]).

*4-(4′-[$^2H_7$]-Methylphenyl)-4,4-dimethylbutanoic Acid (**10**).* A solution of trimethylaluminum (2 M in *n*-hexane, 22.50 mL, 45.00 mmol) and trimethylsilyl trifluoromethanesulfonate (4 mL, 22.50 mmol) were added dropwise to a cooled (0 °C) solution of **9** (5.10 g, 22.46 mmol) in dry dichloromethane (26 mL) under an argon atmosphere. This solution was stirred for 15 h at room temperature. The reaction mixture was then diluted with diethyl ether (20 mL), and excess reagent was destroyed by dropwise addition of methanol and water. The solution was acidified with concentrated hydrochloric acid. The ethereal layer was separated and the aqueous phase was extracted with diethyl ether. The combined organic phases were washed with brine and dried over magnesium sulfate, and finally, the solvent was removed. The oily residue was chromatographed using FC on silica gel (*n*-hexane/ethyl acetate 80:20, v/v). Relevant fractions were pooled and concentrated, yielding the product, **10** (2.24 g, yield 44%, 93% by GC), as a yellowish oil. IR ($\tilde{\nu}$, $cm^{-1}$): 2962 (w), 1703 (s), 1420 (w), 1296 (m), 826 (w), 606 (w), 507 (m). $^1H$ NMR (300 MHz, $CDCl_3$, $\delta$, ppm): 2.12−1.92 (m, 4H, H-2, H-3), 1.30 (s, 6H, H-5, H-6). $^{13}C$ NMR (75 MHz, $CDCl_3$, $\delta$, ppm): 180.3 (Cq, C-1), 144.8 (Cq, C-1′), 135.1 (Cq, C-4′), 38.8 ($CH_2$, C-3), 37.0 (Cq, C-4), 30.1 ($CH_2$, C-2), 28.9 (2 × $CH_3$, C-5, C-6).

*1,1-Dimethyl-6-([$^2H_3$]-methyl)-1,2-dihydro-([5,7,8-$^2H_3$])-naphthalene-4-one (**11**).* Compound **10** (2.24 g, 10.52 mmol) was dissolved in trifluoroacetic acid (10 mL), and the solution was cooled to 0 °C. Trifluoroacetic anhydride (2.2 mL, 15.78 mmol) was added to this solution. After removal of the ice bath, the mixture was stirred for 4 h at room temperature. Then the reaction mixture was diluted with dichloromethane (10 mL) and treated with water (10 mL). The aqueous phase was extracted with dichloromethane (3 × 20 mL), and the combined organic phases were first washed with sodium hydroxide solution (1 N, 20 mL) and then with brine. The organic phase was dried over sodium sulfate and filtered, and the solvent was removed by evaporation under reduced pressure. The product, **11** (1.90 g, yield 86%, 92% by GC), was obtained as a yellowish oil. IR


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($\tilde{\nu}$, $cm^{-1}$): 2961 (w), 1799 (w), 1735 (w), 1682 (m), 1591 (w), 1464 (w), 1422 (w), 1378 (w), 1244 (w), 1166 (w), 1014 (w), 564 (w), 486 (w). $^1H$ NMR (300 MHz, $CDCl_3$, $\delta$, ppm): 2.12−1.92 (m, 4H, H-2, H-3), 1.30 (s, 6H, CH-10, CH-11). $^{13}C$ NMR (75 MHz, $CDCl_3$, $\delta$, ppm): 180.1 (Cq, C-4), 144.6 (Cq, C-10), 135.0 (Cq, C-6), 125.6 (Cq, C-9), 38.7 ($CH_2$, C-3), 36.9 (Cq, C-1), 30.0 ($CH_2$, C-2), 28.8 (2 × $CH_3$, C-11, C-12).

