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Method for Determining Nitrogenous Heterocycle Compounds in Wine

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Qualitative and quantitative analyses of N-heterocycle compounds were conducted by using a liquid−liquid extraction followed by additional chemical silica gel purification and injection into a gas chromatographic (GC) column coupled to a nitrogen−phosphorus detector (NPD). The purified extract fraction was investigated by GC-NPD and by GC−mass spectrometry. The compounds identified and quantified in wines were 2,4,5-trimethyloxazole, 2,4-dimethylthiazole, and 4-methylthiazole by GC-NPD and both 2-acetylthiazole and 2-acetyl-2-thiazoline by GC-MS. The procedure was used to analyze different wines and gave reliable and reproducible results.

KEYWORDS: GC-NPD; GC-MS; 2,4,5-trimethyloxazole; 2,4-dimethylthiazole; 4-methylthiazole; 2-acetylthiazole; 2-acetyl-2-thiazoline; wine

INTRODUCTION

α-Dicarbonyl compounds occur in all types of wine, particularly after malolactic fermentation. Owing to their participation in the direct aroma of wines, the most important substances are diacetyl and pentane-2,3-dione, whereas glyoxal and methylglyoxal have little importance. We have already presented a method for the determination of all dicarbonyls in wine by the formation of quinoxaline derivatives, thereby allowing the evolution of these compounds in wine to be monitored (*1*). Recent work in our laboratory has shown that dicarbonyl compounds are very reactive with amino acids and could play a role in the future aging aroma (*2*). Most of the products that we have identified come from the Maillard reaction and are alkylpyrazines, aldehydes, and N-heterocycles. Marchand (*3*) investigated reaction products between cysteine and carbonyls and identified sulfured, oxygenated, and nitrogen-containing heterocyclics such as thiazole, 4-methylthiazole, 2,4-dimethylthiazole, 2-acetylthiazole, 2-acetyl-2-thiazoline, trimethyloxazole, 2-methyl-3-furanthiol, 2-furanmethanethiol, and thiophene-2-thiol (*2*). These compounds generate notes of "very ripe fruit" for trimethyloxazole; "popcorn", "roasted", and "peanuts" for thiazoles; and "roasted coffee" or "burned rubber" for furans and thiophenes. They are known products of the Maillard reaction that occurs in the agribusiness and leads to roasted food flavors (*4*).

To determine the concentrations of all these compounds, quantitative methods have been widely described in the literature using gas chromatography (GC) and various detectors. The present paper proposes a method for recovering and quantifying N-heterocycles by using GC coupled with a nitrogen−phosphorus detector (NPD) or with a mass spectrometry (MS) detector. Before injection, we use liquid−liquid extraction, silica gel purification, and concentration under a nitrogen flow. The first applications of this quantitative method to measure 4-methylthiazole, 2,4-dimethylthiazole, 2-acetylthiazole, 2-acetyl-2-thiazoline, and trimethyloxazole in wine are reported.

MATERIALS AND METHODS

Reagents. Trimethyloxazole and thiazole derivatives (4-methylthiazole, 2-acetylthiazole, 2-acetyl-2-thiazoline, and 2-thiazolecarboxaldehyde) were purchased from Sigma Aldrich Chemical Co., and 2,4-dimethylthiazole was obtained from Lancaster. Inorganic reagents and solvents were all commercial products of analytical grade. The silica gel was a silica gel 100 for a Fluka chromatographic column; particle size = 0.063−0.200 (70−230 mesh ATSM).

Experimental Procedures. *Preparation of Sample.* We analyzed wines or spiked wines with different N-heterocycles. Two hundred microliters of internal standard (10 mg/L of 2-thiazolecarboxaldehyde in hydroalcoholic solution 50% volume) was added to 100 mL of sample. Each wine was brought to pH 5.0 with 1 M NaOH and extracted by dichloromethane (10 mL × 3 × 5 min × 1200 rpm); the organic phase was recovered by centrifugation (10000 rpm × 5 min) and dried on sodium sulfate. The extract was purified by trapping the substances of interest on silica gel (0.5 g) and elution in a silica gel (1.2 g) column by a mixture of hexane/ethyl acetate, 90:10 v/v. Eighteen milliliters of the solvent was concentrated under a nitrogen flow to 450 μL. Two microliters of extract was injected into the chromatograph.

