

# Combinatorial Synthesis and Sensorial Properties of Polyfunctional Thiols

Catherine Vermeulen, Jérôme Pellaud,<sup>†</sup> Laurence Gijs, and Sonia Collin\*

Unité de Brasserie et des Industries Alimentaires, Faculté d'Ingénierie biologique, agronomique et environnementale, Université Catholique de Louvain, Croix du Sud, 2 bte 7, 1348 Louvain-la-Neuve, Belgium

Over the past few years, polyfunctional thiols present as trace components have been found to play a major role in many food flavors, due to their exceptionally low odor thresholds. Unfortunately, their presence in minute concentration (in ng/kg to a few  $\mu\text{g}/\text{kg}$ ) and their high reactivity make it very difficult to extract and identify them. Furthermore, most of them are not yet commercially available. The aim of this work was to characterize the chromatographic and sensorial properties of 10 synthetic mercaptoketones and mercaptoalcohols. Combinatorial chemistry proved to be a very useful way to synthesize them rapidly. Sulfur-selective sulfur chemiluminescence detection chromatograms coupled with mass spectroscopy enabled the target compounds to be identified. Flavor profiles and best estimate gas chromatography lowest amount detected by sniffing (BE-GC-LOADS) values were further determined by GC-olfactometry. As expected, new, exceptionally odorant molecules (BE-GC-LOADS < 0.1 ng) were revealed by this unusual approach.

**Keywords:** *Combinatorial chemistry; polyfunctional thiols; flavors; BE-GC-LOADS; sulfur compounds*

## INTRODUCTION

Due to their extremely low threshold values, polyfunctional thiols, like other sulfur-containing molecules, contribute significantly to the sensory properties of food flavors. So far >40 of them have been identified as relevant aroma components in various foods such as beef (1), pork (2), chicken (1), tuna fish (3), cheese (4), olive oil (5), syrup (6), durian (7), passion fruit (8), grapefruit (9), grape (10), black currant (11), asparagus (12), onion (11), scallion (13), sesame (14), popcorn (15), bread (16), yeast extract (17), buchu (18), hops (19), wine (20), beer (19), coffee (21), and tea (22). Mercaptoketones and mercaptoalcohols are probably among the most relevant, due to their threshold values.

For lack of commercially available substances, there is very little information about the flavor properties of analogues with different alkyl chain lengths. Often, in food aromatic extract analysis, no chromatographic peak is evident by mass spectrometric detection in fractions displaying the most interesting smells, so it is difficult to identify the responsible compounds in a real matrix on the sole basis of retention indices and odor. The aim of the present work was to establish a strategy for quickly obtaining retention indices and flavor properties for a greater number of polyfunctional thiols. Recently, combinatorial chemistry was used successfully by Khan et al. (23) to obtain a series of thioester analogues. This original approach permitted us to intensify the process of flavor synthesis and analysis by handling "arrays" of homologous compounds rather than individual fla-

vorants; we obtained mercaptoketones by simple addition of hydrogen sulfide to five  $\alpha,\beta$ -unsaturated ketones mixed in the same vessel. Further reduction of the mixture led to the corresponding mercaptoalcohols.

## MATERIALS AND METHODS

**Chemicals.** The starting materials for the syntheses were of the highest purity commercially available and were not further purified before use. The solvents were anhydrous and stored over molecular sieves.

The following compounds were provided by Aldrich Chemicals (Bornem, Belgium): 65% 3-penten-2-one, 95% 4-hexen-3-one, 98% 4-methyl 3-penten-2-one, 75% 5-methyl 3-hexen-2-one, and 99% piperidine. Ninety-five percent 3-methyl 3-penten-2-one was supplied by Fluka (Bornem, Belgium). Hydrogen sulfide was obtained from Praxair (Antwerp, Belgium).

Tetrahydrofuran (THF; 99.9%), 98% pentane, 99.5% diethyl ether, 0.2-mm-thick silica plates, and 5,5-dithiobis(2-nitrobenzoic acid) were purchased from Aldrich Chemicals. Dichloromethane (99.9%) was from Romil (Cambridge, U.K.). Ethanol (99.8%), 2,4-dinitrophenylhydrazine, and sodium borohydride ( $\text{NaBH}_4$ ) were supplied by Merck (Overijse, Belgium).

