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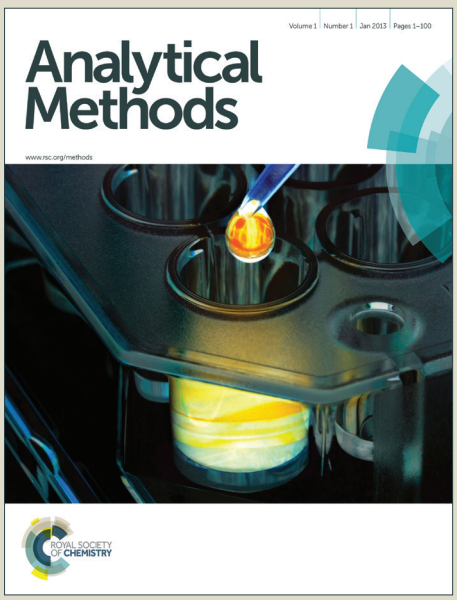

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Sensitive determination of glycerol by derivatization using HPLC-DAD method in Biodiesel Samples

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ABSTRACT

Biodiesel is mainly produced through transesterification reaction which yields biodiesel and glycerol, a by-product. This article describes quantification of glycerol by high performance liquid chromatography (HPLC) using diode array detector (DAD) in various biodiesel samples through post-derivatization. Glycerol was converted into a UV active product i.e. glyceryl tribenzoate (GTB) through a simple and effective esterification reaction using benzoyl chloride and copper chloride as catalyst under mild condition. Optimized reaction conditions (afford 100% yield) were obtained by using copper chloride (17 mol %), benzoyl chloride (12.0 equiv) and triethyl amine (11.0 equiv). The limit of detection and limit of quantification were found to be 0.23 µg/mL and 0.76 µg/mL, respectively. The developed and validated HPLC-DAD method is sensitive, selective, reproducible and successfully applied for the quantification of glycerol in biodiesel of various sources.

Keywords:

Biodiesel, glycerol, derivitization, glyceryl tribenzoate, high performance liquid chromatography

1.0.Introduction

Biodiesel is obtained by transesterification of neutral lipids from many biological sources which gives mixture of fatty acid methyl esters (FAMEs) (biodiesel) and a by-product glycerol [1]. Although glycerol is removed from biodiesel during purification step by washing, incomplete washing can leave behind traces of glycerol in the biodiesel. Presence of glycerol biodiesel can cause various problems such as corrosion of tank, clogging of fuel filter, damage to combustion system and production harmful gases during combustion such as acrolein. American Society for Testing and Materials (ASTM), and European Union standards and Brazilian regulatory agency "Agência Nacional do Petróleo" (National Petroleum Agency, ANP) for biodiesel have established the limit of maximum amount of free glycerol in biodiesel as 200 mg per Kg (0.02%)[2]. Therefore, it is necessary to determine the concentration of glycerol in purified biodiesel as quality assurance to check the quality of biodiesel. Various methods for the determination of free glycerol in biodiesel using thin layer chromatography (TLC) [3], supercritical fluid chromatography (SFC)[4], high performance liquid chromatography (HPLC)[5-8], gas chromatography (GC)[9], capillary electrophoresis (CE)[10], spectrophotometry[2,11,12], fluorimetery[13], potential cycling technique (voltammetry)[14] and amperometry[15] have been reported. Literature survey reveals that reported HPLC methods quantify glycerol in biodiesel are based on refractive index[6] and evaporative light scattering detections[5, 7, 8] while HPLC along with DAD/PDA detector is a common configuration for most HPLC systems operating in industries and research laboratories. Glycerol molecule lacks a chromophore to be detected by DAD/PDA and this puts a restriction on its quantification in biodiesel samples using HPLC-DAD/PDA. This paper describes the quantification of glycerol by conversion into a UV active derivative, glyceryl tribenzoate (GTB) using benzoyl chloride and copper chloride as a catalyst under mild condition.

Although, glycerol has been derivatized by using various methodologies[16, 17] and also quantified through derivatization in various biological samples including plasma, urine and tissues[18, 19] but to the best of our knowledge, this is the first report describing sensitive analysis of glycerol by derivatization in various biodiesel through HPLC-DAD.

