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ARTICLES

Stir Bar Sorptive Extraction Combined with GC-MS Analysis and Chemometric Methods for the Classification of South African Wines According to the Volatile Composition

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A simple method for the analysis of major wine volatiles and semivolatiles by stir bar sorptive extraction in combination with thermal desorption and gas chromatography−mass spectrometry (SBSE-TD-GC-MS) was developed. Significant experimental parameters such as extraction time, temperature, salt addition, pH, and thermal desorption parameters were optimized to provide a sensitive and robust analytical method. The method provided good repeatability (%RSD < 10%) for 38 major wine volatile compounds, including alcohols, acids, esters, phenols, aldehydes, ketones, and lactones. Quantitative data for 62 South African red and white wines were used to study the suitability of major volatile data for the differentiation of wine samples according to grape variety or cultivar. Principal component analysis (PCA) and cluster analysis (CA) showed that most of the variation in volatile composition between wine samples could be ascribed to differences in wine age, wood contact, and fermentation practices. Despite the contribution of these factors, discriminant analysis (DA) was successfully applied to the classification of red and white wine samples according to cultivar.

KEYWORDS: Wine; volatiles; stir bar sorptive extraction; GC-MS; chemometrics

INTRODUCTION

Volatile and semivolatile compounds present in wine determine the perceived flavor and aroma and have a definitive influence on the quality and therefore consumer acceptance of the final product (*1*). As the content of aroma compounds in grapes and wine depend on many factors such as the climatic and geographical origin as well as viticultural and wine-making practices, the volatile composition may be used for purposes of quality control as well as for authentication and classification purposes (*2*).

Analysis of wine flavor compounds is commonly performed by gas chromatography (GC). As the influential volatiles exist in wine at levels ranging from ng/L (ppt) to mg/L (ppm), sample preparation prior to GC analysis is crucial. The most common methods of sample preparation reported in the literature for wine volatile analysis include liquid−liquid extraction (LLE) (*3*), solid phase extraction (SPE) (*4*), and solid phase microextraction (SPME) (*5*) either performed in the immersion or in the headspace mode. Sorption-based sample preparation techniques offer advantages of solventless extraction, high sensitivity, limited matrix interference, ease of use, and the option of automation (*6*). As a result, these techniques have been applied extensively for wine analyses, including target analyses (*7*) and in screening methods (*8*).

Relatively recently, stir bar sorptive extraction (SBSE) was developed as an alternative sorption-based sample preparation technique. SBSE offers increased sensitivity compared to SPME because of an increased amount of stationary phase (*9*). Application of SBSE to wine analysis has been reported, including the determination of haloanisoles and halophenols involved in cork taint (*10*), volatile phenols related to *Brettanomyces* spoilage (*11*), oak-derived volatiles (*12*), and monoterpenes (*13*). Recent reports have also indicated the suitability of this technique for screening of a broad range of wine

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Table 1. Summary of the South African Wines Analyzed in the Current Study

red wine cultivar	vintage (*number*)	white wine cultivar	vintage (*number*)
Cabernet Sauvignon	1996 (1), 1997 (1), 1998 (2), 2003 (3)	Chardonnay	1999 (1), 2000 (1), 2001 (3), 2003 (6)
Pinotage	1999 (1), 2001 (2), 2002 (1), 2003 (2)	Chenin Blanc	2000 (1), 2003 (2)
Merlot	1999 (1), 2003 (6)	Sauvignon Blanc	2000 (1), 2001 (2), 2003 (2)
Ruby Cabernet	2003 (5)		
Shiraz	1999 (3), 2000 (2), 2001 (1), 2003 (7)		
blends	1998 (2), 1999 (1), 2000 (2)		

Table 2. Summary of the Wine Volatiles Quantified by SBSE-TD-GC-MS, Together with Repeatability Data and Ions Used for Quantitation

number[a]	compound	supplier, quality	%RSD ($n = 5$)	quantitation ion (*m*/*z*)
1	ethyl butyrate	Fluka, 99%	8.2	88
2	ethyl isovalerate (ethyl 3-methylbutanoate)	Fluka, 99%	5.6	88
3	isoamyl acetate (isopentyl acetate)	Riedel de Haën, 99%	4.3	87
4	1-butanol	Fluka, 99%	6.2	56
5	isoamyl alcohol (3-methylbutan-1-ol)	Sigma-Adrich, 99.5%	5.9	55
6	ethyl hexanoate	Sigma-Adrich, 99.5%	3.4	88
7	hexyl acetate	Fluka, 99%	4.6	84
8	2-octanone	Merck, 99%	6.4	99
9	ethyl lactate (ethyl 2-hydroxypropanoate)	Fluka, 99%	3.7	75
10	1-hexanol	Merck, 99.5%	7.9	56
11	ethyl octanoate	Fluka, 99%	2.3	88
12	acetic acid	Merck, 99.5%	9.0	60
13	furfural (furan-2-carbaldehyde)	Merck, 99.5%	2.9	96
14	formic acid	Merck, 99.5%	9.8	46
15	propanoic acid	Fluka,99%	8.2	74
16	1-octanol	Sigma-Adrich, 99.5%	7.1	84
17	γ-butyrolactone (dihydrofuran-2(3*H*)-one)	tentative identification[b]	5.6	86
18	ethyl decanoate	Fluka, 99%	4.5	88
19	furfuryl alcohol (furan-2-ylmethanol)	Fluka, 99%	4.7	98
20	diethyl succinate	Fluka, 99%	2.5	101
21	ethyl 9-decenoate (ethyl dec-9-enoate)	tentative identification[b]	5.5	88
22	ethylphenyl acetate (ethyl 2-phenylacetate)	Fluka, 99%	6.4	91
23	phenylethyl acetate	Fluka, 99%	3.1	91
24	ethyl dodecanoate	Fluka, 99%	6.7	88
25	guaiacol (2-methoxyphenol)	Sigma-Adrich, 99.5%	4.6	109
26	ethyl isopentyl succinate	tentative identification[b]	5.8	101
27	phenylethyl alcohol	Sigma-Adrich, 99.5%	4.0	91
28	(*Z*)-whiskey lactone ((*Z*)-5-butyl-4-methyldihydro-2(3*H*)-furanone)	Fluka, 98.5%	5.1	99
29	octanoic acid	Sigma-Adrich, 99.5%	4.1	60
30	4-vinylguaiacol (2-methoxy-4-vinylphenol)	Sigma-Adrich, 99.5%	4.6	150
31	decanoic acid	Sigma-Adrich, 99.5%	5.8	60
32	*cis*-isoeugenol ((*Z*)-2-methoxy-4-(prop-1-enyl)phenol)	tentative identification[b]	6.9	164
33	*trans*-isoeugenol ((*E*)-2-methoxy-4-(prop-1-enyl)phenol)	tentative identification[b]	7.3	164
34	dodecanoic acid	Sigma-Adrich, 99.5%	8.2	60
35	5-hydroxymethylfurfural (5-(hydroxymethyl)furan-2-carbaldehyde)	Sigma-Adrich, 99.5%	6.4	97
36	vanillin (4-hydroxy-3-methoxybenzaldehyde)	Sigma-Adrich, 99.5%	4.1	152
37	ethyl vanillate (ethyl 4-hydroxy-3-methoxybenzoate)	tentative identification[b]	5.2	151
38	acetovanillone (1-(4-hydroxy-3-methoxyphenyl)ethanone)	Sigma-Adrich, 99.5%	5.1	151

[a] Peak numbers correspond to **Figure 3**. [b] Tentative identification performed by using mass spectra and retention index data (*24, 25*).

volatiles (*14–17*). In addition, headspace sorptive extraction (HSSE) has been recently successfully applied for screening of wine volatiles (*18*).

