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Analytical Methods

Improved sample preparation for GC–MS–SIM analysis of ethyl carbamate in wine

Ian C.C. Nóbrega [a,*], Giuliano E. Pereira [b], Marileide Silva [c], Elainy V.S. Pereira [a], Marcelo M. Medeiros [c], Danuza L. Telles [c], Eden C. Albuquerque Jr. [c], Juliane B. Oliveira [b], Dirk W. Lachenmeier [d]

[a] Universidade Federal Rural de Pernambuco, Departamento de Tecnologia Rural, CEP 52.171-900, Recife, PE, Brazil
[b] Embrapa Uva e Vinho/Semiárido, BR 428, km 152, CEP 56.302-970, Petrolina, PE, Brazil
[c] Laboratório de Agrotóxicos e Contaminantes em Alimentos e Bebidas Alcoólicas (LabTox), Instituto de Tecnologia de Pernambuco (ITEP), CEP 50.740-540, Recife, PE, Brazil
[d] Chemisches und Veterinäruntersuchungsamt (CVUA) Karlsruhe, Weissenburger Strasse 3, 76187 Karlsruhe, Germany

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ABSTRACT

An improved sample preparation procedure for analysis of carcinogenic ethyl carbamate (EC) in wine by GC–MS–SIM is proposed. Differences over AOAC reference procedure were: (1) use of EC-d_5 as internal standard instead of less similar propyl carbamate; (2) extraction by diethyl ether instead of more toxic dichloromethane, and (3) concentration by vacuum automated parallel evaporation instead of more time and work consuming rotary evaporation. Mean recovery was 104.4%, intraday precision was 6.7% (3.4 μg L^{-1}) and 1.7% (88.5 μg L^{-1}), regression coefficient was 0.999 in the linear working range of 3–89 μg L^{-1}, and limits of detection and quantification were 0.4 and 1.2 μg L^{-1}. Applicability was demonstrated by analysis (in triplicate) of 5 wine samples. EC concentration ranged from 5.2 ± 0.2 to 29.4 ± 1.5 μg L^{-1}. The analytical method is selective, accurate, repeatable, linear, and has similar method performance as the reference method along with the several mentioned advantages.

1. Introduction

Ethyl carbamate (EC, $C_2H_5OCONH_2$), a multi-site carcinogen in experimental animals and probably carcinogenic to humans (IARC group 2A), occurs in many fermented foods, in particular alcoholic beverages, where it is thought to be formed from the reaction between ethanol and nitrogen-containing compounds (EFSA, 2007; Lachenmeier et al., 2010). With respect to wine, urea and citrulline – derived mainly from the yeast and lactic acid bacteria metabolisms of arginine – are considered important nitrogen-containing precursors; the rate of EC formation in wine increases with temperature and storage time (Butzke & Bisson, 1997; Monteiro, Trousdale, & Bisson, 1989; Uthurry, Suarez Lepe, Lombardero, & Garcia del Hierro, 2006).

According to data of the European Food Safety Authority, a median of 5 μg L^{-1} and a P95 (95th percentile of values) equal to 78 μg L^{-1} were found in 23,278 wine samples from EU Member States. There are currently no harmonized maximum EC levels for table wine in the EU, but Canada and USA recommend maximum values of 30 μg L^{-1} and 15 μg L^{-1}, respectively (EFSA, 2007).

The standard method for EC determination in wine is the AOAC method 994.07 (Canas, Burns, Joe, & Diachenko, 1994), also adopted by OIV (method MA-AS315-04; OIV, 2013a), and as reference method in the European Union (Commission Regulation, 1999). The AOAC method involves analysis by GC–MS–SIM (gas chromatography coupled to mass spectrometry in selected ion monitoring) after the following sample preparation procedures: (1) addition of propyl carbamate as internal standard; (2) cleanup through diatomaceous earth columns; (3) EC extraction by dichloromethane, and (4) eluate concentration using vacuum rotary evaporation. This technique has been used by several authors (Masqué et al., 2011; Romero, Reguant, Bordons, & Masqué, 2009; Uthurry et al., 2004, 2006) for EC analysis in table wine in recent years. Limiting steps in the standard sample preparation procedures are: (1) use of considerable amounts of a chlorinated toxic solvent (dichloromethane) for extraction; (2) use of intensive labor effort and prolonged time during the concentration step, and (3) use of an internal standard with a lower degree of similarity to control extraction and chromatographic responses. To overcome the solvent limitation, some alternative wine preparation procedures have been proposed, such as the use of solid-phase microextraction (SPME) with a carbowax/divinylbenzene (CW/DVB) fiber (Whiton & Zoecklein, 2002) or solid-phase extraction (SPE) with minimal use of solvents (Jagerdeo, Dugar, Foster, & Schenck,