2.0. Experimental

2.1. Chemical and reagents

Standard glycerol (assay ≥99.5%), glyceryl tribenzoate (GTB) (95%) and triethyl amine were purchased from Sigma-Aldrich (USA). Benzoyl chloride (synthesis grade) was purchased from Scharlau (Spain). HPLC grade methanol, acetonitrile, hexane and ethyl acetate were purchased from Tedia (USA). Tetrahydrofuran (THF) with sealed septa dried over molecular sieves (<0.001% H_2O) was purchased from ACROS organic (Belgium). Copper chloride dihydrate, ammonium chloride and magnesium sulfate anhydrous were purchased from Wako (China). Water was purified using a Millipore® Milli-Q Plus system (Bedford, USA). Sunflower and peanut oil seeds were purchased from local market. Dry biomass of mixed microalgal culture was obtained from Pakistan Council of Scientific and Industrial Research (PCSIR).

2.2. Esterification of glycerol into glycerol tribenzoate (GBT)

2.2.1. Standard protocol (Table-1, entry 9)

A dry 5.0 mL microwave vial equipped with a magnetic stirring bar containing 1.0 mmol (92 mg) of glycerol was sealed and flushed with nitrogen. After addition of anhydrous THF (3.0 mL) and Et_3N (1.5 mL, 11 mmol) through vial septum, BzCl (1.4 mL, 12 mmol) was added drop-wise with a flow rate of 15 drops/minute to the reaction mixture at room temperature with constant

stirring. After total 1 h (stirring), the resulting mixture was quenched by a solution of saturated NH_4Cl (5 mL). The resulting mixture was extracted twice with EtOAc (10 mL X 2). The EtOAc extracts were combined and dried over $MgSO_4$. Then evaporate on under reduced pressure.

2.2.2. Microwave procedure (Table-1, entry 10)

Microwave-assisted synthesis was carried out in an Initiator 8 single-mode microwave instrument producing controlled irradiation at 2.450 GHz (Biotage AB, Uppsala), including proprietary Workflow Manager software (version 2.1). Experiments were carried out in sealed microwave process vials (2 to 5 mL filling volume) utilizing the standard absorbance level (400 W maximum power). Reaction times under microwave conditions refer to hold times at the temperatures indicated, not to total irradiation times. The temperature was measured with an IR sensor on the outside of the reaction vessel.

2.3. Purification and spectroscopic data of GTB

The crude product was purified by preparative recycling HPLC system (LC-908, Japan Analytical Industry) on a preparative column Sil-D-60-10 (250 x 20 mm x 5µm) using Hexane/EtOAc (1:4) to provide the desired product GTB as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.05- 7.99 (m, t, J = 7.2 Hz, 6H), 7.57-7.52 (m, 3H), 7.44-7.38 (m, 6H), 5.81 (m, 1H), 4.69 (dd, J = 12.0, 4.5 Hz, 4H). HR-ESI-MS (*m/z*): 405.1321 (405.1338 calcd. for $C_{24}H_{21}O_6$). ^{1}H NMR spectra were recorded on a 300 MHz instrument Bruker AM-300. Chemical shifts (δ) are expressed in ppm downfield from TMS as internal standard. HR-ESI-MS analysis was carried out by using Qq-TOF-MS/MS instrument (QSTAR XL mass spectrometer, Applied Biosystem/MDS Sciex, Darmstadt, Germany).

2.4. Preparation of stock and calibration standard solutions

Stock solutions of glycerol and GTB were prepared by accurately weighing 5.0 mg of each into a 5 mL volumetric flask separately and making up the volume with water and acetonitrile, respectively. Calibration standard solutions ranging from 3-100 µg/mL of glycerol and GTB were prepared by dilution of stock solution with water and acetonitrile, respectively.

2.5. Preparation of biodiesel samples

Biomass (1kg) of plants (seeds)/microlagae including sunflower and peanut were soaked for three days in petroleum ether (2L each). Extracts were filtered and the solvent was removed with rotatory evaporator to obtain oil. The biodiesel was produced by basic methanolysis of all oils using a methanol/oil molar ratio of 12:1, with 1% potassium hydroxide by weight as the catalyst. The reaction temperature and time were 60 °C and 1 h, respectively [20]. After completion, the reaction mixture was transferred into a separating funnel, washed thoroughly with water and biodiesel was extracted with hexane (3x 1L) and dried over anhydrous magnesium sulfate. Hexane was evaporated from the biodiesel by using rotary evaporator (N-1000, Eyela, Japan).