Screening methods are typically used to quantify the common grape- and fermentation-derived volatiles, which are present in all wines and therefore play a relatively minor role in determining the so-called varietal character (*19*). Nevertheless, screening methods are often used for quality control and in authentication and classification studies because of the large amount of information provided in a single analysis. As vast quantities of data are typically generated in such studies, it is often problematic to meaningfully interpret the chemical data. Multivariate analysis methods have been extensively used as valuable aids for extracting relevant information from large data sets (*20*).

Exploratory analysis is frequently performed using unsupervised techniques such as principal component analysis (PCA) and cluster analysis (CA). PCA allows the reduction of data dimensionality for visualization purposes, whereas CA is used to evaluate similarity between samples based on their distances in an *n*-dimensional space (*21*). In contrast, supervised pattern recognition techniques are used to derive classification rules for categorization of unknown samples. In the case of wine samples this can be a common denominator such as cultivar, age, origin, etc. In linear discriminant analysis (LDA) a set of canonical variables are derived which describe a multivariate space on which predefined classes of samples are plotted. Classification of unknown samples is then based on the shortest distance to a particular class (*21*).

The efficacy of multivariate methods for classification of wines has been demonstrated by several authors using diverse sets of chemical data, including volatiles (*22*) and nonvolatiles (*23*). Chemometric methods in combination with chemical data can in this way be applied to unequivocally determine whether a specific wine is indeed of the claimed cultivar, origin, or even vintage. In addition, comprehensive data on volatile wine

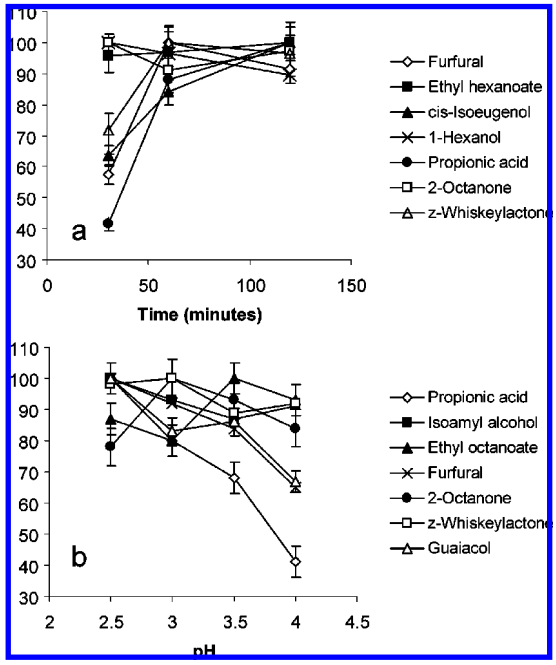

Figure 1. Effect of sampling time (**a**) and sample pH (**b**) on SBSE extraction of selected wine volatiles.

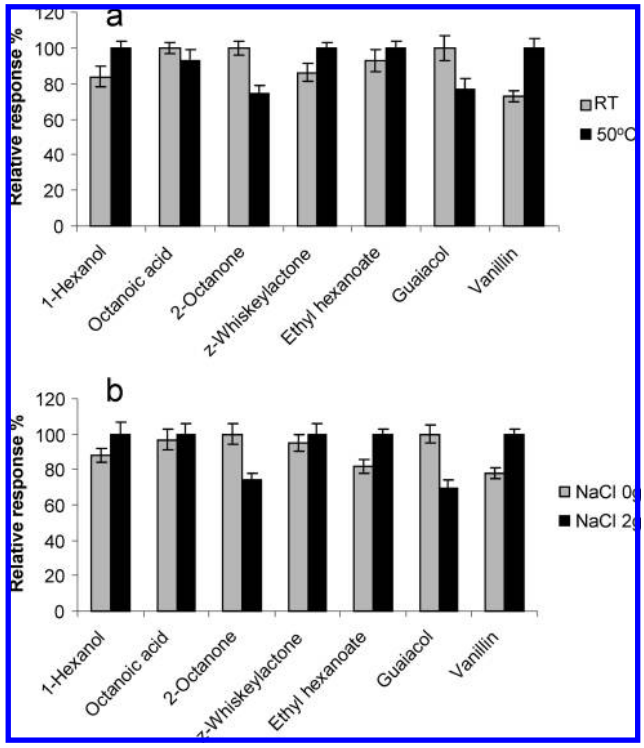

Figure 2. Effect of sampling temperature (**a**) and addition of salt (**b**) on SBSE extraction of selected wine volatiles.

constituents may serve to identify causes of defects in wine and/or be used to provide insight into the effect of oenological practice. Specifically, comparison of data for the volatile composition of South African wines could provide insight into the unique climatological- and cultivar-dependent characteristics of these wines.

The ability to perform statistical classification of wines based on relatively simple analytical techniques is therefore of significant interest. Within this context, the aim of the current study was 2-fold: (a) to develop a simple and robust sampling method exploiting the potential benefits of SBSE for the analysis of major wine volatiles and semivolatiles, and (b) to evaluate the differentiation of wines according to cultivar, independent of geographical origin, vintage, or oenological practice, based on volatile data.

MATERIALS AND METHODS

Materials. Polydimethylsiloxane (PDMS)-coated stir bars (Twisters) of 10 mm length and 0.5 mm film thickness were obtained from Gerstel, Mullheim a/d Ruhr, Germany. Standards were obtained from Riedel de Haën (Seelze, Germany), Merck (Darmstadt, Germany), Fluka (Buchs, Switzerland), Sigma-Aldrich (Steinheim, Germany) (**Table 2**). Hydrochloric acid was purchased from Merck and 2-octanol from Fluka (99%). Deionized water was obtained from a Millipore Elix water purification system (Supelco, Bellefonte, PA).

A total of 43 red and 19 white wines of vintages ranging from 1996 to 2003 were analyzed (**Table 1**). Wines were either purchased commercially, obtained from the KWV (Koöperatieve Wijnbouwers Vereniging, Paarl, South Africa) or the South African National Wine Show Association. The wines originated from most of the major wine-producing regions in South Africa. Samples were transferred under nitrogen from freshly opened bottles to completely filled amber vials for storage (4 °C) prior to analysis.

Sample Preparation. The sample preparation procedure was optimized as outlined in Results and Discussion. The optimized procedure was as follows: to a 15 mL vial were added 10 mL of deionized water, 0.5 mL of wine (pH previously adjusted to 3.0 using 0.1 M hydrochloric acid), and 5 μL of an internal standard solution (500 mg/L 2-octanol in ethanol). A preconditioned stir bar was introduced to the vial, which was covered with aluminum foil and stirred at 1200 rpm for 1 h at a temperature 22 °C (thermostatted room). Following extraction, the stir bar was removed, washed with a small amount of deionized water, and dried with a lint-free paper towel before being introduced in a thermal desorption tube (180 mm length, 4 mm OD, 3 mm ID, Gerstel). Stir bars were reconditioned (in a desorption tube installed in a GC oven) at 300 °C under a constant flow of nitrogen (100 mL/min) for 1 h.

