A simple fluorescence-based method for detecting total water, methanol, and glycerol in biodiesel.

by

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Abstract

As biodiesel production and consumption levels rapidly increase around the world, there is a need to monitor the process and the fuel quality. This is essential in order to increase the efficiency of its production and to ensure that products are of high quality and meet regulatory standards. Various factors can influence biodiesel fuel quality due to the nature of the starting material, the production process, and subsequent handling. Additionally, developing an analytical method for quality assessment in biodiesel is of great importance for biodiesel production quality control. This will improve the lifespan of the fuel and the engine, thereby enhancing the economic and environmental sustainability of biodiesel. The whole process of biodiesel analysis could be made more efficient by screening undesirable contaminants in the fuel using a simple, rapid, quicker, fluorescence-based method. These contaminants include methanol, glycerol, water, etc. They have been previously reported to be determined in biodiesel by several spectroscopic methods (like nuclear magnetic resonance and infrared spectroscopy) and chromatographic methods, but these methods require may expensive equipment and sophisticated sample pretreatment procedures. They are also time-consuming and require a high level of technical knowledge. In contrast, fluorescence methods have the main advantage that they can be quickly used to determine whether any or all of these contaminants are present, together, at concentrations above the specified standard level.

In this study, Nile red was used as a fluorescent dye for the detection of total water, methanol, and glycerol in biodiesel. The fluorescent probe showed good fluorescence emission and a solvatochromic red shift sufficient to detect polar liquids as they were incorporated into the biodiesel. The calibration curves as well as some figures of merit such as limit of detection, (LOD), and limit of quantification (LOQ) were also reported. The results of this report suggest

that the biodiesel used for this experiment incorporates appreciable polar liquids contaminants which may exceed ASTM standards. However, the result needs to be further tested by more selective methods.

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Chapter 1 - Introduction

1.1 Background of the Study

Climate change, rising crude oil prices, the depletion of the fossil fuel supply and the pollution caused by consumption of fossil fuels in the environment, particularly greenhouse gas emissions, have put pressure on society to find renewable alternatives and efficient utilization of agro-based products aimed at obtaining value-added bio-products, including biofuels, bio chemicals and biomaterials.[1,2,3,4] Additionally, the increase in global population requires additional resources to deliver energy for human consumption.[5,6] Most of the energy we use today is derived from fossil fuels. But fossil fuels have a disadvantage that they are limited in nature and the burning of fossil fuels also releases massive amounts of pollutants such as CO_2 which are detrimental to the environment.[7] In many countries of the world, America inclusive, energy consumption is based on imported refined fossil fuel, but there is a need for alternative sources of energy which are more secure and produce less greenhouse gases and which can successfully compete with fossil fuels, in terms of cost and quantity.[3,8] Many countries of the world, especially the advanced nations, devote millions of dollars to research in the quest for alternative energy that can meet the needs of the populace in terms of adequate energy provision and environmental safety. Thus, scientists are considering renewable energy to fulfil these demands.[5,7,9] Renewable energy is referred to as clean energy that comes from sources which are natural, such as wind, solar power, and biomass. They are replenished by natural processes and do not cause environmental pollution.[5] Biofuels such as biodiesel from renewable resources are today already viable alternatives to fossil fuels. Biodiesel releases less new carbon dioxide to the atmosphere than do petroleum-based diesel fuels because the former is made from plants that

absorb carbon from the atmosphere as they grow.[9] Biodiesel can either be used alone, or combined with petroleum diesel in certain proportions.[10]

As biodiesel production and consumption levels rapidly increase around the world, there is a need to monitor the production process and the fuel quality. This is essential in order to increase the efficiency of its production and to ensure that products are of high quality and meet ASTM (American Society for Testing and Materials) standards. While extensive testing is already performed, these tests are often complicated, expensive, time consuming, and require a high level of technical knowledge. The whole process could be made more efficient by implementing simple tests that can be used to quickly determine whether a fuel meets ASTM standards or whether more involved testing is required. This could be achieved by screening for undesirable contaminants in the fuel using a simple, rapid, fluorescence-based method. Common polar liquids which are contaminants in biodiesel include water, methanol, and glycerol. These polar liquids have been previously reported to be determined in biodiesel by spectroscopic methods (like nuclear magnetic resonance and infrared spectroscopy) and chromatographic methods, but these methods require expensive equipment or are time consuming.[15,16,19]

This thesis explores the use of a novel fluorescence-based method as a simpler, and quicker alternative. A solvatochromic dye called Nile red is applied as an optical sensor to detect the possible presence of polar liquid contaminants such as water, glycerol, and methanol in biodiesel. The method has the main advantage that it can be quickly used to determine whether any or all of these contaminants are present, together, at aggregate above the ASTM level. The benefits of the use of fluorescence as a technique for detecting undesirable polar liquids as contaminants (i.e., water, methanol, and glycerol) in biodiesel are presented.

1.2 Nile Red

Nile red is a lipophilic fluorescent dye that is commonly used for staining lipids with varying colors from deep red to strong yellow-gold emission.[11,12] It is strongly fluorescent, but only in relatively non-polar environments. While Nile red is very poorly soluble in aqueous media, it is appropriately soluble in organic solvents such as biodiesel. The absorption and emission spectra of Nile red depend on the polarity of its environment.[32] Furthermore, its fluorescence is much stronger in apolar and aprotic solvents than in polar and protic solvents.[1,12] It has been used in various fluorescence-based methods, including flow cytometry, fluorescence microscopy, and spectrofluorometry.

In flow cytometry, Nile red is commonly used as a stain to quantify the lipid content of cells.[29] The fluorescence intensity of Nile red is proportional to the amount of lipids present, allowing for the quantification of lipid accumulation in cells.[30] For example, Nile red has been used to measure the lipid content of microalgae cells in order to optimize their lipid production for biofuel purposes. In fluorescence microscopy, Nile red has been used to visualize and quantify lipid droplets in cells.[30,31] It has been used to study lipid metabolism in various cell types, including adipocytes and hepatocytes.[31] Nile red can be used to label lipid droplets in live cells, allowing for real-time visualization of lipid accumulation and mobilization.[31] This technique has been used to study the effects of various factors on lipid metabolism, such as hormones, drugs, and dietary changes.[11,12] Nile red has also proven to be a versatile tool in various fields of research, including biochemistry, environmental science, and microbial ecology, due to its ability to selectively bind to hydrophobic regions of lipids.[32] Its fluorescence and solvatochromic properties make it a valuable asset in these fields.

The emission spectrum of Nile red shifts to shorter wavelengths in the presence of lipids, allowing for the quantification of lipid content. This technique has been used to measure lipid accumulation in various tissues and organs, such as the liver, adipose tissue, and the brain.[12]

1.3 Biodiesel Production

Biodiesel is a liquid fuel typically consisting of long chain fatty acid esters. The long hydrocarbon chains are similar to those in petroleum diesel. Biodiesel is a renewable, clean-burning diesel which can be made domestically from vegetable or agricultural oils, animal fats, micro and macro algal oil.[4,20,21] It has also been confirmed that biodiesel can be produced from used cooking oil or waste oil.[21,22,25] However, these waste oils present special challenges for biodiesel production because they contain contaminants such as water, meat scraps, and breading that must be filtered out before the oil is converted to biodiesel.[22] In addition, the used oils also contain high percentage of free fatty acids (FFAs) which tend to react with the alkali catalyst in biodiesel production to form soap instead of biodiesel. This reduces the level of free catalyst as well as the speed of the transesterification reaction.[22] Biodiesel is also known for its renewability, biodegradability, nontoxicity, and carbon neutrality and as a result, has long been researched as an alternative fuel for diesel engines.[4] Biodiesel has also found application in domestic and commercial boilers as a heating fuel where a blend of heating oil and standardized biofuel is used.[5,27]

The term biodiesel signifies the substitution of traditional petroleum energy sources with an inexhaustible renewable biofuel. Biodiesel is obtained from vegetable oils through a process known as transesterification.[1,4,15,18] This process involves reaction of a triglyceride molecule (oil or fat) made of three fatty acids and a glycerol molecule with an alcohol (usually methanol or

ethanol added in excess) producing three molecules of biodiesel (called “fatty-acid methyl esters” or “ethyl esters”) and one molecule of glycerol that can be removed for use in chemical applications, food processing, and other settings.[1,3,4,13] The reaction is usually run in the presence of catalysts such as NaOH, KOH and enzymes. The catalyst is usually used to activate the transesterification process and to improve the reaction rate and yield.[4,18] Due to its low cost and relatively smaller size, methanol is the most common alcohol used for biodiesel production.[3,16] As the trans-esterification process goes to completion, the reaction flow is separated into two phases: the biodiesel phase (top layer) and glycerol rich phase (bottom layer).[3,19] Figures 1.1 and 1.2 show the chemical equation and schematic diagram respectively for fatty acid methyl ester production from triglycerides by the transesterification process in the presence of a catalyst.

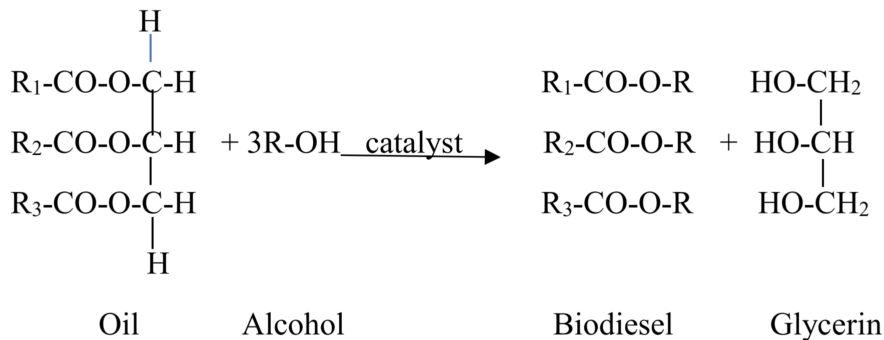

Figure 1.1. Transesterification of triglyceride with alcohol in the presence of catalyst.

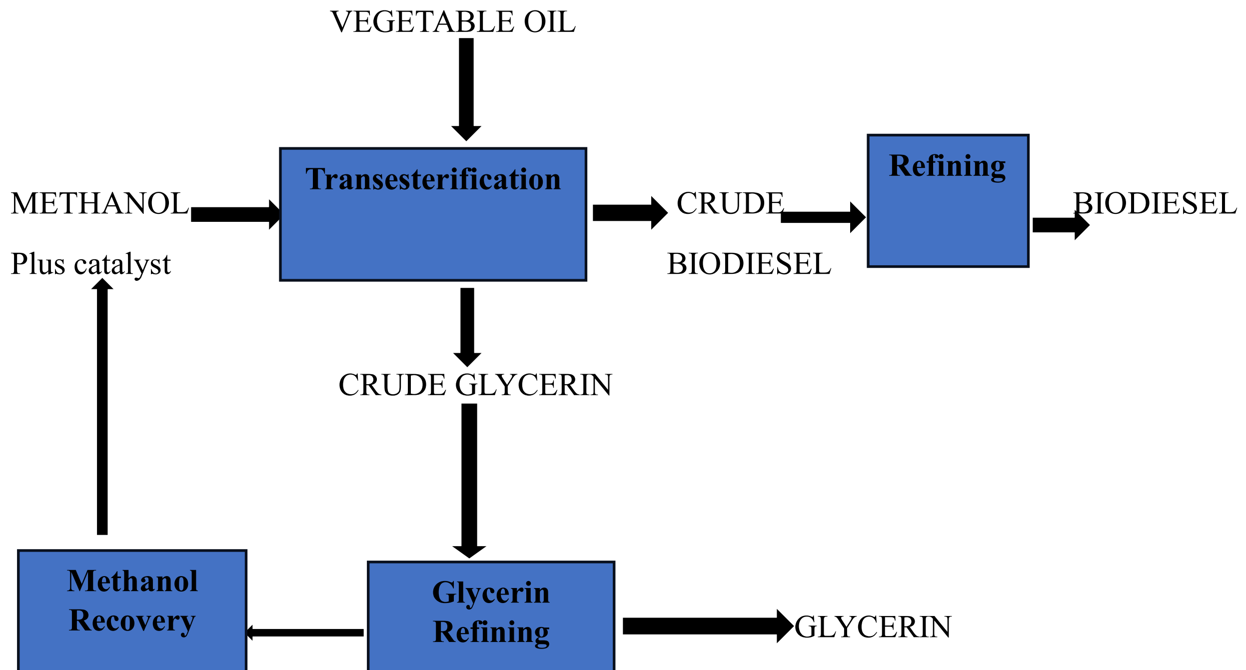

Figure 1.2. Schematic of biodiesel production path.

The transesterification process consists of a series of three consecutive reversible reactions. The initial step involves the conversion of triglycerides to diglycerides, which is further reduced to monoglycerides. In the third step, monoglycerides are converted to glycerol. Thus, one molecule of ester (biodiesel) is produced in each of the three steps.[14,17] The present study has shown that the high polar liquids content of the biodiesel might be due to the presence of residual water, methanol, glycerol or mono- and di-glycerides generated duriing the transesterification process. The mechanism of the reaction is shown in Figure 1.3 below.

