INCIDENCE OF NITROGENOUS COMPOUNDS OF MUST ON ETHYL CARBAMATE FORMATION INDUCED BY LACTIC ACID BACTERIA

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Abstract

Aims: Ethyl carbamate (EC) is a toxic compound of major concern for the hygiene quality of wines. Its natural synthesis in wine is connected to the ethanolysis of molecules such as urea (alcoholic fermentation metabolite) and citrulline and carbamyl phosphate (malolactic fermentation metabolites) during ageing. This study reports the effect of adenine, arginine, urea and ammonium phosphate of *Vitis vinifera* L. cv. Tempranillo must, on EC synthesis in fermented wine using two lactic acid bacteria strains under different microbiological and physicochemical conditions.

Methods and results: EC was quantified by gas chromatography-mass spectrometry detection. The *Lactobacillus hilgardii* strain evaluated was associated with particularly high EC production. No significant effect on EC formation was observed for nitrogen enrichment of the starting must.

Conclusion: Residual arginine levels at the end of alcoholic fermentation from arginine- and ammonium-enriched musts showed no direct relationship to EC levels found after incubation with either of the LAB strains. Overall results suggest that the different nitrogenous substrates added to *V. vinifera* L. cv. Tempranillo must do not have any influence on EC formation due to LAB growth in wines under the experimental conditions used.

Significance and impact of study: The composition of the nitrogen status of must has frequently been said to be a factor of concern on the final urethane concentration of wines. High contents of arginine coming from over fertilised vineyards are known to render significant levels of urea after alcoholic fermentation if conducted by arginase (+) yeast strains. This urea is always likely to undergo ethanolysis. No significant correlations were found between any of the nitrogenous compounds tested and final EC. High levels of arginine in the starting must did not lead to greater EC concentrations in the resulting wines.

Key words: ethyl carbamate, must nitrogenous status, ethyl carbamate precursor, malolactic fermentation, lactic acid bacterium

Résumé

Objectif : Le carbamate d'éthyle est un composé toxique qui déprécie la qualité hygiénique des vins. Sa synthèse normale dans les vins est liée à l'éthanolyse des molécules comme l'urée (métabolite de la fermentation alcoolique), le citruline et le carbamyle - phosphate (métabolites de la fermentation malolactique) pendant le vieillissement. Dans cette étude, il a été examiné l'effet de quatre composés azotés, adenine, arginine, urée et phosphate d'ammonium, dans le moût de *Vitis vinifera* L. cv. Tempranillo sur la synthèse du carbamate d'éthyle (EC), au cours de la fermentation du vin, en utilisant deux souches de bactéries lactiques sous différentes conditions microbiologiques et physicochimiques.

Méthodes et résultats : Le carbamate d'éthyle a été mesuré par détection en chromatographie en phase gazeuse couplée à la spectrométrie de masse. La souche *Lactobacillus hilgardii* Lb76 testée est caractérisée par une production d'EC particulièrement importante. Il n'a pas été observé d'effets significatifs sur la formation d'EC après l'enrichissement en azote du moût initial.

Conclusion : Des niveaux résiduels d'arginine en fin de la fermentation alcoolique et des moûts enrichis en ammonium ne sont pas en relation directe avec les niveaux du carbamate d'éthyle trouvés après incubation avec des levures ou des bactéries lactiques. Dans l'ensemble, les résultats suggèrent que les différents substrats azotés ajoutés au moût de *V. vinifera* L. cv. Tempranillo n'ont pas d'influence sur la formation du carbamate d'éthyle par les levures ou les bactéries lactiques dans les vins dans les conditions expérimentales testées.

Importance et impact de l'étude : Il a fréquemment été indiqué que la fraction azotée du moût est un facteur lié à la concentration d'uretane (urée) des vins. Il est connu que d'importantes concentrations d'arginine provenant de la sur-fertilisation des vignobles sont responsables de niveaux significatifs d'urée après la fermentation alcoolique si celle-ci est conduite par des souches de levure arginase (+). Cette urée subit probablement l'éthanolyse. Il n'a pas été observé de corrélation significative entre les composés azotés testés et la concentration finale de carbamate d'éthyle. Des niveaux élevés d'arginine dans le moût initial ne mènent pas à d'importantes concentrations de carbamate d'éthyle dans les vins résultants.

Mots clés : carbamate d'éthyle, composés azotés, précurseur du carbamate d'éthyle, fermentation malolactique, bactérie lactique

manuscript received: 19 July 2007 - revised manuscript received: 4th October 2007

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J. Int. Sci. Vigne Vin, 2007, **41**, n°4, 215-223

INTRODUCTION

Ethyl carbamate (EC), or urethane, is a toxic compound found in many fermented foods such as bread, cheese, liquors, distillates and wine. Its carcinogenicity in experimental animals is well documented (Stoewsand *et al.*, 1991; Stoewsand *et al.*, 1996), although this has not been confirmed in humans. EC may be formed naturally in wines due to alcoholic and malolactic fermentation, and especially during the ageing of wines. Efforts have been made to determine the possible pathways involved (Arena *et al.*, 1999; Ingledew *et al.*, 1987; Liu *et al.*, 1995; Mira de Orduña *et al.*, 2001; Ough *et al.*, 1988). The presence of EC in wine is important from a hygiene and health point of view. Its concentration in wine has been regulated in Canada since 1985 by the Health Protection Branch (30 µg/L), whereas in the USA a voluntary limit of 15 µg/L has been established (US FDA, 2000).

