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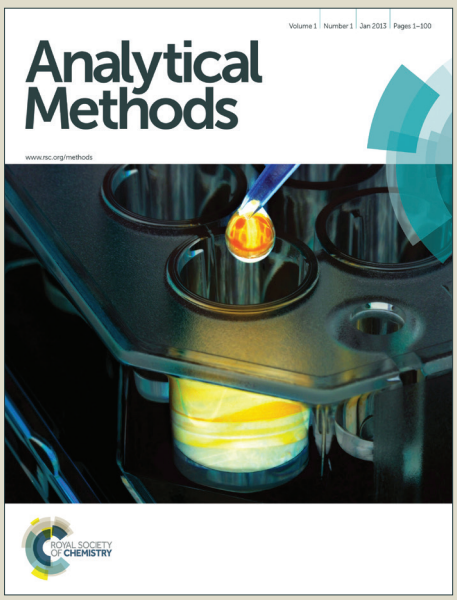

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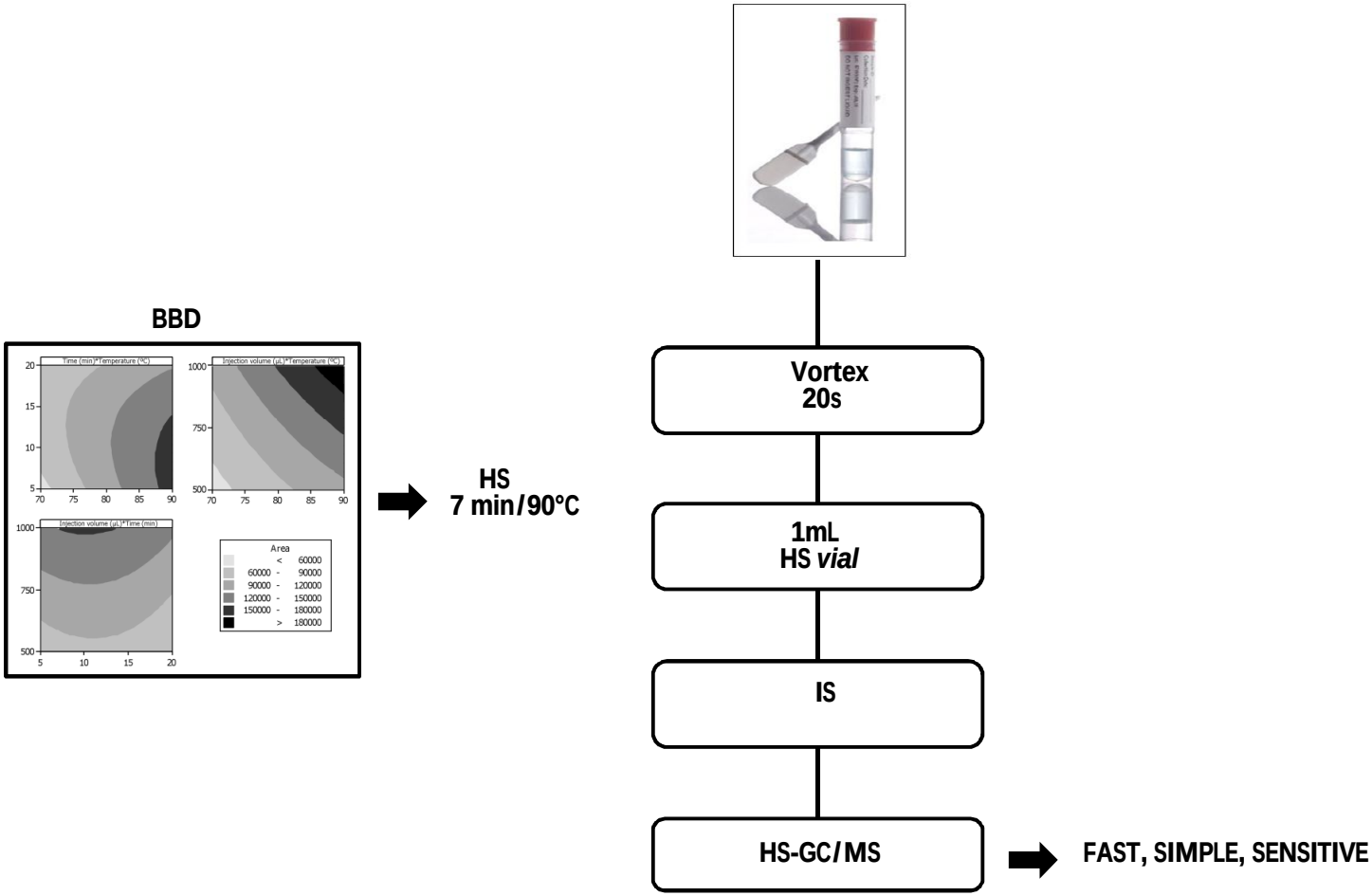

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EXTRACTION OPTIMIZATION USING BOX-BEHNKEN DESIGN AND METHOD VALIDATION FOR ETHANOL IN ORAL FLUID