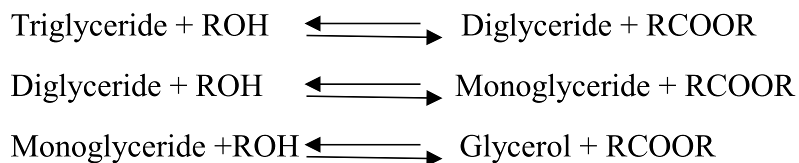

Figure 1.3. General equation for transesterification of triglyceride.

1.4 Advantages of Biodiesel over Conventional Diesel

Biodiesel is environmentally friendly and has several advantages over conventional petroleum diesel which is a non-renewable fuel derived from crude oil. First, biodiesel comes from renewable resources i.e., plants and not from finite resources - resources that are not easily replenished by the environment (i.e coal, natural gas, petroleum).[4] Another benefit over fossil fuel is the mitigation of greenhouse gases. This is because the carbon dioxide released during combustion of biodiesel is offset by the carbon dioxide absorbed while growing the feedstocks used for the production of the fuel.[23] Additionally, utilization of biodiesel results in significant reductions in emissions of carbon monoxide, particulate matter, unburned hydrocarbons, sulfates, and carcinogenic compounds compared to petroleum diesel fuel.[20] This is because biodiesel is an oxygenated fuel, unlike petroleum diesel. The added oxygen helps in completing the combustion process.[20] Furthermore, biodiesel is biodegradable, safer, easy to store and far less toxic than fossil fuels. It degrades about four times faster than petroleum diesel both in soil and water. In an aquatic environment, biodiesel is 15 times less toxic to common species of fish than petroleum diesel.[5,20,23] Although biodiesel has physical properties that are similar to those of petroleum diesel, it's cetane number and lubricity are higher and as a result of this, it combusts easier and lubricates the fuel system better.[2]

Furthermore, utilization of biodiesel as an energy resource will boost the domestic economy as it will offer new energy-related markets to local farmers. In other words, it reduces the use of foreign oils and helps to keep energy money at home.[5] In general, using biodiesel as a transportation fuel shows great potential as an alternative liquid fuel, enhances energy security, improves the quality of air and environment as well as provides safety benefits.[20]

1.5 Biodiesel Blends

Biodiesel is most commonly used by blending it into petroleum diesel fuel. This reduces the quantity of petroleum diesel used, while also taking advantage of the benefits of biodiesel mentioned above. Blending is the process whereby biodiesel and petroleum diesel fuel are mixed.[26] The two liquids are usually mixed in a certain proportion to meet the requirements of either ASTM D975 (blends up to 5%) or ASTM D7467 (blends between 6 and 20% biodiesel).[20] The blend obtained is usually designated by BXX where XX represents the biodiesel content of the blend. Typical blends are listed below.

- B100 (100% or pure biodiesel)
- B20 (20% biodiesel, 80% petroleum diesel)
- B7 (7% biodiesel, 93% petroleum diesel)
- B5 (5% biodiesel, 95% petroleum diesel)
- B2 (2% biodiesel, 98% petroleum diesel).

Pure biodiesel (B100) is generally used as a blendstock to produce lower blends which are commonly distributed for use in the retail diesel fuel marketplace.[12,20] While these biodiesel blends are readily available in developed countries, B20 is mostly used and commercially available in gas stations due to its numerous benefits such as cold-weather performance, good balance of cost, materials compatibility, and ability to act as a solvent.[20] Biodiesel blends such as B20, usually have a slightly lower energy content than petroleum diesel, but the effect on fuel economy is negligible.

In general, using biodiesel blends is a promising alternative to traditional fossil fuels. The development of new and innovative methods for biodiesel production and quality control, such as the simple fluorescence method for the determination of total water, methanol, and glycerol in biodiese explored here, can help to ensure the optimal performance and compliance of biodiesel blends with industry standards.

1.6 Biodiesel Standards

In the United States the quality requirements of biodiesel fuels are typically based on the specifications of ASTM International. These specifications are of various types for different biodiesel concentrations. Biodiesel blends of low-level up to B5 must meet the specification of ASTM D975. These biodiesel blends are often called diesel fuel because they can operate in any compression-ignition engine designed to be operated on petroleum diesel.[20] Thus, they have physical properties similar to that of conventional diesel fuel.[21] The specifications based on ASTM D6751 and ASTM D7467 are assigned to B100 and B6-B20 respectively. Tables 1.1 shows selected ASTM International requirements for B100.

Table 1.1. Selected ASTM D6751 requirements for 100% biodiesel (B100).

Cold soak filterability, s, max	D7501	200	200	360		360
Requirements for All Grades						
Calcium and Magnesium, combined, ppm, max		EN14538	5	5	5	5
Flash point (closed cup), °C, min		D93	93	93	93	93

Alcohol Control: **One of the following must be met**					
(1) Methanol content, mass%, max	EN14110	0.2	0.2	0.2	0.2
(2) Flash point, °C, min	D93	130	130	130	130
Water and sediment, % volume, max	D2709	0.050	0.050	0.050	0.050
Kinematic viscosity, mm^2/s, 40°C	D445	1.9-6.0	1.9-6.0	1.9-6.0	1.9-6.0
Cetane number, **min**	D613	47	47	47	47
Cloud point, °C	D2500	Report	Report	Report	Report
Carbon residue[a], % mass, max	D4530	0.050	0.050	0.050	0.050
Acid number, mg KOH/g, max	D664	0.50	0.50	0.50	0.50
Free glycerin, % mass, max	D6584	0.020	0.020	0.020	0.020
Total glycerin, % mass, max	D6584	0.240	0.240	0.240	0.240

Source: Biodiesel Handling and Use Guide (*Fifth Edition*). *National Renewable Energy.*[20]

1.7 Contaminants found in Biodiesel

Biodiesel production involves a simple chemical process, as described in section 1.3, and its application as an alternative fuel for diesel engines has rapidly increased due to reduction in lifecycle carbon emissions and emissions of toxic air pollutants.[20] The quality assessment of biodiesel before consumption is essential to comply with international standards. The quality of

biodiesel is dependent on the purity and composition of the feedstocks and the production process. Impurities, such as water, glycerol, and methanol, can affect the performance and stability of the fuel.[27] Therefore, accurate and reliable methods for determining these impurities in biodiesel are necessary.[9,10] When biodiesel is produced, it is usually washed with water to remove residual catalyst (sodium hydroxide), glycerol, and methanol.[4,24,28] Although, the process is broadly adopted in the biodiesel industry, it takes time for the water to separate from the fuel, and certain conditions may lead to the formation of an emulsion, possibly preventing separation.[4] Research has also shown that the presence of excess methanol in biodiesel makes glycerol and water somewhat difficult to separate from the fuel. Thus, the fuel may be contaminated with water, glycerol, and methanol.[20] Glycerol and methanol content are usually quantified or determined by gas chromatography (GC) or high-performance liquid chromatography (HPLC), while water content is determined by centrifugation.[16] Fluorescence based methods have recently been used to determine these parameters with the aim of ensuring high quality fuels are obtained with less time and effort spent on testing. Other parameters such as residual catalyst, bound glycerin, free fatty acids, soaps, and the products of oxidation have also been reported as potential contaminants found in biodiesel.[16] These contaminants generally can cause undesirable engine problems such as carbon deposits, lacquer deposits on the injector tips, injector tip coking, and deposits in the combustion chamber.[15]

However, quality standards and specifications such as ASTM and EN 14214 have been developed to place limits on the amounts of contaminants that may be present in biodiesel. In this context water, methanol, and glycerol are considered to be major contaminants in biodiesel.

1.7.1 Contamination by Water

The main important contaminant encountered during biodiesel production is water because biodiesel is hygroscopic in nature.[3] The quantity may be small, but its content is problematic and is a major issue of concern.[3,4] Water typically comes in contact with fuel tanks through vents and seals as humidity in the air. Water is present in the fuel as a dissolved substance and the amount dissolved depends on its solubility. It is also present in the fuel as free water collected at the bottom of the sample.[15] A major problem known with free water is microbial growth.[16] The presence of water can also cause decomposition of esters through their hydrolysis, thereby changing the composition of the fuel and its properties (Figure 1.4). The degradation level, however, increases to a great extent when the fuel is exposed to air and high temperature.[24]

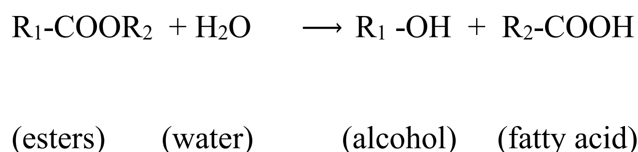

Figure 1.4. Hydrolysis of ester.

When biodiesel is contaminated by water, it can also cause corrosion of fuel system components such as the engine, pumps, fuel lines, etc. Prolonged water contamination can lead to acid corrosion, which may attack fuel storage tanks. The presence of organisms such as yeast, fungi, and bacteria facilitated by water contamination usually convert the sulphur in the fuel to sulphuric acid which can corrode metal fuel tanks. In addition, the presence of water reduces the heat of fuel combustion.[12]

During the production process, water contamination also reduces the activity of the catalyst which aids the reaction of free fatty acids with the catalyst to produce saponified product. Thus, biodiesel yield is reduced by the formation of soap leading to significant difficulty in product separation and purification.[17,18]

1.7.2 Contamination by Methanol

Methanol is the simplest form of alcohol used in biodiesel production, although ethanol is used in some cases as well. Alcohol has the general formula $C_nH_{2n+1}OH$, where n=1 (i.e. CH_3OH) in the case of methanol. Methanol is often abbreviated as MeOH. Figure 1.5 shows the chemical structure of methanol.

Methanol is a colorless and highly volatile liquid. It is one of the major contaminants encountered in biodiesel.[13] The methanol content of biodiesel must be < 0.2% (by mass) according to ASTM D6751.When soybean oil is treated with 100% excess methanol during the transesterification process, about half of the excess methanol is distributed in each phase (biodiesel, top layer and glycerin, bottom layer).[3,18] When the transesterification reaction is complete, the biodiesel is washed with water and there is likely little alcohol left since the alcohol is more soluble in the water than in the biodiesel. However, if even a little alcohol remains as a contaminant in biodiesel, it can alter its flash point, and heating value as well as degrade rubber components in the fuel system. The formation of carbon deposits in the engine can also be traced to the presence of excess alcohol in biodiesel.[15] It has been reported that methanol in biodiesel at high concentrations (>5%) could affect fuel lubricity and cetane number but low concentrations show no measurable effects.[16] Furthermore, an excess of methanol

increases the cost of biodiesel production and prevents complete separation of glycerol from biodiesel in the presence of excess catalyst.[15]

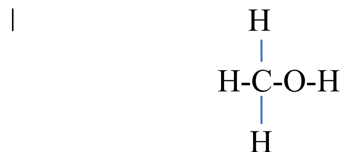

Figure 1.5. Chemical structure of methanol.

1.7.3 Contamination by Glycerol

Glycerol, also called glycerin, is a byproduct of biodiesel production and of the saponification process. In the soap making process, glycerol is produced as a byproduct from the reaction between fats and oils and a strong base.[13] In biodiesel production, it is one of the main byproducts after transesterification that determines fuel quality and shows the effectiveness of the production process.[1] It is a common substance found in almost all cosmetics and many pharmaceuticals. Its molecular formula is $C_3H_8O_3$ and its structure is shown in Figure 1.6. It is a colorless hygroscopic liquid and contributes to processes such as corrosion and fuel oxidation. It has no characteristic odor.[16] It is an organic compound with the IUPAC name of 1,2,3-Propanetriol. The 3 hydroxyl groups (-OH) make it soluble in water. Glycerol is present in biodiesel due to insufficient separation of the glycerin phase or insufficient washing by water after separation. The presence of glycerol in biodiesel can cause problems associated with fuel filters when it settles in the fuel tank. Namely, it can clog fuel filters and accelerate the wear and tear of the entire combustion system. In addition, glycerol may accumulate around the injector valve heads and hinder engine performance.[20,24] It has been reported that deposit formation in the engine may be caused by glycerol contamination in biodiesel. High concentrations of free

glycerol were one of the main causes in the past for filter blocking and general failure in diesel systems.[17]

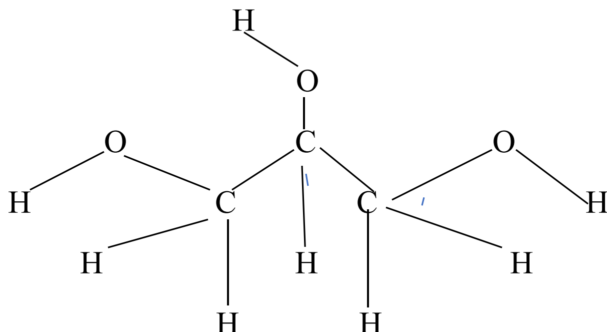

Figure 1.6. Chemical structure of glycerol.

