Available online at www.sciencedirect.com
SCIENCE DIRECT®
Food Chemistry 94 (2006) 262–270
Food Chemistry
www.elsevier.com/locate/foodchem

Ethyl carbamate production by selected yeasts and lactic acid bacteria in red wine

C.A. Uthurry [a,*], J.A. Suárez Lepe [a], J. Lombardero [b], J.R. García Del Hierro [b]

[a] *Departamento de Tecnología de los Alimentos, E.T.S. Ingenieros Agrónomos, Universidad Politécnica de Madrid, 28040 Ciudad Universitaria, Madrid, Spain*

[b] *Laboratorio Arbitral del Estado Español (Ministerio de Agricultura, Pesca, Ganadería y Alimentación de España), 28023 Aravaca, Madrid, Spain*

Received 7 April 2004; received in revised form 1 November 2004; accepted 4 November 2004

Abstract

This study was designed to investigate the formation of ethyl carbamate during the fermentation of musts of *Vitis vinifera* L. cv. Tempranillo and Cabernet Sauvignon by selected yeasts in different conditions of temperature and pH. Secondary malolactic fermentation was then induced by selected lactic acid bacteria. The results indicate an increased concentration of ethyl carbamate after malolactic fermentation, irrespective of the bacteria used or the prevailing physicochemical conditions.

Keywords: Ethyl carbamate; Red wines; Alcoholic fermentation; Malolactic fermentation; Yeasts; Lactic acid bacteria; Urea; Citrulline; Carbamyl phosphate

1. Introduction

In the 1970s, the possible origin of ethyl carbamate (EC) in wines became a subject of investigation due to the potential toxicological effects of this known carcinogen. The use of the product *Baycovin* (DEPC, diethylpyrocarbonate) as a microbiocidal agent in semisweet and sweet wines attracted much attention, when it was shown that this product's slow reaction with ammonium ions in the wine yielded EC as the main product, along with compounds such as ethanol and carbon dioxide (Ough, 1976b). In the mid 1970s, other natural sources of EC in wine were identified (Ough, 1976a). The appearance of several of ECs precursors during alcoholic fermentation (e.g., urea) and malolactic fermentation (e.g., citrulline and carbamyl phosphate) enabled researchers to establish the biochemical mechanisms used by yeasts and lactic acid bacteria to generate EC (Arena, Saguir, & Manca de Nadra, 1999; Granchi, Paperi, Rosellini, & Vincenzini, 1998; Henschke & Ough, 1991; Ingledew, Magnus, & Patterson, 1987; Kodama, Suzuki, Fujinawa, De la Teja, & Yotsuzuka, 1994; Liu, Pritchard, Hardman, & Pilone, 1994; Mira de Orduña, Liu, Patchet, & Pilone, 2000; Mira de Orduña, Patchet, Liu, & Pilone, 2001; Monteiro, Trousdale, & Bisson, 1989; Ough, Crowell, & Mooney, 1988; Ough, Stevens, Sendovski, Huang, & An, 1990; Tegmo-Larsson, Spittler, & Rodriguez, 1989).

From a practical perspective, it was found that the physicochemical conditions used for wine ageing and storing have a particular effect on EC production (Kodama et al., 1994).

In Canada, the legal limit for EC levels in table wines is 30 μg/l. Further, in 1988 the Food and Drug Administration (FDA) in the US accepted a plan to reduce EC levels in wine proposed by the country's largest wine producers represented by the Wine Institute and the Association of American Vintners. It was ruled that table wines

* Corresponding author. Tel.: +34 91 336 57 45/50; fax: +34 91 336 57 46.
E-mail address: cuthurry@latinmail.com (C.A. Uthurry).

0308-8146/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2004.11.017

(⩽14% alcohol) produced after the 1988 harvest should have a mean EC level below 15 μg/l, and that for dessert wines (>14% alcohol) the limit after the 1989 harvest should be a mean EC concentration no greater than 60 μg/l (US FDA, 2000).

The aim of the present study was to investigate the formation of EC during the alcoholic and malolactic fermentation of musts of Tempranillo and Cabernet Sauvignon using selected strains of yeasts and bacteria under different conditions of temperature and pH.

2. Materials and methods

2.1. Musts

Tempranillo and Cabernet Sauvignon musts were provided during the 2001 vintage by Campos de Prácticas, E.T.S. Ingenieros Agrónomos, Universidad Politécnica de Madrid, Madrid, Spain. Grapes were harvested during the morning, crushed and pressed in order to obtain the musts. Both musts were sulphited at 5 g/Hl and decanted into 50-l stainless steel deposits. Each must was evenly mixed and poured into 2-l plastic containers. Micro fermentation was performed in 100-ml Erlenmeyer flasks equipped with a Müller valve to gravimetrically monitor the process.

Each variety was subjected to 32 tests including quadruplet alcoholic fermentations and duplicate malolactic fermentations as outlined in the experimental protocol provided in Table 1.

2.2. Microorganisms

The following yeasts were selected for alcoholic fermentation: *Saccharomyces bayanus* strain 9CVG2r from our collection at E.T.S. Ingenieros Agrónomos of the Universidad Politécnica de Madrid and *Saccharomyces cerevisiae* from Uvaferm CM (LSA, Lallemand Inc., Canada). Malolactic fermentation was induced by the bacteria: *Lactobacillus hilgardii* strain Lb76 of the Colección Española de Cultivos Tipo (CECT) provided by the Universidad de Valencia and *Oenococcus oeni* from Uvaferm MLD (MBR, Lallemand Inc., Canada).