**Synthesis. Mercaptoketones.** Seventy-five milliliters of THF, 12 drops of piperidine, and the five  $\alpha,\beta$ -unsaturated ketones (final individual concentrations = 25 mM) were mixed in a 100-mL three-neck flask. The flask was then immersed in an acetone/liquid  $\text{N}_2$  bath at  $\sim -15^\circ\text{C}$  and the mixture stirred magnetically. Hydrogen sulfide was then continuously and gently bubbled into the liquid phase for 6 h. After this period, thiols were detected by thin-layer chromatography (TLC). The solution was stored at  $-80^\circ\text{C}$ .

**Mercapto secondary alcohols.** Thirty milliliters of the above-mentioned mercaptoketone solution and 30 mL of water were placed in a 200-mL flask fitted with a reflux condenser, and 1 N NaOH was added to attain pH 8. A slight excess of  $\text{NaBH}_4$  (1.5 equiv) was then added under magnetic stirring. After 4 h, 90 mL of water was added and the reaction mixture adjusted to pH 3 with 6 N HCl. Thiols were then extracted with

\* Author to whom correspondence should be addressed [telephone +32-(0)10-47 29 13; fax +32-(0)10-47 21 78; e-mail collin@inbr.ucl.ac.be].

<sup>†</sup> Present address: Interbrew Technology and Development, Vaartstraat 137, B-3000 Leuven, Belgium.

**Table 1. GC-MS and Sensorial Properties of Mercaptoketones Synthesized**

Name	Structure	MS fragments [relative percentages]	Kovats index (CP-Sil5 CB)	Odor at the sniffing port	Odor described in the literature
4-Mercaptopentan-2-one		43 [100]; 85[29]; 41 [26]; 118 <sup>a</sup> [26]; 61 [19]	884	Greenery, potato, black currant	-
4-Mercapto 4-methylpentan-2-one		43 [100]; 132 <sup>a</sup> [26]; 75 [21]; 55 [19]; 99 [12]	915	Black currant, catty, broom, vinaigrette, citrus fruit	Broom, box tree, catty, black currant (31)
4-Mercapto 3-methylpentan-2-one		43 [100]; 99 [69]; 55 [50]; 61 [19]; 132 <sup>a</sup> [6]	959 <sup>b</sup> 967 <sup>b</sup>	Sweat Cooked milk	-
5-Mercaptohexan-3-one		57 [100]; 61 [48]; 75 [44]; 132 <sup>a</sup> [30]; 99 [28]	984	Box tree, fresh, empyreumatic	-
5-Methyl 4-mercaptohexan-2-one		43 [100]; 112 [22]; 113 [19]; 55 [17]; 146 <sup>a</sup> [6]	1069	Exotic fruit, sweet, sulfury	Sulfury, cabbage (7)

<sup>a</sup> Molecular ion (M<sup>+</sup>). <sup>b</sup> The two values correspond to diastereoisomers.

**Table 2. GC-MS and Sensorial Properties of Mercapto Secondary Alcohols**

Name	Structure	MS fragments [relative percentages]	Kovats index (CP-Sil5 CB)	Odor at the sniffing port	Odor described in the literature
4-Mercaptopentan-2-ol		45 [100]; 86 [68]; 71 [63]; 61[53]; 69 [37] (120 <sup>a</sup> [11] present)	914 <sup>b</sup> 926 <sup>b</sup>	Broom, black currant, catty Raw onion	-
4-Mercapto 4-methylpentan-2-ol		57 [100]; 85 [97]; 45 [68]; 100 [58]; 41 [42] (134 <sup>a</sup> [16] present)	952	Broom, black currant, solvent, fresh, sweet	Lemon (31)
5-Mercaptohexan-3-ol		61[100]; 59 [96]; 71 [46]; 100 [43]; 116 [32] (134 <sup>a</sup> [11] present)	1012 <sup>b</sup> 1023 <sup>b</sup>	Sweat, meat broth, citrus fruit Sweat, cooked milk	-
4-Mercapto 3-methylpentan-2-ol		56 [100]; 45 [96]; 55[71]; 61 [67]; 100 [58] (134 <sup>a</sup> [13] present)	1037	Onion, leek, sweat, soup	-
5-Methyl 4-mercaptohexan-2-ol		71 [100]; 45 [90]; 55 [76]; 61 [67]; 114 [45] (148 <sup>a</sup> [14] present)	1097 <sup>b</sup> 1107 <sup>b</sup>	Rhubarb, lemon Spicy, peppery, meaty	-