*1,1-Dimethyl-6-([$^2H_3$]-methyl)-1,2-dihydro-([5,7,8-$^2H_3$])-naphthalene-4-ol (**12**).* Compound **11** (1.90 g, 9.79 mmol) was dissolved in methanol (100 mL). After addition of cerium(III) chloride heptahydrate (1.31 g, 3.52 mmol) and sodium borohydride (0.26 g, 6.85 mmol), the reaction mixture was stirred for 30 min at room temperature. Water (50 mL) was added, and the solution was saturated with sodium chloride and extracted with dichloromethane (50 mL). The aqueous phase was extracted with dichloromethane (3 × 30 mL). The pooled extracts were dried with sodium sulfate and concentrated. The product, **12** (1.74 g, yield 90%, 94% by GC), was obtained as a colorless oil. IR ($\tilde{\nu}$, $cm^{-1}$): 3331 (w), 2934 (w), 1456 (w), 1179 (w), 1062 (m), 1035 (m), 996 (m), 950 (m), 685 (w), 486 (m). $^1H$ NMR (300 MHz, $CDCl_3$, $\delta$, ppm): 4.69 (t, 1H, H-4), 2.11−1.54 (m, 4H, H-2, H-3), 1.31, 1.23 (2x s, 6H, H-11, H-12). $^{13}C$ NMR (75 MHz, $CDCl_3$, $\delta$, ppm): 142.6 (Cq, C-9), 137.5 (Cq, C-10), 135.2 (Cq, C-6), 68.9 (CH, C-4), 34.4, 28.9 (2 × $CH_2$, C-2, C-3), 33.6 (Cq, C-1), 31.5, 31.4 (2 × $CH_3$, C-11, C12).

*1,1-Dimethyl-6-([$^2H_3$]-methyl)-1,2-dihydro-([5,7,8-$^2H_3$])-naphthalene (**1.2**).* Compound **12** (1.69 g, 8.62 mmol) was added to a mixture of calcium chloride (2 g) and *p*-toluenesulfonic acid (TsOH, 0.85 mg, 4.49 mmol) in cyclohexane (250 mL). The reaction mixture was stirred at 50 °C for 2 h. The reaction mixture was then diluted with dichloromethane (100 mL) and saturated sodium bicarbonate solution (100 mL). The organic phase was separated, and the aqueous phase was extracted with dichloromethane (3 × 50 mL). The combined organic phases were washed with water (50 mL) and saturated sodium chloride solution (50 mL) and dried over sodium sulfate. The solvent was removed with a rotary evaporator. The residue was purified on silica gel by CC (*n*-hexane, 100%). After concentration of the fractions, the product, **1.2** (0.73 g, yield 43%, 91% by GC), was obtained as a yellowish oil. IR ($\tilde{\nu}$, $cm^{-1}$): 3034 (w), 2958 (m), 2820 (w), 2251 (w), 1590 (w), 1456 (m), 1428 (w), 1381 (w), 1359 (m), 1327 (w), 1268 (w), 1092 (w), 1054 (w), 995 (w), 850 (m), 808 (w), 766 (w), 725 (w), 694 (w), 679 (s), 481 (s). $^1H$ NMR (300 MHz, $CDCl_3$, $\delta$, ppm): 6.40 (dt, $J$ = 9.6/1.8 Hz, 1H, H-4), 5.90 (dt, $J$ = 9.6/4.5 Hz, 1H, H-3), 2.29 (s, 3H, H-13), 2.20 (dd, $J$ = 4.4/1.8 Hz, 2H, H-2), 1.25 (s, 6H, H-11, H-12). $^{13}C$ NMR (75 MHz, $CDCl_3$, $\delta$, ppm): 141.1 (Cq, C-9), 135.2 (Cq, C-6), 132.7 (Cq, C-10), 127.6 (CH, C-3), 127.2 (CH, C-4), 39.0 ($CH_2$, C-2), 33.1 (Cq, C-1), 28.5 (2 × $CH_3$, C-11, C-12).

**Synthesis of Isomeric Vitispiranes and Vitispiranes-$d_5$.** The synthesis was performed according to the methods previously described by Anderson et al.,[27] Nilsson et al.,[28] and Eggers et al.[29] The MS and NMR data were identical with published data.[27−29]

**Stability of Deuterated Internal Standards at Wine pH (TDN-$d_6$ and Vitispiranes-$d_5$).** The stability of the labeled standards was investigated as described by Kotseridis et al.[30] Internal standards were dissolved in a 12% ethanol/water mixture, pH-adjusted to 3, and stirred at room temperature for 24 h. Volatiles were extracted with diethyl ether and concentrated. Subsequent GC-MS analysis was done to monitor possible H−D back-exchange.

**Model Wine and Wine Samples.** For calibration standards and dilution of the wine samples, a model wine was prepared by dissolving 4 g of tartaric acid and 120 mL of ethanol in deionized water to a volume of 1 L. The pH value was adjusted to 3.2 by dropwise addition of a 5 M sodium hydroxide solution.