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ABSTRACT

In Brazil, the routine verification of alcohol use among drivers is performed through breath alcohol analyzers and confirmation of ethanol in blood by the headspace (HS) technique associated to gas chromatography with flame ionization detector (GC/FID). Oral fluid (OF) is an alternative that once collected can be used both for screening and confirmation and has many advantages. We propose an optimization of the extraction of ethanol from OF by HS through experimental design and subsequent development and validation of an analytical method by HS-GC/FID and HS-GC/MS (mass spectrometry), using Quantisal® as a collection device. Experimental design was performed using the Box-Behnken Design and the evaluated parameters were heating temperature, stirring time and injection volume. Selectivity, residual effect, matrix effect, linearity, precision, accuracy, limit of detection and quantification, stability and recovery were evaluated in validation process. The optimized conditions for extraction were: temperature of 90 °C, injection volume of 1000 µL and stirring time of 7 min. Linearity was obtained with an R^2 greater than 0.99. Accuracy of quality control samples remained within 101.56 and 111.2 of the target concentrations, while precision has not exceeded 12% of their relative standard deviation. The developed method showed full viability of running, proving to be rapid, sensitive, as it does not require sample preparation steps. The HS-GC/MS method reached detection limits lower than those found by analysis on HS-GC/FID, and can be easily applied for routine confirmation of ethanol in traffic.

Keywords: alcohol, ethanol, gas chromatography, headspace, oral fluid, BBD.

1 INTRODUCTION

Several studies reveal that there is a relationship between alcohol consumption and traffic accidents.[1] In Brazil, the Institute for Applied Economic Research of Brazil (IPEA) estimated a total cost of 6.5 billion Brazilian reais due to accidents on federal highways between the years 2004 and 2005.[2] In the city of Porto Alegre, southern Brazil, according to the Center for Studies and Research in Traffic and Alcohol (NEPTA), between the years of 2007 and 2008, the costs of traffic accidents reached 65 million Brazilian reais, with approximately 31 million due to consumption of alcohol.[3]

One of the measures adopted by Brazil to refrain the high number of accidents was the change in the Brazilian Traffic Code (CTB) by Law nº 11.705 of 2008 and most recently by Resolution nº 432 of 2013 established by the National Traffic Council (CONTRAN). The new legislation determined a decrease in the allowable limit of breath alcohol analyzer to 0.05 milligrams of alcohol per liter of breath (0.05 mg/L) and zero tolerance for blood alcohol concentration (BAC). When the BAC measured is higher than 0.6 g/L more severe punishments including prison, as well as civil and criminal liability are applied to the drunk driver.[4]

The usual way to measure alcohol consumption among drivers in Brazil is through the breath analyzer, by the local police approach.[5] The critical points regarding the use of the breath analyzer is the fact that they are based on the measurement of alcohol content only along the respiratory tract through the exhaled air (alveolar air) and not the bloodstream.[6] Some volatile substances that are expelled as exhaled air produced during normal metabolic activity or present in foods and beverages, such as acetone, may lead to false-positive results.[7,8] Moreover, workers exposed to organic solvents, for example, may achieve positive results arising from occupational exposure, without having consumed alcohol.[9,10] Thus, it is extremely important to confirm positive results by blood analysis and, when possible, by other analytical techniques based on different principles and in different biological matrices.[11]

In recent years, the use of oral fluid (OF) to monitor the consumption of alcohol and drugs in traffic has increased significantly in many countries worldwide.[12-16] The OF has many advantages over the blood, such as non-invasive collection, it may be performed by the traffic agent through commercial devices, and it is easy to apply. Furthermore, it has a correlation with ethanol levels in plasma, providing information on recent use and confirming that the suspected driver is under the influence of alcohol.[1, 12, 13] When blood is used as matrix, ethanol is metabolized leading to an extrapolation of the analytical window time due to the delay between the traffic agent approach and the collection of blood in a laboratory. Then, the possibility of collecting OF just after the use of the breath analyzer prevents analyst from missing the confirmation result,

therefore it is an excellent alternative for monitoring ethanol in traffic and its refusal rate is significantly lower than blood.[17-19]

The gas chromatography mass spectrometry (GC/MS) is considered the "gold standard" for confirmation of forensic results, because it allows the analysis of lower levels of analytes, enables the qualitative confirmation of the analyte, and it has the requirements to support a lawsuit. Although the GC/MS is present in most forensic laboratories it is still not widely used in the routine analysis of ethanol in blood and OF. To the best of our knowledge there are only GC/MS methods for the analysis of ethanol in blood considering the current literature.[20,21] Therefore, this paper proposes the optimization of the extraction step using the experimental design methodology - Box-Behnken design (BBD), in order to obtain optimal conditions for extraction by HS technique for the determination of ethanol in oral fluid samples by GC/FID and GC/MS, using Quantisal® as collecting device, and the comparison of sensitivity obtained by the both methods.

2 EXPERIMENTAL

2.1 Chemicals and materials

Ethanol, *n*-propanol (IS), acetone, methanol, ethyl acetate, dichloromethane, toluene, ethyl ether, and isopropanol were purchased from Tedia Company (Fairfield, OH, USA).