The aim of the present work was to determine the influence of four nitrogenous compounds - adenine, arginine, urea and ammonium phosphate - added to the must on EC formation after inoculating two selected lactic acid bacteria (LAB) strains in wines under different experimental conditions. These compounds were chosen on the basis of their involvement in EC synthesis throughout the winemaking process: adenine may be a source of urea (Trioli and Colagrande, 1992), urea has been used as a fermentation activator (Ingledew *et al.*, 1987; US FDA, 2000), arginine is a major source of urea (via degradation by yeast during alcoholic fermentation) and a precursor of other different carbamyl compounds (via degradation by LAB during malolactic fermentation) (Arena *et al.*, 1999; Ingledew *et al.*, 1987; Mira de Orduña *et al.*, 2001; US FDA, 2000), and ammonium ions (NH_4^+) may influence the formation of EC since they form part of the easily assimilable nitrogen (EAN) fraction and may cause loss of yeast arginine permease activity, thus making arginine metabolism more difficult and supplying arginine as substrate for LAB involved in malolactic fermentation (Henschke and Ough, 1991). Data were pooled to assess the global effect of these different nitrogenous substrates under an identical set of conditions.

MATERIALS AND METHODS

1. Must preparation and fermentations

Must from *Vitis vinifera* L. cv. Tempranillo grapes was obtained during the 2001 vintage in Madrid, Spain. Skins were discarded after short-term maceration with the must. The must was sulfited to 5 g/hL and properly homogenised. The total volume was divided into five batches. Each of four batches was enriched with a different nitrogenous substrate: adenine (1 mg/L), arginine (500 mg/L), urea (10 mg/L) or ammonium ions (139 mg/L) as ammonium phosphate. These enrichments were chosen to significantly increase the concentration of each substrate considered in the study. The fifth batch was used as a control. Table 1 shows the composition of the experimental musts. Each must batch had a low L-malic acid level (0.05 g/L), which was not corrected so as to increase nitrogen substrate availability for bacteria in the fermented wines.

Micro-vinifications were performed with 75 mL of these prepared musts in 100-mL Erlenmeyer flasks equipped with a Müller valve to gravimetrically monitor alcoholic fermentation. This was performed with the yeast strain *Saccharomyces cerevisiae* Uvaferm CM (dried active yeast, Lallemand Inc., Canada), which was kept as a pure culture. Must was inoculated with a starter culture of the yeast in yeast peptone dextrose (YPD) broth ($8.3x10^7$ cfu/mL) representing 2 % of the fermenting volume. Fermentations were performed at 25 °C and 30 °C and pH 3.2 and 3.8. All fermentations were carried out in duplicate for controls and in quadruplicate for nitrogen-enriched assays (tables 2a and 2b).

After alcoholic fermentation, two selected LAB strains were used for inoculation: *Lactobacillus hilgardii* strain Lb76 from the Colección Española de Cultivos Tipo (CECT) (Universidad de Valencia) or *Oenococcus oeni* (formerly *Leuconostoc oenos*) Uvaferm MLD (MBR,

Table 1 - Experimental must batches of *Vitis vinifera* L. cv. Tempranillo

Composition	Control	Adenine enriched (1 mg/L)	Arginine enriched (500 mg/L)	Urea enriched (10 mg/L)	Ammonium enriched (139 mg/L)
Urea (mg/L)	1.8	1.8	1.8	12.0	1.8
Ammonium (mg/L)	42.0	42.0	42.0	42.0	181.0
Total Nitrogen (mg/L)	375.7	376.2	535.7	380.4	483.8
EAN (mg/L)	91.9	91.9	251.9	96.6	200.0
Arginine (mg/L)	50.1	50.1	550.0	50.1	50.1

Note: other physicochemical parameters of the musts remained stable such as sugar (195.0 g/L), total acidity (3.2 g/L), pH (3.6), free SO_2 (3.0 mg/L), total SO_2 (20.2 mg/L), TPI (11.4) and malic acid (0.05 g/L). Nitrogenous compounds such as glycine and adenine were not detected.

Lallemand Inc., Canada). Both bacterial strains were kept as pure cultures. The fermented wines were inoculated with synchronised starter cultures of $3.9x10^7$ cfu/mL for *L. hilgardii* and $1.3x10^7$ cfu/mL for *O. oeni*. The inoculum size for both bacteria was 5 % of the wine volume. Bacterial growth was allowed to proceed at 25 °C and 30 °C and at pH 3.2 and 3.8 (tables 2a and 2b). Depletion of the little malic acid left after alcoholic fermentation (ranging from 0.05 to 0.1 g/L) was monitored and sufficient time to ensure the completion of bacterial growth (overall time nearly 1 month) was allowed.

2. Culture media

Malt extract-agar medium for pure yeast cultures was prepared by dissolving 33 g of malt extract (Pronadisa, Lab. Conda, Madrid, Spain) and 6 g of European bacteriological agar (Pronadisa) in 250 mL of distilled water. Its final pH was around 4.7. YPD medium was prepared by dissolving 1 g of yeast extract (Pronadisa), 2 g of bacteriological peptone (Pronadisa), and 2 g of glucose (J.T. Baker Chemicals B.V., Deventer, Netherlands) in 100 mL of distilled water. The final pH was around 6.8. Man Rogosa Sharpe (MRS) lactobacillus medium was prepared by dissolving 13 g of the commercial powder (Pronadisa) in 250 mL of distilled water. When preparing MRS agar, 6.25 g of European bacteriological agar was added. The final pH of the medium was 6.2.