Riesling wines of different vintages, regions, and wineries were supplied from the wine competition "Best of Riesling 2015" (131 samples, vintage 1990−2014, mean age 4.7 years), which included wines originating from Germany (125), Austria (4), the United States (1), and New Zealand (1), and the wine competition "Mundus Vini 2015" (119 samples, vintage 2012−2014, mean age 1.2 years), which included wines originating from Germany (94), Luxembourg (8), the Czech Republic (4), Austria (4), Hungary (2), the United States (2), New Zealand (2), Australia (1), Bulgaria (1), and France (1). The wines were stored at 4 °C in glass vials lined with PTFE septa until analysis.

**Automated HS-SPME/GC-MS Analysis for Quantitation of TDN and Vitispiranes.** For sample preparation, automated headspace−solid-phase microextraction (HS-SPME) was used. Analysis was performed by gas chromatography−mass spectrometry (GC-MS) with selected-ion-monitoring (SIM, method A) and selected-reaction-monitoring (SRM, method B) modes for wines from "Best of Riesling 2015" and "Mundus Vini 2015", respectively. The parameters for method B are given in square brackets. The quantitation of free TDN and vitispiranes was carried out in 20 mL glass vials with 4.50 mL [4.00 mL] of model wine solution, to which 2 g of sodium chloride, 500 $\mu$L [1000 $\mu$L] of the wine, and 50 $\mu$L of the internal standard (ISTD target concentrations: method A, 0.43 $\mu$g/L TDN-$d_6$ and 0.98 $\mu$g/L vitispiranes-$d_5$; method B, 0.41 $\mu$g/L TDN-$d_6$ and 0.72 $\mu$g/L vitispiranes-$d_5$) had been added.

For the quantitation of the hydrolytically released compounds, 5 mL of the wine sample was hydrolyzed in a 20 mL glass vial lined with a PTFE septum for 36 h at 100 °C (the headspace within the vial was air). After cooling, 50 $\mu$L [100 $\mu$L] of the sample in 4.95 mL [4.9 mL] of model wine was used for the measurement. Calibration standards were prepared in 5 mL of model wine. (Calibration ranges are given in section Calibration and Analytical Validation of GC-MS Methods A and B.) After incubation of the samples for 5 min at 40 °C, the samples were extracted for 20 min [30 min] (SPME fiber, Sigma-Aldrich; polydimethylsiloxane (PDMS), 1 cm, 100 $\mu$m $d_f$). Desorption was performed in a programmable-temperature vaporizing (PTV) inlet at 240 °C for 3 min in splitless mode and purged after 2 min. This injector was equipped with a 2.0 mm (i.d.) metal liner (ThermoFisher Scientific, Waltham, MA). The fiber penetration depth was set to 35 mm. Cleaning and conditioning of the PDMS fiber was conducted at 250 °C for 3 min under a helium flow of 1.2 mL/min in a split−splitless (SSL) inlet equipped with a 2.0 mm (i.d.) metal liner (ThermoFisher Scientific) with a split of 1:100 before and after each sampling. The GC analysis was performed with a Trace 1300 gas chromatograph equipped with a TriPlus RSH autosampler (both ThermoFisher Scientific) on a VF-WAXms column (Agilent, Waldbronn, Germany, 30 m × 0.25 mm i.d. × 0.25 $\mu$m $d_f$); helium was used as the carrier gas with a constant flow of 1.2 mL/min. To minimize contamination of the analytical column, a polar-deactivated precolumn (Restek, Bellefonte, PA, 2.5 m × 0.53 mm i.d.) was coupled via a "T" connector (ThermoFisher Scientific) to the analytical column, and a backflush was set to 5 min. The oven-temperature program started isothermally at 50 °C for 1 min, then increased to 240 °C at 10 °C/min, and was held isothermally for 10 min. The mass-spectrometric (MS) detection was carried out with a TSQ Duo triple-quadrupole mass spectrometer (ThermoFisher Scientific). Temperatures for the MS transfer line and ion source were set to 250 °C. In SIM mode, the ions $m/z$ 157 (TDN), 163 (TDN-$d_6$), 177 (vitispiranes), and 182 (vitispiranes-$d_5$) were used as quantifier ions. The qualifier ions were $m/z$ 142, 172 (TDN); 148, 178 (TDN-$d_6$); 149, 192 (vitispiranes); and 154, 197 (vitispiranes-$d_5$), respectively. In SRM mode, argon (purity ≥99.999%) was used as the collision gas. The mass resolution was set to 1 amu for Q1 and Q3 (cycle time of 200 ms). Mass transitions (SRMs, $m/z$) and collision energies are listed hereafter (quantifier SRMs are underlined): <u>157.1 → 142.1 (14 V)</u>, 157.1 → 115.1 (38 V), and 172.1 → 157.1 (8 V) for TDN; <u>163.2 → 148.2 (10 V)</u>, 145.1 → 144.1 (15 V), and 178.2 → 163.2 (10 V) for TDN-$d_6$; <u>177.1 → 93.1 (15 V)</u>, 177.1 → 121.1 (10 V), and 192.1 → 177.2 (10 V) for vitispiranes; and <u>182.2 → 93.1 (15 V)</u>, 182.2 → 121.1 (10 V), and 197.2 → 182.1 (10 V) for vitispiranes-$d_5$. Xcalibur software (version 3.0.63) was used for instrument control and data acquisition (ThermoFisher Scientific).

