

Artigo

Determination of Free Glycerol in Biodiesel Using UV-Visible Spectroscopy: a Validation Study

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Determinação de Glicerina Livre em Biodiesel Utilizando Espectroscopia UV Visível: Estudo de Validação do Método

Resumo: Concentrações elevadas de glicerina livre em biodiesel causam danos em motores. Pela legislação brasileira, (ANP Resolução 45/2014), o limite máximo permitido para glicerina livre em biodiesel é 0,02 % em massa. A norma ASTM D6584 estabelece que o método padrão para se determinar glicerina livre em biodiesel é cromatografia gasosa, uma técnica demorada e cara. Como alternativa, a glicerina livre pode ser quantificada, após derivatização, por espectroscopia de absorção na região do UV-Visível (410 nm). No presente trabalho usamos o protocolo de validação para o método espectroscópico. Resultados indicam que o método é linear ($r^2 = 0.995$) para o intervalo de concentrações estudado e possui exatidão e precisão aceitáveis. O método apresentou valores de desvio padrão relativos para repetibilidade e exatidão abaixo de 5,0 %. Para o cálculo dos valores de limite de detecção e quantificação foram empregados três diferentes métodos. Dois deles apresentaram limites de quantificação apropriados para glicerina. Entretanto, o método proposto pelo INMETRO forneceu um valor maior para o limite de quantificação. Considerando que o método é baseado em reação química de derivatização, com parâmetro colorimétrico, a robustez do método foi analisada para pequenas alterações na reação em função da temperatura, do comprimento de onda e do material da cubeta. Os resultados mostraram que o método é robusto para pequenas variações na temperatura de reação, mas não é robusto para mudanças no comprimento de onda e para mudança de material da cubeta (plástico ou vidro).

Palavras-chave: Método de validação; biodiesel; Espectroscopia UV-visível.

Abstract

High glycerol content in biodiesel impairs engine performance. Brazilian legislation, (ANP Resolution 45/2014), limits the maximum of 0.02 % w/w for free glycerol in biodiesel. The ASTM D6584 standard method for glycerol determination is gas chromatography, a time consuming and expensive technique. As alternative, free glycerol in biodiesel can be determined, after derivatization, by absorption spectroscopy in the visible region (410 nm). In this work, we have used a validation protocol for the spectroscopy method. Results indicate that the proposed method is linear ($r^2 = 0.995$) for the studied concentration range and has acceptable accuracy and precision. The method presented relative standard deviations values of intermediate repeatability and accuracy below 5.0 %. To calculate the limits of detection and quantification, three different calculation methods were used. Two of them resulted in appropriate quantification limits for glycerol. However, for the INMETRO calculation method, we found a higher value of quantification limit. Considering that the method is based on a chemical derivatization reaction, to produce a color product, the robustness of the method was analyzed for small changes in reaction temperature, wavelength and type of cuvette used in measurements. The results have shown that the method is robust for small variations in the reaction temperature, but not for small changes in wavelength and type of cuvette used (glass or plastic cuvette).

Keywords: Validation method; biodiesel; UV-visible spectroscopy.

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1. Introduction

A snapshot of the global population in 2017 numbers the world's population to be nearly 7.6 billion. This implies that the world has added approximately one billion

inhabitants over the past twelve years.¹ The growth of the world's population has led to the expansion of the industrial and transport sectors, gradually increasing the demand for food and energy sources.^{2,3} The shortage of oil, coal and natural gas coupled with rising prices and a series of environmental problems caused by their combustion, demands a search for alternative energy sources that will partially or completely substitute fossil fuels.^{2,4,5} Under the conceptual view of NEXUS for cities, general efforts should focus on sustainable uses of water, food and energy to ensure that there will be enough resources for the generations.^{6,7}