Medium for *Oenococcus oeni* (MLO; Caspritz and Radler, 1983) was prepared by dissolving 10 g of tryptone (Pronadisa), 5 g of yeast extract, 10 g of glucose, 5 g of fructose (Panreac, Montplet & Esteban S.A., Barcelona, Spain), 3.5 g of acid ammonium citrate (Panreac), 1 mL of Tween 80 (Merck-Schuchardt, Schuchardt, Germany), 0.5 g of cysteine hydrochloride (Fluka Biochemika, Buchs, Switzerland), 0.2 g of $MgSO_4.7H_2O$ (D'Hemio Laboratorios, Madrid, Spain), 0.05 g of $MnSO_4.H_2O$ (Panreac), and 300 mL of tomato juice in 1,000 mL of distilled water. The solution was adjusted to pH 4.8. The tomato juice was prepared by macerating 100 mL of natural juice with 300 mL of distilled water overnight at 4 °C. The resulting suspension was then centrifuged at 10,000 rpm and the supernatant was filtered through Whatman n° 1 filter paper (Whatman International Ltd., Maidstone, UK) before use. To prepare solid MLO, the liquid medium and melted agar were independently sterilised to avoid agar hydrolysis (due to the low pH of tomato juice and the high temperature). European bacteriological agar (20 g) was suspended in 500 mL of distilled water and melted in a 1.5-L Erlenmeyer flask. The remaining components including the tomato juice were dissolved in 500 mL of distilled water in another

Table 2a - Experimental protocol for control assays.

Alcoholic fermentation	Temperature	pH	Assays	Bacterium	Assays
S. cerevisiae	T_1 = 25° C	pH_1 = 3.2	AC	B1: *L. hilgardii*	AC1
				B2: *O. oeni*	AC2
		pH_2 = 3.8	BC	B1: *L. hilgardii*	BC1
				B2: *O. oeni*	BC2
	T_2= 30° C	pH_1= 3.2	CC	B1: *L. hilgardii*	CC1
				B2: *O. oeni*	CC2
		pH_2 = 3.8	DC	B1: *L. hilgardii*	DC1
				B2: *O. oeni*	DC2

Note: assays are denoted by codes referenced in text in order to make table usage easier.

Table 2b - Experimental protocol for nitrogen enriched assays.

Nitrogen substrate	Alcoholic fermentation	Temperature	pH	Assays	Bacterium	Assays
Adenine Arginine Urea Ammonium	*S.cerevisiae*	T_1 = 25° C	pH_1 = 3.2	(A, E, I, M) N	B1: *L. hilgardii*	(A, E, I, M) N1
					B2: *O. oeni*	(A, E, I, M) N2
			pH_2 = 3.8	(B, F, J, N) N	B1: *L. hilgardii*	(B, F, J, N) N1
					B2: *O. oeni*	(B, F, J, N) N2
		T_2 = 30° C	pH_1 = 3.2	(C, G, K, O) N	B1: *L. hilgardii*	(C, G, K, O) N1
					B2 : *O. oeni*	(C, G, K, O) N2
			pH_2 = 3.8	(D, H, L, P) N	B1 : *L. hilgardii*	(D, H, L, P) N1
					B2 : *O. oeni*	(D, H, L, P) N2

Note: assays are denoted by codes referenced in text in order to make table usage easier.

Table 3 - Urea, ammonium ions and arginine concentrations after alcoholic fermentation of nitrogen-enriched musts.

Nitrogen substrate	Yeast	Temperature	pH	Urea (mg/L)	Ammonium ions (mg/L)	Arginine (mg/L)	Assays
Adenine	*S. cerevisiae*	T_1 = 25° C	pH_1 = 3.2	0.5 (0.2)	0.7 (0.5)	2.7 (1.6)	**AN**
			pH_2 = 3.8	1.0 (1.2)	0.7 (0.7)	3.8 (2.1)	**BN**
		T_2 = 30° C	pH_1 = 3.2	1.9 (2.7)	0.7 (0.9)	8.2 (3.1)	**CN**
			pH_2 = 3.8	1.1 (1.6)	0.4 (0.3)	8.3 (4.0)	**DN**
Arginine		T_1 = 25° C	pH_1 = 3.2	0.7 (0.9)	1.1 (0.7)	16.7 (5.4)	**EN**
			pH_2 = 3.8	0.7 (1.0)	1.4 (1.6)	14.2 (5.3)	**FN**
		T_2 = 30° C	pH_1 = 3.2	1.9 (0.9)	0.5 (0.4)	19.6 (6.4)	**GN**
			pH_2 = 3.8	0.2 (0.1)	1.5 (1.0)	17.9 (11.5)	**HN**
Urea		T_1 = 25° C	pH_1 = 3.2	1.7 (2.0)	0.5 (0.4)	2.1 (1.1)	**IN**
			pH_2 = 3.8	0.9 (1.1)	0.4 (0.2)	3.9 (2.4)	**JN**
		T_2 = 30° C	pH_1 = 3.2	0.9 (1.2)	1.4 (1.4)	6.6 (1.9)	**KN**
			pH_2 = 3.8	1.2 (1.3)	1.5 (0.5)	10.1 (3.3)	**LN**
Ammonium ions		T_1 = 25° C	pH_1 = 3.2	0.7 (0.6)	0.9 (0.7)	13.2 (5.2)	**MN**
			pH_2 = 3.8	0.8 (0.4)	0.2 (0.1)	11.4 (6.7)	**NN**
		T_2 = 30° C	pH_1 = 3.2	0.7 (0.5)	0.9 (0.9)	11.6 (4.4)	**ON**
			pH_2 = 3.8	1.0 (1.8)	0.9 (0.6)	15.9 (8.9)	**PN**

Note: assays are denoted by codes referenced in text in order to make table usage easier; data is displayed in terms of means plus standard deviations in parentheses.

Table 4 - Urea, ammonium ions, arginine and ethyl carbamate concentrations after incubation of wines with both bacterial strains.

