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Application of a Headspace Sorptive Extraction Method for the Analysis of Volatile Components in South African Wines

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A headspace sorptive extraction (HSSE) in combination with thermal desorption gas chromatography-mass spectrometry (TD-GC-MS) method for the analysis of volatile components (alcohols, esters, carbonyls, acids, phenols and lactones) in wine samples was developed. Extraction conditions such as salting-out effects, sorption time, stirring speed, phase ratio, extraction temperature, and effect of pH were thoroughly evaluated as part of method validation. The method was very sensitive with LODs and LOQs between 50 pg/L to 299 μg/L and 0.2 ng/L to 0.996 μg/L, respectively. Repeatability for all the compounds was between 3 and 22%. The intermediate repeatability was obtained within the acceptable range. Out of 39 volatile compounds selected, 37 were detected and quantitated. The method was found to be simple, cost-effective, sensitive, and use a small sample volume. The method was successfully applied for the routine analysis of 79 young red and white wine samples from various South African districts.

KEYWORDS: Stir bar; headspace extraction; CIS; TDS; volatile compounds; wine; GC; and MS

INTRODUCTION

Wine is one of the most complex alcoholic beverages, resulting from enzymatic transformation of grape juices and its aroma responsible for much of such complexity (*1, 2*). Describing the wine aroma is far from a simple task for researchers because more than 800 components have been identified in the volatile fraction of wine including alcohols, esters, carbonyls, acids, phenols, lactones, acetals, thiols, terpenols, etc. It comprises different chemical characteristics covering a wide range of polarity, solubility and volatility. Furthermore, the existence of some of these constituents at a very low concentration (</= mg/L) in wine and the unstable nature of some of these compounds giving rise to the appearance of artefacts due to oxidizing being in contact with air or degraded by heat or extreme pH, makes their analyses more complex (*2–4*).

Aroma of a wine is one of the major factors that determine the nature and quality of wine (*3, 5*) and it can be influenced by the climate, soil type, geographical location, type of grape, fermentation processes, the container where fermentation and ageing takes place, to name a few (*6–8*). Many of the aroma compounds in wine already exist in the grape but several are also formed during fermentation as well as maturation such as esters and higher alcohols (*8–11*). Furthermore, a significant number of volatiles are formed during ageing or extraction from oak such as phenols, furans, oak-derived-vanillic compounds, to mention few (*12*).

Because of the complex nature of wine matrices and the low levels of some of the volatile compounds which are partially responsible for the aroma and flavor, sample enrichment is crucial for identification and quantification. This sample enrichment should allow for extraction, concentration, and isolation of analytes, thereby greatly influencing the consistent and accurate analysis of wine.

In the last few years several sample enrichment techniques have been developed that partially fulfill the above needs. Classical liquid-liquid extraction (LLE) (*1–4, 6, 13*) based on organic solvent extraction and solid phase extraction (SPE) (*12, 14, 15*) based on adsorbent materials where analytes are bound to active sites on a surface have been successfully applied to wine analyses. However, these methods may suffer from disadvantages such as time constraints, labor intensiveness and may involve multistep processes which may lead to analyte loss, as well as the use of toxic organic solvents.

In the early 90s a solvent free method called solid phase micro extraction (SPME) was developed by Pawliszyn and co-workers (*16*). SPME has successfully been applied for the analyses of different wines (*4, 17–19*). Recently, a new extraction procedure for aqueous samples, named stir bar sorptive extraction (SBSE) was developed by Baltussen and Sandra (*20, 21*). SBSE is based

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on the same principle as SPME except that higher PDMS phase in SBSE (50 to 250 times greater amounts of extraction phase) (*8*, *22*) provides higher sample capacity. SBSE extraction can be done either in the headspace (*23*–*25*) or by introducing directly into the aqueous sample (*5*, *26*, *27*) and stirring for a given time. Regardless of the increasing in equilibrium time compared to direct SBSE, the headspace extraction is very advantageous in reducing the risk of contamination. Furthermore, it increases the lifetime of the PDMS coated stir bar when used in the headspace as a complex matrix and the presence of sugar, particularly in sweet wines, can lead to a faster degradation of the PDMS layer (*28*). SBSE has been applied successfully for the analyses of aroma compounds in wine (*5*, *8*, *23*).

In this contribution we present a simple and cost effective extraction technique that allows analysis of a large number of volatile compounds which can potentially contribute to the aroma and flavor of wines in a single chromatographic run. This was done based on headspace stir bar sorptive extraction method. It involves sorption of volatile compounds into the PDMS phase of the stir bar from the headspace of the sample followed by desorption, cryo-trapping, and gas chromatograph-mass spectrometry analysis. Furthermore, given the lack of existing information, on volatile components in South African wines the developed method was applied to a select wine samples. The results are used to verify differences in the aroma constituents among similar or different cultivars according to their region and production technology. Moreover, it is important to underline that, as to the best of our knowledge the method is new with a new approach to sample preparation technique for screening volatiles in wine.

MATERIAL AND METHODS

Wine Samples. A total of 79 young wines of vintage 2005 were supplied by different cellars from various South African districts. The 64 are red wines of different cultivars (Pinotage, Shiraz, Cabernet Sauvignon, and Merlot), 16 from each cultivar of six different regions and produced by different cellars. The other 15 are Chardonnay white wine from different regions and produced by different cellars.