2.3. Chemical analysis

Total acidity, total and easily assimilated nitrogen (EAN), and free and total sulphur dioxide determinations in musts were performed according to the official methods of the Office International de la Vigne et du Vin, (OIV) (1990). Arginine was quantified by the colorimetric method of Sakaguchi (Gilboe & Williams, 1956). Malic acid, urea and ammonium levels in the musts were determined using the enzyme method of the Boehringer–Mannheim Kit (Roche, R-Biopharm GmbH, D-64293 Darmstadt, Germany). This method for malic acid has a precision of 2.0–5.0 mg/l and for urea and ammonium a precision of 0.4–1.0 mg/l for ammonium and 0.7–2.0 mg/l for urea, respectively. The polyphenol index used in determinations in musts was that of total polyphenols, or TPI (total polyphenol index).

The course of malolactic fermentation was monitored by paper chromatography using the following mixture as the eluent-developer: 0.18 g bromophenol blue dissolved in 180 ml *n*-butanol + 72 ml acetic acid diluted 50% in distilled water.

2.4. Standard reagents

EC (99.0% pure) used for calibrating the method was supplied by Fluka Chemika (Fluka Chemie, AGCH-9471 Buchs, Switzerland) and propyl carbamate (PC) (98.0% pure) used as the internal standard was obtained

Table 1
Experimental protocol for each grape variety

Alcoholic fermentation	Temperature (°C)	pH	Assays	Malolactic fermentation	Assays
L1: *S. bayanus*	t_1: 25	pH_1: 3.2 (×4)	A	B1: *L. hilgardii* (×2)	A1
				B2: *O. oeni* (×2)	A2
		pH_2: 3.8 (×4)	B	B1: *L. hilgardii* (×2)	B1
				B2: *O. oeni* (×2)	B2
	t_2: 30	pH_1: 3.2 (×4)	C	B1: *L. hilgardii* (×2)	C1
				B2: *O. oeni* (×2)	C2
		pH_2: 3.8 (×4)	D	B1: *L. hilgardii* (×2)	D1
				B2: *O. oeni* (×2)	D2
L2: *S. cerevisiae*	t_1: 25	pH_1: 3.2 (×4)	E	B1: *L. hilgardii* (×2)	El
				B2: *O. oeni* (×2)	E2
		pH_2: 3.8 (×4)	F	B1: *L. hilgardii* (×2)	F1
				B2: *O. oeni* (×2)	F2
	t_2: 30	pH_1: 3.2 (×4)	G	B1: *L. hilgardii* (×2)	G1
				B2: *O. oeni* (×2)	G2
		pH_2: 3.8 (×4)	H	B1: *L. hilgardii* (×2)	H1
				B2: *O. oeni* (×2)	H2

Note: ×4: quadruplet; ×2: duplicate.

from Aldrich (Aldrich Chemical Company, Inc., Milwaukee, WI 53233, USA).

Acetone used to prepare the working solutions of EC and PC (10 μg/ml each) was of Panreac PRS quality (Panreac Química SA, E-08110 Montcada/Reixac, Barcelona, Spain). Dichloromethane used in the elutions was of chromatographic quality and supplied by Merck (Merck KGaA, 64271 Darmstadt, Germany).

The solid-phase extraction columns filled with diatom packing were Extrelut NT20 of 40 ml capacity from Merck (Merck KGaA, 64271 Darmstadt, Germany). Sodium chloride was Panreac PA grade (Panreac, Montplet & Esteban SA, Barcelona, Spain).

2.5. Equipment

Ethyl carbamate determination in the fermentation mixtures was performed according to a normalised procedure, Procedimiento Normalizado de Trabajo (PNT) that was validated by Laboratorio Arbitral del Estado Español (Ministerio de Agricultura, Pesca y Alimentación, MAPA). This method involves the following steps:

(a) Solid-phase extraction (SPE) of the sample. Fifty microlitre of a 10 μg/ml solution of PC in acetone (internal standard) is added to 20 g of sample and mixed well. The sample is then extracted on an Extrelut NT 20 column filled with a mixture of a diatom resin + 10 g NaCl to promote water absorption, and left to act for 10 min. This procedure serves to remove the interfering matrix, mainly comprised of polyphenols and residual sugars in the sample.
(b) Sample elution. Once subjected to SPE, the sample is eluted with 90 ml of dichloromethane for gas chromatography. The eluent collected contains the internal standard, possible EC and compounds of similar polarity that are soluble in dichloromethane.
(c) Eluent concentration. The eluent is then evaporated in a rotavapor at 40 °C under vacuum to an approximate volume of 1 ml. The concentrated eluent is then transferred to a 2-ml vial.
(d) Gas chromatography/mass detection. The equipment used for gas chromatography (GC) was an HP 5890 with selective mass detector and automatic HP 6890 injector. (Hewlett Packard Company, USA). The chromatography column was a FFAP capillary of length 30 m, internal diameter 0.25 mm and 0.25 μm film thickness.