GC-NPD Analysis. To develop the method, a gas chromatograph (Hewlett-Packard 5890) was coupled with an NPD (or thermoionic) at 220 °C. The NPD detector (HP) was a flame ionization detector with a rubidium silicate cylindrical salt placed in the flame to eliminate most of the organic compounds and to activate the emission of ions from the nitrogenous and phosphorus substances, thereby providing great sensitivity. Separations were carried out with an HP5 column (50 m ×

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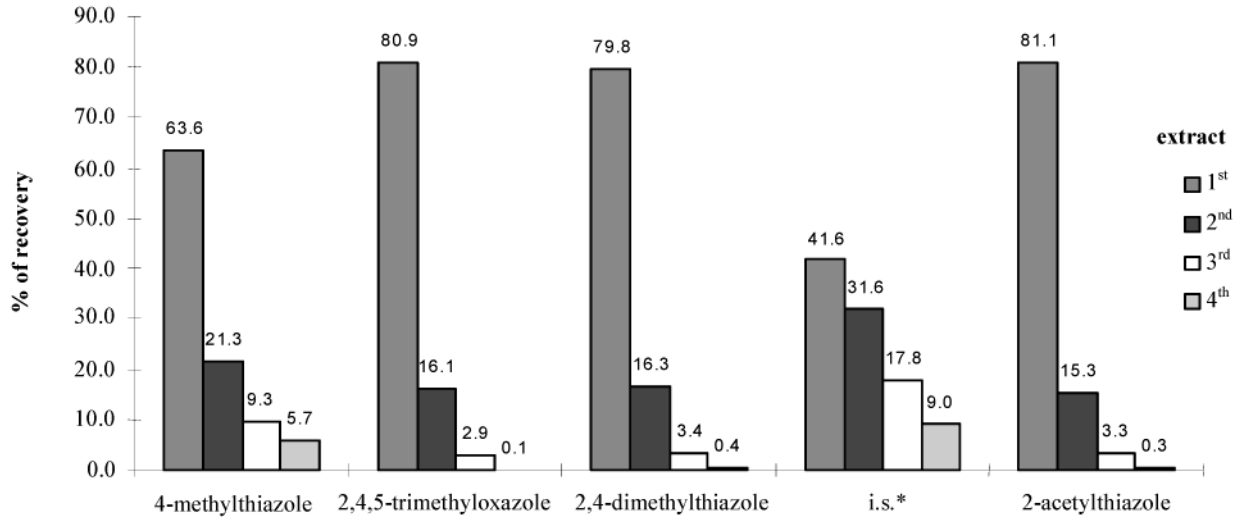

Figure 1. N-Heterocyclic recovery of a spiked wine (10 μg/L) with four successive dichloromethane extractions. *, internal standard (2-thiazolecarboxaldehyde).

0.32 mm × 0.52 μm phase thickness). The oven temperature was programmed from 60 °C for 1 min to 120 °C at a rate of 2 °C/min and then to 220 °C at a rate of 5 °C/min, and the final isothermal time was 15 min. The carrier gas was helium; the splitless time was 30 s, and the split vent was 30 mL/min. The temperature of the injector was 220 °C. The gas flows were as follows: for hydrogen, 3.5 mL/min; for nitrogen/oxygen, 110 mL/min; makeup gas (nitrogen), 44 mL/min; carrier gas (helium), 1.8 mL/min.

