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FREE GLYCERIN DETERMINATION IN ETHANOL BIODIESEL UTILIZING SPECTROPHOTOMETRIC AND CHROMATOGRAPHIC (GC/FID) METHODS

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ABSTRACT

According to the Resolution number 7 from the Brazilian National Agency of Oil, Natural Gas and Biofuels (ANP), the official methodologies for free glycerin determination in biodiesel are the ASTM D 6584, EN 14105 and EN 14106 methods. However, these procedures are limited to the analysis of free glycerin in methyl esters and they are not suitable for the analysis of esters from lauric oils. In the present work, a gas chromatographic method was developed for the determination of the amount of free glycerin in ethyl esters from lauric oils, aiming at overcoming the limitations present in the official methods. Moreover, the present method can also be used for ethanol and methanol biodiesel samples from other oily sources. Besides, a methodology that ascertains the content of free glycerin by UV-Vis spectrophotometry was also applied. Both methods were sensitive enough to determine the content of free glycerin in biodiesel, below the limit specified by the ANP, fixed at 0.02 % (200 mg/L), achieving the detection limits of 5.69 mg/L and 2.17 mg/L for the chromatographic and spectrophotometric methods, respectively.

Keywords: Biodiesel, Glycerin, Spectrophotometry, Chromatography.

RESUMO

De acordo com a Resolução número 7 da Agência Nacional de Petróleo, Gás Natural e Biocombustíveis (ANP), as metodologias oficiais para determinação de glicerina livre no biodiesel são os métodos ASTM D 6584, EN 14105 e EN 14106. No entanto, estes procedimentos são limitados à análise de glicerina livre em ésteres metílicos, e eles não são adequados para a análise dos ésteres de óleos láuricos. No presente trabalho, um método por cromatografia a gás foi desenvolvido para determinar o teor de glicerina livre em ésteres etílicos de óleos láuricos, visando superar as limitações presentes nos métodos oficiais. Assim, o presente método também pode ser usado para amostras de biodiesel etílico e metílico, de qualquer oleaginosa. Além disso, foi aplicada uma metodologia que verifica o teor de glicerina livre por espectrofotometria UV-Vis. Ambos os métodos foram suficientemente sensíveis para determinar o teor de glicerina livre no biodiesel, abaixo do limite especificado pela ANP, fixada em 0,02 % (200 mg/L), com os seguintes limites de detecção 5,69 mg/L e 2,17 mg/L para o métodos cromatográfico e o espectrofotométrico, respectivamente.

Palavras-chave: Biodiesel, Glicerina, Espectrofotometria, Cromatografia.

1. Introduction

In the most recent years, a growing demand has been occurring for renewable energy sources, which do not pollute the environment and at the same time reduce the greenhouse gas emissions. Among these sources, biodiesel, a renewable fuel obtained from vegetable oils and animal fats, deserves a special emphasis.

Most of biodiesel are produced by the transesterification process, which yields a mixture of fatty acid esters and glycerin. Thus, after the glycerin separation, the biodiesel is purified. Nevertheless, even after the purification process traces of dissolved glycerin may be present.

The amount of free glycerin is an important parameter in the biodiesel quality. The resolution number 7/2008 of ANP, Brazilian Agency for Petroleum, Natural Gas and Biofuels, establishes the maximum allowed limit of 0.02 mass % of free glycerin in biodiesel [1]. The presence of these glycerin traces, in the biodiesel medium, may harm the fuel injection system, due to the increase in the viscosity. Glycerin may also interact with deposits from the bottom of the fuel tanks and with water, increasing the engine corrosion and thus lowering the engine life. Furthermore, the burning of the glycerin contained in biodiesel can cause the emission of acrolein, a highly toxic pollutant for humans [2].

It is expected that such limit will be reduced in the future. Thus, analytical methodologies sensitive enough to determine increasingly lower glycerin concentrations in biodiesel will be required.