<sup>a</sup> Molecular ion (M<sup>+</sup>). <sup>b</sup> The two values correspond to diastereoisomers.

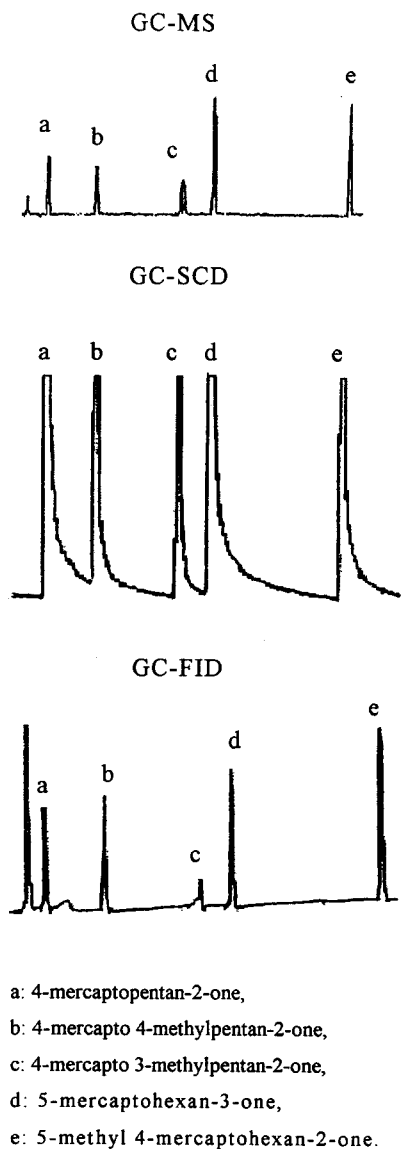
dichloromethane (4 × 40 mL). After evaporation of solvent under vacuum, 30 mL of a liquid with a very strong odor was obtained and stored at -80 °C.

**Analytical Methods.** *Thin-Layer Chromatography (TLC).* Pentane/diethyl ether (60:40) was used as chromatographic eluent with 0.2-mm silica plates. The colorless non-UV-absorbing thiols were revealed with two different reagents: 5,5-dithiobis(2-nitrobenzoic acid) for thiols (24) and 2,4-dinitrophenylhydrazine for mercaptoketones (25).

*Gas Chromatography Coupled with Sulfur Chemiluminescence Detection (GC-SCD).* GC was performed using a Chrompack CP9001 chromatograph equipped with a splitless injector maintained at 250 °C and opened after 0.5 min. Analysis of sulfur compounds was performed using a 50 m × 0.32 mm i.d., wall-coated open tubular (WCOT) apolar CP-

SIL 5 CB capillary column (film thickness = 1.2 μm) connected to a sulfur chemiluminescence detector (Sievers, model 355 SCD) and a Shimadzu CR3A integrator. An initial oven temperature of 40 °C was maintained for 4 min and then programmed to rise from 40 to 132 °C at 2 °C/min followed by 132–250 °C at 10 °C/min. The final temperature was then held for 45 min. Helium carrier gas was used at a flow of 32.0 cm/s (flow rate = 1.0 mL/min). Air and hydrogen flows were maintained at 40 and 100 mL/min, respectively, in the 800 °C combustion chamber. The air flow rate in the ozone generator was 6 psi, and a vacuum of 150–275 Torr was applied to the entire system.

