



VALIDATION AND UNCERTAINTY MEASUREMENT OF HIGHER ALCOHOLS DETERMINATION IN CACHAÇA BY GAS CHROMATOGRAPHY

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Abstract: This paper presents the implementation of a procedure for higher alcohols determination in sugar cane spirit. Method validation was carried out determining the performance parameters linearity, matrix effects, accuracy, limit of detection (LOD), limit of quantification (LOQ), and uncertainty which obtained values were acceptable.

Keywords: In house validation, measurement uncertainty, sugar cane spirit, higher alcohols.

1. INTRODUCTION

Sugar cane spirit, known as *cachaça*, is a typical and exclusive type of distilled beverage with peculiar sensory characteristics from Brazil. It is the third most consumed alcoholic beverage in the world. While *cachaça* holds a small share in total Brazilian export, it has been gaining increasing space on it.

Nowadays, the consumer demand for safe food is imperative and to maintain the position in a strongly competitive global market, the product must be considered to be reliable for consumers.

Dedicated efforts have been made to improve the quality of *cachaça*. As part of our research efforts, the Brazilian legislation states that the amounts of higher alcohols in *cachaça*, expressed in the form of propyl alcohol, isobutyl alcohol and isoamyl and amyl alcohols must be within the limit of 300 mg·100 mL⁻¹ [1]. The formation of excess higher alcohols is directly related to asepsis during the manufacturing process and they are reported as potential toxic agents.

Thus, it is essential that laboratories provide criteria to show that the test methods for their determination lead to reliable and appropriate results. There is a need of validated methods and with an estimated uncertainty of measurement calculated.

The purpose of this paper is to present a detailed procedure for in-house method validation, to evaluate the uncertainty sources for a classic method for detection higher alcohols in *cachaça* by gas chromatography.

2. MATERIALS AND METHOD

2.1 Materials and Reagents

Reagents propyl alcohol, isobutyl alcohol, isoamyl alcohol and amyl alcohol were all from Sigma-Aldrich brand with chromatographic grade. The chromatographic analyses were carried out in Autosystem XL Perkin Elmer Gas Chromatograph, equipped with a flame ionization detector and a capillary column (30 m - 0.25 mm - 0.25 µm) with polyethylene glycol as stationary phase.

2.2. Method procedure

The method 14/05 of the Brazilian agriculture government department (MAPA) was adapted for use with external standardization [2]. The determination of higher alcohols in *cachaça* is effected by direct injection of samples in gas chromatography with flame ionization detector. The concentration of each component is determined based on standards analytical curves performed in conditions identical to those followed in the chromatographic analysis of the sample.

2.3 Method validation

2.3.1 Linearity

Three analytical curves were prepared in different days, with three independent replicates at the levels 4; 10; 25; 37; 44; 59 mg·100 mL⁻¹ for propyl alcohol - 0.3; 0.9; 2; 3; 4; 5 mg·100 mL⁻¹ for amyl alcohol - 14; 35; 88; 133; 159; 213 mg·100 mL⁻¹ for isoamyl alcohol - 4; 10; 37; 44; 59 mg·100 mL⁻¹ for isobutyl alcohol. Blanks were also prepared, in triplicate, for each curve [3].

After an exploratory fit by the ordinary least squares method (OLSM), the regression residuals were examined for obvious research profile, with the outliers indicated by points outside the range $\pm t_{(1-\alpha/2; n-2)} S_{res}$. The outliers were treated by Jackknife test of standardized residuals, which was applied sequentially until they were no longer detected or even an exclusion of up to 22.2% in the original data.

Residual normality was tested using Ryan-Joiner [4], for independence, it was used Durbin-Watson [5], homoscedasticity and adjustment to the linear model were checked using Levene test [6].

2.3.2 Matrix effects

The standard addition method was used to check the matrix effects. All the tests for linearity assumption were also done for these curves. The slopes and interceptions were compared by t-test for α 0.05.

