"Developement of NMR based derivation for quantification of epoxy methyl oleate"

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In the partial fulfilment of the requirement for the degree of

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IN

CHEMISTRY

Submitted By

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UNDER THE SUPERVISION OF

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Certificate

This is to certify that the Thesis entitled "Developement of NMR based derivation for quantification of epoxy methyl oleate" being submitted by Ms. Neha in partial fulfilment of the requirements for the award of degree of Masters in Chemistry in the School of Chemistry and Biochemistry, Thapar Institute of Engineering and Technology, Patiala is a bonafide work carried out under the supervision of Professor, Dr. Ranjana Prakash and Associate Professor and Head, Dr. Amjad Ali, that no part of this project has been submitted for the award of any other degree.

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Candidate's Declaration

I hereby declare that the thesis entitled "Developement of NMR based derivation for quantification of epoxy methyl oleate" is an authentic record of my work carried out as requirements for the award of degree of **Masters of Science** in **Chemistry** at, **Thapar Institute, Patiala** under the supervision of **Dr. Ranjana Prakash** (Professor) & **Dr. Amjad Ali** (Associate Professor and Head), School of Chemistry and Biochemistry, Thapar Institute of Engineering and Techology, Patiala during January, 2018 to July, 2018. No part of the matter embodied in this report has been submitted to any other university or institute for the award of any degree.

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It is certified that the above statement made by the student is correct to the best of my knowledge and belief.

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Abbreviations

OA	Oleic acid
EMO	Epoxy methyl oleate
NMR	Nuclear magnetic resonance
TLC	Thin Layer Chromatography
OA	Oleic acid
MO	Methyl oleate
H_2O_2	Hydrogen peroxide
HCOOH	Formic acid
HCOOOH	Peroxyacetic acid
EMO	Epoxy methyl oleate
H_2SO_4	Sulfuric acid
NaOH	Sodium hydroxide
CH_3OH	Methyl alcohol or methanol
CH_2Cl_2	Dichloro-methane
R_f	Retention factor
$CDCl_3$	Deuterated chloroform
TMS	Tetra methyl silane
FAME	Fatty acid methyl ester
qHNMR	quantification of proton nuclear magnetic resonance
^{1}HNMR	Proton nuclear magnetic resonance
^{13}CNMR	Carbon nuclear magnetic resonance

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Abstract

Lipid oxidation is the degradation of oxidative lipids which leads to toxic, sour flavours of lipid containing foods. Cis-9-Octadecenoic acid (Oleic acid) derivatives were prepared and characterized. The processes involved were esterification of oleic acid and epoxidation of methyl oleate to oxirane ring. The epoxidation of methyl oleate was carried out by using *in-situ* epoxidation *via* generated peroxyformic acid (HCOOOH) to produce epoxy methyl oleate. The degree of temperature, the molar ratio of formic acid or hydrogen peroxide and types of catalyst was considered. The products' structures were confirmed and characterized by ^{1}H-NMR and ^{13}C-NMR. Quantification of the products epoxidised vegetable oils acts as a raw material helps in the synthesis of various chemicals including glycols, polyols, carbonyl compounds and biolubricants because of their oxirane oxygen content and high reactivity of oxirane ring.

Chapter 1

INTRODUCTION

Nuclear magnetic resonance spectroscopic analysis is distinctively and conjointly the most quantitative analytical analysis technique applicable for determining the structure and purity of organic compounds. Therefore, it is employed for the recognition and quantification in structure clarification of natural product, proteins, medicine and alternative biomolecules [1]. Proton nuclear magnetic resonance has conjointly been applied as a quantitative analytical technique because the nuclear magnetic resonance signal intensity is proportional to the amount of nuclei under specific controlled conditions [2]. Quantitative nuclear magnetic resonance (qNMR) provides correct and precise quantitative results for analytes once internal standards square measure used [3]. Due to the generic nature of ^{1}H-NMR and qNMR as a quantitative tool, the technique has been used to verify purities of product by assessing over two separated signals on the product [4].^{1}H-NMR spectroscopy widely used for the study of human blood plasma, animal blood plasma and some other biological fluids which includes bile, urine and cerebrospinal fluids [3]. However, qNMR analytical analysis of blood plasma is hampered due to the presence of broad resonances arise from lipo-proteins and macro-molecules such as albumin. As a result, these higher molecular weight moleculas are often removed prior to ^{1}H-NMR by traditional physico-chemical methods like extraction, ultrafiltration and precipitation [5]. Most of the methods are time consuming, results in loss of metabolites, and give incomplete removal of macro-molecules [5]. A physico-chemical separation of less or high molecular weight molecules always prevents the analysis of various possible interactions, like comlexation of metals, compartmentation of micellar, and physical or chemical exchange processes [3].

