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ORIGINAL ARTICLE

Validation of an analytical technique for the dosage of ethanol in biological fluids

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Abstract. The widespread consumption of alcoholic beverages in modern society has impelled forensic toxicology laboratories to improve on the already high standards of the analytical techniques routinely employed by optimizing how judicially relevant samples are managed, given that it is essential to expedite the delivery time of results. According to the World Health Organization (WHO): "*Total alcohol per capita consumption in the world's population over 15 years of age rose from 5.5 litres of pure alcohol in 2005 to 6.4 litres in 2010 and was still at the level of 6.4 litres in 2016".* To that effect, the dosage of ethanol in biological fluids allows judges, through retrospective calculations, to assess the level of impairment of an individual at the time of the event, which is fundamental for its elucidation. This study proposes the validation of an analytical method that makes it possible to obtain reliable results with a brief analysis.

Key words: *Validation; Mass spectrometry; Ethanol; Biological fluids; Quantification.*

Abbreviations: C_{et}: Ethanol concentration; C_{is}: Concentration of internal standard; A_{et}: Ethanol area; A_{is}: Internal standard area; mV.s: Millivolts per second; Run: Chromatography run; R^2: Coefficient of determination; b*: Absolute bias; R´%: Relative recovery percentage per fortification level; R.T.: Retention time; R.R.T.: Relative retention time; m/z: mass-to-charge ratio; S/N: signal-to-noise ratio; Mx: Slope of the line; B: Intercept; LOD: Limit Of Detection; LOQ: Limit Of Quantification; S.D.: Standard deviation; Swg: Sum of squares within the group; Sbg: Sum of squares between groups; CV%: Percent coefficient of variation.

According to the World Health Organization (WHO): *"Total alcohol per capita consumption in the world's population over 15 years of age rose from 5.5 litres of pure alcohol in 2005 to 6.4 litres in 2010 and was still at the level of 6.4 litres in 2016".*[1] To that effect, the dosage of ethanol in biological fluids allows judges, through retrospective calculations, to assess the level of impairment of an individual at the time of the event, which is fundamental for its elucidation. This study proposes the validation of an analytical method to separate, detect and quantitate ethanol between 0.1 and 5.0 g/L in blood and urine samples through "headspace" (HS) gas chromatography mass spectrometry (GC-MS).

MATERIALS AND METHODS

Control

A water-ethanol Cerilliant® calibration kit (Round Rock, Texas, USA) at a concentration of 1.0 g/L was used in order to compare its results to an ethanol-water solution at the same concentration (Biopack®, City of Buenos Aires, Argentina). No significant differences were found.

Reagents

The following substances were used to test for possible interferences of volatile compounds: Acetone ACS grade (Biopack®), PA grade n-Propanol (Stanton®), HPLC grade methanol (Sintorgan®), Isopropanol ACS grade (Sintorgan®), PA grade ethyl acetate (Biopack®). ACS grade T-butanol (Carlo Erba®) was used as internal standard. Relevant analyte solutions were prepared with ACS grade ethanol absolute (Sintorgan®) and 18 MΩ.cm ultrapure water from a BioSan brand model Labaqua (Biosystems) deionization water purification system.

Instruments

The research was conducted with a Shimadzu GCMS-QP2020 high-end single quadrupole (Kyoto, Japan) gas chro-

matograph mass spectrometer equippd with a Headspace sampler Shimadzu HS-20 (Kyoto, Japan). An Rtx-5MS (30m x 0,25mm x 0,25µm) column provided by Restek Corporation (Bellefonte, Pennsylvania, USA) was also used. For system control, data gathering and processing, LabSolution's GCMS Real Time Analysis and GCMS Postrun -both in LabSolution version 4.52- were employed. The sample temperature and incubation time in the Head-Space module were 75 °C for 10 minutes. The injection and transfer lines were at 150 °C with a 1:100 split ratio. The oven module was set at a 40 °C isothermal. The ionizer of the mass module was at 220 °C, with a voltage relative to its tuning plus a 0.1 kV gain. The run time was 4.5 minutes. The determination of the analytes was performed using the SCAN mode (29-100 m/z) and SIM mode (ethanol: 31*-45-46, t-butanol: 59*-31-41).