In the literature several methods are applied for glycerin determination in biodiesel matrices. Bondioli et al. [3] suggested a method for ascertaining the amount of free glycerin in biodiesel by glycerin extraction with water/ethanol and a small concentration of formic acid. Soon after, glycerin is analyzed by a gas chromatograph equipped with a flame ionization detector.

Mittelbach [4] measured free glycerin in biodiesel, performing analyses by gas chromatography in a device equipped with flame ionization detector and mass spectrometer, using a 1,4-butanediol internal standard. Glycerin was derivatized with N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA); the samples were inje cted with a split ratio of 1:10 and the analyses were performed in a column DB-5 for high temperatures.

Plank and Lorbeer [5] simultaneously ascertained the free and total glycerin in biodiesel, by gas chromatography using 1,2,4-butanetriol and tricaprine as internal standards, on-column injection, derivatization with N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA). The analyses were carried out in a column DB-5, for high temperatures, using a flame ionization detector.

Four official methods are cited in the Resolution ANP number 7, ABNT NBR 15341, ASTM D 6584, EN 14105 and EN 14106. The method ABNT NBR 15341 is recommended for a biodiesel produced mainly from castor oil, and the other techniques are not recommended for biodiesel from lauric acid oils, such as babassu, palm and coconut. Furthermore, in all cases, such methodologies are only capable to evaluate methyl esters, in detriment to ethyl esters [6,7,8].

The official method ASTM D 6584 [6] deals with the determination of free and total glycerin in biodiesel (B 100) by gas chromatography. Glycerin is derivatized with MSTFA, 1,2,4-butanetriol and tricaprine is used as an internal standard. The samples are directly fed by an on-column injector; the analyses are performed in a column DB-5 for high temperatures coupled to a flame ionization detector.

The method EN 14105 [7] proposes to ascertain the free and total glycerin by gas chromatography with derivatization with MSTFA, using on-column injection and a flame ionization detection (FID).

In Brazil, the method ABNT NBR 15341 [8] is concerned with the quantification of free glycerin in biodiesel by gas chromatography, using flame ionization detector and ethylene glycol as an internal standard. The sample is diluted in ethanol and directly fed by an on-column injector. The analyses are performed in a cyanopropyl phenyl/dimethylsiloxane polymer phase capillary column (1:1).

Bondioli and Bella [9] developed an alternative route to the chromatographic methods – a UV/visible spectrophotometric method for the determination of free glycerin in biodiesel. Initially, glycerin is extracted from biodiesel by centrifugation and later is oxidized by sodium metaperiodate to formaldehyde. Formaldehyde reacts with acetylacetone, giving rise to a colored compound that absorbs in the UV/visible region at 410 nm.

In the present work, a gas chromatographic method was developed for the determination of free glycerin in ethanol biodiesel. This methodology is possible to be applied to methyl and ethyl lauric esters, besides esters from other oily plants. Also, a method developed by Bondioli and co-workers was applied, which employs spectrophometry in the UV/visible region.

2 Experimental

2.1 Reagents

All reagents used in the present work were of analytical degree. They are ammonium acetate (Merck), acetylacetone (Merck), ehylene glycol (Merck), sodium metaperiodate (Merck), acetic acid (Quimex), ethanol (Quimex), hexane (Quimex), glycerin (QM Reagentes), N,N-dimethylformamide (Carlo Erba) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Aldrich).

2.2 Instrumentation

A gas chromatograph VARIAN, CP 3800, was used, equipped with split/splitless injector 1175, VARIAN capillary column CP-Sil 8 CB (5% phenyl, 95 % polydimethylsiloxane), with dimensions of 30 m length, 0.25 mm of internal diameter and 0.25 μm of film thickness and a flame ionization detector (FID). A UV/visible spectrophotometer VARIAN, Cary 50, was also employed.