Chromatographic Conditions. A 6890 GC coupled to a 5972 MS (Agilent Technologies, Palo Alto, CA) equipped with a thermal desorption system (TDS2) and a programmed temperature vaporizing injector (CIS4), both from Gerstel, were used throughout the study. For thermal desorption, the TDS was programmed as follows: 60 °C, held for 5 min, ramped to 300 °C (10 °C/min), held for 5 min. The TDS was operated in the solvent vent mode for the first 2 min and splitless mode thereafter. The transfer capillary temperature was kept constant at 300 °C. Analytes were trapped in the PTV cooled to −100 °C with liquid nitrogen and subsequently injected onto the column by ramping the injector to 280 at 600 °C/min (held at this temperature for 2 min). The PTV injector was kept in solvent vent mode during desorption and splitless mode (2 min) during injection. The split flow was adjusted to 50 mL/min. Separation was performed on an HP-INNOWAX capillary column (30 m length, 0.25 mm ID and 0.25 μm d_f, Agilent Technologies) with helium as carrier gas at a constant pressure of 50 kPa. The oven program was as follows: 40 °C held for 8 min, ramped at 3 °C/min to 60 °C, 5 °C/min to 200 °C, and at 20 °C/min to 250 °C (held for 5 min). The transfer line to the MS was kept at 280 °C with the MS scanning from 30−350 m/z at a rate of 2.5 scans/s.

Data Analysis. The relative peak areas of the 38 target analytes were used, after correction for the peak area of the internal standard, to construct matrices for red, white, and red and white wines together. All analytical data were autoscaled to produce variables with zero means and unit standard deviation. ANOVA, PCA, and LDA were performed using Statistica v.6.0 (Statsoft Inc., Tulsa, OK).

RESULTS AND DISCUSSION

Optimization of the Sample Preparation Procedure. Optimization was performed using real wine samples instead of model solutions in order to take into account possible matrix effects commonly encountered with sorptive sample preparation

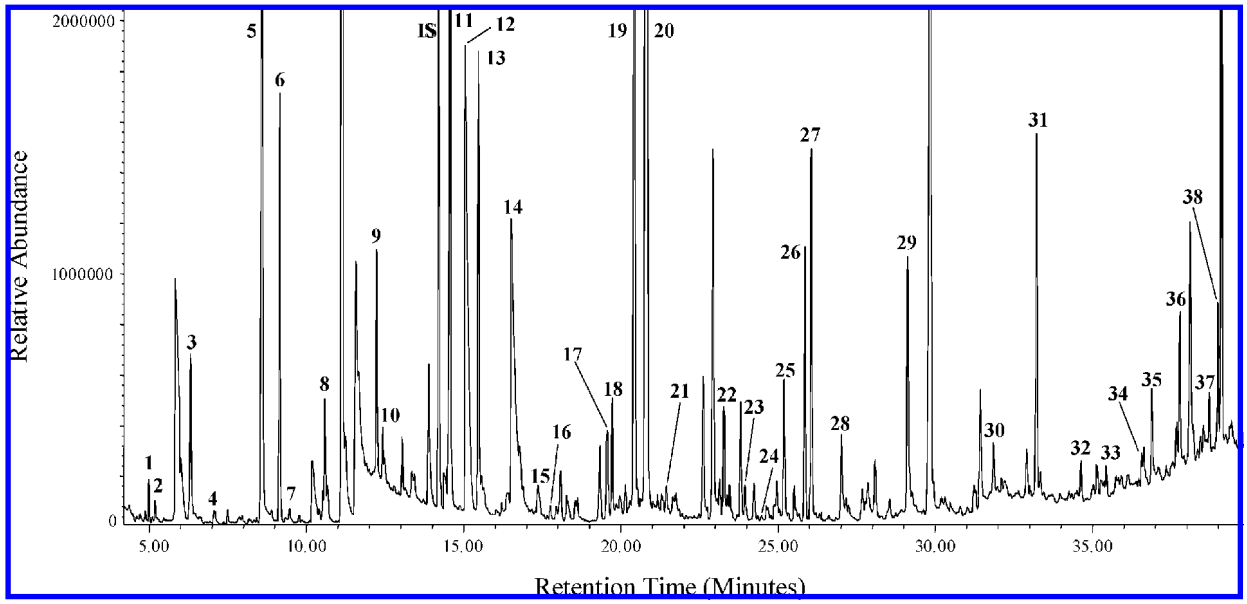

Figure 3. Total ion chromatogram for the SBSE-TD-GC-MS analysis of a South African red wine (Shiraz, 2000). Peak numbers correspond to **Table 2**. For experimental details, refer to Materials and Methods.

methods (*6*). The same red wine sample was used for all optimization experiments, and the stirring speed was kept constant at 1200 rpm throughout. All analyses were performed in triplicate using preconditioned stir bars, and mean values are presented. Note that since most of the influential parameters are well-known from extensive SPME investigations, a full experimental design was omitted.

A consequence of this increased sensitivity of SBSE (*9*) is that for undiluted wine samples, overloading of the column is observed, often obscuring other trace level volatile compounds. We found that diluting 0.5 mL wine with 10 mL of water provided the largest quantity of identifiable compounds without overloading the column. In this manner, the increased sensitivity of SBSE is exploited to reduce matrix effects. As an added benefit, dilution increases the lifetime of the stir bars; following extended use, stir bars become discolored, and together with increased detection of PDMS degradation products, this indicates that a particular stir bar is no longer usable. We have found that use of undiluted wine samples speeds up this degradation, probably because of small amounts of nonvolatile compounds remaining on the surface of the stir bar even after rinsing with water. Our findings for SBSE are in agreement with previous reports utilizing SPME and SPE (*7*).

The optimal extraction time at 22 °C was determined using a diluted sample adjusted to pH 3. The results for seven compounds representative of different chemical classes are summarized in **Figure 1a**. For most compounds, equilibrium was reached between 1 and 2 h, although it seems that longer extraction times favor less volatile compounds (larger molecules) at the expense of highly volatile analytes. A 60 min time was selected as optimal, also taking into account practical considerations such as the total analysis time. Extraction efficiency was evaluated at pH values of between 2.5 and 4.0. As is evident from **Figure 1b**, recovery for most compounds was optimal at pH 3.0, whereas the recovery of volatile organic acids decreases at higher pH values. Since most wines have pH values between 3.1 and 4.0, it was decided to standardize the pH to 3.0 for all samples. Extraction at 22 °C (thermostatted room) and at 50 °C (in a GC oven) were also compared (**Figure 2a**). Higher temperatures resulted in a minor positive effect on vanillin, presumably due to faster extraction kinetics. However, the slight benefit for selected compounds was not considered sufficient to warrant sampling at elevated temperature, especially taking into account practical implications for the routine application of the method. Finally, the effect of addition of 2 g of NaCl prior to extraction was also evaluated (**Figure 2b**), although the minor changes observed were not considered sufficient to include the addition of salt in the procedure.

