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Ethyl carbamate concentrations of typical Spanish red wines

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Abstract

The ethyl carbamate concentrations of four typical *appelation contrôlée* Spanish red wines (Rioja, Ribera del Duero, Valdepeñas and Vinos de Madrid) were studied, and correlations sought with the alcoholic, volatile, acid and mineral concentrations. Data were analysed by principal components analysis (PCA) using either all eighteen variables studied or eight supposedly better correlated with ethyl carbamate concentration. Maximum wine ethyl carbamate levels were <25 μg/L; in some samples, levels of 3 or 4 μg/L were registered, in others no ethyl carbamate was detected at all. In the analysis involving eight variables, the strongest correlations were seen between ethyl carbamate and ethyl lactate and volatile acidity. Suggestions are made regarding the origin of ethyl carbamate in these wines.

Keywords: Ethyl carbamate; Red wines; Alcoholic fermentation; Malolactic fermentation; Yeasts; Lactic bacteria; Urea

1. Introduction

Between 1970 and 1980, the detection of high levels of ethyl carbamate in alcoholic beverages, such as fruit distillates and some brandies, caused concern in Canada about possible dangers to health. A great deal of attention was then paid to ethyl carbamate levels in wines, and in 1985 the Health Protection Branch of Canada passed legislation regarding wines and alcoholic beverages in general. Almost simultaneously, the US Food and Drug Administration (FDA) produced its own regulations, tightening controls on the import and production of wine (US FDA, 2000).

According to Canadian legislation, ethyl carbamate levels in wines should not exceed 30 μg/L. For its part, in 1988 the FDA accepted a plan proposed by the largest American wineries and presented by the Wine Institute and the Association of American Vintners to reduce ethyl carbamate levels in table and dessert wines. The agreement stated that table wines (⩽ 14° alcohol) produced from the 1988 vintage onwards should have an average of no more than 15 μg/L urethane, while for dessert wines (>14° alcohol) this should not exceed 60 μg/L from the 1989 vintage onwards. The goals were that from the 1995 vintage, no more than 1% of total table wine production would have >25 μg/L urethane, and that no more than 1% of dessert wine production would have >90 μg/L (US FDA, 2000).

In the 1970s, during the early stages of research into the origin of ethyl carbamate, great attention was paid to the potential of the microbicide product Baycovin (DEPC, diethylpyrocarbonate) for preventing spoilage defects in sweet wines. The slow reaction between this product and ammonium ions (NH_4^+) in wine forms ethyl carbamate as well as other minor compounds such as ethanol and carbon dioxide (Ough, 1976a). In the middle of the decade, other natural ways of forming ethyl carbamate in wine were demonstrated (Ough, 1976b). The production of certain precursors during alcoholic fermentation (such as urea) and malolactic fermentation

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(such as citrulline and carbamyl phosphate) led to the discovery of the biochemical pathways used by yeasts and bacteria to produce ethyl carbamate (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; MiradeOrduña, Patchet, Liu, & Pilone, 2001; Mira de Orduña, Liu, Patchet, & Pilone, 2000; Monteiro, Trousdale, & Bisson, 1989; Ough, Crowell, & Mooney, 1988a; Ough, Stevens, Sendovski, Huang, & An, 1990; Tegmo-Larsson, Spittler, & Rodriguez, 1989). It was also demonstrated that physicochemical conditions during the aging and storage of wines noticeably influence the formation of ethyl carbamate (Kodama et al., 1994).

The goal of the present work was to investigate the levels of ethyl carbamate in typical Spanish red wines and to determine the variables that best correlate with them in young, *cru* and *reservé* wines. The possible origins of ethyl carbamate in these wines are discussed.

2. Materials and methods

2.1. Wines

The young, *cru* (6 months aging in oak barrels) and *reservé* (12 months aging in oak barrels) wines examined came from the Spanish *appelation contrôlée* areas of La Rioja, Ribera del Duero, Valdepeñas and Vinos de Madrid. The young wines were from the 1999 vintage, the *cru* wines from the vintages 1997–1998, and the *reservé* wines from the vintages 1994–1996. Some of the samples were obtained at wine retailers in Madrid, the rest were kindly provided by different wineries of the *appelation controlée* areas.