2.3.3 Accuracy, selectivity and limits

The method accuracy, selectivity and experimental limits of detection and quantification were established by spiked samples in four different days, with three independent replicates at the same levels of linearity test. Blank samples were also prepared, in triplicate, for each curve and day. Repeatability and within-reproducibility were estimated by ANOVA. LOD and LOQ were determined from analytical curve following methodology described by Peter C. Meyer and Richard E. Zünd in "Statistical Methods in Analytical Chemistry" [7]. The inaccuracy was evaluated in terms of mean recovery by the standard addition method.

2.3.4 Measurement uncertainty

Uncertainties were estimated according to the Guide to the Expression of Uncertainty in Measurement [8]. Relevant sources for the uncertainty assessment have been divided into four groups: a) the uncertainty associated with determining the alcohol content, b) preparation of standard solutions, c) the analytical curve, d) precision and uncertainty of the method. To calculate the expanded uncertainty (U), a coverage factor (k) of 2 was used which corresponds to a 95% confidence interval for a normal probability distribution.

3. RESULTS AND DISCUSSION

The residuals were plotted and are shown in Figure 1 with outliers. Outliers were removed before statistical tests. The assumptions that the errors are normally distributed, homoscedastic and independent were confirmed by test results shown in Table 1.

Ryan-Joiner correlation coefficients were 0.9835; 0.9438 and 0.9528 for propyl alcohol, amyl alcohol and isoamyl alcohol and isobutyl alcohol respectively, indicating no significant ($p > 0.05$) deviation from normality.

The assumption of homoscedasticity of the regression residuals was confirmed. The values of the Levene Statistic t_L 0.864, 0.949 and 0.495, with significance of 0.40, 0.36 and 0.63, were estimated to isoamyl alcohol and amyl alcohol, propyl alcohol and isobutyl alcohol, respectively, and were not significant as shown in Figure 2

Table 1. Normality, homoscedasticity and independency evaluation for higher alcohols.

Statistic	Isoamyl alcohol and amyl alcohol	Propyl alcohol	Isobutyl alcohol
n^1	14	14	14
R^2	0.948	0.9534	0.9528
t_L^3	0.864	0.949	0.495
P^4	0.40	0.361	0.630
d^5	2.300	2.319	2.212

¹Number of observations.

²Ryan-Joiner correlation coefficient for $p > 0.05$

^{3,4}Levene t-statistics

⁵Durbin-Watson statistic for $p > 0.10$

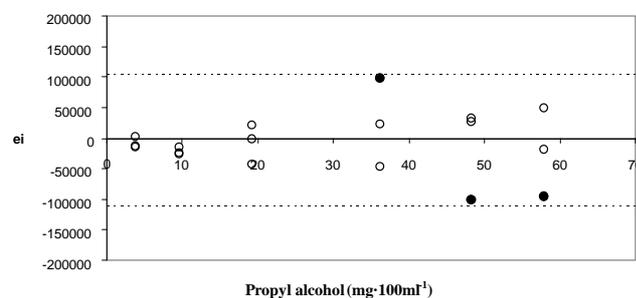
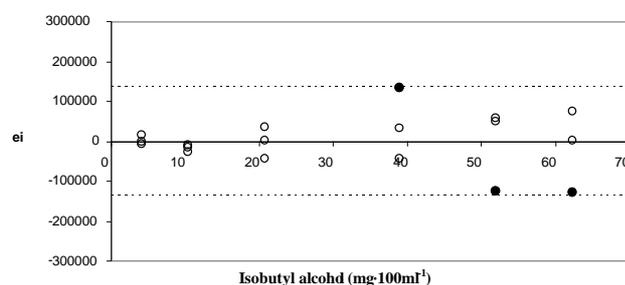
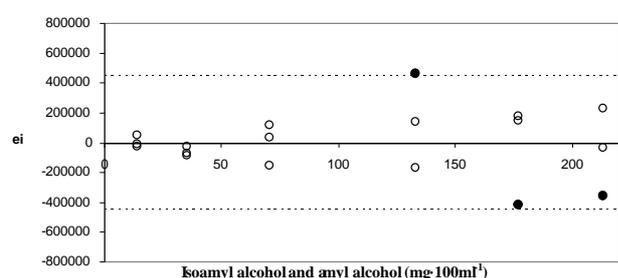