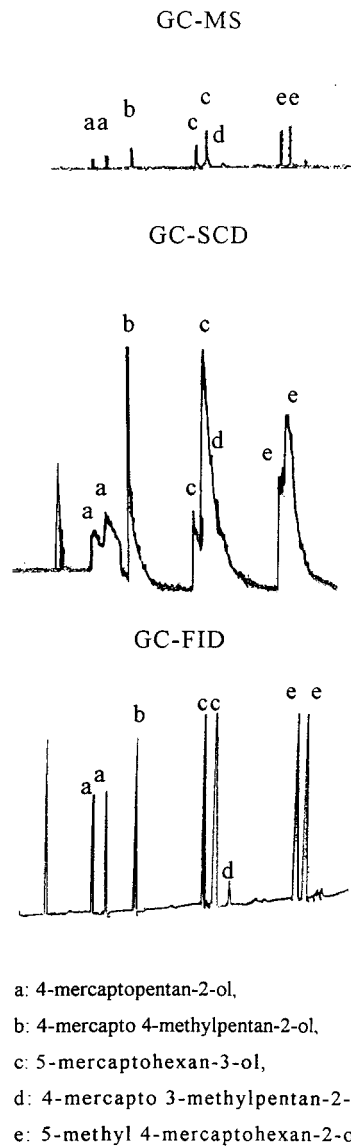
*Gas Chromatography Coupled with Electronic Impact Mass Spectrometry (GC-MS).* Mass spectra were recorded at 70 eV on an HP 5988 quadrupole mass spectrometer connected to a



**Figure 1.** MS, FID, and SCD GC chromatograms of the mercaptoketone combinatorial synthesis medium.

Hewlett-Packard model 5890 gas chromatograph equipped with a splitless injector and the previously described column. Oven temperature, initially kept at 40 °C for 4 min, was programmed to rise from 40 to 132 °C at 2 °C/min and, thereafter, from 132 to 250 °C at 10 °C/min, remaining at the maximum temperature for 15 min. Spectral recording was automatic throughout elution using HP 59970C software. The compounds were identified on the basis of their fragmentation patterns.

*Gas Chromatography Coupled with dual Flame Ionization Detection and Olfactometry (GC-FID-O).* This was performed using a Chrompack CP9001 gas chromatograph, which was equipped with a splitless injector maintained at 250 °C and opened after 0.5 min. Sulfur compounds were analyzed using a 50 m × 0.32 mm i.d., WCOT apolar CP-Sil 5 CB capillary column (film thickness = 1.2 μm). An initial oven temperature of 40 °C was maintained for 4 min and then programmed to rise from 40 to 132 °C at 2 °C/min followed by 132–250 °C at 10 °C/min. The final temperature was held for 15 min. A T-junction was used at the end of the capillary column. Fifty percent of the eluent was sent to an FID detector maintained at 250 °C and connected to a Shimadzu C-R3A integrator, while the other part was directed to a GC-odor port at 250 °C. In the latter case, the eluent was diluted with a large volume of air (20 mL/min) previously humidified in an aqueous copper(II) sulfate solution to improve the transport of the effluent out of the funnel (26, 27). For each solution, 2 μL was injected



**Figure 2.** MS, FID, and SCD GC chromatograms of the mercaptoalcohol combinatorial synthesis medium.

to determine the polyfunctional thiol flavor quality. As described by Berger et al. (28), the best estimated GC lower amount detected by sniffing (BE-GC-LOADS) is defined as the geometric mean between the lowest mass of compound perceived at the outlet of the GC-odor port and the highest undetected amount injected onto the column. Experiments were performed using dilutions of a compound as follows: 1/50, 1/100, 1/200, 1/500, 1/1000, 1/2000, 1/5000, and so on up to the 1/50000 dilution. Sensory analysis was performed by two judges working independently, and a verbal description of the odor was obtained at the same time.

## RESULTS AND DISCUSSION

Five mercaptoketones (Table 1) and five mercaptoalcohols (Table 2) were synthesized by combinatorial chemistry from the corresponding commercially available  $\alpha,\beta$ -unsaturated ketones. Due to its weak nucleophilic properties, hydrogen sulfide added quickly onto the  $\beta$  position to produce mercaptoketones, whereas subsequent reduction by NaBH<sub>4</sub> led to the corresponding alcohols.