2.5.1. Chromatography conditions

Injector temperature: 200 °C
Carrier gas: ultra pure helium at a constant pressure of 7 ψ.
Injection volume: 2 μl.
Injection mode: split-splitless; time: 1 min.
Column temperature programme:
40 °C for 0.75 min.
10 °C/min ramp to 60 °C
3 °C/min ramp to 140 °C
20 °C/min ramp to 220 °C
220 °C for 3 min.
Under these conditions, EC shows a retention time of 21.3 ± 0.2 min and PC one of 24.7 ± 0.2 min.
Interface temperature: 280 °C.

2.5.2. Detection and quantification conditions

SIM acquisition of 62, 74, and 89 m/z ions. Quantification was performed in terms of the 62 ion and was based on an internal standard procedure. Five standards using the working solutions of EC and PC (10 μg/ml) and diluting up to 10 ml with dichloromethane were prepared to get: (a) 100 μg/l EC (500 μg/l PC), (b) 200 μg/l EC (500 μg/l PC), (c) 400 μg/l EC (500 μg/l PC), (d) 800 μg/l EC (500 μg/l PC), (e) 1600 μg/l EC (500 μg/l PC). A calibration curve was constructed and in any case it showed a good linear response.

Responses of ion 62 for EC and PC were represented as a function of their concentration for each standard. The squared coefficient of correlation (R^2) was = 0.98, reflecting the linear response for the above mentioned five standards ($N = 5$).

The precision of this method is given by a CV $\leqslant$ 30%.

2.6. Statistical software package

The Statgraphics 4.0 (Manugistics, Inc., 9715 Key West Avenue, Rockville, MD 20850, USA) package was used to perform multiple range tests to compare sample means.

3. Results

Table 2 shows the chemical composition of the Tempranillo and Cabernet Sauvignon musts.

Urea, ammonium, arginine and EC levels after each fermentation step are provided in Tables 3 and 4 for Tempranillo, and Tables 5 and 6 for Cabernet Sauvignon.

In 75% and 62.5% of the fermentation mixtures of Tempranillo and Cabernet Sauvignon, respectively, no EC was detected. In Tempranillo, fermentation conducted at 25 and 30 °C using *S. bayanus* 9CVG2r at pH 3.2 gave rise to EC levels exceeding 1 μg/l. For Cabernet Sauvignon, fermentation at 25 and 30 °C using *S. bayanus* 9CVG2r at pH 3.2 gave rise to levels under 1 μg/l while fermentation by *S. cerevisiae* Uvaferm CM at 30 °C and pH 3.8 generated EC concentrations

Table 2
Chemical composition of Tempranillo and Cabernet Sauvignon musts

Parameters	Grape variety	
	Tempranillo	Cabernet Sauvignon
Sugar (g/l)	195	207
Total acidity (g TH2/l)	3.52	3.42
pH	3.6	3.6
Urea (g/l)	n.d.	n.d.
Ammonium (g/l)	0.049	0.026
Total Nitrogen (mg/l)	639.9	653.4
EAN (mg/l)	63.53	39.73
Arginine (mg/l)	42.27	12.37
Glycine (mg/l)	0.1	0.4
Free sulphur dioxide (mg/l)	n.d.	n.d.
Total sulphur dioxide (mg/l)	n.d.	n.d.
TPI	13.2	13.2
Malic acid (g/l)	1.52	0.53

References: EAN, easily assimilable nitrogen; TPI, total polyphenol index; n.d., non-detected.

around 1.5 μg/l. However, the high coefficients of variation in each of these tests: 1.191, 0.669, 2.00, 1.155 and 0.673, respectively, indicate that EC production is not dependable in these conditions.

In contrast, significant yet variable production of urea was observed regardless of the fermenter or the conditions. Maximum production, 6 mg/l, was shown by Cabernet Sauvignon fermented with *S. cervisiae* Uvaferm CM at 30 °C and pH 3.8 (Table 5). This suggests a greater capacity of this strain to yield urea under the conditions examined, which is most likely related to a higher capacity for EC production.

The levels of ammonium ions and of the amino acid arginine detected indicate the consumption of both these nitrogenated nutrients by the yeasts employed. Arginine intake through the action of arginase is common in yeasts and expressed in varying degrees by a large

Table 3
Urea, ammonium, arginine and ethyl carbamate levels (coefficients of variation between brackets) after alcoholic fermentation of Tempranillo in varying conditions of temperature and pH (data represent means of quadruplet experiments)

Tempranillo. alcoholic fermentation

Yeast	Temperature (°C)	pH	Urea (mg/l)	Ammonium (mg/l)	Arginine (mg/l)	EC (μg/l)
S. bayanus 9CVG2r	25	3.2	3.9 (0.61)	0.6 (0.76)	1.8 (0.57)	1.4 (1.19)
		3.8	2.4 (0.86)	1.1 (1.43)	5.4 (0.70)	n.d.
	30	3.2	1.9 (0.59)	0.5 (0.33)	5.3 (0.41)	1.3 (0.67)
		3.8	2.6 (0.30)	0.3 (0.68)	3.4 (0.39)	n.d.
S. cerevisiae Uvaferm CM	25	3.2	2.1 (0.36)	0.1 (1.51)	1.3 (1.19)	n.d.
		3.8	1.6 (0.52)	0.2 (0.76)	1.9 (1.28)	n.d.
	30	3.2	1.3 (1.70)	1.2 (1.07)	4.5 (0.25)	n.d.
		3.8	0.9 (2.00)	1.8 (0.90)	6.6 (0.45)	n.d.

