

High Precision Method for the Determination of Ethyl Carbamate in Cachaça by HRGC-MS

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ABSTRACT

HRGC-MS-SIM was used for determination of ethyl carbamate in commercial *cachaça* by a simple, fast and reliable technique for direct analysis, without the previous stage of extraction of the analyte of interest. The quantification technique used was the internal standardization with ethyl carbamate-d5. The following validation parameters were evaluated: Limit of Detection, Limit of Quantification, Linearity, Repeatability, Reproducibility, Accuracy and Uncertainty. The contributions that influenced the uncertainty in a great extent came from the repeatability and the accuracy. The characterization of the uncertainty sources and the ethyl carbamate determination, allowed Inmetro to certify its Certified Reference Material (CRM) for contaminants in *cachaça*.

1. INTRODUCTION

Cachaça is the typical denomination for the sugarcane beverage (spirit) produced in Brazil with alcohol strength between 38-48 mL/100mL, at 20 °C, obtained by the distillation of fermented sugarcane juice, being possible to add up to 6 g/L of sugar, expressed as sucrose [1].

The annual volume of Brazilian *cachaça* production is near 1,300 million liters. From this, 300 millions liters are produced in artisan way in copper distillers (alambiques), and 1 billion liters are industrialized. Near 1 % of this production is exported to countries such as Paraguay, Portugal, Italy, England, Chile, United States and Germany. The annual profit of the production sector is around US\$ 500 million [2].

The relevance of *cachaça* for Brazilian economy is related to its high export potential. *Cachaça* is the second fermented-distilled alcoholic beverage most consumed in Brazil and the third in the world [3], what makes the consumer market more exigent in the search of a final product of high quality as to its physicochemical and sensorial aspects. Ethyl carbamate or urethane, one of the substances usually present in *cachaça*, is classified as potentially carcinogenic (Group 2B) by IARC (International Agency for Research on Cancer) [4], and it is naturally formed during fermentation of foods such as yogurt, bread, beer,

wine, sake and mainly in fermented-distilled beverages such as whiskey, rum, vodka, grappa, tiquira (cassava spirit) and *cachaça* [5-9]. The presence of this substance in distilled beverages at concentration levels high than 125 ng/mL is considered dangerous for consumption by the Canadian public health authorities. Besides this public health aspect, this substance constitutes a technical barrier to export *cachaça* to Europe and the United States, where law does not allow concentrations above 150 ng/mL. This way, developing analytical methods to identify and quantify different compounds presented in *cachaça* is extremely important to monitor the constituents of *cachaça* as well as its contaminants, frequently present as traces, thus resulting an adequate product for human consumption. In relation to the external market, the trading relations between countries are demanding more rigorous quality standards and therefore to develop new methodologies to differentiate the quality of the *cachaça* in a practical, reliable and objective way, is becoming more and more relevant [10,11,12]. The importance of metrology in the reliability and accuracy of analytical results is unquestionable for chemical measurements, which should be -traceable to the International System of Units (SI).

To overcome this challenge, Inmetro, the Brazilian National Institute of Metrology (Brazilian NMI), has an important role providing measurement

traceability and reliability. To reach this objective in the chemical sector, the Chemical Metrology Division (Dquim) has the incumbency to develop reference procedures techniques (i.e., Isotope Dilution), to develop and certify reference materials, to implement measurement primary methods and to organize proficiency tests.

This work is also part of a certification process of a reference material for *cachaça*, where ethyl carbamate is quantified through internal standardization with ethyl carbamate-d5. This Certified Reference Material (CRM) will be a tool to ensure the traceability to the SI and a useful tool for laboratories of analyses of distilled beverages, as analysis control or as calibration standard, being essential for those laboratories that are certified by Inmetro on the NBR/ISO IEC 17025 standard [13]. This norm requires that the analytical laboratories use certified reference materials in their analyses and, when a CRM is used in an analytical essay, its uncertainty must be considered to compose the measurement uncertainty of the whole process.