Figure 1. Residual plots for outlier diagnostic by Jackknife standardized residuals test in higher alcohols from *cachaça*. e_i = residual of regression, • = outliers

The independence of regression residuals was observed by the Durbin-Watson statistic. It was obtained values of 2.30 for isoamyl alcohol and amyl alcohol, 2.32

for propyl alcohol and 2.21 for isobutyl alcohol. The profile of homogeneous distribution of points in all four quadrants of the graphs of Durbin-Watson suggested that there was no positive or negative trend and no autocorrelation were showed in Figure 3.

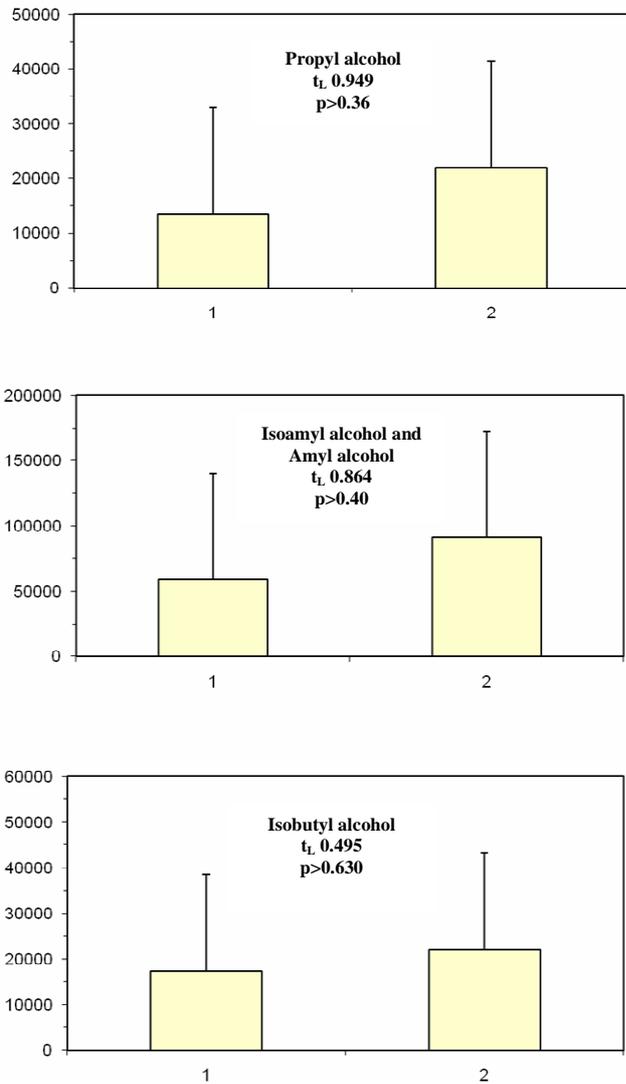


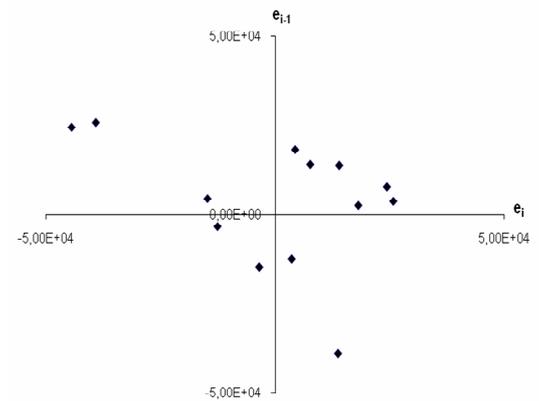
Figure 2 – Representation of the homogeneity of the variances of higher alcohols in the *cachaça* modified Levene test. t_L = t statistic Levene, p = significance

Significant regressions ($p < 0.001$) and no significant ($p > 0.05$) deviations from linearity were observed for the F tests shown in Table 2.