References: EC, ethyl carbamate; n.d., non-detected.

Table 4
Urea, ammonium, arginine and ethyl carbamate levels (coefficients of variation between brackets) after malolactic fermentation of Tempranillo wine in varying conditions of temperature and pH (data represent means of duplicate experiments)

Tempranillo. malolactic fermentation

Wine fermented with	Bacterium	Temperature (°C)	pH	Urea (mg/l)	Ammonium (mg/l)	Arginine (mg/l)	EC (μg/l)
S. bayanus	*L. hilgardii*	25	3.2	2.2 (0.36)	16.2 (0.12)	15.5 (0.11)	4.2 (0.31)
	O. oeni			3.0 (0.14)	22.1 (0.04)	11.0 (0.02)	4.6 (0.26)
	L. hilgardii		3.8	2.0 (0.49)	11.4 (0.01)	18.3 (0.09)	2.7 (0.06)
	O. oeni			2.8 (0.35)	25.6 (0.01)	10.3 (0.18)	4.8 (0.17)
	L. hilgardii	30	3.2	2.6 (0.19)	28.9 (0.13)	22.6 (0.07)	7.6 (0.07)
	O. oeni			1.8 (0.36)	18.5 (0.07)	26.8 (0.04)	4.7 (0.05)
	L. hilgardii		3.8	0.1 (1.41)	14.6 (0.26)	15.7 (0.05)	3.4 (0.06)
	O. oeni			5.8 (0.20)	20.4 (0.23)	14.0 (0.08)	7.4 (0.05)
S. cerevisiae	*L. hilgardii*	25	3.2	4.0 (0.28)	12.9 (0.24)	19.4 (0.05)	3.2 (0.07)
	O. oeni			3.7 (0.28)	21.2 (0.19)	14.8 (0.09)	5.0 (0.001)
	L. hilgardii		3.8	0.9 (0.92)	11.2 (0.04)	17.5 (0.05)	3.0 (0.05)
	O. oeni			3.9 (0.64)	20.3 (0.20)	13.1 (0.33)	6.4 (0.18)
	L. hilgardii	30	3.2	4.5 (0.44)	31.3 (0.02)	23.7 (0.01)	6.4 (0.32)
	O. oeni			2.7 (0.16)	21.3 (0.17)	25.6 (0.07)	9.2 (0.03)
	L. hilgardii		3.8	1.2 (0.80)	13.2 (0.01)	18.8 (0.01)	4.0 (0.40)
	O. oeni			3.0 (0.02)	21.0 (0.21)	16.6 (0.31)	3.9 (0.15)

References: EC, ethyl carbamate.

Table 5
Urea, ammonium, arginine and ethyl carbamate levels (coefficients of variation between brackets) after alcoholic fermentation of Cabernet Sauvignon in varying conditions of temperature and pH (data represent means of quadruplet experiments)

Cabernet sauvignon. alcoholic fermentation						
Yeast	Temperature (°C)	pH	Urea (mg/l)	Ammonium (mg/l)	Arginine (mg/l)	EC (μg/l)
S. bayanus 9CVG2r	25	3.2	1.2 (0.79)	0.2 (2.00)	0.7 (1.37)	0.4 (2.00)
		3.8	0.2 (0.76)	1.9 (1.60)	1.1 (0.91)	n.d.
	30	3.2	2.0 (0.34)	0.0 (2.00)	4.6 (0.83)	0.9 (1.15)
		3.8	2.1 (0.76)	0.4 (1.17)	3.5 (0.26)	n.d.
S. cerevisiae Uvaferm CM	25	3.2	0.9 (0.83)	1.3 (1.02)	0.0 (1.59)	n.d.
		3.8	0.5 (2.00)	1.7 (0.72)	2.0 (1.36)	n.d.
	30	3.2	1.4 (0.80)	0.5 (2.00)	2.7 (0.82)	n.d.
		3.8	6.0 (0.80)	0.2 (0.91)	6.8 (0.43)	1.5 (0.67)

References: EC, ethyl carbamato; n.d., non-detected.

Table 6
Urea, ammonium, arginine and ethyl carbamate levels (coefficients of variation between brackets) after malolactic fermentation of Cabernet Sauvignon wine in varying conditions of temperature and pH (data represent means of duplicate experiments)