To encourage the incorporation of biodiesel into the Brazilian energy matrix, the Federal Government, in 2004, created the National Program for the Production and Use of Biodiesel, which instituted Law 11,097, in 2005. The latter, states the intermediate addition of 2 % of biodiesel (B2) in all diesel marketed domestically as of January 2008 and the percentage of 5 % in the blend (B5) from the beginning of 2013.^{8,9} Since 2017, according to law 13.263/2016, the percentage is 8 % of biodiesel B100 in volume.¹⁰

Biodiesel can be obtained from many renewable sources¹¹ through different methods.¹² Particularly, vegetable oil or animal fats¹³ are the main sources for biodiesel production, by both interesterification reaction (between triglycerides and methyl acetate),¹⁴ and transesterification reaction¹⁵ (triglycerides reacting with methanol or ethanol under alkaline catalysis). The latter process presents higher yields, being thus more appropriate for industrial processes.⁵ The end products of this reaction produce a biphasic system. The oil phase (biodiesel) stays on the top layer, whereas the side-product (glycerol), which has a density of 1.3 g/mL, stays at the bottom phase.¹⁵⁻¹⁷

1.1. Free glycerol in biodiesel

An extremely important parameter for asserting the quality of biodiesel samples is the mass percentage of free and total glycerol. Glycerol in solution can be either dispersed (free) or an additional side-product of the transesterification reaction.¹⁷

The high content of free or bound glycerol in biodiesel can cause accumulation in fuel tanks, valve deposits, injector contamination, fuel lines. In short, a series of problems that may compromise the proper functioning of the vehicle's engine. In addition, above 180 °C, the combustion of glycerol together with biodiesel generates acrolein, which is toxic.¹⁸

The resolution 45/2014 from ANP established through the maximum percentage of traces of free glycerol of 0.02 % m/m for all biodiesel that is commercialized in Brazil.^{18,19}

The American Society of Tests and Materials released the technical standard ASTM 6584 to determinate the percentage of free and / or total glycerol in biodiesel using Gas Chromatography with flame ionization detection.²⁰

However, to attend several legislations around the world, alternative methods have been proposed to determinate the amount of biodiesel in fuels, as ¹H-NMR and ATR-FTIR.²¹⁻²³

Determination of free glycerol by visible absorption spectrophotometry was proposed by Bondioli and Bella.²⁴ Glycerol was extracted from the biodiesel and reacted with sodium metaperiodate. Under this conditions, glycerol is oxidized to form formaldehyde, iodate and formic acid.^{15,24} The method proposed is cheap, uses a small amount of sample and easy to handle.

1.2. Analytical validation

A method is considered validated after complying with all steps of the validation

process. The main analytical parameters studied are: selectivity, linearity, sensitivity, limit of detection, limit of quantification, precision, accuracy and robustness.²⁵⁻²⁹

The aim of this work was to validate the method for free glycerol determination in biodiesel samples, using absorption spectroscopy in the visible region. In addition, to use the new method to evaluate the glycerol content in two biodiesel samples from two Brazilian companies located at Campinas (SP), Brazil.

2. Materials and Methods

2.1. Materials

The equipment used were: Ohaus Adventurer Pro Precision Scale, Chronometer, Vortex Phoenix V56, Ultra-thermostatic Bath NT-281 Nova Técnica, Centrifuge Excelsa II Model 206 Fanem and HP UV / Vis Spectrophotometer Agilent 8453. The reagents used were glacial acetic acid (Nuclear), ammonium acetate (Vetec), ethyl alcohol (Chemco), sodium metaperiodate (Nuclear), acetylacetone (Merck), n-Hexane (Nuclear) and glycerol (Chemco).

2.2. Selectivity

No selectivity analysis was performed. The biodiesel samples were set free from all interferents before derivatization. The reaction product, 3,5-Diacetyl-1,4-hydroxylutidine absorbs at 410 nm, which was selected for absorption measurements.

