FEMS Microbiology Letters 240 (2004) 125–129

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Variation in 4-mercapto-4-methyl-pentan-2-one release by *Saccharomyces cerevisiae* commercial wine strains

Kate S. Howell [a,b], Jan H. Swiegers [a], Gordon M. Elsey [a,c], Tracey E. Siebert [a], Eveline J. Bartowsky [a], Graham H. Fleet [b], Isak S. Pretorius [a,*], Miguel A. de Barros Lopes [a,d]

[a] *The Australian Wine Research Institute, PO Box 197, Glen Osmond, Adelaide, SA 5064, Australia*
[b] *Food Science and Technology, School of Chemical Engineering and Industrial Chemistry, University of New South Wales, Sydney NSW 2052, Australia*
[c] *School of Chemistry, Physics and Earth Sciences, Flinders University, GPO Box 2100, Adelaide, SA 5100, Australia*
[d] *School of Pharmacy and Medical Sciences, University of South Australia, GPO Box 2471, Adelaide, SA 5001, Australia*

Received 11 July 2004; received in revised form 16 September 2004; accepted 16 September 2004

First published online 29 September 2004

Edited by L.F. Bisson

Abstract

The volatile thiol 4-mercapto-4-methylpentan-2-one (4MMP) is a potent contributor to wine aroma. In grape juice, 4MMP is bound to cysteine as a non-volatile compound and requires the action of yeast during fermentation to release the aroma active thiol. A method was developed to measure 4MMP release from the precursor by headspace solid-phase microextraction and separation by gas chromatography with atomic emission detection to screen the ability of wine yeast to release 4MMP. Yeast commonly used in white wine making were grown with the precursor at two different temperatures, and the amount of 4MMP released was measured. The results demonstrate that yeast strain selection and fermentation temperature can provide an important tool to enhance or modulate the grape-derived aromas formed during wine fermentation.

Keywords: Wine; Aroma; Sulfur compounds; Thiol; Wine yeast; *Saccharomyces cerevisiae*

1. Introduction

The aroma of wine is composed of numerous chemical compounds, which are derived from the grape, produced during fermentation of the grape juice, and evolved during ageing or storage [1–4]. One group of these compounds, the volatile thiols, have very low perception threshold concentrations [5], and one in particular, 4-mercapto-4-methylpentan-2-one (4MMP), has been shown to be an important impact odorant. The compound 4MMP is present in wines made from Scheurebe, Sauvignon blanc, Gewürztraminer, Riesling, Colombard, Petit manseng, Semillon, Cabernet sauvignon and Merlot grapes [6–11]. The aroma of 4MMP is reminiscent of box tree and blackcurrant, but presents a cat urine odour at higher concentrations [7,12,13]. The perception threshold of 4MMP has been reported as 0.1 and 3 ng L^{-1} in water and wine, respectively, and it typically occurs in wine in the range of 0–30 ng L^{-1} [10,13]. In one study, 4MMP was found to be the most potent aroma compound of 42 odorants isolated from a wine (7).

* Corresponding author. Tel.: +61 8 8303 6600; fax: +61 8 8303 6601.
E-mail address: sakkie.pretorius@awri.com.au (I.S. Pretorius).

0378-1097/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Microbiological Societies.
doi:10.1016/j.femsle.2004.09.022

Although important in contributing to the varietal character of wine, the volatile thiols are not found in grape juice prior to fermentation. Instead, 4MMP is derived from a non-volatile cysteinylated precursor present in grapes, 4-(4-methylpentan-2-one)-L-cysteine (Cys-4MMP) [14]. Studies on the release of a related thiol, 3-mercaptohexanol (3MH), showed that the precursor can be cleaved by yeast during wine fermentation to release the volatile thiol [15]. As a result, there is the potential to modulate the extent of 4MMP release by fermenting with particular wine yeast strains and, by doing so, dramatically modify wine aroma [16].

The quantification of 4MMP in complex mixtures, such as wine, is challenging due to its presence at very low concentrations. Initial studies on the measurement of wine thiols used an enrichment technique by a mercury trap [9,17], but recently, stable isotope dilution assays using gas chromatography coupled to ion trap tandem mass spectrometry or atomic emission detection (GC–AED) have been published [18]. GC–AED, especially, provides a sensitive method to selectively measure sulfur compounds in wine. Previous authors have used liquid extraction and pre-analysis chromatography methods to prepare samples for volatile thiol analysis [8,16,18]. However, for routine analysis of many samples, minimal sample preparation is preferable. Sulfur compounds in wine can be accurately measured using headspace solid-phase microextraction (HS-SPME) coupled to GC–MS [19–22], but this technique has not been applied to volatile thiols such as 4MMP.

