

Optimal Conditions for the Formation of Sotolon from α -Ketobutyric Acid in the French "Vin Jaune"

Thu Thuy Pham,[†] Elisabeth Guichard,^{*,†} Pascal Schlich,[†] and Claudine Charpentier[‡]

Laboratoire de Recherches sur les Arômes, Institut National de la Recherche Agronomique, 17 Rue Sully, 21034 Dijon Cedex, France, and Laboratoire d'Oenologie, Institut Universitaire de la Vigne et du Vin, Campus Universitaire, 21000 Dijon Cedex, France

Sotolon (3-hydroxy-4,5-dimethyl-2(5*H*)-furanone) is a key flavor compound in the French flor-sherry Vin Jaune. Sotolon in Vin Jaune (120–268 $\mu\text{g/L}$) is produced during the development of yeast film over a period of 6 years. In a synthetic medium resembling wine, the formation of sotolon was studied for different organic acids and acetaldehyde. Then, sotolon was quantified by HPLC on a Lichrospher 100 Diol column. Under the conditions of temperature, pH, and alcoholic content similar to the ageing of wine in barrels, sotolon was formed by a purely chemical mechanism from α -ketobutyric acid and acetaldehyde. The formation of sotolon increases by increasing temperature and decreasing pH and alcoholic content.

Keywords: Sotolon; Vin jaune; flor-sherry; formation

INTRODUCTION

Sotolon was identified by Dubois et al. (1976) and Martin et al. (1992) as a key compound in the French flor-sherry wine Vin Jaune. The threshold of sotolon in wine is very low (10 $\mu\text{g/L}$). This molecule is produced after the formation of the yeast film, which occurs during 6 years of maturation of the wines in barrels. The amount of sotolon in wine is <300 $\mu\text{g/L}$. Its odor is described as "nutty" at low concentrations and "curry" at higher levels. Masuda et al. (1984) postulated that sotolon could be responsible for the typical sweet aroma of botrytized wines (5–20 $\mu\text{g/L}$). Takahashi et al. (1976) found that sotolon gave the burnt note to old Saké (140–430 $\mu\text{g/L}$). This molecule was also found in roasted tobacco (Matsukura et al., 1985), in soy sauce (Nunomura et al., 1976), in sugar molasses (Kobayashi, 1989), and in fenugreek seeds (Girardon et al., 1986).

Several hypotheses exist concerning the formation of sotolon. In fenugreek seeds, the major amino acid (2*S*,3*R*,4*S*)-4-hydroxyisoleucine is supposed to be its precursor. Chiral separation of the enantiomers of sotolon showed that only the 4*S* isomer, which is the same configuration of 4-hydroxyisoleucine (Sauvaire et al., 1992), was found in the seeds. Separation of the enantiomers of sotolon was also realized in flor-sherry wine. The two enantiomers were found in racemic ratios (Guichard et al., 1992). From this result, the mechanism of formation of sotolon in wine should be different from that in fenugreek seeds. Sotolon could be formed from a nonchiral precursor. Different ways of formation of sotolon are proposed in the literature. During the manufacturing of cane sugar, the formation of sotolon was confirmed in a model system composed of glutamic acid and pyruvate (Kobayashi et al., 1989). Takahashi et al. (1976) proposed a way of formation of sotolon by condensation of α -ketobutyric acid and acetaldehyde, which are produced by acidic degradation of threonine (H_2SO_4 , 12 N; 110 °C; 3 h) in the presence of fumaric acid. Dubois et al. (1976) produced sotolon

and 3-hydroxy-4-methyl-5-ethyl-2(5*H*)-furanone from α -ketobutyric acid and pyruvic acid (HCl, 6 N; 100 °C; 24 h).

All these experiments were carried out in media that differed greatly from a real wine. The aim of this work was to determine precursors of sotolon in wine, and to define the optimal conditions of formation of this molecule in a medium that simulates the maturation of Vin Jaune under the yeast film.

MATERIALS AND METHODS

Medium. Medium was composed of tartaric acid (4 g/L), malic acid (3 g/L), acetic acid (0.1 g/L), potassium sulfate (0.1 g/L), magnesium sulfate (0.025 g/L), sodium azide (0.1 g/L), and absolute ethanol. The pH was adjusted with NaOH (5 N). For each experiment, 70 mL of the medium was agitated (100 trs/min) in 100-mL flasks after addition of the different compounds (acetaldehyde, pyruvic acid, fumaric acid, purified α -ketobutyric acid). All chemicals were from Aldrich (France).