**Statistical Analysis.** Analyses were performed in duplicate for each wine. Statistical analyses on volatile concentrations and determinations of statistical significance were performed using OriginPro 9.0.0.G (OriginLab Corporation, Northampton, MA).


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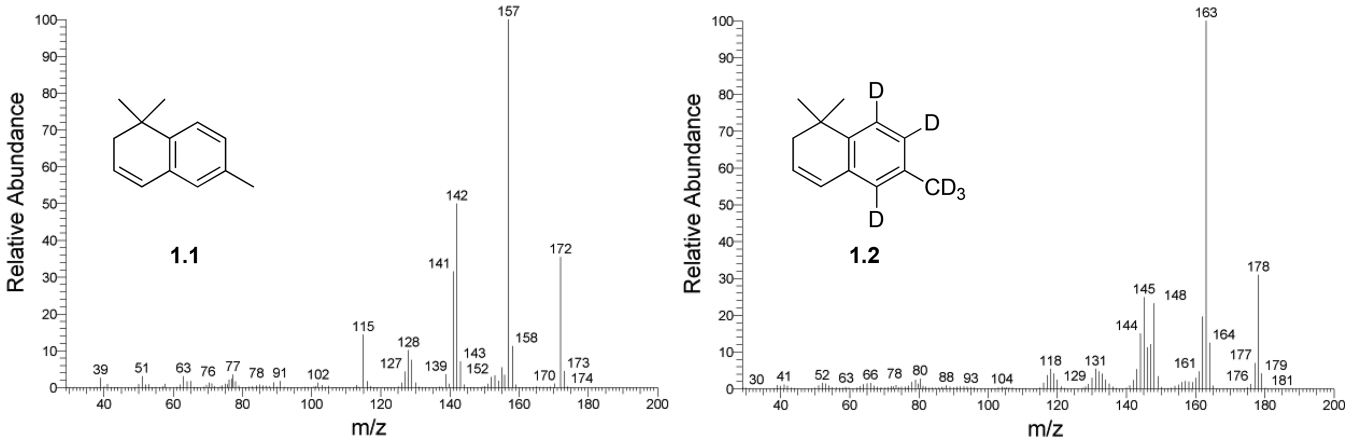


**Figure 3.** Mass spectra and chemical structures of TDN (**1.1**) and TDN-$d_6$ (**1.2**).

## RESULTS AND DISCUSSION

**Total Synthesis of TDN and TDN-$d_6$.** Because various labeling experiments[31,32] starting from $\beta$-ionone-$d_3$[23,24] have given rise to the formation of isotopologue mixtures and because a loss of almost 35−40% of the deuterium has been observed in the following synthetic steps as a result of deuterium−hydrogen back-exchange reactions,[26] we decided to develop a completely new synthetic strategy for the preparation of a stable deuterium-labeled TDN specimen.