2. EXPERIMENTAL

2.1. Materials and Methods

The following solvents and standards were used in this study: Ethyl carbamate (Sigma-Aldrich, Steinheim, Germany, 99 %), propyl carbamate (Sigma-Aldrich, Steinheim, Germany, 98 %), deuterated ethyl carbamate (ethyl-d5, Cambridge Isotope Laboratory, Andore, USA, 98 %), ethanol Lichrosolv (Merck, Darmstadt, Germany, 99.9 %). Ultra pure water with conductivity under 0.056 mScm-1, was purified through a Milli-Q system (Millipore Inc., Paris, France, Type I).

The analyses were carried out on a gas chromatograph (GC) coupled to a mass spectrometer (MS), Shimadzu, model GCMS-QP2010 (Tokyo, Japan). Each point of the calibration curve resulted from triplicate injections in the splitless mode (1 μ L) in a capillary column HP-FFAP (50 m length x 0.2 mm de internal diameter x 0.33 mm film thickness) at flow rate of carrier gas (He White Martins, RJ, Brazil, 5.0) of 1.2 mL/min and injector at 250 °C. The temperature was programmed as follows: initial temperature of 90 °C (1 min), then heated to 150 °C at a rate of 10 °C/min and to 230 °C at a rate of 30 °C/min. The ionic source and interface temperatures were maintained at 220 °C,

operating in the electron impact ionization mode (EI), ionization energy at 70 eV.

The purity determination of Ethyl Carbamate was made by Differential Scanning Calorimetry (DSC) in the *Divisão de Metrologia de Materiais*.

2.2. Stocks Solutions

2.2.1. Ethyl Carbamate

In a 4 mL glass flask, previously tared, weight 0.23 mg ethyl carbamate and the actual mass weighed is recorded. In another 1 L glass flask, previously tare discounted, weight 800 g of the hydro-alcoholic solution (40:60, v/v, ethanol: water), and the actual mass weighed is recorded. Next, carefully insert the 4 mL flask inside the flask containing the hydro-alcoholic solution and mix the final solution during 15 seconds.

2.2.2. Deuterated Ethyl Carbamate

In a 4 mL glass flask, previously tare discounted, weight 0.16 mg deuterated ethyl carbamate and the actual mass weighed is recorded. In another 1 L glass flask, previously tared, weight 800 g hydro-alcoholic solution, and the actual mass weighed is recorded. Next, insert carefully the 4 mL flask inside the flask containing the hydro-alcoholic solution and mix the final solution during 15 seconds.

2.3. Preparation of the Calibration Curve

Eight points were used to obtain the calibration curves, in the following concentration range: 50 ng/g, 75 ng/g, 100 ng/g, 125 ng/g, 150 ng/g, 175 ng/g, 200 ng/g and 225 ng/g. The calibration curves were established from the gravimetric dilution of the ethyl carbamate stock solution in hydro-alcoholic solution. In a previously tare discounted 10 mL glass flask, weight the desired amount of ethyl carbamate stock solution and record the weighted value. Next, add hydro-alcoholic solution up to the relative mass to the desired concentration and record the final mass. Mix in a vortex (Phoenix) during 30 seconds. Repeat the procedure for each point of the calibration curve. Calculate the final concentration for each point.

The internal standard (IS), deuterated ethyl carbamate, was added to the curve points weighting 1.50 g IS solution to 1.50 g point solution of the calibration curve. Mix in vortex (Phoenix) during 30 seconds and transfer 1.00 mL to a 1.5 mL vial flask.

2.4. Quality Controls (QC) Sample Preparation

In a previously tare discounted 10 mL glass flask, weight the desired amount of ethyl carbamate stock solution and record the weighted value. Next, add hydro-alcoholic solution up to the relative mass to the desired concentration (65 ng/g, 137 ng/g and 210 ng/g, Low, Medium and High, respectively) and record the final mass.

2.5. Validation

The following validation parameters were evaluated: Limit of Detection (LOD), Limit of Quantification (LOQ), Linearity, Repeatability, Reproducibility, Accuracy and Uncertainty.

