STUDIA UBB CHEMIA, LXI, 3, Tom II, 2016 (p. 345-353)
(RECOMMENDED CITATION)

Dedicated to Professor Emil Cordoș
on the occasion of his 80th anniversary

VALIDATION OF A METHOD FOR DETERMINATION OF FREE GLYCEROL IN BIODIESEL

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ABSTRACT. In this study, validation of a method for determination of free glycerol from biodiesel samples by using gas chromatography coupled with flame ionization detector (GC-FID) and the measurement uncertainty estimation was described. The derivatization reaction with N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was used to volatilize the glycerol prior to GC analysis. The 1,2,3-butanetriol was used as internal standard. Linearity, limit of detection, limit of quantification, precision and accuracy of the method were determined for the validation of the method. The limit of detection, estimated from chromatograms was 0.0006 % (w/w), while limit of quantification was 0.002 % (w/w). The recovery of free glycerol was determined by using certified reference material (CRMs) and was 102.4 ± 13.0 %. Also, the measurement uncertainty was estimated based on the bottom-up approach. The expanded uncertainty of the determination of free glycerol from biodiesel by GC-FID method was 16%.

***Keywords**: free glycerol, validation, measurement uncertainty, GC-FID*

INTRODUCTION

Biodiesel is the most important biofuel in the world and a promising alternative to conventional diesel [1]. The properties of biodiesel are very similar to those of diesel; have high flash point and cetane number comparative to diesel and does not contain sulphur or aromatics [2, 3].

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Biodiesel (also known as fatty acid methyl esters) is obtained through transesterification of vegetable oil or animal fats with methanol using a catalyst. The most used vegetable oils for biodiesel production are: rapeseed, sunflower and soy oil [4, 5]. Currently, microalgae and used cooking oil are tested for biodiesel production [6, 7]. The transesterification reaction produces glycerol as the main by-product and unreacted triacylglycerides (TAGs), monoacylglycerides (MAGs) and diacylglycerides (DAGs) [8, 9]. Production of biodiesel is expected to increase in the next few years [10].

There are several analytical methods that ca be used for glycerol determination in fuels sample, based mainly on chromatographic techniques [11-14]. The European Standard EN 14105 describe the standardized procedure for the analysis of free glycerol, TAGs, MAGs, DAGs and total glycerol from fat and oil derivatives [15].

The method for determination of free glycerol and derivatives is based on transformation of these compounds in more volatile compounds by derivatization with MSTFA in presence of pyridine, followed by gas chromatography coupled with flame ionization detector (GC-FID) analysis on a non-polar column. The silylation reaction with MSTFA implies the replacing of acidic hydrogen with the more volatile trimethylsilyle derivatizing group.

Validation and uncertainty estimation of free glycerol determination in biodiesel is necessary to produce reliable analytic data related to compliance of biodiesel quality with the European standards [16]. Based on information obtained in the validation process (limit of detection and limit of quantification, linear range, accuracy and precision) the uncertainty of the method can be estimated. Therefore, identification and quantification of all uncertainty sources that occur in the method is obligatory [17,18].

The rules for estimation of uncertainty are established in the International Organization for Standardization (ISO) guide [19]. In EURACHEM document is defined how the ISO guide are applied in chemical measurements [20]. For the measurement uncertainty estimation there are two approaches: top-down or bottom-up. In bottom-up approaches all the uncertainty sources are estimated and included in the uncertainty, while in top-down approach only the major uncertainty sources are take into account [17].

The aim of this study was to validate and to estimate the uncertainty for the determination of free glycerol in biodiesel samples using the standardized method EN 14105 [15]. The measurements uncertainty was evaluated based on bottom-up approach.

RESULTS AND DISCUSSIONS

Validation of free glycerol determination method

The validation of the determination of free glycerol method was performed by evaluation of the main figures of merit: limit of detection (LOD), limit of quantification (LOQ), working and linear range, accuracy and precision (both repeatability and reproducibility) according to the EURACHEM guide requirements [20].

The European standard EN 14214 requires the maximum limit of free glycerol of 0.02 % (w/w) in biodiesel in Europe [16]. The limit of quantification was targeted to be ten times smaller than the maximum amount of free glycerol (0.002 % (w/w)).