GC-MS Analysis. A gas chromatograph (Hewlett-Packard 6890) was coupled with a mass spectrometric detector (HP 5972; electronic impact, 70 eV, 2.7 kV). The column was a BP21 (SGE) (50 m × 0.25 mm × 0.25 μM film thickness). The oven temperature was programmed from 50 °C for 1 min to 150 °C, at a rate of 1.5 °C/min and then at a rate of 5 °C/min to 220 °C, and the final isothermal time was 10 min. The carrier gas was helium (1.5 mL/min). The injector was a split−splitless system (splitless time was 30 s) with a gas flow at 30 mL/min at the split vent. The temperature was 220 °C.

A quantitative determination was performed in the selected ion monitoring (SIM) mode by selecting ions *m*/*z* 127 and 99 for 2-acetylthiazole, *m*/*z* 129 and 87 for 2-acetyl-2-thiazoline, and *m*/*z* 113 and 85 for 2-thiazolecarboxaldehyde (i.s.). The first ion was used for quantification, and the others were used as qualifiers.

RESULTS AND DISCUSSION

Optimization of Analytical Conditions. The principle of the method is as follows: the nitrogenous heterocycles are extracted from the medium by a solvent, and then they are trapped by silica gel added in the extract. The solvent is then evaporated, and the silica gel is placed on the top of a silica gel column. Then the N-heterocycles are eluted by another solvent that is concentrated and injected into GC-NPD or GC-MS. Each stage of the method is optimized by working with a red wine of constant composition spiked with 10 μg/L of each heterocycle, to obtain a concentration sufficient to measure the peaks with good precision despite the complexity of the matrix.

Extraction. The prime objective was to compare the various extraction solvents [i.e., ethyl acetate, ethyl ether, hexane, ethyl ether/hexane (50:50 v/v) or dichloromethane] by injecting them after extraction directly into the GC-NPD. We finally chose dichloromethane, which is of medium polarity, because it made it possible to recover most of the N-heterocycles but has the drawback of extracting many substances and giving a rather complex chromatogram. Consequently, we performed a second stage of purification. If we had used hexane, this stage would not have been necessary, but the extraction of the N-heterocycles would have been incomplete, even after four successive extractions. **Figure 1** presents the progressive extraction of the spiked wine with dichloromethane. For all of the compounds, it was decided to perform three extractions with dichloromethane (10% volume = 10 mL) by stirring at 1200 rpm for 5 min. The rates of recovery were between 91% (i.s.) and 99.9% (2,4,5-trimethyloxazole). The pH is one of the properties that control liquid−liquid extractions (*5*). The extraction was the most complete when the wine was brought to pH 5.0 by the addition of 1 M NaOH (**Figure 2**).

Adsorption of N-Heterocycles on Silica Gel. As stated previously, it was necessary to purify the extract by fixing the N-heterocycles on 0.5 g of silica gel placed directly in the extract in an evaporating flask. The solvent and the nonpolar compounds were eliminated by using a vacuum rotary evaporator.

Elution of the Compounds Adsorbed on Silica. In the second stage, the silica gel having fixed the compounds of interest was placed at the top of a column prepared by adding 1.2 g of silica gel to 3 mL of hexane. Elution started with the addition of 6 mL of hexane followed by successive additions of 6 mL of hexane/ethyl acetate, 90:10. We chose this combination after experiments with combinations of these solvents (100:0 to 70:30) because of the purity of the hexane chromatogram and the capability of ethyl acetate to modify the polarity. Thus, we obtained an ideal coelution of the compounds. Having rejected the first fraction corresponding to the dead volume of the column (the first 3 mL), we visualized the recovery of the compounds nitrogenized by analyzing each 6 mL fraction of eluant (**Figure 3**). The first fraction (6 mL) did not contain any N-heterocycle, whereas the second and third fractions were the richest. The fourth and fifth fractions still contained the compounds but at lower amounts. Thus, the fractions used for chromatographic quantification were the second, third, and fourth fractions, which corresponded to a percentage of recovery ranging from 82% (2,4,5-trimethyloxazole) to 99% (2-acetylthiazole). The fifth fraction was not retained because the volume of recovered solvent was too great compared to its low thiazole content (effect of dilution).