2.3 Solutions

2.3.1 Spectrophotometric Method

100.000 mg/L glycerin in ethanol; 1.6 mol/L aqueous acetic acid; 4 mol/L aqueous ammonium acetate; working solvent (aqueous 50 % ethanol); glycerin standard solutions: 100, 200, 300, 400 and 500 mg/L in babassu biodiesel produced by the ethanol route; 0.2 mol/L acetylacetone in a pH 5.50 acetate buffer (equal volumes of the aqueous solutions of acetic acid and ammonium acetate); 0.01 mol/L sodium metaperiodate in a pH 5.50 acetate buffer.

2.3.2 Chromatographic Method

100.000 mg/L glycerin in DMF; 1000 mg/L ethylene glycol in DMF; glycerin standard solutions: 50, 100, 150, 200 and 250 mg/L in babassu biodiesel produced by the ethanol route.

2.4 Methodology

The following procedures were used to ascertain the contents of free glycerin in biodiesel samples, obtained from several vegetable oils - cotton, babassu, soybean and "pequi" (*Caryocar brasiliense*).

2.4.1.1 Spectrophotometric Method

The procedure described by Bondioli and Bella [9] was followed for the analysis of biodiesel samples of several vegetable oils – cotton, babassu, soybean and "pequi" (*Caryocar brasiliense*).

2.4.1.2 Chromatographic Method

Initially a sample of babassu biodiesel produced from the ethanol route was purified, according to Mittelbach [4], washing three times with a 0.1 mol/L HCl solution, with the purpose of removing the traces of free glycerin. Afterwards, 100 g of such purified and glycerin-free biodiesel were weighted. Adequate volumes of 100.000 mg/L glycerin stock solution were added, in order to prepare glycerin standard solutions in biodiesel in the range from 50 mg/L to 250 mg/L.

Next, 0.1 g of each sample was weighted, to which 0.1 mL of the 1000 mg/L ethylene glycol solution was added, followed by 0.1 mL BSTFA. The contents were stirred and then allowed to rest for 15 to 20 minutes at 70 ºC. Then 1 μL was introduced in the heated gas chromatograph injector. The temperature of injector 1177 was 290

ºC, with a 1:20 split ratio. Helium was the carrier gas with a flow rate of 1.2 mL/minute.

The furnace temperature program started at 50 ºC for 1 minute, later a heating rate of 15 ºC/minute was used, up to 180 ºC, and increased thereafter to 50 ºC/minute up to 300 ºC, with a soaking time of 10 minutes. A flame ionization detector (FID) was employed with a temperature of 300 ºC, hydrogen flow of 30 mL/minute, synthetic air flow of 300 mL/minute and flow of nitrogen make up gas of 30 mL/minute, with an overall analysis time of 22.07 minutes.

3 Results and Discussion

*3.1 Spectrophotometric A*nalyses

In the spectrophotometric method, the free glycerin contained in biodiesel is oxidized with sodium metaperiodate, forming formaldehyde, followed by the Hantzsch reaction, in which formaldehyde reacts with acetylacetone in the presence of ammonium ions to form the compound 3,5-diacetyl-1,4-dihydrolutidine, which absorbs at 410 nm.

*3.2 Chromatographic A*nalyses

Table 1 shows the main differences between the official method and the proposed method. It is noticed that one advantage of the method developed, as compared with the official method, is the fact that the proposed method matrix is biodiesel, thus avoiding matrix effects and the use of toxic substances, such as pyridine. Moreover, the developed method is also faster, spending a total of 22.07 minutes, against 31.81 minutes in the case of the official method. The injection mode was split, rather than on column, avoiding contamination of substances directly into the column. Another difference is that it was possible to inject concentrated samples in the chromatographic system, avoiding the use of solvents for the sample dilution.

In the chromatographic method developed, glycerin and the internal standard (ethylene glycol) were derivatized by silanization with N,O-bis(trimethylsilyl)-trifluoracetamide (BSTFA). This procedure aims to reduce the polarity for a better peak resolution in the chromatogram, upon elution in a polar chromatographic column.