The aim of this work was to screen yeast strains commonly used in white winemaking for their ability to produce 4MMP from the non-volatile precursor, Cys-4MMP. For this purpose, an accurate, sensitive and routine method was developed for measuring 4MMP in synthetic media by utilising HS-SPME for sample enrichment, and GC–AED for separation and quantification. The results show that there is significant diversity in the ability of wine yeast strains to release 4MMP. The release of 4MMP is also dependent on fermentation temperature. These findings provide important knowledge for winemakers to make informed choices when selecting wine strains and fermentation conditions to improve and modulate the varietal aroma of wine.

2. Materials and methods

2.1. Chemicals

All the chemicals used were of analytical grade or higher, and purchased from Sigma, unless otherwise stated. ^{1}H Nuclear Magnetic Resonance (NMR) spectra were recorded on a Varian Gemini spectrometer operating at 300 MHz.

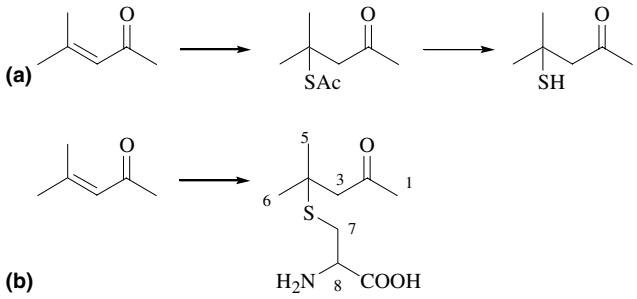

Fig. 1. Synthesis and structures of: (a) 4-mercapto-4-methylpentan-2-one (4MMP): (b) *S*-4-(4-methylpentan-2-one)-L-cysteine (Cys-4MMP). Synthetic procedures are given in Section 2.

2.2. Synthesis and confirmation of 4MMP and Cys-4MMP

4MMP was synthesised using a procedure modified from Vermeulen and Collin [23,24] described in Fig. 1(a). Thiolacetic acid (11.65 g, 153 mM) was added to mesityl oxide (5.0 g, 51 mM) in tetrahydrofuran (50 mL) containing triethylamine (1 mL) and the mixture was stirred at room temperature overnight. After this time, the mixture was diluted with ether (50 mL) and washed successively with water, 10% sodium hydroxide solution (×2), water, and dried with Na_2SO_4. The solvent was removed by gentle distillation to give the thioacetate as an orange oil (8.4 g, 94%). Purity was confirmed by NMR.

Thioacetate (1.44 g, 8.3 mM) in ether (25 mL) was stirred at room temperature with hydrazine hydrate (0.83 g, 16.6 mM). After 2 h, the mixture was washed with water and dried. After concentration, the residue was chromatographed on silica gel (5% ether:pentane) to give the thiol as a colourless oil (0.78 g, 71%). The purity of 4MMP was determined by NMR.

The grape precursor to 4MMP, Cys-4MMP, was synthesised using a modification of the method of Tominaga and co-workers [15] as described in Fig. 1(b). Mesityl oxide (700 mg, 71 mM) and pyridine (1.13 g, 142 mM) were added to a solution of L-cysteine (875 mg, 72 mM) in water (15 mL). The mixture was stirred at room temperature for 48 h before being filtered. The filtrate was concentrated under reduced pressure to give a white solid (1.05 g, 66%). Purity was confirmed by NMR.

2.3. Yeast strains and fermentation conditions

Eight commercial wine yeasts commonly used in the production of white wine, were sourced from the culture collection of the Australian Wine Research Institute (strain numbers AWRI 123–AWRI 130 corresponding to CWY 1–8, respectively). Genomic analysis showed these strains to be *S. cerevisiae* (data not shown).

Triplicate starter cultures were produced for these yeasts by growing cells in YPD [yeast extract (10 g L^{-1}) peptone (20 g L^{-1}) and D-glucose (20 g L^{-1})] (Difco) to stationary phase at 28 °C at 200 rpm. The starter cultures were inoculated at a 100-fold dilution into synthetic medium [25] consisting of 0.67% yeast nitrogen base (Difco), a complete amino acid mix and 8% D-glucose. Cys-4MMP was accurately added to 100 mg L^{-1}. Fermentations were conducted in 250 mL conical flasks fitted with an air lock and side arm septa for sampling. Fermentations were incubated at 28 or 18 °C, and sampled regularly for analysis of biomass accumulation (absorbance at 600 nm) and sugar concentration (refractive index). Completed fermentations (40 h) were clarified by centrifugation (4000 rpm for 5 min) to remove yeast cells and supernatants were stored at −20 °C until further analysis of 4MMP. Fermentations were performed in triplicate.