Concentration. The eluant recovered was concentrated under a nitrogen flow, which gave a better result than vacuum evaporation. The compounds most affected by the loss of evaporation under nitrogen flow were 4-methylthiazole (61%

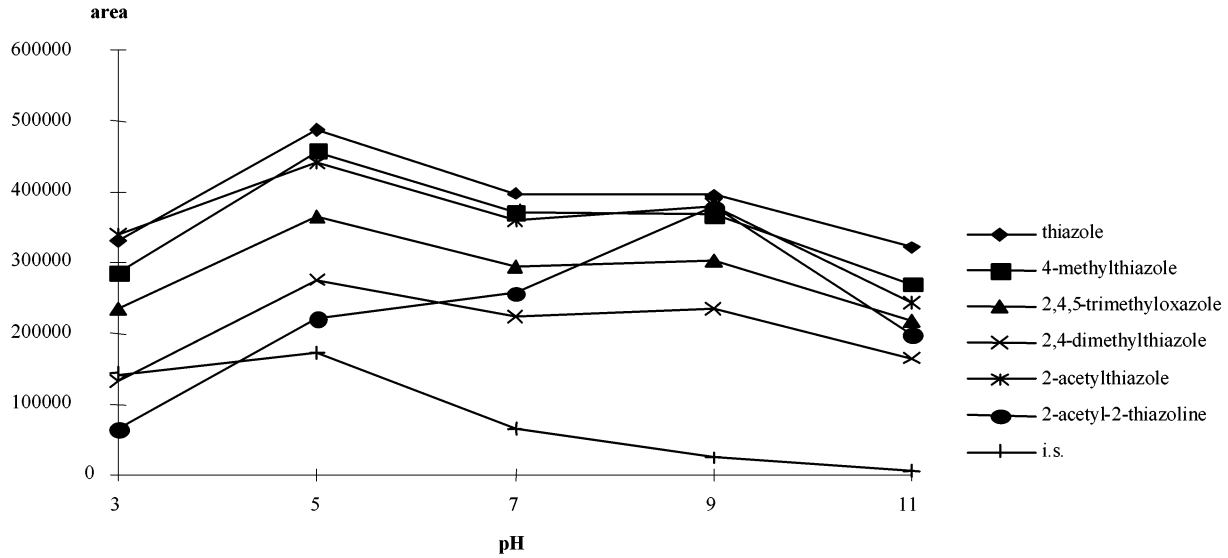

Figure 2. Influence of pH on the extractibility of N-heterocycles in wine by dichloromethane.

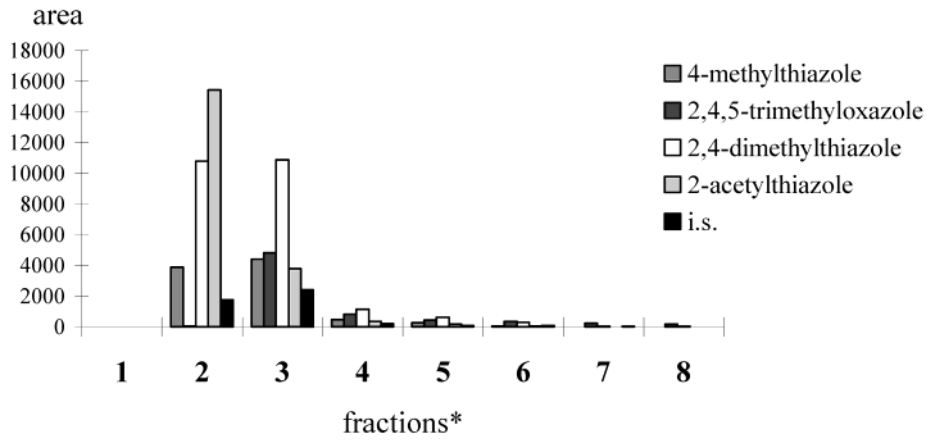

Figure 3. N-Heterocycle recovery in a spiked wine during elution with solvent (*, one fraction = 6 mL) in a silica gel column.

loss of surface) and thiazole (96% loss of surface). Thus, the latter could not be determined by using this method.