The LOD and LOQ were calculated using the 3s criteria by measuring the glycerol peak area in ten parallel samples with very low glycerol content. The LOQ for free glycerol was estimated to be nine times of standard deviation. LOD for free glycerol was established to be 0.0006% (w/w) and LOQ is 0.002 % (w/w). In Figure 1 is presented the chromatogram for a standard solution.

For evaluation of precision and accuracy of LOQ, six solutions of glycerol at a concentration of 0.002 % (w/w) were prepared and measured. The relative standard deviation (RSD) was 6.3 % and the recovery was 105%, and complies with the imposed target (RSD < 20% and recovery between 80 and 120%).

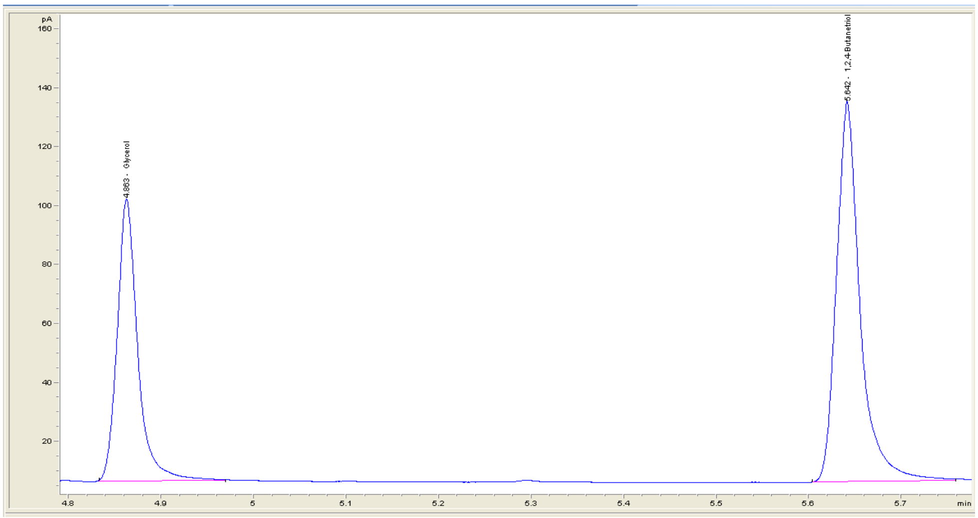

Figure 1. The chromatogram of the TMS derivatives of the standard solution

Working and linear range

Free glycerol was identified by comparison of the obtained retention time with the ones observed for standard solutions analyzed. Four calibration solutions (0.005 -0.05 mg of free glycerol) were prepared by diluting the stock solution of glycerol (500 µg.mL^{-1}). The derivatization procedure was applied for each standard solution. For the lowest and the highest concentrations ten measurements were made for evaluation of homogeneity of variance. The standard deviation was used to calculate PG ratio (s_1/s_4) which was compared with the Fischer value F(9;9;0.99) = 5.35. The determination coefficient of $r^2 > 0.9999$ proved the good linearity of the calibration curve. The statistical parameters of calibration curve for free glycerol were presented in Table 1.

Table 1. Statistical parameters of calibration curve for free glycerol

Parameter	**a**	**b**	**S_y**	**S_{x0}**	**V_{x0}**	**r^2**	**PG**
Free glycerol	-0.0027	1.22	0.013	0.0112	3.25	0.9999	4.3

a- intercept, b-slope, S_y – residual standard deviation, S_{x0} –standard deviation of the method (S_y/b), V_{x0} –coefficient of variation (S_{xo}*100/$X_{average}$), r^2- determination coefficient, PG- test value factor

Accuracy and precision

B100 Biodiesel (Soy-based) was used as certified reference material for evaluation of accuracy. For analysis of CRMs, the difference between certified value and measured value (Δm) must be lower than the expanded uncertainty obtained by combining the certified uncertainty (u_{crm}) with the uncertainty of the repeated glycerol measurements (u_m). The results obtained for analysis of CRM are presented in Table 2.