Figure 1 shows the chromatogram of a biodiesel sample, analyzed after derivatization with BSTFA. As can be seen, the peaks of the internal standard, bis-o-trimethylsilyl-1,2-ethanediol (silanized ethylene glycol) and tris-o-trimethylsilyl-1,2,3-propanetriol (silanized glycerine), are well defined and separated from the peaks of the other compounds.

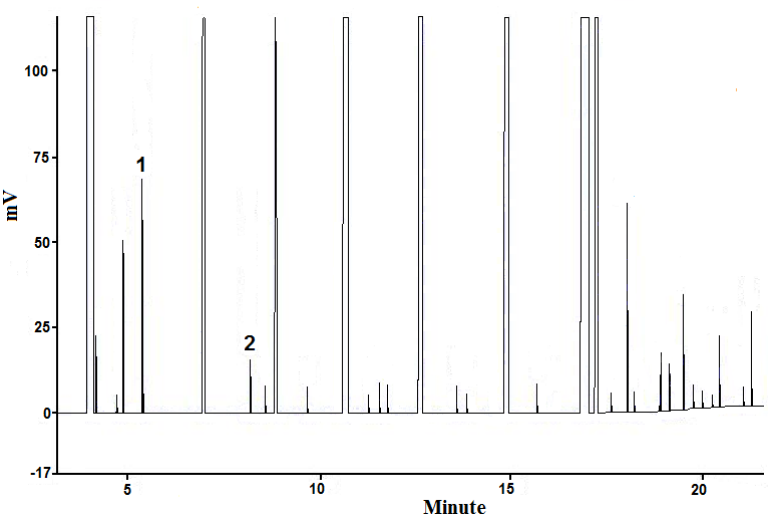


Figure 1. Chromatogram of ethylene glycol (internal standard) (**1**) and free glycerin (**2**) in biodiesel after silanization with BSTFA.

The precision of the two methods was assessed by their repetitivity (Table 2), expressed by an estimate of the standard deviation and coefficient of variation, after having followed all the procedures established in the methods.

In the spectrophotometric method, a standard 200 mg/L glycerin solution in biodiesel was prepared. Seven 1 g aliquots of this standard solution were weighed and analyzed, obtaining the responses in the form of absorbance (abs). In the chromatographic method, a standard 150 mg/L glycerin solution in biodiesel was prepared. Five 0.1 g aliquots were weighed and analyzed. The absorbances of the samples are determined and the results were expressed in terms of the different glycerin/internal standard area ratios (GA/ISA) obtained.

The results obtained for the two methods are presented in Table 2, which also includes the values of the averages.

It can be noticed that the precisions of both methods were found within acceptable limits, that is, variation coefficients below 15 % [10].

3.4 Accuracy

The accuracy of the methods was calculated by the recovery index (**R**) of glycerin. For the spectrophotometric method, the calculation of this parameter used a biodiesel with a negligible free glycerin concentration, which was spiked in order to prepare glycerin standards whose concentrations were 100 mg/L and 200 mg/L. Next three 1 g aliquots of these standards had their concentrations determined, using the method being evaluated.

In the chromatographic method a sample of purified biodiesel, displaying a negligible free glycerin concentration, was strengthened, in order to obtain glycerin concentrations of 150 mg/L and 250 mg/L. Soon after, three 0.1g aliquots of these standards were silanized and analyzed, following the aforementioned chromatographic method. The values of the recovery indices are shown in Table 3.

According to the literature, these values are within an acceptable range. According to Ribani and co-workers [11], an acceptable recovery range is between 70 to 120 %, but Huber [12], suggests that for a concentration of the substance of interest of about 0.01 %, or 100 mg/L, the acceptable recovery rate is between 90 and 107 %.

