Quantitative Determination of Potent Flavor Compounds in Burgundy Pinot Noir Wines Using a Stable Isotope Dilution Assay†

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A specific experimental procedure suitable for the quantification of four esters recently identified in a wine of *Vitis vinifera* cv. Pinot Noir, ethyl dihydrocinnamate (A), ethyl cinnamate (B), methyl anthranilate (C), and ethyl anthranilate (D), was developed and applied to 33 Burgundy wines (calculated on three replicates). The method, involving a stable isotope dilution assay, allows the determination of concentrations from 0.05 μg L^{-1}, with a repeatability better than 10%. The mean, maximum, and minimum amounts found for the four esters were as follows (in μg L^{-1}): (A) 1.6, 3.2, 0.8; (B) 0.8, 1.6, 0.5; (C) 0.2, 0.6, 0.06; (D) 2.4, 4.8, 0.6. Differences between wines, according to their concentrations and the nature of the esters, were visualized by principal component analysis. An analysis of variance indicated that ethyl anthranilate was the most important for wine differentiation.

Keywords: *Pinot noir; wine; aroma; stable isotope SIM-MS*

INTRODUCTION

Four esters, ethyl dihydrocinnamate (A), ethyl cinnamate (B), methyl anthranilate (C), and ethyl anthranilate (D), identified recently in a Burgundy wine of *Vitis vinifera* cv. Pinot Noir as minor components by gas chromatography/mass spectrometry (GC/MS), were suspected to contribute to the typical aroma of Pinot noir wines, according to the results of a gas chromatography/olfactometry analysis (Moio and Etiévant, 1995). The descriptors related to these substances, established during the sensory analysis of this previous study, correspond to desirable fruity notes characteristic of this type of wine. These compounds are interesting since, for the first time, ethyl dihydrocinnamate is identified in the aroma of wine, ethyl cinnamate is reported as a key aroma compound of red wine, and ethyl anthranilate and methyl anthranilate, previously found in American wines made with *Vitis labrusca*, were found in *V. vinifera* wines (Pinot noir).

To evaluate the importance of those potent odorants and to confirm their occurrence in other Pinot noir wines, this study aimed at the isolation and determination of the concentrations of these esters in a selection of different Burgundy Pinot noir wines. We report here a simplified extraction method and a GC/MS stable isotope dilution assay suitable to quantify the four esters in wine.

MATERIALS AND METHODS

Wines. Thirty-three Pinot noir wines were analyzed. Nine of them were provided by the Bureau Interprofessionnel des Vins de Bourgogne, and the others were purchased from local supermarkets, all of them sold as Burgundy wines. To preserve their anonymity, the wines were randomly coded with letters.

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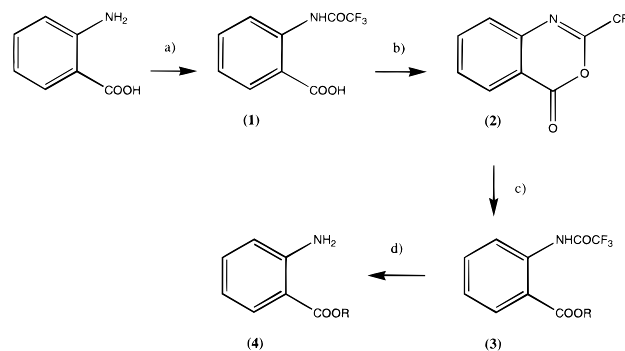

R = CD_3, CH_2CD_3

a) : $(CF_3CO)_2O$; **b)** : $(CH_3CO)_2O$; **c)** : ROH, RONa; **d)** : K_2CO_3

Figure 1. Synthesis of the deuteriated anthranilates.

Chemical Standards. The four target esters were obtained from reliable retailers. The deuteriated standards, ethyl-d_3 dihydrocinnamate (ethyl-d_3 phenyl-3-propionate) and ethyl-d_3 cinnamate (ethyl-d_3 phenyl-3-propenoate), further referred to as (A-d_3) and (B-d_3), were prepared from ethanol-d_3 and the suitable acyl chloride. The alcohol-d_3 (0.2 mL) in pyridine (1 mL) was added at 0 °C to a solution of acyl chloride (5 mmol) in pyridine (5 mL). The reaction mixture was then stirred at room temperature (RT) for 2 h and poured into 50 mL of 1 M HCl. The aqueous layer was extracted with ether (2 × 50 mL). The combined organic phases were washed with saturated aqueous $NaHCO_3$, dried over Na_2SO_4, and evaporated. The crude product was purified on silica gel (pentane/diethyl ether, 80/20). Molar yield: 80−90%.