2.2. Chemical analysis

Total wine acidity was determined by the official method of the *Office International de la Vigne et du Vin* OIV (1990). Volatile acidity was determined using an automatic DEE Gibertini distillation unit attached to a VADE 3 Gibertini steam unit (Gibertini Elettronica SRL, 20026 Novate, Milano, Italy), and titrating the distillate as described by the OIV (1990). The degree of alcohol was again determined by the OIV official method (OIV, 1990) using an automatic DEE Gibertini distillation unit (Gibertini Elettronica SRL, 20026 Novate, Milano, Italy). Potassium was determined by flame photometry as described by the OIV (1990), using an Eppendorf Netheler&Hinz photometer. Polyphenols were estimated by the total polyphenol index (TPI) measured at wavelength 280 nm. Urea and ammonia were determined enzymatically using the urea–ammonia enzymatic test from Boehringer–Mannheim® (Roche) (R-Biopharm GmbH, D-64293 Darmstadt, Germany). Iron and copper were determined by atomic absorption spectrometry using a Perkin–Elmer 3300 apparatus (Perkin Elmer Corporation, Norwalk, CT, USA). Calcium was determined by the gravimetric Webster method (Monograph 141, Universidad Politécnica de Madrid, 2000).

2.3. Standard reagents

Ethyl carbamate (99.0% purity) for calibration was obtained from Fluka Chemika® (Sigma-Aldrich Chemie GmbH, Riedstraße 2, D-89555 Steinheim, Switzerland). Internal standard propyl carbamate (98.0% purity) was provided by the Aldrich Chemical Company, Inc. (Milwaukee, WI 53233, USA).

2.4. Equipment

Lactic acid, malic acid, tartaric acid and succinic acid were determined by HPLC as specified by the OIV (1990) using a Waters 600 E chromatograph equipped with a Waters 717 Plus Autosampler and a Waters 996 UV diode array detector (PDA) (Waters, Milford, MA 01757, USA). Ethyl lactate and (+) amilic and isoamilic alcohols were determined by gas chromatography (GC) using an HP 5890 Series II apparatus with a flame ionization detector (CG/FID) (Hewlett Packard Company, USA) according to an internal standard quantification procedure (4-methyl 2-penthanol used as internal standard).

Ethyl carbamate concentrations were determined according to the work procedure norm Procedimiento Normalizado de Trabajo (PNT) (Laboratorio Arbitral del Estado, [MAPA], Madrid, Spain). Briefly, this involves the solid phase extraction (SPE) of the sample previously added with the internal standard (*n*-propyl carbamate), elution with dichloromethane, and subsequent determination of the concentration of the eluent. The concentrated eluent is transferred to a 2 mL GC screwcap vial for analysis.

2.4.1. Gas chromatography/mass detection

The equipment used for GC was an HP 5890 with a selective mass detector and automatic HP 6890 injector (Hewlett Packard Company, USA). The chromatography column was an FFAP capillary (30 m long, internal diameter 0.25 mm, 0.25 μm film thickness).

2.4.2. Chromatography conditions

Injector temperature: 200 °C.

Carrier gas: ultra pure helium at a constant pressure of 7 psi.

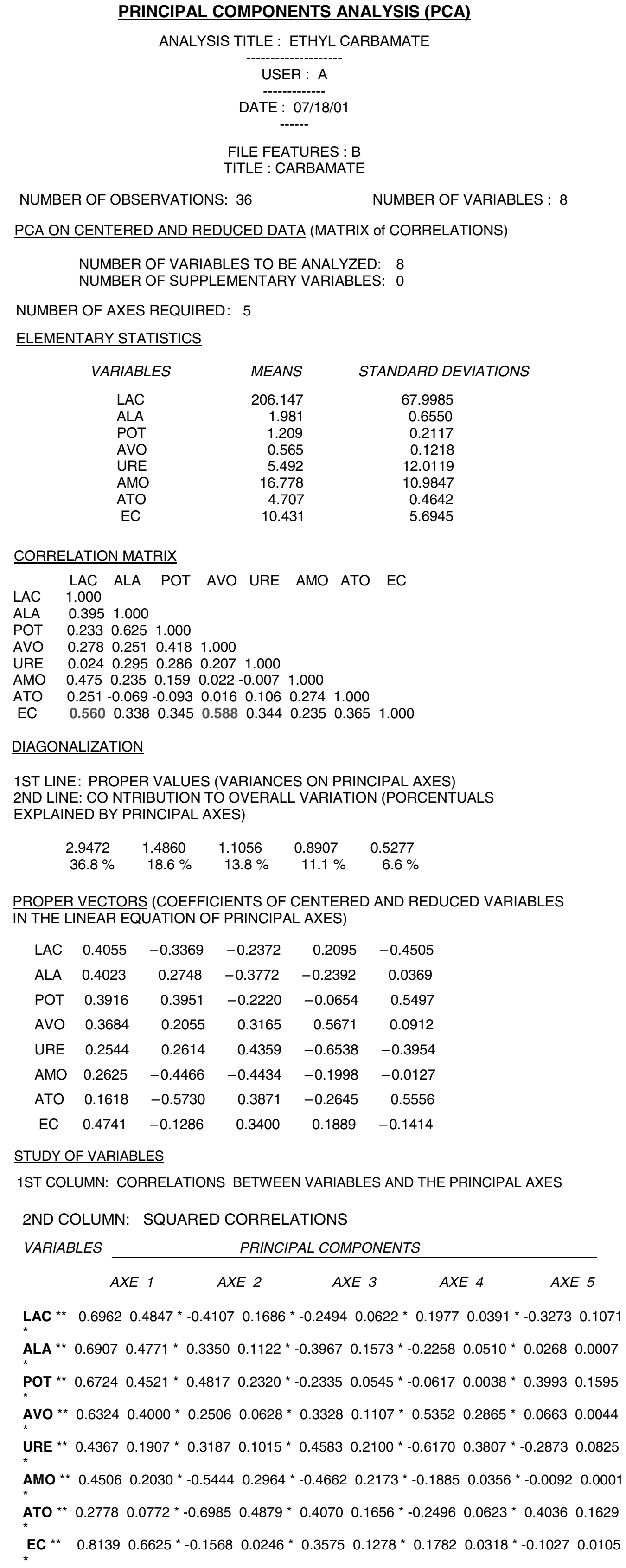
Injection volume: 2 μL.