1.8 Justification

Biodiesel is an increasingly popular alternative to traditional fossil fuels due to its renewable nature, lower carbon footprint, and reduced emissions of harmful pollutants. However, quality control of biodiesel production is crucial to ensure its optimal performance, stability, and compliance with industry standards. One of the critical quality parameters in biodiesel production is the determination of the total water, methanol, and glycerol content. These compounds are known to affect biodiesel's physical and chemical properties, leading to potential engine problems, such as corrosion, fuel filter plugging, and decreased fuel efficiency.

Several analytical methods have been developed for the quantification of water, methanol, and glycerol in biodiesel. These methods usually employ gas chromatography, high-performance liquid chromatography, or titration procedures. These methods may require expensive equipment, complex sample preparation, and lengthy analysis time, which may not be suitable for routine quality control purposes, especially in small-scale biodiesel production facilities.[15,16]

Therefore, development of a simple, rapid, and cost-effective analytical method for determining water, methanol, and glycerol in biodiesel is highly desirable. Fluorescence spectroscopy is a promising technique widely used for analyzing various organic compounds due to its high sensitivity, selectivity, and simplicity.[33,34] However, the application of fluorescence spectroscopy for the analysis of water, methanol, and glycerol in biodiesel has been limited.

In this context, the research performed for this thesis aims to develop a simple fluorescence method for the determination of total (i.e.; aggregate) water, methanol, and glycerol in biodiesel. The proposed method involves the use of a fluorescence probe that exhibits selective and sensitive responses to polar liquids such as water, methanol, and glycerol in biodiesel samples. The fluorescence intensity of the probe will be measured using a fluorescence spectrometer, and the total water, methanol, and glycerol content will be determined based on the fluorescence signal. The vision is that this method could be used to detect excess polar contaminants on the whole, based on a single measurement of a dyed sample. As will be shown below, it cannot be used to determine individual concentrations of polar contaminants, or to identify these contaminants. It is meant only as a means to quickly determine whether the fuel meets ASTM standards in this regard, or if additional testing by other methods is needed.

1.9 Aim of the Research

The aims of this research are:

1. to develop a rapid, simple, semi-quantitative means of determining whether a sample of biodiesel meets ASTM standards for polar liquid (i.e., aggregate, or total glycerol, methanol, and water) content.

2. to evaluate the sensitivity and selectivity of the fluorescence method for the detection of water, methanol, and glycerol in biodiesel samples.
3. to assess the potential application of the proposed method for routine quality control purposes in small-scale biodiesel production facilities.
4. to investigate the limit of detection, limit of quantification and linearity of the proposed method.

1.10 Objectives of the Research

The main objective of the research is to develop a simple, rapid, and cost-effective method for the determination whether the biodiesel meets standards with respect to total content of polar liquids such as water, methanol, and glycerol. This method can contribute to the improvement of biodiesel quality control and ensure its optimal performance and compliance with ASTM standards. Namely, this method was developed to allow for a single measurement to be used to verify if a sample of fuel meets ASTM standards for polar liquids. A failure of this test would indicate that further testing with other common chemically selective methods are required to determine the source of the contamination.

Chapter 2 - Materials and Method

2.1 Materials

2.1.1 Glassware Cleaning

All glassware used (Erlenmeyer flasks, measuring cylinders, beakers, volumetric flasks, and vials) were properly washed with non-ionic liquid detergent, and rinsed with distilled water. They were then air dried and cleaned in a Harrick Plasma Cleaner (PDC-32G) prior to use. Figure 2.1 shows the basic plasma cleaner used during the experiment. Each glassware item was treated for at least 3 minutes in the plasma cleaner. These processes are required to get rid of contaminants that may stick to the materials probably from the surrounding environment. Micropipettes as well as glass pipettes were also used to measure and transfer accurate volumes of liquid.

Figure 2.1. Harrick Plasma Cleaner photo by Samson Adegbite.

2.1.2 Reagents and Sources

All reagents used were of analytical grade and were directly used without further purification. Methanol was purchased from Avantor Performance Materials, Inc, (Pennsylvania, United States). Glycerol and Nile red dye were both purchased from Sigma Aldrich, (United States). The Nile red dye, which is one of the most polarity-sensitive fluorescent probes known was selected and used to determine its sensitivity to trace amounts of glycerol, methanol, and water in biodiesel. Additionally, methyl decanoate was purchased from Fisher Scientific (Portland, America). It was used along with Nile red dye to determine the fluorescence properties of the polar liquids prior to determination in biodiesel by the fluorescence-based method. Methyl decanoate, often called methyl caprate, belongs to the class of organic compounds known as fatty acid methyl esters and is produced via esterification of decanoic acid with methanol in a reactive distillation. It was used here as a simple model for biodiesel. Table 2.1 and Figure 2.2 show the properties and chemical structure of methyl decanoate.

2.1.2.1 Physicochemical Properties of Methyl Decanoate

Table 2.1. Properties of methyl decanoate.

Boiling point	224 °C (1013 Pa)
Density	0.87 g/cm3 (20 °C)
Explosion limit	0.7 %(V)

Flash point	110.5 °C
Formula	$C_{11}H_{22}O_2$
Molar Mass	186.3 g/mol
Melting Point	-18 °C
Vapor pressure	0.0493 Pa (25 °C)
Solubility	0.0038 g/l

Source: Millipore Sigma.[44]

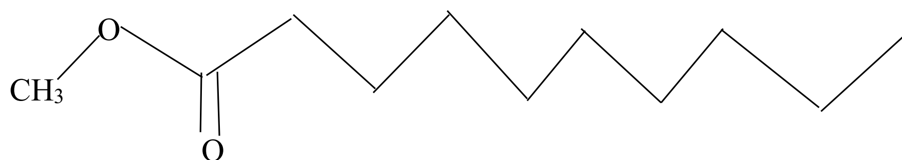

Figure 2.2. Chemical structure of methyl decanoate.

2.1.3 Instrumentation

A Branson ultrasonic bath was used for preparation of homogenous mixtures of the solutions and samples used. Fluorescence emission spectra were obtained from Nile red in the sample using a benchtop fluorescence spectrophotometer (FluoroMax-2). The bench system is based on Czerny-Turner spectrometers, a photomultiplier (R928P), and an ozone-free xenon lamp. An excitation wavelength of 532 nm and scan range of 540 nm-700 nm were used in all spectra. The data was acquired at 1 nm increment with 1 second integration times. The slit width was set at 3 nm (excitation) and 3 nm (emission). The instrument was first calibrated prior to analysis of the samples. The excitation spectrometer was calibrated first, followed by the emission spectrometer. A narrow feature in the xenon lamp spectrum was used to calibrate the excitation monochromator. Water Raman scattering was used to confirm the calibration of the emission monochromator. The excitation monochromator and emission monochromator were considered to be well calibrated if the maximum peak wavelengths were 467±0.5 and 397 nm, respectively. All fluorescence spectra were recorded using 1 cm pathlength quartz cells. The built-in sample holder in the instrument that was designed to hold the quartz cell in the optical path was employed for reproducible positioning of the cell. Figures 2.3 and 2.4 show photographs of the Branson ultrasonic bath and spectrofluorometer used for this research.

Figure 2.3. Branson Ultrasonic photo by Samson Adegbite.

Figure 2.4. Spectrofluorometer (FluoroMax-2) photo by Samson Adegbite.

2.1.4 Sample Collection

The B100 biodiesel used in these experiments was obtained from waste cooking oil collected from campus kitchens and local restaurants. The biodiesel is being produced on the Kansas State University (KSU) campus by students of the KSU Biodiesel Initiative. It is used as

a fuel for transportation especially for recycling trucks and other vehicles on campus. The production of biodiesel from the waste cooking oil is obtained through a transesterification process, like that described in Chapter 1.[1,4,15,17] This process involves reaction of a triglyceride molecule (oil) to form the desired fatty acid methyl esters. Transesterification is catalyzed by the addition of NaOH (or KOH) to a mixture of waste oil and methanol.

2.1.5 Sample Preparation

2.1.5.1 Preparation of Nile red Dye and Biodiesel Solutions

This process involves doping a known sample of biodiesel with a very low concentration of a fluorescent dye that is highly sensitive to the polarity of the liquid medium. The biodiesel solution was prepared by weighing out an appropriate quantity (3 mg) of pure Nile red dye into a beaker and adding 99.805 g of biodiesel (B100). All samples were weighed using analytical balance with 0.1 mg precision. The solution was gently stirred until the dye had dissolved and was then transferred into a storage container. The solution was again mixed thoroughly for about 15 minutes using the Branson ultrasonic bath to obtain a homogeneous mixture. This solution was used as a stock solution of approximately100 µM Nile red in biodiesel. It was diluted to 100 nM by adding 0.1 g of the stock solution to 99.870 g of biodiesel sample using a disposable glass pipette. This low concentration was used for the preparation of other samples and was designated in this experiment as Solution A. The exact concentration of Nile red is not critical to these experiments, only that it does not vary significantly between samples. Following solution preparation, the response of the dye to glycerol, methanol, and water (polar liquids) in the biodiesel (a nonpolar liquid) was determined by adding known quantities of each to the fuel and

then recording the spectrum of the fluorescent dye. Any observed solvatochromic shifts were quantified and related to the polar liquid content of the fuel.

Note: Solution A is a mixture of Nile red solution (0.1 g) and pure biodiesel sample (99.870 g).

2.1.5.2 Preparation of Nile red Dye and Methyl decanoate Solutions

A standard sample of known composition was required to investigate the fluorescence properties of Nile red and to show its effectiveness in detecting the presence of polar liquids. As biodiesel is a complex mixture of many different fatty acid methyl esters that is difficult to reproduce, a much simpler model liquid in the form of methyl decanoate was selected for use as a standard instead. Nile red fluorescence spectra were measured here in methyl decanoate with different fractions of the polar liquids (methanol, glycerol, and water). A stock solution was prepared by measuring a known quantity (3 mg) of pure Nile red dye into a beaker and adding 100.005 g of methyl decanoate. The solution was gently mixed, transferred into a sealed container and then thoroughly mixed for about 15 minutes in the ultrasonic bath to obtain a homogeneous mixture that is suitable for analysis. This is a stock solution of 100 μM Nile red in methyl decanoate. It was diluted to approximately 100 nM by adding 0.1g of the stock solution to 100.008g of methyl decanoate using a disposable glass pipette. This is a low concentration which was used for the preparation of other samples and was designated in this experiment as Solution B.

Note: Solution B is a mixture of Nile red solution (0.1g) and pure methyl decanoate (100.008 g).

2.2 Methods

In this research, the concentrations of total water, methanol, and glycerol in biodiesel were investigated using a simple fluorescence-based method. This research was specifically based on the use of the solvatochromic probe Nile red as a means to sense the total polar liquid content (i.e.; methanol, water, and glycerol together) of biodiesel using a bench spectrofluorometer (FluoroMax-2). Figure 2.5 shows a simplified block diagram showing the layout of the spectrofluorometer. The FluoroMax-2 is a high resolution, time-tested and fully automated spectrofluorometer suitable for a wide variety of applications. The instrument functions under the control of the DataMax spectroscopy software which is a Windows-based program that helps to acquire, display, and process the data. Fluorescence is obtained by exciting dye molecules with light from a 150W xenon arc lamp which after passage through the excitation monochromator delivers monochromatic light to the dye solution. Absorption of incident light by the dye prepares excited state dye that subsequently emits fluorescence. The fluorescence emitted from the sample is dispersed by the emission spectrometer and directed to the photomultiplier tube (PMT) which serves as the photon detector. The PMT signal is then amplified and displayed on the computer. Before reaching the sample compartment, about 8% of the light from the excitation spectrometer is directed to a reference photodiode via a quartz beam splitter and the remaining light continues to the sample. This instrument allows for the change in the Nile red emission spectrum as a function of solvent composition to be investigated.

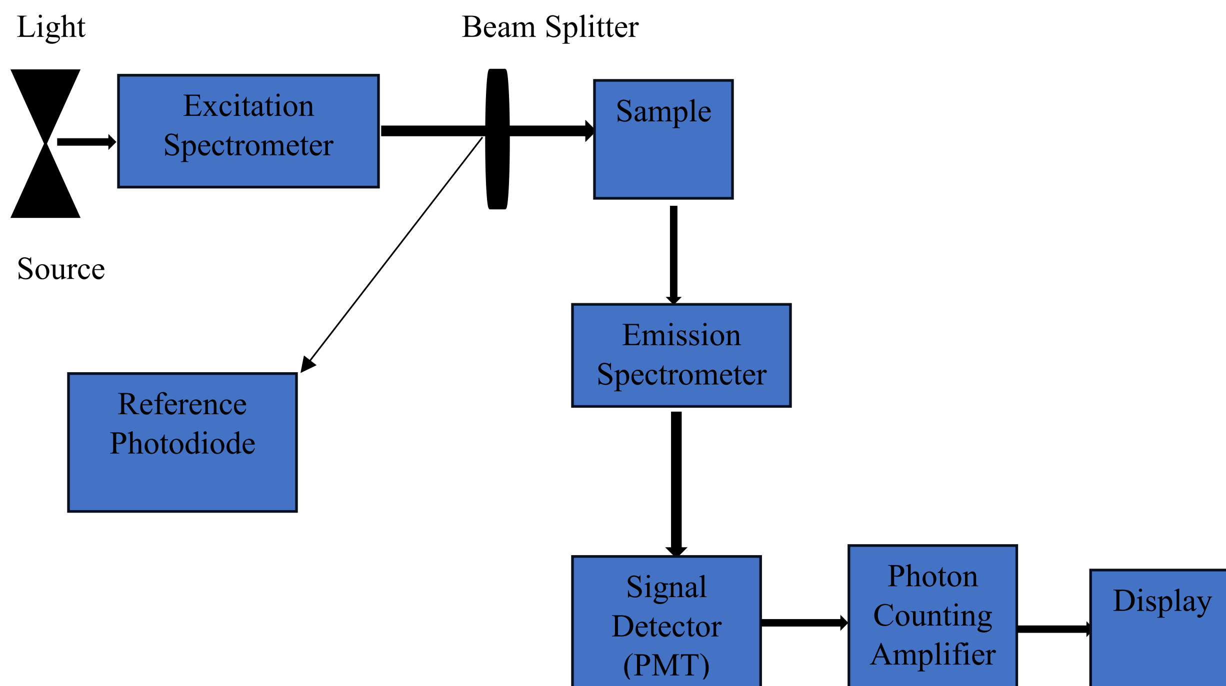

Figure 2.5. A simplified block diagram showing the layout of the spectroflurometer.