Chemicals and Reagents. The following standards of volatile compounds and solvents were used: acetoin, *n*-propanol, *n*-butanol, isobutanol, furfural, diethyl succinate, ethyl butyrate, ethyl-D-lactate, hexyl acetate, 2-phenylethyl acetate, 2,6-dimethoxy phenol, eugenol, 5-(hydroxymethyl)furfural, propionic acid, *n*-butyric acid, isobutyric acid, *n*-valeric acid, isovaleric acid, ethyl octanoate, 4-methyl-2-pentanol (internal standard), acetone (pestanal), and NaCl (Fluka, Zwijndrecht, Netherlands); isoamyl alcohol, *n*-hexanol, 2-phenylethyl alcohol, 5-methylfurfural, ethyl hexanoate, *o*-cresol, *p*-cresol, whiskey lactone, vanillin, hexanoic acid, decanoic acid and ethyl decanoate (Aldrich, Steinheim, Germany); isoamyl acetate, methanol and absolute ethanol (HPLC grade) [(Riedel-de Haën (Steinheim, Germany)]; phenol and guiaiacol (Sigma, Steinheim, Germany); acetic acid (Merck, Darmstadt, Germany), and Milli-Q water (University of Stellenbosch, Stellenbosch, South Africa) were used.

Preparation of Synthetic Wine. A global stock solution containing all the analytes was prepared in a synthetic wine matrix (12% ethanol in Milli-Q water) using different concentrations of analytes ranging from 1 mg/L for ethyl-octanoate and ethyl decanoate to 1.6 g/L for acetic acid based on the collected data from different authors and VCF 2000 volatile compounds in food database [(1996–1999 Boelens Aroma Chemical Information Service) (BACIS)] to make it as close as possible to real wine samples.

Equipment and Apparatus. A 15 mL amber vial coupled with solid PTFE (polytetrafluoroethylene) line screw cap, (Supelco, Bellefonte, PA), 2 mL vials with green caps (Agilent, Technologies, Palo Alto, CA), 20 mL Twister headspace vials with glass inserts Twister (Gerstel, Müllheim a/d Ruhr, Germany), 20 mm magnetic aluminum crimp cap and 20 mm PTFE white silicone molded septa (Agilent Technologies, Palo Alto, CA), and JENWAY 4330 pH meter (Janway Ltd., Felsted, Dunmow, Essex, CM6 3LB, U.K.) were used.

Experimental Conditions. The instrumental set-up was done in a similar way as described by Sandra et al. (*6*, *29*). GC-MS analysis was carried out with an Agilent 6890 GC coupled to a 5973N MS (Agilent Technologies, Palo Alto, CA). A 30 m HP-INNOWax capillary column [(0.250 mm I.D. × 0.5 μm film thickness) (Agilent Technologies)] was used for separating the volatile compounds. The GC oven was held at 30 °C for 2 min, increased to 130 °C at a rate of 4 °C/min and then at 8 °C/min to 250 °C where it was kept for 5 min. Helium was used as the carrier gas with a flow of 1 mL/min in the constant pressure mode. The MS was operated in a scan mode with a scan range of 30–350 amu at 4.45 scans/sec. Spectra were recorded in the electron impact mode (EI) at 70 eV. The MS transfer line, source and quadrupole were at 250, 230, and 150 °C, respectively. Quantitation was performed with total ion chromatograms (TICs) using the sum of all ions for well-separated compounds after careful examination of the peak purity and single ion extraction was applied for closely eluting and minor peaks (**Table 1**). Identification was based on comparison of mass spectra with Wiley 275 and NIST 98 libraries as well as retention times of known standards in synthetic wine for all compounds. For comparison with literature data, retention indices (RI) were experimentally determined using a mixture of *n*-alkanes (**Table 2**).

The TDS 2 was carried out with a temperature program from 30°C held for 1 min and raised at 20 °C/min to 260 °C where it was held for 10 min. It was operated in solvent vent mode with a purging time of 3 min and equilibrium time of 1 min. The heated transfer line was set at 300 °C. After desorption, the analytes were cryofocused in a programmed temperature vaporizing injector (PTV) at −100 °C using liquid nitrogen prior to injection. An empty baffled glass liner was used in the PTV. Solvent vent injection with splitless time of 2 min and purge time of 0.1 min was performed by ramping the PTV from −100 to 270 °C at 12 °C/sec and held for 10 min.