2.5.1. Limit of Detection and Quantification

These parameters were evaluated using the signal to noise ratio (S/N). The ethyl carbamate solution was gravimetrically diluted, obtaining a LOD when the S/N ≥ 3 and a LOQ when the S/N ≥ 10 .

2.5.2. Linearity

Linearity was determined using the linear regression analysis ($y = a \cdot x + b$), correlation coefficient (r) and analysis of variance (ANOVA), allowing to estimate the quality of the curve.

2.5.3. Repeatability and Reproducibility

Repeatability and reproducibility were evaluated from the calculation of the relative standard deviation (RSD) of the quality control. This value was obtained by analysis of variance (ANOVA) from each result of three analysts in three different days (nine replicates). The variance error was associated to repeatability and the sum of errors and analyst variances were associated to reproducibility. The standard deviation of repeatability (s_r) and reproducibility (s_R) were calculated by expected mean squares (EMS) for the terms in the ANOVA model. The limit of repeatability (r) and the limit of reproducibility (R) were calculated from Eq. (1),

$$r = R = t_{(95\%,df)} * \sqrt{2} * s_x, \quad (1)$$

where s_x is the standard deviation of x ($x = r$ or R).

2.5.4. Accuracy

The accuracy of the method was evaluated through the analysis of quality controls (QC) by their normalized error (Eq. (2)),

$$N_{error} = \frac{(X_{lab} - X_{true})}{\sqrt{U_{lab}^2 + U_{true}^2}}, \quad (2)$$

where X_{lab} is the measured value; X_{true} is the certified value; U_{lab} is the expanded uncertainty of measured value; and U_{true} is the expanded uncertainty or certified value.

2.5.5. Measurement Uncertainty

The uncertainty determination for ethyl carbamate analysis in commercial *cachaça* was evaluated based on the Guide to the Expression of Uncertainty in Measurement and on the EURACHEM/CITAC [14, 15].

Since the uncertainty that we want to determine is analytical, the measurand is the concentration of ethyl carbamate, in other terms [analyte], and the equation that defines this value is:

$$[analyte] = \left(\frac{A_{analyte}}{A_{IS}} - b \right) * \frac{p * m_{IS}}{a * m_{sample}}, \quad (3)$$

where $A_{analyte}$ is the area of analyte; A_{IS} is the area of internal standard; b is the linear coefficient of regression curve; a is the slope of regression curve; p is the purity; m_{IS} is the mass of internal standard; and m_{sample} is the mass of sample.

Besides the uncertainties deriving from Eq. (3) we also have the accuracy as an uncertainty source.

The purity of the internal standard, different from the purity of the analyte standard, is not a source of uncertainty in this analysis because, independently of its value, the internal standard is added to the calibration curve points and to the sample in the same concentration. Both being divided by the same factor will cancel their contribution to the uncertainty of the analysis process.

We can express the combined standard uncertainty as:

$$u_c = \sqrt{(u_{Aratio})^2 + (u_{cc})^2 + (u_{sm})^2 + (u_{ism})^2 + (u_p)^2 + (u_{acc})^2}, \quad (4)$$

where u_{Aratio} is the area ratio; u_{cc} is the calibration curve uncertainty; u_{sm} is the sample mass uncertainty; u_{ism} is the internal standard mass uncertainty; u_p is the analyte purity uncertainty; and u_{acc} is the accuracy uncertainty.

In this work, the derivative concept is employed to uniform the input quantity units related to the measurand unit, in this case the accuracy. By applying the classical method, the uncertainty unit of each of the input quantities, turns to be expressed in ng/g, which is the measurand unit.

3. RESULTS

3.1. Analytical Method

The association of high resolution gas chromatography coupled to mass spectrometry offers high sensitivity and selectivity when used together with selective ion monitoring (SIM), consequently, detection becomes still more sensitive by increasing the signal/noise ratio (S/N), once it is capable to select only the ions of interest among others present in the matrix.