2.3. Sensitivity

To calculate the limits of detection (LD) and quantification (LQ), seven blank samples were prepared. Using the mean absorbance

values of the blanks and the analytical curve, the LD and LQ were calculated in three different ways: by the equation of Ribani *et al.*,²⁷ by the method of Ribeiro *et al.*²⁹ and by following INMETRO guidelines.²⁶

2.4. Preparation of blank samples

The amount of 2.00 mL of working solvent (water: ethanol, 1:1) was added to test tubes. Then, 1.2 mL of sodium metaperiodate was added. The solution was stirred for 30 seconds. Acetylacetone (1.2 mL) was added. The tubes were heated at 70 °C for 1 minute with manual stirring. After the reaction time, they were cooled to room temperature and absorption was determined at 410 nm.

2.5. Linearity

For the linearity test, a calibration curve was constructed, in triplicate, containing seven points, from 9.0×10^{-4} mg/mL to 6.3×10^{-3} mg/mL.

Using test tubes, 2.00 mL of each point of the analytical curve was added and the solutions were treated as described above.

2.6. Analysis of biodiesel samples

Two companies from Campinas (SP, Brazil) provided biodiesel samples; one sample each. In a 10 mL volumetric flask, 1.0 g of the biodiesel sample was weighed. Four mL of hexane were added. The flask was then treated with the working solvent (water: ethanol, 1:1). The contents were transferred to the test tube and sealed with a stopper. It was stirred for 5 minutes and centrifuged at 2,000 rpm for 15 minutes. After centrifugation the supernatant was removed using a Pasteur pipette. Upon extraction, exactly 0.5 mL of lower layer was transferred to a second test tube. 1.5 mL of working solvent and 1.2 mL of 10 mmol/L Sodium

Metaperiodate were added. The solution was treated as described above.

2.7. Accuracy

The accuracy analysis was performed by a recovery rate, consisting of the addition of free glycerol to the biodiesel sample provided by company 1. Glycerol was added at three different concentration levels (50, 100 and 150 %), that is, at the final concentrations of 1.8×10^{-3} mg/mL; 3.6×10^{-3} mg / mL and 6.3×10^{-3} mg/mL.

2.8. Repeatability

For the repeatability, seven different analyzes of the biodiesel samples were performed by analyst number 1. The results were evaluated by calculating the relative standard deviation (RSD) of the obtained absorbance values at 410 nm.

2.9. Intermediate accuracy

For the intermediate accuracy, using the same method and equipment, the same biodiesel sample was also analyzed seven times, but by t analyst number 2. The intermediate precision was evaluated by comparing the relative standard deviations (RSD) between the two different analysis.

2.10. Robustness

For robustness measurements, Full Factorial Planning 2^3 was used with a glycerol solution of 3.6×10^{-3} mg/mL (corresponding to the midpoint of the analytical curve). Table 1 presents the factors studied in this planning. Table 2 shows the matrix associated to the factorial design experiments.

From the absorbance values obtained from all these eight tests, a normal probability plot was generated (Teófilo *et al.* ²⁸). This graph allowed us to infer how significant are the parameter changes for this study.

Table 1. Parameters for Full Factorial Planning 2^3

Parameter	Lower level (-)	Upper level (+)
1 - Wavelength	405 nm	420
2 - Derivatization temperature	67 °C	73 °C
3 - Cuvette	Glass	Plastic