2.6. Derivatization of biodiesel samples

1.0 g of each biodiesel was weighed and transferred to a dry 10.0 mL microwave vial equipped with a magnetic stirring bar. The vial was sealed and flushed with nitrogen. The sample was derivitized according to method described in section 2.2.1. The resulting mixture was quenched with a solution of saturated NH_4Cl (5 mL) and extracted with EtOAc (10 mL). The EtOAc extracts were combined and dried over $MgSO_4$. The solvent was evaporated and the residue was dissolved in 5 mL acetonitrile. The solution was filtered with 0.45 µm PTFE fluoropore syringe

driven filter unit (Millipore, Bedford, USA). Samples were preserved at 4 ºC prior to LC analysis.

2.7. HPLC analysis and method validation

HPLC analysis were performed with Agilent 1200 Series Rapid Resolution LC (RRLC) system comprising Agilent binary pump SL with degasser, high performance auto sampler SL with thermostat, thermostatted column compartment (TCC), diode-array detector SL (DAD SL) and evaporating light scattering detector (ELSD). Data acquisition and integration was controlled by Agilent Technologies Chem Station software. Agilent Poroshell 120 E-C18 column (50×3 mm I.D., 2.7 µm) and Zorbax XDB-C8 column (50×4.6 mm I.D., 1.8 µm) were used. The mobile phase was a binary gradient system prepared from water (eluent A) and acetonitrile (eluent B), properly filtered and degassed for 15 minutes in ultra sonic bath before use.

Method was validated by using various parameters including accuracy, precision, sensitivity, robustness, recovery study and specificity. The limit of detection (LOD) and limit of quantification (LOQ) were estimated by using calibration curve. The LOD and LOQ were calculated according to the following equation: LOD = 3.3δ/S and LOQ = 10δ/S, where δ = the residual standard deviation of a regression line or the standard deviation of Y-intercepts of regression line and S = the slope of the calibration curve. Precision and accuracy were determined at three different standard concentration levels including 20, 50 and 80 µg/mL. Method repeatability was evaluated in terms of coefficient of variance (CV) by repeating the analysis on the same day for intra-day precision. Intermediate precision was assessed by the analysis of same standard on different day (inter-day precision). Robustness of the method was determined by varying in the mobile phase composition, flow rate and temperature of column.

All parameters were varied within a range of ±5%. Mobile phase composition and flow rate were varied at initial and final state of gradient system. The effect of these variations on the result was examined as robustness of the method. For recovery studies, pre-analyzed samples were again prepared and spiked with known amount of the standard glycerol and the mixtures were analyzed by the developed method. Microalgal biodiesel samples (contained 0.05% *w/w* glycerol) were selected for conducting recovery studies. Five microalgal biodiesel samples were spiked with known amount of additional glycerol (0.5, 0.2, 0.1, 0.02 and 0.01% *w/w* glycerol) followed by derivatization. Samples were diluted before HLPC anlaysis to make in linear range. Recovery was calculated by the following equation: Recovery (%) = (sample contents after adding - original contents)/contents of added standard x 100. The selectivity of the method was ascertained by overlaying the chromatograms of standard and various biodiesel samples.

3.0. Results and Discussion

3.1. Reaction optimization for esterification

For reaction optimization, a well-planned examination by varying reactants amount at different temperatures was performed. Reaction was started at 1 mmol scale with pure glycerol sample while using 3.5 equiv of esterification reagent (benzoyl chloride), 12 mol% $CuCl_2$. $2H_2O$ as a catalyst and 1.5 equiv Et_3N as a base. Initial experiments showed that three possible esterified products of glycerol can be observed as glycerol tribenzoate (**3**), dibenzoate (**4**) and monobenzoate (**5**). However, presence of a single UV active esterification product for quantification of glycerol in biodiesel samples was necessary. Experiments showed that stoichiometric amount of benzoyl chloride (BzCl) and Et_3N were required for complete conversion of glycerol into glyceryl tribenzoate (**3**) as shown in Table 1 (entry 9). The reaction