2.3 Experimental Procedure

2.3.1 Preparation of Nile red in Biodiesel (Solution A) with Polar Liquids

A 100 g aliquot of Solution A was divided into two equal parts where each part was used for the serial dilution of solutions used in this experiment. The latter involves gradual addition of polar liquids to obtain a series of Nile red solution in biodiesel having polar liquid concentrations ranging from 0% to 0.2%.

2.3.1.1 Preparation of Nile red in Biodiesel (Solution A) with Pure Methanol

A total of 50 g of Solution A (mixture of Nile red solution (0.1 g) and pure biodiesel sample (100 g)) was weighed and transferred into a 100 ml conical flask and 0.1g of pure methanol was added using a disposable glass pipette. The 0.2% weight percent (wgt %) solution obtained is listed as Solution C. This solution was subsequently used to prepare serial dilutions

(0.005% -0.1%) in vials (29 x 65mm). The contents were measured up to total mass of 10g as shown in Table 2.2 and then mixed thoroughly for about 15 minutes using the Branson ultrasonic bath. Each solution vial was sealed with a stopper after preparation. After homogenization, the solution was transferred into a 1 cm pathlength quartz cell and fluorescence spectra were obtained in the range of 540 to 700 nm on the fluorescence spectrophotometer. All fluorescence measurements were performed at room temperature, using an excitation wavelength of 532 nm.

Note: Solution C is a mixture of 50 g of Solution A (Nile red in biodiesel) and 0.1 g of pure methanol.

Table 2.2. Preparation of sample concentrations involving Solution A and Solution C (serial dilutions) used for the experiment.

Mixture/Solution (g)	Concentration (wgt %)
5.005g Solution A + 5.003g Solution C	0.1
7.504g Solution A + 2.502g Solution C	0.05
9.007g Solution A + 0.993g Solution C	0.02
9.500g Solution A + 0.506g Solution C	0.01
9.750g Solution A + 0.257g Solution C	0.005

2.3.1.2 Preparation of Nile Red in Biodiesel (Solution A) with Water

Similar procedures as described above were also carried out with other polar liquids (water and glycerol). A total of 50 g of Solution A was weighed and transferred into a 100 ml conical flask and 0.1g of water was added using a disposable glass pipette. At this stage, a 0.2 wgt % solution (Solution D) has been prepared from this mixture. This was subsequently used to

prepare serial dilutions (0.005% -0.1%) in vials (29 x 65mm). The contents were measured up to a total mass of 10g as shown in Table 2.3. Each vial was sealed with a stopper afterwards and was subsequently mixed thoroughly for about 15 minutes using the Branson ultrasonic bath. After homogenization, the solution was transferred into a 1 cm pathlength quartz cell and fluorescence spectra were obtained in the range of 540 to 700 nm on the fluorescence spectrophotometer. All fluorescence measurements were performed at room temperature, using an excitation wavelength of 532 nm.

Note: Solution D is a mixture of 50 g of Solution A (Nile red in biodiesel) and 0.1 g deionized water.

Table 2.3. Preparation of sample concentrations involving Solution A and Solution D (serial dilutions) used for the experiment.

Mixture/Solution (g)	Concentration (wgt %)
5.006g Solution A + 4.992g Solution D	0.1
7.507g Solution A + 2.506g Solution D	0.05
9.005g Solution A + 1.002g Solution D	0.02
9.503g Solution A + 0.504g Solution D	0.01
9.754g Solution A + 0.249g Solution D	0.005

2.3.1.3 Preparation of Nile red in Biodiesel (Solution A) with Pure Glycerol

A total of 50 g of Solution A was weighed and transferred to a 100 ml conical flask, followed by the addition of 0.1g of pure glycerol using a disposable glass pipette. At this point, 0.2 wgt % solution (Solution E) has been prepared from the mixture and was used to make

successive dilutions (0.005% -0.1%) in vials (29 x 65mm). The contents were measured to a total mass of 10g as given in Table 2.8. Each vial was sealed with a stopper and subsequently mixed for about 15 minutes using the Branson ultrasonic bath. Following homogenization, the solution was placed into a 1 cm pathlength quartz cell, and fluorescence spectra in the range of 540 to 700 nm were collected from the fluorescence spectrophotometer. All fluorescence experiments were carried out at room temperature using a 532 nm excitation wavelength.

Note: *Solution E is a mixture of 50g of Solution A (Nile red in biodiesel) and 0.1g of pure glycerol.*

Table 2.4. Preparation of sample concentrations involving Solution A and Solution E (serial dilutions) used for the experiment.

Mixture/Solution (g)	Concentration (wgt %)
5.003g Solution A + 4.995g Solution E	0.1
7.499g Solution A + 2.500g Solution E	0.05
9.008g Solution A + 0.996g Solution E	0.02
9.501g Solution A + 0.506g Solution E	0.01
9.749g Solution A + 0.254g Solution E	0.005

2.3.2 Preparation of Nile red in Methyl decanoate (Solution B) with Polar Liquids

Prior to analysis of polar liquid content in biodiesel, the sensitivity of fluorescent probe towards the polar liquids was also demonstrated and this was achieved by performing the fluorescent experiments of Nile red solution in methyl decanoate which was chosen to serve as a model for biodiesel. In this case, fluorescence experiments were again performed with gradual

addition of polar liquids from 0% to 0.2%. A 100 g aliquot of Solution B was divided into two equal parts where each part was used for the serial dilution of solutions used in this experiment.

2.3.2.1 Preparation of Solution of Nile red in Methyl decanoate (Solution B) with Pure Methanol

A total of 50 g of Solution B was weighed and transferred into a 100 ml conical flask and 0.1g of pure methanol was added using a disposable glass pipette. The 0.2% weight percent (wgt %) solution obtained is listed as Solution F. This solution was subsequently used to prepare serial dilutions (0.005% -0.1%) in vials (29 x 65mm). The contents were measured up to total mass of 10g as shown in Table 2.2 and then mixed thoroughly for about 15 minutes using the Branson ultrasonic bath. Each solution vial was sealed with a stopper after preparation. After homogenization, the solution was transferred into a 1 cm pathlength quartz cell and fluorescence spectra were obtained in the range of 540 to 700 nm on the fluorescence spectrophotometer. All fluorescence measurements were performed at room temperature, using an excitation wavelength of 532 nm.

Note: Solution F is a mixture of 50g of Solution B (Nile red in methyl decanoate) and 0.1g of pure methanol.

Table 2.5. Preparation of sample concentrations involving Solution A and Solution F (serial dilutions) used for the experiment.

Mixture/Solution (g)	Concentration (wgt %)
5.003g Solution B + 5.007g Solution F	0.1
7.504g Solution B + 2.507g Solution F	0.05
9.00g Solution B + 1.004g Solution F	0.02
9.503g Solution B + 0.504g Solution F	0.01
9.749g Solution B + 0.254g Solution F	0.005

2.3.2.2 Preparation of Solution of Nile Red in Methyl Decanaote (Solution B) with Water

Similar procedures as described above were also carried out with other polar liquids (water and glycerol). A known quantity (50g) of Solution B was weighed and transferred into a 100 ml conical flask and 0.1g of water was added using a disposable glass pipette. At this stage, a 0.2 wgt % solution (Solution G) has been prepared from the mixture. This was subsequently used to prepare serial dilutions (0.005% -0.1%) in vials (29 x 65mm). The contents were measured up to a total mass of 10g as shown in Table 2.3. Each vial was sealed with a stopper afterwards and was subsequently mixed thoroughly for about 15 minutes using the Branson ultrasonic bath. After homogenization, the solution was transferred into a 1 cm pathlength quartz cell and fluorescence spectra were obtained in the range of 540 to 700 nm on the fluorescence spectrophotometer. All fluorescence measurements were performed at room temperature, using an excitation wavelength of 532 nm.

Note: Solution G is a mixture of 50g of Solution B (Nile red in methyl decanoate) and 0.1g of distilled water.

Table 2.6. Preparation of sample concentrations involving Solution B and Solution G (serial dilutions) used for the experiment.

Mixture/Solution (g)	Concentration (wgt %)
5.008g Solution B + 5.004g Solution G	0.1
7.501g Solution B + 2.488g Solution G	0.05
9.000g Solution B + 1.007g Solution G	0.02
9.503g Solution B + 0.503g Solution G	0.01
9.750g Solution B + 0.248g Solution G	0.005

2.3.2.3 Preparation of Solution of Nile Red in Methyl Decanoate (Solution B) with Pure Glycerol

A total of 50 g of Solution B was weighed and transferred to a 100 ml conical flask, followed by the addition of 0.1g of pure glycerol using a disposable glass pipette. At this point, 0.2 wgt % solution (Solution H) has been prepared from the mixture and was used to make successive dilutions (0.005% -0.1%) in vials (29 x 65mm). The contents were measured to a total mass of 10g as given in Table 2.8. Each vial was sealed with a stopper and subsequently mixed for about 15 minutes using the Branson ultrasonic bath. Following homogenization, the solution was placed into a 1 cm pathlength quartz cell, and fluorescence spectra in the range of 540 to 700 nm were collected from the fluorescence spectrophotometer. All fluorescence experiments were carried out at room temperature using a 532 nm excitation wavelength.

Note: Solution H is a mixture of 50g of Solution B (Nile red in methyl decanoate) and 0.1g of pure glycerol.

Table 2.7. Preparation of sample concentrations involving Solution B and Solution H (serial dilutions) used for the Experiment.

Mixture/Solution (g)	Concentration (wgt %)
5.005g Solution B + 5.007g Solution H	0.1
7.504g Solution B + 2.503g Solution H	0.05
9.007g Solution B + 0.998g Solution H	0.02
9.505g Solution B + 0.503g Solution H	0.01
9.752g Solution B + 0.253g Solution H	0.005

2.4 Linearity Test

Linearity is an essential parameter for determining the accuracy and precision of analytical methods used to measure the concentration of analytes in a sample.[35] The linearity test assesses the relationship between the concentration of the analyte and the response of the analytical instrument.[36,37] However, in this experiment, the linearity test was performed by recording fluorescence spectra of Nile red in different biodiesel-polar liquid mixtures. The readings were measured in triplicate, containing six points from 0.005 wgt % to 0.2 wgt % with a blank i.e., pure biodiesel (non-polar liquid) to set the baseline fluorescence. The mixture (solution) was prepared in a series of standard solutions containing varying concentrations of polar liquids (water, methanol, and glycerol), ranging from 0.005 wgt % to 0.2 wgt % as described above. Each standard solution was transferred into a vial and the fluorescence was measured using the spectroflurometer.

A fluorescence intensity ratio (see 2.5) was then plotted against the known concentration of each component to obtain a calibration curve for each component. The resulting calibration curve

exhibited a significant linear correlation, with a correlation coefficient of at least 0.98. This observation implied that Nile red was competent for the quantitative determination of biodiesel samples containing up to 0.2 wgt % polar liquids. From the equation obtained from the calibration plot, the added methanol, water, and glycerol concentrations were calculated.

2.5 Calculation of Fluorescence Intensity Ratio

The fluorescence intensity ratio $(I_{580}-I_{640})/(I_{580}+I_{640})$ was calculated by integrating the emission from 560-600 nm (580±20 nm) and from 620-660 nm (640±20 nm). Specifically, this emission ratio was chosen because Nile Red emits near 640 nm in polar solvents and near 580 nm in nonpolar solvents. It was also selected based on its broad use in previous experiments from our laboratory. It was originally derived from the use of bandpass filters to selectively detect Nile red fluorescence in these two bands.[43] In these experiments, the spectral selection of these bandpass filters is mimicked by instead integrating the spectrum over each of these two spectral ranges.