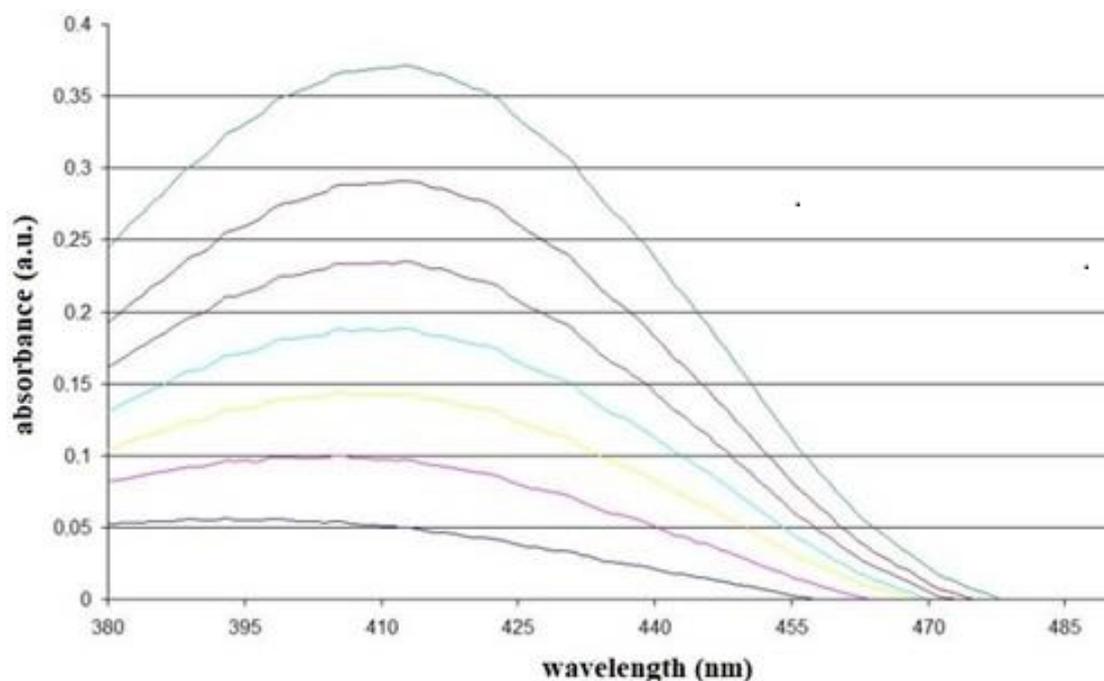
Table 2. Matrice of signals for the Full Factorial Planning 2^3

Essay	1	2	3
1	-	-	-
2	+	-	-
3	-	+	-
4	+	+	-
5	-	-	+
6	+	-	+
7	-	+	+
8	+	+	+

3. Results and Discussion

3.1. Linearity and Sensitivity

From the absorbance values (Figure 1), three analytical curves were obtained, each containing six points (Figure 2).