Methyl-d_3 anthranilate (methyl-d_3 amino-2-benzoate) and ethyl-d_3 anthranilate (ethyl-d_3 amino-2-benzoate), further referred to as (C-d_3) and (D-d_3), were not prepared according to the classical method, as alcohol-d_3 was judged too expensive. They were synthesized from the mild hydrolysis of methyl-d_3 and ethyl-d_3-2-trifluoroacetamidobenzoates as described in Figure 1 (Errede *et al.*, 1977).

*Synthesis of the N-Nitrofluoroacetylanthranilic Acid (**1**).* Trifluoroacetic anhydride (5.3 g, 25 mmol) was added to a cooled and stirred solution (0 °C) of 2.8 g of anthranilic acid (20 mmol) in trifluoroacetic acid (100 mL). The suspension was stirred for 3 h and then poured into 100 mL of water. The solid was filtered, washed with water, and then dissolved in

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100 mL of ethyl acetate; the organic layer was washed with aqueous 1 M HCl (50 mL) and water and dried over Na_2SO_4. The solvent was evaporated, and the solid thus obtained was used without purification.

*Synthesis of 2-(Trifluoromethyl)-1,3-(4H)benzoxazin-4-one (**2**).* The crude solid (**1**) was converted to benzoxazine by treatment with refluxing acetic anhydride for 2 h. The solution was evaporated *in vacuo* and the product crystallized from heptane to give 2.66 g of **2** . Molar yield: 62%, mp 50 °C [51−52 °C according to Errede *et al.* (1977)].

*Synthesis of Methyl-d_3 and Ethyl-d_3 2-Trifluoroacetamidobenzoate (**3**).* Thirty milligrams of sodium was added to 0.4 mL of alcohol-d_3, and after 10 min, 1.02 g of **2** was added. After 4 h of stirring, 0.5 M HCl (20 mL) was added. The solution was extracted with diethyl ether (2 × 50 mL); the organic layer was washed with water, dried over Na_2SO_4, and evaporated. The crude product was purified on silica gel (pentane/diethyl ether, 80/20). Molar yield: 66%.

*Synthesis of Methyl-d_3 Anthranilate and Ethyl-d_3 Anthranilate (**4**).* An aqueous solution of 10% K_2CO_3 (5 mL) was added to a solution of 0.55 g of **3** in 3 mL of acetonitrile; the mixture was stirred for 24 h at RT and then poured into 50 mL of water. The aqueous layer was extracted with diethyl ether (2 × 50 mL); the organic layer was washed and dried (Na_2SO_4), and the solvent was evaporated. The crude product was purified on silica gel (pentane/diethyl ether, 80/20). Molar yield: 80%.

Analysis of Model Mixtures. *Stability of the Esters in Model Ethanolic Mixtures.* An aqueous solution with 12% alcohol, buffered with citric acid/sodium citrate at pH 5 and containing 50 mg L^{-1} of each ester, was prepared. To follow if a hydrolysis of the esters occurred in a model mixture, hermetically sealed tubes containing 10 mL of the hydroalcoholic solution were immersed in a bath regulated at 30 °C. At different periods, from 3 h to 7 days, a 10 mL sample was extracted with 5 mL of dichloromethane. The organic extract, dried over Na_2SO_4, was spiked with a known amount of pentadecane, and the concentrations of the esters were then determined, using the following formula: $m_i = K_{i/s} m_s (A_i/A_s)$ (i = A, B, C, D; m_i = concentration of ester in μg L^{-1}; $K_{i/s}$ = FID response factor; m_s = concentration of pentadecane in μg L^{-1}; A_i/A_s = ester/pentadecane GC surface ratio).

Optimization of the Extraction. The extraction efficiency of two solvents of different polarity, pentane and dichloromethane, was tested, as the former is reported to be selective for esters and the latter is one of the most efficient solvents for the liquid−liquid extraction of aroma compounds (Nykänen and Suomalainen, 1984). Ten miililiters of the hydroalcoholic solution was extracted once by 10 mL of solvent. The organic phase was spiked with a known amount of internal standard (pentadecane) and directly injected in GC. The exact amounts of the esters extracted by each solvent were determined using the FID response factors previously determined. The minimum time of agitation of the two phases necessary to reach the maximum recovery was also estimated. Each experiment was duplicated.