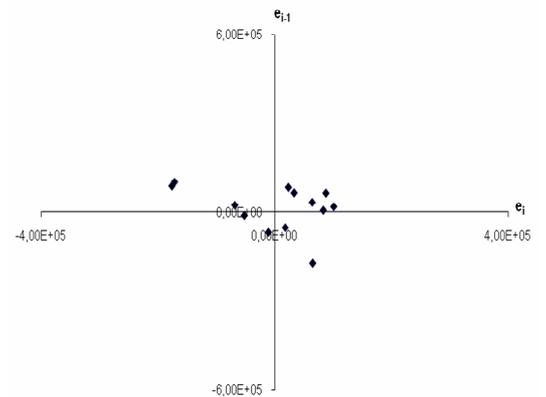
Table 2 - ANOVA statistics for regression including lack-of-fit test for the solvent of higher alcohols in the *cachaça*

Statistic	Isoamyl Alcohol and Amyl Alcohol	Propyl Alcohol	Isobutyl Alcohol
<i>Regression</i>			
F	$1.26 \cdot 10^{-3}$	$1.04 \cdot 10^{-3}$	$1.38 \cdot 10^{-3}$
p	$1.56 \cdot 10^{-13}$	$5.01 \cdot 10^{-13}$	$9.22 \cdot 10^{-14}$
<i>Lack-of-fit</i>			
F	0.60	0.49	0.64
p	$6.74 \cdot 10^{-1}$	$7.43 \cdot 10^{-1}$	$6.51 \cdot 10^{-1}$

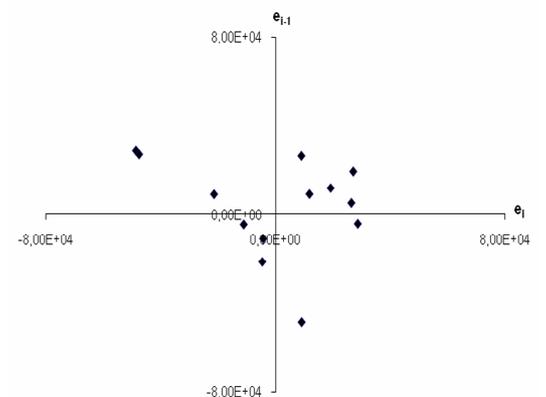
F: variance ratio, p : significance.



A- Plot of residuals autocorrelation for Propyl alcohol; $d = 2.319$; $p > 0.10$



B- Plot of residuals autocorrelation for Isoamyl alcohol and Amyl alcohol; $d = 2.300$; $p > 0.10$



C- Plot of residuals autocorrelation for Isobutyl alcohol; $d = 2.212$; $p > 0.10$

Figure 3 – Plot of residuals autocorrelation of higher alcohols in the *cachaça*. e_i = residual, d = Durbin- Watson statistic, P = significance.

Calibration curves with their equations and determination coefficients are shown in Figure 4.