2.4. Analysis of 4MMP

The GC–AED analysis was carried out on an Agilent 6890 gas chromatographer (GC) coupled to a G2350A atomic emission detector. The GC column was a SolGel Wax column (15 m, ID 0.32 mm, film thickness 0.5 μm, SGE, Australia) connected to a VB-5 column (60 m, ID 0.25 mm, film thickness 0.5 μm, Vici Gig Harbor Group, USA). The carboxen-polydimethylsiloxane fibre (85 μm Stable Flex, Supelco) was desorbed in the GC inlet at 220 °C (23.34 psi) for 7 min. The inlet was fitted with a 0.75 mm (internal diameter) liner (Supelco) and was in pulse splitless mode. The carrier gas was helium (UHP grade, AirLiquide), with a flow rate of 2.7 mL min^{-1} in constant flow mode. The oven program was as follows; initial temperature 40 °C held for 5 min, then increased at 7 °C/min to 110 °C; and further increased at 10 °C/min to 260 °C and held for 5 min. The transfer line and cavity of the AED were at 260 °C. Data were collected for carbon (193 nm) and sulfur (181 nm). The plasma used helium (UHP grade and further purified with a SAES GC50 getter) at 35 mL min^{-1}. The reagent gases were oxygen (N5.5 grade, Air Liquide) and hydrogen (UHP grade, BOC), with helium as the make up gas at 20 mL min^{-1}. The discharge tube was cooled with water at 65 °C.

Fermentation samples for the measurement of 4MMP were processed for headspace solid-phase microextraction following the methods of Mestres et al. [19]. EDTA (0.1 g) and NaCl (2 g) were added to 10 mL of sample in a 20 mL sample vial. The internal standard (0.1 μg L^{-1} propyl thioacetate) (Lancaster Synthesis) was injected through the septum. The vial was placed in a 45 °C water bath and the fibre exposed for 45 min before manual injection into the GC.

Standard curves were constructed in a model wine-like medium (5% ethanol, tartaric acid added to pH 3.2), a non-fermented synthetic medium and a spent synthetic medium (yeast allowed to ferment model medium, then centrifuged to clarify). Replicates were measured at each point. Fermentation samples were appropriately diluted in distilled water or spent media to fit the calibration range, and aliquots analysed in duplicate.

3. Results and discussion

3.1. Development of an analytical method to measure 4MMP

The methods used to measure volatile thiols in wine have been technically challenging and not suitable for routine use (8–10). In this study, a SPME–GC–AED method was developed to quantify the amount of 4MMP present in the headspace of liquid solutions. The advantage of this method is that it does not involve enrichment of the sample by solvent extraction, large volumes of sample or derivatisation of the sample. The method was validated by construction of a linear calibration function and provides accurate results in a model wine-like medium (Table 1). The amount of ethanol in wine or wine-like media can interfere with SPME analyses [26–29], and initial experiments also suggested that there might be a matrix effect with regards to quantification of 4MMP. Therefore, for the experiments reported here, calibration curves were constructed in both a non-fermented and spent synthetic medium (medium that had been fermented by yeast) (Table 1). The linear regression in each medium investigated was found to be different (Table 1). For quantification, the non-fermented synthetic medium calibration function was used to measure the amount of 4MMP in the experimental control medium, while the spent synthetic medium calibration function was used to quantify 4MMP in the media fermented by yeast.

Table 1
Calibration parameters and concentration range for 4MMP quantification in different types of media using HS-SPME–GC–AED

Media type[a]	Linear regression[b]	R^2	Concentration range (μg L^{-1})
Model	$y = 0.005x + 0.0105$	0.9989	0.1–100
Non-fermented	$y = 0.005x + 0.129$	0.9742	5–100
Spent	$y = 0.02x + 0.4226$	0.9753	5–100

[a] Details of media composition are given in Section 2.
[b] y is the area ratio and x the concentration ratio relative to the thiol and internal standard, respectively.