Apart from qNMR, high performance liquid chromatography (HPLC), and gas chromatography (GC) also widely used chromatographic techniques for the quantification of molecules [5]. GC is used for the volatile substances like terpenes and HPLC can be used with less volatile or non-volatile substances. GC with its destructive nature requires complex operational procedures, volatile substances and mass spectra for product conformation [6]. On the other hand HPLC technique is very

time consuming and needs costly HPLC grade solvents, set of standards, equilibration of the columns of the analyte [7].

Absolutely different from natural method, qNMR, doesn't need a high purity reference for the correct quantification of the compounds of interest. This technique for quantification needs no extended analytical time or sample reference. qNMR is non-destructive and compound may be recovered after the analysis [8]. The amount of solvent (Deuterated chloroform, $CDCl_3$) for qHNMR analysis is minimum (less than or equal to 0.5ml) which is very less as compared to HPLC and GC

Quantitative measure means determination of the absolute and relative abundance (expressed as a concentration) of one or all substances present in the solution to observe the kind of species. In basic nuclear magnetic resonance spectroscopic analysis, the identification of various compounds is predicated by the observation of signals with specific frequencies comparable to distinct/different molecular structures. Thus in its simplest form, quantitative nuclear magnetic resonance means the intensity of every signal is directly proportional to the concentration of analyte. Getting quantitative results does not need correct concentrations that may be calculated from numerous varieties of spectral information. Whereas it could be easier because it does not need extra computational and standardization factors. Any single signal in nuclear magnetic resonance is in most cases proportional to concentration of analyte because the acquisition method is at centre of nucleus, linear and non linear errors measures rarely introduced. However, a quantitative spectrum sometimes implies that every one of the signals square measure proportional to the concentration within the same manner [9]. If the concentration comparable to one signal is understood (usually the internal standard), the concentration of the other signal may be calculated by multiplying the concentration with the intensity quantitative relation of the signals [10].

Oleic acid is mono-unsaturated fatty acid is odorless, colorless and occur naturally in animal and vegetable fat oils used in many foods, in the form of triglycerides. Oleic acid with its sodium salt is a major component of soap as an emulsifying agent or as an emollient. Methyl oleate could be prepared via esterification of oleic acid with methanol in presence of mineral acid as catalyst. The prepared methyl oleate was then converted into epoxy methyl oleate using peroxyacetic acid as catalyst and toulene as a solvent.

Epoxidised methyl oleate will open a wide range of feasible reactions, carried out under moderate reaction conditions due to high reactivity of oxirane ring. It is colorless transparent liquid and soluble in many organic solvent. Synthetic biolubricant basestocks with improved low temperature and oxidative stability were prepared by chemical modification of EMO [5]. Epoxy fatty acid methyl ester is mainly used in the production of high quality poly vinyl chloride (PVC) products like disposable PVC gloves, children toys, perfusion tube, food package film, medical equipment, refrigerator seal etc.

All the experimental results are given during these analysis were performed on 400-MHz or 800-MHz nuclear magnetic resonance spectrometers. The aim of the study was to formulate and use NMR based derivation for quantification of methyl oleate and its corresponding epoxide.

Chapter 2

Literature review

Lipids are one of the major components in foods, along with water, carbohydrates, and proteins including organic compounds like fatty acids and their derivatives, steroids, cartenoids, etc [11]. Lipids are the group of compounds which are soluble in organic solvents but this type of definition is not sufficient as lipids have different structures [13]. The main food ingredients in lipids are fats and oils. The Canadian Food Inspection Agency (CFIA) has defined fats as 'total lipid fatty acids expressed as triglycerides' [12].

Oils remains in a liquid state at room temperature and fats are solid at room temperature [14]. Fats and oils contributions in human dietary include calories, necessary fatty acids including vitamins A, D, E and K etc. They also add some desired flavores and textures to the foods [14].

Different types of oils are found/derived from different sources, as vegetables oils are derived from the beans and seeds and marine oils are usually fats extracted from the marine animals. Oils shows varying physical, chemical and nutritional properties because of the presence of various chemical compounds [19].

Fatty acids are of two types, either saturated or unsaturated. Monounsaturated fatty acids (containing one double bond) and polyunsaturated fatty acids (containing more than one double bond) are the class of unsaturated fatty acids. Unsaturated fatty acids undergoes lipid oxidation due to the presence of double bond. Lipid-containing foods when exposed to radical initiators and oxygen, at high temperatures, oxidation can takes place [15].