Sample Preparation. One mL of wine, 100 μL (1.7 mg/L) of 4-methyl-2-pentanol (internal standard), and 1.5 g NaCl was transferred into a 20 mL headspace vial. The volume was made to 6 mL with ultra-pure water of 12% ethanol mixture. The pH was adjusted to 3.2 using a formate buffer. A glass coated magnetic stirrer was added to the mixture. A preconditioned SBSE stir bar Twister (Gerstel, Müllheim a/d Ruhr, Germany) of 10 mm length coated with a 0.5 mm PDMS layer (25 μL) was suspended in the headspace using a glass insert. The vial was sealed with 20 mm aluminium crimp cap and PTFE/silicone molded septa using a hand crimper. The mixture was stirred for 1 hour at room temperature and 1200 rpm. Then the vial was left standing for 3 hours at room temperature. After sampling, the stir bar was removed, dried gently with lint free tissue, and placed in a glass tube of 187 mm length, 6 mm o.d. and 4 mm i.d., which then was placed in the TDS-A auto-sampler tray (Gerstel, Müllheim a/d Ruhr, Germany). It was followed by thermal desorption, cryo-trapping, and gas chromatography-mass spectrometry analysis. The stir bars were reconditioned for 30 min at 280 °C under a nitrogen stream flow, and no carry-over was observed. Regularly system blanks were run to confirm cleanliness of the system.

RESULTS AND DISCUSSIONS

Method Optimization. To characterize the aroma and flavoring compounds in South African wines using HSSE-TD-GC-MS, standard parameters such as ionic strength, sorption time, stirring speed, pH, sample volume, extraction temperature, TDS 2 (desorption), and CIS 4 (cryo-trapping) conditions were thoroughly investigated to evaluate sorption and desorption conditions of the method as well as separation of the analytes. The synthetic wine was used to get the optimum conditions that give an adequate number of chromatographic peaks and quantifiable peak areas in a single run.

TDS 2 and CIS 4 Conditions. Taking the number of chromatographic peaks and total chromatographic areas as an experimental response for optimization (*30*) TDS-2 and CIS-4

Table 1. Method Validation Data Obtained by HSSE-TD-GC-MS (See Text for Conditions)

no.	compounds	($y = mx + c$)[a]	(R^2)[b]	LODs (ng/L)	LOQs (ng/L)	repeatability[c]	intermediate repeatability[d]	relative %Recovery[e]	quantitative signal[f]
1	ethyl butyrate	$y = 1.6075x + 1.063$	0.9710	10.50	34.90	12	5	94	TIC[i]
2	isobutanol	$y = 0.011x + 0.0138$	0.9911	126.20	420.80	4	10	108	TIC[i]
3	isoamyl acetate	$y = 2.7946x + 1.3326$	0.9929	1.90	6.40	13	8	70	TIC[i]
4	*n*-butanol	$y = 0.02x + 0.0202$	0.9919	0.60	2.10	5	8	73	TIC[i]
5	isoamyl alcohol	$y = 0.0933x + 0.1105$	0.9938	43.50	145.10	8	7	43	TIC[i]
6	ethyl hexanoate	$y = 13.561x + 0.9322$	0.9946	0.80	2.60	3	6	67	TIC[i]
7	hexyl acetate	$y = 12.985x + 0.2265$	0.9991	0.70	2.20	5	5	72	TIC[i]
8	acetoin	$y = 0.0013x + 0.0001$	0.9949	9.54[g]	31.78[h]	5	16	42	45[j]
9	ethyl-D-lactate	$y = 0.0056x + 0.0571$	0.9934	2.42[g]	8.06[h]	12	24	91	TIC[i]
10	1-hexanol	$y = 0.3255x + 0.4976$	0.9899	1.80	6.00	5	7	94	TIC[i]
11	ethyl octanoate	$y = 134.01x + 0.0557$	0.9995	0.08	0.30	6	4	54	TIC[i]
12	acetic acid	$y = 0.0023x + 0.2174$	0.9900	5.40	17.80	8	7	91	TIC[i]
13	furfural	$y = 0.0482x + 0.0466$	0.9964	2.10	7.00	20	15	90	TIC[i]
14	propionic acid	$y = 0.0046x + 0.0033$	0.9992	2.80	9.40	8	13	126	74[j]
15	isobutyric acid	$y = 0.0355x - 0.0087$	0.9994	5.80	19.50	21	11	95	TIC[i]
16	5-methylfurfural	$y = 0.2064x + 0.012$	0.9996	2.00	6.70	13	14	99	TIC[i]
17	*n*-butyric acid	$y = 0.0239x + 0.0147$	0.9996	3.00	10.10	11	14	120	60[j]
18	ethyl decanoate	$y = 88.471x - 0.0148$	0.9999	0.05	0.20	16	20	34	88[j]
19	isovaleric acid	$y = 0.0559x + 0.0304$	0.9996	7.40	24.60	9	19	121	60[j]
20	diethyl succinate	$y = 0.1362x + 0.3446$	0.9924	39.10	130.30	13	11	95	101[j]
21	*n*-valeric acid	$y = 0.0676x - 0.0009$	0.9998	2.60	8.60	16	17	92	TIC[i]
22	2-phenethyl acetate	$y = 1.0095x + 0.235$	0.9914	5.10	17.00	13	21	58	104[j]
23	hexanoic acid	$y = 0.5482x + 0.0251$	0.9997	0.40	1.20	12	12	24	60[j]
24	guaiacol	$y = 0.3434x + 0.0314$	0.9957	4.10	13.80	19	20	102	109[j]
25	cis-oak-lactone	$y = 0.2291x + 0.0039$	0.9994	0.35[g]	1.16[h]	12	9	87	99[j]
26	2-phenylethyl alcohol	$y = 0.0989x + 0.0676$	0.9965	18.50	61.60	15	5	88	TIC[i]
27	trans-oak-lactone	$y = 0.1258x + 0.0134$	0.9997	0.64[g]	2.15[h]	18	18	109	99[j]
28	o-cresol	$y = 0.3668x + 0.0131$	0.9995	13.10	43.70	20	18	97	TIC[i]
29	phenol	$y = 0.0746x - 0.0034$	0.9997	3.70	12.50	13	14	125	TIC[i]
30	4-ethylguaiacol	$y = 0.6471x + 0.0328$	0.9991	38.80	129.20	10	7	123	137[j]
31	octanoic acid	$y = 0.2385x + 0.0726$	0.9918	0.30	0.90	19	21	80	TIC[i]
32	*p*-cresol	$y = 0.3182x + 0.001$	0.9986	0.37[g]	1.25[h]	18	18	97	TIC[i]
33	eugenol	$y = 0.4531x + 0.034$	0.9991	110.80	369.30	15	16	115	164[j]
34	decanoic acid	$y = 0.1034x + 0.0179$	0.9977	9.80	32.70	19	20	103	60[j]
35	2,6-dimethoxy phenol	$y = 0.0094x + 0.0043$	0.9933	19.00[g]	62.00[h]	22	22	82	154[j]
36	5-(hydroxymethyl)furfural	$y = 0.0008x + 0.0002$	0.9958	299.00[g]	996.00[h]	5	26	68	126[j]
37	vanillin	$y = 0.0019x + 0.0002$	0.9978	31.00[g]	103.00[h]	18	6	94	151[j]