time was monitored for glyceryl tribenzoate (**3**) at room temperature and completion was found in 1 hour. With considerable experiments, it was quickly realized that $CuCl_2$. $2H_2O$ catalyst was required 17 mol% in optimum. Stoichiometric amount of BzCl (12 equiv) was needed for promoting complete conversion to glyceryl tribenzoate (**3**). Reducing the amount of BzCl, resulted into predominant formation of mono- and di-tribenzoate by-products (Table-1, entry 2-3). To our gratification, we explored that the amount of Et_3N was effectively controlling the reaction completion and selectivity of product and by-products. After substantial efforts, it was identified that 11 equiv of Et_3N provided complete conversion (100%) into a single tribenzoate (**3**) product. Noticeably, HPLC conversion reflected the comparable isolated yield of compound (**3**) (Table 1). For example in entry 9, 90% yield of glycerol tribenzoate (**3**) was obtained after preparative HPLC. HPLC-ELSD chromatogram also confirmed the complete conversion of glycerol into GTB and showed peak for standard glycerol at RT 0.59 ± 0.03 whereas this peak disappeared from reaction mixture chromatogram of after derivitization (supplementary material Figure S1). GTB was identified by correlating NMR spectra and mass spectrometric data of the product with the standard compound. The use of higher reaction temperatures using microwave irradiation did not improve the efficiency of this transformation and led to a diminished amount of product due to the formation of undesired by-products (Table 1, entry 10 vs 5-6).

3.2. HPLC Method Optimization

The HPLC procedure was optimized to develop an accurate, reliable and rapid method for the analysis of GTB (derivitized glycerol) in the biodiesel samples. GTB showed λ_{max} at 238 nm through UV-Vis. scanning. The standard GTB and biodiesel samples were run in different gradient solvent systems of water, methanol and acetonitrile in which the water/acetonitrile gradient system was found optimum. The best results were obtained by using the stationary

phase as Agilent Poroshell 120 E-C18 column (50×3 mm I.D., 2.7 µm). This system showed sharp and symmetrical peak for GTB at retention time 5.95 ± 0.02 (Figure 1). Biodiesel samples become a complex mixture after derivitization reaction but the developed optimized HPLC conditions provide well separated peak of GTB from other constituents present in the samples for such a complex mixture. (Supplementary material Figure S2)

3.3. Method Validation

The linear concentration range for GTB was from 3-100 µg/mL with correlation coefficient of 0.9999. This linearity was evaluated by six standard working solutions. The method validation data including retention time, correlation coefficient, regression equation, LOD and LOQ are given in Table 2. LOD was found to be 0.23 µg/mL while LOQ was found to be 0.76 µg/mL for GTB. For the developed method, the precision was expressed in terms of percentage relative standard deviation (% R.S.D.). Precision was determined with two different analysts while reproducibility was determined by intra-day and inter-day analysis of GTB. The % R.S.D. for intra-day analysis for was found to be 0.39% in all cases. The accuracy of the method was in between 99.01-100.04% for intra-day analysis. The % R.S.D. for intra-day analysis for GTB was found to be < 0.43% in all cases. The accuracy of the method was in between 99.04-100.62% for inter-day analysis. The detailed data of accuracy and precision are summarized in Table S1.

In our method, robustness was evaluated by the tuning various parameters such as mobile phase composition, flow rate and temperature within ±5% variation from the proposed method. The standard deviation of peak area was calculated for each parameter and % R.S.D. was found to be less than 2.4% which indicates validity and robustness of the method. The data of all parameters for robustness are summarized in supplementary material Table S2.

Recovery studies were checked by the estimation of GTB from microalgal biodiesel sample after spiking with known amount of additional glycerol followed by derivatization. Recovery percentage was found to be 96-111% with percent relative standard deviation 0.5-3 (Table 3). Selectivity of GTB was assessed by comparing the spectra of standard and biodiesel of various sources. The overlaid spectra showed that there is no other peak at the retention time of GTB (Supplementary Figure S3).