**Figure 1.** Set of spectra in the visible region (380-500 nm) for the reaction product

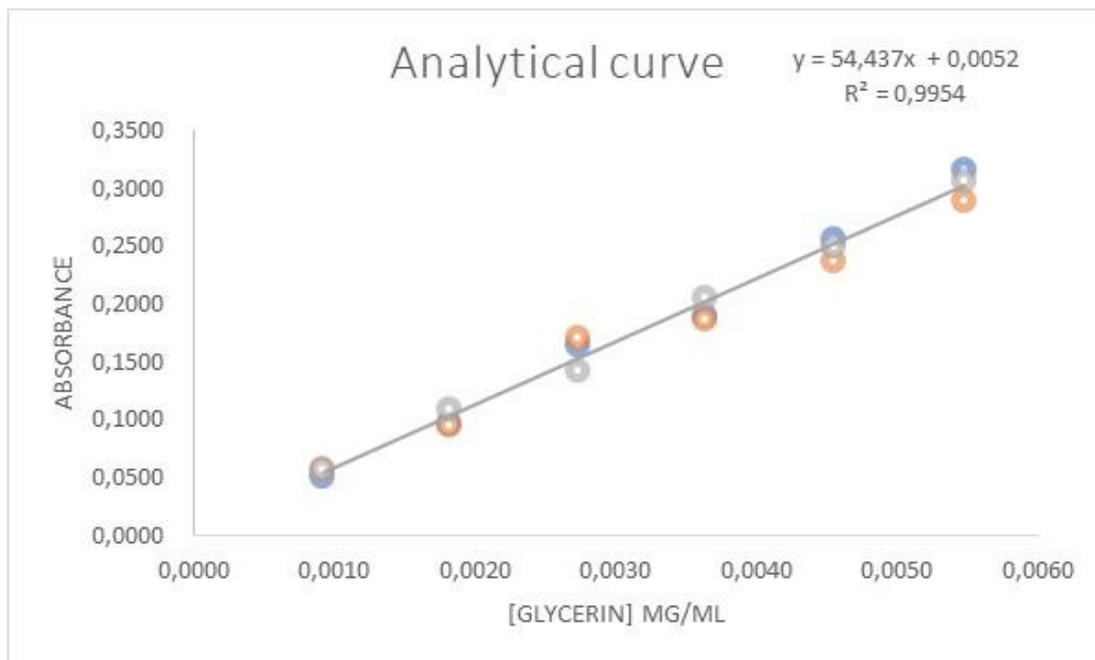


Figure 2. Calibration curves for the determination of free glycerol in biodiesel

Linear regression was applied to the standard curves to confirm the type of curve. The obtained correlation coefficient (r^2) = 0.9954 confirms that the analytical curve obtained corresponds to a straight line.

Table 3 shows that the linear regression model is statistically adjusted, since the specific value (F-calculated), of 1276.1 is higher than F Table ($F_{1, 4}$) of 7.71 and the obtained coefficient of determination (variance explained) is 99.68 %.

Table 3. Analyses of Variance (ANOVA) in 95 % confidence interval

Source	Sum of Squares	Degrees of Freedom	Variance estimate (mean square)	F cal	F tab _{1,4}
Regression	0.04231	1	0.04231	1276.1	7.71
Residue	0.00013	4	3.32×10^{-5}		
Total	0.04244	5			
% Variance explained (R^2)	99.68				

Figure 3 presents the residuals plot, corresponding to the points of the curve, in connection to the linear model for standard curves. The random distribution, without

trend and with the constant variation (homoscedasticity) is close to zero, demonstrating that the linear model is satisfactorily applied to this analytical curve.

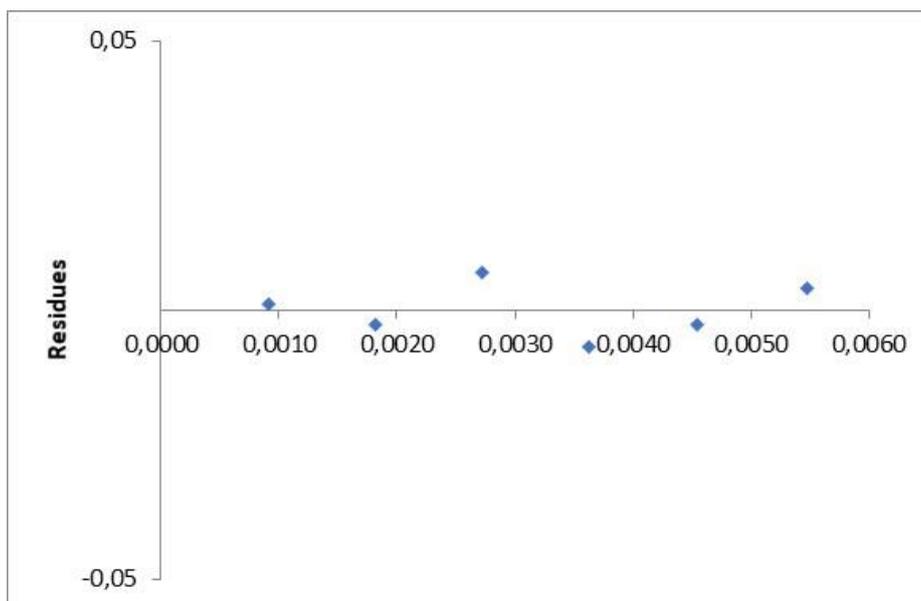


Figure 3. Residuals plot of the linear model analytical curve

Table 4 shows the concentrations of free glycerol for the biodiesel samples supplied by two companies from Campinas, SP (Brazil). The mean concentrations of glycerol were

obtained by linear regression using equation $y = 54.437 x + 0.0052$, which was selected based on the coefficient of determination.