2.6 Detection limit determination

The detection limit is the lowest concentration of a substance that can be reliably detected using a given analytical method.[35,36,37] It helps to evaluate the sensitivity of the fluorescence spectroscopy method for the analyte of interest.[37] The limit of detection (LOD) for each of the polar liquids, using the Nile red fluorescent probe was calculated in this experiment from the fluorescence data using the equation of 3SD/S, where SD is the standard deviation on the intercept of the fitted data and S is the slope obtained from the linear calibration plot, respectively. The standard deviation used for the LOD was calculated by considering the pooled standard deviation (SD) from all the results. This involves squaring the individual SD values and multiplying them by the number of degrees of freedom in each case. This is followed by adding

the values and dividing by the total number of degrees of freedom. Finally, the SD was obtained by taking the square root of the resulting value. The calculated LOD values are discussed in the next chapter.

Chapter 3 - Results and Discussion

The results of this research demonstrate the development of a new, sensitive, rapid, and reliable method for the detection of total polar liquid content in biodiesel. Polar liquid contaminants can have a significant impact on the quality and performance of the final product. The method is based on the principle of fluorometry and relies on the use of a fluorescent dye that is sensitive to the presence of water, methanol, and glycerol. The method was tested on real-world samples of biodiesel to determine its practicality and applicability. All results were based on the average of three replicate measurements. Notably, the present study showed a strong solvatochromic red shift for Nile red based on a fluorescence intensity ratio (see 2.5) that displayed a significant linear correlation with polar liquid content over the range of concentrations investigated. The calibration as well as some figure of merits such as limit of detection, (LOD), limit of quantification (LOQ) were also reported.

A similar study by Qin et al.[38] used a fluorescence-based method to detect methanol in biodiesel and reported a linear calibration curve as obtained in the present study with a similar positive correlation between the concentration of methanol and the emission intensity ratio. However, the range of concentrations tested was much larger (0-10 wgt%), which may affect the slope and intercept of the calibration curve. Sathish et al.[39] also employed a fluorescence-based approach to detect methanol in biodiesel and observed a linear calibration curve with a comparable positive correlation between methanol concentration and emission intensity ratio. They tested a range of concentrations from 0 to 50 wgt%, which is again a much larger range than the one used in the present study, and much higher than would be expected in any production process. They reported a lower LOD of 1.0×10^{-3} wgt% compared to the LOD of 0.052 wgt% reported in the present study. Variation in detection limits may be attributed to a combination of factors such as sample

size and dilution, the sensitivity of the analytical instruments used, the presence of interfering substances, instrument calibration and the complexity of the sample matrix.

3.1 Fluorescence Emission Ratio Method for Detecting Polar Liquids in Biodiesel

The polar-liquid contaminant sensing properties of Nile red in biodiesel were studied by fluorescence spectroscopy. The fluorescence spectrum of Nile red in biodiesel/methanol, biodiesel/water, and biodiesel/glycerol mixtures undergoes a detectable solvatochromic red shift in each case, as the polar liquid concentration increases. This red shift is detected and quantified by determining the fluorescence intensity ratio $(I_{580}-I_{640})/(I_{580}+I_{640})$. The intensity ratio was calculated by integrating the emission from 560-600 nm (580±20 nm) and from 620-660 nm (640±20 nm). In each case (see below), when the polar liquid fraction was increased from 0 wgt% to 0.2 wgt%, the emission intensity in the 580±20 nm band gradually decreased, while the signal observed in the 640±20 nm band increased or remained constant. Nile red is also known to exhibit a polarity sensitivity in its quantum yield.[40,41] Thus, the observed signal level in each case (see below) was also observed to correlate well with an increase in the polarity of the solution. The results provided below show the effectiveness of the probe towards the sensing of methanol, water, and glycerol. It suggests that the wgt% of these polar liquids in biodiesel can be followed quantitatively by recording the fluorescence emission spectrum of Nile red in their presence. Note, however, that the purpose of this work is not to quantify the individual amounts of these contaminants, since Nile red only reports on the total polar liquid content of the fuel. The method reported is again meant only as a means to determine whether a particular sample meets ASTM standards for total polar liquid content based on a single measurement.

The experiments reported below were performed using biodiesel (B100) obtained from the Kansas State University Biodiesel Initiative. As such, the polar liquid content of the biodiesel

was initially unknown and uncontrolled. To overcome this limitation, methyl decanoate was selected as a standard model for biodiesel. Note that because biodiesel mixtures vary significantly in exact fatty acid methyl esters composition, it is not possible to obtain a proper standard solution otherwise. Pure methyl decanoate (98%+) was purchased from Fisher Scientific and was used as received in these studies. Polar liquids were added to the methyl decanoate along with Nile red and its spectrum was measured in a manner identical to the measurements made with biodiesel. The methyl decanoate results are discussed first in the sections below, followed by the results obtained in biodiesel.

3.2 Measurement of Methanol in Methyl decanoate and in Biodiesel

The sensitivity of Nile red to methanol was first investigated by measuring its fluorescence spectrum in methyl decanoate (which was used as a standard sample) for different methanol concentrations (i.e from 0 wgt% to 0.2 wgt%). Figure 3.1 shows the results obtained and the data are given in Table 3.1. The Nile red fluorescence spectrum in methyl decanoate is very similar to that obtained in biodiesel. As in biodiesel, the emission intensity near 580 nm steadily decreased as the methanol fraction was increased from 0 wgt% to 0.2 wgt%. Also, as in biodiesel, the fluorescence intensity in the 640±20 nm increased as methanol was added. As shown in Figure 3.3 and Figure 3.4, the Nile red fluorescence spectra in both biodiesel and methyl decanoate follow a similar trend with increasing methanol. The linear regression analysis of the data reveals a strong positive correlation between the concentration of methanol in methyl decanoate and the emission intensity ratio.

The methanol-sensing properties of Nile red in biodiesel are displayed in Figure 3.2 and the data are given in Table 3.2. The fluorescence spectrum of Nile red in biodiesel/methanol mixtures undergoes a solvatochromic red shift as the methanol concentration increases. This red

shift is detected and quantified by determining the fluorescence intensity ratio $(I_{580}-I_{640})/(I_{580}+I_{640})$ as described in section 3.1. This intensity ratio showed a significant linear correlation (Figure 3.4) with methanol concentration from 0 wgt% to 0.2 wgt% methanol (i.e. the peak position shifted to the red). When the methanol fraction was increased from 0 wgt% to 0.2 wgt%, the emission intensity in the 580±20 nm gradually decreased, while the signal observed in the 640±20 nm increased or remained nearly constant. As seen from Table 3.2 and Figure 3.4, the higher the concentration of methanol in the biodiesel sample, the lower the intensity of the fluorescence signal. Nile red is known to exhibit a polarity sensitivity in its quantum yield.[40,41] Thus, the observed decrease in signal also correlates well with an increase in the polarity of the solution. This shows the effectiveness of the probe towards the sensing of methanol. It suggests that the wgt% of methanol in biodiesel can be followed quantitatively by recording the fluorescence emission spectrum of Nile red.

The equation of the calibration curve was determined by fitting a straight line to the data points using linear regression analysis. The slope and intercept of the line represent the sensitivity and offset of the method, respectively. The calibration curve generated from the emission intensity ratio data depicts a linear relationship between the concentration of methanol in biodiesel and the corresponding emission intensity ratio values, demonstrating that the method responds to methanol as a contaminant. Note that the method cannot be used to directly quantify methanol in biodiesel because Nile red also responds to the presence of other polar liquids (see below). This method has important applications in the field of biodiesel production and quality control, as the presence of methanol can affect the performance of biodiesel in engines and can also have environmental implications.[15] The result also showed that the method has a linear response range of 0 wgt% to 0.2 wgt% (with 0.2 wgt% as the highest concentration tested) for

methanol. This means that Nile red provides a detectable response to the presence of methanol in biodiesel within this range, which closely matches to the limit 0.2 wgt% set by ASTM (American Society for Testing and Materials). The precision of the method was also high, with relative standard deviations (RSDs) of less than 1% for replicate methanol measurements.

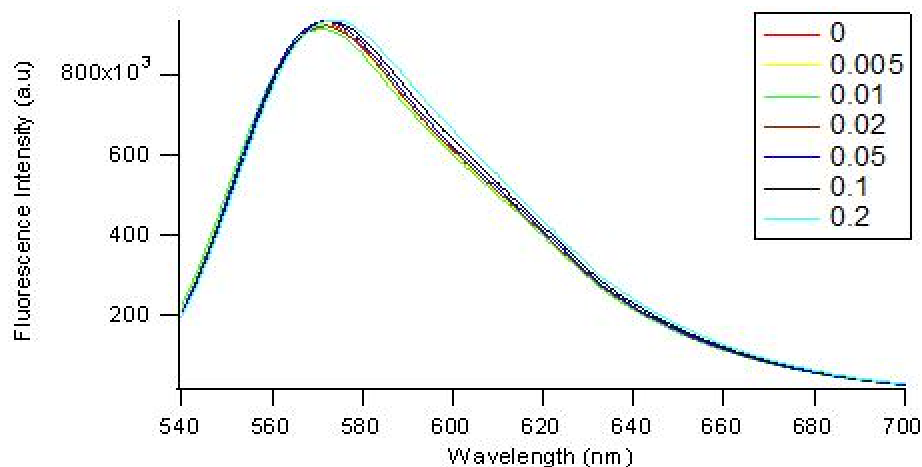

Figure 3.1. Fluorescence spectra of Nile red in methyl decanoate with an incremental addition of methanol. The Nile red concentration was 100nM. The excitation wavelength was 532 nm. The spectra are representative spectra from single experiments.

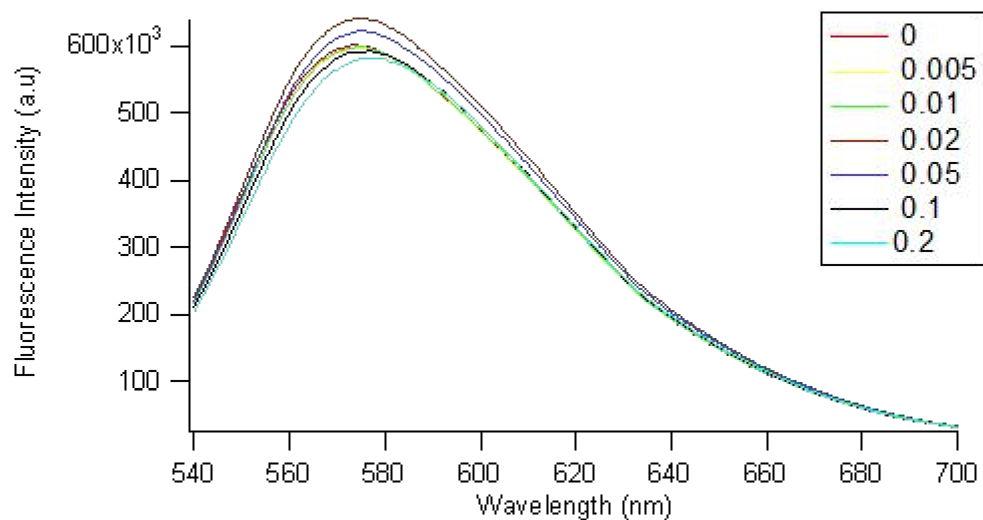

Figure 3.2. Fluorescence spectra of Nile red in biodiesel with an incremental addition of methanol. The Nile red concentration was 100nM. The excitation wavelength was 532 nm. The spectra are representative spectra from single experiments.

Table 3.1. Data from the measurements of methanol in methyl decanoate.

Concentration (%w/w)	**Fluorescence Intensity Ratio**	**Standard Deviation**
0	0.5538	0.0004
0.005	0.5519	0.0022
0.01	0.5520	0.0014
0.02	0.5498	0.0017
0.05	0.5496	0.0020
0.1	0.5429	0.0017
0.2	0.5334	0.0018

Table 3.2. Data from the measurements of methanol in biodiesel.

Concentration (%w/w)	**Fluorescence Intensity Ratio**	**Standard Deviation**
0	0.4739	0.0023
0.005	0.4716	0.0026
0.01	0.4713	0.0029
0.02	0.4715	0.0030
0.05	0.4691	0.0035
0.1	0.4658	0.0048
0.2	0.4578	0.0051

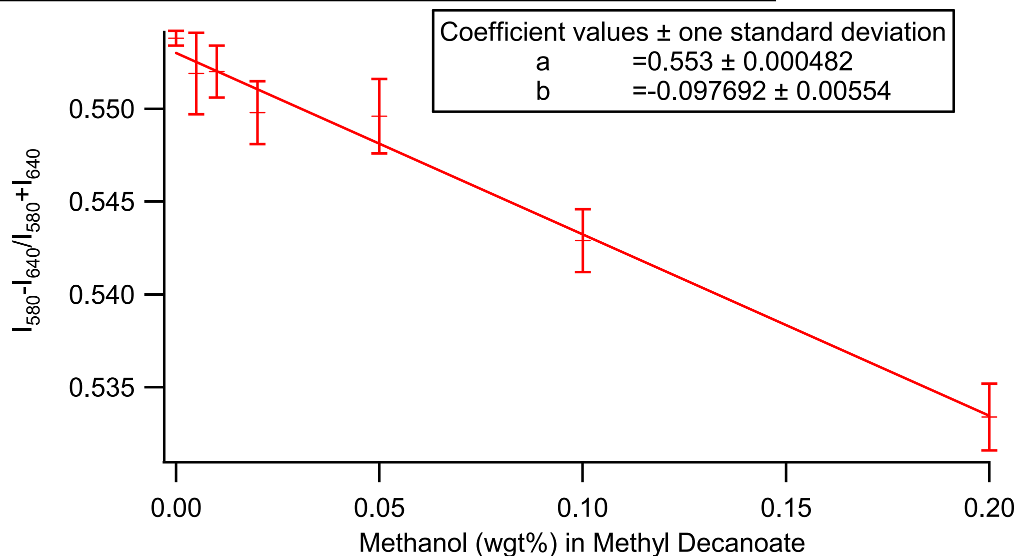

Figure 3.3. Fluorescence intensity ratio $(I_{580}-I_{640})/(I_{580}+I_{640})$ of Nile red in methyl decanoate with an incremental addition of methanol. The Nile red concentration was 100nM. The excitation wavelength was 532 nm. The data are presented as mean ± SD, n = 3. Here, a = y-intercept and b = slope of the calibration curve.