3.4. Analysis of biodiesel samples

The developed method was applied for quantification of glycerol in various biodiesel samples. These samples include microalgal biodiesel, peanut biodiesel and sunflower biodiesel. All biodiesel were prepared in the laboratory by transestrification reaction (section 2.5). In all six biodiesel samples, GTB peak was observed at retention time (RT) 5.96 ± 0.01 min in the chromatogram for along with other components. GTB appears in the chromatogram at significantly different retention time as shown in figure S1 (supplementary material). The free glycerol content in the biodiesel samples was found to be 0.004-0.359% (%w/w) of biodiesel (Table 4). According to the United states and European Union standard, free glycerol limit is <0.02% for biodiesel only MB-1 and SB-1 found within the limit whereas other biodiesels have much higher concentration of free glycerol, which could be due to the improper washing of the biodiesel during purification step.

3.5. Comparison of developed HPLC method with other reported techniques

Comparative analysis of glycerol using HPLC-DAD after post derivatization with other reported techniques is summarized in Table 5. The comparison shows that in our HPLC-DAD method, limit of detection (LOD) is better than other reported techniques with acceptable coefficient of

variance (CV) except automatized flow-batch method for fluorescent determination of free glycerol[13]. Gratifyingly, the developed method is ten times more sensitive than other HPLC methods employing evaporative light scattering detector and refractive index detector.

4.0. Conclusion

The developed HPLC-DAD method is accurate, precise and robust for the determination of glycerol in the biodiesels. Statistical data showed that the method is reproducible and selective for the quantification of target analytes. Moreover, this method can be used as an alternative method for HPLC-RI and HPLC-ELSD with ten times enhanced sensitivity. All types of biodiesel samples can be analyzed within 8 minutes run by using a single HPLC method and followed by simple and low cost derivatization procedure for sample preparation.

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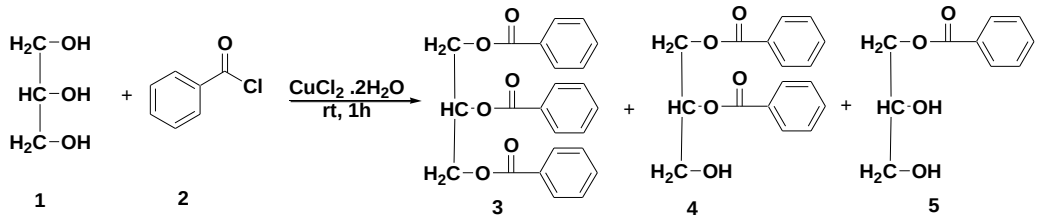
References

1. M. Stelmachowski, *Ecol. Chem. Eng. S*, 2011, **18**, 9-30.
2. M. S. Ribeiro and F. R. P. Rocha, *Microchemical Journal*, 2013, **106**, 23-26.
3. K. Bansal, J. McCrady, A. Hansen and K. Bhalerao, *Fuel*, 2008, **87**, 3369-3372.
4. J. Cole, J. Lefler and R. Chen, *Lc Gc Europe*, 2008, 44-46.
5. R. Li, N. Liang, S.-g. Li, W.-s. Wang, B. Chen and X.-x. Ma, *Fenxi Shiyanshi*, 2012, **31**, 36-39.
6. M. Hajek, F. Skopal and J. Machek, *European Journal of Lipid Science and Technology*, 2006, **108**, 666-669.
7. K. A. Blacksmith and L. Ott, American Chemical Society, 2013, **7**, 1419.
8. R. Li, W. Wang, X. Ma and B. Chen, *Fenxi Ceshi Xuebao*, 2011, **30**, 1167-1170.
9. C. Plank and E. Lorbeer, *Journal of Chromatography A*, 1995, **697**, 461-468.
10. L. C. Goncalves Filho and G. A. Micke, *Journal of Chromatography A*, 2007, **1154**, 477-480.
11. P. Bondioli and L. Della Bella, *European Journal of Lipid Science and Technology*, 2005, **107**, 153-157.
12. S. G. Silva and F. R. P. Rocha, *Talanta*, 2010, **83**, 559-564.
13. M. B. Lima, M. Insausti, C. E. Domini, M. F. Pistonesi, M. C. Ugulino de Araujo and B. S. Fernandez Band, *Talanta*, 2012, **89**, 21-26.
14. L. M. Lourenco and N. R. Stradiotto, *Talanta*, 2009, **79**, 92-96.
15. A. H. Maruta and T. R. L. C. Paixao, *Fuel*, 2012, **91**, 187-191.
16. P.Wipf and W.Xu. *Organic Chem*istry, 1993, **58**, 5880.
17. B. Jung, M. Sook Hong and S. H. Kang, *Angewandte Chemie*, 2007, **46**, 2616-2618.
18. Y. Dong, Y. Ma, K. Yan, L. Shen, X. Wang, Y. Xu, G. He, Y. Wu, J. Lu, Z. Yang, F. Feng, J*ournal of Chromatography B*, 2014, **957**, 30-35.
19. R.A. Frieler, D.J. Mitteness, M.Y. Golovko, H.M. Gienger, T.A. Rosenberger, *Journal of Chromatography B*, 2009, **877**, 3667-3672.
20. S. T. Keera, S. M. El Sabagh and A. R. Taman, *Fuel*, 2011, **90**, 42-47.
21. S. G. Silva, A. Morales-Rubio, M. de La Guardia and F. R. P. Rocha, *Anal. Bioanal. Chem.*, 2011, **401**, 365-371.