Most of the oxidation products were obtained from single fatty acids which includes hydroperoxides, conjugated dienes, carbonyls, alcohols and epoxides [16]. However, hydroperoxides and bound carbonyl group further undergoes different reactions and generate more volatile compounds even while they bound to the original molecule. Dietary oxidized lipids are absorbed by the animals resulting in loss of appetite, cause harmful impact on blood circulation and inflammation [17]. The very first primary oxidation products are conjugated dienes arising from the poly-unsaturated fatty acids. However, this is not possible with the oxidation of monounsaturated fatty acids such as Oleic acid. Conjugated dienes arise when first radical forms and double bonds tends to rearrange themselves to stabilize the radicals [18].

The concentrations of oxidation products such as epoxides and alcohols have been reported rarely because while studying lipid oxidation in foods, there is lack of suitable and applicable methods to predict these products [19].

During epoxidation, an oxygen atom is inserted into the one bond of the double bond and shared by the two carbon atoms which results the formation of three membered ring.In lipid epoxidation, when peroxy radical during epoxidation process adds to the double bonds results into peroxy dimer which decomposes to give an epoxide and an alkoxy radical. By the cyclization of alkoxy radicals epoxides also can be produced. Neff and Byrdwell (1998) observed that there are two classes of the epoxides in trilinolein and trilinolenin [20]. One class include epoxy group that are formed at position of original double bond and other include epoxy group that are formed distant from the double bond.

During the last 30 years there have been reports on the toxicity and carcinogenicity of epoxides [21]. The earliest studies indicating the use of epoxides in industrial processes but their hazardous activity for biological systems is still not clear [22]. Epoxides are used extensively, in industries and also reported in biochemical pathways [21]. Some of them are quite toxic, a few of these are carcinogenic, at least in animals. However, epoxides vary in their reactivity and therefore not all are equally hazardous. Epoxides can be in many forms, such as solids, liquids and gases. Ethylene oxide (simplest aliphatic epoxide) a gas, the vapours of which when mixed with air causes explosion, but it gets inactivated when mix with CO_2 or fluorocarbons (Freon) [23]. Several epoxides are liquids having larger boiling points like epichlorohydrin, diepoxybutane and styrene oxide [24]. Naturally occurring solid epoxides are 9,10 Epoxystearic acid and fusarenon-X [25]. The major characteristics of epoxides are due to electro-philic and lipo-philic nature and also due to reactivity of three memered ring. The ring opening of the epoxides can be initiated by the attack of several nucleophiles i.e. NH_2, OH^-, Cl^-, and S^- [26].

In 1978, another class of epoxides were selected by the US Environmental Protection Agency i.e. halogenated alkyl epoxy compounds, as priority chemicals for the epidemiologcal studies and test the health and environmental effevts under 1976 Toxic Substances Control Act. [27].

^{1}H-NMR spectroscopy widely used for the study of human blood plasma, animal blood plasma and some other biological fluids which includes bile, urine and cerebrospinal fluids [3]. However, qNMR analytical analysis of blood plasmais

hampered due to the presence of broad resonances arise from lipo-proteins and macro-molecules such as albumin. As a result, these higher molecular weight moleculas are often removed prior to ^{1}H-NMR by traditional physico-chemical methods like extraction, ultrafiltration and precipitation [5]. Most of the methods are time consuming, results in loss of metabolites, and give incomplete removal of macro-molecules [5]. A physico-chemical separation of less or high molecular weight molecules always prevents the analysis of various possible interactions, like comlexation of metals, compartmentation of micellar, and physical or chemical exchange processes [3].

2.2 Epoxides occurrence and exposure

In industries, generation of epoxides occur in vast quantities. The high reactivity of epoxy group makes epoxides useful for alkylating and cross-linking agents. Mostly used aliphatic compounds are ethylene oxide, propylene oxide, epoxybutane and epichlorohydrin. Tanaka *et. al* [21] reviewed list of epoxides that are used in industries.

A few of them are useful as end products e.g. as sterilising agents, and are the most important stabilisers. Many others are used for the manufacturing of special solvents, cements, fine chemicals and surface active agents [10]. Some commercially available products like PVC and tri-chloroethylene tends to degrade steadily with the evolution of hydrochloric acid. Supplementation of marginal amounts (< 1%) of several epoxy compounds in the combination with other classes prevents or slowdown the degradation process [21]. Many patents are known on use of epoxides as stabilisers, but a few of them are commercially applicable [29]. A brand of technical grade trichloroethylene was stabilised with the combination of 0.22 % epichlorohydrin and 0.20 % epoxybutane [29]. Epoxidised soyabean oil at a concentration of 1.5-3.0 % is used as stabilisr for most of the PVC formulations, and at high concentrations used as a plasticiser in PVC commonly used in furniture, wire coatings, cars, coverings of floor etc. Epoxy compounds being used as polymer stabilisers and plasticisers again has been discussed by Port [23] and Szczeczek [24].