* Corresponding author. Tel.: +55 8133206280; fax: +55 8133206260.
E-mail address: ian@dtr.ufrpe.br (I.C.C. Nóbrega).

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2002). Although these alternative preparations have advantages over the standard procedure, they have not been extensively adopted for EC analysis in wine and are not without problems. For instance, the CW/DVB fiber is no longer commercially available (Liu, Xu, & Zhao, 2012). Furthermore, the alcohol part in the sample may influence the SPME extraction yield (Lachenmeier, Nerlich, & Kuballa, 2006) and the method proposed by Jagerdeo et al. (2002) involves a previous time-consuming step for ethanol removal from wine by vacuum.

From a conventional perspective of analysis (AOAC method 994.07), this paper introduces and validates the time and work efficient use of a vacuum automated parallel evaporator for EC analysis in table wine, which allows for the simultaneous evaporation of various wine eluates to a specified volume. Other changes in the AOAC method were carried out, such as the use of the more similar deuterated ethyl carbamate (EC-d_5) as internal standard (which was not commercially available at the time when the AOAC procedure was developed) and the less toxic diethyl ether (instead of dichloromethane) as extraction solvent, which have been suggested in some previous studies (Fauhl & Wittkowski, 1992; Huang et al., 2013).

2. Materials and methods

2.1. Wine samples

Five different bottled, recorded, commercial table wines (W01–W05) were collected in triplicate (same batch code) at Brazilian wineries in May 2012. Three wines (W01, W02, and W03) were representative of two large wineries located in the wine producing region of São Francisco Valley, Northeast Brazil; two wines (W04 and W05) were representative of one large winery located in the wine producing region of Campanha Gaúcha, South Brazil. According to label information, the wine varieties/vintages were the following: Chenin blanc/2010 (W01), Syrah/2010 (W02), Syrah/2008 (W03), Merlot/2010 (W04), and Merlot/2010 reserve (W05). Once collected, the bottles were stored horizontally in a wine cellar at 18 ± 1 °C until analysis.

2.2. Physicochemical characterization of wine samples

The following parameters (principles of methods are given in brackets) were determined (in duplicate analysis) as described by OIV (2013b): total acidity (potentiometric titration using sodium hydroxide), volatile acidity (steam distillation and titration with sodium hydroxide), pH (potentiometry), alcoholic strength by volume at 20 °C (steam distillation followed by measurement using a hydrostatic balance), density (densimetry using a hydrostatic balance), total dry extract (calculated indirectly from the specific gravity of the alcohol-free wine), free sulfur dioxide (direct titration with iodine), total sulphur dioxide (free sulphur dioxide + iodometric titration after alkaline hydrolysis), and reducing sugars (wine clarification by lead acetate; determination by iodometric titration after reducing action on an alkaline copper salt solution). Polyphenols, as total polyphenol index (TPI), were estimated by spectrophotometry at 280 nm (Harbertson & Spayd, 2006). Equipments used in the analyses included: oenochemical electronic distilling unit, model Super DEE, attached to steam distillation unit, model VADE 3 (Gibertini Elettronica SRL, Milano, Italy); hydrostatic balance, model Super Alcomat (Gibertini Elettronica SRL, Milano, Italy); and a spectrophotometer SP 220 (Biospectro Ltda, Curitiba, Brazil).

2.3. Ethyl carbamate analysis in wine

2.3.1. Chemicals and materials

Extrelut NT 20 columns (they contain a mixture of diatom resin and NaCl), Uvasol *n*-pentane (for spectrometry), and ethanol (absolute, pro analysi) were purchased from Merck (Darmstadt, Germany). Diethyl ether (for spectrometry) from Vetec Química/Sigma–Aldrich (Duque de Caxias, Brazil), ethyl carbamate (98.5%) from Dr Ehrenstorfer (Augsburg, Germany), and ethyl-d_5 carbamate (99%; isotopic purity 98 atom % D) from Sigma–Aldrich (St. Louis, USA). Ultra-pure water (Milli-Q system) was used throughout to prepare solutions.