Purification of α -Ketobutyric Acid. Commercial α -ketobutyric acid already contained a non-negligible amount of sotolon (0.1%). Purification was realized by crystallization in hexane (SDS Peypin, France for HPLC) to obtain <0.01% sotolon.

Extraction of Sotolon. Extraction was performed according to the method of Guichard et al. (1993). A solution of 50 mL of synthetic medium was agitated with 2 g of XAD4 resin (90 min), then poured into a glass column (40 cm length, 1 cm i.d.). After washing with 100 mL of purified water, adsorbed compounds were eluted with 30 mL of diethyl ether. This organic extract was dried over anhydrous sodium sulfate and concentrated to 3 mL.

HPLC Analysis. The HPLC analysis method has been previously published (Guichard et al., 1993). A Waters 6000 pump, equipped with a Perkin Elmer UV spectrophotometer, was used. The separation was realized on a Lichrospher 100 Diol (250 mm length, 4 mm i.d., 5 μm , Merck) with UV detection at 232 nm. Peak integrations were realized with an ENICA 10 (DELSI Instruments). Extracts were filtered with syringe filters (DynaGard, 0.45 μm) before injection of 20 μL . Elution was realized with dichloromethane/hexane (60/40), at a flow rate of 0.6 mL/min.

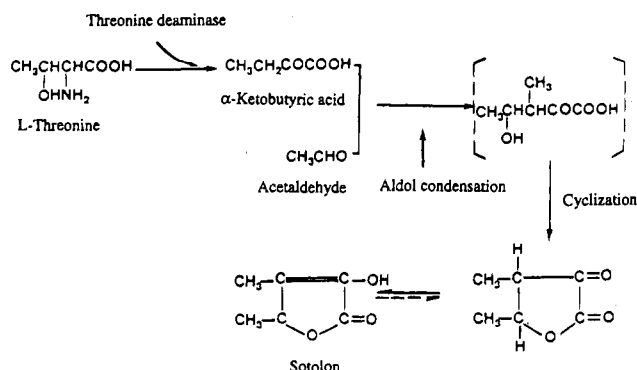
Experimental Design. A complete factorial design with three factors was realized. Temperature and pH were defined by three different levels (respectively, 15, 25, and 35 °C; and 3, 3.25, and 3.5), to determine quadratic effects of these factors. Alcoholic content was defined by two levels (12 and 15 °GL).

[†] Laboratoire de Recherches sur les Arômes.

[‡] Laboratoire d'Oenologie.

Table 1. Formation of Sotolon from Organic Acids with Acetaldehyde^a

ingredients (mg/L)				
α -ketobutyric acid	acetaldehyde	pyruvic acid	fumaric acid	amount of sotolon (μ g/L)
100	260	0	0	251
100	0	150	0	0
100	0	0	100	0
100	0	0	0	0

^a Determined at 35 °C; 10 days.**Figure 1.** Formation of sotolon.

One temperature (35 °C) was higher than those found in the cellars (0–25 °C) to accelerate the reaction.

The total number of experiments was 18. To take into account the effect of time on the production of sotolon, the design was replicated at 6 and 10 days of reaction. The repeatability of the experiments was evaluated with eight experiments realized in duplicate. The measured response was the concentration of formed sotolon in the medium. Analysis of variance was realized on the three factors, including three principal effects and three interactions of first order between the factors.

RESULTS AND DISCUSSION

Formation of Sotolon from α -Ketobutyric Acid and Acetaldehyde. The formation of sotolon was studied from different organic acids and acetaldehyde. Sotolon was quantified every 2 days in the medium as a function of time. The amounts of sotolon in the different media after 10 days are shown in Table 1. In a synthetic medium similar to a wine and at a temperature slightly higher than that found in the cellars, sotolon was only found when α -ketobutyric acid and acetaldehyde were simultaneously present. These results were suggested by Roedel et al. (1974) and Takahashi et al. (1976) in experiments held at higher temperatures and lower pH. Sulser et al. (1967) obtained another lactone (3-hydroxy-4-methyl-5-ethyl-2(5H)-furanone) from α -ketobutyric acid (HCl, 6 N, 110 °C, 24 h). In our study, we identified this second lactone as an impurity in α -ketobutyric acid, and its amount did not increase when sotolon was formed. In the present experiment, no sotolon was produced from α -ketobutyric acid and fumaric acid (Takahashi et al., 1976).