Study of Method. The different steps consisted of the search for linearity, the determination of repeatability, the recovery of the additions of known quantities of substances, and the determination of detection and quantification thresholds. Our aim was to validate this method for the relatively low concentrations observed in wine (*3*). For 4-methylthiazole, 2,4-dimethylthiazole, and 2,4,5-trimethyloxazole, the NPD method can be used; however, for 2-acetylthiazole and 2-acetyl-2-thiazoline, the presence of contamination peaks required a method based on MS detection by selecting the specific ions of the molecules.

Linearity of Quantification. This was studied by adding increasing quantities (six levels of concentration) of each of the compounds to a hydroalcoholic solution of water/ethanol, 12% v/v, added with 4 g/L of tartaric acid and brought to pH 3.5 with 0.1 M NaOH. The results were satisfactory for additions ranging between 0 and 11 μg/L (**Table 1**).

Repeatability. The repeatability of the method was established by starting from 100 mL of a wine supplemented with 100 μL of a reference solution (water/ethanol, 50% v/v) composed of the following: 1.12 mg/L 4-methylthiazole, 1.03 mg/L 2,4,5-trimethyloxazole, 1.40 mg/L 2,4-dimethylthiazole, 1.12 mg/L 2-acetylthiazole, and 2.65 mg/L 2-acetyl-2-thiazoline. The experiment was repeated eight times. The statistical data [average, standard deviation, and coefficients of variation (CV % = (variation type/mean) × 100)] concerning the heterocycles are presented in **Table 2**. The CV was minimal (3.1%) for 4-methylthiazole and reached 9.1% for 2,4,5-trimethyloxazole. These values are acceptable for concentrations near 1 μg/L.

Recovery of Added Substances. **Table 3** shows the quantification of 2-acetylthiazole and 2-acetyl-2-thiazoline in a supple-

Table 1. Study of Linearity

compound	linear calibration function[a]	*r*
4-methylthiazole	$y = 40.877x - 0.3127$	0.994
2,4,5-trimethyloxazole	$y = 46.836x - 0.1393$	0.995
2,4-dimethylthiazole	$y = 22.564x - 0.0953$	0.997
2-acetylthiazole	$y = 11.12x + 0.0117$	0.997
2-acetyl-2-thiazoline	$y = 9.4389x + 0.1625$	0.996

[a] y = concentration (μg/L); x = compound area/internal standard area.

Table 2. Study of the Repeatability and Performance of the Method (Levels in Micrograms per Liter in Modified Wine)

compound	mean	SD	CV (%)
4-methylthiazole	1.20	0.037	3.1
2,4,5-trimethyloxazole	1.12	0.102	9.1
2,4-dimethylthiazole	1.50	0.119	8.0
2-acetylthiazole	1.21	0.066	5.4
2-acetyl-2-thiazoline	2.73	0.141	5.2

Table 3. Recovery of Heterocycles from Spiked Wines (Levels in Micrograms per Liter)

2-acetylthiazole, initial level[a] = 0.02		2-acetyl-2-thiazoline, initial level = 0.00	
uantity added	recovery (%)	quantity added	recovery (%)
0.1	107.3	0.3	105.5
0.6	100.6	1.3	94.1
1.1	97.5	2.6	98.3
5.6	90.7	13.2	97.0
11.2	92.9	26.5	99.4

[a] As determined by standard addition.

mented wine at various concentrations. These results show that the products added to the wine were correctly found by this method. Data for the other compounds were comparable (not shown).

Determination of Detection and Quantification Thresholds. We used a graphical approach (*6*) to obtain a chromatogram-like recording. The thresholds of detection (DOT) and quantification (QOT) were estimated by starting from the background noise of the recording of the analysis of a sample:

$$\mathrm{DOT} = 3 \times H \times R \text{ (the associated risk remains } <0.13)$$

$$\mathrm{QOT} = 10 \times H \times R \text{ (the associated risk remains } <0.05)$$

H is the average of maximum amplitude of the signal on a window corresponding to 10 times half the width of the peak obtained for a low concentration, and R is the response factor quantity of substance/signal.