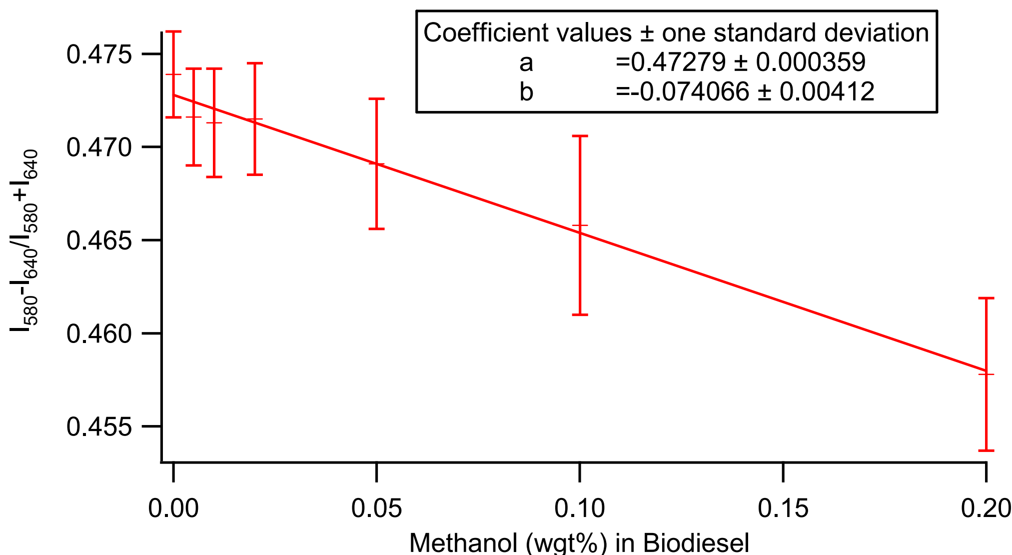

Figure 3.4. Fluorescence intensity ratio $(I_{580}-I_{640})/(I_{580}+I_{640})$ of Nile red in biodiesel with an incremental addition of methanol. The Nile red concentration was 100nM. The excitation wavelength was 532 nm. The data are presented as mean ± SD, n = 3. Here, a = y-intercept and b = slope of the calibration curve.

Methyl decanoate also provided an initial test to know if there are any polar impurities in biodiesel prior to the addition of methanol. The data obtained from the experiment as presented in Table 3.1 and Table 3.2 show that the biodiesel intensity ratios are much smaller than that of methyl decanoate. Thus, the results reported suggest that the biodiesel may be contaminated by other polar liquids. Because no good standard liquid could be obtained, it is also possible that the biodiesel tested is simply more polar than methyl decanoate.

3.2.1 Limit of Detection (LOD) of Methanol

The detection limit values are important because they allow researchers and industry professionals to accurately quantify the amount of impurities present in biodiesel samples, which is critical to ensuring the quality and performance of the final product.[36] The methanol detection limit (LOD) was calculated from the fluorescence data obtained from the series of methyl

decanoate solutions, using the equation of LOD = 3s/m, where s is the standard deviation on the intercept of the fitted data and m is the slope obtained from the linear calibration plot, respectively. The detection limit of 0.052 wgt% obtained is indicative of the sensitivity and the potential of this method as a tool for detecting small amounts of methanol in biodiesel samples. Similarly, the limit of quantitation (LOQ) is determined to be 0.173 wgt%. This value is found to be lower than the ASTM standards of 0.2 wgt% recommended for methanol. Again, because Nile red responds to all polar liquids present in the biodiesel, it is not possible to determine the concentration of methanol in these samples by this method. Note, however, that comparison of the biodiesel and methyl decanoate results suggests that the biodiesel may be contaminated with appreciable quantities of polar liquids, and thus needs to be tested further by other means, such as gas chromatography, to determine the exact concentration of methanol that may be present.

Table 3.3. Limit of detection (LOD) and Limit of quantification (LOQ) for methanol in methyl decanoate (left) and biodiesel (right).

LOD = 3s/m	LOD = 3s/m
s= Standard Deviation= 0.00169	s= Standard Deviation= 0.00320
m= Slope= 0.0977	m= Slope= 0.0741
LOD= 3x 0.00169/0.0975= 0.052 wgt%	LOD= 3 x 0.00320/0.0741= 0.129 wgt%
LOQ= 10s/m=10 x 0.00169/0.0977=0.173 wgt%	LOQ=10s/m=10 x 0.00320/0.0741=0.432 wgt%

3.2.2 R-squared Value of Measurement of Methanol in Biodiesel

The R-squared value, also known as the coefficient of determination, is a statistical measure that represents the proportion of the variation in the dependent variable (the fluorescence signal) that can be explained by the independent variable(s) (methanol

concentration in biodiesel).[42] An R-squared value of 1.0 indicates a perfect fit of the model, while a value of 0 indicates no relationship between the independent and dependent variables. The high R-squared value of 0.98 obtained from the calibration curve (Figure 3.4) indicates a strong linear relationship between the fluorescence signal and the concentration of the target analyte (methanol). Thus, the high value provides strong evidence that the fluorescence-based method is effective in detecting the methanol concentration in biodiesel samples with a high degree of accuracy and reliability.

3.3 Measurement of Water in Methyl Decanoate and Biodiesel

The sensitivity of the Nile red probe to water was first explored by performing fluorescent experiments of Nile red in a model for biodiesel (methyl decanoate) with gradual addition of water from 0 wgt% to 0.2 wgt%. As noted above, the Nile red emission yield is also known to depend on the polarity of the solvent in which it is dissolved. Thus, the fluorescence intensity ratio decreased as the concentration of water increased (Table 3.4 and Figure 3.5). The fluorescence spectral variations of Nile red in both biodiesel and methyl decanoate followed a similar trend, as illustrated in Figure 3.7 and Figure 3.8. When the volume fraction (wgt %) of water increased from 0 wgt% to 0.2 wgt%, the emission gradually shifted to the red. The slope of the plot for methyl decanoate as shown in the calibration curve is about the same as that for biodiesel which suggests that the sensitivity of Nile red in the two solvents is very close.

The water sensing properties of Nile red in biodiesel are revealed in Figure 3.6 and the data are given in Table 3.5. Following the gradual addition of pure water directly into the methyl decanoate and biodiesel samples, a decrease in fluorescence intensity in the 580±20 nm band along with an increase in fluorescence in the 640±20 nm band and a good linear relationship between the intensity ratio $(I_{580}-I_{640})/(I_{580}+I_{640})$ was obtained. This analysis shows a detectable

solvatochromic red shift as water was added to both methyl decanoate and biodiesel. This result implies that the fluorescence-based method can also be used to detect water in biodiesel. Based on the data obtained in Table 3.5, a linear calibration curve was obtained by plotting the emission intensity ratio $(I_{580}-I_{640})/(I_{580}+I_{640})$ against the concentration of water added to the biodiesel. The calibration curve was found to have a strong positive correlation between the concentration of water in biodiesel and the corresponding emission intensity ratio values. The slope of the linear regression line was negative, indicating that as the concentration of water increased, the emission intensity ratio decreased. The equation of the linear calibration curve was determined by fitting a straight line to the data points using linear regression analysis.

While the calibration curve shown in Figure 3.7 and 3.8 cannot be used to determine the concentration of water present in the biodiesel, because as noted above, Nile red responds to all polar liquids, the slope of the calibration curve does reveal information on the sensitivity of Nile red to each polar liquid. In this case, the slope of the methanol calibration curve was found to be -0.0975 while that for water was found to be -0.2155. These number suggests that Nile red is two-fold more sensitive to water than to methanol contamination of biodiesel. However, it is important to recognize that Nile red is clearly sensitive to both of these polar liquids. The difference in sensitivity of Nile red to water and methanol likely result from the different polarities of the two liquids as water is more polar than methanol. This is because the structure of water has two hydrogen atoms attached to one oxygen atom unlike methanol that has only one hydrogen and an alkyl group (which is non polar in nature) attached to one oxygen atom. Thus, due to the difference in electronegativity of hydrogen and oxygen, coupled with higher dielectric constant, water is more polar.

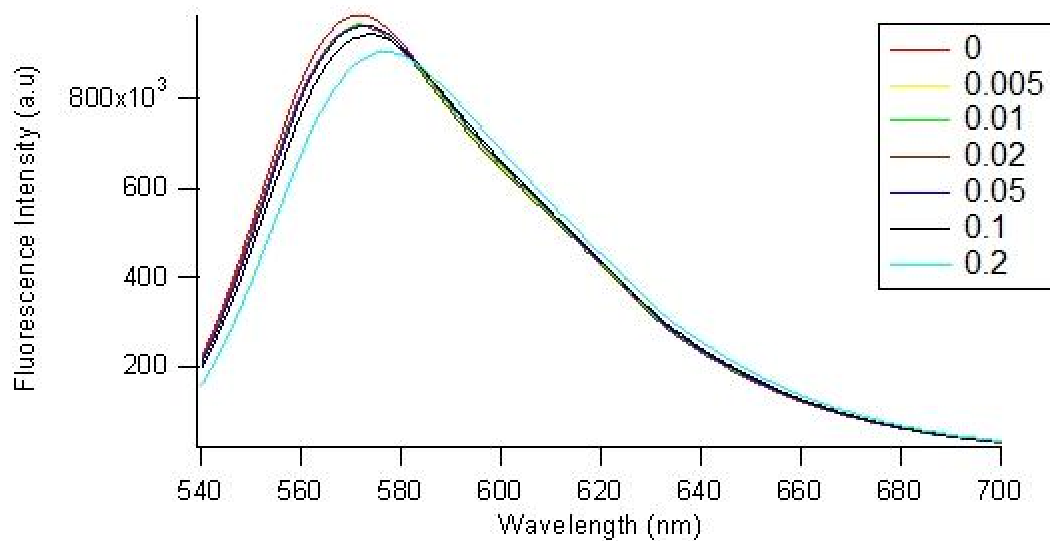

Figure 3.5. Fluorescence spectra of Nile red in methyl decanoate with an incremental addition of water. The Nile red concentration was 100nM. The excitation wavelength was 532 nm. The spectra are representative spectra from single experiments.

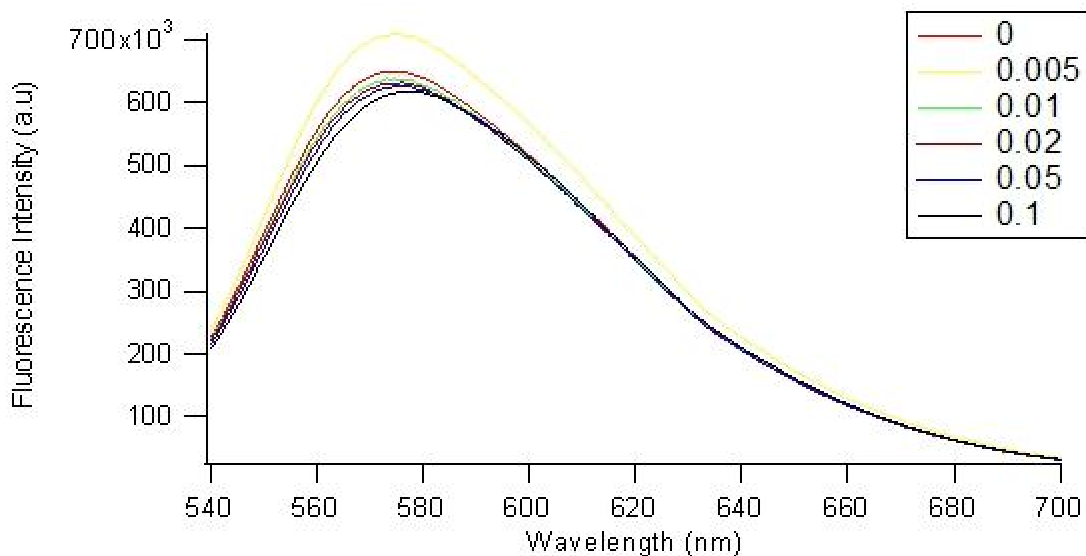

Figure 3.6. Fluorescence spectra of Nile red in biodiesel with an incremental addition of water. The Nile red concentration was 100nM. The excitation wavelength was 532 nm. The spectra are representative spectra from single experiments.