Table 4. Average concentrations of glycerol for biodiesel samples collected from two companies

Biodiesel sample	Average Absorbance (a.u.)	Average Glycerol Concentration (mg.mL ⁻¹)	Average Free Glycerol Percentage (% m/m)
Company 1	0.305	$5.29 \times 10^{-3} \pm 9.67 \%$	0.0230 ± 0.009
Company 2	0.483	$8.29 \times 10^{-3} \pm 2.02 \%$	0.0360 ± 0.013

From the results presented in Table 3 we concluded that the biodiesel supplied by Company 2 presents a free glycerol content that is above the allowed limit of 0.02 % w/w.

3.2. Sensitivity

To evaluate sensitivity the limits of detection and quantification were calculated. There are different ways to calculate these limits depending on the regulatory body. Table 5 presents the LD and LQ using three different calculation methods.

Table 5. Comparison of three different calculations methods for the limit of detection (LD) and limit of quantification (LQ)

Reference	(RIBANI <i>et. al.</i> , 2004) ²⁷	(RIBEIRO <i>et. al.</i> , 2008) ²⁹	INMETRO* ²⁶
LD (mg/ mL)	0.990×10^{-3}	1.29×10^{-3}	3.04×10^{-3}
LQ (mg/ mL)	2.99×10^{-3}	1.59×10^{-3}	6.03×10^{-3}

* table T Student within 99 % confidence for 6 degrees of freedom: $t_{(7-1, 99\%)}$ is 3.143

By Comparing the results from the three calculation methods, the obtained values of LD and LQ differ substantially.

These parameters are calculated in different manners. INMETRO²⁶ calculates LD and LQ using the signal obtained for the blank. Ribani *et al.*²⁷ uses the standard deviation of the blank and the slope of the analytical curve to obtain the same parameters. RIBEIRO *et al.*²⁹ calculates LD and LQ from the deviation of the analytical curve.

Considering the LQ of 2.99×10^{-3} mg/mL (Ribani) and 1.59×10^{-3} mg/mL (Ribeiro) these may be considered appropriate LQ values since the sample concentration (within the range of 0.02 % m/m free glycerol) is 5.29×10^{-3} mg/mL.

However, in this work, the method suggested by INMETRO was used, which gave a LQ value of 6.03×10^{-3} mg/mL.

Therefore, using the Quantification Limit proposed by method of INMETRO, it is concluded that only those biodiesel samples that have a glycerol concentration higher than 6.03×10^{-3} mg/mL, are statistically acceptable for analysis.

3.3. Accuracy and precision

The recovery rates for the accuracy analysis are presented in Table 6.

Table 6. Recovery rates calculated for the three studied levels.

Level	Average Absorbance (a.u.)	Glycerol Reference Average (mg/mL)	Average Predicted Glycerol (mg/mL)	T (%)
50 %	0.403 ±0.002	7.08×10^{-3}	6.94×10^{-3}	98.0
100 %	0.422 ±0.031	8.30×10^{-3}	7.28×10^{-3}	87.7
150 %	0.573 ±0.021	1.13×10^{-2}	9.82×10^{-3}	86.7

According to INMETRO for concentrations ranging between 1 to 10 mg/L, the recovery rate value should be between 80 and 110%. In this work the sample concentration range was between 0.9 and 6 mg/L. For the three levels analyzed, the recovery rate of the

method was within the expected range, being 98.8, 87.7 and 86.7%, respectively.

The results of the accuracy (repeatability and intermediate precision) are presented in Table 7.

Table 7. Relative Standard Deviation (RSD) for repeatability and intermediate accuracy

Type of Accuracy	RSD (%)
Intermediate (2 analysts)	2.02
Repeatability	4.76

For precision analysis, the value of relative standard deviation (RSD%) of the repeatability is expected to be less than that of the intermediate precision. However, the relative standard deviation (RSD%) of the repeatability test was 4.76 % and the intermediate precision was 2.02 %. Even with higher RSD value for repeatability, both values obtained can be considered acceptable (up to 5 %) since there is no relative standard deviation limit established for this analysis.

3.4. Robustness

For robustness analysis, considerable variations were observed between the measured absorbance values. To evaluate which variations were significant, the normal probability plot was produced (Figure 4).