2.3.2. Standards and solutions

For ethyl carbamate (EC) stock solution, 2.8 mg were placed into a 500 mL volumetric flask, diluted to volume in ethanol (abs.) and stored at −20 °C protected from light. For deuterated ethyl carbamate (EC-d_5, internal standard) stock solution, 5.1 mg were place into a 250 mL volumetric flask, diluted to volume in ethanol (abs.) and stored at −20 °C protected from light. For calibration solutions, 15 μL, 35 μL, 75 μL, 155 μL and 395 μL of the EC stock solution were given into five 25 mL volumetric flasks which were then filled to volume using a freshly prepared 13% vol. ethanol solution (simulating a table wine matrix) and stored at 3 ± 1 °C. For matrix effect assessment, the ethanol solution was replaced by wine sample W04 (the lowest in terms of EC concentration found in a previous round of analyses). Final EC concentrations in each calibration standards were 3.4 μg L^{-1}, 7.8 μg L^{-1}, 16.8 μg L^{-1}, 34.7 μg L^{-1} and 88.5 μg L^{-1} which cover the concentration range most likely to be found in table wines (EFSA, 2007). All calibration solutions were treated similar to wine samples prior to measurement (i.e. calibration solutions were extracted and concentrated in the same way as the wine samples).

2.3.3. Extraction

Extraction and concentration procedures were adapted from Lachenmeier et al. (2009). Each calibration solution or wine sample (25 mL) were spiked with 40 μL of internal standard stock solution (final concentration of EC-d_5 equal to 32.6 μg L^{-1}) and directly applied to the Extrelut column. After 15 min of equilibration, the column was washed with 2 × 20 mL of *n*-pentane (aiming at reducing non-polar interferences of the wine matrix). The washing was discarded. Next, the analytes were extracted using 4 × 30 mL diethyl ether and the eluate collected in a 250 mL glass bottle (Schott Duran, Germany) and closed with screw PTFE-lined cap. The bottle was then left at −20 °C for 48 h for the removal of residual moisture. It may be of interest to note that the elution flows of *n*-pentane and diethyl ether were increased considerably by manually applying an over-pressure on top of column with a small hand rubber bellow.

2.3.4. Eluate concentration using a vacuum automated parallel evaporator

Parallel evaporation was performed using a Syncore Analyst with a 6 position rack attached to a vacuum pump/controller V-700/V-855, and recirculating chiller F-108 (Büchi Labortechnik AG, Flawil/Switzerland). Six sample glass vessels with working volumes of 25–250 mL were used. The Syncore Analyst was equipped with a flushback module that condensed the vapor at the top of the vessels, gently rinsing the glass walls. The sample vessels had 3 mL appendices at the bottom, cooled during evaporation, to facilitate the collection of the defined volume and to avoid evaporation to dryness (Fig. 1).

The ethereal eluates at −20 °C were filled into the Analyst sample vessels, leaving behind (attached to the glass bottles) residual moisture as ice. The heating temperature was set at 40 °C and

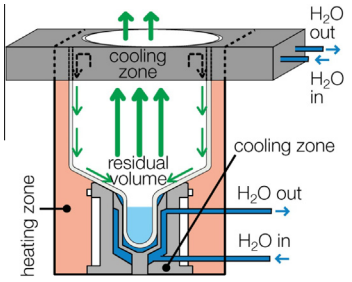

Fig. 1. Principle of the automated evaporative concentration to a pre-defined residual volume. The small appendix at the bottom is locally cooled, which stops the evaporation process as the solvent level reaches the top of the cooling zone, and prevents thermal decomposition. The cooling zone at the top facilitates a constant rinse of the glass wall preventing a loss of analytes (Lachenmeier et al., 2009).

cooling temperature at 5 °C. The evaporation was then started using the vacuum pump/controller at a constant pressure of 300 mbar and a horizontal orbital movement (sample rack) at 180 rpm throughout. Evaporation time was 30 min, after which the appendix residual volume (3 mL) was achieved. Approximately 1.5 mL of the residual volume was then transferred to 2 mL crimp glass vials which were sealed with a PTFE/rubber septa cap (Agilent Technologies, USA) and immediately submitted to GC–MS–SIM analysis.