Optimization of Thermal Desorption and Injection Parameters. Following optimization of the sample preparation step, the thermal desorption and injection parameters were fine-tuned. For thermal desorption, it was found that using a trapping temperature of −100 °C instead of −150 °C greatly improved the peak shapes for early eluting compounds. It is believed that faster heating of the liner to the injection temperature leads to reduced injection times and therefore less band broadening. The occasional occurrence of distorted and even split peaks, especially for ethyl esters, was observed. This is thought to be due to a small amount of water remaining on the stir bar or between the PDMS layer and the glass sleeve after drying with a paper towel. In order to avoid this, a 'solvent venting' step was performed by raising the TDS temperature to 60 °C in the solvent vent (2 min) mode prior to thermal desorption. The potential loss of some highly volatile compounds such as methanol, ethyl acetate, and ethyl propionate was considered of less significance compared to the benefit of avoiding split peaks.

Evaluation of the SBSE-TD-GC-MS Method. In **Figure 3** a typical total ion chromatogram obtained for the SBSE-TD-GCMS analysis of red wine is presented. Unambiguous identification of 32 of the 38 relevant volatile compounds was performed using authentic standards, NIST 98 and Wiley 275 mass spectral databases, and correlation with retention indices (RIs) reported in the literature (*24, 25*). The remaining 6 were tentatively identified by using mass spectral databases and RIs only (**Table 2**). These 38 compounds, representing the bulk of the major wine volatile constituents were selected for quantitative and chemometric analyses (**Table 2**). Some major compounds were not considered since no conclusive identification could be obtained from the MS spectra and RIs in the absence of standards.

Peak areas relative to the IS (2-octanol) were used for

Table 3. ANOVA Results for the Volatile Compounds Quantified in Red Wines[a]

peak[b]	variety (*n*)[c]	blend (5)	Cabernet Sauvignon (7)	Merlot (7)	Pinotage (6)	Ruby Cabernet (5)	Shiraz (13)	F_{calc}
Alcohols								
5	isoamyl alcohol	2.72	3.52	3.23	1.60	3.09	2.89	**4.76**
4	1-butanol	0.17	0.13	0.11	0.16	0.14	0.14	0.84
10	1-hexanol	0.56	0.51	0.46	0.50	0.81	0.52	**2.97**
16	1-octanol	0.91	0.99	0.66	0.93	0.76	0.98	0.88
27	β-phenylethyl alcohol	6.84	12.91	7.92	2.77	9.42	7.66	**5.76**
19	furfuryl alcohol	2.55	3.74	2.20	3.02	2.98	3.21	0.62
Phenols								
25	guaiacol	0.29	0.28	0.26	0.31	0.24	0.36	0.40
30	4-vinylguaiacol	0.37	0.39	0.36	0.43	0.34	0.47	0.32
32	*cis*-isoeugenol	0.08	0.08	0.05	0.08	0.06	0.09	0.77
33	*trans*-isoeugenol	0.23	0.24	0.28	0.32	0.23	0.33	0.30
Aldehydes								
13	furfural	18.92	23.69	18.61	21.37	18.97	23.93	0.37
35	5-hydroxymethylfurfural	0.45	0.62	0.61	0.67	0.66	0.70	0.42
36	vanillin	0.39	0.50	0.50	0.56	0.45	0.57	0.27
Ketones								
8	2-octanone	0.51	0.60	0.54	0.59	0.59	0.56	1.77
38	acetovanillone	0.29	0.34	0.31	0.37	0.29	0.36	0.20
Acids								
14	formic acid	18.68	34.04	25.23	29.95	24.89	36.29	0.80
12	acetic acid	42.35	43.72	43.67	48.22	32.35	53.54	0.44
15	propanoic acid	1.33	1.79	1.06	1.79	1.42	2.32	1.72
29	octanoic acid	0.26	0.25	0.29	0.35	0.38	0.26	**2.57**
31	decanoic acid	0.30	0.40	0.53	0.58	0.63	0.37	**2.98**
34	dodecanoic acid	0.01	0.03	0.03	0.03	0.05	0.02	**3.48**
Esters								
3	isoamyl acetate	0.10	0.18	0.23	0.27	0.31	0.18	1.29
7	hexylacetate	0.03	0.06	0.09	0.17	0.17	0.09	**2.65**
22	ethylphenyl acetate	0.25	0.21	0.09	0.07	0.09	0.12	**15.73**
23	β-phenylethyl acetate	0.12	0.30	0.23	0.18	0.40	0.27	1.56
9	ethyl lactate	10.26	7.09	4.13	6.55	4.22	5.06	**4.17**
1	ethyl butyrate	0.06	0.06	0.07	0.08	0.08	0.06	1.40
2	ethyl isovalerate	0.16	0.14	0.08	0.06	0.03	0.08	**5.19**
6	ethyl hexanoate	3.03	3.229	3.68	3.97	4.30	2.30	1.51
11	ethyl octanoate	7.17	8.967	11.19	12.34	12.28	7.86	**2.31**
18	ethyl decanoate	1.16	2.177	2.67	2.69	3.68	1.65	**4.52**
21	ethyl 9-decenoate	0.02	0.016	0.05	0.06	0.04	0.04	1.13
24	ethyl dodecanoate	0.02	0.107	0.13	0.06	0.32	0.06	**3.72**
20	diethyl succinate	170.56	146.664	81.77	62.04	94.91	105.50	**3.02**
26	ethyl isopentylsuccinate	16.19	25.026	15.17	5.76	19.93	16.40	**4.20**
37	ethyl vanillate	0.22	0.17	0.09	0.13	0.08	0.19	**3.12**
Lactones								
17	γ-butyrolactone	0.20	0.158	0.11	0.15	0.15	0.19	1.43
28	(*Z*)-whiskey lactone	0.11	0.108	0.10	0.12	0.10	0.13	0.25

[a] Mean values for each variety are listed together with calculated *F* ratios. *F* ratios above the critical value ($F_{5,42,0.05} = 2.47$) are presented in bold. [b] Peak numbers refer to **Figure 3** and **Table 2**. [c] Numbers in parentheses refer to the number of wine samples of each cultivar.

quantitation as reported previously, since certain reference standards were unavailable (*26, 27*). Also, for statistical techniques absolute concentrations are not required. Attempts to find a suitable IS for each of the different classes of compounds were unsuccessful due to failure to obtain sufficient separation for the complex wine extracts obtained by SBSE. 2-Octanol was therefore selected as IS based on the fact that it elutes in the middle of the chromatogram and possesses polar as well as nonpolar properties.

The method demonstrated good repeatability as reflected by relative standard deviations (%RSD, $n = 5$) ranging from 2.3% for ethyl octanoate to 9.8% for formic acid. These results demonstrate that SBSE provides acceptable sensitivity and good repeatability for a wide range of wine volatiles and therefore presents a viable alternative to SPME analysis. Comparison of the proposed methodology to published SPME methods for wine volatile analysis is complicated by the fact that results are highly dependent on sampling conditions. Nevertheless, both SPME and SBSE screening methods are applicable to the same compounds (i.e., the major wine volatiles). As mentioned earlier, the inherent sensitivity of SBSE for especially nonpolar analytes may be exploited by diluting wine samples prior to extraction, thereby reducing matrix concerns. On the other hand SPME offers the possibility of selecting a stationary phase based on the chemical properties of the compounds of interest (alternative phases for SBSE are not commercially available as yet). Both techniques offer the advantages of solventless extraction and higher sensitivity compared to LLE, and both benefit from ease of use, although the sampling step for SBSE cannot be fully automated as is the case for SPME. In productivity terms this is not a limitation, bearing in mind that multiple simultaneous SBSE extractions can be performed prior to subsequent automated injection. We have recently reported a headspace sorptive extraction (HSSE) method for the analysis of wine (*18*). The volatiles extracted in both the headspace and immersion modes using PDMS-coated stir bars are similar. SBSE compares favorably to the HSSE approach in terms of reduced matrix effects, repeatability, and sensitivity while advantages of the