N substrates	Wine	Bacterium	Temperature	pH	Urea (mg/L)	Ammonium (mg/L)	Arginine (mg/L)	EC (µg/L)	Assays
Adenine	*S. cerevisiae*	B1: *L. hilgardii*	T_1 = 25° C	pH_1 = 3.2	1.8 (20.9)	20.9 (3.7)	22.3 (6.4)	11.1 (1.5)	**AN1**
		B2: *O. oeni*			0.7 (24.8)	24.8 (7.1)	20.2 (0.8)	7.9 (2.3)	**AN2**
		B1: *L. hilgardii*		pH_2 = 3.8	0.6 (18.0)	18.0 (3.9)	21.1 (4.3)	11.6 (3.4)	**BN1**
		B2: *O. oeni*			7.7 (18.9)	18.9 (2.7)	24.5 (4.9)	6.3 (2.2)	**BN2**
		B1: *L. hilgardii*	T_2 = 30° C	pH_1 = 3.2	0.9 (22.0)	22.0 (7.4)	25.2 (4.6)	18.0 (9.5)	**CN1**
		B2: *O. oeni*			2.8 (36.6)	36.6 (4.5)	13.4 (6.7)	13.6 (6.0)	**CN2**
		B1: *L. hilgardii*		pH_2 = 3.8	1.0 (20.7)	20.7 (5.8)	23.3 (3.1)	20.1 (9.4)	**DN1**
		B2: *O. oeni*			0.7 (37.9)	37.9 (11.7)	21.7 (7.0)	14.6 (7.4)	**DN2**
Arginine		B1: *L. hilgardii*	T_1 = 25° C	pH_1 = 3.2	0.5 (17.9)	17.9 (8.5)	35.1 (10.0)	21.3 (9.5)	**EN1**
		B2: *O. oeni*			1.5 (26.6)	26.6 (14.7)	9.6 (0.6)	6.3 (3.5)	**EN2**
		B1: *L. hilgardii*		pH_2 = 3.8	1.5 (19.8)	19.8 (9.2)	27.2 (2.7)	22.0 (3.6)	**FN1**
		B2: *O. oeni*			3.2 (39.7)	39.7 (6.3)	22.5 (4.2)	6.0 (1.0)	**FN2**
		B1: *L. hilgardii*	T_2 = 30° C	pH_1 = 3.2	1.7 (26.1)	26.1 (2.7)	34.0 (3.7)	26.0 (9.5)	**GN1**
		B2: *O. oeni*			5.1 (26.9)	26.9 (2.5)	31.6 (1.6)	12.1 (1.1)	**GN2**
		B1: *L. hilgardii*		pH_2 = 3.8	4.0 (21.7)	21.7 (5.4)	31.6 (7.2)	21.5 (5.3)	**HN1**
		B2: *O. oeni*			0.1 (27.5)	27.5 (10.3)	26.5 (8.4)	10.6 (0.6)	**HN2**
Urea		B1: *L. hilgardii*	T_1 = 25° C	pH_1 = 3.2	1.3 (16.5)	16.5 (0.6)	24.4 (6.7)	11.2 (2.1)	**IN1**
		B2: *O. oeni*			1.5 (21.0)	21.0 (6.6)	22.2 (4.7)	8.7 (0.7)	**IN2**
		B1: *L. hilgardii*		pH_2 = 3.8	1.2 (25.2)	25.2 (3.6)	19.5 (4.6)	7.6 (1.9)	**JN1**
		B2: *O. oeni*			n.d.	27.6 (15.2)	22.9 (4.1)	5.0 (0.5)	**JN2**
		B1: *L. hilgardii*	T_2 = 30° C	pH_1 = 3.2	2.2 (21.0)	21.0 (1.1)	26.0 (1.8)	23.1 (2.4)	**KN1**
		B2: *O. oeni*			1.0 (23.5)	23.5 (1.9)	30.8 (19.4)	12.5 (6.3)	**KN2**
		B1: *L. hilgardii*		pH_2 = 3.8	1.0 (25.1)	25.1 (5.7)	27.2 (1.0)	29.8 (6.0)	**LN1**
		B2: *O. oeni*			0.8.(26.4)	26.4 (1.9)	21.8 (1.2)	10.4 (1.7)	**LN2**
Ammonium ions		B1: *L. hilgardii*	T_1 = 25° C	pH_1 = 3.2	n.d.	21.2 (0.8)	32.4 (2.6)	13.4 (2.2)	**MN1**
		B2: *O. oeni*			1.5 (24.6)	24.6 (6.7)	18.1 (8.0)	10.4 (8.0)	**MN2**
		B1: *L. hilgardii*		pH_2 = 3.8	2.8 (16.0)	16.0 (5.1)	29.0 (1.3)	15.9 (9.9)	**NN1**
		B2: *O. oeni*			2.5 (39.2)	39.2 (2.9)	26.3 (1.7)	5.4 (1.1)	**NN2**
		B1: *L. hilgardii*	T_2 = 30° C	pH_1 = 3.2	0.6 (31.0)	31.0 (0.2)	34.3 (3.6)	18.7 (4.2)	**ON1**
		B2: *O. oeni*			1.0 (33.2)	33.2 (0.9)	32.7 (4.3)	8.7 (3.2)	**ON2**
		B1: *L. hilgardii*		pH_2 = 3.8	0.2 (22.0)	22.0 (2.6)	31.1 (4.8)	20.1 (1.8)	**PN1**
		B2: *O. oeni*			2.3 (33.6)	33.6 (11.0)	29.2 (4.3)	9.3 (2.5)	**PN2**

Note: assays are denoted by codes referenced in text in order to make table usage easier; data is displayed in terms of means plus standard deviations in parentheses. References: n.d.: non-detected.

500-mL Erlenmeyer flask. The resulting solution was adjusted to pH 4.8. Both preparations were sterilised at 121 °C for 20 min, mixed under a sterile laminar flow, and shaken vigorously to achieve homogenisation.

3. Chemical analysis

Must total acidity, total nitrogen, EAN, and free and total sulfur dioxide were determined according to the official methods of the Office International de la Vigne et du Vin (OIV, 1990).