From these results, a mechanism of formation of sotolon in the French Vin Jaune was proposed, as shown in Figure 1. A release of amino acids due to the autolysis of the yeasts occurs during maturation of the wine under a yeast film (Feuillat and Charpentier, 1982; Charpentier et al., 1986). The formation of α -ketobutyric acid by deamination of threonine with threonine deaminase from *Saccharomyces* (yeasts dominating in

Table 2. Repeatability for the Quantification of Sotolon

time (days)	mean (μ g/L)	ASDAR ^a (μ g/L)	CVR ^b (%)
6	47	4.58	10
10	80	4.69	6

^a Average standard deviation among replicates. ^b Coefficient of variation of replicates.

Table 3. Experimental Design and Results

no.	factor			sotolon (μ g/L)	
	temp (°C)	pH	alcohol (°GL)	at 6 days	at 10 days
1	15	3.5	15	9	10
2	15	3.5	12	7	10
3	15	3.25	15	10	9
4	15	3.25	12	13	12
5	15	3.0	15	10	14
6	15	3.0	12	16	28
7	25	3.5	15	21	23
8	25	3.5	12	21	27
9	25	3.25	15	20	23
10	25	3.25	12	29	38
11	25	3.0	15	30	32
12	25	3.0	12	32	45
13	35	3.5	15	40	80
14	35	3.5	12	71	115
15	35	3.25	15	43	118
16	35	3.25	12	86	145
17	35	3.0	15	68	135
18	35	3.0	12	155	251

Table 4. Analysis of Variance at 6 Days^a

source	degrees of freedom	mean square	Fisher	probability
temp	2	7285	54.00	0.0013
alcohol	1	1780	13.20	0.0221
pH	2	925	6.86	0.0510
temp \times alcohol	2	1284	9.52	0.0301
temp pH	4	474	3.52	0.1254
alcohol \times pH	2	184	1.37	0.3531
residual	4	135		

^a $R^2 = 0.977$; grand mean = 37.83; root mean square of error = 11.61; model coefficient of variation = 31%.

yeast film) was already demonstrated by Chen (1977) and Fukuda et al. (1993). Moreover, a great amount of acetaldehyde is produced by the yeasts during aging of wine (Milan and Ortega, 1988). Sotolon would then be produced by a chemical reaction between acetaldehyde and α -ketobutyric acid.

Repeatability of the Experiment. The results shown in Table 2 show that experimental errors are 10 and 6% after 6 and 10 days, respectively, and negligible due to the variations caused by the factors studied.

Effect of Experimental Conditions on the Formation of Sotolon. The experimental design and the raw data are presented in Table 3. Analysis of variance was realized after 6 and 10 days. The results in Tables 4 and 5 show that temperature has the strongest effect on the formation of sotolon (probabilities of 0.0013 for 6 days and 0.0006 for 10 days), followed by alcohol (0.0221 and 0.0448), and pH (0.0510 and 0.0469). No influence of pH was found on the actions of the other two factors (probabilities > 0.1). Only a weak interaction exists between the actions of temperature and alcohol (probabilities of 0.0301 and 0.1176). Temperature has a positive effect, whereas pH and alcohol have negative effects (Table 6). For temperature, the mean amounts of sotolon obtained at 15 and 25 °C are not significantly different, whereas they increase significantly between 25 and 35 °C. The effect of pH is significant at the 5% level only between pH 3.0 and 3.5.

Table 5. Analysis of Variance at 10 Days^a

source	degrees of freedom	mean square	Fisher	probability
temp	2	28347	82.37	0.0006
alcohol	1	2863	8.32	0.0448
pH	2	2489	7.23	0.0469
temp × alcohol	2	1318	3.83	0.1176
temp × pH	4	1183	3.44	0.1294
alcohol × pH	2	568	1.65	0.3001
residual	4	344		

^a $R^2 = 0.981$; grand mean = 61.94; root mean square of error = 18.55; model coefficient of variation = 30%.

Table 6. Mean Comparisons (lsd 5%)^{a,b}

days	amount of sotolon ($\mu\text{g/L}$) at							
	temp ^c ($^{\circ}\text{C}$)			pH ^c			alcohol ^d ($^{\circ}\text{GL}$)	
	15	25	35	3.0	3.25	3.5	12	15
6	10.83 ^a	25.50 ^a	77.17 ^b	51.83 ^a	33.50 ^{ab}	28.17 ^b	47.78 ^a	27.89 ^b
10	13.83 ^a	31.33 ^a	140.67 ^b	84.17 ^a	57.50 ^{ab}	dd.17 ^b	74.56 ^a	49.33 ^b

^a Means with the same letter are not significantly different. ^b Least significant differences for temperature and pH are 18.62 and 29.73 for 6 and 10 days, respectively; and for alcohol are 15.20 and 24.27 for 6 and 10 days, respectively. ^c $n = 6$. ^d $n = 9$.