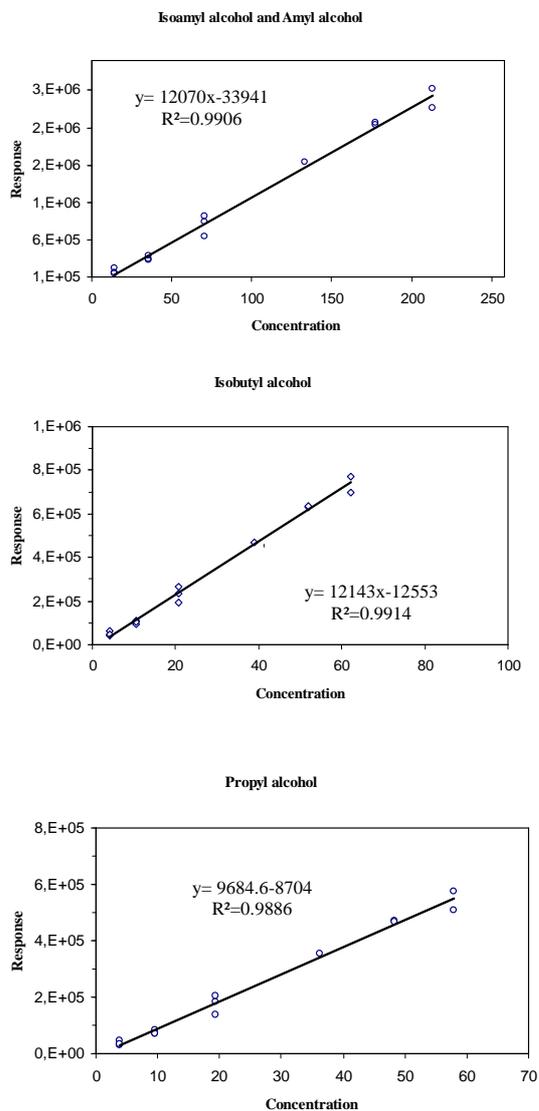


Figure 4 – Calibration curves of higher alcohols in testing and evaluating the linearity in the ranges previously tested, with their equations and correlation coefficients.

Since the variances of residuals from all pairs of curves were compared by the test considered homogenous F ($p > 0.05$), t-test combined with variances was used to compare the intercept and slope parameters in testing and evaluating the matrix effects. No matrix effects were detected, the intercepts were not significantly different from zero ($p > 0.05$), and no significant differences were observed between the intercepts of the solvent and matrix matched curves ($p > 0.05$), in both ranges.

No outliers were identified when the results of apparent recovery, obtained for each concentration level, were analyzed by Grubbs for $p > 0.05$. The mean recovery values ranged from 92.8% to 115.5%, 87.3% to 102.2% and 91.9% to 108.7% for amyl alcohol and isoamyl alcohol, propyl alcohol and isobutyl alcohol, respectively.

Normality and homoscedasticity of result residuals were observed by apparent recovery and Ryan-Joiner test and Levene modified, which allowed estimation of the relative standard deviations of repeatability and reproducibility by analysis of partial variance. Relative standard deviations of, 7.2% and 13.1% for amyl alcohol and isoamyl alcohol, 8.4% and 11.5% for propyl alcohol and 7.4% and 12.9% for isobutyl alcohol which are acceptable values for repeatability and within-reproducibility, respectively, were obtained. The LOD and LOQ estimated values were 8.8 - 16.70 $\text{mg} \cdot 100\text{mL}^{-1}$ for amyl alcohol and isoamyl alcohol, 2.69 - 5.25 $\text{mg} \cdot 100\text{mL}^{-1}$ for propyl alcohol and 0.86 - 1.49 $\text{mg} \cdot 100\text{mL}^{-1}$ for isobutyl alcohol.

The individual components of total uncertainty were calculated by the error propagation approach. Figure 5, shows diagram of cause and effect.

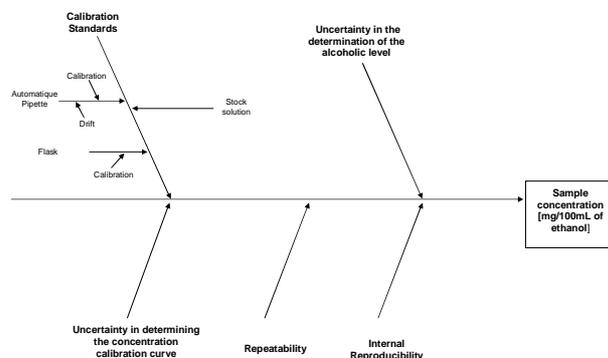


Figure 5 – Cause and effect diagram for the determination of higher alcohols in cachaca.