Table 4. Wines Analyzed (Levels in Micrograms per Liter)

wine	vintage	2,4,5-trimethyl-oxazole	4-methyl-thiazole	2,4-dimethyl-thiazole	2-acetyl-thiazole	2-acetyl-2-thiazoline
Champagnes						
Pinot Noir[a]	1999		0.2			
Pinot Meunier[a]	1999		0.2			
Brut 1			0.4	0.3	0.3	0.2
Brut 2					0.4	
Rosé-Brut				0.2	0.3	0.2
Ultra Brut			0.2		0.4	
Blanc de Noirs[a]	1988				0.2	
	1998					
Blanc de Blancs[a]	1988				0.3	
	1998			0.3	0.3	
fortified wines						
Porto Ruby		0.4		0.4	0.2	
Tauny		0.3			0.3	
10 years		0.6		0.4	0.5	
20 years				0.2	0.4	0.4
Madeira, 10 years		0.7		0.2		
Rivesaltes	1994	0.3		0.4	0.2	
	1984	1.3			0.2	0.2
Saint Emilion						
wine 1	1994	0.5	0.2	0.4		
	1995	0.3		0.2		
	1996	0.4		0.2	0.2	
	1997	0.3		0.2		
wine 2	1994	0.7		0.4		0.2
	1995			0.2		
	1996	0.4				
	1997	0.7		0.3		
Pomerol						
wine 1	1969		0.4	0.3		
wine 2	1979	0.2			0.2	0.2
	1981	0.2		0.3		0.2
	1982	0.3				0.3
	1983	0.2			0.2	0.2
	1985	0.3		0.6	0.2	0.2
wine 3	1993			0.2		
Premières Côtes de Blaye	1997			0.4		
Mèdoc						
wine 1	1992		0.7	0.2		
wine 2	1992		0.9		0.4	
wine 3	1994			0.2	0.3	
white wines						
Chinon	1995		0.3			
Graves 1	2000		0.2			
Graves 2	2000					
Entre Deux-Mers	2000	0.3		0.6	0.3	
Sancerre	1998		0.2	0.2	0.3	
botrytized wines						
Sainte Croix du Mont	1985					0.2
	1996			0.3	0.2	
Sauternes	1988					
Jurançon	1990					0.2

[a] Base wines.

By using MS detection, we calculated a DOT = 0.4 μg/L and a QOT = 1.34 μg/L for 2-acetylthiazole. With NPD detection we calculated a DOT = 0.3 μg/L and a QOT = 0.95 μg/L for 2,4-dimethylthiazole and a DOT = 0.5 μg/L and a QOT = 1.7 μg/L for 2,4,5-trimethyloxazole. The OIV (*6*) method is not always adapted to these concentrations, and because it is difficult to find a chromatogram with a constant baseline for each peak, we did not calculate these thresholds for 2-acetyl-2-thiazoline or 4-methylthiazole. Their values are probably of the same order as those of the other three. We thus validated a method for quantifying thiazoles and trimethyloxazole at a level of 0.5 μg/L detection threshold. Hérent and Collin (*5*) developed a method for quantifying thiazoles based on vacuum distillation followed by liquid−liquid extraction and concentration with a Kuderna−Snyder device. In this way, they were able to quantify 50 μg/L in hydroalcoholic solutions of a composition similar to that of beer. The maximum number of samples analyzed under the current conditions of our laboratory was six wines in two days.

Quantification of Thiazoles and 2,4,5-Trimethyloxazole in Wines. We applied our method to determining nitrogenous heterocycles in wines of different origins (**Table 4**). The values were lower than those measured by Marchand (*3*). In the latter paper, recovery of wine compounds was performed by a direct liquid−liquid extraction with various solvents. The authors presented the distribution of N-heterocycles and found a maximum value higher than in the present paper. The initial results were due to the complexity of the chromatograms. A large number of N-heterocycles in wine have now been evaluated, thanks to the large number of samples analyzed (45) and their diversity (red wines, white wines, Champagnes, Port wines, and others).