Cabernet sauvignon. malolactic fermentation							
Wine fermented with	Bacterium	Temperature(°C)	pH	Urea (mg/l)	Ammonium (mg/l)	Arginine (mg/l)	EC (μg/l)
S. bayanus	*L. hilgardii*	25	3.2	0.9 (1.41)	16.8 (0.35)	17.1 (0.07)	3.2 (0.06)
	O. oeni			3.2 (0.40)	32.4 (0.03)	13.5 (0.11)	5.3 (0.18)
	L. hilgardii		3.8	1.8 (0.30)	13.7 (0.01)	17.4 (0.18)	1.9 (0.59)
	O. oeni			3.6 (0.13)	15.5 (0.11)	21.1 (0.14)	2.1 (0.61)
	L. hilgardii	30	3.2	2.9 (0.63)	12.6 (0.04)	19.5 (0.30)	2.5 (0.77)
	O. oeni			0.4 (1.41)	25.2 (0.15)	29.2 (0.04)	12.6 (0.73)
	L. hilgardii		3.8	1.1 (1.28)	13.3 (0.14)	20.3 (0.12)	3.3 (0.07)
	O. oeni			2.4 (0.48)	19.9 (0.31)	26.3 (0.18)	6.4 (0.06)
S. cerevisiae	*L. hilgardii*	25	3.2	5.7 (0.45)	18.3 (0.05)	21.1 (0.11)	3.4 (0.07)
	O. oeni			4.0 (0.34)	20.1 (0.09)	21.3 (0.40)	5.6 (0.07)
	L. hilgardii		3.8	2.2 (0.58)	13.1 (0.14)	14.3 (0.05)	3.0 (0.03)
	O. oeni			2.2 (0.67)	21.7 (0.19)	19.0 (0.17)	5.2 (0.12)
	L. hilgardii	30	3.2	1.8 (0.44)	18.7 (0.14)	21.0 (0.15)	4.9 (0.58)
	O. oeni			2.5 (0.00)	19.6 (0.02)	29.8 (0.08)	6.9 (0.17)
	L. hilgardii		3.8	n.d.	14.7 (0.19)	19.1 (0.05)	4.6 (0.20)
	O. oeni			2.7 (1.10)	17.5 (0.34)	12.9 (0.25)	7.8 (0.03)

References: EC, ethyl carbamato; n.d., non-detected.

number of vinicultural yeasts (Henschke & Ough, 1991; Ingledew et al., 1987; Kodama et al., 1994; Ough, Crowell, & Gutlove, 1988; Ough et al., 1988; Ough, Huang, & Stevens, 1991; Ough et al., 1990; US FDA, 2000). The relationship between arginine consumption and urea production observed in the Tempranillo assays might be explained by the fact that these yeast strains do not reabsorb the urea released into the medium during certain stages of fermentation, as reported for some strains (Ough et al., 1991).

Accordingly, the higher the arginine consumption, the greater the amount of urea excreted into the fermentation medium. In contrast, for the Cabernet fermentation mixtures, this relationship does not hold; using *S. cerevisiae* Uvaferm CM in conditions of 30 °C and pH 3.8, arginine consumption was minimal yet urea release reached its peak. This suggests different mechanisms to those induced by arginase that also give rise to urea, e.g., the use of other nitrogenated compounds whose catabolism generates this product.

Tables 4 and 6 indicate substantial EC production during malolactic fermentation induced by the bacterial strains *L. hilgardii* Lb76 and *O. oeni* Uvaferm MLD.

For Tempranillo, we observed EC production ranging from 2.7 μg/l, using *L. hilgardii* Lb76 at 25 °C and pH 3.8, to 9.2 μg/l, using *O. oeni* Uvaferm MLD at 30 °C and pH 3.2 ($\bar{x}$ = 5.0 μg/l; [s] = 1.9 μg/l; CV = 0.37) (Fig. 1).

For Cabernet Sauvignon, EC yields ranged from 1.9 μg/l, using *L. hilgardii* Lb76 at 25 °C and pH 3.8, to 12.6 μg/l, using *O.oenos* Uvaferm MLD in an assay conducted at 30 °C and pH 3.2 ($\bar{x}$ = 4.9 μg/l; [s] = 2.7 μg/l; CV = 0.55) (Fig. 1).

Many heterofermentative lactic acid bacteria, such as several *Oenococcus oeni* strains, can – in low carbohydrate conditions – obtain energy in the form of ATP

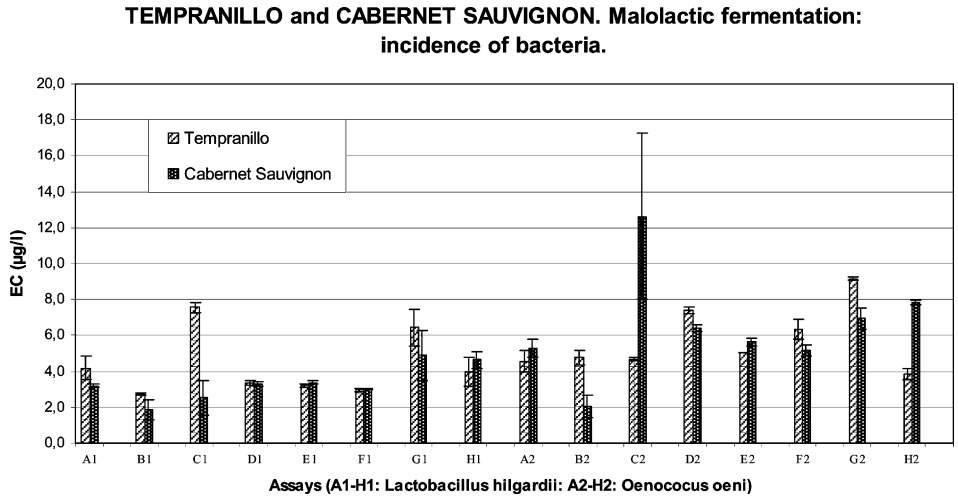

Fig. 1. Incidence of bacteria used in the malolactic fermentation of Tempranillo and Cabernet Sauvignon wines on ethyl carbamate (EC) production.