Table 3.4. Data from the measurements of water in methyl decanoate.

Concentration (%w/w)	**Fluorescence Intensity Ratio**	**Standard Deviation**
0	0.5537	0.0002
0.005	0.5484	0.0028
0.01	0.5464	0.0025
0.02	0.5482	0.0028
0.05	0.5428	0.0026
0.1	0.5320	0.0035
0.2	0.5073	0.0012

Table 3.5. Data from the measurements of water in biodiesel.

Concentration (%w/w)	**Fluorescence Intensity Ratio**	**Standard Deviation**
0	0.4740	0.0031
0.005	0.4744	0.0034
0.01	0.4701	0.0029
0.02	0.4692	0.0031
0.05	0.4631	0.0022
0.1	0.4521	0.0028

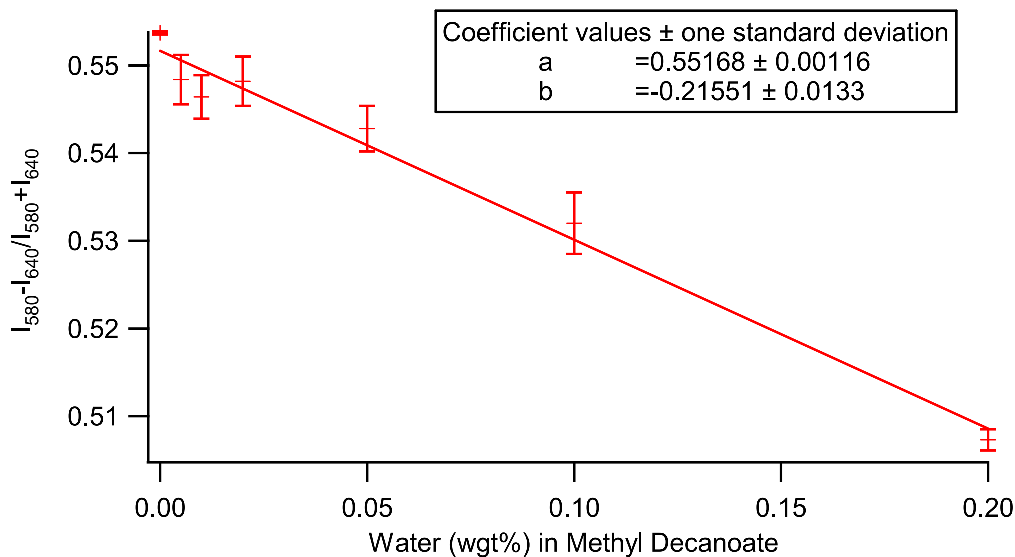

Figure 3.7. Fluorescence intensity ratio (I_{580}-I_{640})/(I_{580}+I_{640}) of Nile red in methyl decanoate with an incremental addition of water. The Nile red concentration was 100nM. The excitation wavelength was 532 nm. The data are presented as mean ± SD, n = 3. Here, a = y-intercept and b = slope of the calibration curve.

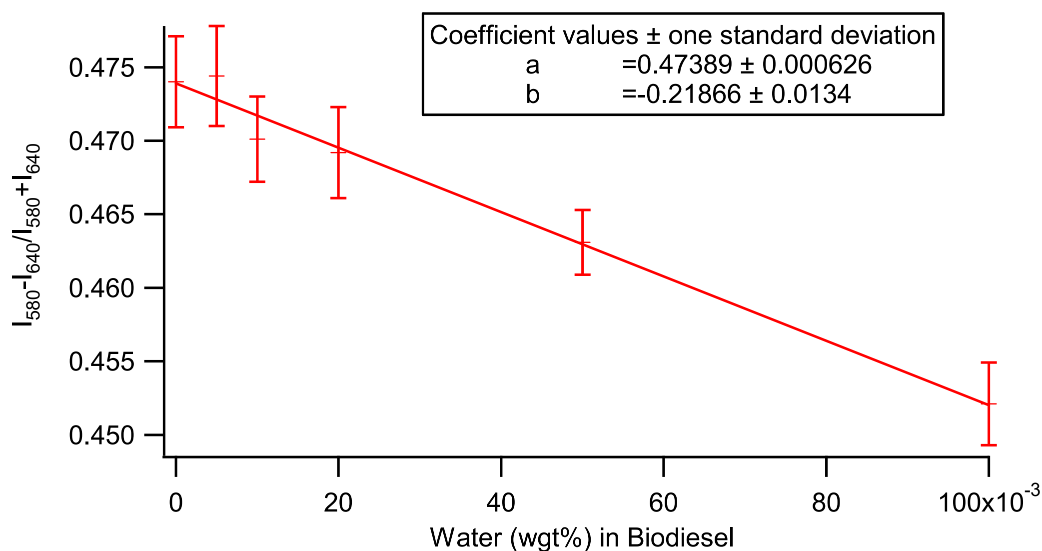

Figure 3.8. Fluorescence intensity ratio (I_{580}-I_{640})/(I_{580}+I_{640}) of Nile red in biodiesel with an incremental addition of water. The Nile red concentration was 100nM. The excitation

wavelength was 532 nm. The data are presented as mean ± SD, n = 3. Here, a = y-intercept and b = slope of the calibration curve.

As with the case of methanol, comparison of the biodiesel and methyl decanoate results again suggests that the biodiesel may be contaminated with appreciable quantities of polar liquids, and thus needs to be tested further. A high content of water might result from poor removal of water during the production process and inadequate purification step after transesterification reaction as biodiesel needs to go through purification steps to remove any impurities remaining including methanol and water.

3.3.1 Water Detection Limit

The determination of LOD for water in biodiesel was based on the methyl decanoate data. A detection limit of 0.034 wgt% was obtained and means that the method can detect water in biodiesel at a concentration as low as 0.034 wgt%. This is a very low concentration, equivalent to only 340 parts per million (ppm). This high level of sensitivity is crucial in the biodiesel industry, where even small amounts of water can cause significant problems during production, such as the formation of soap, which can reduce the quality and performance of the final product. The value of LOQ (0.114 wgt%) and that of LOD (0.034 wgt%) are significantly lower than the ASTM standard of 0.2 wgt%. The method's linear response range of 0 wgt% to 0.2 wgt% (with 0.2 wgt% as the highest concentration tested) further supports its use in the biodiesel industry for regulatory compliance and quality control.

Table 3.6. Limit of detection (LOD) and Limit of quantification (LOQ) for water in methyl decanoate (left) and biodiesel (right).

LOD= 3s/m	LOD= 3s/m
s= Standard Deviation=0.00246	s= Standard Deviation=0.00294
m= Slope = 0.2155	m= Slope= 0.2187
LOD= 3 x 0.00246/0.2155 = 0.034 wgt%	LOD= 0.00294 x 3/0.2187 = 0.040 wgt%
LOQ= 10s/m=10x0.00246/0.2155=0.114 wgt%	LOQ= 10s/m=10 x 0.00294/0.2187= 0.134 wgt%

3.3.2 R-squared Value of Measurement of Total Water in Biodiesel

The high R-squared value of 0.9843 indicates that the fluorescence-based method is highly reliable in detecting any water that might be present in biodiesel samples. A R-squared value of 0.9843 means that 98.43% of the variance in the dependent variable (fluorescence signal) can be explained by the independent variable (total water concentration in biodiesel) and it shows a good linear relationship between the emission ratio and the concentration of water in biodiesel.

3.4 Measurement of Glycerol in Methyl Decanoate and Biodiesel

The sensitivity of Nile red towards glycerol was also investigated by measuring its fluorescence spectrum in methyl decanoate for different glycerol concentrations (i.e from 0 wgt% to 0.2 wgt%). This is illustrated in Figure 3.9 and the data are displayed in Table 3.7. It was observed that the Nile red fluorescence spectrum in methyl decanoate is very similar to that obtained in biodiesel and the emission intensity near 580 nm gradually decreased as the glycerol fraction was increased from 0 wgt% to 0.2 wgt%. Also, the fluorescence intensity in the 640±20 nm increased as glycerol was added. As shown in Figure 3.11 and Figure 3.12, the Nile red fluorescence spectra in both biodiesel and methyl decanoate follow a similar trend with

increasing glycerol. The calibration plot of the data reveals a strong positive correlation between the concentration of glycerol in methyl decanoate and the emission intensity ratio.

Nile red was again used to measure the glycerol content of biodiesel samples, and the corresponding spectra and data are provided in Figure 3.10 and Table 3.8. The fluorescence-based method used to detect glycerol in biodiesel relies on the emission intensity ratio $(I_{580}-I_{640})/I_{580}+I_{640}$. This ratio was calculated based on the fluorescent properties of the Nile red probe in the biodiesel sample, which changes in the presence of glycerol. Similarly, upon addition of glycerol, emission by the dye gradually decreased, accompanied by a solvatochromic red shift. To generate a calibration curve, a range of glycerol concentrations (0, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2 wgt%) were tested and the corresponding emission intensity ratios were measured. A linear regression analysis was then performed to determine the relationship between the concentration of glycerol and the emission intensity ratio. The calibration curve obtained from the fluorescence-based method provides a reliable and sensitive approach for detecting glycerol in biodiesel. The method can be used for quality control and monitoring of glycerol levels during biodiesel production. The resulting linear calibration curve has several important characteristics. First, it has a strong positive correlation between the concentration of glycerol and the emission intensity ratio (Figure 3.12). This means that as the concentration of glycerol increases, the emission intensity ratio decreases. This relationship is represented by the slope of the line, which was used to calculate the concentration of glycerol based on the measured emission intensity ratio. The intercept of the linear regression line represents the offset of the method, which is the emission intensity ratio value when the concentration of glycerol is zero.

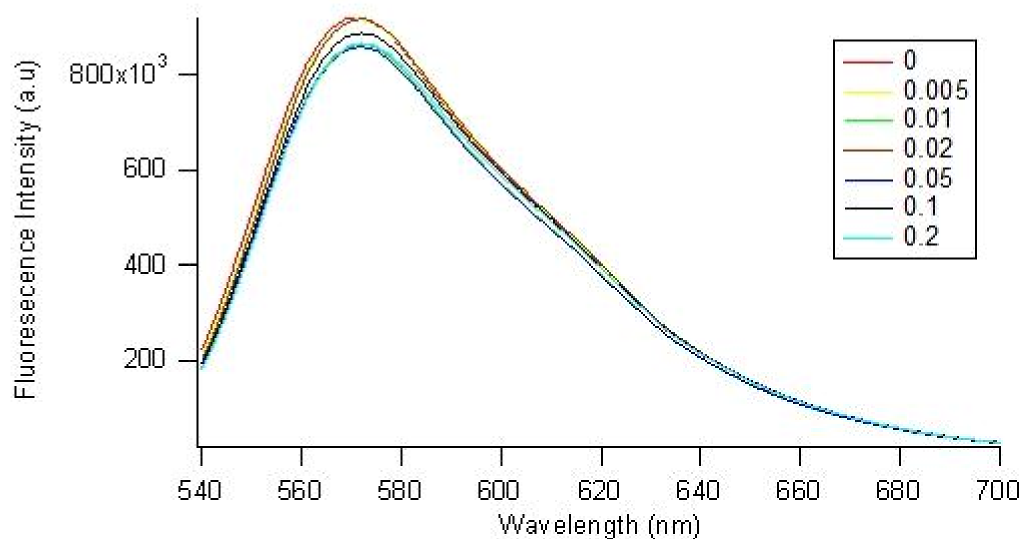

Figure 3.9. Fluorescence spectra of Nile red in methyl decanoate with an incremental addition of glycerol. The Nile red concentration was 100nM. The excitation wavelength was 532 nm. The spectra are representative spectra from single experiments.

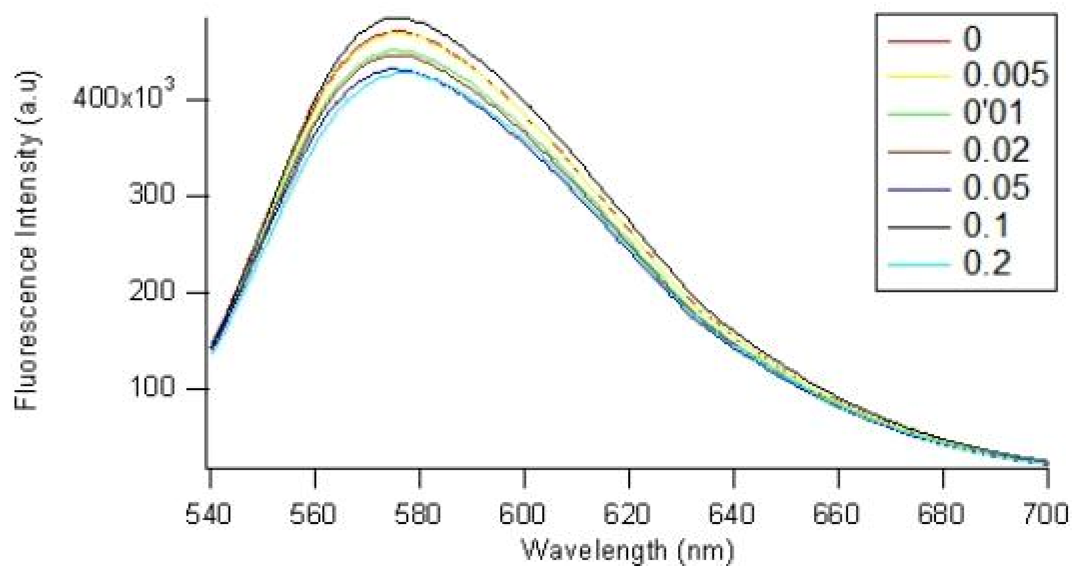

Figure 3.10. Fluorescence spectra of Nile red in biodiesel with an incremental addition of glycerol. The Nile red concentration was 100nM. The excitation wavelength was 532 nm. The spectra are representative spectra from single experiments.