The arginine content was determined according to the Sakaguchi colorimetric method described by Gilboe and Williams (1956), and adapted for the coloured samples to be analysed. For red musts and wines, this requires preparation of a colour blank and a reagent blank, since anthocyanins interfere with the coloured reaction product at 490-510 nm. Thus, the reagent blank was used to prepare a calibration curve, and water was used as the colour blank. The possible interference of ammonium ions and glycine with the colorimetric reaction was taken into account during calibration.

Malic acid, urea and ammonium ions in the must and wines were determined enzymatically using Boehringer-Mannheim kits (Roche, R-Biopharm GmbH, Darmstadt, Germany).

Polyphenols in the must were estimated by the total polyphenol index -TPI- at 280 nm.

The bacterial growth was monitored indirectly by paper chromatography of the residual malic acid using the following mixture as the mobile phase: 0.18 g bromophenol blue dissolved in 180 mL of n-butanol and 72 mL of acetic acid diluted 50 % in distilled water. The stationary phase used was Whatman n° 1 filter paper.

4. Standards and reagents

Ethyl carbamate (99.0 % purity) was supplied by Fluka Chemika (Buchs, Switzerland), and n-propyl carbamate (98.0 % purity) by Aldrich Laboratories (Milwaukee, WI, USA). Acetone for preparing EC and PC working solutions was from Panreac PRS (Barcelona, Spain). Chromatographic quality dicholoromethane was supplied by Merck (Darmstadt, Germany). Extrelut NT 20-40-mL solid phase extraction (SPE) columns (filled with diatomaceous material with traces of silicic acid) were also from Merck. Sodium chloride added to the column to absorb water was from Panreac.

5. Apparatus

EC concentrations were determined according to the standard procedure norm Procedimiento Normalizado de Trabajo PNT (Laboratorio Arbitral del Estado, MAPYA, Madrid, Spain) (Uthurry *et al.*, 2007). Peaks were detected in single ion mode for acquisition of m/z 62, 74 and 89 ions. Quantification was performed in terms of the m/z 62 ion (target ion) and was based on an internal standard (PC) procedure. Ions m/z 74 and 89 were used as qualifiers. The calibration curve showed a good linear response and the squared coefficient of correlation (R^2) was ≥0.98 for the range 100-1,600 µg/L EC. Recovery was in the range 80-110 %, and the repeatibility was good (CV <0.3). The limit of detection (LOD) was 1.0 µg/L.

6. Statistical analysis

Data were evaluated by multiple range tests using the least significant difference (LSD) method. Statgraphics 4.0 software (Manugistics, Inc., Rockville, MD, USA) was used for all calculations.

RESULTS AND DISCUSSION

Table 3 shows the concentrations of urea, ammonium ions and arginine after alcoholic fermentation of nitrogen-enriched musts.

Bacterial inoculation of the wines was performed using *L. hilgardii* Lb76 or *O. oeni* Uvaferm MLD. Table 4 shows the concentrations of urea, ammonium ions, arginine and EC in the wines after incubation.

Urea concentrations following alcoholic fermentation were generally relatively low, ranging from non-detectable levels to almost 2 mg/L. Since the urea concentration in the must was only 1.8 mg/L in every assay, except for those to which extra urea was added (approx. 12.0 mg/L), these results were expected. Yeast depletes the urea, and there is only a low level of urea excretion into the medium as a result of arginine metabolism. In the urea-enriched assays, the results again suggest that urea is depleted and that excretion levels are low.

The maximum urea levels (1.9 mg/L) were recorded for the assays at 30 °C and pH 3.2 for either adenine (assay CN) or arginine (assay GN) enrichment of must.

In the wine industry, urea concentrations obtained following alcoholic fermentation are important in terms of its role as a potential precursor of EC during ageing and conservation (Ingledew *et al.*, 1987; Ough *et al.*, 1988; Daudt *et al.*, 1992; Kodama *et al.*, 1994; Monteiro *et al.*, 1989; Ough, 1976; Stevens and Ough, 1993). The concentrations found in this work were <2 mg/L, which are low enough to expect final EC concentrations below the legal Canadian limit (30 µg/L) after a 4-year ageing period (Kodama *et al.*, 1994).

There is no experimental evidence that urea could play a role as a nitrogenous substrate for LAB in

malolactic fermentation, thus rendering carbamyl precursors likely to undergo ethanolysis.

The ammonium ion levels detected at the end of alcoholic fermentation in both the control and nitrogen-enriched musts suggest remarkable ammonium depletion by the yeast. The ammonium ion concentration ranged from 0.2 mg/L (s=0.1 mg/L) for the ammonium-enriched assay at 25 °C and pH 3.8 (assay NN) to 2.1 mg/L (s=2.0 mg/L) for the control assay carried out at 25 °C and pH 3.2 (assay AC). Ammonium ions (NH_4^+) make up a high percentage of the must EAN fraction, so the intake rate is high. The ammonium ion itself is not an EC precursor like urea, but may be a readily available nitrogenous source for LAB.

Arginine is also a readily assimilable nitrogenous compound and makes up a variable percentage of the EAN fraction of must, depending on the grape variety and other viticultural factors. In the experiments, higher final arginine levels were recorded in the arginine-enriched assays (table 3). The maximum level registered was 19.6 mg/L (s=6.4 mg/L) (assay GN). The arginine concentrations detected after alcoholic fermentation of ammonium-enriched musts were slightly lower. The maximum level recorded for these ammonium-enriched assays was 15.9 mg/L (s=8.9 mg/L) (assay PN). Figure 1 shows graphical results of the multiple range test performed for arginine content after alcoholic fermentation of each experimental must batch.