3.2. Release of 4MMP by different yeast strains

To investigate the capacity of different yeast strains to release 4MMP during fermentation, fermentations were performed with Cys-4MMP added to a synthetic medium. The wine yeast strains used were selected from commonly used yeast in commercial white winemaking. There was no difference in biomass accumulation or sugar utilisation, with the ferments completed after 40 h (data not shown). The control synthetic medium with no addition of yeast showed very little 4MMP, confirming the view that release of 4MMP is largely dependent on yeast growth [15]. The spontaneous release that occurred appears to be temperature dependent (0.5 μg L^{-1} was released at 28 °C and 0.14 μg L^{-1} at 18 °C) and the results are expressed as fold difference to the spontaneous release of the media at the temperature of fermentation (Fig. 2(a) and (b)).

The amount of 4MMP measured with different wine yeast strains shows that there is extensive variation in a strain's capability to release 4MMP from the cysteine precursor, with strains releasing between 5-fold and up to 135-fold more 4MMP than the control medium when fermented at 28 °C (Fig. 2(a)). Yeast strains CWY1, 2, 3, 5 and 6 form a group that released minor quantities of 4MMP during fermentation (less than 10-fold more than that released in the control medium). Strains CWY7 and CWY4 released 20 and 65-fold more than the control medium. In the CWY8-fermented medium, 138-fold more 4MMP was measured than the control synthetic medium.

To investigate whether yeast strains release similar amounts of 4MMP under conditions more closely resembling wine fermentation temperatures, a subset of the yeast were grown at 18 °C with Cys-4MMP (Fig. 2(b)). At the lower temperature, CWY1 and CWY7 released 5-fold more, while CWY8 released 35-fold more than the control synthetic medium incubated at 18 °C. Strain CWY8 released 100-fold more 4MMP when grown at 28 °C than at 18 °C. However, as yeast CWY1 released the same amount of 4MMP with respect to the control synthetic medium whether the fermentations were performed at 28 or 18 °C, it is likely that other factors are also involved in influencing the amount of 4MMP release.

The data confirm and extend a previous study by Murat and co-workers [16] that indicated release of 4MMP was strain dependent. These authors [16] showed that strain VL3c produced high amounts of 4MMP; here it is shown that VL3c (CWY7) releases less 4MMP when compared to the strains CWY4 and CWY8. Clearly, the ability of yeast strains to release 4MMP is more diverse than previously identified, and other strains could produce higher levels of 4MMP in wine than the commonly used winemaking strain VL3c. Development of a high throughput analytical method has meant that more yeast strains and different fermentation conditions can be screened for 4MMP release.

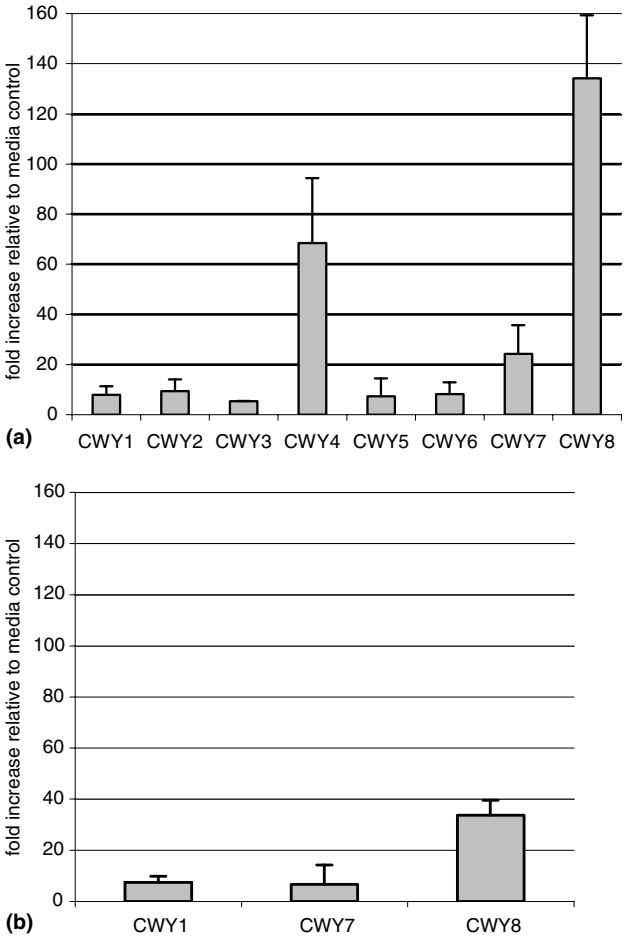

Fig. 2. Release of 4MMP is dependent on wine yeast strain and fermentation temperature. Fermentations containing commercially available wine yeast and Cys-4MMP were performed in synthetic media at (a) 28 and (b) 18 °C. The amount of 4MMP in the triplicate fermentation samples was quantified by HS-SPME–GC–AED (see Section 2). Results are expressed as fold difference with respect to the control synthetic medium at the temperature of fermentation. Results show means of triplicate fermentations, and error bars give standard error of the mean.