Table 2. Certified and measured values of free glyceron in B100 Biodiesel (Soy-based) CRM (mean ± expanded uncertainty, n = 6 parallel samples)

	Measured	**Certified**
Free glycerol	168 ± 28.1 mg kg^{-1}	164 ± 16.0 mg kg^{-1}

The methods recovery estimated by determination of free glycerol in CRM was 102.4 ± 13.0 %.

For estimation of the methods precision, the repeatability and reproducibility were determined. For the estimation of repeatability, six samples were analyzed. The limit of repeatability (r) represent the difference between two individual results, obtained by the same method and the same operator and must be below r = 0.1615*x + 0.0003, where x is the average of two results). The limit of repeatability (r) was 0.0012 % (w/w). The repeatability standard deviation was 0.00045 % (w/w).

The reproducibility of the method was determined by analyzing six real samples of biodiesel in ten different days using the same equipment. The limit of reproducibility (R) was 0.0062 % (w/w) and reproducibility standard deviation was 0.0025 % (w/w) and was below the reproducibility limit R = 0.1866*x + 0.0061. The precision of the method comply with that imposed by the EN 14214 standard (r < 0.002 % (w/w) and R below 0.0096 % (w/w)).

In Table 3 are presented the results obtained for validation of free glycerol from biodiesel samples.

Table 3. Results of method validation for the measurement of free glycerol

Validation Parameter	Results
Limit of detection	0.0006 %(w/w)
Limit of quantification	0.002 %(w/w)
Linear range	0.005 -0.05 mg
Accuracy for CRMs (recovery)	102.4 %
Precision (limit of repetability)	0.0012 %(w/w)
Precision (limit of reproductibility)	0.0096%(w/w)

Uncertainty estimation

For estimation of method's uncertainty all the uncertainty sources were identified and quantified and the combined uncertainty was calculated. The sources of uncertainty for glycerol determination are: the concentration of reference material, the concentration of internal standard, standard preparation (volumetric flasks, pipettes), the weight of samples, the uncertainty given by the calibration curve, precision of the method.

The concentration of glycerol is given on the certificate as 502.0 ± 2.5 ug mL^{-1}. Because in certificate is no additional information about the expanded uncertainty, a rectangular distribution is supposed. To obtain the standard uncertainty u_{rm} the value was divided by $\sqrt{3}$. The standard uncertainty u_{si} for internal standard was calculated by dividing by $\sqrt{3}$ the value given in certificate and the results is 2.89 ug.mL^{-1} (the concentration of 1,2,4-butanetriol given in certificate is 1003 ± 2.5 ug.mL^{-1}).

The preparation of stock and working solution for calibration curve gives a major source of uncertainty. The volumes of solution have three source of uncertainty: the uncertainty from the certificate of volumetric flask, the uncertainty given by the variation of the temperatures and standard deviation of repeated filling of the volumetric flask.

The weight of the sample and the analytic balance contribute also to the uncertainty of the method. The uncertainty given by the calibration curve (a linear function of first order) is calculated using Equation (1) [21,22]:

$$S_{x0} = \frac{S_y}{b} \bullet \sqrt{\frac{1}{N} + \frac{1}{m} + \frac{\left(\overline{y_0} - \overline{y}\right)^2}{b^2 \sum_{i=1}^{N} \left(x_i - \overline{x}\right)^2}} \qquad (1)$$

where: S_y –residual standard deviation; *b*- is the calculated best-fit gradient of the calibration curve; *N* – number of repeat measurements made on the sample; *m* –the number of paired calibration points ($x_i y_i$);$\overline{y_0}$ – the mean of N repeat measurements of y for the sample; $\overline{y}$ – the mean of the y value for the calibration standards, x_i – a value on the x-axis; $\overline{x}$ - the mean of the x_i axis. In Table 4 is presented the uncertainty components for determination of free glycerol by GC-FID.