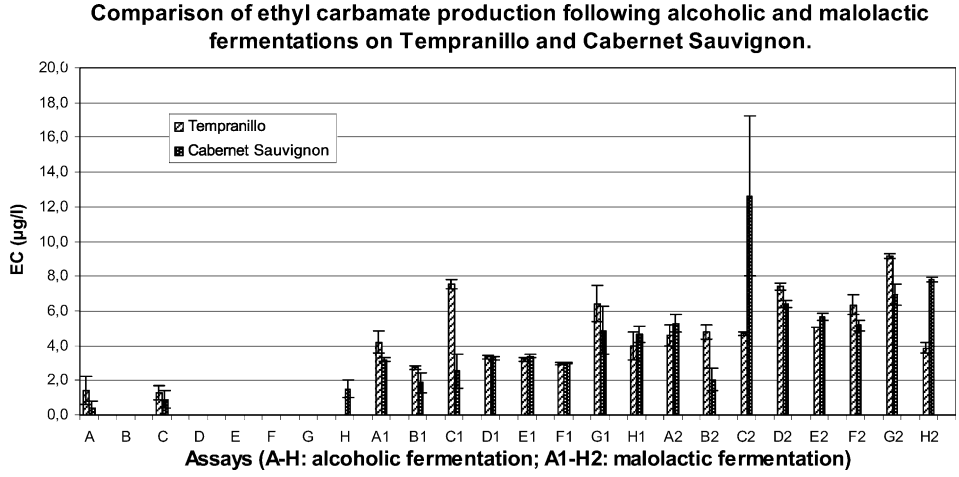

Fig. 2. Ethyl carbamate (EC) production following alcoholic and malolactic fermentations on Tempranillo and Cabernet Sauvignon.

via arginine degradation by the arginine deiminase (ADI) pathway (Arena et al., 1999; Granchi et al., 1998; Liu et al., 1994; Liu, Pritchard, Hardman, & Pilone, 1995; Mira de Orduña et al., 2000; Mira de Orduña et al., 2001). This mechanism gives rise to increased ammonium levels and those of EC precursors such as citrulline, and in smaller measure, to carbamyl phosphate. (See Fig. 2)

Our experimental data suggest that malolactic fermentation by some strains of *O. oeni* at a given pH can give rise to greater EC production at higher temperatures. Moreover, this tendency to increase EC production was noted in most of the fermentation mixtures containing *O. oeni*, irrespective of the physicochemical conditions, probably through its ADI activity (Fig. 1). ANOVA of data for Tempranillo indicated no significant difference between EC production by *L. hilgardii* and *O. oeni* (Fig. 3). However, a significant difference between the bacteria was recorded for Cabernet Sauvignon (Fig. 3), *O. oeni* showing a tendency to produce higher EC levels.

Higher ammonium levels were detected in fermentations induced by *O. oeni* compared to *L. hilgardii* (Tables 4 and 6), corresponding to the data for EC. This tendency was most marked for the Cabernet Sauvignon, as revealed by the ANOVA in which a significant difference was shown for this variety but not for the Tempranillo (Fig. 4). It can thus be inferred that *O. oeni* shows ADI activity leading to an increased ammonium ion concentration in the medium (Mira de Orduña et al., 2000; Mira de Orduña et al., 2001). In turn, raised levels of citrulline react with the alcohol present to generate EC.

Increased arginine levels were detected after malolactic fermentation, with mean values of 17.73 mg/l ([*s*] = 4.91 mg/l) recorded for Tempranillo and 20.18 mg/l ([*s*] = 4.94 mg/l) for Cabernet Sauvignon. These arginine levels can be explained by the presence of lees. The prolonged period of almost two months of

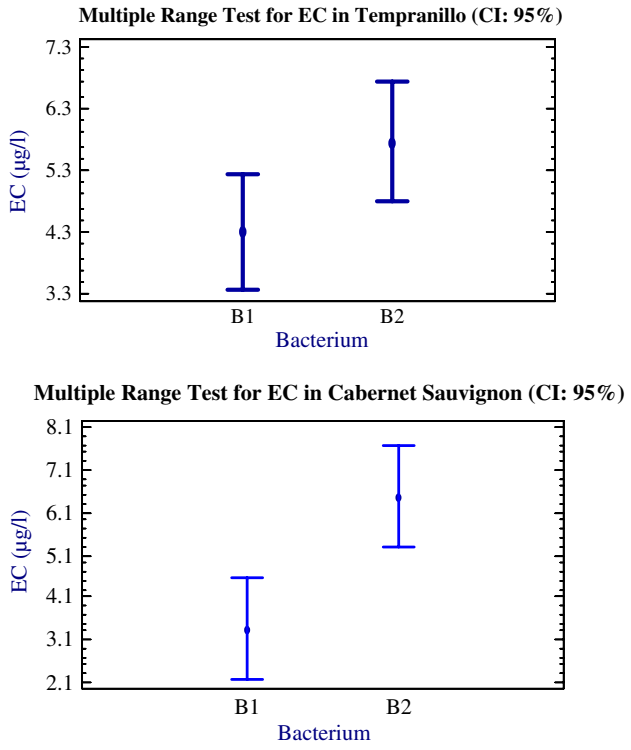

Fig. 3. ANOVA comparison between mean ethyl carbamate (EC) levels (LSD, CI: 95%) following malolactic fermentation induced in Tempranillo and Cabernet Sauvignon wines. References: B1: *Lactobacillus hilgardii*; B2: *Oenococcus oeni*.