Injection mode: split–splitless; time: 1 min.

Table 1
Original data showing all the variables determined. This was later simplified to a data matrix taking into account eight variables (Table 2) thought to be better correlated with ethyl carbamate

WINES	Alcoholic degree (%vol)	Ethyl lactate (mg/L)	(+) Amilic (mg/L)	Isoamilic (mg/L)	Total acidity (g · TH2/L)	Ammonium (mg/L)	Malic acid (g/L)	Lactic acid (g/L)	Tartaric acid (g/L)	Succinic acid (g/L)	Volatile acidity (g · AcOH/L)	Urea (mg/L)	Copper (mg/L)	Potassium (g/L)	Calcium (mg/L)	Iron (mg/L)	T. polyphenols (mg · gallic acid/L)	Ethyl carbamate (μg/L)
YWR1	12.1	162.7	25.3	143.3	4.69	18.9	0.19	1.20	1.51	0.38	0.42	3.4	0.2	0.83	85.7	4.0	787.0	7.8
CWR1	12.3	213.2	27.2	165.8	4.94	24.2	0.17	1.53	1.91	0.19	0.58	3.2	0.4	1.05	72.1	4.8	818.3	9.6
RWR1	11.3	293.3	25.3	149.4	4.67	34.9	0.10	1.80	1.15	n.d	0.53	n.d.	0.1	1.01	59.2	7.1	820.0	10.0
YWR2	12.4	110.3	24.1	169.0	4.55	16.8	0.42	3.80	1.26	0.60	0.50	3.2	0.2	1.24	104.3	4.7	854.3	5.8
CWR2	12.7	167.9	29.7	187.0	5.13	25.2	0.19	1.57	1.35	0.38	0.67	5.1	0.3	1.07	99.5	3.2	1012.3	8.5
RWR2	13.1	294.4	32.8	234.0	4.76	18.6	n.d	2.22	1.24	0.35	0.63	2.5	0.4	1.22	61.8	1.6	1538.0	8.2
YWR3	14.1	123.2	34.7	270.5	3.66	10.3	0.03	2.26	1.08	0.60	0.41	6.4	1.5	1.27	55.9	0.9	1288.3	3.8
CWR3	13.8	227.6	38.3	231.6	6.11	21.4	0.02	2.05	1.37	0.51	0.57	1.9	0.4	1.16	64.8	1.5	1247.3	17.4
RWR3	13.8	255.3	28.9	165.8	4.87	21.5	0.06	3.42	1.63	0.40	0.72	0.6	0.2	1.79	83.6	1.5	1156.3	11.6
YWD1	13.0	203.7	41.9	252.5	4.43	18.2	n.d	2.82	1.14	0.76	0.46	2.5	0.1	1.43	42.5	0.6	1128.0	5.8
CWD1	13.4	303.3	31.2	289.9	5.41	26.9	0.15	2.33	1.57	0.79	0.48	4.5	0.1	1.14	67.9	1.5	1436.3	14.0
RWD1	13.2	416.5	29.0	293.3	5.64	46.5	n.d	2.95	1.51	0.31	0.56	11.2	0.1	1.22	79.3	1.5	1482.3	24.7
YWD2	12.1	62.7	32.8	196.0	5.57	17.7	0.14	1.00	1.18	0.69	0.48	29.6	0.1	1.15	75.6	2.8	1519.0	7.2
CWD2	12.8	267.8	25.5	183.0	4.63	67	0.02	3.47	1.29	0.53	0.76	70.1	0.2	1.60	60.4	2.8	1536.0	22.6
RWD2	12.6	243.1	23.4	153.9	4.68	42.1	0.08	2.22	1.47	0.77	0.72	5.6	0.1	1.67	59.1	2.1	1465.7	17.2
YWD3	12.6	165.5	35.2	193.1	4.14	22.9	0.14	2.03	1.52	0.58	0.55	5.2	n.d.	1.41	66.4	1.7	1294.3	4.7
CWD3	13.2	252.0	23.5	165.9	4.08	20.2	0.07	2.44	1.32	0.63	0.61	2.3	n.d.	1.29	51.1	1.4	1132.3	10.3
RWD3	13.2	183.0	25.3	161.2	4.08	30.9	0.11	2.18	1.36	0.50	0.63	4.9	0.1	1.26	44.6	1.9	1160.3	16.0
YWM1	12.4	232.3	28.5	186.8	4.67	39.1	0.04	1.47	1.19	0.48	0.44	8.4	0.3	1.21	60.9	2.6	1804.7	3.4
CWM1	12.7	243.5	24.6	161.2	5.26	8.9	0.01	2.00	1.14	0.67	0.48	1.3	0.5	1.31	79.8	4.6	1258.3	14.5
RWM1	11.9	160.5	34.6	225.6	4.73	8.7	0.10	1.65	1.75	0.31	0.49	1.6	0.5	1.32	113.4	8.5	1493.7	14.9
YWM2	12.1	151.7	29.3	170.1	4.77	7.5	0.20	1.12	1.62	0.41	0.60	1.0	0.2	1.08	69.6	8.2	988.0	8.5
CWM2	12.5	188.9	35.7	243.7	4.87	7.2	0.21	1.17	1.31	0.59	0.67	1.9	0.2	1.02	76.3	5.0	836.3	15.6
RWM2	12.3	247.0	23.7	192.5	4.48	6.3	0.02	2.18	2.57	0.29	0.88	2.6	0.2	1.34	82.1	3.3	960.3	23.5
YWM3	12.1	171.7	45.0	234.7	4.26	9.0	n.d	1.71	1.63	0.48	0.70	3.5	n.d.	0.92	63.6	4.3	909.3	5.8
CWM3	12.4	129.6	33.3	208.2	4.47	9.2	0.03	1.72	1.53	0.37	0.63	3.9	n.d.	1.11	54.1	4.8	1057.0	11.5
RWM3	12.6	185.4	31.6	203.1	4.83	8.6	0.04	1.60	1.79	0.40	0.57	3.3	0.2	1.15	56.4	3.4	1254.7	7.9
YWV1	11.9	140.6	28.7	202.4	4.49	4.5	0.08	1.46	1.65	0.49	0.30	n.d.	1.4	1.15	63.7	2.3	1417.0	n.d
CWV1	12.9	175.8	34.6	192.8	4.71	4.8	0.05	1.25	2.07	0.40	0.47	n.d	n.d.	0.86	68.7	2.4	1125.0	4.1
RWV1	12.1	94.1	32.7	191.5	4.57	5.5	0.09	1.34	1.53	0.36	0.43	2.7	0.1	1.14	67.3	3.0	1277.0	5.3
YWV2	11.6	232.0	30.2	161.1	4.60	9.7	0.36	1.45	1.40	0.09	0.68	2.0	0.2	1.23	89.6	8.9	898.0	12.9
CWV2	11.9	228.0	27.6	167.0	4.70	9.7	0.23	2.14	1.06	0.34	0.64	0.7	0.2	1.48	96.5	4.6	1651.0	8.8
RWV2	11.9	203.5	24.8	168.1	4.60	7.3	0.10	1.96	2.79	0.31	0.72	n.d.	0.1	1.35	87.0	4.7	1051.3	8.9
YWV3	11.9	165.8	30.9	190.7	4.61	11.4	n.d	1.82	1.51	0.66	0.39	2.1	0.2	0.92	87.7	1.9	979.7	8.1
CWV3	12.8	241.5	37.3	235.6	4.68	14.4	n.d	2.05	3.36	0.50	0.51	0.5	0.2	0.94	94.4	1.8	1298.0	6.1
RWV3	13.8	294.2	35.1	266.3	4.12	8.3	0.12	1.92	0.92	0.88	0.45	n.d.	0.1	1.19	49.0	1.9	1897.0	10.5

Y: young wine; C: *cru* wine; R: *reservé* wine; W: winery; R: *appelation contrôleé* Rioja; D: Ribera del Duero; M: Vinos de Madrid; V: Valdepeñas; 1: first winery; 2: second winery; 3: third winery.