3.5 Detection Limits and Quantification Limits of the Methods

In order to ascertain the aforementioned limits in the spectrophotometric method, at first a 5 mg/L standard glycerin biodiesel solution was prepared. Next, seven 1 g aliquots of such standard were analyzed following the spectrophotometric method. Soon after, the standard deviation of these seven analyses was estimated. The detection limits and the quantification limits were calculated by the procedure suggested by Csuros [13].

The Detection Limit of the Method (*DLM*) was determined by multiplying the standard deviation (*SD*) by the value of t_{n-1}, in which n = 7, from the table of Student ($t_{7-1} = 3.143$), with confidence limit of 98% ($DLM = SD \times t_{7-1}$).

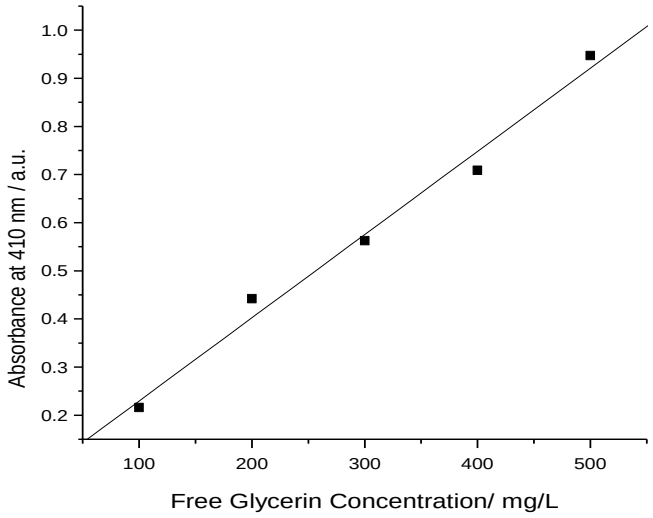
The Quantification Limit of the Method (QLM) was determined as ten times the standard deviation ($QLM = SD \times 10$). The same procedure was employed for the calculation of these two parameters in the chromatographic method.

The differences are that for the spectrophotometric method, a strengthening of 5 mg/L was used for a biodiesel with a negligible concentration of free glycerin, seven 1 g aliquots were analyzed using the procedure described in method. In the case of the chromatographic method, seven 0.1 g aliquots of the 2 mg/L standard solution were taken and analyzed, following thereafter the aforementioned described procedure for this method.

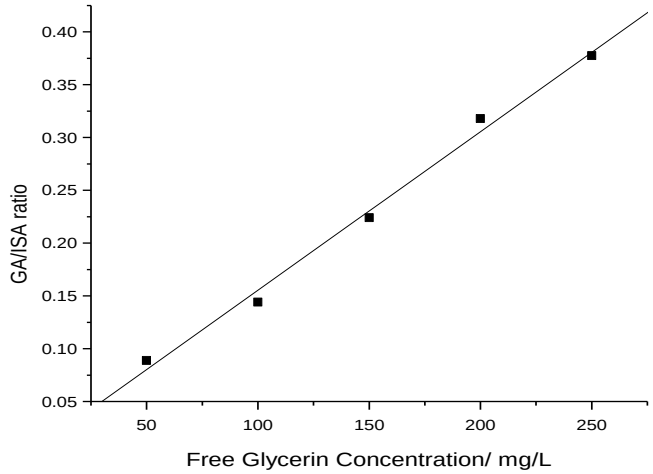
Table 4 displays the detection and the quantification limits for both methods evaluated. It can be noticed that these methods are sensitive enough to determine free glycerin concentrations below the value of 200 mg/L, the upper limit stated by ANP in the biodiesel specification.

3.4 Linearity of the Method

The linearity of the methods was determined analyzing glycerin standard solutions in biodiesel in the concentration ranges of 100 mg/L to 500 mg/L and of 50 mg/L to 250 mg/L for the spectrophotometric method and chromatographic method, respectively. Figure 2 illustrates the relationship between the free glycerin concentrations and the responses for the two evaluated methods.