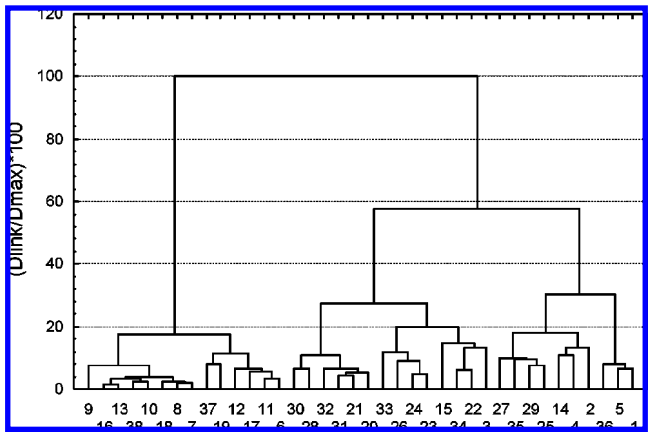

Figure 4. Tree diagram obtained for the volatile compounds in red wines using Ward's method and Manhattan distances. Numbers refer to the compounds specified in **Figure 3** and **Table 2**.

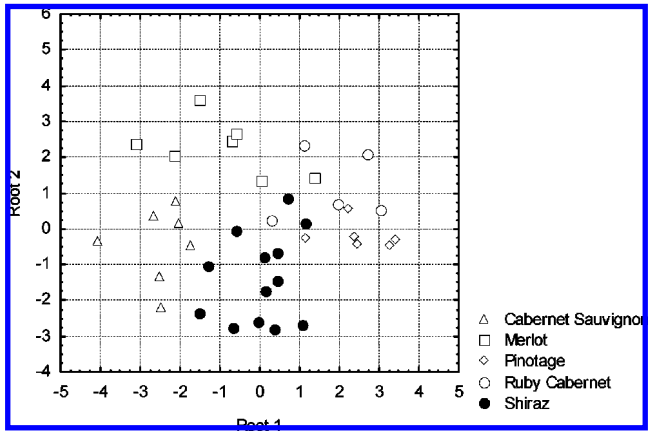

Figure 5. Two-dimensional scatter plot of the scores for red wine samples depicted on canonical roots 1 and 2.

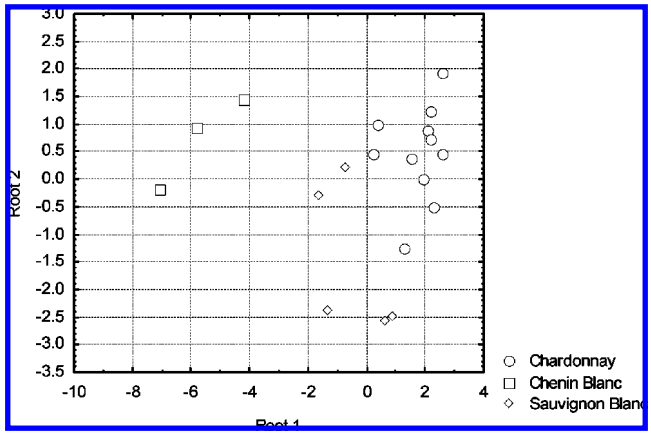

Figure 6. Scatter plot of the canonical scores for the white wines on the two canonical roots obtained by standard LDA.

proposed SBSE method include reduced extraction time and ease of use (no salt addition or specialized glassware is required).

In terms of the compounds quantified using the described SBSE-TDS-GCMS method (**Table 2**), these represent the common wine volatiles present in most wines and are responsible for the base of the flavor profiles of wines. All the compounds investigated in the current study have previously been identified in wine. Concerning alcohols, all red wines contain relatively high amounts of isoamyl and β-phenethyl alcohol; their levels are largely determined by fermentation conditions. In terms of flavor contribution, the higher (or fusel) alcohols produce a negative effect at high concentrations, although their effect can be positive at normal levels.

Wine acids are derived both from the grape and the yeast during fermentation. Volatile, low molecular weight compounds (formic, butyric, and especially acetic acid) are important contributors to the so-called "volatile" acidity; excess amounts are indicative of bacterial spoilage (*19, 28*). Higher molecular weight fatty acids are yeast-derived and only indirectly affect wine flavor by leading to the production of fatty acid esters, although octanoic acid has been associated with a fatty and unpleasant odor (*19, 29*).

Esters are formed by either enzymatic or chemical esterification of organic acids and alcohols, and in wine the most common are ethyl esters. Levels normally increase with age as chemical esterification occurs. However, for some ethyl wax esters (e.g., ethyl hexanoate, octanoate, and decanoate), levels decrease with age as the excess fatty acid esters formed by yeast are hydrolyzed during aging. Fatty acid esters contribute mainly fruity aromas (*19, 29, 30*) but also flowery and rose flavor notes (β-phenethyl acetate) (*19, 28*). Isoamyl acetate produces a banana aroma important for especially young wines. Ethyl esters of the main organic acids in wine (tartaric, malic, lactic, succinic, acetic, and citric) are formed in all wines during aging. These compounds are thought to contribute little to the improvement of wine aroma. The exception is ethyl lactate, the formation of which is related to malolactic fermentation.

Furfuryl compounds are derived from wood aging. Reduction of furfural to furfuryl alcohol (and further products) takes place during wine aging. A similar process leads to the formation of 5-hydroxymethylfurfural from 5-methylfurfural. Thus the content of these three compounds is strongly determined by wine-making practice (i.e., wood aging) and by inference by wine age. Aside from the furfural-derived products, vanillin also enters wine during barrel aging and produces a distinctive vanilla aroma. Vanillin undergoes reduction during further aging, leading to the formation of vannilyl alcohol and further products. The vanillin-derived compounds ethyl vanillate and acetovanillone were also quantified in the current study.

γ-Butyrolactone is produced during fermentation while the whiskey lactones are released from oak during wine aging. The trans isomer is associated with a coconut flavor (*28*). The content of volatile phenols is also associated with wood aging. Guaiacol and the isoeugenol isomers are directly extracted from oak. The latter two compounds contribute spicy, smoky aromas to wine, although these compounds are also associated with 4-vinyl- and 4-ethylphenols, the latter compounds being linked to largely negative aromatic properties (*28*).

Classification of South African Wines According to Cultivar Based on the Selected Volatile Compounds. The developed SBSE-TDS-GCMS method was applied to the quantitative analysis of 62 South African wines (**Table 1**). For semiquantitative data, peak areas relative to the internal standard were used for reasons outlined above. In order to study the suitability of volatile data for the classification of wines according to cultivar, the results were separated into three data sets containing volatile information for red, white, and red and white wines, respectively. Matrices of the autoscaled data were constructed containing the wine samples (objects) as rows, and the chemical compounds (variables) as columns. These data sets were subsequently investigated using chemometric methods as outlined below.