Quantisal® OF collection devices, filters, and preservative buffer solution were purchased from Immunalysis Corporation (Pomona, CA, USA). Each device contained a collection pad with an indicator that turns blue when 1 mL of OF has been collected, and a plastic transport tube with 3 mL of preservative buffer, with a final specimen volume of 4 mL.

Headspace vials and aluminum screw caps with PTFE/silicone septum were purchased from Agilent Technologies (Agilent J&W Scientific, Folsom, CA).

2.2 Blank oral fluid

The blank OF used in the experiments consisted of ethanol free specimens collected from six volunteers. The specimens were pooled and frozen until the time of analysis.

2.3 Standard and work solutions

Working solutions of ethanol and IS were prepared in distilled water from a stock solution. The concentration range of working solutions was 0.5-40 g/L. The IS

was prepared at a concentration of 2 g/L. After preparation all solutions were stored in a refrigerator.

2.4 Sample preparation

Calibration curve and the quality controls were obtained by spiking 1 mL of blank OF with 100 µL of ethanol stock solutions. The calibration curve was obtained in a final concentration range of 0.05-2 g/L, and samples of LLOQ (lower limit of quantification), LQC (low quality control), MQC (medium quality control), HQC (high quality control), and DQC (dilution quality control), at final concentrations of 0.05, 0.1, 0.5, 1.5 and 4.0 g/L, respectively. As recommended, for being outside the calibration curve, the DQC was subjected to a pre-defined dilution, reaching a concentration of 1g/L.

Final solutions were diluted with Quantisal® preservative buffer (3 mL of buffer to each 1 mL of sample) in order to mimic a collection procedure with Quantisal® device, and then vortexing for 20s. Subsequently, aliquots of 1 mL were transferred to 10 mL HS vials and spiked with 50 µL of IS. The HS vials were sealed with PTFE/silicone septa and aluminum screw caps, and placed into the vial rack of the auto-sampler.

2.5 Instrument

GC analyses were performed at a GC 5975C coupled to flame ionization detector and mass detector 7890A (Agilent Technologies, CA, USA), equipped with an automatic HS auto-sampler (CTC Analytics Combipal, Basel, Switzerland). A ZB-BAC1 column - Zebron (Phenomenex) of 30m x 0.32mm x 1.80µm, was used for chromatographic separation.

2.5.1 GC/FID and GC/MS analysis

GC analyses were performed at the same equipment, separately. The oven temperature was programmed starting at 40 ºC for 3 min, with an increase of 5 °C/min to 70 ºC, for 1 min. The total run was 10 min. The post-run temperature was maintained at 200 °C for 3 min. Helium ultra pure was used as carrier gas at flow rate of 1.4 mL/min. The injector was maintained at 200 ºC and operated in split mode 25/1.

For GC/FID analysis, Nitrogen was used as make up gas, and Synthetic Air and Hydrogen were used to ignite the flame detector.

The MS system was operated in electron impact ionization mode at 70 *e*V, and in selected-ion monitoring (SIM). The ions monitored were *m/z* 31, 45, 46 for ethanol, and *m/z* 60, 59, 31 for IS. The underlined ions were used for quantification. The ratios

of peaks areas of ethanol to IS were calculated. Temperatures of the interface, ion source, and quadrupole were 220 °C, 230 °C and 150 °C, respectively.

2.6 Experimental design

The experimental conditions of the HS technique were optimized using the Box–Behnken design (BBD). Data were processed by Minitab 14 statistical software (State College, PA, USA). The design was conceived with three factors in three levels: heating temperature 70 (-1), 80 (0) and 90 °C (+1), stirring time 5 (-1), 12.5 (0) and 20 min (+1), and injection volume 500 (-1), 750 (0) and 1000 µL (+1).

Experiments were carried out in randomized order and performed in replicate (two blocks), totaling 30 runs (Table 1). The experiment aimed to evaluate the sensitivity of analyte under different conditions tested during the extraction by HS technique.

Experimental data were fitted following a second-order polynomial model (equation I), where *Yi* generically represents each response, *n* is the number of factors or variables, b_0 is the regression coefficient of the intercept, and b_i, b_{ii} and b_{ij} are regression coefficients for linear, quadratic and interaction of each factor A_i, respectively.

$$Yi = b_0 + \sum_{i=1}^{n} biAi + \sum_{i=1}^{n} biiA^2i + \sum_{i\leq 1\leq j}^{n} bijAij \qquad (I)$$

The validity and predictive capacity of the mathematical model was evaluated under optimal conditions comparing the optimum responses obtained by the mathematical model with the experimental results.

Table 1

2.7 Method Validation

Validation was performed according to the USA Food and Drug Administration Center (FDA) and the Brazilian Health Surveillance Agency (ANVISA) recommendations for bioanalytical methods, with the following parameters: selectivity, residual effect, matrix effect, linearity, precision, accuracy, limit of detection and quantification, stability and recovery.[22, 23]

2.7.1 Carryover and Matrix effects

To evaluate the residual effect after injection of samples at high concentrations, six blank samples were analyzed after processing the ULOQ (upper limit of quantification) and the HQC, and results were compared with those of the samples processed in the LLOQ.