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                 PRINCIPAL COMPONENTS ANALYSIS (PCA)

                  ANALYSIS TITLE :  ETHYL CARBAMATE
                        --------------------
                             USER :  A
                           -------------
                         DATE :  07/18/01
                              ------

                        FILE FEATURES : B
                         TITLE : CARBAMATE

 NUMBER OF OBSERVATIONS:  36                 NUMBER OF VARIABLES :  8

PCA ON CENTERED AND REDUCED DATA (MATRIX of CORRELATIONS)

          NUMBER OF VARIABLES TO BE ANALYZED:    8
          NUMBER OF SUPPLEMENTARY VARIABLES:  0

 NUMBER OF AXES REQUIRED :   5

 ELEMENTARY STATISTICS

           VARIABLES              MEANS          STANDARD DEVIATIONS

              LAC                206.147                  67.9985
              ALA                  1.981                   0.6550
              POT                  1.209                   0.2117
              AVO                  0.565                   0.1218
              URE                  5.492                  12.0119
              AMO                 16.778                  10.9847
              ATO                  4.707                   0.4642
               EC                 10.431                   5.6945

CORRELATION MATRIX
        LAC   ALA    POT   AVO   URE    AMO   ATO   EC
LAC    1.000
ALA     0.395  1.000
POT     0.233  0.625  1.000
AVO     0.278  0.251  0.418  1.000
URE     0.024  0.295  0.286  0.207  1.000
AMO    0.475  0.235  0.159  0.022 -0.007  1.000
ATO     0.251 -0.069 -0.093  0.016  0.106  0.274  1.000
 EC     0.560  0.338  0.345  0.588  0.344  0.235  0.365  1.000

DIAGONALIZATION

1ST LINE :  PROPER VALUES (VARIANCES ON PRINCIPAL AXES)
2ND LINE: CO NTRIBUTION TO OVERALL VARIATION (PORCENTUALS
EXPLAINED BY PRINCIPAL AXES)

        2.9472       1.4860       1.1056       0.8907       0.5277
         36.8 %       18.6 %       13.8 %       11.1 %        6.6 %

PROPER VECTORS (COEFFICIENTS OF CENTERED AND REDUCED VARIABLES
IN THE LINEAR EQUATION OF PRINCIPAL AXES)

    LAC   0.4055    −0.3369    −0.2372     0.2095    −0.4505
    ALA   0.4023     0.2748    −0.3772    −0.2392     0.0369
    POT   0.3916     0.3951    −0.2220    −0.0654     0.5497
    AVO   0.3684     0.2055     0.3165     0.5671     0.0912
    URE   0.2544     0.2614     0.4359    −0.6538    −0.3954
    AMO   0.2625    −0.4466    −0.4434    −0.1998    −0.0127
    ATO   0.1618    −0.5730     0.3871    −0.2645     0.5556
     EC   0.4741    −0.1286     0.3400     0.1889    −0.1414

STUDY OF VARIABLES

 1ST COLUMN:  CORRELATIONS  BETWEEN VARIABLES AND THE PRINCIPAL AXES

  2ND COLUMN:   SQUARED CORRELATIONS
  VARIABLES                        PRINCIPAL COMPONENTS
                 ______________________________________________________