The novel synthetic route for labeled TDN developed in this study consists of six steps, as shown in Figure 2. The first and important key step of our synthetic route was the introduction of deuterium via [$^2H_8$]-toluene (**6**) in a Friedel−Crafts acylation.[33] The resulting carboxylic acid, **8**, was esterified to protect the carboxyl group with a hydrogen chloride−ethanol solution, yielding compound **9**.[34] The second key step was the preparation of gem-dimethyl carboxylic acid, **10**, which was realized via treatment with trimethyl aluminum.[35] Friedel−Crafts cyclization[36,37] yielded ketone **11**, which was reduced with sodium borohydride.[38] TDN-$d_6$ (**1.2**) was finally obtained by dehydration of the resulting alcohol, **12**.[39] The total overall yield of **1.2** was in the range of 8%. For the synthesis of nonlabeled TDN, [$^2H_8$]-toluene can be replaced by toluene. The main advantage of this new route is that intermediates with acidic C−H positions are avoided. In addition, the labeling on the aromatic ring was found to be stable in our analytical conditions, as no proton exchange was observed during storage and analysis under winelike conditions. The mass spectra (EI+, 70 eV) of labeled and nonlabeled TDN are shown in Figure 3.

**Quantitative Analysis of Free and Total TDN and Vitispiranes.** The concentration levels of TDN and vitispiranes in Riesling wines were determined by means of SIDA-based quantitation, both directly (free forms) and after acid hydrolysis also comprising the respective precursor forms (total TDN and vitispiranes, predominantly glycosidically bound forms). In the course of this study, automated HS-SPME was applied to 250 wine samples from the wine competitions "Best of Riesling 2015" and "Mundus Vini 2015" and analysis was performed by GC-SIM-MS (method A) and GC-SRM-MS (method B). HS-SPME analysis was done after slight modifications according to the method of Pozo-Bayón et al.,[40] whereas the extraction parameters for minor wine volatiles were extensively evaluated. PDMS was used as fiber material because the two target substances are quite nonpolar. This ensured high extraction efficiencies for both compounds and consequently allowed the use of low amounts of sample volumes in a defined model wine solution. In this way, it was possible to carry out the quantitation in a standardized matrix.

The quantitative determination of the nonvolatile progenitors of $C_{13}$-norisoprenoids is more challenging and conventionally carried out by means of simultaneous distillation−extraction (SDE) or acid hydrolysis of precursors in ampules or microreaction vessels with subsequent extraction of the liberated volatiles.[41,42] However, SDE is a time- and solvent-consuming method, especially in cases where a high number of samples have to be analyzed. For this reason, a standardized, rapid acid-hydrolysis method was developed to release the target compounds TDN and vitispiranes from their progenitors. To the best of our knowledge, no related systematic study on the formation kinetics of TDN and vitispiranes from their precursors in wine has been published yet. In order to fill this gap, model acid hydrolysis of the precursors in a Riesling wine sample were carried out in a closed system (10 mL glass vials) at genuine wine pH (3.4) and at 100 °C for 72 h (with method B). Figure 4 clearly shows that increasing the time of hydrolysis initially causes a strong increase in TDN and vitispirane formation. After a certain amount of time (~36 h), a plateau is formed. Further heating of the sample for more than 36 h

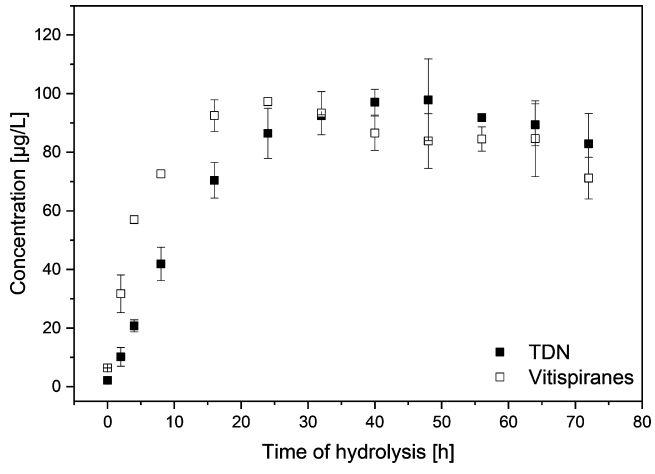


**Figure 4.** Kinetic study of the formation of TDN and vitispiranes in a Riesling wine sample (pH 3.4, genuine; hydrolysis temperature, 100 °C; $n$ = 2 if standard deviations are provided).