In Figure 6 it is shown the contribution for expanded uncertainty by each alcohol. The values of the standard uncertainty contribution from each source from all alcohol are shown in Figure 7. It can be seen that the uncertainties associated with the analytical curve are the main contribution in the method.

Although the uncertainty associated with the repeatability is much smaller than analytical curve uncertainty, it should be considered especially if there is a change of staff or equipment.

As example, for the analyzed sample, whose concentration determined was 157.24 $\text{mg} \cdot 100\text{mL}^{-1}$, the value of expanded uncertainty (U) for the determination of higher alcohols was 14.72 $\text{mg} \cdot 100\text{mL}^{-1}$.

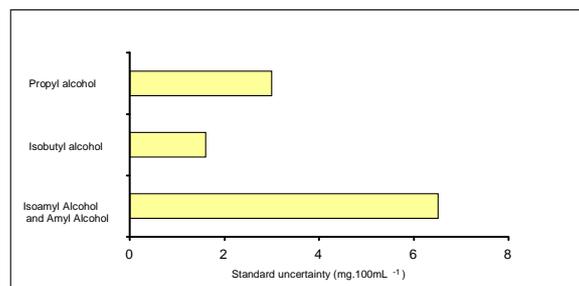


Figure 6 – Contribution of each alcohol in the expanded uncertainty.

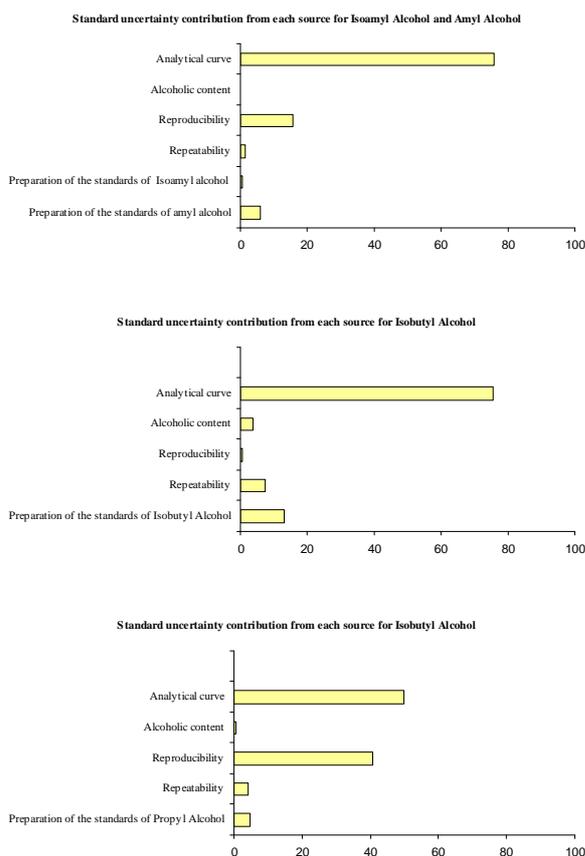


Figure 7 – Standard uncertainty contribution from each source for the determination of higher alcohols in *cachaça*.

3. CONCLUSIONS

The methodology showed no significant matrix effects. The method had low values of LOD and LOQ and good precision. Inaccuracy was not observed and the uncertainty was acceptable.

Although Normative Instruction No. 13 of MAPA [4] does not established acceptable values for the uncertainties, the expanded uncertainties calculated values showed satisfactory results for the methodology used. It was observed that the main source of uncertainty contribution is the analytical curve.

Almost all performance parameters of method were set out using statistical tools well established. Thus, the method described here is an important tool on quality assessment of *cachaça* and can be a reference for other laboratories which intend to implement this method.

ACKNOWLEDGEMENTS

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