                 AXE  1          AXE  2          AXE  3          AXE  4          AXE  5

  LAC **   0.6962  0.4847 * -0.4107  0.1686 * -0.2494  0.0622 *  0.1977  0.0391 * -0.3273  0.1071
  *
  ALA **  0.6907  0.4771 *  0.3350  0.1122 * -0.3967  0.1573 * -0.2258  0.0510 *  0.0268  0.0007
  *
  POT **  0.6724  0.4521 *  0.4817  0.2320 * -0.2335  0.0545 * -0.0617  0.0038 *  0.3993  0.1595
  *
  AVO **  0.6324  0.4000 *  0.2506  0.0628 *  0.3328  0.1107 *  0.5352  0.2865 *  0.0663  0.0044
  *
  URE **  0.4367  0.1907 *  0.3187  0.1015 *  0.4583  0.2100 * -0.6170  0.3807 * -0.2873  0.0825
  *
  AMO **  0.4506  0.2030 * -0.5444  0.2964 * -0.4662  0.2173 * -0.1885  0.0356 * -0.0092  0.0001
  *
  ATO **  0.2778  0.0772 * -0.6985  0.4879 *  0.4070  0.1656 * -0.2496  0.0623 *  0.4036  0.1629
  *
   EC **   0.8139  0.6625 * -0.1568  0.0246 *  0.3575  0.1278 *  0.1782  0.0318 * -0.1027  0.0105
  *
```

Fig. 1. Numerical Stat-itcf PCA analysis for the samples taking into account the eight most important variables. LAC: ethyl lactate; ALA: lactic acid; POT: potassium; AVO: volatile acidity; URE: urea; AMO: ammonia; ATO: total acidity; EC: ethyl carbamate.

Table 2
Ethyl carbamate in Spanish red wines. Data matrix with eight variables (the analyzed variables) and 36 observations (wine samples). (Note: ethyl carbamate levels are the mean of triplicate sampling.)

WINES	Ethyl lactate (mg/L)	Lactic acid (g/L)	Potassium (g/L)	Volatile acidity (g · AcOH/L)	Urea (mg/L)	Ammonium (mg/L)	Total acidity (g · TH2/L)	Ethyl carbamate (μg/L)
YWR1	162.7	1.20	0.83	0.42	3.4	18.9	4.69	7.8
CWR1	213.2	1.53	1.05	0.58	3.2	24.2	4.94	9.6
RWR1	293.3	1.80	1.01	0.53	n.d.	34.9	4.67	10.0
YWR2	110.3	3.80	1.24	0.5	3.2	16.8	4.55	5.8
CWR2	167.9	1.57	1.07	0.67	5.1	25.2	5.13	8.5
RWR2	294.4	2.22	1.22	0.63	2.5	18.6	4.76	8.2
YWR3	123.2	2.26	1.27	0.41	6.4	10.3	3.66	3.8
CWR3	227.6	2.05	1.16	0.57	1.9	21.4	6.11	17.4
RWR3	255.3	3.42	1.79	0.72	0.6	21.5	4.87	11.6
YWD1	203.7	2.82	1.43	0.46	2.5	18.2	4.43	5.8
CWD1	303.3	2.33	1.14	0.48	4.5	26.9	5.41	14.0
RWD1	416.5	2.95	1.22	0.56	11.2	46.5	5.64	24.7
YWD2	62.7	1.00	1.15	0.48	29.6	17.7	5.57	7.2
CWD2	257.8	3.47	1.6	0.76	70.1	6.7	4.63	22.6
RWD2	243.1	2.22	1.67	0.72	5.6	42.1	4.68	17.2
YWD3	165.5	2.03	1.41	0.55	5.2	22.9	4.14	4.7
CWD3	252.0	2.44	1.29	0.61	2.3	20.2	4.08	10.3
RWD3	183.0	2.18	1.26	0.63	4.9	30.9	4.08	16.0
YWM1	232.3	1.47	1.21	0.44	8.4	39.1	4.67	3.4
CWM1	243.5	2.00	1.31	0.48	1.3	8.9	5.26	14.5
RWM1	160.5	1.65	1.32	0.49	1.6	8.7	4.73	14.9
YWM2	151.7	1.12	1.08	0.60	1.0	7.5	4.77	8.5
CWM2	188.9	1.17	1.02	0.67	1.9	7.2	4.87	15.6
RWM2	247.0	2.18	1.34	0.88	2.6	6.3	4.48	23.5