Red Wines. *Analysis of Variance (ANOVA).* Quantitative results for the red wine volatiles are summarized according to the different classes of compounds in **Table 3**. As a first

Table 4. Summary of Volatile Compounds Utilized as Variables in Discriminant Analyses of Red and White Wines

compound	white	red	red + white	compound	white	red	red + white
alcohols				acids			
isoamyl alcohol		X	X	formic acid			X
1-butanol	X		X	acetic acid			
1-hexanol		X	X	propanoic acid		X	X
1-octanol				octanoic acid		X	X
β-phenylethyl alcohol		X	X	decanoic acid			X
furfuryl alcohol				dodecanoic acid			
phenols				esters			
guaiacol				isoamyl acetate		X	
4-vinylguaiacol	X			hexylacetate			X
cis-isoeugenol		X	X	ethylphenyl acetate	X	X	X
trans-isoeugenol		X		β-phenylethyl acetate			
aldehydes				ethyl lactate		X	X
furfural	X	X	X	ethyl butyrate			
5-hydroxymethylfurfural				ethyl isovalerate			X
vanillin				ethyl hexanoate			
ketones				ethyl octanoate			
2-octanone	X		X	ethyl decanoate			
acetovanillone			X	ethyl 9-decenoate	X		X
lactones				ethyl dodecanoate		X	X
γ-butyrolactone				diethyl succinate	X	X	X
(*Z*)-whiskey lactone				ethyl isopentyl succinate	X		
				ethyl vanillate		X	X

exploratory step analysis of variance (ANOVA) was carried out to determine which compounds display significant differences between cultivars. Fifteen of the 38 quantified analytes showed significant differences between red wine cultivars at the 95% level (**Table 3**).

The following alcohols displayed significant variation between cultivars: β-phenethyl alcohol (**27**), isoamyl alcohol (**5**), and hexanol (**10**). Especially β-phenethyl and isoamyl alcohol may be used to differentiate Pinotage wines from the rest of the cultivars, as this cultivar is characterized by significantly lower amounts of these compounds. Regarding the acids, dodecanoic acid (**34**), decanoic acid (**31**), and octanoic acid (**29**) were found to vary significantly. The ester ethylphenyl acetate (**22**) showed the highest variation between cultivars, with Cabernet Sauvignon wines containing on average more of this compound than the other cultivars. In fact, the content of this compound differs significantly between Cabernet Sauvignon and the rest of the single-cultivar wines as well as between the blended wines and each of the single-cultivar wines. (Blended wines are wines of different cultivar which are mixed together following wine-making.) This latter distinction of blends can probably be ascribed to the fact that blended wines invariably contain Cabernet Sauvignon as the predominant wine. Other esters that showed significant variation between cultivars include ethyl isovalerate (**2**), hexyl acetate (**7**), ethyl decanoate (**18**), and ethyl dodecanoate (**24**). For the last two compounds, differences in mean amounts between cultivars mirror the behavior of the corresponding acids, decanoic and dodecanoic acid: Ruby Cabernet contains on average the highest levels of these compounds while Shiraz, Cabernet Sauvignon, and the blended wines contain on average the lowest. This variation might be ascribed to differences in the average age of each of these classes of wine, as their levels in wine decrease with age. The average age of each of the classes of red wine at the time of analysis (2003) were: 5 years for blends, 3 years for Cabernet Sauvignon, 1 year for Merlot, Pinotage, and Shiraz, and less than 1 year for Ruby Cabernet (**Table 1**). Both esters derived from succinic acid (ethyl isopentyl succinate (**26**) and diethyl succinate (**20**)) displayed similar variations in the analyzed wines: Pinotage and Cabernet Sauvignon wines displayed the lowest and highest mean values of these compounds, respectively. As part of this study, the organic content of the same wines was quantified using an ion-exclusion HPLC method (30). These data indicate that the content of succinic acid also varies significantly between the cultivars, with Cabernet Sauvignon wines containing significantly higher mean levels than Pinotage (results not shown). It thus seems reasonable that the variation in the succinic acid between these cultivars is responsible for the measured variations in the volatile ester derived from this compound. Similarly, significant variation in ethyl lactate levels (**9**) can partially be correlated to the content of lactic acid in the analyzed wines: the lowest mean value of lactic acid was measured in Merlot wines, as is the case for ethyl lactate. High levels of lactic acid in turn are associated with increased incidence of malolactic fermentation. Moreover, the content of these so-called acid-esters have been shown to generally increase during wine aging (*31*). Thus differences in the average ages of wines of each cultivar as outlined above may serve to obscure cultivar-related differences.

The amount of ethyl vanillate (**37**) a compound associated with wood contact, was found to vary significantly between the wines, with lower mean values in Merlot and Ruby Cabernet wines compared to the other three wines. This might be explained by common wine-making practice in South Africa, where Cabernet Sauvignon, Shiraz, and Pinotage are more often exposed to wood aging in order to produce wines with aging potential. In contrast to this observation, however, the content of none of the analyzed phenols, lactones, or ketones were found to differ significantly between the cultivars. Any trends might then be concealed by the varied oenological practices necessarily associated with the diverse wines analyzed in the current study.

The picture for the blended red wines is slightly unclear: the highest content of certain compounds (ethyl vanillate, ethylphenyl acetate, ethyl lactate, ethyl isovalerate, diethyl succinate, γ-butyrolactone) is measured in the blends while for other compounds the content is lower in the blended wines than any of the single cultivar wines (furfuryl alcohol, *trans*-isoeugenol, 5-hydroxymethylfurfural, vanillin, 2-octanone, formic acid, decanoic acid, dodecanoic acid, isoamyl acetate, hexyl acetate, β-phenyl acetate, ethyl octanoate, ethyl decanoate, and ethyl dodecanoate). As alluded to above, especially the concentration of the neutral esters decreases significantly with aging (*31*)

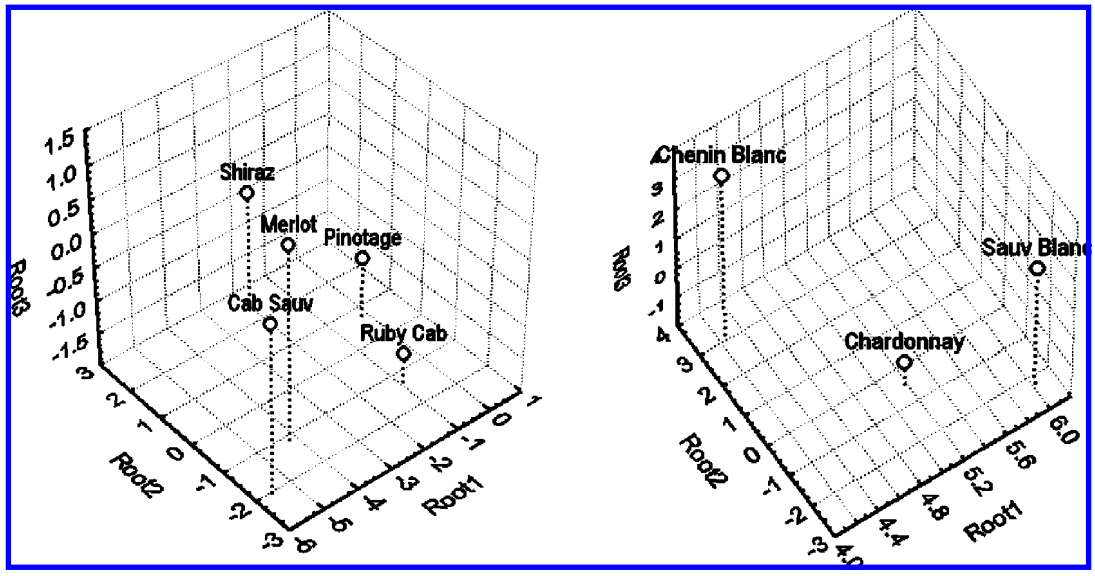

Figure 7. Scatter 3D plot of group centroids for red (left) and white (right) wine cultivars. The red and white wines are separated for the sake of clarity.

whereas concentrations of acid esters such as ethyl lactate and diethyl succinate increase with age. In light of this observation, the differences in volatile content for the blended wines can likely be ascribed to the higher average age of the blended red wines.