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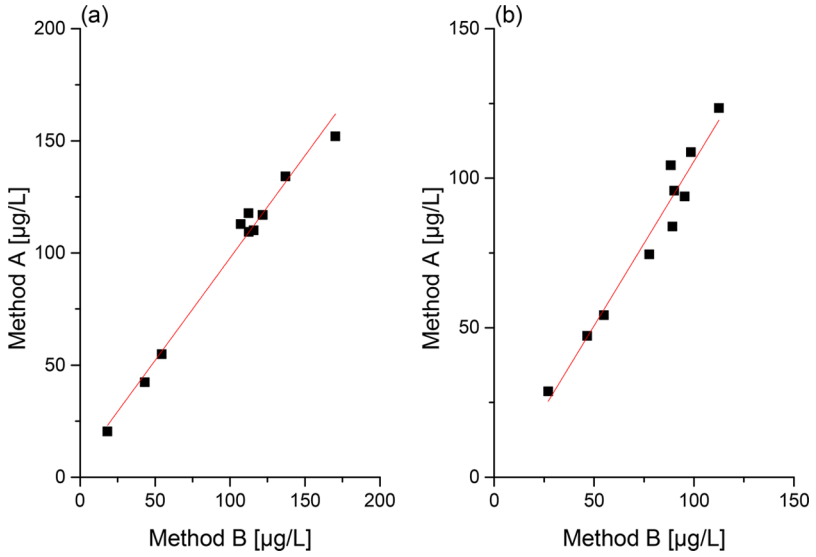


**Figure 5.** Comparison of quantitation methods A and B using wine samples after acid hydrolysis for TDN (a) and vitispiranes (b).

results in a slight decrease in TDN concentration. This indicates possible degradation of TDN and matches the investigations of Marais et al.[41] on the storage and accelerated aging of Riesling wines.

**Calibration and Analytical Validation of GC-MS Methods A and B.** The calibration curves were obtained by linear regression. The following calibration ranges were chosen for a five-point calibration curve: 0.04−4.12 μg/L (TDN, method A), 0.02−2.09 μg/L (vitispiranes, method A), 0.04−3.88 μg/L (TDN, method B), and 0.06−6.19 μg/L (vitispiranes, method B). In all cases, good linearities (coefficients of determination, $R^2 > 0.99$) for the calibrated ranges of methodological relevance were obtained (method A: TDN, $R^2 = 0.9996$, and vitispiranes, $R^2 = 0.9991$; method B: TDN, $R^2 = 0.9997$, and vitispiranes, $R^2 = 0.9997$). The limits of detection (LODs) for method A were 0.140 μg/L (TDN) and 0.099 μg/L (vitispiranes), and for method B, they were 0.099 μg/L (TDN) and 0.140 μg/L (vitispiranes). The limits of quantitation (LOQs) for method A were 0.483 μg/L (TDN) and 0.338 μg/L (vitispiranes), and for method B, they were 0.343 μg/L (TDN) and 0.487 μg/L (vitispiranes). Calculation of LODs and LOQs was done according to the German Institute for Standardization (DIN 32645) methods and only for the calibrated ranges. The LOD and LOQ values achieved in our methods allowed us to measure well below the sensory thresholds of both substances (TDN, 2.3 μg/L; vitispiranes, 101.0 μg/L). Further validation data were acquired with two Riesling wines with low and high analyte concentrations within the calibration range, and data acquisition was done by spiking at two different levels of calibration standards (for TDN with method A, 0.078 and 0.0155 μg/L; for vitispiranes with method A, 0.041 and 0.083 μg/L; for TDN with method B, 0.039 and 0.388 μg/L; and for vitispiranes with method B, 0.062 and 0.619 μg/L). The determined validation data were 108.1 ± 9.9 and 105.5 ± 16.5% for method A and 99.7 ± 2.0 and 102.6 ± 13.3% for method B for TDN and vitispiranes, respectively. In order to determine the repeatability of the methods (intraday), eight independent samples were prepared and analyzed, and the variation coefficients ($V_k$) were calculated as 2.1% for TDN and 3.9% for vitispiranes with method A and as 2.9% for TDN and 6.7% for vitispiranes with method B.