Validation parameters

"*Method validation is basically the process of defining an analytical requirement, and confirming that the method under consideration has capabilities consistent with what the application requires".*[2] It is interesting to look into other definitions of the concept of validation. According to ISO/IEC 17025, it is the "*confirmation by examination and the provision of objective evidence that the particular requirements for a specific intended use are fulfilled*".[3] VIM defines it as "*verification, where the specified requirements are adequate for an intended use*".[4] Taking into account that "*Method validation is usually considered to be very closely tied to method development*"[2], the guidelines drawn up in "Eurachem Guide: The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics (2nd ed. 2014)"[5] were followed. The recommendations in "Eurachem/CITAC Guide CG 4: Quantifying Uncertainty in Analytical Measurement (3rd ed. 2012)" were also adopted.[6]

RESULTS

Model calibration

The selected acceptance criterium was a linear model ranging from 0.1 to 5.0 g/L, with 8 (eight) points in the calibration curve (Fig. 1). An aqueous solution of T-butanol 0.05 g/L was used as internal standard. The linearity of the results was tested by injecting 8 (eight) levels of concentration of the analyte, which was ran three times. In order to ensure the linear regression of the working range, a graphical analysis of the residuals was performed (Fig. 2), with the predictor variable (concentration of analyte) in the X axis and the difference between the observed values and the predictive values in the Y axis. The adjusted regression curve was plotted (Fig. 3) so as to verify if its goodness of fit is adequate for the relation between the predictor and the dependent variables in the regression model. These measurements were performed by 5 (five) analysts on different days.

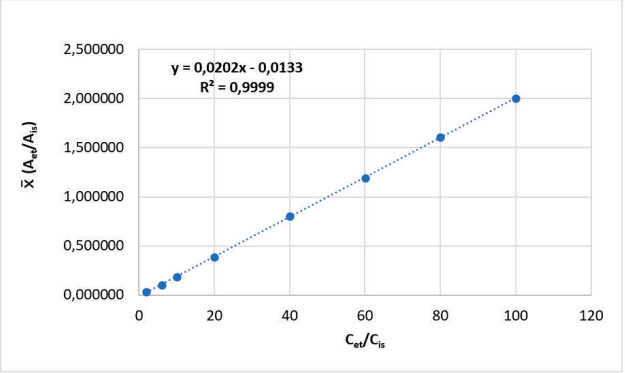

Figure 1. Calibration curve.

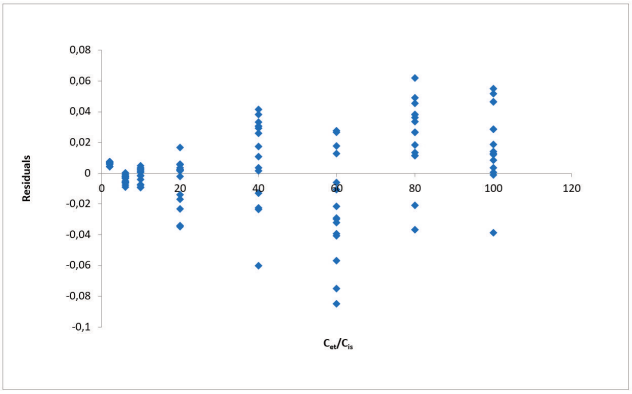

Figure 2. Analysis of residuals.

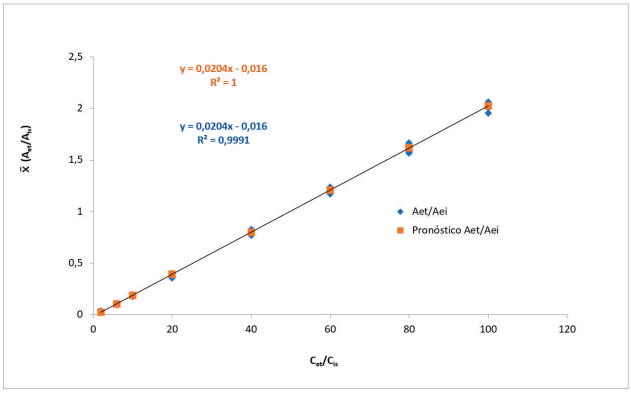

Figure 3. Adjusted regression curve.