Losses of Esters during Concentration. Twenty milliliters of an organic solution containing the four esters (25 mg L^{-1} in pentane) was concentrated as described below and then diluted to the initial volume. The diluted solution was spiked with a known amount of internal standard (pentadecane) and injected in GC to determine the recovery concentrations. The experiment was repeated twice.

Preparation of Wine Extracts. Seven hundred milliliters of a wine was spiked with a mixture containing the deuteriated esters: (A-d_3) 470 ng; (B-d_3) 360 ng; (C-d_3) 250 ng; (D-d_3) 1650 ng. The wine sample was placed in a flask closed and vigorously stirred for 15 min. It was then divided into three samples of 200 mL, each of them being extracted with 2 × 20 mL pentane during 15 min. The organic phase was dried over Na_2SO_4 and concentrated in a Kuderna-Danish apparatus at 55 °C. A further concentration under a nitrogen stream was made down to 100 μL. The final concentration factor was 2000.

Capillary Gas Chromatography. *Determination of Flame Ionization Detector (FID) Response Factors.* An organic solution of the esters (50 mg L^{-1}) was prepared in dichloromethane and injected in GC with pentadecane as internal standard. The flame ionization response coefficients of the esters, $K_{i/s}$, were determined from triplicate injections.

Table 1. Estimation of the Extraction Recovery (Percent) at 30 °C with Time

sampling time (h)	ester code			
	A	B	C	D
0	95	97	95	94
26	97	99	99	98
41	96	99	99	98
140	95	98	100	98

Extracts Analysis. Extracts from model mixtures were analyzed by HRGC on a 30 m × 0.32 mm fused silica capillary column (J&W DB-1701, film thickness = 1 μm) settled in the following conditions: gas chromatograph (GC) HP 5890, splitless injection, temperatures of detector and injector = 220 °C, oven temperature programmed at 5 °C min^{-1} from 40 to 180 °C, hydrogen carrier gas linear velocity = 50 cm s^{-1}.

Capillary Gas Chromatography/Mass Spectrometry. Mass spectrometry was performed on a HP 5970 MSD quadrupole mass spectrometer directly coupled with a GC HP 5890. Samples were injected on-column under the same GC conditions as described above, except for the temperature program (from 40 to 150 °C at 5 °C min^{-1}, from 150 to 200 °C at 3 °C min^{-1}, and from 200 to 220 °C at 5 °C min^{-1}) and the carrier gas (helium, linear velocity = 35 cm s^{-1} at 220 °C). Mass spectra were produced in the electron impact mode (70 eV) and recorded with a HP-UX Chemstation. One microliter of a wine extract was first injected in SCAN mode, and the presence of the four esters was confirmed by comparison of their spectra in the Wiley mass spectra library. The quantification was then done in single ion monitoring mode (SIM) on selected ions. Ninety-nine wine extracts were analyzed, corresponding to 33 different wines (3 replicates).

Standard Curves. Six solutions containing different concentrations of the four esters and fixed amounts of the labeled compounds in pentane were injected in HRGC/MS. For each ester, peak intensity ratios (molecular ion of ester/ molecular ion of corresponding labeled ester) were plotted against the ratio of amounts injected. Standard curves were calculated by linear fitting of these data (no forced zero intercept). Standard curves were renewed every 24 injections. The reliability of the HRGC/MS measurements was controlled by analyzing the same wine extract at different periods, that is five times for the whole study.

Statistical Analysis. The analysis of variance was performed with two softwares: SAS/STAT (SAS Institute Inc.) procedure GLM and Sigma Stat (Jandel Scientific). A principal component analysis (PCA) was then performed on the results, with the Biplot procedure: this program, developed in the laboratory with SAS/STAT, provides the analyst with a representation where observations and variable vectors appear on the same plane.

RESULTS AND DISCUSSION

Extraction. The stability of the esters was first studied in a buffered ethanolic model mixture adjusted at pH 5. This particular pH was chosen since it theoretically permits a 99.7% extraction of the ethyl and methyl anthranilates according to their pK_a values, respectively 2.2 and 2.4 (Casimir and Moyer, 1976; Cumming, 1906). As seen from Table 1, no hydrolysis or degradation of the four unlabeled esters could be observed in the model solution after 140 h at 30 °C, since the percentage of each ester extracted by dichloromethane is fairly constant. This result allows us to perform and to interpret the following experiments and to use the deuteriated esters as standards for the quantification.