malolactic fermentation in many of the assays should also be considered. In the presence of lees, autolytic processes can provide varying amounts of amino acids, including arginine, that act as a source of nitrogen for the bacteria that take part in the process. Malolactic fermentation conducted in the presence of lees can give rise to other EC precursors (Ough et al., 1988), thus increasing the probability of generating higher levels of this compound. The urea in the medium after alcoholic fermentation can supply some of the EC by reacting with the alcohol for prolonged periods. Hence, as the number of precursors rises, there is a greater chance that more EC will be produced.

Following malolactic fermentation of the primary fermentation mixtures of Tempranillo, urea concentrations showed considerable variation compared to the levels recorded after alcoholic fermentation. In 75% of the assays using *O. oeni*, urea levels rose, while this occurred in 50% of the fermentations performed using *L. hilgardii*. This same effect was observed for the Cabernet Sauvignon when *O. oeni* was used; increased urea concentrations being detected in 62.5% of the assays performed in the presence of the *L. hilgardii* strain. This information does not help discern whether the effect is due to bacterial metabolism or perhaps to the possible appearance of autolysis products that generate urea as an intermediate or end product of degradation.

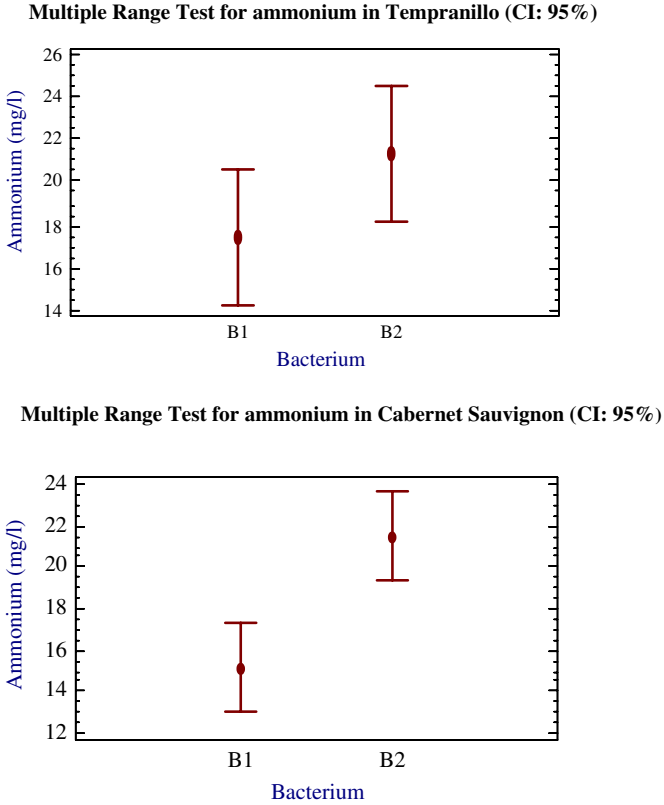

Fig. 4. ANOVA comparison between mean ammonium levels (LSD, CI: 95 %) following malolactic fermentation induced in Tempranillo and Cabernet Sauvignon wines. References: B1: *L. hilgardii* ; B2: *O. oeni*.

4. Conclusions

During the fermentation of Tempranillo and Cabernet Sauvignon musts with the yeast strains used, no EC or only discrete levels of this compound are produced under the conditions of temperature and pH established. These findings are consistent with the changes in urea, ammonium and arginine that occur during fermentation.

In contrast, when malolactic fermentation is induced by selected bacteria in the resultant fermentation mixtures, the build-up of EC is appreciably higher when *O. oeni* is used.

These bacteria can metabolise arginine by the ADI pathway releasing potential EC precursors into the medium, such as citrulline and – to a lesser extent – carbamyl phosphate, which is highly reactive and susceptible to ethanolysis. This mechanism is suggested by the levels of ammonium observed after malolactic fermentation. High wine arginine levels are the result of yeast autolysis, which enables its degradation by participating bacteria. This along with the slow reaction of urea with the alcohol present gives rise to substantial amounts of EC, particularly when fermentation is induced by *O. oeni* rather than *L. hilgardii*. However, in no case do these EC concentrations approach the unacceptable limits set by countries other than Spain.

These conclusions were inferred from experimental results on typical Spanish red musts of Madrid. Further investigation should be made on red musts of Tempranillo and Cabernet Sauvignon from other Spanish regions so as to make the above mentioned conclusions stronger.

Acknowledgements

The corresponding author, Carlos A. Uthurry, was supported by the Agencia Española de Cooperación Internaciona (AECI, the Spanish Agency of International Co-operation) under the auspices of the Ministerio de Asuntos Exteriores (Spanish Ministry of Foreign Affairs). The authors also acknowledge the funds provided by Project AGL-20012723 from the Ministerio de Ciencia y Tecnología (Spanish Ministry of Science and Technology), and the help with the ethyl carbamate determinations provided by the Laboratorio Arbitral del Estado Español (Spanish State Arbiter Laboratory) of the Ministerio de Agricultura, Pesca y Alimentación (Spanish Ministry of Food, Fishing and Agriculture).