Arginine levels were significantly higher for the arginine-enriched assays than for those enriched with adenine or urea and the control assays (CI 95.0 %). No significant difference was recorded between arginine- and ammonium ion-enriched assays. Significant differences were recorded between ammonium-enriched assays and assays enriched with adenine, urea and controls (CI 95.0 %).

This indicates that in musts with high ammonium/arginine or arginine/ammonium ratios, significant arginine levels may appear after alcoholic fermentation, providing a good nitrogen source for the growth of LAB involved in malolactic fermentation. The metabolism of arginine by wine heterofermentative LAB is known to produce carbamyl EC precursors according to Liu *et al.* (1995) and Mira de Orduña *et al.* (2001). Thus, the presence of residual arginine poses a risk with respect to the conservation of young wines. Sterilisation by filtration, a stabilisation procedure performed at most wineries, can protect wine against possible microbiological spoilage.

The results also suggest the order in which yeasts took up the major components of the EAN fraction from the must. The ammonium ion concentration was lower than the arginine concentration in all cases. Therefore, ammonium ions were probably used first.

For wines with the maximum residual arginine levels at the end of alcoholic fermentation (from arginine- and ammonium-enriched musts), no direct relationship with EC levels was found after incubation with tested LAB. Although higher EC concentrations were expected in these wines, no significant differences (CI 95.0 %) were observed, in contrast with wines containing less residual arginine (figure 2).

The EC concentrations detected after bacterial growth must have been derived mainly from ethanolysis of carbamyl phosphate, and to a lesser extent from citrulline and urea. Carbamyl phosphate and citrulline are bacterial metabolites from the degradation of arginine (Arena *et al.*, 1999; Liu *et al.*, 1995; Mira de Orduña *et al.*, 2001; Ough *et al.*, 1988).

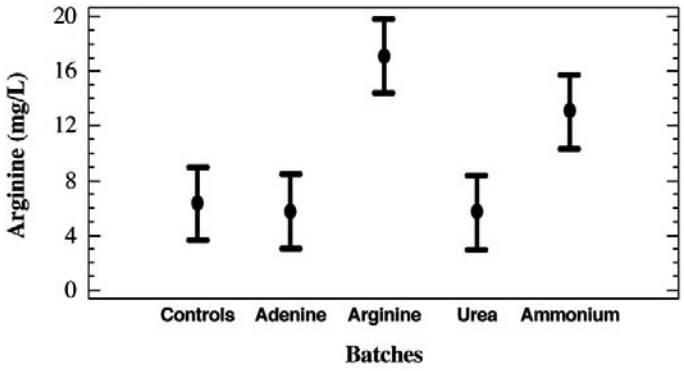

Figure 1 - Comparison of mean arginine levels detected at the end of the alcoholic fermentation of controls and nitrogen enriched musts. (multiple range test; LSD method, CI 95.0 %).

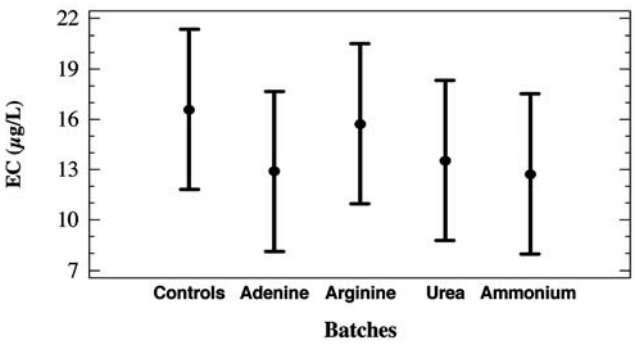

Figure 2 - Comparison of mean EC levels after incubation of wines from controls and nitrogen enriched musts with both selected bacterial strains. (multiple range test; LSD method, CI 95.0 %).

The EC concentrations found after incubation were significant. The data show a greater accumulation of EC in assays performed with *L. hilgardii* Lb76 than in those with *O. oeni* Uvaferm MLD (for all test and control fermentations). With *L. hilgardii* Lb76, EC levels ranged from 7.6 to 29.8 µg/L (mean 18.2 µg/L; s=6.0 µg/L; CV=0.33), while for *O. oeni* Uvaferm MLD these values ranged from 5.0 to 14.6 µg/L (mean 9.2 µg/L; s=3.0 µg/L; CV=0.32). In the control assays, EC levels ranged from 5.9 to 44.4 µg/L (mean 25.0 µg/L; s=19.2 µg/L; CV=0.77) for *L. hilgardii* Lb76, and from 4.2 to 18.4 µg/L (mean 8.2 µg/L; s=6.8 µg/L; CV=0.84) for *O. oeni* Uvaferm MLD.

In the nitrogen-enriched assays, a significant difference (CI 95.0 %) was observed in EC concentrations recorded (figure 3).

EC accumulation was greater with *L. hilgardii* Lb76. These results suggest that this strain induced significant concentrations of EC as a product of the ethanolysis of possible carbamyl compounds such as citrulline and carbamyl phosphate (derived from arginine metabolism). Hetero- and homofermentative species belonging to the *Lactobacillus genus* have been described (Liu *et al.*, 1995; Mira de Orduña *et al.*, 2001). *L. hilgardii* belongs to the former. The arginine deiminase (ADI) pathway for the degradation of arginine is used by many hetero-fermentative bacteria, suggesting that the studied *L. hilgardii* strain might have degraded arginine in this way. *O. oeni*, a heterofermentative LAB, has been shown to degrade arginine by the same pathway (Liu *et al.*, 1995; Mira de Orduña *et al.*, 2001; Uthurry *et al.*, 2007).