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Bias

In order to calculate bias, 4 (four) levels of the working range were tested (0.3, 1.0, 2.0, and 4.0 g/L), in 5 (five) chromatography runs, three times. To that end, blood samples from different volunteers were fortified. Those samples were analyzed beforehand to rule out the presence of analyte in the matrix. The bias was calculated in absolute values (b*) and as relative recovery of the addition (R´%). The acceptance criterium established was a variation of ±5% of the determined real value.

Carryover

With the aim of determining the existence of carryover between chromatography runs, the acceptance criterium established was a relative intensity of the analyte quantifier ion (m/z = 31.00) and of the internal standard (m/z = 59.00) below 10,000. The measures of each point within the working range were taken, and a ninth point was added, equivalent to an ethanol concentration of 10.0 g/L and alternating with blanks within each level.

Interferences

In order to assess the specificity of the method, the possible interferences in the matrix (putrefaction alcohols) (Fig. 4) were tested, as well as the possible influence of preservatives and anticoagulants in the blood samples. For that purpose, the following were analyzed separately: a) an aqueous solution of each of the volatile substances at a concentration of 1.0 g/L with internal standard; b) blank blood samples with internal standard (Figure 5), held in sterile tubes without air chamber, which had different preservative and anticoagulant agents; and c) blank urine samples with internal standard (Figure 6), held in sterile containers. Once the data was gathered, the retention time (T.R.) and relative retention time (R.R.T.) were measured for each substance under analysis. Moreover, 10 (ten) blank blood samples and 10 (ten) blank urine samples were tested: no interfering compounds that affect the ethanol and internal standard retention time were observed. Finally, the analyte results were compared in the different matrixes proposed for the method (Fig. 7, 8 and 9).

Limits

Limit of detection. The calculation of the "*concentration that emits a signal in the instrument that is significantly different from the blank signal or background noise*"[7] or lowest quantity of analyte that can be detected by the device (Limit Of Detection, LOD) was done employing two methods: a) *Formula*: 10 (ten) samples of an aqueous ethanol solution at a concentration of 0.05 g/L were analyzed. Afterwards, the

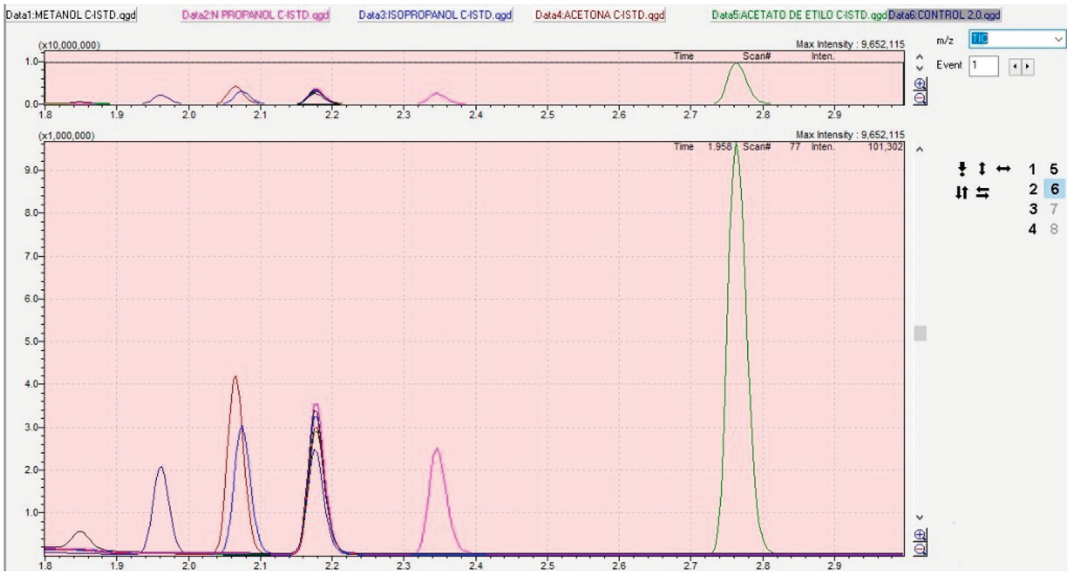


Figure 4. Interference chromatogram.