2.3.5. GC–MS–SIM

A Thermo AS 3000 autosampler was used to introduce in splitless mode 1 μL aliquots of the eluates onto a silica capillary column (Carbowax 20 M, 60 m × 0.32 mm × 1 μm film thickness; Ohio Valley Specialty Co., Marietta, Ohio) installed in a Thermo Trace GC Ultra gas chromatograph (GC) coupled to a Thermo ISQ mass spectrometer (MS), using software Xcalibur. Helium at 1.5 mL min^{-1} was used as carrier gas. The GC oven was initially kept at 90 °C (2 min), followed by an increase to 150 °C at 10 °C min^{-1}, then up to 230 °C at 40 °C min^{-1}. The injector temperature and the MS transfer line were kept at 240 °C. The MS was operated in selected ion monitoring (SIM) mode with electron impact ionization (70 eV). The ions monitored were m/z 62 for EC and m/z 64 for EC-d_5 (internal standard). The calibration curves were constructed following injection of the calibration standard solutions (in both ethanol at 13% and wine sample W04) into the GC–MS–SIM instrument and plotting the peak height ratios of the analyte (m/z 62) to the internal standard (m/z 64) on the y-axis against the concentration of EC (μg L^{-1}) on the x-axis. For the calibration curve using sample W04 as matrix, values of the peak height ratios of the analyte to the internal standard on the y-axis were deduced from the mean value obtained in the non-spiked sample (i.e. sample W04 not containing spiked ethyl carbamate).

2.4. Validation studies

Validation studies were based on Ribani, Bottoli, Collins, Jardim, and Melo (2004) and guidelines for validation of Brazil's National Institute of Metrology (INMETRO, 2007) and Australia's National Association of Testing Authorities (NATA, 2012).

Repeatability in this study refers to multiple measurements of the same sample on the same day (intraday).

Initial studies were carried out to investigate a possible matrix effect, thus calibration solutions of the analyte were prepared in two different matrices: a simple solution of ethanol at 13% vol. (simulating a wine matrix) and wine (sample W04). The effect was assessed by comparing the slopes of both curves (NATA, 2012) as well as by application of F-test (homogeneity of variances) and Student's t test (comparison of two means) (INMETRO, 2007).

For the calibration curve, seven intraday replicate analyses were conducted at each concentration (3.4–88.5 μg L^{-1}, $n = 5$) in each matrix type. The mathematical relationship between the response (y) and the EC concentration (x) was expressed by the linear equation (and its regression coefficient, r) which for a linear model is $y = ax + b$, where a = slope of best line fit and b = y-intercept of best line. The limits of detection (LOD) and quantification (LOQ) were calculated by the ratio of the standard deviation of linear coefficient to the slope of the calibration curve and the result multiplied by 3.3 and 10, respectively (Ribani et al., 2004).

Determinations of standard deviation of repeatability (S_r), precision intraday (expressed in terms of relative S_r, %) and accuracy (expressed in terms of recovery, %) were based on seven intraday replicate analyses in which EC was added to the wine matrix (sample W04) at the lowest and highest concentrations in the calibration range (3.4 and 88.5 μg L^{-1}, respectively).

EC analyses in the sampled wines (W01–W05) were performed in three intraday replicates of the analytical procedure (i.e. each wine sample generated three ethereal extracts which were immediately analyzed by GC–MS–SIM).

2.5. Stability tests

Stability tests of ethereal wine eluates from selected wine samples (W01, W02, and W03) were also performed. Approximately 1.5 mL of residual volume in the appendix of glass vessel (Fig. 1) were transferred to 2 mL clear glass vials which were sealed with a PTFE/rubber septa crimp cap (Agilent Technologies, USA) and stored at −20 °C. After 30 days of storage, a new 5 points calibration curve was prepared and the stored wine eluates submitted to GC–MS–SIM analysis. The data of the stored and fresh eluates were evaluated using the software Statistica® 10.0 (StatSoft Inc., 2010), considering (p) ⩽ 5%. The variance among the means was investigated by ANOVA test. Welch's Robustness test was used for data without homogeneity between samples. The means were compared by Tukey's multiple comparisons and the values of the standard deviation and relative standard deviation at 95% confidence interval were obtained. Pearson's test was used to investigate correlations between the stored and fresh eluates.