The results suggest that *L. hilgardii* Lb76 has greater ADI activity than *O. oeni* Uvaferm MLD. This is contrary to the results of prior experiments performed by us using Cabernet Sauvignon must (Uthurry *et al.*, 2007). Further experiments should be carried out to better assess

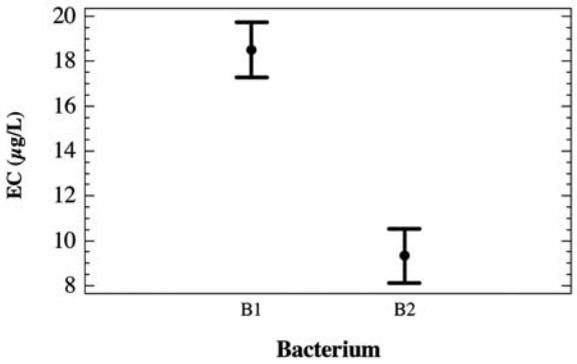

Figure 3 - Comparison of mean EC concentrations after incubation of test assays with *Lactobacillus hilgardii* (B1) and *Oenococcus oeni* (B2). (multiple range test; LSD method, CI 95 %).

differences in ADI activities between these bacteria. The greater the number and concentration of carbamyl precursors released by LAB into the medium, the higher is the potential for EC formation in wine by ethanolysis.

Maximum EC levels - 38.2 and 44.4 µg/L - were detected in controls inoculated with *L. hilgardii* Lb76 and incubated at 30 °C at pH 3.2 and 3.8 (assays DC1 and CC1, respectively), values that exceed the limit set by Canada (30 µg/L) for table wines. The values obtained in the remaining controls and nitrogen-enriched assays were either far from or close to the Canadian limit.

Although no significant differences were observed for EC levels detected after bacterial inoculation of the experimental batches (figure 2), wines with higher residual arginine content (fermented from arginine-enriched must) showed a tendency to build up higher EC concentrations. This effect was not observed for the other set of wines with high arginine content (fermented from ammonium-enriched musts). Unexpectedly, some control fermentations (assays CC1 and DC1) appeared to show the highest EC build-up, but the data for the control batch were too variable (CV=0.97) and are thus not significant (data not shown).

These results suggest that different nitrogenous substrates in *V. vinifera* L. cv. Tempranillo musts at significant concentrations do not influence EC formation due to LAB growth in wines under the experimental conditions used. Wines containing higher residual arginine concentrations after alcoholic fermentation might not necessarily accumulate more EC. The experiments showed that significant arginine enrichment of the starting must (0.5 g/L) did not lead to a significant increase in EC concentration due to the growth of any LAB strain inoculated after alcoholic fermentation. It would be interesting to perform the same experiment with different levels of arginine enrichment (as well as for the other nitrogenous substrates) and a greater initial L-malic acid concentration to evaluate more accurately its effect on the final EC content after a real malolactic fermentation.

Ammonium concentrations were higher after malolactic bacterial growth (table 4) than after alcoholic fermentation in all assays. The increased levels of ammonium ions found after bacterial treatments were possibly due to bacterial metabolism and/or the presence of lees. Both bacterial strains used were inoculated in the presence of lees to encourage their growth. Since yeast autolysis is a very slow natural process, microbial metabolism was probably the cause of this enrichment.

The urea concentration was higher than that detected after alcoholic fermentation (table 4). The high coefficients of variation obtained for the present data do not allow a clear identification of the origin of the urea formed due

to bacteria. The increase in total nitrogen found after growth of both bacterial strains might be related to the increase in urea concentration due to either yeast autolysis (quite unlikely) or other mechanisms.

The arginine concentration detected was higher than that recorded after alcoholic fermentation (table 4). The increase in arginine concentration might only be explained by the start of slow yeast autolysis; this might have started within the time of incubation, releasing simple amino acids into the wine. The arginine provided might be easily assimilated by LAB.

It would be interesting to monitor a possible yeast autolysis under the same physicochemical conditions to measure the enrichment of the wine in terms of ammonia and α-amino acids.

CONCLUSION

Initial high ammonium/arginine or arginine/ammonium ratios in musts may lead to significant arginine levels after alcoholic fermentation, thus providing a good nitrogen source for LAB growth. The presence of residual arginine poses a risk with respect to the conservation of young wines. Sterilisation by filtration can protect wine against microbiological spoilage and higher potential EC concentrations.

L. hilgardii Lb76 produced significant concentrations of EC as a product of the ethanolysis of possible carbamyl compounds such as citrulline and carbamyl phosphate (derived from arginine metabolism).

The results suggest that *L. hilgardii* Lb76 has greater ADI activity than *O. oeni* Uvaferm MLD. Nevertheless, further experiments should be performed to assess the ADI activities of these bacteria more accurately.

Maximum residual arginine levels at the end of alcoholic fermentation (from arginine- and ammonium-enriched musts) showed no direct relationship to EC levels found after incubation with either of the LAB strains under the experimental conditions used. Wines with higher residual arginine content (fermented from arginine-enriched must) showed a tendency to build up higher EC concentrations, but the difference was not statistically significant.

Moreover, the overall results suggest that the different nitrogenous substrates added to *V. vinifera* L. cv. Tempranillo must do not have any influence on EC formation due to LAB growth in wines under the experimental conditions used. It would be interesting to perform new assays with different levels of arginine enrichment (and for the other nitrogenous substrates) and a greater initial L-malic acid concentration to evaluate more accurately the effect on the final EC level after a real malolactic fermentation process.

The increased quantities of ammonium ions found after bacterial treatments were possibly due to microbial metabolism and/or the presence of lees.

The increase in total nitrogen found after growth of both bacterial strains might be related to the increase in urea concentration due to either yeast autolysis (quite unlikely) or other mechanisms.

Higher arginine concentrations recorded after LAB growth might be explained by the start of slow yeast autolysis; this might have started within the time of incubation, releasing simple amino acids into the wine. The arginine provided might be a nitrogen source for LAB.