The presence of a sulfur atom in the molecules was easily checked by GC-SCD (Figures 1 and 2). Because the response is proportional only to the number of sulfur

**Table 3. BE-GC-LOADS of Polyfunctional Thiols**

name	BE-GC-LOADS (ng at the sniffing port)	published threshold (ppt)
4-mercaptopentan-2-ol	0.002 and 0.02 <sup>a</sup>	
4-mercaptopentan-2-one	0.03	
4-mercapto 3-methyl-pentan-2-ol	0.0001	
4-mercapto 3-methyl-pentan-2-one	0.02 and 0.4 <sup>a</sup>	
4-mercapto 4-methylpentan-2-ol	0.009	20 in water (31); 55 in a 12% ethanolic solution (32)
4-mercapto 4-methylpentan-2-one	0.004	0.066–0.165 in water (30); 0.8 in a 12% ethanolic solution (33); 3 in wine (34)
5-mercaptohexan-3-ol	0.02 and 0.06 <sup>a</sup>	
5-mercaptohexan-3-one	0.02	
5-methyl 4-mercaptohexan-2-ol	0.002 and 0.002 <sup>a</sup>	
5-methyl 4-mercaptohexan-2-one	0.03	

<sup>a</sup> The two values correspond to diastereoisomers.

atoms, it was possible to quantify each compound in the complex synthetic medium without a specific calibration curve.

All of the suspected structures were confirmed by mass spectrometry. In all cases, the molecular ion was detected. Most of the other fragment ions were explainable by the loss of hydrogen sulfide.

As depicted in Tables 1 and 2, two peaks were found, as expected, for compounds with two or more chiral carbon atoms, the two diastereoisomers usually being separated by ~10 retention index units. Further investigations should be undertaken to identify the structures more precisely, but our results already show that distinct odors characterize such isomers. Similar odor descriptors were found for many mercaptoketones and mercaptoalcohols, for example, black currant, sweat, and cooked milk. 5-Methyl 4-mercaptohexan-2-one (exotic fruit) and one diastereoisomer of 5-methyl 4-mercaptohexan-2-ol (rhubarb, lemon) were the most pleasant odors perceived at the sniffing port.

As initially suggested by Berger et al. (28) for thioesters and by Gijs et al. (29) for sulfur compounds, we serially diluted our original extract to determine matrix-composition-independent sensorial threshold values. The so-called BE-GC-LOADS value is defined as the lowest amount (in nanograms) of compound arriving at the detector that can be perceived by the panelist. In our case, due to the presence of one sulfur atom in each molecule, quantification of the complex original extract was easily achieved by SCD (signal only proportional the number of S atoms). Odor threshold values were further expressed in nanograms, taking into account that only 1  $\mu$ L arrives at the sniffing port. Among our 14 compounds, 4-mercapto 4-methylpentan-2-one showed the lowest BE-GC-LOADS value: 0.004 ng (Table 3). This black currant–catty–broom flavor has been previously described by Darriet et al. (30) as a determinant in Sauvignon wines, with a threshold between 0.06 and 3 ppt, according to the medium composition. From our results, 4-mercapto 3-methylpentan-2-ol emerges as another exceptionally odorant compound with a BE-GC-LOADS of 0.012 ng (Table 3), well below the value of 1.4 ng obtained for dimethyl trisulfide, the sulfur molecule showing the lowest value reported by Gijs et al. (29).

Combinatorial chemistry coupled to GC-SCD, GC-MS, and GC-O is a very rapid way to screen for new odorant thiols, as long as not too many reagents are mixed in the “one-pot” system, thus avoiding excessive crossed reactions or wrong identifications. As expected, some polyfunctional thiols are exceptionally odorant, compared to other well-known sulfur flavors. More traditional organic syntheses should now be undertaken for

some of them. A similar approach could be used for other polyfunctional thiols, for example, mercaptoaldehydes, provided all of the selected reagents used in the mixture ( $\alpha,\beta$ -unsaturated aldehydes in this case) show similar reactivities.