3. Results and discussion

3.1. Physicochemical characterization of wine samples

Table 1 shows selected physicochemical characteristics of the five wine samples (W01–W05) from Brazil used in this study as well as the corresponding limits established by Brazilian regulations for table wine. All samples complied with Brazilian regulations for alcoholic strength, reducing sugars, total acidity, volatile acidity, total SO_2 and the alcohol/extract ratio.

Results of alcoholic content, sugars, total acidity and pH values were in accordance with expected values (average or range) for table wines in general (IARC, 2010). Although our sampled wines presented higher volatile acidities than maximum level (0.3 g L^{-1}) expected for normal wine (IARC, 2010), the results are still below the acceptable limit (1.2 g/L) according to OIV (2013c). The samples were also compliant with OIV (2013c) acceptable limits for total SO_2 (maximum of 0.15 g L^{-1} and 0.20 g L^{-1} for dry red and dry white wines, respectively).

3.2. Chromatograms, retention times and blank extractions

Typical chromatograms obtained in SIM mode of an authentic wine sample are presented in Fig. 2. Under the chromatographic conditions used in this study, distinct peaks for EC (m/z 62) and EC-d5 (m/z 64, internal standard) appeared. As expected, the

Table 1
Selected physicochemical results[a] (alcoholic strength, reducing sugars, dry extract, total acidity, volatile acidity, total SO_2, pH, alcohol/extract ratio, and total polyphenol index) of sampled wines (W01–W05) from Brazil.

Sample[b]	Alcoholic strength (% v/v at 20 °C)	g L^{-1}					pH	Alcohol/extract ratio[e]	Total poly-phenol index
		Reducing sugars	Dry extract	Total acidity[c]	Volatile acidity[d]	Total SO_2			
W01	13.3	2.25	–[f]	5.85	0.56	0.11	3.4	–[g]	6.8
W02	13.6	2.25	30.0	6.45	0.98	0.11	3.5	3.59	68.6
W03	12.5	1.65	100.4	5.70	0.94	0.05	3.9	0.98	83.1
W04	11.9	1.25	26.2	4.95	0.69	0.07	3.5	3.58	42.9
W05	12.5	2.75	31.6	5.70	0.76	0.08	3.5	3.12	62.0
Limits[h]	8.6–14.0	0.0–4.0[i]	–[j]	3.0–9.75	0.0–7.2	0.0–0.35	–[j]	0.0–4.8[k]	–[j]

[a] Results are the mean of duplicate analysis and the CV never exceeded 1.1%.
[b] For sample information (wine variety, vintage, etc), see Section 2.1.
[c] Total acidity expressed as tartaric acid.
[d] Volatile acidity expressed as acetic acid.
[e] Ratio of alcohol by weight to reduced extract.
[f] Level of dry extract in white wine sample W01 was not quantifiable.
[g] Alcohol/extract ratio not calculated because dry extract data was not available.
[h] Limits (min–max) established by Brazilian regulations for table wine (Lei 10.970, 2004; Portaria 259, 2010).
[i] Limits (reducing sugar) established for dry wines.
[j] Limits not established for the parameter.
[k] Limits (alcohol/extract ratio) established for red wine (for white wine the limit is 0.0–6.5).