4. Conclusion

This study examined the potential of wine yeast to release 4MMP. Compounds 4MMP and Cys-4MMP were synthesised and a HS-SPME-GC–AED method developed to measure release of 4MMP during fermentation in synthetic medium. The results demonstrate that the choice of yeast strain and temperature of fermentation strongly impacted on 4MMP production, and highlights the importance of screening wine yeasts for their potential to release this important wine component. The results indicate that grape varietal aromas can be

significantly modulated by yeast strain selection and fermentation temperature.

Acknowledgements

This project is supported by Australia's grapegrowers and winemakers through their investment body the Grape and Wine Research Development Corporation, with matching funds from the Australian Government. K.S.H. is in receipt of an Australian Postgraduate Award stipend and AWRI scholarship. The authors thank Prof. Peter Høj for advice during the project, and Dr. Mark Sefton and Dr. Markus Herderich and Yoji Hayasaka for helpful discussions.

References

[1] Lambrechts, M.G. and Pretorius, I.S. (2000) Yeast and its importance to wine aroma – a review. S. Afr. J. Enol. Vitic. 21, 97–129.
[2] Dumont, A. and Dulau, L. (1997) The role of yeasts in the formation of wine flavours In: ASVO Seminar – Advances in Juice Clarification and Yeast Inoculation (Allen, M., Leske, P. and Baldwin, G., Eds.), pp. 14–16. Winetitles, Adelaide, Australia.
[3] Berry, D.R. and Watson, D.C. (1987) Production of organoleptic compounds In: Yeast Biotechnology (Berry, D.R., Russell, I. and Stewart, G.G., Eds.), pp. 345–368. Allen and Unwin, London, London.
[4] Rapp, A. and Mandery, H. (1986) Wine aroma. Experientia 42, 873–884.
[5] Mestres, M., Busto, O. and Guasch, J. (2000) Analysis of organic sulfur compounds in wine aroma. J. Chromatogr. A 881, 569–581.
[6] Aznar, M., Lopez, R., Cacho, J.F. and Ferreira, V. (2001) Identification and quantification of impact odorants of aged red wines from Rioja. GC-olfactometry, quantitative GC–MS, and odor evaluation of HPLC fractions. J. Agric. Food Chem. 49, 2924–2929.
[7] Guth, H. (1997) Identification of character impact odorants of different white wine varieties. J. Agric. Food Chem. 45, 3022–3026.
[8] Tominaga, T., Furrer, A., Henry, R. and Dubourdieu, D. (1998) Identification of new volatile thiols in the aroma of *Vitis vinifera* L. var. Sauvignon blanc wines. Flavour Fragrance J. 13, 159–162.
[9] Tominaga, T., Murat, M.L. and Dubourdieu, D. (1998) Development of a method for analyzing the volatile thiols involved in the characteristic aroma of wines made from *Vitis vinifera* L. Cv. Sauvignon blanc. J. Agric. Food Chem. 46, 1044–1048.
[10] Tominaga, T., Baltenweck-Guyot, R., Peyrot de Gachons, C. and Dubourdieu, D. (2000) Contribution of volatile thiols to the aromas of white wines made from several *Vitis vinifera* grape varieties. Am. J. Enol. Vitic. 51, 178–181.
[11] Murat, M.L., Tominaga, T. and Dubourdieu, D. (2001) Assessing the aromatic potential of Cabernet Sauvignon and Merlot musts used to produce rosé wine by assaying the cysteinylated precurser of 3-mercaptohexan-1-ol. J. Agric. Food Chem. 49, 5412–5454.
[12] Pearce, T.J.P., Peacock, J.M., Aylward, F. and Haisman, D.R. (1967) Catty odours in food: reactions between hydrogen sulfide and unsaturated ketones. Chem. Ind.- London 37, 1562–1563.
[13] Darriet, P., Tominaga, T., Lavigne, V., Boidron, J. and Dubourdieu, D. (1995) Identification of a powerful aromatic compound of *Vitis vinifera* L. var. Sauvignon wines: 4-Mercapto-4-methylpentan-2-one. Flavour Fragrance J. 10, 385–392.