(a)



(b)

Figure 2. Analytical concentrations curves of free glycerin in biodiesel. (a) By UV/Vis spectrophotometry, measuring the absorbance at 410 nm. (b) By gas chromatography, using FID, measuring the ratio between the glycerin and the internal standard peak areas.

The fitting of the linear regression models for the free glycerin concentration was evaluated by means of the correlation coefficient (R), the coefficient of determination (R^2) and the statistical significance of the regression models by the analysis of variance (ANOVA). Table 5 shows the values of the correlation coefficient and the coefficient of determination, besides results from the analysis of variance, such as the ratio between Regression Mean Square and the Residual Mean Square, also known as F_{Calc} .

The correlation coefficient (R) represents the correlation between the responses observed and the values predicted by the regression analysis. The coefficient of determination (R^2) is the ratio between the Sum of Squares explained by the regression (SS_{reg}) and the Total Sum of Squares (SS_t). This parameter is frequently used to assess if a regression equation is well adjusted. The closer the coefficient of determination is to 1, the smaller will be the dispersion of the set of experimental points and the better will be fitness of the regression model.

The correlation coefficients for both methods are higher than 99.00% indicating that the response of both methods correlate well with the free glycerin content in biodiesel, although the chromatographic method displayed a higher correlation than the spectrophotometric method (Table 5). The evaluation of the coefficient of determination suggests that the linear regression model for the chromatographic method is able to explain 99.28 % of the variation around the average. However, the spectrophotometric method displayed a R^2 value of 98.63%, below the value recommended by Bondioli and Bella [9], namely a coefficient of determination higher than 99.00%.

Although the correlation coefficient and the coefficient of determination are used as references to assess the validity of models, these parameters alone are not sufficient to conclude on the fitness, or not, of the regression models. Another important evaluation of the statistical significance is the analysis of the residues distribution, utilizing the method ANOVA (Analysis of Variance).

The models were evaluated by means of the ratio between the Regression Mean Square (MS_{reg}) and the Residual Mean Square (MS_r). The best model should present a high value for the $MS_{reg}/MS_{\mathbf{r}}$ ratio, also called F_{Calc}, as compared with the value found in statistical tables for the $F_{1,n-2}$ distribution for the 95% confidence limit ($F_{1,3}$ = 10.13 for n = 5). The higher is the MS_{reg}/MS_r ratio, as compared with the value of $F_{1,n-2}$, the more a linear relationship is indicated between the concentration of glycerin and its responses, in the two evaluated methods. Box and Wetz [14] established that the value of F_{Calc} should be, at least, five times bigger than the value of $F_{1,n-2}$ for a

regression be statistically meaningful and be able to predict results.

The data from Table 5 show that both the regression models are statistically significant, once such condition was satisfied for the two studied methods, pointing out that the models are representative. Therefore, the responses obtained in the methods are proportional to the concentrations of free glycerin in biodiesel.

The patterns of residues, for the different glycerin concentrations, are another parameter that indicates if the regression models are fitted. The residues should present a random distribution, without a defined standard. As shown in Figure 3, this condition was met for both methods evaluated.