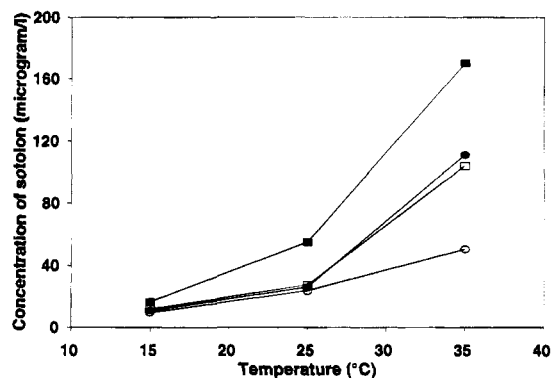


Figure 2. Formation of sotolon as a function of temperature and alcoholic content. Key: at 6 days, (●) 12 °GL, (○) 15 °GL; and at 10 days, (■) 12 °GL, (□) 15 °GL.

A low pH could favor the cyclization of the hydroxyacid, and a high concentration in ethanol could lead to the formation of esters and acetals (Shinohara et al., 1979). These reactions were confirmed by the identification by gas chromatography-mass spectrometry of the following compounds in our medium: butanoic acid, 2-oxo ethyl ester and 1,1-diethoxy ethane. These compounds are produced by condensation of ethanol with acetaldehyde or α -ketobutyric acid.

The curves of formation of sotolon as a function of the temperature at two alcoholic concentrations are not parallel (Figure 2). This could be due to a possible interaction between the actions of two factors: high temperature could increase the formation of sotolon and also the reactions of esterification in the presence of a higher level of ethanol.

Kinetics of Formation of Sotolon. Two kinetics of formation at different pH (3 and 3.5) are shown in Figure 3. The stability of sotolon was tested during the kinetics of formation and we observed that added sotolon was stable. A similar finding was published previously by Martin et al. (1989). The formation of sotolon is slow, and the negative effect of pH on this formation is not negligible. At each studied temperature, the amount of sotolon formed at pH 3 was twice the amount formed at pH 3.5.

Our results show that sotolon can be formed by a chemical pathway between acetaldehyde and α -ketobu-

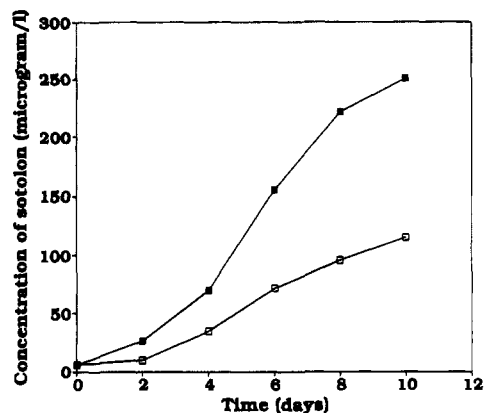


Figure 3. Kinetics of formation of sotolon. α -Ketobutyric acid (100 mg/L) and acetaldehyde (260 mg/L) were heated at 35 $^{\circ}\text{C}$. Key: (■) pH 3; (□) pH 3.5.

tyric acid. As none of these two molecules is chiral, this chemical pathway could explain the enantiomeric ratios of sotolon found in flor-sherry wines (50/50) (Guichard et al., 1993). Positive effect of temperature and negative effect of pH and alcoholic content on the formation of sotolon in wines were observed. Fluctuations of temperature observed in cellars could lead to an increase in the formation of sotolon during summer.