Ethyl carbamate was analyzed directly in the matrix, with no previous preparation of the sample. Therefore without usual steps such as extraction, clean up and concentration of analyte of interest.

Initially ethyl carbamate was analyzed by linear scan (SCAN) with HRGC-MS to obtain and confirm the mass spectrum of the analyte of interest.

Considering that ethyl carbamate is present in low concentrations in the matrix, the use of the selective ion monitoring technique increases the detection capacity of the compound in trace levels, in a complex matrix as *cachaça*, where other organic substances are presented.

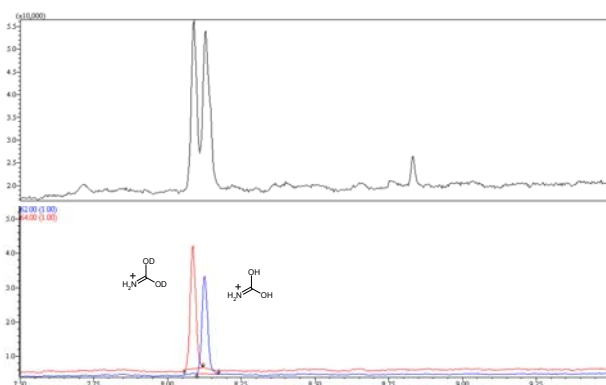


Fig. 1. Total ion chromatogram (top) and selective ion monitoring (down).

Selected ions for the analyte of interest identification and confirmation were m/z 44, m/z 74 and m/z 62, the latter was used in the

quantification. The m/z = 74 ion corresponds to methyl [M-CH3]⁺ loss and the base peak m/z = 62 derives from McLafferty rearrangement, which ion is formed and stabilized by resonance.

In relation to the internal standardization of this work, the ions used in the analyte and internal standard quantification, employing selective ion monitoring (SIM), were m/z = 62 (ethyl carbamate) and m/z = 64 (deuterated ethyl carbamate), respectively. Fig. 1 shows the SIM obtained through the chromatogram of the Medium QC.

3.2. Validation

In Table 1, were summarized the validation's parameters, where can be applied, the acceptance criteria of these ones were in agreement with the European Union Directive [16].

Table 1. Validations parameters.

Parameter	Value	Criteria	Status
LOD	1 ng/g	-	-
LOQ	3 ng/g	150 ng/g ¹	ok
Linearity	0.9985	>0.9900	ok
Repeatability ²	7.3 %	14.2 %	ok
Reproducibility ^{2,3}	7.3 %	21.3 %	ok
Accuracy	0.03	<1 ⁴	ok
Uncertainty ²	16 ng/g ⁵	-	-

- 1 – Normative Instruction n°13, of the Agriculture, Breeding and Supply Ministry [1].
- 2 – For the medium quality control.
- 3 –Intermediate Precision.
- 4 – Normalized error.
- 5 – U for 95 % confidence level, and k=2.

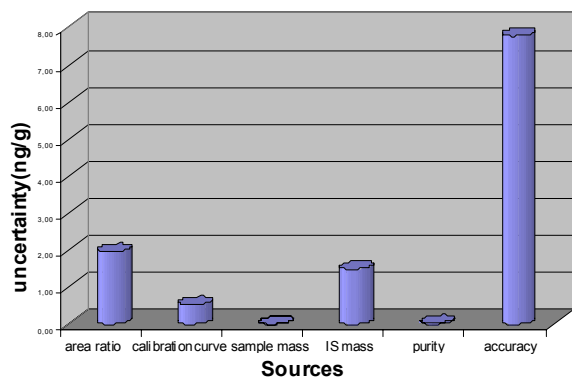


Fig. 2. Uncertainty sources for ethyl carbamate in *cachaça*.

For a typical analysis, the value obtained for a medium quality control was (136±16) ng/g ((137±12) ng/g for a certified value), for one relative expanded uncertainty about 12 % (95 %,)

k=2). The contributions of each source, for the overall uncertainty, are reported in Fig. 2.