[a] Regression equation where y = the relative peak area, m = slope, and c = intercept. [b] Regression coefficient. [c] Repeatability ($n = 8$) and [d] Intermediate Repeatability ($n = 4$) both in terms of % relative standard deviation. [e] Relative recovery (%). [f] Quantitative signal. [i] TIC (total mass). [j] Single ion extract used for quantitation. [g] LODs. [h] LOQs: concentrations presented in μg/L.

(Gerstel, Müllheim a/d Ruhr, Germany) working conditions were thoroughly investigated. Among the many parameters investigated, purging time, desorption time, desorption temperature, as well as inlet initial and final temperatures have showed significant influence on the quality of the analyses. As a result, the above-mentioned desorption and cryo-trapping conditions were selected.

Influence of Ionic Strength (Salting-Out Effect). Salting-out effect on the extraction of flavoring compounds at various concentration levels were described by many authors (*12*, *31*). Based on the data gathered from different authors (*17*, *18*, *28*) only sodium chloride (NaCl), the most common salt used in sample enrichment, was examined at different concentration levels (from 0% to saturation) such as 0.5, 1, 1.5, and 2 g. As the amount of salt increases the peak areas increased proportionally except for acetoin and acids which could be due to their high ionization properties. Since addition of 2 g NaCl saturated the solution and started to negatively affect the early eluting compounds, 1.5 g NaCl was selected as an optimum concentration.

Sorption Time. The amount of analytes from the aroma and flavor of wine samples that can be extracted by HSSE is determined by two partition coefficients (*8*). The partition coefficient of the analytes between the headspace and the PDMS coated stir bar as well as between the headspace and the sample matrix. Therefore, sorption of analytes into the stir bar was investigated by two parameters .i.e. stirring time where the time the headspace vial stirred and standing time where after stirring the vial stands at room temperature. In the former, five different times, namely 30, 60, 90, 120, and 150 min, were tested. Stirring time beyond 1 hour showed a decrease in peak areas for acetoin, ethyl-D-lactate, isobutyric acid, *o*-cresol, phenol, 4-ethylguaiacol, octanoic acid, and *p*-cresol. This behavior could probably be due to them being released from the PDMS phase of the stir bar after being initially sorbed and then replaced by less volatile but more apolar compounds that require a considerably longer time to reach equilibrium between liquid phase and the headspace since equilibrium is not yet reached. Hence, 1 hour stirring time was chosen as the optimum to encompass average sensitivity for all compounds.

The second sorption parameter (standing time), showed a dramatic improvement on the extraction for most of the compounds. This could be due to the time required for the analytes that have already migrated to the headspace of the sample to be fully sorbed into the PDMS coating of the stir bar (*8*). Hence, eight different standing times ranging from 30 min to 12 hours (30 min, 1, 2, 3, 4, 6, 8, and 12 hours) were tested. Beyond 3 hours, the peak areas of the lower alcohols and esters start to decrease as the time increases, probably due to them being released from the PDMS layer to the headspace (*32*). For the rest of the compounds, no significant increase was achieved beyond 4 hours. Consequently, a time of 3 hours that