References

Arena, M. E., Saguir, F. M., & Manca de Nadra, M. C. (1999). Arginine, citrulline and ornithine metabolism by lactic acid bacteria from wine. *International Journal of Food Microbiology, 52*, 155–161.

Gilboe, D. D., & Williams, J. N. Jr., (1956). Evaluation of the Sakaguchi reaction for the quantitative determination of arginine. *Proccedings of the Society of Experimental Biology and Medicine, 91*, 535–536.

Granchi, L., Paperi, R., Rosellini, D., & Vincenzini, M. (1998). Strain variation of arginine catabolism among malolactic *Oenococcus oeni* strains of wine origin. *Italian Journal of Food Science, 10*(4), 351–357.

Henschke, P. A., & Ough, C. S. (1991). Urea accumulation in fermenting grape juice. *American Journal of Enology and Viticulture, 42*(4), 317–321.

Ingledew, W. M., Magnus, C. A., & Patterson, J. R. (1987). Yeast foods and ethyl carbamate formation in wine. *American Journal of Enology and Viticulture, 4*(38), 332–335.

Kodama, S., Suzuki, T., Fujinawa, S., De la Teja, P., & Yotsuzuka, F. (1994). Urea contribution to ethyl carbamate formation in commercial wines during storage. *American Journal of Enology And Viticulture, 1*(45), 17–24.

Laboratorio Arbitral del Estado Español (Ministerio de Agricultura, Pesca y Alimentación –MAPA– de Espa simna). Procedimiento Normalizado de Trabajo (PNT). Padre Buidobro s/n, Aravaca, (28023) Madrid, Spain.

Liu, S.-Q., Pritchard, G. G., Hardman, M. J., & Pilone, G. J. (1994). Citrulline production and ethyl carbamate (urethane) precursor formation from arginine degradation by wine lactic acid bacteria *Leuconostoc oenos* and *Lactobacillus buchneri*. *American Journal of Enology and Viticulture, 45*(2), 235–242.

Liu, S.-Q., Pritchard, G. G., Hardman, M. J., & Pilone, G. J. (1995). Occurrence of arginine deiminase pathway enzymes in arginine catabolism by wine lactic acid bacteria. *Applied and Environmental Microbiology, 61*(1), 310–316.

Mira de Orduña, R., Liu, S.-Q., Patchet, M. L., & Pilone, G. J. (2000). Kinetics of the arginine metabolism of malolactic wine lactic acid bacteria *Lactobacillus buchneri* CUC-3 and *Oenococcus oeni* Lo111. *Journal of Applied Microbiology, 89*, 547–552.

Mira de Orduña, R., Patchet, M. L., Liu, S.-Q., & 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. *Applied and Environmental Microbiology, 67*(4), 1657–1662.

Monteiro, F. F., Trousdale, E. K., & Bisson, L. F. (1989). Ethyl carbamate formation in wine: use of radioactively labeled precursors to demonstrate the involvement of urea. *American Journal of Enology and Viticulture, 1*(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'eédition officielle de juin*. OIV, Paris.

Ough, C. S. (1976a). Ethylcarbamate in fermented beverages and foods. I. Naturally occurring ethylcarbamate. *Journal of Agriculture and Food Chemistry, 2*(24), 323–327.

Ough, C. S. (1976b). Ethylcarbamate in fermented beverages and foods II. Possible formation of ethylcarbamate from diethyl dicarbonate addition to wine. *Journal of Agriculture and Food Chemistry, 2*(24), 328–331.

Ough, C. S., Crowell, E. A., & Gutlove, B. R. (1988). Carbamyl compound reactions with ethanol. *American Journal of Enology and Viticulture, 3*(39), 239–242.

Ough, C. S., Crowell, E. A., & Mooney, L. A. (1988). Formation of ethyl carbamate precursors during grape juice (chardonnay) fermentation. I. Addition of amino acids, urea, and ammonia: effects of fortification on intracellularand extracellular precursors. *American Journal of Enology and Viticulture, 3*(39), 243–249.

Ough, C. S., Huang, Z., & Stevens, D. (1991). Amino acid uptake by four commercial yeasts at two different temperatures of growth and fermentation: effects on urea excretion and reabsorption. *American Journal of Enology and Viticulture, 42*(1), 26–40.

Ough, C. S., Stevens, D., Sendovski, T., Huang, Z., & An, D. (1990). Factors contributing to urea formation in commercially fermented wines. *American Journal of Enology and Viticulture, 1*(41), 68–73.

Tegmo-Larsson, I.-M., Spittler, T. D., & Rodriguez, S. B. (1989). Effect of malolactic fermentation on ethyl carbamate formation in chardonnay wine. *American Journal of Enology and Viticulture, 40*(2), 106–108.

US Food and Drug Administration, Center for Food Safety and Nutrition. (2000). *Information on ethyl carbamate (urethane) in foods and beverages*. Available from: www.cfsan.fda.gov/~frf/ecintro.html.