In order to evaluate the matrix effect, samples of LQC and HQC prepared in OF and distilled water, were analyzed. The matrix effect is evaluated by CV (%) of MFs, which must be below 15%.

$$MF = \frac{\text{Response of the analyte in OF/Response of the IS in OF}}{\text{Response of the analyte in solution/Response of the IS in solution}}$$

2.7.2 Linearity

The linearity of the method was accessed through three standard curves, run at different days and prepared by spiking blank OF with ethanol at concentrations of 0.05, 0.1, 0.2, 0.5, 1.0, 1.5, and 2.0 g/L, and IS at 0.1 g/L.

2.7.3 Detection and quantification Limits

The LLOQ was estimated as the lowest standard on the calibration curve considering accuracy (between 80-120%) and precision (up to 20%) by analyzing five independent fortified OF samples. The limit of detection (LOD) was estimated up to a signal-to-noise ratio (S/N) about three.

2.7.4 Intra and Inter-day accuracy and precision

Intra-day accuracy and precision were evaluated during a single run, analyzing five replicates of LLOQ, LQC, MQC, HQC, and DQC samples (n=25). Inter-day assays were performed over three days, analyzing five replicates of each quality control sample per day. Precision was expressed as relative standard deviation (RSD) and accuracy as percentage of theoretical concentration.

2.7.5 Stability

Stability tests were performed only for GC/MS analysis on three samples of LQC and HQC spiked with IS, and included: cycles of freezing and thawing over three days, post-processing stability (14 h), stability of short duration (24 and 48 h at 4 °C), stability in controlled temperature (20 °C) and in ambient temperature (± 25 °C) for 4 hours before the extraction. Also, a long-term stability was performed for OF samples and working solutions at the same concentrations during 1 month. The analytical

results were assessed by a calibration curve prepared on each day of analysis, comparing the area response of stability sample of analyte and IS with the area response of sample prepared from fresh stock solutions.

2.7.6 Recovery

For the recovery experiment, LQC, MQC and HQC samples (n=9) were prepared in OF and distilled water. Concentrations were calculated based on a calibration curve prepared on the day of analysis as the ratio of the value obtained in OF and the value obtained in water x 100.

2.7.7 Selectivity

The method was evaluated for selectivity by analyzing OF samples collected with Quantisal® device from six volunteers non-ethanol users. The presence of interfering substances in the proposed method was evaluated. The results were compared with those obtained in the samples processed in the LLOQ.

In addition, the selectivity of HS-GC/MS method was evaluated through the analysis of a blank OF sample fortified with ethanol and 0.1 g/L of potential interfering substances including solvents and other substances used as inhalants, with an intent to find interfering peaks at the time of ethanol retention. The substances tested were: methanol, isopropanol, ethyl acetate, ethyl ether, dichloromethane and toluene.

2.8 Statistical analysis

The suitability of ordinary least squares method has been assessed in the statistical inference of data, by applying the model to the three standard curves (n = 21). Data were evaluated without mathematical transformations, through the analysis of residuals and variance (ANOVA) considering 95% of confidence interval.

3 RESULTS AND DISCUSSION

3.1 Optimization of the HS parameters

The assessment of response surface through the BBD design allows estimating the parameters of the quadratic model, the model building sequence, and detecting the lack of fit of the model; it also allows the use of blocks.[24] This design was chosen to optimize the parameters of the HS, thus enhancing the extraction conditions of ethanol from matrix, considering temperature, stirring time, and injection volume, to obtain greater sensitivity in the analysis.

The experimental results obtained were well-fitted by second-order polynomial model (R^2 = 0.90), as only 10% of variance were left unexplained by the model proposed. No evidence of inadequacy was detected by the lack-of-fit test ($p > 0.05$). Thus, the mathematical model generated (equation II) was able to describe the relationship between responses and the factors evaluated.

$$y = -413099 + 6377x_1 + 21882x_2 - 189x_3 - 185x_1x_2 + 4x_1x_3 - x_2x_3 - 18x_1^2 - 287x_2^2 \quad \text{(II)}$$

Where, x_1 represent the heating temperature, x_2 the stirring time, and x_3 the volume of sample injection.

According to response surface, the response increased proportionally with the injection volume (Fig. 1). Thus, the volume of 1000 µL was selected due to the higher response generated. The same occurred with the heating temperature, which an optimum response was obtained at 90 °C. The stirring time was the most important characteristic evaluated and it is considered an independent risk factor, because it is capable of generating a quadratic negative effect in the response surface, ($p < 0.05$). After 7 min of stirring there was a significant decrease in the response (Fig. 1) that was more prominent after 10 min. This finding is opposite to that presented so far in the literature for ethanol analysis, which usually presents a heating time of up to 10 min.[25-27]

Fig. 1

The predictive capacity of the mathematical model generated was assessed by analyzing five replicates of the same sample, evaluated under optimal extraction conditions: heating temperature (90ºC); stirring time (7 min); injection volume (1000 µL). The predictive capacity of the model was attested by high similarity between the predicted area and experimental area obtained (Table 2). By evaluating the predictive capacity it was possible to confirm the validity of the mathematical model generated to determine the optimum extraction conditions considering the combination of the three factors evaluated. Thus, the parameters obtained for the HS in experimental design were used to perform the method validation, ensuring a higher sensitivity of the technique.