YWM3	171.7	1.71	0.92	0.70	3.5	9.0	4.26	5.8
CWM3	129.6	1.72	1.11	0.63	3.9	9.2	4.47	11.5
RWM3	185.4	1.60	1.15	0.57	3.3	8.6	4.83	7.9
YWV1	140.6	1.46	1.15	0.30	n.d.	4.5	4.49	n.d.
CWV1	175.8	1.25	0.86	0.47	n.d.	4.8	4.71	4.1
RWV1	94.1	1.34	1.14	0.43	2.7	5.5	4.57	5.3
YWV2	232.0	1.45	1.23	0.68	2.0	9.7	4.60	12.9
CWV2	228.0	2.14	1.48	0.64	0.7	9.7	4.70	8.8
RWV2	203.5	1.96	1.35	0.72	n.d.	7.3	4.60	8.9
YWV3	165.8	1.82	0.92	0.39	2.1	11.4	4.61	8.1
CWV3	241.5	2.05	0.94	0.51	0.5	14.4	4.68	6.1
RWV3	294.2	1.92	1.19	0.45	n.d.	8.3	4.12	10.5

Y: young wine; C: *cru* wine; R: *reservé* wine; W: winery; R: *appelation contrôleé* Rioja; D: Ribera del Duero; M: Vinos de Madrid; V: Valdepeñas; 1: first winery; 2: second winery; 3: third winery.

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, ethyl carbamate shows a retention time of 21.3 ± 0.2 min. and propyl carbamate one of 24.7 ± 0.2 min.

Interface temperature: 280 °C.

2.4.3. Detection and quantification conditions

SIM acquisition of 62, 74, 89 m/z ions. Quantification was performed in terms of the 62 ion and was based on an internal standard procedure. Using the working solutions of ethyl carbamate (EC) and propyl carbamate (PC) (10 μg/mL) and by diluting to 10 mL, five standards with dichloromethane were prepared: (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 showing a good linear response.

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

3. Statistical software packages

The Stat-itcf statistical software package (*Institut Technique des Céréales et des Fourrages*, 8 Avenue du Président Wilson, 75116 Paris, France) was used to perform principal components analysis (PCA). 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.

4. Results

Table 1 shows the ethyl carbamate concentrations in the tested wines (figures represent the mean of triplicate sampling); values range from 25 μg/L to non-detectable levels, with 3–4 μg/L as the minimum values registered. Statistical treatment and PCA (Fig. 1) of the data matrix considering only those variables of greatest interest (Table 2) showed the closest relationships between ethyl carbamate and ethyl lactate and volatile acidity levels. The greater correlations between these variables can be seen in the correlation matrix generated by the Stat-itcf programme (Fig. 1). The highest correlation coefficients were 0.560 and 0.588, between ethyl carbamate and ethyl lactate and volatile acidity, respectively. Although in absolute terms these values are not particularly large, in relative terms both become more significant (2.38 and 2.50, respectively). This can be seen in the correlation circle generated by the Stat-itcf programme (Fig. 2).