LITERATURE CITED

- Charpentier, C.; Nguyen Van Long, T.; Bonaly, R.; Feuillat, M. Alteration of cell wall structure in *Saccharomyces cerevisiae* and *Saccharomyces bayanus* during autolysis. *Appl. Micro. Biotechnol.* **1986**, *24*, 405-413.
- Chen, E. C. H. Keto acid decarboxylase and alcohol dehydrogenase activities of yeast in relation to the formation of fusel alcohol. *Food Sci. Technol.* **1977**, *10* (1), 27-30.
- Dubois, P.; Rigaud, J.; Dekimpe, J. Identification of 4,5-dimethyltetrahydrofuranedione-2,3 in vin jaune. *Lebensm. Wiss. Technol.* **1976**, *9*, 366-368.
- Feuillat, M.; Charpentier, C. Autolysis of yeasts in champagne. *Am. J. Enol. Vitic.* **1982**, *33* (1), 6-13.
- Fukuda, K.; Muromachi, A.; Watanabe, M.; Asano, K.; Takasawa, S. Mutants producing high concentrations of flavor components active amylalcohol and normal propanol in *Saccharomyces cerevisiae*. *J. Ferment. Bioeng.* **1993**, *75* (4), 288-292.
- Girardon, P.; Sauvaire, Y.; Baccou, J. C.; Bessiere, J. M. Identification of 3-hydroxy-4,5-dimethyl-2(5H)-furanone in the aroma of fenugreek seeds. *Lebensm. Wiss. Technol.* **1986**, *19*, 44-46.
- Guichard, E.; Etiévant, P.; Henry, R.; Mosandl, A. Enantiomeric ratios of pantolactone, solerone, 4-carboethoxy-4-hydroxybutyrolactone and of sotolon, a flavour impact compound of flor-sherry and botrytized wines. *Z. Lebens. Unters. Forsch.* **1992**, *195*, 540-544.
- Guichard, E.; Pham, T. T.; Etiévant, P. Quantitative determination of sotolon in wines by high performance liquid chromatography. *Chromatographia* **1993**, *37*, 539-542.
- Kobayashi, A. Sotolon: identification, formation and effect on flavor. In *Flavor Chemistry: Trends and Developments*; Teranishi, R., Ed.; American Chemical Society: Washington, DC, 1989; pp 49-59.
- Martin, B.; Etiévant, P.; Henry, R. The chemistry of sotolon: a key parameter for the study of a key component of flor sherry wines. In *Flavour Science and Technology*; Bessière, Y., Ed.; Wiley: Chichester, U.K., 1989; pp 53-56.
- Martin, B.; Etiévant, P. X.; Le Quéré, J. L.; Schlich, P. More clues about sensory impact of sotolon in some flor-sherry wines. *J. Agric. Food Chem.* **1992**, *40*, 475-478.
- Masuda, M.; Okawa, E. C.; Nishimura, K. I.; Yunome, H. Identification of 4,5-dimethyl-3-hydroxy-2(5H)-furanone (sotolon) and ethyl 9-hydroxynonanoate in botrytised wine and

- evaluation of the roles of compounds characteristic of it. *Agric. Biol. Chem.* **1984**, *48* (11), 2702–2710.
- Matsukura, M.; Takahashi, K.; Kawamoto, M.; Ishiguro, S.; Matsushita, H. Identification of 3-Hydroxy-4,5-dimethyl-2(5H)-furanone (sotolon) in roasted tobacco volatiles. *Agric. Biol. Chem.* **1985**, *49* (11), 3335–3337.
- Milan, C.; Ortega, J. M. Production of ethanol, acetaldehyde, and acetic acid in wine by various yeast races: role of alcohol and aldehyde dehydrogenase. *Am. J. Enol. Vitic.* **1988**, *39* (2), 107–112.
- Nunomura, N.; Sasaki, M.; Asao, Y.; Yokotsuka, T. Isolation and identification of 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone as a flavor component in shoyu (soy sauce). *Agric. Biol. Chem.* **1976**, *40* (3), 491–495.
- Roedel, W.; Hempel, U. Formation and sensory properties of 2-hydroxybuten-2-oliden-1,4-alkyl derivatives and compounds of similar structures. *Nahrung* **1974**, *18*, 133–142.
- Sauvaire, Y.; Brenac, P.; Guichard, E.; Fournier, N. Relation between composition in free amino acids and flavor quality of fenugreek seeds. Fourth International Meeting on Seeds. *Basic Appl. Aspects Seed Biol.* **1992**, *1*, 201–206.
- Shinohara, K.; Shimizu, J. I.; Shimazu, Y. Esterification rates of main organic acids in wines. *Agric. Biol. Chem.* **1979**, *43* (11), 2351–2358.
- Sulser, H.; Pizzol, J. D.; Büchi, W. A probable flavoring principle in vegetable protein hydrolysates. *J. Food Sci.* **1967**, *32*, 611–615.
- Takahashi, K.; Tadenuma, M.; Sato, S. 3-Hydroxy-4,5-dimethyl-2(5H)-furanone, a burnt flavoring compound from aged sake. *Agric. Biol. Chem.* **1976**, *40* (2), 325–330.

Received for review January 12, 1995. Revised manuscript received June 19, 1995. Accepted July 6, 1995.*

JF950027F

* Abstract published in *Advance ACS Abstracts*, September 15, 1995.