Table 2

3.2 Sample preparation and Method Validation

The method proposed by HS technique is simpler and less expensive when compared to techniques such as solid phase microextraction, as performed by Feltraco et al, 2009,[28] and faster, compared to the methods described by Gubala, Zuba, 2002; [25] Yonamine et al, 2003;[26] Alyev et al, 2011 [27]. Considering the time of extraction by HS and the chromatographic run, the total time required for the analysis of one sample is 17 minutes. This shows the quickness of HS-GC/FID and HS-GC/MS methods, and therefore, the advantage of its implementation in the laboratory routine.

Analyte was identified by assessing retention time (RT) in the GC/FID (Fig. 2), and RT plus mass spectra in the GC/MS (Fig. 2 and 3). Retention times obtained for the ethanol were equal to 2.02 and 1.78 min for GC/FID and GC/MS, respectively. The total analysis time of 10 min was maintained due to the simultaneous analysis of interfering solvents performed in the selectivity test. For GC/MS ethanol and IS monitoring the most abundant ions representative of the molecules (base peak and molecular ion) (Fig. 3) were chosen.

Fig. 2 and Fig. 3

3.2.1 Carryover and Matrix effects

The responses of the interfering peaks in the blank samples injected after the ULOQ and HQC were below 20% and 5% for ethanol by GC/FID and GC/MS, respectively. Thus, for GC/FID analysis, in order to avoid interference on the results obtained for ethanol after the ULOQ analysis, two blank samples were injected.

The matrix effect values obtained by the CV of the samples conducted through HS-GC/FID and HS-CG-MS were below 5% for the HQC and LQC, as recommended by the guidelines.

3.2.2 Linearity

The calibration curve was constructed from seven points in the range of 0.05 to 2.0 g/L on three different days. Linear regression was used to evaluate linearity by the method of ordinary least squares. An R^2 was obtained on the order of 0.99 in all equations, observing a proportional increase of the area ratio over the concentration of analyte (Table 3). Linearity was also evaluated for residual analysis, showing a uniform dispersion of points around the line. According to the obtained residual plots, the homoscedasticity track tested at a 95% confidence interval, and the absence of outlier points, considered atypical observations outside the confidence interval, confirmed the linearity of the method. ANOVA (lack-of-fit test) was also conducted, showing values of

p> 0.05 (Table 3). The combination of results proved that there were no deviations from linearity, and the adequacy of data description to evaluate the linearity of the method, as presented in Fig. 4, which represents one day of analysis by GC/MS and GC/FID.

Table 3

Although it is common to use only the least squares model to assess linearity, we question its viability when analyzed separately. Other statistical tests can indicate a lack of correlation between the proportional areas of the analytes obtained and concentrations tested (heteroscedasticity).[29] Thus, we highlight the importance of conducting further analysis of the least squares in order to present the results of linear regression with confidence, ensuring the linearity of the method developed.

Fig. 4

3.2.3 Limits of Detection and Quantification

The LLOQ of ethanol in OF was 0.0125 g/L for GC/MS, with an intraday precision and accuracy below 15%. The LOD, estimated by signal to noise ratio of about three, was 0.005 g/L, lower than that found in the analysis by GC/FID, which showed an LOD equal to 0.0129 g/L. This demonstrates the increased sensitivity of the GC/MS technique compared to our GC/FID method and the methods already described in the literature.[25-28]

3.2.4 Recovery

Recovery was assessed by comparing the results obtained in water and OF through a calibration curve prepared on the day of analysis for the LQD, MCQ, and HQC, obtaining suitable values for the proposed method. Confirming these results, the recovery data obtained on GC/MS did not vary more than 5%, showing the effectiveness of the extraction by the HS method developed.

3.2.5 Accuracy and Precision

The results for accuracy and intraday and inter-day accuracy did not exceed 20% for LLOQ and 15% for the other QC, as it is suitable for both methods proposed and according to the guidelines for bioanalytical validation of methods (Table 4).

Table 4

3.2.6 Stability

Stability assay was carried out with the intent to reproduce a common routine of OF collection and transportation to laboratory. The LQC and HQC samples were stored in a refrigerator for 24 to 48 h, after standing 4 h at 20 °C and reanalyzed later (longer than the time interval between the end of sample preparation and longer analytical run). All samples showed variation lower than 10%, as well as subjected to cycles of freezing and thawing. Long-term stability testing was also performed, where samples remained frozen during 1 month (-10 °C). The ethanol stock solutions prepared in distilled water were also subjected to the test of long duration for the same period, but were stored in the refrigerator (4 °C) as well as the solutions that remained during the validation test. The OF samples and test solutions used in the long-term test showed variation greater than 15% and 10% respectively, and are not considered stable under the conditions underwent during the test.

Still, in an attempt to reproduce the real collection, the samples remained at room temperature (± 25 °C) without humidity control, during 4 h. The samples showed variation lower than 15% for both quality controls (low and high), as recommended by the validation guides.