Principal Component Analysis (PCA). Application of PCA to the volatile data set for red wines revealed that 83.3% of total variance is extracted by the first seven principal components. PC1 accounts for 30.8% of the variance and correlates positively with all the analyzed phenols, lactones, and furfural compounds as well as vanillin and acetovanillone. All of these compounds are derived from oak cooperage. Therefore it seems that wood aging practices are responsible for most of the variation in the volatile composition of the analyzed wines. Although ANOVA indicated that none of these compounds varied significantly between cultivars, it should be noted that PCA is an unsupervised exploratory technique used to maximize the variance in the complete data set (i.e., not according to predefined classes). This underscores the fact that wood exposure, present for some of the wines of each cultivar, is responsible for most of the variation in the data set. The expectation would thus be that this factor might overshadow variatal differences in major volatile content, thereby complicating their classification according to cultivar.

In addition to the wood-derived products, the low molecular weight organic acids (formic, acetic, and propanoic acid) also show high loading factors on PC1. PC2, responsible for 20.5% of the total variance, and PC3 (9.2%), describe the behavior of the remaining acids and esters. The content of these compounds are known to vary with wine age and were also shown by ANOVA to differ significantly between cultivars. PCs 2 and 3 therefore seem to reflect variations due to wine age. In conclusion, PCA results indicate that the principal variations in the volatile data for the analyzed red wines can be related to corresponding variation in wine age and wood maturation, and not cultivar.

Cluster Analysis (CA). CA largely corroborated the conclusions reached from PCA data. Using Ward's method of agglomeration and Manhattan distances to measure the similarity between variables, two main clusters can be discerned (**Figure 4**). The cluster on the left contains the wood-related compounds and low molecular weight acids, the same compounds highly correlated with PC1. The second group contains the remainder of the acids and esters, correlated with PC2 and PC3, again indicating that wood aging and wine age are largely responsible for the variation in the current data set.

Linear Discriminant Analysis (LDA). To achieve a classification of red wines according to cultivar, stepwise standard LDA was used. Blended red wines were omitted for this study, as the aim was to classify according to grape variety. Fourteen variables were used in the classification function, including alcohols (isoamyl alcohol, 1-hexanol, β-phenylethyl alcohol), phenols (*cis*- and *trans*-isoeugenol), aldehydes (furfural and ethyl vanillate), acids (propanoic acid and octanoic acid), and esters (isoamyl acetate, ethylphenyl acetate, ethyl lactate, ethyl dodecanoate, and diethyl succinate). Most of the compounds were shown to vary significantly between cultivars by ANOVA. Also, these compounds broadly reflect wood aging (eugenols, furfural, ethyl vanillate) and fermentation practices (alcohols, acids, esters) as well as wine age (esters). It was further found that four canonical roots accounted for 100% of the properties of data set.

Because of the relatively limited amount of samples, the complete set of samples was used as training data to derive the classification function in LDA. Accordingly, the same data set was also used to evaluate the recognition ability of the model, thus the *posterior* probabilities were calculated. The classification function in this manner provided correct prediction of all wine samples according to cultivar. This promising result is obtained in spite of the contribution of other parameters such as age and wood maturation to the variability in the volatile data. **Figure 5** presents the scatter plot of red wines on the first two canonical roots, where relatively good discrimination between the different red wine samples according to cultivar is evident in the two-dimensional space.

White Wines. A similar statistical procedure as outlined above was applied to white wines. ANOVA indicated that only three esters displayed significant differences between the three cultivars ($F_{2,18,0.05} = 3.63$). These compounds, ethyl lactate (**9**, $F_{calc} = 4.47$), diethyl succinate (**20**, $F_{calc} = 4.86$), and ethyl isopentyl succinate (**26**, $F_{calc} = 5.75$), are all esters of principal wine organic acids. As alluded to earlier, the formation of especially ethyl lactate can be related to fermentation practices. ANOVA applied to the (unpublished) results for the organic

content of the same wines indicated a significant ($F = 6.16$, $F_{2,37,0.05} = 3.27$) difference in the lactic acid content of the three cultivars. Specifically, Chardonnay wines contained significantly higher levels of lactic acid. This is related to the increased incidence of malolactic fermentation for these wines, as also indicated by significantly higher pH levels ($F = 5.76$) and lower levels of malic acid ($F = 2.76$) measured for Chardonnay wines. It therefore seems reasonable that these differences in fermentation practice are also reflected in the content of related volatile esters for the same wines. No difference in the content of succinic acid was observed from the organic acid data, although this might be related to the coelution of an unkown compound with succinic acid in the HPLC method utilized.

As was the case for red wine, PC1 was highly correlated to all compounds associated with wood aging (1-butanol, guaiacol, 4-vinylguaiacol, *cis*- and *trans*-isoeugenol, furfural, 5-hydroxymethylfurfural, vanillin, acetovanillone, and (*Z*)-whiskey lactone) while PC2 was correlated to fatty acids and esters. In agreement with the discussion for the red wines, these two PCs, describing most of the variation in the analyzed wines, can be related to wood contact and wine age. In addition, cluster analysis (data not shown) reveals two distinct groups of variables. The first contains wood-derived phenols, alcohols, aldehydes, and ketones as well as the low molecular weight acids and the acid esters. The presence of the acid esters (ethyl isopentyl succinate, diethyl succinate, and ethyl lactate) can probably be ascribed to the fact that wooded wines on average are of older vintages (these wines have more aging potential).

Standard LDA applied to the white wine data provided a function containing eight variables, once again providing 100% correct prediction for all white cultivars according to posterior probabilities. The compounds used in the classification of white wines include 1-butanol, 4-vinylguaiacol, furfural, 2-octanone, ethylphenyl acetate, ethyl-9-decenoate, diethyl succinate, and ethyl isopentyl succinate. Note that ethyl lactate is not included in this model, which is unexpected considering the higher incidence of malolactic fermentation of Chardonnay wines referred to above. Nevertheless, two canonical roots cover 100% of the properties for white wines. In **Figure 6** a scatter plots of the canonical scores for the white wines on these two roots are depicted, again illustrating good discrimination between white wines according to cultivar.

Interestingly, wood aging was shown to be largely responsible for variance in the volatile content of red wines by PCA but did not vary significantly between cultivars (ANOVA). In contrast, for white wines differences in volatile content related to fermentation and wood aging are responsible both for significant variation (PCA), but are also useful for classification purposes. This can be ascribed to higher incidence of wood aging and malolactic fermentation for one of the white cultivars in our data set (Chardonnay).

Red and White Wines. The precedent results clearly demonstrate that the content of major volatile compounds can be used for the classification of both red and white wines according to cultivar, despite significant variation in the data set due to extraneous factors such as wood aging, vintage, and geographical origin. However, of more practical importance for routine implementation would be a single classification function to allow simultaneous categorization of both red and white wines (and eventually also special cases such as rosé wines) according to cultivar. This was attempted using the complete data set of all analyzed wines. Use of the complete data set allows the option to remove a wine sample from the training set and to obtain a classification function which can subsequently be used to categorize the unknown sample.