Epoxides were first observed in methyl esters (oxidized) and TAGs by GC [30]. Methyl oleate, methyl linoleate, triolein, and trilinolein were heated for 15 hr at 180^{o}C and samples were tested after every 5 hr for the determination of epoxy fatty acid. After

15 hr of heating of methyl oleate, two methyl mono-epoxystearates (*trans*-9,10 epoxy and *cis*-9,10 epoxy) were present at 20.3 mg/g and 14.9 mg/g, respectively. Under similar conditions, four methyl mono-epoxyoleates (*trans*-9,10, *trans*-12,13, *cis*-9,10, and *cis*-12,13 isomers) were produced by the oxidation of methyl linoleate at different concentrations of 5.9, 6.9, 3.1, 3.2 mg/g, respectively. By the use of sodium methoxide catalyzed transmethylation, the total amounts of mono-epoxy fatty acids in oxidized triolein (35.7 mg/g) and trilinolein (18.3 mg/g) were similar to those present in oxidized methyl oleate (32.3 mg/g) and methyl linoleate (19.3 mg/g) [30]. In addition, less unsaturated substrates (methyl oleate and triolein) produced larger amounts of epoxides in comparison to more unsaturated substrates (methyl linoleate and trilinolein) (Berdeaux et al., 1999). The six monoepoxy fatty acids reported in this study, including two methyl monoepoxystearates and four methyl monoepoxyoleates, have become the six most commonly measured epoxy fatty acids in the literature. Velasco, *et. al* (2004) determined monoepoxy fatty acids in oils heated using the same temperature and heating time as reported by Berdeaux et al. (1999). The total amounts of the six common monoepoxy fatty acids were 14.24 and 9.44 mg/g in olive oil and sunflower oils, respectively [31].

Ten used frying oils were determined for methyl epoxystearates and methyl epoxyoleates by the authors and hence concluded that (1) in mono-unsaturated oils more *trans*-epoxides were formed than *cis*-epoxides (2) in mono-unsaturated oils higher concentration of epoxides were found when compared to polyunsaturated oils. In fresh oils, trans-epoxy fatty acids were present at lower levels than *cis*-epoxy fatty acids.

2.3 Quantification of epoxides

Epoxidised methyl oleate product was quantified by different analytical techniques such as ^{1}H-NMR, high pressure liquid chromatography (HPLC) [32], Gas Chromatography (GC) [33]. HPLC is used in wide range to separate epoxy fatty acids, hydroxy fatty acids and keto fatty acids

Normal Phase HPLC (NP-HPLC), and Reversed Phase HPLC (RP-HPLC) are the two types of HPLC techniques. They are classified on the basis of their polarity in stationary and mobile phase. NP-HPLC consists a polar stationary phase and a non-polar mobile phase. RP-HPLC consists a non-polar stationary phase and a polar

mobile phase. NP-HPLC mainly used for analysing polar lipids/products as in their free or methyl ester forms and for analysing oxidation products such as hydroperoxides. In RP-HPLC, usually non-polar compounds were separate [34].

In ^{1}H-NMR there is no complexity during the data interpretation. A very small amount of sample is needed for the quantification of product. ^{1}H-NMR takes hardly 15-20 mins for analysis of product as HPLC takes more time to quantify the same analysis. NMR is more advantageous than HPLC as it requires a very less amount of solvent (= 0.5 ml). In contrast to this, HPLC needs more amount of solvents in high purity grade. Therefore, the two above techniques were used for the quantification of the epoxy methyl oleate.

Quantification is possible where the signals being integrated are well separated. In comparison to chromatographic techniques, there is a very small possibility to influence the separation due to the direct relation of molecular sturcture with chemical shift. NMR spectroscopy is used in pharmaceutical for the analysis of drugs. qNMR is used for analysing the level of impurities, for the structure determination, to illustrate course of the degradation results in related impurities, for the determination of ratio of diastereomers and the enantiomeric composition. qNMR is also used to determine molar ratios of basic drugs (protonated) and organic acids (deprotonated).