The ethanol solutions and OF samples prepared during validation proved to be stable under most of the conditions tested, except for the assay of long duration. However, other conditions have not been tested for long-term test that may allow their recommended storage conditions, such as freezing at temperatures to -20 °C or -40°C. Still, in other tests that reproduced the routine storage and analysis, the samples showed no significant variation, allowing collection, preparation, and transportation under the usual conditions required for its use to monitor drivers under the influence of ethanol. These results have great importance since the methods described in the literature typically have no data regarding the stability, or have insufficient data, [25-28] despite being recommended by the validation guidelines for bioanalytical methods.[22, 23]

3.2.7 Selectivity

Selectivity was proven by injecting OF samples from six different subjects, which showed no interfering peaks near the retention time of ethanol and IS with responses below 5% for samples obtained in LLOQ, as shown in figure 5.

Fig. 5

Selectivity was also assessed by analysis of methanol, acetaldehyde, isopropanol, ethyl acetate, ethyl ether, toluene, and dichloromethane by HS-GC/MS.

Methanol is one of the solvents most released into the environment and it is present in fuels, resins, dyes, enamels, besides being used in the adulteration of alcoholic beverages. Isopropanol may also be present in alcoholic beverages and domestic glue.[30] The other solvents were chosen because they are used as inhalants and may cause interference in the screening analysis by the breath analyzer.[9] Some solvents are produced illegally or smuggled for purposes of abuse, such as the inhalant ethyl chloride, widely used by young people, and "scent of loló" (a mixture of diethyl ether, ethanol and chloroform).[31]

The simultaneous analysis of ethanol and solvents was performed by GC/MS in scan mode. None of the tested substances interfere with the analysis of ethanol, which obtained an RT of 1.78 min, showing good separation from other composites tested (Fig. 5) and well-defined mass spectra, excluding the possibility of false positives results for the tested solvents. Considering the simultaneous assessment of the mass spectra, the confirmation of ethanol by another chromatographic system is not required. Thus, the method is capable of performing unambiguous identification of the analyte with performance consistent with the forensic analysis.

Fig. 6

Usually methods that assess both licit and illicit drugs consumed in traffic perform the determination of ethanol by GC/FID and other psychoactive substances by GC/MS or liquid chromatography with mass detection, as realized by Yonamine et al, 2003,[26] which monitored the legal and illegal substances used on Brazilian highways.

The method developed showed full viability of running, proving to be rapid, sensitive, and easily applicable in routine laboratory, as it does not require sample preparation steps. Thus, opening up precedents for simultaneous monitoring of ethanol and other solvents, using the technique developed, such as the methods previously developed by our research group.[29, 32]

4 CONCLUSIONS

Validation showed that the method is linear, specific and, sensitive with recovery, precision, accuracy, and stability within the accepted limits for the FDA and ANVISA.

The possibility of collecting OF at the place of police approach avoids the extrapolation of ethanol analytic window as occurred in blood collection, aside from the simplicity and reliability of the analytical method. Thus, it shows this applicability with

the importance required to withstand a lawsuit and, thereby filling a gap in confirming the results of ethanol consumption by conductors in Brazil.

ACKNOWLEDGMENTS

The authors are thankful to the volunteers for providing the blank oral fluid specimens and to CNPq for financial support.

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FIGURES

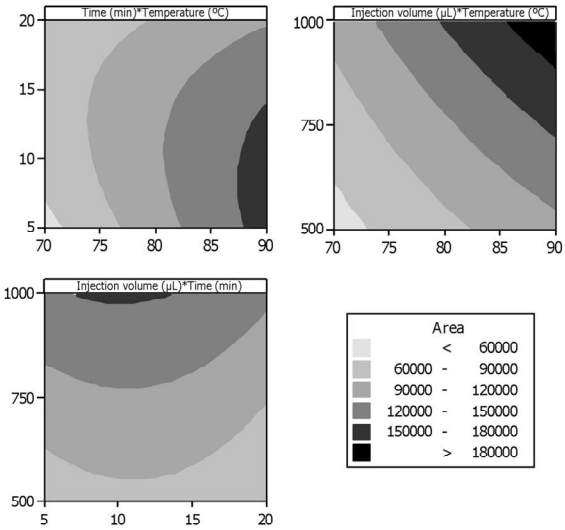

Fig 1. Contour plots by BBD for the three factors evaluated in HS: heating temperature, stirring time, and injection volume. Hold values: heating temperature (80 ºC); stirring time (12.5 min); injection volume (750 µL). (pg 08)

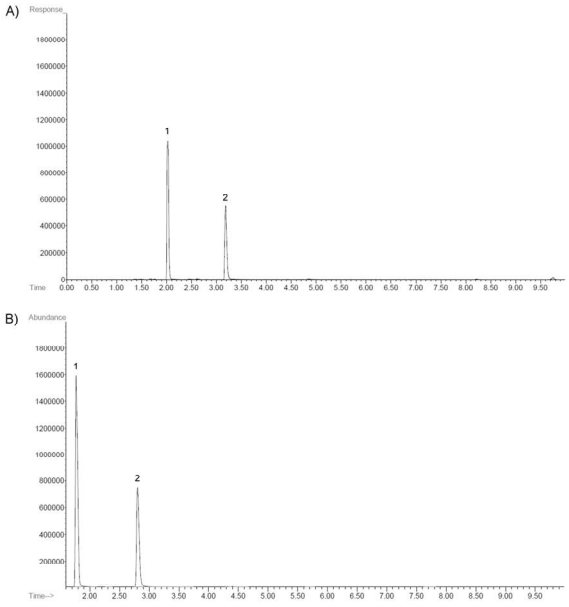

Fig 2. Chromatogram of Ethanol (1) and IS (2) in oral fluid by GC/FID (A) and GC/MS (B). (pg 09)