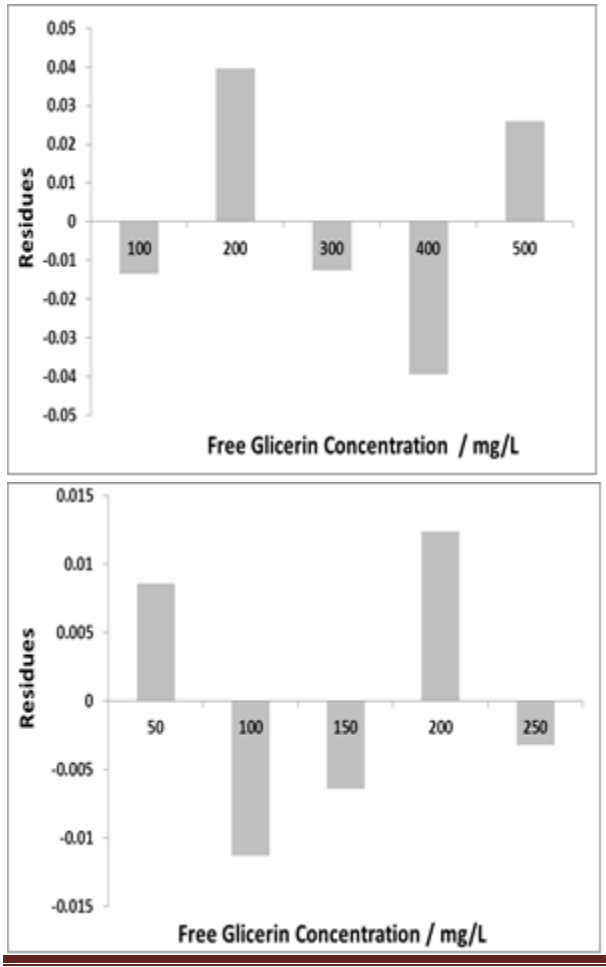


Figure 3. Random distribution of the residues of the regression models, in terms of the glycerin concentrations. (a) for the spectrophotometric method and (b) for the chromatographic method.

3.5 Application of the Methods

Samples of methanol or ethanol biodiesel, obtained from several vegetable oils - cotton, babassu, soybean and "pequi" (*Caryocar brasiliense*) were analyzed. The results of the analyses are shown in Table 6*3.5 Application of the Methods*

Samples of methanol or ethanol biodiesel, obtained from several vegetable oils - cotton, babassu, soybean and "pequi" (*Caryocar brasiliense*) were analyzed. The results of the analyses are shown in Table 6.

According to Table 6, it can be noticed that most of the samples met the ANP requirements, displaying a free glycerin content below 0.02 weight %. However, the spectrophotometric method was not sensible enough to determine smaller contents of free glycerin in samples. Thus, for such samples, the free glycerin can only be ascertained by the chromatographic method. It can be observed that the results for both methods are similar in the sample with a higher free glycerin content.

Table 1. Major differences between the chromatographic method developed and ASTM D 6584

Parameter	Method Developed	Method ASTM D 6584
Injection Mode	Split (1:20)	On Column
Internal Standard	Ethylene glycol	1,2,4-butanetriol
Matrix	Biodiesel	Pyridine
Sample Dilution	Concentrated	Diluted (n-heptane)
Analysis Time	22.07 minutes	31.81 minutes

Table 2. Repetitivity parameters for the Spectrophotometric and Chromatographic Methods Developed

Method	Average	Standard Deviation	Coefficient of Variation
Spectrophotometric	0.4551 abs	0.0361 abs	7.9368 %
Chromatographic	0.2329 GA/ISA	0.0088 GA/ISA	3.7682 %

Table 3. Recovery of free glycerin in biodiesel by UV/Vis spectrophotometry and gas chromatography (GC/FID).

Spectrophotometric Method		Gas Chromatographic Method	
Fortification Level (mg/L)	**Recovery (%)**	**Fortification Level (mg/L)**	**Recovery (%)**
100	100	150	100
200	100	250	90

Table 4. Detection and Quantification Limits of the two Methods

Parameter	Spectrophotometric method	Chromatographic method
DLM	5.69 mg/L	2.17 mg/L
QLM	18.11 mg/L	6.69 mg/L

DLM - Detection Limit of the Method

QLM - Quantification Limit of the Method

Table 5. Fitting of the linear regression models for free glycerin in biodiesel

Adjustment parameters of the regression model	Spectrophotometric method	Chromatographic method
R	99.31%	99.64%
R^2	98.63%	99.28%
F_{Calc}	216.74	416.80
$F_{1,3}$	10.13	10.13
F_{Calc} / $F_{1,3}$	21.40	41.14

Table 6. Free Glycerin Analyses in samples, by the spectrophotometric and chromatographic methods, of ethanol or methanol biodiesel obtained from oils from several species.