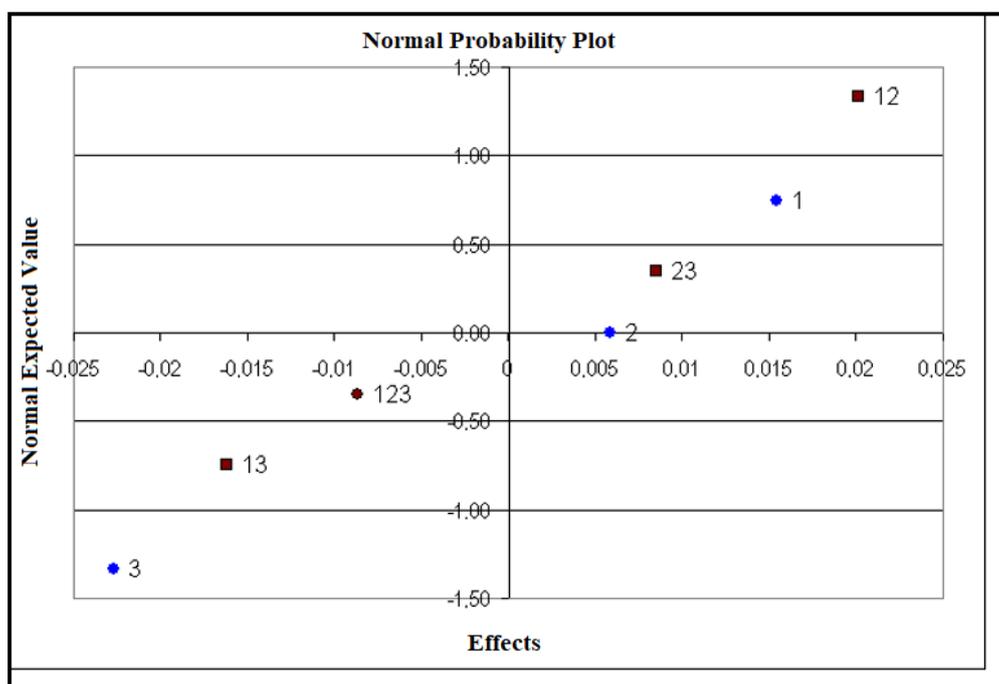


Figure 4. Normal Probability Plot indicating the effects of each factor in the robustness analysis of the method

In this graph one sees that the greater the distance from "zero point", the greater are the effects of the factors in contributing for the analysis' performance. For instance, point 12, which corresponds to the interaction

between wavelength and temperature, and point 1, which is related to the wavelength of analysis, are the ones that have significant effects on the analysis. The results demonstrate that they promoted positive

changes. Yet, point 13 (interaction between wavelength and type of cuvette) and point 3 (type of cuvette) also provoked significant changes, which impact negatively on the analysis. On the other hand, point 2 (derivatization temperature) and point 123 (ternary interaction) show practically no effect on the analysis. Thus, changing the wavelength from 405 nm to 420 nm causes a positive effect, while changing the cuvette material from glass to plastic produces a negative response. Therefore, it is possible to infer that the small variation of wavelength and the material of the cuvette significantly alter the response. Consequently, the method is not robust for the variation of these two parameters. However, the parameter reaction temperature (derivatization reaction), either 67 °C or 73 °C, does not significantly interfere with the response, i.e. for this parameter, the method is robust.

4. Conclusions

The method for determining glycerol by absorption spectroscopy in the visible region (410 nm) shows acceptable linearity and accuracy. Repeatability and intermediate precision did not exceed 5 % of relative standard deviation. The limits of detection and quantification for two calculation methods are considered adequate. For the calculation of INMETRO, the LQ is not adequate. In the evaluation of the robustness, using experimental planning and normal graph it was also possible to conclude that the method is robust for small changes in temperature (67 or 73 °C) of the derivatization reaction, but it is not robust for small changes in the wavelength or in the type of cuvette.

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