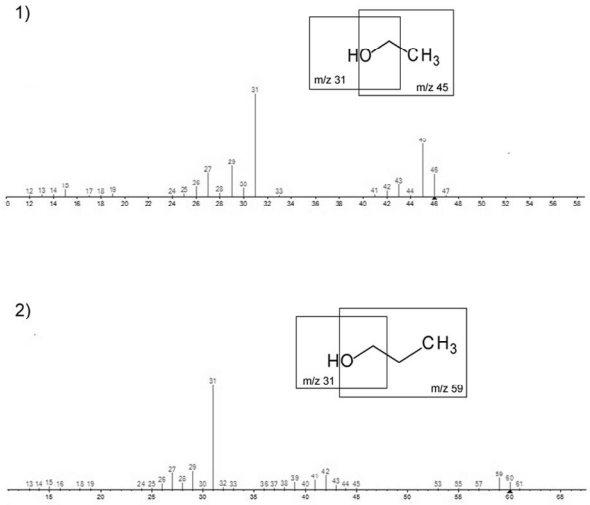

Fig 3. Mass spectra of Ethanol (1) and IS (2) and the respective fragmentation models. (pg 09)

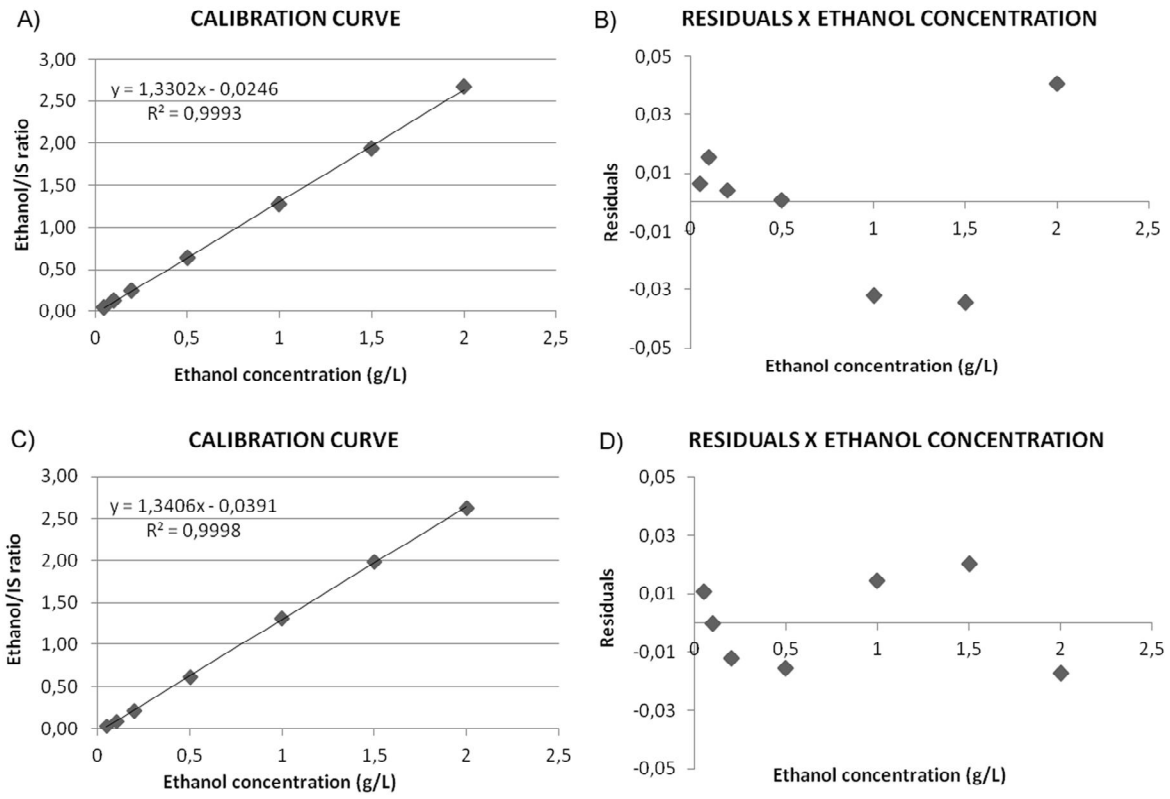

Fig 4. GC/FID (A) and GC/MS (C) calibration curve, and GC/FID (B) and GC/MS (D) residual analysis. (pg 10)