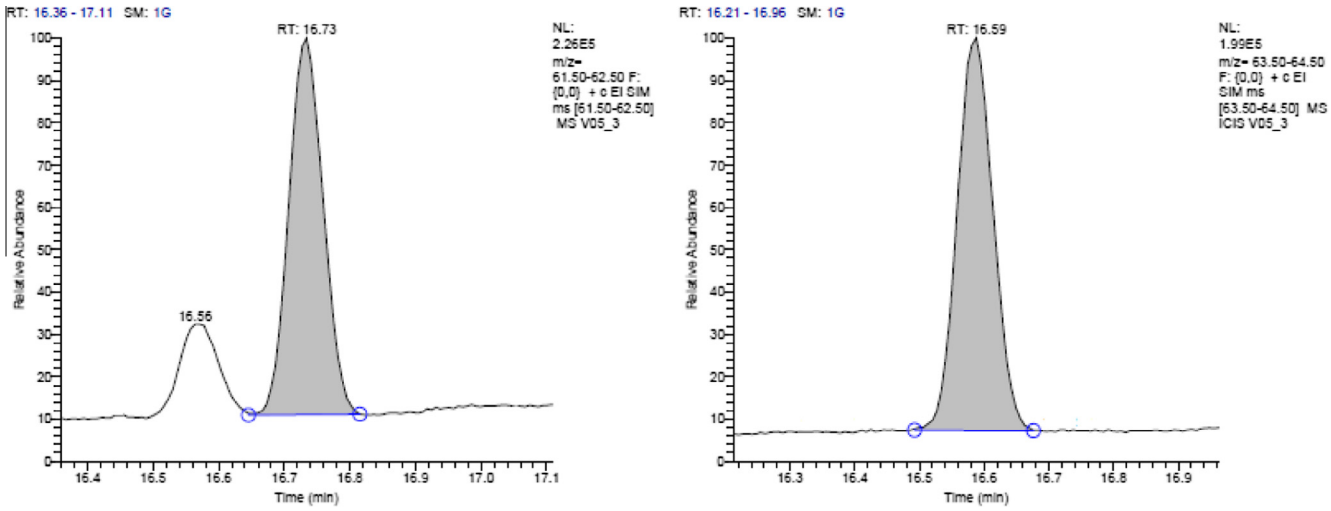

Fig. 2. Sections of GC–MS–SIM chromatograms of an authentic wine sample, showing the monitoring of ions *m*/*z* 62 (left chromatogram, retention time of EC = 16.7 min) and *m*/*z* 64 (right chromatogram, retention time of EC-d_5 = 16.6 min).

retention time of EC-d_5 (16.6 min) was close to the one of EC (16.7 min) and, in this respect, EC-d_5 has a more adequate response than the internal standard (propyl carbamate) used in the AOAC procedure (approximately 5 min separated from EC) (Canas et al., 1994; OIV, 2013a). The ions *m*/*z* 62 and *m*/*z* 64 were used for quantification because they are characteristic for the carbamate structure, have the highest abundance and were found to be non-interfered by matrix (Jagerdeo et al., 2002; Mirzoian & Mabud, 2006). No nearby interferences from blank extractions (ethanol 13% vol. or wine samples) were observed in our investigations as well.

3.3. Matrix effect and limits of detection and quantification

Taking into account our sample preparation procedure (i.e. sample cleanup through Extrelut columns followed by pentane washings), it was considered that the wine matrix would probably have no significant effect on the results. To confirm this hypothesis, calibration solutions were tested in two different matrices: ethanol 13% vol. and red wine (sample W04). Sample W04 was chosen as wine matrix because it contained, among sampled wines, the lowest level of ethyl carbamate (mean of 5.2 μg L^{-1}) in previous rounds of EC analyses. For the construction of the calibration curve using sample W04 as matrix, values of the peak height ratios of EC to the internal standard (EC-d_5) were subtracted from corresponding ratio values obtained from the sample without EC addition (i.e. sample W04 containing internal standard only).

Table 2 presents results for both matrices. The assays were linear in the required concentration range between 3.4 and 88.5 μg L^{-1} with a regression coefficient of 0.9998 (ethanol 13% vol.) and 0.9993 (wine sample W04). Although the limit of quantification (LOQ) in the wine matrix (1.9 μg L^{-1}) was higher than in ethanol 13% vol. (1.2 μg L^{-1}), it is still well below maximum EC limits established or recommended by Canada (30 μg L^{-1}) and the USA (15 μg L^{-1}) for table wines (EFSA, 2007). The LOD and LOQ are generally below values obtained in other works using similar or different procedures (Canas et al., 1994; Mirzoian & Mabud, 2006; Whiton & Zoecklein, 2002).

The difference between the slopes of calibration curves in ethanol 13% vol. and in wine (0.0327 and 0.0330; Table 2) was 1%, thus below the value of 10% above which matrix effect would need to be compensated (NATA, 2012). To confirm the absence of matrix effect, a *F*-test (homogeneity of variances) was performed first. Since the calculated *F* was less than the critical *F* value (Table 2),

Table 2
Selected results of matrix study (ethanol 13% vol. *vs* red wine) and the application of *F* and *t* tests.