**Comparison of Methods A and B for Quantitative Determination.** Methods A and B were applied after acid hydrolysis to 10 Riesling wines that covered a wide range of TDN and vitispirane concentrations (∼20−170 μg/L TDN and ∼30−90 μg/L vitispiranes). The results are shown in Figure 5. The values obtained by both methods were almost comparable. The high correlation between methods A and B was confirmed with $R^2$ values of 0.9806 (TDN) and 0.9486 (vitispiranes). A highly significant positive correlation (Pearson, $p < 0.05$) could be detected and confirmed for the concentrations of TDN ($p = 2.41 \times 10^{-8}$) and vitispiranes ($p = 1.21 \times 10^{-6}$) with both methods. On the basis of these results, the comparability of the results of both methods was ensured.

**Concentrations of TDN and Vitispiranes in Riesling Wines.** The distributions of the concentrations of TDN and vitispiranes (free and total) in a total of 250 Riesling wines of different origins (predominantly from Germany) are shown as box plots in Figure 6. Figure 6a shows the results of Riesling wines from the wine competition "Best of Riesling 2015", and Figure 6b shows the results of Riesling wines from the wine competition "Mundus Vini 2015". The dotted horizontal lines represent the panelist (DT)- and consumer (CDT)-detection thresholds (DT = 2.3 μg/L, CDT = 14.7 μg/L) and the consumer-rejection thresholds (CRT = 60.0 μg/L for young wine and 91.0 μg/L for aged wine) determined for TDN.[6]

For the wines from "Best of Riesling 2015", the mean for the free-TDN concentration was 3.1 μg/L, and that for the total was 94.5 μg/L. In the case of the vitispiranes, values of 17.1 μg/L for free vitispiranes and 84.5 μg/L for total vitispiranes were determined. Except for two wines with high TDN concentrations, there were no wines above the detection threshold of the consumers; however, the majority of the wines were above the detection threshold of the trained panel. It should be underlined that the mean age of the wines in the case of the samples from "Best of Riesling 2015" was 4.7 years.

The observed TDN-levels for the wines from "Mundus Vini 2015" ranged from 0.4 to 14.8 μg/L for free TDN and from 38.5 to 333.0 μg/L for total TDN. The concentrations of free and total vitispiranes varied from 2.1 to 44.6 μg/L and from


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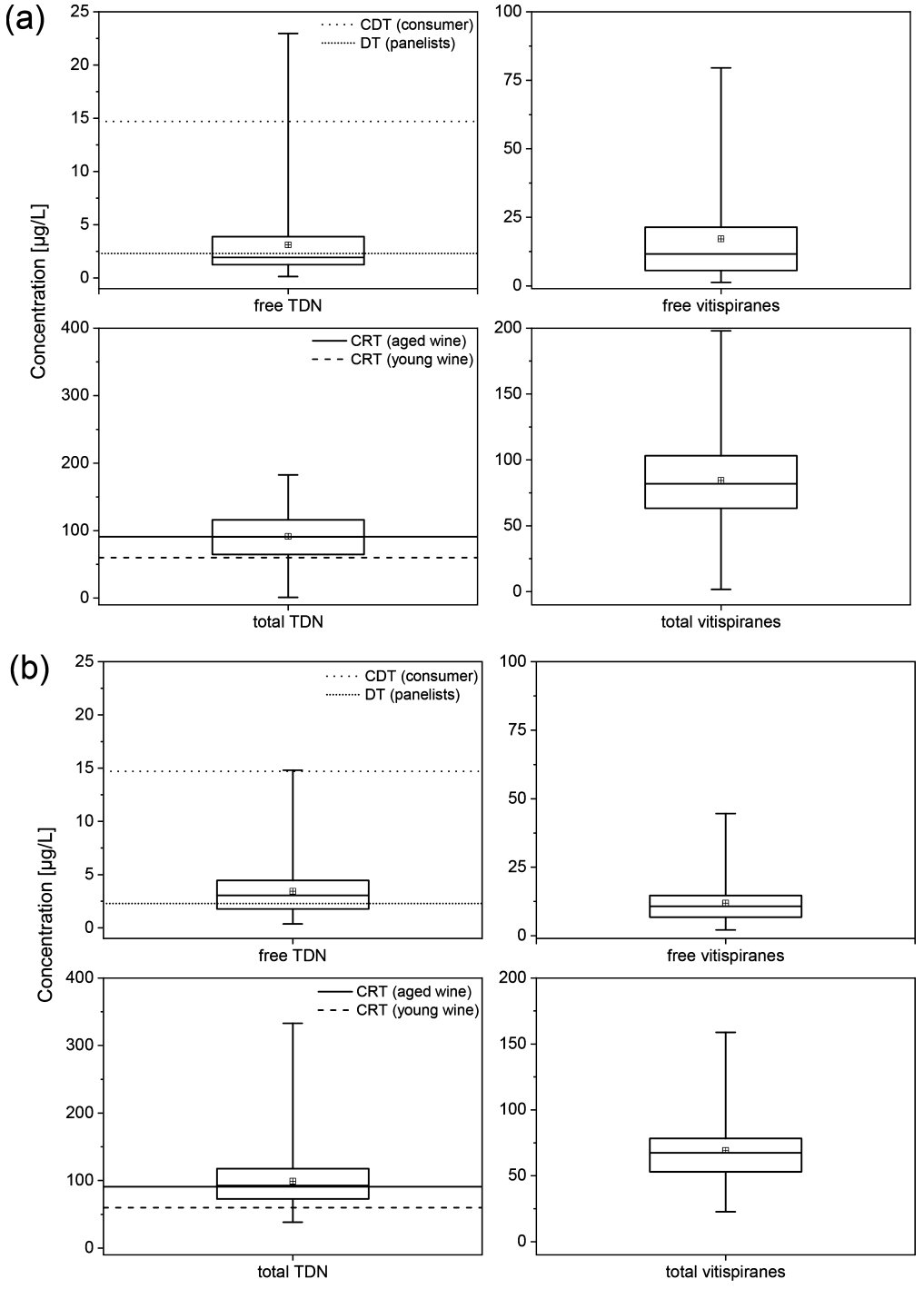