4. DISCUSSION

In the linearity determination, besides the correlation coefficient (r) presented in the Table 1, the results of ANOVA regression also indicates a linear adjustment (F_{tab} = 2.74; F_{calc} = 1.70; average of 3 experiments) and a high correlation between the two variables x and y (F_{tab} = 4.3; F_{calc} = 8620; average of 3 experiments). The low value of accuracy leads to a small difference between the true value (137 ng/g) and the experimental value (136 ng/g).

Because of the statistical model used in ANOVA calculation it was found identical values of repeatability and reproducibility (Intermediate Precision) parameters. The first approach was with a one-way model, where the operators were sources of variance. The replicates were not considered as sources of variance thus they were not true replicates. The result of this calculation is presented in the Table 2.

Table 2. One-way ANOVA for operator – model 1.

	DF	SS	MS	F	p-value
Constant	1	1.7e05	1.7e05	1.7e04	< 1e-08
Operator	2	13.539	6.7693	0.70105	0.53258
error	6	57.936	9.656		

Table 3 shows the components of variance of the result in the Table 2.

Table 3. Components of variance for model 1.

	Estimate	SE ¹	DF
Operator	-0.9622	2.9231	0.2167
error	9.656	5.5749	6

1 – Standard error

According to Table 3, it is observed that the estimated variance is negative for the operator. Negatives values are not common, but in some cases it could happen. According to Montgomery [17] negatives values could be associated with a null variance. In this case this is reasonable because standard error embody zero. Thus, adding the operator in the total error model and recalculating ANOVA, results are presented in Table 4.

In this case, the component of variance of repeatability is equal to the variance of reproducibility (Intermediate Precision) = 8.9343 (ng/g)². In accordance with this value and with the Eq. (1) the result of this parameter is 7.3 %.

Table 4. One-way ANOVA – model 2.

	DF	SS	MS	F	p-value
Constant	1	1.7e05	1.7e05	1.9e04	< 1e-08
error	8	71.474	8.9343		

Generally speaking, all the validation parameters analyzed showed adequate purpose.

From the results showed in Fig. 2, we can conclude that the contributions that influence the uncertainty in a greater extent come from the accuracy and from the process repeatability. Those values are reasonable because the target concentration is relativity low, for this technique.

Among the uncertainties of the input quantities, that deriving from the gravimetric primary method presented the smallest values of all uncertainties, except the one that comes from IS mass. This result is in accordance with what is expected from this technique, since its only uncertainty source is the balance resolution.

Differently to other uncertainties deriving from gravimetric method, IS mass show a high value due the low mass weighted (0.16 mg). This mass is close to the balance precision and the expression (Eq. 5) that calculate this uncertainty must consider the solvent mass and mass of the internal standard added to the sample uncertainties. Thus, propagation of these uncertainties sources on the uncertainty of IS mass increases this value. This IS mass does not impact significantly in the analysis result, unfortunately, this must be considered, since it comes from the equation that defines the measurement,

$$u_{ISm} = \sqrt{(u_{ISweighed})^2 + (u_{Solventmass})^2 + (u_{ISadded})^2} \tag{5}$$

where $u_{ISweighed}$ is the mass of IS in stock solution; $u_{Solventmass}$ is the mass of solvent in stock solution; $u_{ISadded}$ is the mass of stock solution added into sample; and u_{ISm} is the internal standard mass uncertainty.

Since the construction of the calibration curve reflects a ratio analyte/IS and since the deuterated standard has almost the same retention time and physic-chemical properties than the analyte ethyl carbamate, this uncertainty is propagated through all the curve construction points, reflecting on a smaller uncertainty value for an uncertainty calibration curve when a isotope internal standard is used.

The value of the uncertainty of the area ratio, that may be considered small in relation to uncertainty of accuracy (less than 20 %), denotes a good repeatability of the chromatograph used in this work, once this uncertainty is associated to the standard deviations of the analyses.

5. CONCLUSIONS

HRGC-MS-SIM is a simple, fast and reliable technique for direct analysis of ethyl carbamate in commercial *cachaça*, without the previous stage of extraction of the analyte of interest, using as internal standard deuterated ethyl carbamate.