In a second step, the efficiency of the extraction of the four esters with two solvents, pentane and dichloromethane, was evaluated in the same model solution. A batch extraction was chosen as it was proved to be

Table 2. Extraction Recovery (Percent)

type of solvent	time for agitation (min)	ester code A	B	C	D
dichloromethane	20	95	85	90	95
	2	93	85	92	95
pentane	20	75	73	65	69
	2	76	72	65	70

the most rapid and efficient method for flavor isolation (Leahy and Reineccius, 1984). As expected, Table 2 shows that dichloromethane allows better recovery of the esters than pentane. However, some interference was observed later in the SIM-GC/MS analysis of the dichloromethane wine extracts, due to a lack of selectivity. Pentane was therefore chosen, as its affinity for polar substances susceptible to create interference is lower and because it allows the extraction of the four esters with an acceptable yield >65%. The same table shows that ester partition between the two phases is completed after only 2 min of agitation. Practically, 15 min of agitation was applied.

Concentration of the Extract. The concentration was then tested with pentane as the solvent. The losses by evaporation or degradation during this step were estimated to be 5% for A, 15% for B, 12% for C, and 10% for D. Taking into account the extraction recovery, the global recovery of each ester is therefore higher than 71% for A, 66% for B, 55% for C, and 61% for D. These recovery yields were considered to be good enough to perform a stable isotope dilution assay, since the principle of the measurement is based on the close physical properties of the deuterium-labeled and unlabeled molecules (especially the partition coefficients, boiling points, and FID responses) (Gilbert, 1987).

Optimization of the SIM-GC/MS. As the amounts of these esters in wine were suspected to be low, a stable isotope dilution assay was chosen for the quantification. The standard esters were deuteriated on their alcohol moiety, as their mass spectra were known to include a strong molecular ion peak, which was chosen for the SIM-GC/MS study. The final purities of the deuteriated esters (internal standards) were estimated by GC/MS to be, respectively, 98.5% for A-d_3, 99.8% for B-d_3, 100% for C-d_3, and 99.6% for D-d_3. As previously noticed in this type of analysis, the deuteriated compounds were eluted just before the unlabeled esters (Schieberle and Grosch, 1987). The choice of the ions for GC/MS quantification was made on their abundance and their *m*/*e* value to minimize the interference with similar ions belonging to the spectra of unknown compounds coeluted and to optimize the signal/noise ratio. The ions 178 and 181, 176 and 179, 151 and 154, and 165 and 168 were thus selected to quantify, respectively, A, B, C, and D using the standards A-d_3, B-d_3, C-d_3, and D-d_3.

The amounts of the labeled compounds added to the wine sample before the isolation and quantification were determined from one injection in SIM-GC/MS of a wine extract to which an arbitrary amount of labeled standards was added. The surface ratio obtained (from the ion peaks of the unlabeled and labeled substances) was determined, and the theoretical amount of labeled compound to be added to the wine was calculated to obtain an unlabeled/labeled ester ion ratio equal to 1.

A model organic solution containing the esters at concentrations as low as 0.08 ng μL^{-1} (80 ppb), injected in SIM-GC/MS mode, gave quantifiable peaks for the four esters, with a signal/noise ratio of 10. Thus, taking into account the concentration factor involved in the preparation of the wine extract (×2000), ester amounts in wine as low as 50 ng L^{-1} could be determined with this method. The theoretical SIM-GC/MS detection limit is therefore 40 ng L^{-1} for the cinnamates and 15 ng L^{-1} for the anthranilates. Compared to the methods already published, the stable isotope dilution assay thus appears to be among the most sensitive methods to determine methyl anthranilate concentrations, previously considered responsible for the negative "foxy" note of certain American wines from *V. labrusca* (Primus and Griffin, 1996; Nelson *et al.*, 1978).

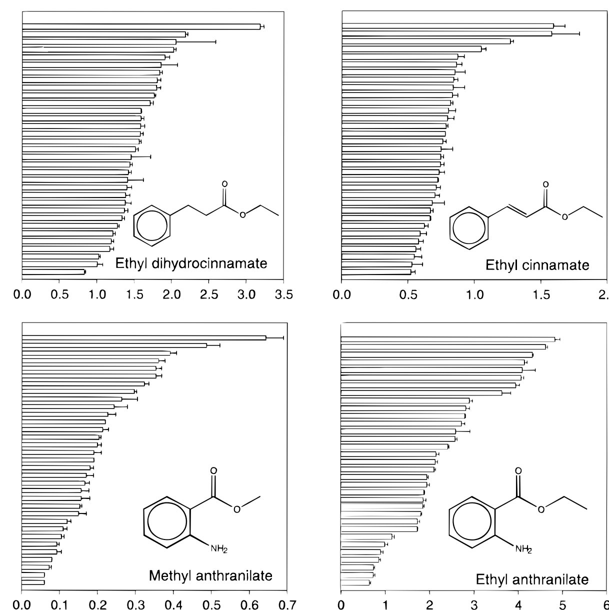

Figure 2. Concentrations (mg L^{-1}) determined in 33 wines. (In each bar chart, samples are ranked according to their content in the corresponding ester, so that the order of the samples varies from one graph to another.)