**Figure 6.** Concentrations of free and total TDN and vitispiranes of investigated Riesling wines from wine competitions "Best of Riesling 2015", which had 131 wine samples (a), and "Mundus Vini 2015", which had 119 wine samples (b). The bottoms and tops of the boxes represent the 25th and 75th percentiles, respectively. The whiskers represent the extrema, without statistical outliers ($n$ = 2). The medians are represented by the horizontal lines, and the means are represented by the small squares in the boxes. In addition, for a better discussion of the analytical data with respect to the sensory data, the dotted horizontal lines represent the panelist (DT)- and consumer (CDT)-detection thresholds (DT = 2.3 $\mu$g/L, CDT = 14.7 $\mu$g/L) and consumer-rejection thresholds (CRT = 60.0 $\mu$g/L for young wine and 91.0 $\mu$g/L for aged wine) determined for TDN in a recent study.[6]

22.7 to 158.8 $\mu$g/L, respectively. As the mean age of the wines was in the range of only 1 year, free-TDN concentrations in almost half of the wines were below the detection threshold of a trained panel. Nevertheless, approximately 50 and 75% of the investigated wines were prone to developing a petrol off-flavor during bottle aging, because with values up to 333 $\mu$g/L for total TDN, these wines are clearly above the consumer-rejection thresholds[6] for both young wines (60 $\mu$g/L) and aged wines (91 $\mu$g/L). With other words, the fates of these wines will strongly depend on the further storage conditions, especially the storage temperature. The results obtained in this study are quite similar to those obtained for free TDN in previous studies for Riesling wines from Germany (1−2 $\mu$g/L),[12] New York state (7−20 $\mu$g/L[13] and 1−17 $\mu$g/L[5]), and


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Australia (1−10 μg/L).[43] However, median values for free TDN obtained in this study are far from the median values published for Australian Riesling wines (12−25 μg/L).[44]

It is expected that because of global warming and enhanced sunlight exposure of grapes, the number of wines exhibiting a petrol off-flavor will continue to increase even in European countries. For this reason, further studies concerning the isolation and quantitation of naturally occurring norisoprenoid aroma precursors in grapes are required to understand the mechanisms of precursor formation of TDN and vitispiranes in the course of carotenoid metabolism. Also, technological and enological measures (clone selection, vine-training system, etc.) that allow minimization of the development of TDN off-flavor in Riesling wines are subjects of our ongoing research.

## AUTHOR INFORMATION


### Corresponding Author

*Tel.: +49 531 391 7200. Fax: +49 531 391 7230. E-mail: p.winterhalter@tu-braunschweig.de.

### ORCID

Hans-Georg Schmarr: 0000-0003-1443-1162

Peter Winterhalter: 0000-0003-3417-6023


### Notes

The authors declare no competing financial interest.

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