The wines that in general have the highest concentrations for all of the compounds are the fortified wines such as Port, Madeira, and Rivesaltes. Among the heterocycles sought, 2,4,5-trimethyloxazole, 2,4-dimethylthiazole, and 2-acetylthiazole have been found to be the most abundant in these types of wines. One of the hypotheses to explain this phenomenon is the early stopping of alcoholic fermentation by alcohol addition, which leaves a high concentration of the precursors of these compounds, such as the amino acids and the dicarbonyl compounds, by preventing their consumption or their reduction. Vernin and Metzger (*7*) suggested that if a precursor is cysteine, Strecker degradation leads to the formation of an α-aminocetone, which can condense with the ethanal and produce a oxazoline, which oxidizes to form an oxazole. When the α-dicarbonyl starting compound is diacetyl, trimethyloxazole is formed.

Two types of wines present high concentrations for one particular compound, that is, Saint Emilion wines for 2,4,5-trimethyloxazole (0.1−0.7 μg/L) and Champagne wines for 2-acetylthiazole (0.1−0.5 μg/L). For the latter, aging in the presence of yeast lees might explain this phenomenon, whereas the contents remain lower than the sensory thresholds determined by Marchand (*3*), that is, 3 μg/L for 2-acetylthiazole and 17 μg/L for 2,4,5-trimethyloxazole. 4-Methylthiazole has too a high sensory threshold of 55 μg/L. However, Schieberle and Hofmann (*8*) demonstrated a possible synergy between molecules present in concentrations lower than their sensory threshold when they are chemically similar. Moreover, the sensory threshold of 2-acetyl-2-thiazoline is low at 0.01 μg/L, and in wines in which this compound is present, the concentrations range from 10- to 40-fold the threshold.

LITERATURE CITED

(1) de Revel, G.; Pripis-Nicolau, L.; Barbe, J. C.; Bertrand, A. The detection of α-dicarbonyl compounds in wine by formation of quinoxaline derivatives. *J. Sci. Food Agric.* **2000**, *80*, 102−108.

(2) Pripis-Nicolau, L.; de Revel, G.; Bertrand, A.; Maujean, A. Flavor components formation by the reaction of amino acids and carbonyl compounds in mild conditions. *J. Agric. Food Chem.* **2000**, *48*, 3761−3766.

(3) Marchand, S.; de Revel, G.; Bertrand, A. Approaches to wine aroma: release of aroma compounds from reactions between cysteine and carbonyl compounds in wine. *J. Agric. Food Chem.* **2000**, *48*, 4890−4895.

(4) Potman, R. P.; van Wijk, Th. A. Mechanistic studies of the Maillard reaction with emphasis on phosphate-mediated catalysis. In *Phosphate-Mediated Catalysis*; ACS Symposium Series 409; American Chemical Society: Washington, DC, 1989; pp 182−195.

(5) Hérent, M.; Collin, S. Pyrazine and thiazole structural properties and their influence on the recovery of such derivatives in aroma extraction procedures. *J. Agric. Food Chem.* **1998**, *46*, 1975−1980.

(6) OIV. Resolution OENO 7/2000. Estimation de la limite de détection et de quantification d'une méthode d'analyse. 80ème Assemblée générale de l'O.I.V., Paris, France, June 2000.

(7) Vernin, G.; Metzger, J. La chimie des arômes: Les hétérocycles. *Bull. Soc. Chim. Belg.* **1981**, *90*, 553−587.

(8) Schieberle, P.; Hofmann, T. Identification of the key odorants in processed cysteine Maillard mixtures by instrumental analysis and sensory studies. In *Flavour Science, Recent Developments*; Taylor, A. J., Mottram, D. S., Eds.; Royal Society of Chemistry: Cambridge, U.K., 1997; pp 175−181.

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