Table 4. Uncertainty components for determination of free glycerol by GC-FID

Source	Unit	Value	Standard uncertainty	Interven-tions	Total standard uncertainty	Relative uncertainty
Concentration of standard	µg mL^{-1}	502	1.443	1	1.4433	0.0029
Concentration of internal standard	µg mL^{-1}	1003	2.886	1	2.8860	0.0029
Pipette	µL	10	0.360	1	0.3600	0.0360
Pipette	µL	40	0.360	1	0.3600	0.0090
Pipette	µL	70	0.361	1	0.3610	0.0052
Pipette	µL	100	0.363	1	0.3630	0.0036
Pipette	µL	80	0.361	5	1.8050	0.0226
Pipette	µL	150	0.301	4	1.2049	0.0080
Pipette	mL	8	0.023	5	0.1150	0.0144
Pipette	µL	200	0.301	3	0.9036	0.0045
Weight of sample	g	0.1	0.00005	1	0.0001	0.0005
Equipment	%	100	0.0010	1	0.0500	0.0005
Calibration	mg	0.343	0.0112	1	0.0112	0.0327
Reproducibility	%	0.017	0.0025	1	0.0025	0.1479

After measurement of all relative uncertainty for each source of uncertainty, the combined standard uncertainty (U_c) is calculated by combining all the uncertainty components by using law of propagation of uncertainty. The combined standard uncertainty was calculated to be 8.0 %. The expanded uncertainty (U_E) is obtained by multiplying combined standard uncertainty by a coverage factor (k) which is 2 for level of confidence of 95 % [23]. In Table 4 can be observed that the biggest contributors to uncertainty comes from reproducibility and calibration curve.

The concentration of standard, concentration of internal standard and weight of sample has a low contribution to the uncertainty. The expanded uncertainty for free glycerol determination in biodiesel by GC-FID method is 16 %.

CONCLUSIONS

In this paper, a GC-FID method has been applied for the analysis of free glycerol in biodiesel samples according to the EN 14105 standard. The validation of this method and measurement uncertainty evaluation was made by quantification of all uncertainty sources based on bottom-up approach. The accuracy was studied by evaluating the recovery of biodiesel using certified reference material. It was demonstrated that the method can be applied for determination of free glycerol in biodiesel samples by GC-FID.

EXPERIMENTAL SECTION

All chemicals were analytical reagent grade. N-heptane and pyridine were purchased from Merck (Darmstadt, Germany). Glycerol (glycerin standard), 1,2,4-butanetriol (internal standard) and derivatization agent N-Methyl-N-(trimethylsilyl)trifluoroacetamide were purchased from Sigma–Aldrich. Biodiesel 100 (Soy-based) SRM 2772 was used as CRM for the validation procedure. The volumes were measured using calibrated glassware (Hirschman, Germany).

The transformation of free glycerol into more volatile silylated derivative is based on the procedures described in EN 14105 [15].

The method consists in weighting approximately 100 mg of samples in a 10 mL vial and mixing it with 80 µL 1,2,3-butanetriol, 100 µL pyridine and 150 µL MSTFA under continuous shaking. After keeping 15 min at room temperature, 8 mL n-heptane was added to the solution. The solution was analyzed by GC-FID.

Working standard solutions of glycerol 500 µg.mL^{-1} were prepared by diluting glycerol stock standard solution (0.5 mg mL^{-1}) with pyridine. Calibration standard solution in the range of 0.005 - 0.05 mg (glycerol) were prepared by adding 10, 40, 70, 100 µL from working solution of glycerol. In each solution, 80 µL 1,2,3-butanetriol (as internal standard) and 150 µL MSTFA were added.

GC-MS analysis

Analyses were performed using a gas chromatograph (Agilent Technologies, 7890N GC) coupled with flame ionization detector (Agilent Technologies, 7683) and capillary column of 15 m length × 0.32 mm I.D. × 0.1 µm DB-5HT film thickness. The temperature program was as following: the initial oven temperature 50 °C, held for 1 min, from 50 to 180 °C via a ramp of 15°C/min, 180 to 230 °C at a ramp of 7°C/min and 230 to 370 °C at a ramp of 10 C/min for 15 min.

ACKNOWLEDGMENTS

This work was funded by Core Program, under the support of ANCS, project OPTRONICA IV (PN 16.40), Sectoral Operational Programme "Increase of Economic Competitiveness" Priority Axis II, (PN 1887, code SMIS-CSNR 49164) INOVA-OPTIMA and Romanian National Agency for Scientific Research, Project Number PN-II-PT-PCCA-2011-3.2-0452: "*Hydrogen production from hydroxylic compounds resulted as biomass processing wastes*" – HYCAT.

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