Table 2. Average Concentration (mg/L ± SD) of Volatile Compounds Obtained in 79 Young South African Wine Samples of Vintage 2005 Using the Validated Method HSSE-TD-GC-MS (See Text for Conditions)

compounds	Pinotage average[a] ± SD[b]	Shiraz average[a] ± SD[b]	Cabernet Sauvignon average[a] ± SD[b]	Merlot average[a] ± SD[b]	Chardonnay average[a] ± SD[b]	RI[c]
Ethyl butyrate	51.04 ± 0.73	50.60 ± 0.17	50.75 ± 0.24	50.79 ± 0.27	52.09 ± 0.59	990
isobutanol	23.64 ± 11.68	38.87 ± 15.79	36.32 ± 12.74	45.53 ± 29.89	9.61 ± 5.52	1072
isoamyl acetate	6.27 ± 2.88	4.00 ± 1.55	4.25 ± 1.70	3.65 ± 1.88	10.34 ± 3.53	1098
n-butanol	3.13 ± 0.80	6.81 ± 3.54	6.22 ± 3.01	5.01 ± 2.34	10.09 ± 5.66	1145
isoamyl alcohol	183 ± 36.96	207 ± 23.13	268 ± 44.62	264 ± 69.38	159 ± 24.92	1216
ethyl hexanoate	0.45 ± 0.26	0.30 ± 0.08	0.36 ± 0.10	0.34 ± 0.14	1.14 ± 0.37	1233
hexyl acetate	0.01 ± 0.01	0.01 ± 0.01	0.006 ± 0.004	0.003 ± 0.003	0.12 ± 0.05	1278
acetoin	19.71 ± 10.25	28.51 ± 16.38	19.93 ± 12.12	21.87 ± 11.72	26.54 ± 21.20	1307
ethyl-D-lactate	230 ± 62.73	184 ± 72.89	220 ± 74.73	208 ± 75.80	51.81 ± 69.80	1364
1-hexanol	3.55 ± 2.97	4.15 ± 0.98	4.79 ± 1.02	4.06 ± 1.78	6.31 ± 13.49	1372
ethyl octanoate	0.04 ± 0.03	0.02 ± 0.01	0.024 ± 0.01	0.023 ± 0.01	0.12 ± 0.04	1455
acetic acid	996 ± 999	1344 ± 846	1395 ± 763	1509 ± 1014	901 ± 499	1476
furfural	3.73 ± 1.99	7.90 ± 4.15	7.68 ± 3.81	10.39 ± 4.13	15.54 ± 6.29	1495
propionic acid	6.30 ± 3.99	9.33 ± 6.82	17.02 ± 7.59	23.85 ± 10.49	28.44 ± 10.88	1570
isobutyric acid	0.56 ± 0.34	0.64 ± 0.20	0.59 ± 0.20	0.89 ± 0.39	0.29 ± 0.09	1597
5-methylfurfural	0.14 ± 0.09	0.18 ± 0.06	0.20 ± 0.10	0.24 ± 0.08	0.28 ± 0.08	1610
n-butyric acid	2.40 ± 4.95	0.99 ± 0.52	1.00 ± 0.44	1.28 ± 0.60	1.40 ± 0.38	1659
ethyl decanoate	0.01 ± 0.01	0.006 ± -0.003	0.006 ± 0.003	0.005 ± 0.003	0.03 ± 0.01	1665
isovaleric acid	1.03 ± 0.54	1.33 ± 0.52	2.17 ± 0.97	2.02 ± 0.70	0.37 ± 0.09	1707
diethyl succinate	17.38 ± 8.15	24.61 ± 7.37	28.14 ± 11.88	22.83 ± 8.91	2.06 ± 1.03	1716
n-valeric acid	0.44 ± 0.22	0.32 ± 0.30	0.24 ± 0.19	0.26 ± 0.21	0.20 ± 0.19	1772
2-phenethyl acetate	0.16 ± 0.10	0.23 ± 0.16	0.20 ± 0.11	0.12 ± 0.06	0.21 ± 0.15	1863
hexanoic acid	0.24 ± 0.09	0.16 ± 0.08	0.18 ± 0.06	0.16 ± 0.06	0.47 ± 0.16	1876
guaiacol	0.21 ± 0.15	0.13 ± 0.04	0.20 ± 0.08	0.14 ± 0.05	0.014 ± 0.01	1909
cis-oak-lactone	0.01 ± 0.003	0.01 ± 0.01	0.01	0.02 ± 0.01	nd[e]	1949
2-phenylethyl alcohol	13.80 ± 4.11	36.72 ± 14.37	67.05 ± 45.20	49.82 ± 19.25	6.89 ± 2.35	1968
trans-oak-lactone	0.08 ± 0.04	0.07 ± 0.05	nd[e]	nd[e]	nd[e]	2030
o-cresol	0.03 ± 0.02	0.04 ± 0.03	0.053 ± 0.03	0.07 ± 0.03	0.005 ±	2053
phenol	0.20 ± 0.10	0.30 ± 0.09	0.29 ± 0.08	0.32 ± 0.10	0.24 ± 0.07	2059
4-ethylguaiacol	0.013 ± 0.01	0.02 ± 0.01	0.02 ± 0.02	0.015 ± 0.01	0.009 ± 0.01	2090
octanoic acid	0.92 ± 0.34	0.72 ± 0.45	0.87 ± 0.45	0.97 ± 0.31	3.01 ± 1.30	2097
p-cresol	0.09 ± 0.07	nd[e]	nd[e]	nd[e]	0.007 ± 0.01	2134
eugenol	0.05 ± 0.03	0.05 ± 0.02	0.07 ± 0.04	0.05 ± 0.03	0.008 ± 0.01	2225
decanoic acid	nd[e]	nd[e]	nd[e]	nd[e]	nd[e]	2255[d]
2,6-dimethoxy phenol	nd[e]	nd[e]	nd[e]	nd[e]	nd[e]	2274[d]
5-(hydroxymethyl)furfural	56.98 ± 19.37	111 ± 47.31	113 ± 64.23	114 ± 56.37	154 ± 62.81	2528
vanillin	47.35 ± 27.63	55 ± 31.89	92.83 ± 56.53	34.19 ± 17.73	47.46 ± 23.74	2568