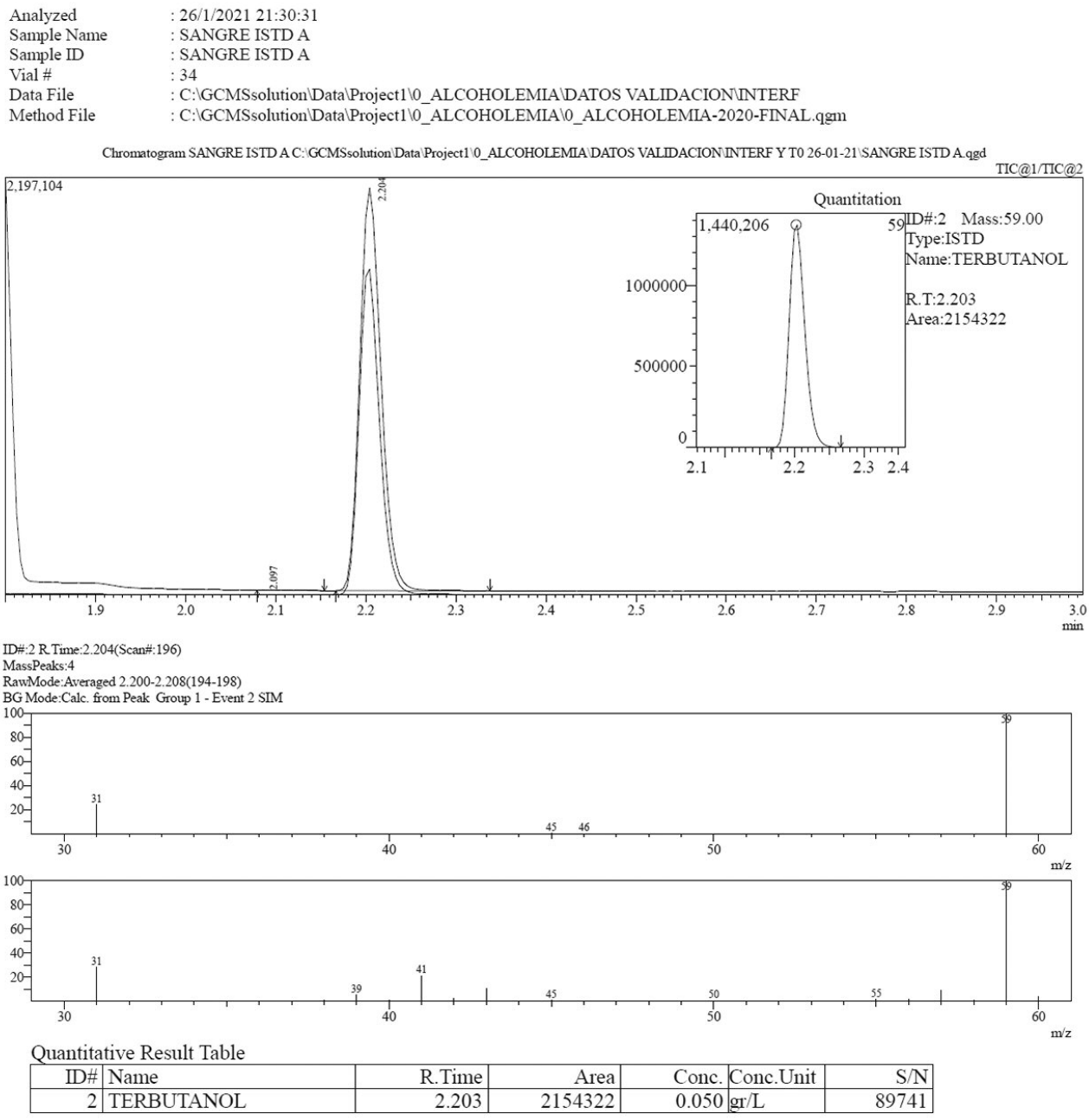


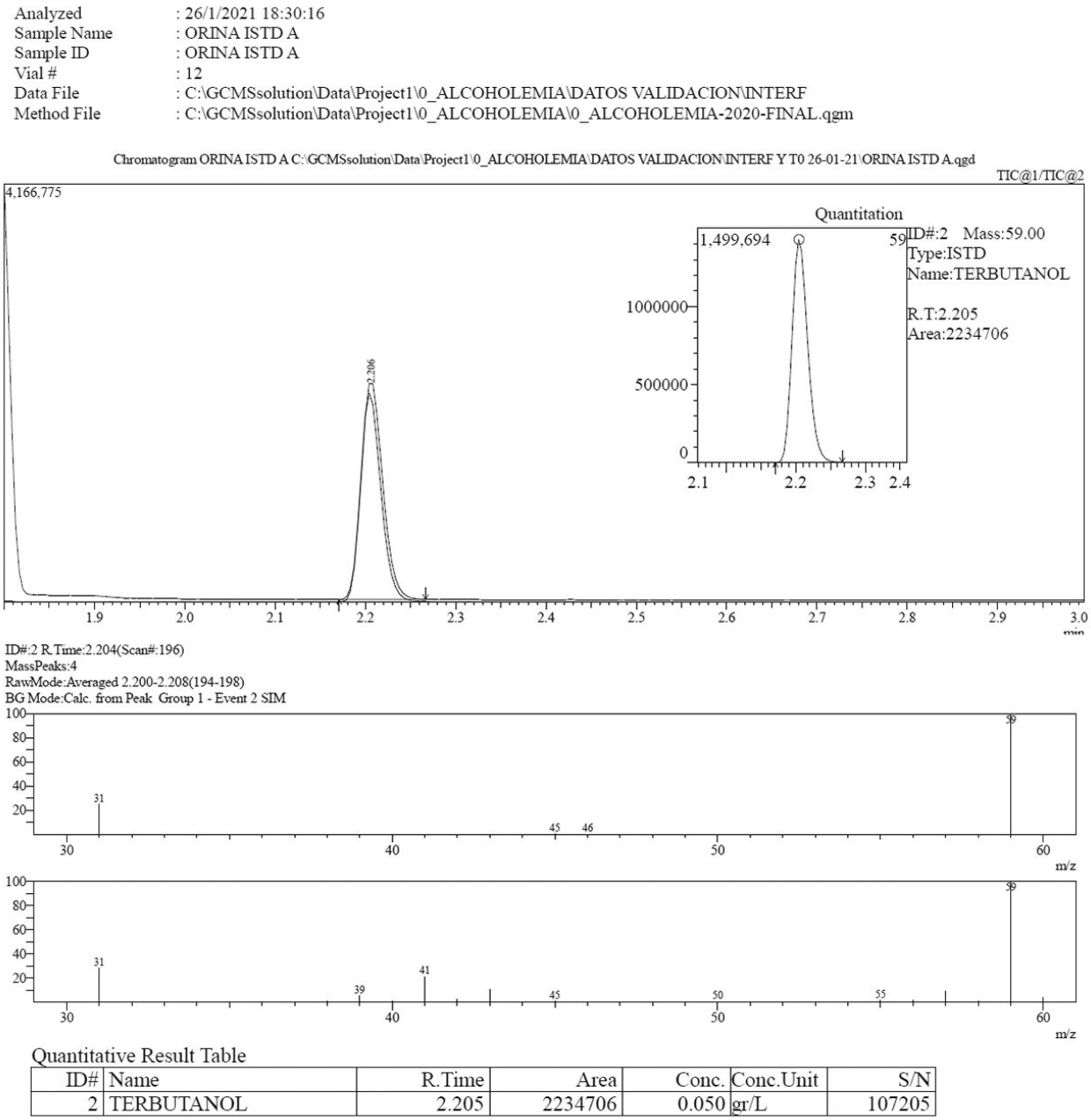
Figure 5. Chromatogram of a blank blood sample.

linear regression curves were plotted to estimate the values of the slope and the intersect for each sample; b) *S/N ratio*: the S/N ratio for each sample was calculated, with an acceptance criterium of a S/N ratio > 3.

Limit of quantification. In order to calculate the "*lower limit of precise quantitative measurements, as opposed to qualitative detection*"[7] (Limit Of Quantification, LOQ), the acceptance criteria set was a S/N ratio > 10 for a solution at a concentration of 0.1 g/L. To that end, 10 (ten) samples of a solution of ethanol at a concentration of 0.1 g/L were tested. The LOQ value proposed for this method is 0.1 g/L.