Table 3. Calibration Characteristics

	ester code A	B	C	D
mean slope	0.49	0.90	0.80	0.86
slope standard deviation (%)	2	4	4	5

Determination of the Ester Concentrations. All 99 measurements were made within 3 weeks. As seen from Table 3, the calibration was fairly constant during this period. The R^2 coefficients of the standard curves were never lower than 0.995. This point seems to be most important to ensure the quality of the results as a bias was recently proved to be frequently found in stable isotope dilution assays through the use of incorrectly fitted linear standard curves (Troost and Olavesen, 1996). The results of the repeatedly analyzed extract (five times) gave the following coefficients of variation: 7% for A, 5% for B, 14% for C, and 6% for D, over the period of the quantification of the 99 samples.

The results of the quantification are illustrated in Figure 2. The four esters were always found in the wine samples, but at low concentrations. These concentrations vary between 0.8 and 3.2 μg L^{-1} (mean 1.6 μg L^{-1}) for A, between0.5 and 1.6 μg L^{-1} (mean 0.8 μg L^{-1}) for B, between 0.06 and 0.6 μg L^{-1} (mean 0.2 μg L^{-1}) for C, and between 0.6 and 4.8 μg L^{-1} (mean 2.4 μg L^{-1}) for D. As can be seen from the standard error bars, despite these low concentrations, the variation of the quantification estimated from three replicates is always <10%.

Figure 2 shows that ethyl dihydrocinnamate and ethyl cinnamate vary quantitatively less between wines than anthranilates do. A "wine" factor of variation is confirmed by the analysis of variance (Table 4). It is interesting to note that ethyl anthranilate was found

Table 4. Significance of the Differences between Wines (ANOVA)

	ester code			
	A	B	C	D
F ratio[a]	35	53	145	453
associated probability $P(H_0)$	10^{-30}	10^{-35}	10^{-49}	10^{-65}

[a] Critical value: $F = 2.12$ [$P(H_0) < 0.01$].

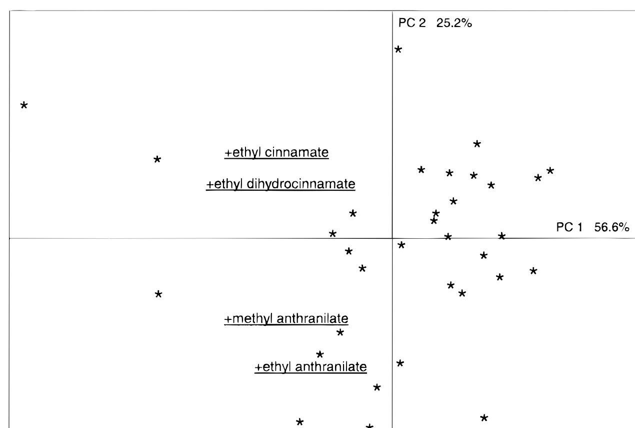

Figure 3. Projection of Pinot noir compositional data on PC1 and PC2 compositional loadings (vectors) and factor scores for 33 wines.

Table 5. Variation of the Four Ester Concentrations Explained by PC1 and PC2 (Sum of the Squared Correlations of the Four Variables with PC1 and PC2)

	ester code			
	A	B	C	D
% explained	83	86	73	82

to have the highest olfactory index among the four esters in a previous CHARM analysis of Pinot noir wine extracts (Moio and Etiévant, 1995); it is also the one whose concentration varies the most between wines.

A principal component analysis performed on the concentration means of the four esters measured in the 33 wines shows (Figure 3) 81.8% of the variance of the initial analytical data set along the first two principal components. As can be seen from Table 5, the four variables (concentrations in esters) are well correlated with PC1 and PC2 . The first PC is primarily a function of concentrations, with all four variables pointed in the same direction, and PC2 allows differentiation of cinnamate from anthranilate abundances. Wines, represented by stars on the graph, are well separated from each other, which is not surprising as concentrations were found to vary significantly (cf. ANOVA results). As can be seen in the figure, seven wines near the anthranilate variables seem to contain high concentrations in those compounds. The loadings for the four esters also indicate a significant correlation between the two cinnamates and the two anthranilates, most probably due to common precursors (respectively, $r = 0.51$ and $= 0.68$, critical value $r = 0.41$ at $\alpha = 0.01$).