Table 3.7. Data from the measurements of glycerol in methyl decanoate.

Concentration (%w/w)	Fluorescence Intensity Ratio	Standard Deviation
0	0.5618	0.0042
0.005	0.5578	0.0044
0.01	0.5570	0.0050
0.02	0.5569	0.0047
0.05	0.5544	0.0036
0.1	0.5516	0.0040
0.2	0.5433	0.0003

Table 3.8. Data from the measurements of glycerol in biodiesel.

Concentration (%w/w)	Fluorescence Intensity Ratio	Standard Deviation
0	0.4675	0.0029
0.005	0.4665	0.0025
0.01	0.4666	0.0030
0.02	0.4658	0.0019
0.05	0.4656	0.0041
0.1	0.4613	0.0006
0.2	0.4542	0.0015

Once again, the concentration of glycerol cannot be determined because Nile red responds to all polar liquids present. The difference between the methyl decanoate and biodiesel results may suggest methyl decanoate is not a good model for biodiesel and further studies should be explored to determine if this is the case. However, it is likely that the red shift observed in biodiesel instead indicates the presence of appreciable polar liquids as contaminants. These may include mono- and di-glycerides as well as methanol, water, and glycerol.

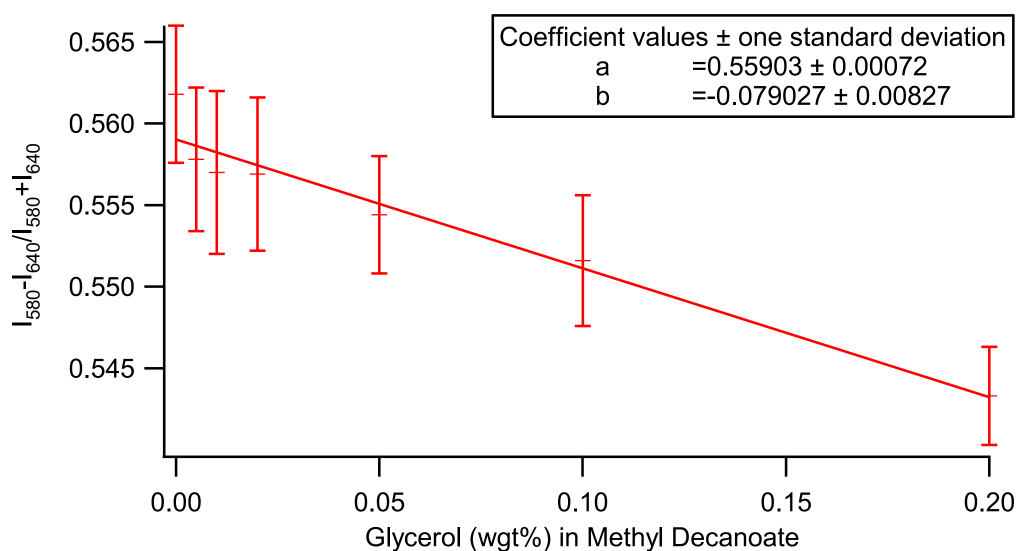

Figure 3.11. Fluorescence intensity ratio $(I_{580}-I_{640})/(I_{580}+I_{640})$ of Nile red in methyl decanoate with an incremental addition of glycerol. The Nile red concentration was 100nM. The excitation wavelength was 532 nm. The data are presented as mean ± SD, n = 3. Here, a = y-intercept and b = slope of the calibration curve.

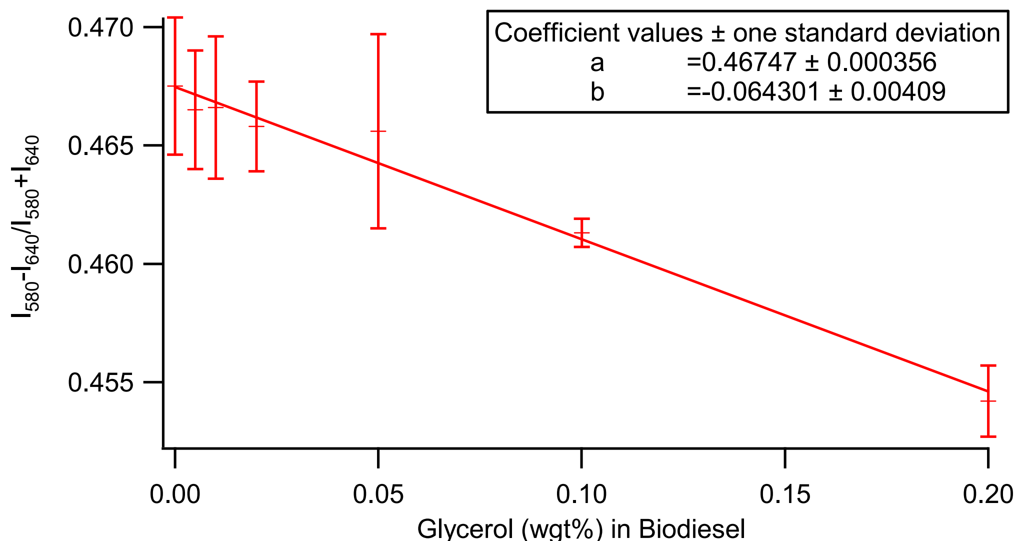

Figure 3.12. Fluorescence intensity ratio $(I_{580}-I_{640})/(I_{580}+I_{640})$ of Nile red in biodiesel with an incremental addition of water. The Nile red concentration was 100nM. The excitation wavelength was 532 nm. The data are presented as mean ± SD, n = 3. Here, a = y-intercept and b = slope of the calibration curve.

3.4.1 Limit of Detection (LOD) of Glycerol

The reported LOD of 0.153 wgt% indicates that the fluorescence-based method is capable of detecting glycerol in biodiesel samples at concentrations as low as 0.153 wgt%. This means that the method is sensitive enough to detect small amounts of glycerol, which is an important impurity in biodiesel that can affect its properties and performance. It also means that if a sample of biodiesel contains glycerol at a concentration below 0.153 wgt%, the fluorescence-based method may not be able to detect it with sufficient accuracy or reliability. The reported values of LOQ (0.509 wgt%) and LOD (0.153 wgt%) show the lowest glycerol concentrations that can be quantitatively detected in biodiesel and are found to be significantly higher than the ASTM standard of 0.02 wgt% for free glycerol. However, the value of LOD is lower than the 0.24 wgt% limit recommended by ASTM for total glycerol.

A study by Silva et al.[45] employed a fluorescence-based method to detect free and total glycerol in biodiesel and observed a linear calibration curve with linear response (5ppm-70ppm) much lower than the one reported in the present study. The LOD value (0.5ppm) of their studies was also found to be much lower compared to the LOD of 1530 ppm reported in the present study. Lima et al.[46] also reported that the use of fluorometric methods provides a trustworthy and low-cost method to determine the presence of undesirable amounts of glycerol in biodiesel. They observed a linear response within the range 0.10–5.00 ppm and the detection limit and quantification limit were estimated at 0.036 ppm and 0.121ppm respectively. However, the reported range of linear response was slightly higher than the one employed in this study while the LOD and LOQ values were lower. Again, the difference in detection limit may stem from a combination of factors including sample preparation, sample size, sample matrix interference and other experimental considerations.

Table 3.9. Limit of detection (LOD) and Limit of quantification (LOQ) for glycerol in methyl decanoate (left) and biodiesel (right).

LOD= 3s/m s= Standard deviation = 0.00402 m= Slope = 0.0790 LOD= 3 x 0.00402/0.0790= 0.153 wgt% LOQ= 10s/m=10x0.00402/0.0790=0.509 wgt%	LOD= 3s/m s= Standard Deviation= 0.00258 m= Slope= 0.0643 LOD= 0.00258 x 3/0.0643 = 0.120 wgt% LOQ= 10s/m=10 x 0.00258/0.0643= 0.401 wgt%

3.4.2 R-squared Value of Measurement of Glycerol in Biodiesel

A correlation coefficient (R^2) of 0.9788 was obtained after fitting the experimental data as displayed in Figure 3.12. In this case, the high R-squared value indicates a good fit of the data to the line, and that the Nile red emission ratio is a good indicator of the concentration of glycerol in biodiesel.

3.5 Conclusions

In general, the results presented in this chapter are consistent with other recent studies that have used fluorescence-based methods for detecting polar liquids (methanol, glycerol, and water) in biodiesel. However, the range of concentrations tested and the LODs as well as LOQs reported vary between studies depending on the specific analytical techniques, equipment, and procedures used. It is also important to note that the accuracy and precision of the method may depend on various factors such as the type of biodiesel, the fluorescent properties of the sample, and the analytical equipment used. Therefore, it is important to carefully evaluate the performance of the method in each specific application as each method has its own advantages and limitations.

Using methyl decanoate as a standard suggests that the biodiesel used for this experiment incorporates appreciable polar liquids contaminants and needs to be further tested. The high polar liquids content of the biodiesel might be due to the presence of residual mono- and di-glycerides, residual water, and methanol during production and after the transesterification process. However, it remains uncertain if one or all of the three contaminants are present in problematic concentrations. This represents the exact situation in which the results of this thesis based on a single measurement of the biodiesel sample would be used to motivate further investigations of contaminant by more selective methods.

Chapter 4 - Conclusion

Fluorescence-based methods have demonstrated their effectiveness and versatility in giving initial evidence of the level of biofuel contamination by polar liquids as revealed by total water, glycerol, and methanol in biodiesel. These methods offer several advantages including high sensitivity, selectivity, and speed, making them valuable assets and highly suitable for routine analysis in biodiesel production, quality control and research. These methods typically require minimal sample preparation and exhibit higher sensitivity, allowing for more accurate and efficient analysis. This method can provide significant advantages over traditional methods such as distillation and gas chromatography, which can be time-consuming, complex, and expensive. The fluorescence-based method proposed in this research offers a faster, simpler, and less expensive option for detecting the total water, methanol, and glycerol concentrations in biodiesel samples based on a single measurement from an unknown sample. The method is based on the principle of fluorometry, which involves measuring the solvatochromic shift of the fluorescence by a fluorescent dye in response to the presence of water, methanol, and glycerol in the biodiesel sample.

The determination of total water content in biodiesel is crucial because excessive water content can lead to fuel instability, decreased combustion performance, and reduced storage life. Similarly, the quantification of glycerol and methanol in biodiesel is essential for assessing fuel purity and compliance with industry standards. In this study, the fluorescence-based technique being investigated was shown to be sensitive to water, methanol, and glycerol contaminants in biodiesel samples based on the solvatochromism properties of the Nile red dye employed. The

fluorescent dye displayed considerable changes in emission bands with an incremental addition of methanol, water, and glycerol.

The linear calibration curve generated from the emission intensity ratio data and the high R-squared values obtained from the experimental data in the research demonstrate the validity and reliability of the fluorescence-based method for detecting total water, methanol, and glycerol concentrations in biodiesel samples. The method can provide accurate and reliable results for quality control purposes, allowing biodiesel producers to ensure that their product meets industry standards and regulations. The fluorescence-based method proposed in this research can be easily adapted for use in different biodiesel samples. The method's versatility and simplicity make it an attractive option for various related fields.

Moreover, the methyl decanoate used as a standard indicates that the biodiesel employed in this experiment may have considerable amounts of polar liquid contaminant and needs to be further analyzed. However, it is unknown whether any of the three contaminants investigated (water, methanol, glycerol) are present in problematic concentrations. This is the exact situation in which the results of this thesis would be utilized to encourage further investigations of contaminant using more selective techniques. This includes the use of advanced analytical techniques such as mass spectrometry and high-performance liquid chromatography (HPLC). These advancements are expected to lead to more accurate and reliable methods for detecting impurities in biodiesel and other types of fuels, which will have important implications for environmental protection, public health, and industry sustainability.

In general, as the demand for sustainable fuels continues to grow, fluorescence-based methods will undoubtedly play a pivotal role in ensuring the quality and compliance of biodiesel

products. Their contributions will help drive the advancement and widespread adoption of biodiesel as a viable and environmentally friendly fuel source.

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