These results suggest possible connections between the presence of ethyl carbamate and microbiological processes that increase volatile acidity and the concentrations of esters (such as malolactic fermentation), and/or certain spoilage phenomena that produce lactic acid from residual sugars. Since the wines did not appear to have the symptoms of the latter, and taking into account that mean lactic and malic acid levels were 1.98 and 0.1 g/L, respectively, it can be deduced that the evident malolactic fermentation in these wines could increase volatile acidity as well as the possibility of an esterification reaction occurring between ethanol and lactic acid. The metabolism of arginine via the arginine-deaminase pathway (ADI) (Arena et al., 1999; Granchi et al., 1998; Liu, Pritchard, Hardman, & Pilone, 1995; Mira de Orduña et al., 2001; Mira de Orduña et al., 2000) (an alternative for the bacterial generation of ATP) could form citrulline and small quantities of carbamyl phosphate, two compounds which, if they reacted in time with ethanol, would yield ethyl carbamate.

The ethyl carbamate levels resulting from the slow reaction between urea (a nitrogen metabolite of yeast) and ethanol during aging should logically be included with those produced by malolactic fermentation. The notably higher ethyl carbamate content of most of the *cru* and *reservé* wines compared to the young wines supports this (Table 2): older wines are more likely to have generated significant levels of ethyl carbamate (Kodama et al., 1994; Stevens & Ough, 1993). Multiple range test analysis of the sorted data for each subsample of young, *cru* and *reservé* wines shows the oldest to have the highest ethyl carbamate concentrations (Fig. 3). Significant differences in ethyl carbamate levels were seen between *cru* and *reservé* wines and young wines.

Young wines have a tendency towards lower concentrations of ethyl carbamate since the only precursor source appears to be the urea which comes from arginine metabolism. This is made by several yeast strains during alcoholic fermentation. In the short time that elapses between bottling and consumption, there is no real chance for ethanolysis to occur. In contrast, *cru* and *reservé* wines have more chance of generating ethyl

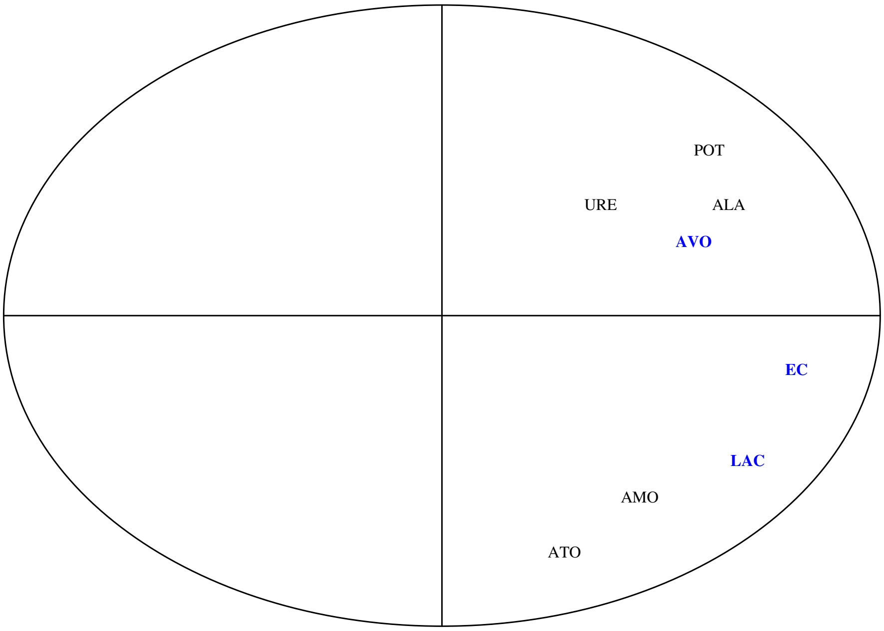
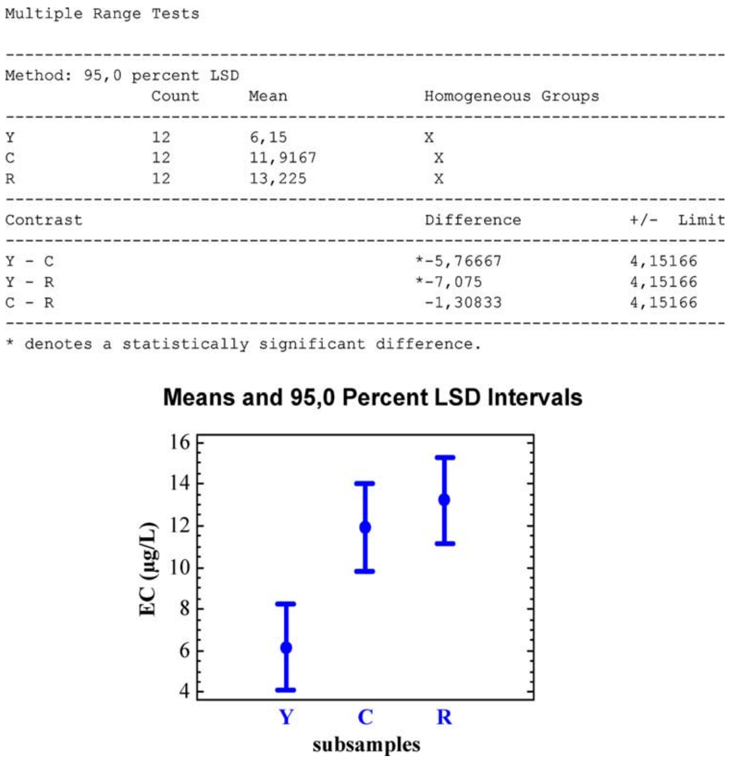


Fig. 2. Ethyl carbamate in Spanish red wines. Mapping of the eight variables explained in Fig. 1.