Table 1: Esterification of glycerol into glyceryl tribenzoate.



1 2 3 4 5

Entry	$CuCl_2$. $2H_2O$ (mol %)	BzCl (equiv)	Et_3N (equiv)	Product **3**[b] (%)	By-products **4**+**5**[b] (%)
1	12	3.5	1.5	<10	24
2	15	3.5	1.5	<10	28
3	15	6.0	3.0	15	35
4	15	12.0	3.0	19	45
5	30	12.0	3.0	20	48
6	17	12.0	5.5	35	50
7	17	12.0	7.0	56	28
8	17	12.0	10.0	95	traces
9	17	12.0	11.0	100[c]	–
10	30	12.0	5.0	<10[d]	–

[a]Reaction conditions: 1 mmol of glycerol **1**, 3.0 mL THF, stirring at room temperature for 1 h. [b]Product distribution refers to relative peak area (%)ratios of crude HPLC-UV (239 nm) traces.[c]Product isolation by preparative HPLC provided a 90% yield of glyceryl tribenzoate **3**. [d]Reaction conditions: sealed-vessel, single-mode, microwave irradiation at 120°C for 15 min.

Table 1: Retention time, correlation coefficient, regression equation, LOD and LOQ of GTB.

Retention time (min)	LOD (µg/mL)	LOQ (µg/mL)	Regression equation	r^2
5.99 ± 0.02	0.23	0.76	y = 4.787x + 0.912	0.999

Table 3: Recovery studies (n=3).

Added conc. of standard glycerol (% *w/w*)	Recovery (%)			Mean Recovery (%)	R.S.D. (%)
	1	2	3		
0.50	97	96	96	96.7 ± 0.5	0.5
0.10	106	101	107	105 ± 3	3.0
0.05	98.0	99	97	98 ± 1	1.1
0.02	106	105	110	107 ± 3	2.4
0.01	110	110	114	111 ± 2	2.2

*Original microalgal sample concentration was 0.05% *w/w*

Table 4: Analysis of biodiesel samples.

Biodiesel samples (Code)	Glycerol (%w/w)
Microalgal biodiesel 1 (MB-1)	0.004 ± 0.001
Microalgal biodiesel 2 (MB-2)	0.327± 0.008
Sunflower biodiesel 1(SB-1)	0.005 ± 0.001
Sunflower biodiesel 2 (SB-2)	0.36 ± 0.01
Peanut biodiesel 1(PB-1)	0.086± 0.004
Peanut biodiesel 2 (PB-2)	0.229± 0.001

Table 5: Comparison of glycerol analysis using HPLC-DAD after post derivatization with reported methods.