Parameter	Matrix	
	Ethanol 13% vol.	Red wine (sample W04)
Range	3.4–88.5 μg L^{-1}	3.4–88.5 μg L^{-1}
Calibration curve[a]	$y = 0.0327x + 0.0289$	$y = 0.0330x + 0.0136$
Regression coefficient	0.9997	0.9993
Standard error of regression	0.0165	0.0273
Standard deviation of linear coefficient	0.0039	0.0064
Slope	0.0327	0.0330
Limit of detection (LOD)[b]	0.3920	0.6423
Limit of quantification (LOQ)[b]	1.1880	1.9461
Mean of response[c]	1.0129	1.0177
Variance of response[c]	1.0844	1.0611
Calculated value of *F*	1.0218	
Critical value of *F*[d]	1.7721	
Calculated value of *t*	0.0194	
Critical value of *t*[e]	1.9954	

[a] Calibration curve given as $y = ax + b$, where y = response [peak height ratio of the analyte (m/z 62) to the internal standard (m/z 64)], x = analyte concentration, a = slope, and b = y-intercept.
[b] LOQ and LOD were calculated by the ratio of the standard deviation of linear coefficient to the slope and the result multiplied by 3.3 and 10, respectively (Ribani et al., 2004).
[c] Mean and variance obtained from 35 observations (responses) in each matrix.
[d] Critical value of *F* obtained with 34 degrees of freedom (DF) in both the numerator and denominator, using a one-tailed 95% confidence interval.
[e] Critical value of *t* obtained from the *t*-distribution table for 68 DF, using a two-tailed 95% confidence interval.

the matrix has no important effect on the method's precision. The standard deviations of series were then grouped and the means tested with a *t*-distribution. As the calculated *t* value was less than the critical *t* value (Table 2), it is concluded that the matrix has no significant effect (INMETRO, 2007).

3.4. Precision and recovery

Table 3 presents precision (repeatability) and recovery (accuracy) intraday results (7 replicates) for two spiked concentrations of ethyl carbamate (3.4 and 88.5 μg L^{-1}) in a wine matrix (sample W04). Taking into account that sample W04 contains a previously known concentration of EC, values of the peak height ratios of the analyte to the internal standard (y-axis) were deduced from the mean ratio value obtained in the non spiked sample.

The average intraday precision of our procedure (4.2%; Table 3) was lower than results obtained by Canas et al. (1994) in the standard AOAC methodology (average 4.9%; added EC levels of 0, 15, and 40 μg L^{-1}) and, thus, entirely satisfactory. However, our average result was similar to the precision intraday reported by Mirzoian and Mabud (2006) using an optimized SPE/GC–MS–SIM procedure proposed by Jagerdeo et al. (2002), which is characterized by minimal use of solvents and previous removal of wine's ethanol by vacuum.

Our intraday recovery results (average of 104.4%; Table 3) were also satisfactory. It was higher than the average value obtained in the standard AOAC procedure (93%; for added EC levels of 15 and 40 μg L^{-1}) and similar to the average recovery (104.4%; for added EC levels in the range of 50–500 μg L^{-1}) obtained by Mirzoian and Mabud (2006).

3.5. Ethyl carbamate concentration in wines and stability tests

Table 4 present the concentrations of ethyl carbamate found in fresh (W01–W05) and stored wine eluates (stability tests).

Each wine sample was extracted and concentrated simultaneously in triplicate, resulting in three ethereal eluates (R_1, R_2, and R_3; Table 4), which were analyzed by GC–MS–SIM just after their preparation (fresh wine eluates) or after 30 days of storage at −20 °C (stored wine eluates). The average coefficient of variation (CV) in the EC results was 4.8% and never exceeded 9.2%, which is entirely acceptable in terms of repeatability of the procedure.

With regard to EC levels in the five samples of Brazilian wines, values (mean ± standard deviation) ranged from 5.2 ± 0.2 to 29.4 ± 1.5 μg L^{-1} (overall mean of 14.4 μg L^{-1}). Due to our limited sample collective, it is not possible to present an adequate assessment of the EC situation in Brazilian wines. However, all sampled wines were compliant to the maximum level established by Canada (30 μg L^{-1}), but one of them (W03) has twice as much EC as

Table 3
Results[a] of precision and recovery intraday assays.