Precision

Aiming to determine the precision of the method and its repeatability and reproducibility, the one factorial analysis of variance (ANOVA) was used within the group and across groups. 4 (four) levels of the working range (0.3, 1.0, 2.0, and



Quantitative Result Table

ID#	Name	R.Time	Area	Conc.	Conc.Unit	S/N
2	TERBUTANOL	2.205	2234706	0.050	gr/L	107205

Figure 6. Chromatogram of a blank urine sample.

4.0 g/L) were used across different days, by different analysts and was ran three times. The acceptance criterium established was a CV% of ±5%.

DISCUSSION

According to the data gathered, the linearity of the working range was verified, as was its homoscedasticity. When checking for heteroscedasticity, there seems to be no trend among the residuals, which can lead to the assumption that errors have a constant variance. Therefore, the model adequately follows all assumptions.

The results of the evaluation of bias were within the proposed acceptance criteria. According to the data, there is no carryover, even after the 10 g/L solution.

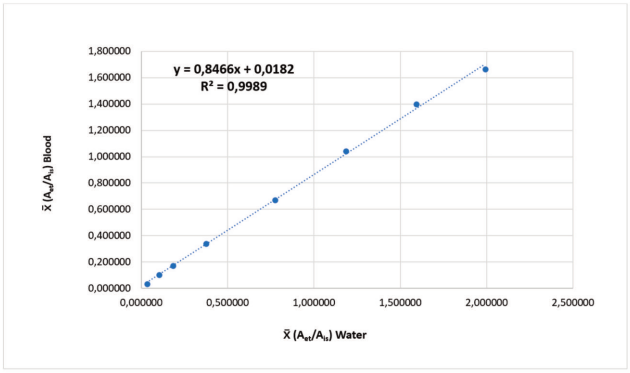


Figure 7. Water against blood curve.

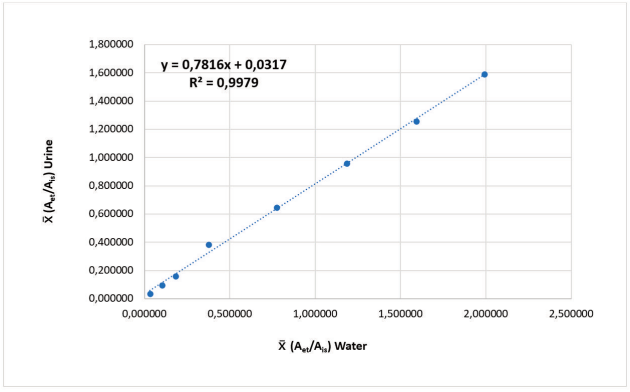


Figure 8. Water against urine curve.

The data gathered when testing the specificity shows no interference in the retention time of the analyte and of the internal standard and a correlation is found between the 3 (three) matrixes that were tested ($R^2 \approx 1$).

The sodium citrate molecular ions observed in the spectrometry were similar to the results obtained for acetone,

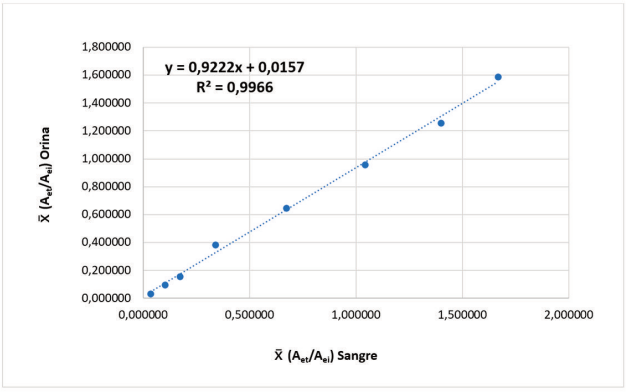


Figure 9. Blood against urine curve.

with similar retention times as well. In contrast, no signals were detected during the acquisition time of the method for EDTA, Heparin, and NaF.

Finally, it was verified that the results obtained for the limit parameters of the method were within the acceptance criteria: LOQ = 0.1 g/L and LOD 0.05 g/L.

CONCLUSIONS

Taking into account the validated parameters and the results previously presented, the method is suitable for the purposes intended. Furthermore, the use of sodium citrate as an anticoagulant is discouraged for this analysis method, EDTA and NaF being recommended as anticoagulant and preservative respectively.

Declaration of interest

The authors declare no conflicts of interest.

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