[a] Average of the detected values only. [b] SD: standard deviation of the determined values only. [c] RI: retention indices from real wine samples and [d] RI from synthetic wine calculated on HP-INNOWax column. [e] nd: not detected.

satisfies the sensitivity of all the compounds was selected as an optimum time for the sorption process. Thus, by combining the two parameters discussed, a total of 4 hours were taken as an optimum sorption time.

Stirring Speed. Stirring speed of the sample (solution) during extraction where it gives rapid equilibrium between the liquid sample and the gas phase was the most influential variable. Stirring speeds of 500, 900, 1100, and 1200 rpm were tested. With the exception of few compounds such as the lower alcohols (isobutanol, 1-butanol, and isoamyl alcohol), C_2 to C_5 acids and acetoin, increasing stirring speed showed good improvement on the peak areas of all analytes. Increasing stirring speed not only increases extraction efficiency but also lowers the equilibrium time. Thus, 1200 rpm was selected as an optimum stirring speed for extraction.

Effect of pH. Compounds can exist in solution as either neutral or charged species depending on the pH of the solution (*33*). As the pH of wine samples range between 2.8 and 4.0 (*34*), it was decided to adjust the pH to 3.2 (*17*). Hence, HCl and format buffer were investigated. Both HCl (1 M, drop-wise) and a formate buffer (400 μL, 1 M, pH 4.1) showed good improvement of extraction mainly for the acids, although a decrease in extraction efficiency was measured for the lower alcohols and esters. The evident effect on the acids is mainly due to protonation changing them from ionized to neutral species (*33*) which allow their migration to the gas phase as well as their interaction with the PDMS phase of the stir bar. Since adding a fixed amount of formate buffer adjusts the sample pH to the desired value while eliminating the need for continuous pH measurements, this method is less labor-intensive and was therefore selected.

Volume (Phase) Ratios. During the extraction three different total volumes of samples, namely 3, 6, and 9 mL, were investigated. Beyond 6 mL increasing the volume of sample and decreasing the headspace volume shows no significant improvement for any of the analytes, as previously reported (*30*). Moreover, increasing the sample volume decreases the stirring power of the magnetic stirrer, thus increasing the equilibration time. In addition the probability the matrix coming in contact with the PDMS coated stir bar suspended in a headspace was high. As a result, 6 mL was selected as an optimum volume for the sample.

Extraction Temperature. The effect of extraction temperature on analytical response was evaluated at three levels: room temperature, 40, and 50 °C. Increasing extraction temperature showed a negative effect for most of the analytes, this could be due to shifting of the equilibrium between the gas phase and the PDMS favoring the former (*4*) or between the sample matrix and the headspace.

Although initially 39 volatile compounds were selected for this work, two of them (methanol and 1-propanol) were excluded at the end of the method optimization because under all the

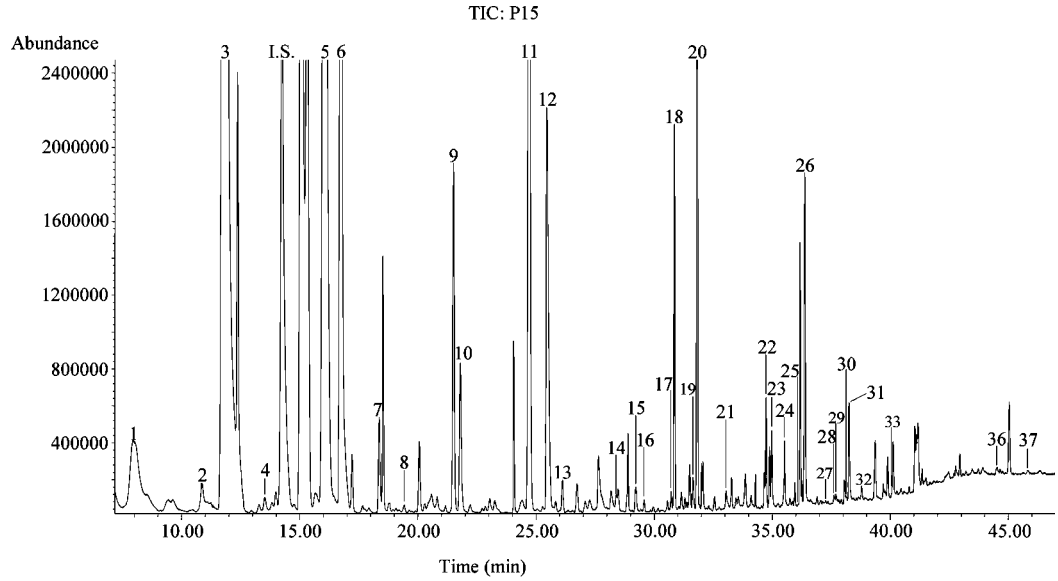

Figure 1. A TIC Chromatogram of Pinotage wine vintage 2005 obtained using the optimized method HSSE-TD-GC-MS. For compound identification, see **Table 1**, and for quantitative information see **Table 2**. Concentration of I.S. 1.7 mg/L. (See Text for Conditions).