```
Multiple Range Tests

--------------------------------------------------------------------------
Method: 95,0 percent LSD
               Count     Mean              Homogeneous Groups
--------------------------------------------------------------------------
Y              12        6,15              X
C              12        11,9167            X
R              12        13,225             X
--------------------------------------------------------------------------
Contrast                                   Difference          +/-  Limit
--------------------------------------------------------------------------
Y - C                                     *-5,76667            4,15166
Y - R                                     *-7,075              4,15166
C - R                                      -1,30833            4,15166
--------------------------------------------------------------------------
* denotes a statistically significant difference.
```

Fig. 3. Multiple range test for comparison of wine subsamples Ethyl carbamate. Ref.: Y: young wine; C: *cru* wine; R: *reservé* wine; EC: ethyl carbamate.

carbamate since precursors such as urea and certain carbamyl compounds (nitrogen metabolites of malolactic fermentation e.g., citrulline and carbamyl phosphate) may all be present (Arena et al., 1999; Kodama et al., 1994; Mira de Orduña et al., 2001; Mira de Orduña et al., 2000; Ough, Crowell, & Gutlove, 1988b; Tegmo-Larsson et al., 1989). The ADI pathway hypothesis is only valid if arginine-degrading lactic acid bacteria take part in malolactic fermentation, and this depends not only on the strains present but also on conditions such as sugar content (Liu et al., 1994; Liu et al., 1995; Mira de Orduña et al., 2001; Mira de Orduña et al., 2000). The longer time over which reactions proceed, plus a wider variety of precursors, would be a combination sure to render higher concentrations of ethyl carbamate in the final wine.

5. Conclusions

In the studied wines, ethyl carbamate levels ranging from 0 to 25 µg/L were found. These correlated best with ethyl lactate and volatile acidity, suggesting that higher ethyl carbamate concentrations must be derived partly from urea and partly from carbamyl compounds produced by heterofermentative and other bacteria during malolactic fermentation (Figs. 1 and 2). These carbamyl compounds, along with urea, are potential precursors of ethyl carbamate since they react with ethanol in the medium as shown by Ough et al. (1988b). The ethyl carbamate levels found in the *cru* and *reservé* wines support this hypothesis. As ethyl carbamate in aged wine comes mostly from urea, time must play an important role, whether malolactic fermentation takes place or not. This justifies the higher levels of the compound in *cru* and *reservé* wines (Fig. 3).

Undoubtedly a larger sample size is needed to strengthen these conclusions. However, this survey of typical Spanish red wines is orientative with regard to screening for ethyl carbamate since samples came from the best known winemaking areas of the country. In all cases, ethyl carbamate levels were below the target limit of 30 ppb established by the Canada authorities, but not all are in line with the US industry's voluntary limit of 15 ppb.

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