The determination of analyte standard purity, allowed the traceability to the international system of units (SI).

This process could also be used by other countries in the certification of their typical distilled beverages (for ex. Brandy, Bourbon, Whiskey, Vodka, Tequila, Pisco, Kirsh, Grappa, Marc, Cognac, Calvados, Sake, etc.) [18]. After matrix extension, it could also be applied to food prone to this contamination.

The characterization of the uncertainty sources, the validation procedure and the ethyl carbamate determination in *cachaça* by HRGC-MS/SIM using the isotope dilution technique, allowed Inmetro to certify its Certified Reference Material (CRM) for contaminants in *cachaça*; making a Certified Reference Material with a reduced measurement uncertainty.

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REFERENCES

- [1] Instrução Normativa nº13, de 29 de junho de 2005 <<http://www.agricultura.gov.br/>>, April 2007.
- [2] ABRARE <<http://www.abrabe.org.br/>>, April 2007.
- [3] National Institute of Metrology, Standardization and Industrial Quality - INMETRO: <<http://www.inmetro.com.br/>>, August 2005.
- [4] International Agency for Research on Cancer, IARC Monographs on the Evaluation of Carcinogenic Risks to Humans 1974; 7:111.
- [5] B. Zimmerli and J. Schlatter, "Analytical Methodology Occurrence, Formation, Biological-Activity and Risk Assessment" *Mutat. Res.*, **259** (1991) 325-350.
- [6] R. Battaglia, *et al*, Ethyl Carbamate (Urethane) in Alcoholic Beverages and Foods – A review, *Food Addit. Contam.*, **7**, 1990, 477-496.
- [7] C.S. Ough, Ethylcarbamate in fermented beverages and foods. 1. Naturally occurring ethylcarbamate, *J. Agr. Food Chem.*, **24**, 1976, 323-328.
- [8] H.B.S. Conacher, *et al*, Column Gas-Chromatographic Determination of Ethyl Carbamate in Alcoholic Beverages With Confirmation by Gas-Chromatography Mass-Spectrometry, *J. AOAC INT.*, **70**, 1987, 749-751.
- [9] L.A.F. Nagato, *et al*, Quantitation of ethyl carbamate (EC) by gas chromatography and mass spectrometric detection in distilled spirits, *Alimentaria*, **311**, 2000, 31-36.
- [10] B.P.Y. Lau, *et al*, Gas-chromatographic mass-spectrometric determination of Ethyl Carbamate in alcoholic beverages, *J. Chromatogr.*, **402**, 1987, 233-241.
- [11] W.M. Pierce, *et al*, Determination of Ethyl Carbamate in distilled alcoholic beverages by gas-chromatography with flame ionization or mass-spectrometric detection, *J. AOAC INT.*, **71**, 1988, 781-784.
- [12] L.G. Andrade-Sobrinho, *et al*, Carbamato de etila em bebidas alcoólicas (cachaça, tiquira, uísque e grapa), *Quim. Nova*, **25**, 2002, 1074-1077.
- [13] ISO/IEC 17025:2005. General requirements for the competence of calibration and testing laboratories. ISO - International Organization for Standardization, Switzerland:Geneva, 2005.
- [14] ISO GUM. Guide to the expression of uncertainty of measurement. ISO - International

- Organization for Standardization, Switzerland: Geneva, 1993.
- [15] EURACHEM/CITAC Guide CG4. Quantifying Uncertainty in Analytical Measurement. EURACHEM/CITAC, London, 2a edition, 2000.
- [16] Commission Decision 2002/657/EC, *OJ L*, **221**, 2002.
- [17] D.C. Montgomery, *Design and Analysis of Experiments*, 6th edition, John Wiley & Sons, 2005.
- [18] D.W. Lachenmeier, *et al*, Automated determination of ethyl carbamate in stone-fruit spirits using, headspace solid-phase microextraction and gas chromatography-tandem mass spectrometry, *J. Chromatogr. A*, **1108**, 2006, 116-120.