#### LITERATURE CITED

- Shankaranarayana, M. L.; Raghavan, B.; Abraham, K. O.; Natarajan, C. P. Sulphur compounds in flavours. In *Food Flavours*; Elsevier Scientific Publishing: Amsterdam, The Netherlands, 1982; pp 169–281.
- Kerscher, R.; Grosch, W. Quantification of 2-methyl-3-furanthiol, 2-furfurylthiol, 3-mercapto-2-pentanone, and 2-mercapto-3-pentanone in heated meat. *J. Agric. Food Chem.* **1998**, *46*, 1954–1958.
- Huber, U. A.; Bergamin, D. Novel access to furan-3-thiols and derivatives, impact meat-flavor compounds. *Helv. Chim. Acta* **1993**, *76*, 2528–2536.
- Polak, E.; Fetison, G.; Fombon, A.-M.; Skalli, A. Structure–odor relationships for “catty”-smelling mercapto compounds in humans. *J. Agric. Food Chem.* **1988**, *36*, 355–359.
- Reiners, J.; Grosch, W. Concentration of 4-methoxy-2-methyl-2-butanethiol in Spanish virgin olive oils. *Food Chem.* **1999**, *64*, 45–47.
- Maga, J. A. The role of sulfur compounds in food flavor: thiophenes. *Crit. Rev. Food Sci. Nutr.* **1975**, 241–270.
- Weenen, H.; Koolhaas, W. E.; Apriyantono, A. Sulfur-containing volatiles of durian fruits (*Durio zibethinus* Murr.). *J. Agric. Food Chem.* **1996**, *44*, 3291–3293.
- Gora, J.; Brud, W. Progress in synthesis of sensory important trace components of essential oils and natural flavours. *Nahrung* **1983**, *27*, 413–428.
- Buettner, A.; Schieberle, P. Characterization of the most odor-active volatiles in fresh, hand-squeezed juice of grapefruit (*Citrus paradisi* Macfayden). *J. Agric. Food Chem.* **1999**, *47*, 5189–5193.
- Boelens, M. H.; van Gemert, L. J. Volatile character-impact sulfur compounds and their sensory properties. *Perfum. Flavor.* **1993**, *18*, 29–39.
- Maga, J. A. The role of sulfur compounds in food flavor: thiols. *Crit. Rev. Food Sci. Nutr.* **1976**, 147–192.
- Yanagawa, H.; Kato, T.; Kitahara, Y. Asparagusic acid, dihydroasparagusic acid and S-acetyldihydroasparagusic acid, a new plant growth inhibitor in etiolated young *Asparagus officinalis*. *Tetrahedron Lett.* **1972**, *25*, 2549–2552.
- Block, E. The organosulfur chemistry of the genus *Allium*. Implications for the organic chemistry of sulfur. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 1135–1178.
- Hofmann, T.; Schieberle, P. Evaluation of the key odorants in a thermally treated solution of ribose and cysteine by aroma extract dilution techniques. *J. Agric. Food Chem.* **1995**, *43*, 2187–2194.
- Schieberle, P. Primary odorants in popcorn. *J. Agric. Food Chem.* **1991**, *39*, 1141–1144.