Biodiesel	Spectrophotometric Method (mg/L)	Chromatographic Method (mg/L)
Methanol / Cotton	nd*	14.41
Ethanol / Babassu	335.81	368.29
Ethanol / Soybean	nd*	13.67
Methanol / "Pequi"	nd*	7.59
Methanol / Babassu	nd*	19.99

*nd – not detected

4 Conclusion

The chromatographic method was shown to be sensible enough to determine free glycerin in ethanol biodiesel from lauric oils, in concentrations below the limit required by ANP. This method was also faster than the official method, spending a total of 22.07 minutes, against 31.81 minutes in the case of the ASTM D 6584 method. Such method can also be used for ethanol and methanol biodiesel samples from other oily sources. The spectrophotometric method is less sensible than the chromatographic method. According to the analysis of variance, it was observed that both methods fit the regression models, obtaining answers which are proportional to concentration of free glycerin in biodiesel.

Acnowledgements

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References

[1] ANP – Brazilian National Agency of Petroleum, Natural Gas and Biofuels Resolution number 07 (March 19, 2008).DOU 20.03.2008. In Portuguese.

[2] Gardner R, Kazi S, Ellis EM. Detoxication of the environmental pollutant acrolein by a rat liver aldo-keto reductase. Toxicol Lett 2004;148:65–72.

[3] Bondioli P, Mariani C, Lanzani A, Fedeli E, Veronese E. Vegetable oil derivatives as diesel fuel substitutes. Analytical aspects. Note 2: determination of free glycerol. Riv Ital Sost Gras 1992;69:7-9.

[4] Mittelbach M. Diesel fuel derived from vegetable oils: Gas chromatographic determination of free glycerol in transesterified vegetable oils. Chromatographia 1993; 37:623-6.

[5] Plank C, Lorbeer E. Simultaneous determination of glycerol, and mono-, di- and triglycerides in vegetable oil methyl esters by capillary gas chromatography, J Chromatogr, A 1995;697:461-8.

[6] ASTM Standard D 6584, 2000, Test method for determination of free and total glycerin in B-100 biodiesel methyl esters by gas chromatography, ASTM International, West Conshohacken, PA, USA, 2000.

[7] EN 14105. Fat and oil derivatives – Fatty acid methyl esters (FAME) – Determination of free and total glycerol and mono-, di-, triglyceride contents (Reference Method). European Committee for Standardization, 2003.

[8] ABNT NBR 15341, Biodiesel – Determination of free glycerol in castor oil biodiesels by gas chromatography, ABNT, Brazilian Association of Technical Standards, 2006.

[9] Bondioli P, Bella LD. An alternative spectrophotometric method for the determination of free glycerol in biodiesel. Eur J Sc Technol 2005;107:153-7.

[10] Causon R. Validation of chromatographic methods in biomedical analysis. Viewpoint and discussion. J Chromatogr, B: Anal Technol Biomed Life Sci 1997;689:175-80.

[11] Ribani M, Bottoli C B G, Collins C H, Jardim I C S F. Validation for chromatographic and electrophoretic methods. Quim Nova, 2004; 27 (5): 771-80. In Portuguese.

[12] Huber L. Validation of Analytical Methods and Procedures. Available at: <http://www.labcompliance.com/tutorial/methods/default.aspx>. Accessed in February 17, 2011.

[13] Csuros M. Environmental sampling and analysis. In: Taylor and Francis editors, Lab Manual, Cleveland: CRC Press; 1997.

[14] Box GEP, Wetz J. (University of Wisconsin). Criteria for judging adequacy of estimation by an approximate response function. University of Wisconsin Technical Report. 9; 1973.