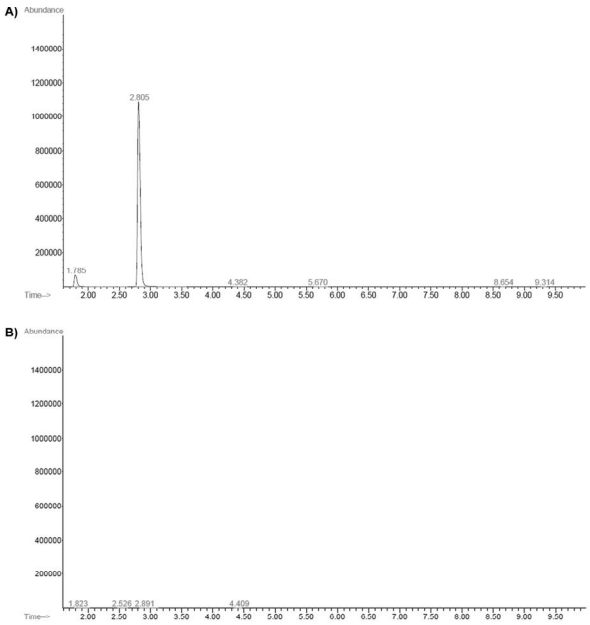

Fig 5. Chromatogram of Ethanol LLOQ (A) and blank oral fluid (B). (pg 11)

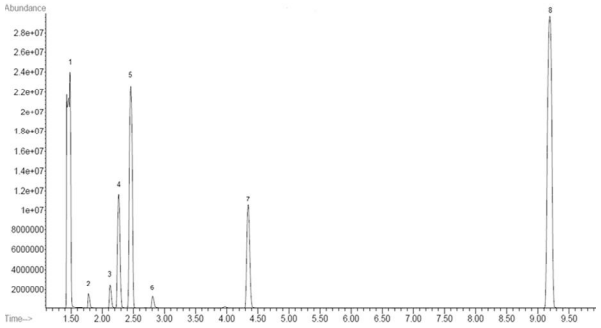

Fig 6. Chromatogram of Ethanol in oral fluid and possible interfering solvents. (1) Methanol, (2) Ethanol, (3) Isopropyl Alcohol, (4) Dichloromethane, (5) Ethyl Ether, (6) IS, (7) Ethyl Acetate and (8) Toluene. (pg 12)

TABLES

Table 1. BBD design showing the three factors evaluated and their levels. (pg 5)

Run	Heating temperature (°C)	Stirring time (min.)	Volume of sample injection (µL)
1	70 (-1)	5.0 (-1)	750 (0)
2	90 (+1)	5.0 (-1)	750 (0)
3	70 (-1)	20.0 (+1)	750 (0)
4	90 (+1)	20.0 (+1)	750 (0)
5	70 (-1)	12.5 (0)	500 (-1)
6	90 (+1)	12.5 (0)	500 (-1)
7	70 (-1)	12.5 (0)	1000 (+1)
8	90 (+1)	12.5 (0)	1000 (+1)
9	80 (0)	5.0 (-1)	500 (-1)
10	80 (0)	20.0 (+1)	500 (-1)
11	80 (0)	5.0 (-1)	1000 (+1)
12	80 (0)	20.0 (+1)	1000 (+1)
13	80 (0)	12.5 (0)	750 (0)
14	80 (0)	12.5 (0)	750 (0)
15	80 (0)	12.5 (0)	750 (0)

Table 2. Results obtained by evaluating the predictive capacity of mathematical model generated. (pg 8)

	Predicted area [a]	Experimental area [b] (n=5)	Predictive capacity (%)
	208800	224520	107.53
SD		28389	13.60
RSD (%)		12.64	12.64

[a] Predicted value from the mathematical model generated
[b] Experimental area obtained from optimized conditions

Table 3. Results obtained from ordinary least squares and ANOVA. (pg 10)

	GC/FID			GC/MS		
	Equation [a]	**R^2** [b]	**p** [c]	**Equation** [a]	**R^2** [b]	**p** [c]
1	y = 1.3302x – 0.0246	0.9993	0,1884	y = 1.3081x – 0.0217	0.9995	0.6594
2	y = 1.4203x – 0.0387	0.9975	0,2332	y = 1.31x – 0.0343	0.9994	0.6817
3	y = 1.3797x – 0.0311	0.9978	0,3461	y = 1.3406x – 0.0391	0.9998	0.6534

[a:] Equation obtained from calibration curve
[b:] R^2 obtained from calibration curve
[c:] p value obtained from ANOVA (lack-of-fit-test)

Table 4. Intra and inter-day accuracy and precision by GC/MS and GC/FID. (pg 10)

	GC/MS				GC/FID			
	Intraday		Inter-day		Intraday		Inter-day	
QC sample (n=5)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
LLOQ	111.3	1.9	103.5	2.8	114.0	5.3	106.0	11.9
LQC	97.9	1.4	93.4	3.5	106.0	4.7	107.0	2.1
MQC	97.5	2.2	97.9	1.5	92.2	6.0	95.0	3.4
HQC	105.2	4.7	101.9	4.2	95.7	4.4	96.7	0.9
DQC	101.6	2.4	103.1	10.3	93.0	6.4	98.7	5.4

QC: Quality Control
LLOQ: Lower limit of quantification (0.05 g/L); LQC: Low QC (0.1 g/L); MQC: Medium QC (0.5 g/L); HQC: High QC (1.5 g/L) and DQC: Dilution QC (4.0 g/L).