Further experiments should be performed to assess possible yeast autolysis under the same physicochemical conditions to measure enrichment of the wine in terms of ammonia and α-amino acids.

Under winemaking conditions, conducting malolactic fermentation over lees could lead to increased levels of residual arginine, thus providing an enriched substrate for the development of possible spoilage phenomena. The formation of EC and/or other undesired metabolites might be feasible. Thus, it is better to rack wine and conduct malolactic fermentation in another vessel to avoid microbiological instability in the final wine.

Acknowledgements : This work was financially supported by project AGL 2001-2723 assigned by Ministerio de Ciencia y Tecnología (MCYT) of Spain under the R&D National Plan. We wish to thank to Dr. Augusto Arce, Departamento de Química, E.T.S. Ing. Agrónomos (Universidad Politécnica de Madrid) for permission to carry out total nitrogen determinations. We are also grateful to Dra. Encarnación Pueyo, Instituto de Fermentaciones Industriales (Consejo Superior de Investigaciones Científicas CSIC) for glycine analyses of musts and advice on adapting the Sakaguchi method for our samples.

REFERENCES

ARENA M.E., SEGUIR F.M. and MANCA DE NADRA M.C., 1999. Arginine, citrulline and ornithine metabolism by lactic acid bacteria from wine. *Int. J. Food Microbiol.*, **52**, 155-161.

CASPRITZ G. and RADLER F., 1983. Malolactic enzyme of *Lactobacillus plantarum*. Purification, properties, and distribution among bacteria. *J. Biol. Chem.*, **258**, 4907-4910.

DAUDT C.E., OUGH C.S., STEVENS D. and HERRAIZ T., 1992. Investigations into ethyl carbamate, n-propyl carbamate, and urea in fortified wines. *Am. J. Enol. Viticult.*, **43**, 318-322.

GILBOE D.D. and WILLIAMS J.N. Jr., 1956. Evaluation of the Sakaguchi reaction for the quantitative determination of arginine. *P. Soc. Exp. Biol. Med.*, **91**, 535-536.

HENSCHKE P.A. and OUGH C.S., 1991. Urea accumulation in fermenting grape juice. *Am. J. Enol. Vitic*., **42**, 317-321.

INGLEDEW W.M., MAGNUS C.A. and PATTERSON J.R., 1987. Yeast Foods and Ethyl Carbamate Formation in Wine. *Am. J. Enol. Vitic*., **38**, 332-335.

KODAMA S., SUZUKI T., FUJINAWA S., DE LA TEJA P. and YOTSUZUKA F., 1994. Urea contribution to ethyl carbamate formation in commercial wines during storage. *Am. J. Enol. Vitic*., **45**, 17-24.

LABORATORIO ARBITRAL DEL ESTADO ESPAÑOL (MINISTERIO DE AGRICULTURA, PESCA Y ALIMENTACIÓN -MAPYA - SPAIN). Procedimiento Normalizado de Trabajo (PNT), Aravaca, Madrid, Spain.

LIU S.Q., PRITCHARD G.G., HARDMAN M.J. and PILONE, G.J., 1995. Occurrence of arginine deiminase pathway enzymes in arginine catabolism by wine lactic acid bacteria. *Appl. Environ. Microb.*, **61**, 310-316.

MIRA DE ORDUÑA R., PATCHETT M.L., LIU S.Q. and PILONE G.J., 2001. Growth and arginine metabolism of the wine lactic acid bacteria *Lactobacillus buchneri* and *Oenococcus oeni* at different pH values and arginine concentrations. *Appl. Environ. Microb*., **67**, 1657-1662.

MONTEIRO F.F., TROUSDALE E.K. and BISSON L.F., 1989. Ethyl carbamate formation in wine: use of radioactively labeled precursors to demonstrate the involvement of urea. *Am. J. Enol. Vitic*., **40**, 1-8.

OFFICE INTERNATIONAL DE LA VIGNE ET DU VIN, 1990. Recueil des méthodes internationales d'analyse des vins et des moûts. *Complément nº 1 à l'édition officielle de juin 1990. OIV*, Paris.

OUGH C.S., 1976. Ethyl carbamate in fermented beverages and foods. I. Naturally occurring ethyl carbamate. *J. Agr. Food Chem*., **24**, 323-327.

OUGH C.S., CROWELL E.A. and GUTLOVE B.R., 1988. Carbamyl compound reactions with ethanol. *Am. J. Enol. Vitic*., **39**, 239-242.

STEVENS D.F. and OUGH C.S., 1993. Ethyl carbamate formation: reaction of urea and citrulline with ethanol in wine under low to normal temperature conditions. *Am. J. Enol. Vitic*., **44**, 309-312.

STOEWSAND G.S., ANDERSON J.L. and MUNSON L., 1991. Inhibition by wine of tumorigenesis induced by ethyl carbamate (urethane). *Food Chem. Toxicol*., **29**, 291-295.

STOEWSAND G.S., ANDERSON J.L. and MUNSON L., 1996. Wine and the effect of body weight on ethyl carbamate-induced tumorigenesis in mice. *J. Wine Res*., **7**, 207-211.

TRIOLI G. and COLAGRANDE O., 1991-2. El carbamato de etilo en el vino. *Vitivinicultura*, 45-49.

U.S. FOOD AND DRUG ADMINISTRATION, CENTER FOR FOOD SAFETY and NUTRITION, 2000. Information on ethyl carbamate (urethane) in foods and beverages. Web site: http:// www.cfsan.fda.gov/<frf/ecintro.html.

UTHURRY C.A., SUÁREZ LEPE J.A., LOMBARDERO J. and GARCIA DEL HIERRO J.R., 2007. Ethyl carbamate production induced by selected yeasts and lactic acid bacteria in red wine (*Erratum to Food Chem.*, **94**, 2006, 262-270).