[14] Tominaga, T., Masneuf, I. and Dubourdieu, D. (1995) A S-cysteine conjugate, precursor of aroma of white sauvignon. J. Int. Sci. Vigne Vin 29, 227–232.
[15] Tominaga, T., Peyrot des Gachons, C. and Dubourdieu, D. (1998) A new type of flavour precursors in *Vitis vinifera* L. cv. Sauvignon Blanc: S-cysteine conjugates. J. Agric. Food Chem. 46, 5215–5219.
[16] Murat, M.L., Masneuf, I., Darriet, P., Lavigne, V., Tominaga, T. and Dubourdieu, D. (2001) Effect of *Saccharomyces cerevisiae* yeast strains on the liberation of volatile thiols in Sauvignon blanc wine. Am. J. Enol. Vitic. 52, 136–139.
[17] Bouchilloux, P., Darriet, P., Henry, R., Lavine-Cruege, V. and Dubourdieu, D. (1998) Identification of volatile and powerful odorous thiols in Bordeaux red wine varieties. J. Agric. Food Chem. 46, 3095–3099.
[18] Schneider, R., Kotseridis, Y., Ray, J.L., Augier, C. and Baumes, R. (2003) Quantitative determination of sulfur-containing wine odorants at sub parts per billion levels. 2. Development and application of a stable isotope dilution assay. J. Agric. Food Chem. 51, 3243–3248.
[19] Mestres, M., Sala, C., Marti, M.P., Busto, O. and Guasch, J. (1999) Headspace solid-phase microextraction of sulphides and disulphides using carboxen-polydimethylsiloxane fibers in the analysis of wine aroma. J. Chromatogr. A 835, 137–144.
[20] Mestres, M., Busto, O. and Guasch, J. (2002) Application of headspace solid-phase microextraction to the determination of sulphur compounds with low volatility in wines. J. Chromatogr. A 945, 211–219.
[21] Mestres, M., Marti, M.P., Busto, O. and Guasch, J. (2000) Analysis of low-volatility organic sulphur compounds in wines by solid-phase microextraction and gas chromatography. J. Chromatogr. A 881, 583–590.
[22] Mestres, M., Busto, O. and Guasch, J. (1998) Headspace solid-phase microextraction analysis of volatile sulphides and disulphides in wine aroma. J. Chromatogr. A 808, 211–218.
[23] Kotseridis, Y., Ray, J.L., Augier, C. and Baumes, R. (2000) Quantitative determination of sulfur containing wine odorants at sub-ppb levels. 1. Synthesis of the deuterated analogues. J. Agric. Food Chem. 48, 5819–5823.
[24] Vermeulen, C. and Collin, S. (2002) Synthesis and sensorial properties of mercaptoaldehydes. J. Agric. Food Chem. 50, 5654–5659.
[25] Ausubel, F.M., Brent, R. and Kingston, R.E. (1994) Current protocols in molecular biology In: Current Protocols in Molecular Biology (Ausubel, F.M., Brent, R. and Kingston, R.E., Eds.), pp. 13.0.1–13.14.17. Wiley, New York.
[26] Legrand, S., Dugay, J. and Vial, K. (2003) Use of solid-phase microextraction coupled with gas chromatography for the determination of residual solvents in pharmaceutical products. J. Chromatogr. A 999, 195–201.
[27] Alzaga, R., Ortiz, L., Sachez-Baeza, F., Marco, M.P. and Bayona, J.M. (2003) Accurate determination of 2,4,6-trichloroanisole in wines at low parts per trillion by solid-phase microextraction followed by GC–ECD. J. Agric. Food Chem. 51, 3509–3514.
[28] Millan, S., Sampedro, M.C., Unceta, N., Goicolea, M.A., Rodriguez, E. and Barrio, R.J. (2003) Coupling solid-phase microextraction and high-performance liquid chromatography for direct and sensitive determination of halogenated fungicides in wine. J. Chromatogr. A 995, 135–142.
[29] Rodriguez-Bencomo, J.J., Conde, J.E., Garcia-Montelongo, F. and Perez-Trujillo, J.P. (2003) Determination of major compounds in sweet wines by headspace solid-phase microextraction and gas chromatography. J. Chromatogr. A 991, 13–22.