- (16) Hofmann, T.; Schieberle, P.; Grosch, W. Model studies on the oxidative stability of odor-active thiols occurring in food flavors. *J. Agric. Food Chem.* **1996**, *44*, 251–255.
- (17) Güntert, M.; Bruning, J.; Emberger, R.; Köpse, M.; Kuhn, W.; Thielmann, T.; Werkhoff, P. Identification and formation of some selected sulfur-containing flavor compounds in various meat model systems. *J. Agric. Food Chem.* **1990**, *38*, 2027–2041.
- (18) Sundt, F.; Willhalm, B.; Chappaz, R.; Ohloff, G. Das organoleptische prinzip von cassis-flavor in buccublätteröl. *Helv. Chim. Acta* **1971**, *54*, 1801–1805.
- (19) Peppard, T. I.; Laws, D. R. J. Hop derived sulphur compounds and their effect on beer flavour. *Eur. Brew. Convention* **1979**, 91–104.
- (20) Lavigne, V.; Henry, R.; Dubourdieu, D. Identification et dosage de composés soufrés intervenant dans l'arôme "grillé" des vins. *Sci. Aliments* **1998**, *18*, 175–191.
- (21) Tressl, R.; Silwar, R. Investigation of sulfur-containing components in roasted coffee. *J. Agric. Food Chem.* **1981**, *29*, 1078–1082.
- (22) Kumazawa, K.; Masuda, H. Identification of potent odorants in Japanese green tea (Sen-cha). *J. Agric. Food Chem.* **1999**, *47*, 5169–5172.
- (23) Khan, J. A.; Gijs, L.; Berger, C.; Martin, N.; Piraprez, G.; Spinnler, H. E.; Vulfson, E. N.; Collin, S. Combinatorial approach to flavor analysis 1. Preparation and characterization of S-methyl-thioesters library. *J. Agric. Food Chem.* **1999**, *47*, 3269–3273.
- (24) Glaser, C. B.; Maeda, H.; Meienhofer, J. Chromatographic detection of thiols, disulfides and thioesters with 5,5'-dithiobis(2-nitrobenzoic acid). *J. Chromatogr.* **1970**, *50*, 151–154.
- (25) Kirchner, J. G. Thin-layer chromatography. In *Technique of Organic Chemistry*; Perry, E. S., Weissberger, A., Eds.; Interscience Publishers: New York, 1967; pp 151–176.
- (26) Hanaoka, K.; Sieffermann, J.-M.; Giampaoli, P. Effects of the sniffing port air makeup in gas chromatography-olfactometry. *J. Agric. Food Chem.* **2000**, *48*, 2368–2371.
- (27) Van Ruth, S. M.; O'Connor, C. H. Influence of assessors' qualities and analytical conditions on gas chromatography-olfactometry analysis. *Eur. Food Res. Technol.* **2001**, *213*, 77–82.
- (28) Berger, C.; Martin, N.; Collin, S.; Gijs, L.; Khan, J. A.; Piraprez, G.; Spinnler, H. E.; Vulfson, E. N. Combinatorial approach to flavor analysis 2. Olfactory investigation of a library of S-methyl-thioesters and sensory evaluation of selected components. *J. Agric. Food Chem.* **1999**, *47*, 3274–3279.
- (29) Gijs, L.; Piraprez, G.; Perpète, P.; Spinnler, E.; Collin, S. Retention of sulfur flavours by food matrix and determination of sensorial data independent of the medium composition. *Food Chem.* **2000**, *69*, 319–330.
- (30) Darriet, P.; Tominaga, T.; Lavigne, V.; Boidron, J.-N.; Dubourdieu, D. Identification of a powerful aromatic component of *Vitis vinifera* L. Sauvignon wines: 4-mercapto-4-methylpentan-2-one. *Flavour Fragrance J.* **1995**, *10*, 385–392.
- (31) Tominaga, T.; Furrer, A.; Henry, R.; Dubourdieu, D. Identification of new volatile thiols in the aroma of *Vitis vinifera* L. var. Sauvignon blanc wines. *Flavour Fragrance J.* **1998**, *13*, 159–162.
- (32) Bouchilloux, P.; Darriet, P.; Henry, R.; Lavigne-Cruège, V.; Dubourdieu, D. Identification of volatile and powerful odorous thiols in Bordeaux red wine varieties. *J. Agric. Food Chem.* **1998**, *46*, 3095–3099.
- (33) Tominaga, T.; Darriet, P.; Dubourdieu, D. Identification de l'acétate de 3-mercaptohexanol, composé à forte odeur de buis, intervenant dans l'arôme des vins de Sauvignon. *Vitis* **1996**, *35*, 207–210.
- (34) Dubourdieu, D. Les arômes: facteurs de qualité, les arômes du Sauvignon, valorisation par la vinification. Presented at the *9th Colloque Viticole et Oenologique Euroviti*, Montpellier, France, 1995.

Received for review June 18, 2001. Revised manuscript received August 31, 2001. Accepted September 3, 2001.

JF010816Z