To this end, a random wine sample was removed from the data set, and stepwise standard LDA was performed. Seven roots that cover 95.2% of the data properties and formed by 20 volatile components were obtained (**Table 4**). This classification function once again provides 100% correct posterior prediction of all red and white wines (excluding the omitted sample) according to cultivar.

The discriminant function allows the calculation of the coordinates of each group (or cultivar) centroid on each of the canonical roots from the coefficients of the discriminant functions and values of each variable. A three-dimensional scatter plot of the cultivar centroids plotted on the first three discriminant roots is presented in **Figure 7**, where the red and white varieties are separated to differently scaled sections for clarity purposes.

The variables of the unknown wine were subsequently inserted into the discriminant function and the resultant scalar values compared to the centroid coordinates for each of the cultivars. The unknown wine is then assigned to the group to which the Euclidian distance is shortest, in this case the Chardonnay wines (Euclidean distance 1.39, compared to other white wines >4.73 and red wines >6.12).

To determine the probability of this assignment, the variance radii for each centroid was calculated using Fisher's *F*-statistics and the risk value (α) determined. From the risk factor, the probability *P* can be calculated ($P = 1 - \alpha$). Using this method, the unknown wine sample is identified as Chardonnay with a 97.3% probability. (The probability for Sauvignon Blanc is 2.0% and less than 0.1% for the other cultivars.) Following this prediction, the unknown sample was inserted into the modeling data set, and new classification was performed. From this stepwise standard LDA, the posterior probability of this wine being a Chardonnay was calculated as 99.99%, thereby confirming the previous prediction.

In conclusion, a summary of the volatile compounds used in each of the classifications presented above is depicted in **Table 4**. *trans*-Isoeugenol and isoamyl acetate were used for the classification of red wines but not the complete data set. The content of the former is on average higher, and for the latter lower, in red wines compared to white wines. Similarly, 4-vinylguaicol and ethyl isopentyl succinate were used to classify white wines but were not used for the complete data set. Mean values for the latter compound were on average lower in white wines while for the former, levels were similar for red and white wines. These observations can be ascribed to significant variation in levels of these compounds within the complete data set, which serves to obscure any cultivar-related differences and thereby precludes their utility in an overall classification function for red and white wines.

Other volatile compounds were used in the classification function of the complete set of wines, although they were not used to classify either red or white wines separately. These compounds generally display differences in mean levels between red and white cultivars. This increases the discriminatory power of these compounds for all wine cultivars, even though they might not be suitable to differentiate between only red or only white cultivars. Compounds included in this class are acetovanillone (higher levels in red), decanoic acid (higher in white), hexyl acetate (higher in white), ethyl isovalerate (higher in red), and formic acid (higher in Chardonnay and Sauvignon Blanc).

The following compounds were not used in any of the LDA functions: γ-butyrolactone, (*Z*)-whiskey lactone, furfuryl alcohol,

guaiacol, 5-hydroxymethylfurfural, vanillin, acetic acid, dodecanoic acid, β-phenylethyl acetate, ethyl butyrate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate. With the exception of ethyl decanoate and dodecanoic acid, none of these compounds showed significant differences between cultivars by ANOVA for red or white wines. This indicates that, at least for the current data set, fewer compounds may be quantified while still providing successful differentiation of wine samples according to cultivar. Contrary to our findings for red and white wines, ethyl decanoate has previously been used to successfully classify Nebbiollo-based Italian wines according to geographical origin by SLDA (*31*) while ethyl octanoate has been used to classify white and rosé Spanish wines according to cultivar (*32*).

It is important to note that the content of major wine volatiles analyzed in the current study do not necessarily have a significant bearing on the perceived flavor characteristics of the wines. A number of these compounds are typically present at levels below their odor threshold values, in other words with odor activity values (OAVs) below 1. Depending on the nature of wine sample, examples of compounds with OAVs below 1 include β-phenylethyl acetate, 1-hexanol, guaiacol, ethyl vanillate, acetovanillone, decanoic acid, furfural, furfuryl alcohol, and diethyl succinate. Examples of compounds typically present above their threshold values include isoamyl alcohol, ethyl decanoate, ethyl butyrate, ethyl hexanoate, isoamyl acetate, ethyl octanoate, hexanoic acid, butyric acid, octanoic acid, hexanoic acid, 4-vinylguaiacol, *Z*-whiskey lactone, and vanillin (*29*). Relation of aroma characteristics to chemical composition is further complicated by the fact that high OAVs do not guarantee an impact on wine flavor. Aroma model and emission experiments indicate that especially compounds such as fusel alcohols, acids, esters, and some volatile phenols (i.e. the majority of the compounds analyzed here) often do not contribute to wine aroma individually, even though they are present at levels significantly above their thresholds (*19*). Rather, it is commonly accepted that cultivar-specific flavor can often be ascribed to trace-level "varietal" aroma compounds such as monoterpenes (Muscat wines), norisoprenoids (*33*), pyrazines (*34*) (Sauvignon Blanc, Cabernet Sauvignon, and Cabernet Franc), thiols, and mercaptans (*26*). However, analysis of these so-called impact odorants is significantly more labor- and time-intensive and expensive (*26*). From this perspective, the simple screening method presented here should prove advantageous in studies where the aim is to classify large amounts of wine samples according to cultivar and as such competes with screening methods utilizing SPME and LLE.

The same set of wines used in the current study has previously been used to classify wine cultivars by anthocyanin- (*35*) and noncolored phenolic content (*23*). Compared to classification according to nonvolatile phenolics, the current method based on the major wine volatiles offers the advantages of simple (although time-consuming) sample preparation and straightforward quantitation and provides a better overall classification of wines according to cultivar.

In conclusion, the principal value of the current classification lies in the fact that wine samples were not selected according to predefined criteria in order to reduce variability due to age, oenological practice, or geographic origin. It is well-known that each of these factors significantly affects the volatile composition of wines. In fact, PCA and CA have shown that most of the variability in the volatile data for the selected set of wines can be related to wood aging, fermentation practice, and wine age. It should furthermore be noted that in South Africa, by law, wine may be labeled as a single cultivar if it contains at least 85% of the specified cultivar. Therefore any number of the analyzed wines may contain up to a maximum of 15% of a different variety, which may logically serve to hamper attempts at classifying these wines according to cultivar. However, despite these contributions to variability, we have shown that it is possible to extract the information from the data set to allow successful classification of wine samples according to cultivar. It would seem that the major volatile composition contains a substantial amount of information that can fruitfully be studied with chemometric methods in combination with simple and reliable screening methods as developed in this paper. Further work is required to increase the number of wine samples to confirm the conclusions drawn here for a relatively small sample set as well as to investigate the suitability of major volatile data for the classification of wines according to alternative criteria (geographical origin, vintage, sensory data, detection of adulteration).

ABBREVIATIONS USED

SBSE, stir bar sorptive extraction; HSSE, headspace sorptive extraction; TDS, thermal desorption system; PDMS, poly(dimethylsiloxane); SPME, solid phase microextraction; LLE, liquid−liquid extraction; OAV, odor activity value; ANOVA, analysis of variance; PCA, principal component analysis; LDA, linear discriminant analysis; CA, cluster analysis.

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