Theoretically, the method developed allows the determination of ethyl cinnamate and methyl anthranilate at subthreshold concentrations since their thresholds in water were published to be, respectively, 16 μg L^{-1} (Etiévant *et al.*, 1983) and 3 μg L^{-1} (Hirvi and Honkanen, 1982). The amounts of these two esters in the wine studied were lower than those values. Their contribution to the flavor of wine, suggested by the GC-olfactometry analysis of a Grand Echezeau wine (Moio and Etiévant, 1995), is therefore not obvious since their detection thresholds are most probably higher in wine (Etiévant *et al.*, 1983). Concerning the two other esters, no data could be found concerning their olfactory thresholds. More work has therefore to be done to check and to measure their thresholds in this particular type of wine. For anthranilates though, the concentrations determined do not correspond to the total amount that could be revealed at the pH of the mouth. However, the correction factor that should be applied, of 9% maximum, can be considered as negligible.

A quantitative descriptive analysis of the flavor of 30 other Pinot noir wines is currently being performed to observe if a correlation could be established between the concentrations of these four esters and some of the wine sensory characteristics.

LITERATURE CITED

Casimir, D. J.; Moyer, L. Fluorometric detection of methyl anthranilate in Concord grape juice. *J. Assoc. Off. Anal. Chem.* **1976**, *59*, 269–272.

Cumming, A. Anthranilsauremethylester, Anthranilsaureäthylester. *Z. Phys. Chem.* **1906**, *57*, 579–598.

Errede, L. A.; Oien, H. T.; Yarian, D. R. Acylanthranils. Part 3. The influence of ring substituents on reactivity and selectivity in the reaction of acylanthranils with amines. *J. Org. Chem.* **1977**, *42*, 12–18.

Etiévant, P. X.; Issanchou, S. N.; Bayonove, C. L. The flavour of Muscat wines: the sensory contribution of some volatile compounds. *J. Sci. Food Agric.* **1983**, *34*, 497–504.

Gilbert, J. Applications of Quantitative Mass Spectrometry in Food Science. In *Applications of Mass Spectrometry in Food Science*; Gilbert, J., Ed.; Elsevier Applied Science: London, 1987.

Hirvi, T.; Honkanen, E. The volatiles of two new strawberry cultivars, "Annelie" and "Alaska pioneer". *Z. Lebensm. Unters. Forsch.* **1982**, *175*, 113–116.

Leahy, M.; Reineccius, G. Comparisons of methods for the isolation of volatile compounds from aqueous model system. In *Analysis of Volatiles: Methods, Applications, and Procedures—International Workshop*; Schreier, P., Ed.; De Gruyter: Berlin, 1984.

Moio, L.; Etiévant, P. X. Ethyl Anthranilate, Ethyl Cinnamate, 2,3-dihydrocinnamate, and Methyl Anthranilate: four important odorants identified in Pinot noir Wines of Burgundy. *Am. J. Enol. Vitic.* **1995**, *46*, 392–397.

Nelson, R. R.; Acree, T. E.; Butts, R. M. Isolation and Identification of volatiles, from Catawba wines. *J. Agric. Food Chem.* **1978**, *26*, 1188–1190.

Nykänen, L.; Suomalainen, H. Determination of esters of monocarboxylic acids. In *Aroma of Beer, Wine, and Distilled Alcoholic Beverages*; Reidel Publishing: Dordrecht, The Netherlands, 1984.

Primus, T.; Johnston, J.; Griffin, D. Solid phase extraction/high performance liquid chromatography method for the determination of methyl anthranilate residues in blueberries. *J. Liq. Chromatogr. Relat. Technol.* **1996**, *19*, 393–402.

Schieberle, P.; Grosch, W. Quantitative Analysis of Aroma Compound in Wheat and Rye Bread Crusts Using a Stable Isotope Dilution Assay. *J. Agric. Food Chem.* **1987**, *35*, 252–257.

Troost, J. R.; Olavesen, E. Y. Gas chromatographic/mass spectrometric calibration bias. *Anal. Chem.* **1996**, *68*, 708–711.

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