EC added (μg L^{-1})	Mean EC found[a] (μg L^{-1})	Standard deviation of repeatability (S_r)	Relative S_r (precision intraday, %)	Recovery intraday (%)
3.4	3.6	0.2	6.7	105.9
88.5	91.2	1.6	1.7	103.0

[a] Results were obtained from seven intraday replicate GC–SIM–MS analyses of each added level to a red wine matrix (sample W04).

Table 4
Concentrations of ethyl carbamate in three replicates (R_1, R_2, and R_3) of fresh and stored ethereal wine eluates.

Wine sample	Ethyl carbamate (μg L^{-1})[a]					
	Fresh wine eluate[b]			Stored wine eluate (stability tests)[c]		
	R_1	R_2	R_3	R_1	R_2	R_3
W01	11.5	13.5	11.6	14.3	15.2	12.7
W02	14.3	14.6	14.1	15.5	15.7	15.3
W03	27.9	29.4	30.8	28.3	29.0	30.9
W04	5.1	5.4	5.0	–[d]	–[d]	–[d]
W05	10.5	11.3	10.7	–[d]	–[d]	–[d]

[a] Ethyl carbamate concentrations were obtained from three replicates (R_1, R_2 and R_3) of the procedure (i.e. each wine sample generated three ethereal eluates); average CV of replicates was 4.8% and never exceeded 9.2%.
[b] Freshly obtained eluates (i.e. eluates just concentrated by the vacuum automated parallel evaporator) were immediately analyzed by GC–SIM–MS using a specially prepared 5 points calibration curve in ethanol 13% vol. ($y = 0.0341x + 0.0427$, $r^2 = 0.9998$, linear range 3.4–88.5 μg L^{-1}).
[c] Stored eluates were analyzed by GC–MS–SIM after 30 days of storage at −20 °C, using a freshly prepared 5 points calibration curve in ethanol 13% vol. ($y = 0.0334x + 0.0258$, $r^2 = 0.9993$, linear range 3.4–88.5 μg L^{-1}).
[d] Eluates from samples W04 and W05 were not submitted to stability tests.

the limit recommended by the USA (15 μg L^{-1}) for table wines (EFSA, 2007). A deeper assessment of EC levels in Brazilian wines was reported by Francisquetti, Vanderlinde, Carrau, and Moyna (2002), in which 124 wine samples produced in the South of Brazil (85 samples were *Vitis vinifera* wines) were investigated by the standard AOAC/OIV procedure; values ranged from 1.0 to 70.0 μg L^{-1} and the overall mean concentration was 10.0 μg L^{-1}.

Little differences in the EC concentrations of fresh and stored eluates were observed (Table 4), suggesting EC stability in the ethereal eluates for 30 days at −20 °C. To confirm this observation, data from both groups (fresh and stored) were evaluated as described previously (Section 2.5). The mean EC content of fresh eluates was 18.6 μg L^{-1} and the one of stored eluates was 19.7 μg L^{-1}. ANOVA results show that there was no significant difference between the two groups of eluates ($p = 0.880$).

4. Conclusions

Results have shown that the proposed analytical method is selective, accurate, repeatable and linear over the EC concentration range (3.4–88.5 μg L^{-1}). Major advantages over the standard AOAC/OIV procedure include: (1) reduced work effort and time due to the automated evaporation; (2) use of a less toxic extraction solvent, and (3) use of an internal standard (EC-d_5) with a higher degree of similarity to control extraction and chromatographic responses. Besides theses advantages, EC concentrations were shown to be stable in the ethereal wine eluates when stored for 30 days at −20 °C, providing greater time flexibility for the GC–MS–SIM analyses. On the other hand, the proposed procedure may be further improved by trying to reduce the amount of solvents (*n*-pentane and diethyl ether) and the storage time at −20 °C (required to remove residual moisture as ice from ethereal eluates).

Our sample collective was too small to draw conclusions about the situation of EC in Brazilian table wines. However, considering the good performance and validation results obtained in the proposed procedure, another study is currently underway to expand the EC assessment to table wines produced in São Francisco Valley, Northeast Brazil.

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