Technique	Linear range (ppm)	LOD (ppm)	CV (%)	Reference
HPLC-DAD	0.6-22.7	0.2	3.0	Present work
HPLC-ELSD	7.1-307.3	2.5	2.0	[5]
HPLC-RI	-	2	1.0	[6]
GC	1.0-6.0	-	1.3	[9]
CE	12-82	4.3	1.1	[10]
	4-80	0.4	2.1	[2]
Spectrophotometry	25-150	-	-	[11]
	5-50	1.0	1.5	[12]
TLC	-	2000	-	[3]
Fluorimetry	5-75	0.5	1.0	[21]
	0.1-5.0	0.04	1.5	[13]
Voltammetry	15-150	2.3	0.7	[14]
Amperometry	3-160	0.25	5.0	[15]

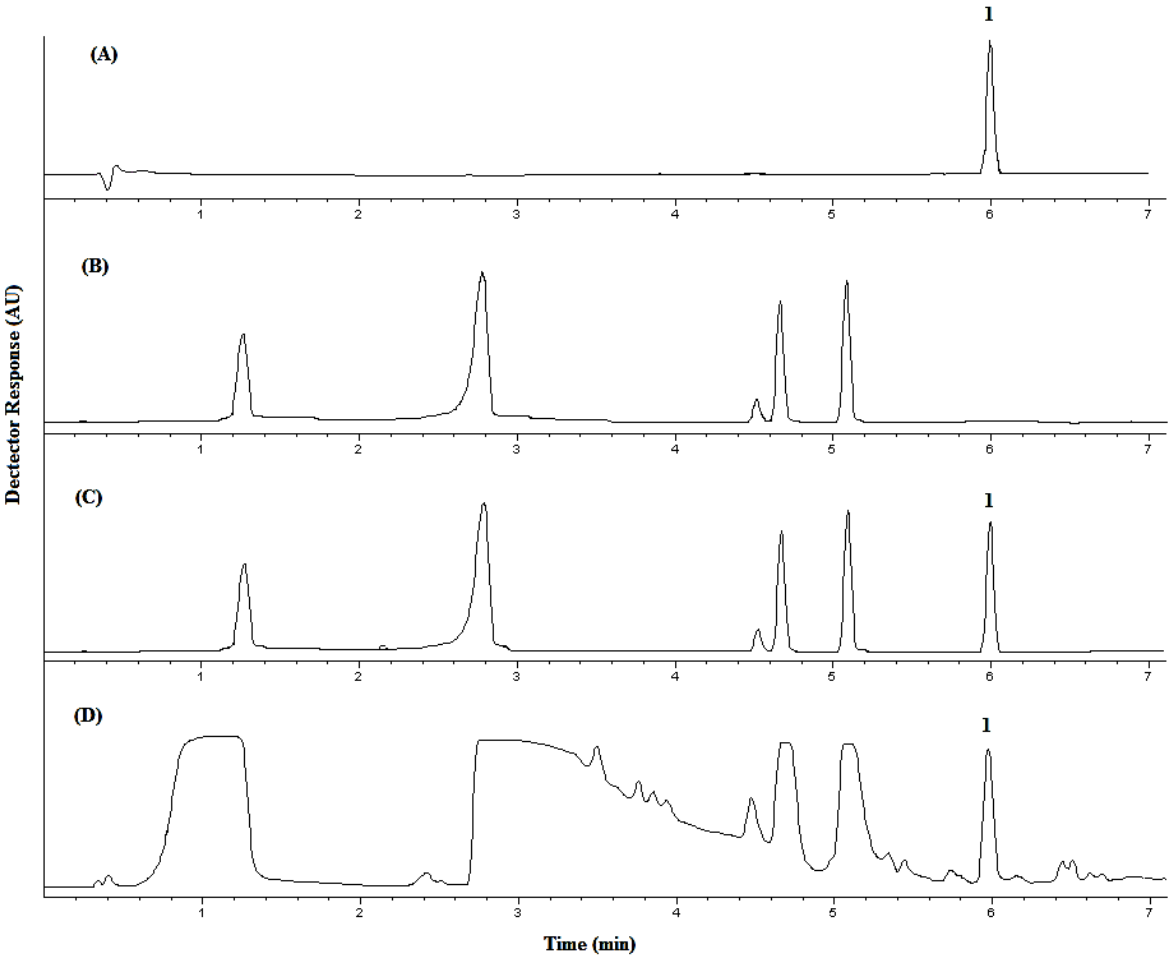


Figure 1: Chromatogram of (A) standard (B) blank (C) derivatized standard glycerol (D) derivatized sunflower biodiesel, Peak 1 GTB (Rt: 5.95 ± 0.02)