conditions examined during extraction, they failed to be detected. This could be due to the high solubility in water which keeps them from migrating to the headspace (*1*) or lack of interaction with the PDMS phase of the stir bar due to their very low partition coefficients. Fore this reason, subsequent work was done based on the thirty seven remaining compounds (**Table 1**).

Method Validation. As the importance of method validation is a requisite for a good method, the optimized method was validated thoroughly using the synthetic wine. The calibration lines of each compound were prepared by dilution of the global stock solution to different concentrations ranging between 8.3 ng/L and 333 mg/L for esters, 250 ng/L and 667 mg/L for acids, 625 ng/L and 31.25 mg/L for alcohols, 125 ng/L and 25 mg/L for phenols, 167 ng/L and 33.3 mg/L for furans, 250 ng/L and 280.3 mg/L for carbonyls, as well as 420 ng/L and 20.8 mg/L for lactones. After the addition of 1.7 mg/L internal standard (4-methyl-2-pentanol) for each of the above calibration concentrations, the previously mentioned HSSE extraction procedure and TD-GC-MS conditions were applied.

Each concentration level used for calibration was repeated four times (four replicates). The average peak areas relative to internal standard obtained against the different concentrations used were applied to construct the calibration curves. From each calibration curve, the regression coefficient (R^2), linearity and other analytical characteristics were calculated. The regression coefficient (R^2) for most of the compounds was greater than 0.99 except for ethyl butyrate (0.9710) (**Table 1**). A wide range of linearity ($\approx 10^5$) was obtained for most of the compounds.

The limit of detections (LODs) and limit of quantitations (LOQs) were calculated from the calibration graphs constructed for each volatile compound as 3 and 10 times the signal to noise ratio (S/N), respectively (*4*). The method proved very sensitive, achieving low LODs ranging between 50 pg/L to 299 μg/L for ethyl decanoate and low LOQs between 0.2 ng/L to 996 μg/L for 5-(hydroxymethyl)furfural (**Table 1**).

The repeatability was evaluated using eight replicates of a synthetic wine of the same batch using different stir bars assuming all PDMS coated stir bars are the same and calculated as percent relative standard deviation (%RSD). The repeatability was between 3 and 22% for ethyl hexanoate and 2.6-dimethoxy phenol, respectively (**Table 1**). The intermediate repeatability was evaluated by analyzing four replicates of different batches using different stir bars and calculated in terms of %RSD. With the exception of ethyl lactate (24%), 2-phenylethyl acetate (21%), octanoic acid (21%), 2,6-dimethoxy phenol (22%), and 5-(hydroxymethyl)furfural (26%) it was within the acceptable range ($\leq$20%) (*35*) (**Table 1**).

In an extraction based on sorptive techniques the recovery, expressed as the ratio of the extracted amount of solute (m_{PDMS}) over the original amount of solute in the water ($m_o = m_w + m_{PDMS}$), is dependent upon the distribution coefficient (*22*). Since the developed method involves three phases (liquid, gas, and PDMS), it is expected for the analytes to experience different partition properties among the different phases (*8*). Hence, it was impossible to calculate the absolute recovery as the original concentration of the analytes is distributed among the three phases. Nevertheless, the relative recovery (**Table 1**) was carried out from a spiked synthetic wine. i.e. spiked with known amount and recovery calculated in a similar fashion as reported (*1*).

The method was very selective and applicable to compounds that can migrate to the headspace of the vial (volatile and semivolatiles) as well as compounds that can have good interaction with the PDMS phase of the stir bar.

In all the parameters tested, isoamyl acetate, isoamyl alcohol, ethyl hexanoate, hexyl-acetate, ethyl octanoate, ethyl decanoate, diethyl succinate, and 2-phenylethyl acetate were easily and efficiently extracted. This could be due to their higher distribution coefficient (*K*o/w) compared to the rest of the compounds (*22*). Moreover, even the concentration increases the peak intensity of the lower alcohols (isobutanol, and 1-butanol), acetoin, C_3 to C_5 acids, 5-(hydroxymethyl)furfural, and vanillin remain very small, whereas their area increases proportionally. For the rest of the compounds, the analytical responses were proportional to their concentrations and relatively good. This variation might be related to the response factor of each compound.

It is also essential to highlight that the very low detection and quantitation limits of almost all the analytes using MS detector makes the technique suitable for sample screening and multicompound analysis.

Application to Real Wine Samples. After optimizing and validating the method thoroughly, it was applied to the analysis of 64 red wine samples from four different red wine cultivars

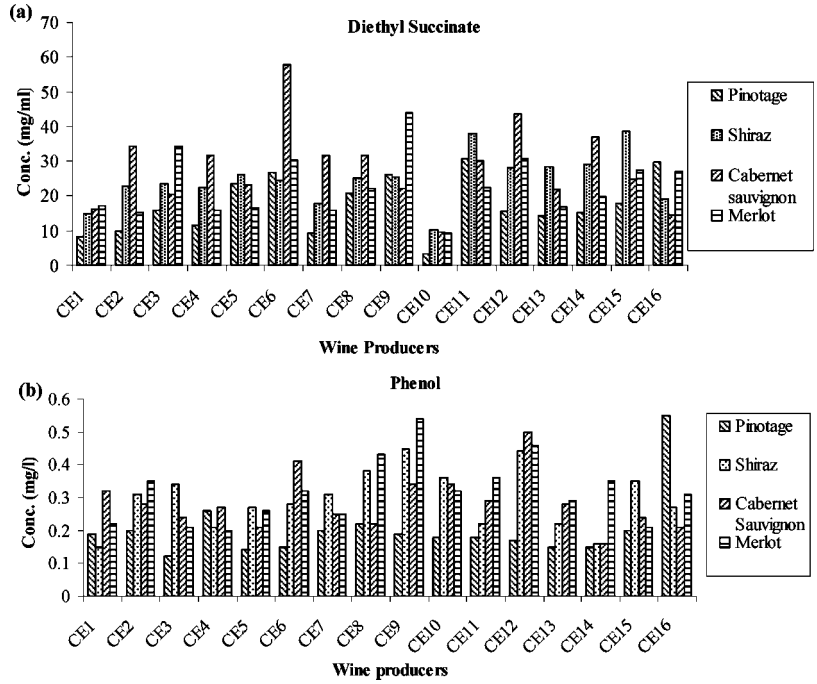

Figure 2. Chart representation of (**a**) Diethyl succinate (**b**) phenol measured in Pinotage, Shiraz, Cabernet Sauvignon, and Merlot wine samples, 16 from each cultivar obtained by HSSE-TD-GC-MS. (see text for conditions). CE1 to CE16 = Cellar 1 to Cellar 16 suppliers of the wine samples. Each cellar represents same region but different cultivar.

(Pinotage, Shiraz, Cabernet Sauvignon, and Merlot, 16 samples of each cultivar), and 15 Chardonnay wines, all of vintage 2005. A typical chromatogram of Pinotage wine is presented in **Figure 1**.

The summary of all the volatile components identified in the wine samples are presented in **Table 2**. Theses compounds mainly belong to esters, alcohols, lower acids, and furans as well as other compounds in lesser amounts belonging to carbonyls, lactones, and phenols. With the current method decanoic acid and 2,6-dimethoxy phenol were unable to identify in all the samples. Moreover, *p*-cresol was below the LOD in all wines of Shiraz, Cabernet Sauvignon, and Merlot cultivars. The cis-oak-lactone was unidentified in the white wines. Furthermore, it was not detected in all the samples of Cabernet Sauvignon cultivars except in one. Its racemic isomer, trans-oak-lactone, was not determined in all the samples of Cabernet Sauvignon, merlot, and chardonnay cultivars.

Small, but in some cases observable, differences were found in the measured amounts of the analytes in wines, even among those from the same cultivar, producer, and region. For instance in **Figure 2**, the amount of diethyl succinate and phenol measured in four different red wine cultivars is presented for sixteen different producers in South Africa. From this figure it would seem that wine making procedures, geographical origin, and cultivar plays a more detrimental role in the quality of the wines and not the age since all the wines analyzed were from the 2005 vintage. The data in **Figure 2** suggests that the method and data generated would prove useful to study the volatile composition of wines and possibility to classify them according to certain criteria such as geographical origin, production technology, or grape variety. This will be the focus of subsequent statistical investigations in future.

In conclusion the developed analytical technique based on stir bar technology was found very sensitive and suitable for the analysis of trace and ultra-trace compounds. HSSE extraction was very advantageous in reducing the risk of contamination and increasing the lifetime of the PDMS coated stir bar.

The overall results are satisfactory for the analysis of volatile compounds in wine responsible for its aroma achieving low detection and quantification limits. The methodology proposed in this paper allowed us to determine the 37 most important volatile compounds partially responsible for the aroma of wines in a relatively quick and easy procedure with a low sample volume and cost effectively.

Although SBSE is a very sensitive technique, PDMS, a nonpolar phase, is the only polymer at present adopted as coating of stir bars. This results in poor recoveries of polar compounds with low octanol–water partition coefficients ($K_{o/w}$). This was improved by pH adjustment especially for the organic acids. However a dual-phase twister approach could bring some solution to the limitation of the current stir bar technology by utilizing a material which retains both polar and nonpolar compounds.

ABBREVIATIONS

HSSE, headspace sorptive extraction; SBSE, stir bar sorptive extraction; TDS, thermal desorption system; PTV, programmed temperature vaporization inlet; CIS, cooled injection system; PDMS, poly(dimethylsiloxane); m_{PDMS}, mass in the PDMS phase; m_w, mass in the aqueous phase; m_o, original mass in the sample.

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