Quantifying methyl oleate and epoxidised methyl oleate by the above two techniques, it has been observed that ^{1}H-NMR is better than HPLC and GC as it requires least time, and less quantity of solvents.

Here,the information regarding the occurrences of epoxides and alcohols in oxidized lipids has been very limited in the literature, mainly due to the limitations of the currently available methods. Therefore, the overall goal of this thesis is to develop methods to measure epoxides and hydroxy fatty acids in edible oils to enable the study of the formation of minor compounds during lipid oxidation.

The specific objectives of the study are the discussed in Chapter 3.

Chapter 3

Objectives

1. Synthesis of methyl oleate ($C_{19}H_{36}O_2$) from oleic acid ($C_{18}H_{34}O_2$).
2. Synthesis of methyl oleate epoxide ($C_{19}H_{36}O_3$).
3. Developement of NMR based derivation to quantify the mixture of methyl oleate and methyl oleate epoxide.

Chapter 4

Experimental Methods

4.1 Experimental Apparatus

The experimental set up consisted of two neck round bottom flasks (RBF, 500ml). A bulk condenser and Thermometer

4.2 Materials

Oleic acid ($C_{18}H_{34}O_2$), Sulfuric acid (H_2SO_4), Sodium hydroxide (NaOH), hydrogen peroxide (H_2O_2), formic acid (HCOOH), Glacial acetic acid (CH_3COOH) were purchased from Sigma Aldrich Private Limited (India) and are used furthur for experimental work. n-hexane (C_6H_6), ethyl acetate ($CH_3COOC_2H_5$) methyl alcohol (CH_3OH), Dichloro-methane (CH_2Cl_2), an anisaldehyde solution were purchased from Spectrochem Private Limited (India). Metal silicon oxide plates are purchased from S-D Fine-Chem Limited, India. All reagents used for experimental purpose were analytical grade except formic acid (LR grade).

4.3 Methods

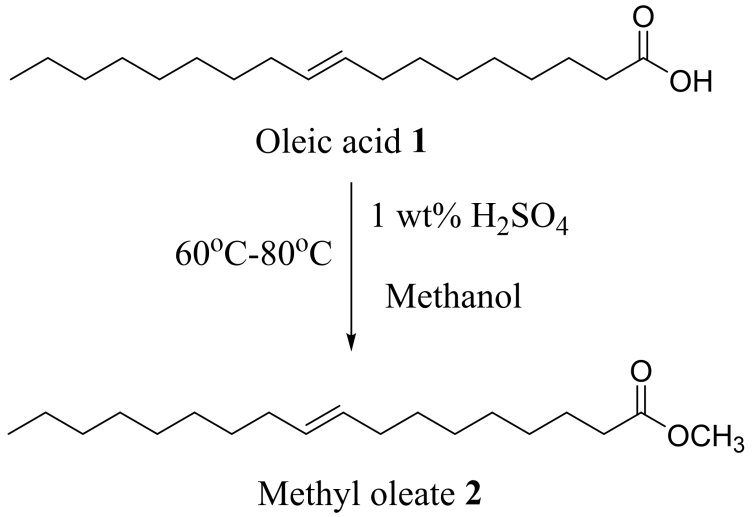
4.3.1 Synthesis of methyl oleate (MO)

The esterification reaction (Scheme 1) was carried out in the experimental apparatus described in section **4.1**. Oleic acid **1** (20g, 70.803 mmol) was taken in the round bottomed flask attached with reflux condenser and to it Methanol (25.81 ml, 637.25 mmol) was added as acyl acceptor and sulphuric acid (1 wt%) as a catalyst . The reaction was stirred for 120 min and the samples of the reaction mixture were collected for analysis after every 15 min to determine optimum temperature, catalyst concentration.

Variables such as temperature ranging from 80^{o}C to 100^{o}C and catalyst concentration ranging from 1.0% to 1.5% w/w were considered for the study. The molar ratio of alcohol to fatty acid was set at 9:1.

Progress of the reaction was monitored through thin layer chromatography. After the completion of the reaction, the product was extracted with ethyl acetate. The acidic organic layer was neutralized with saturated solution of sodium bicarbonate ($NaHCO_3$). After maintaining pH>7, the aqueous layer was again extracted with ethyl

acetate ($CH_3COOC_2H_5$). The organic layer was then dried over anhydrous sodium sulfate (Na_2SO_4). and concentrated under vacuum as a colourless oil.



Scheme 1 : Synthesis of methyl oleate in methanol by acid catalyst.

4.3.2 Synthesis of epoxy methyl oleate

4.3.2.1 Using peroxyacetic acid

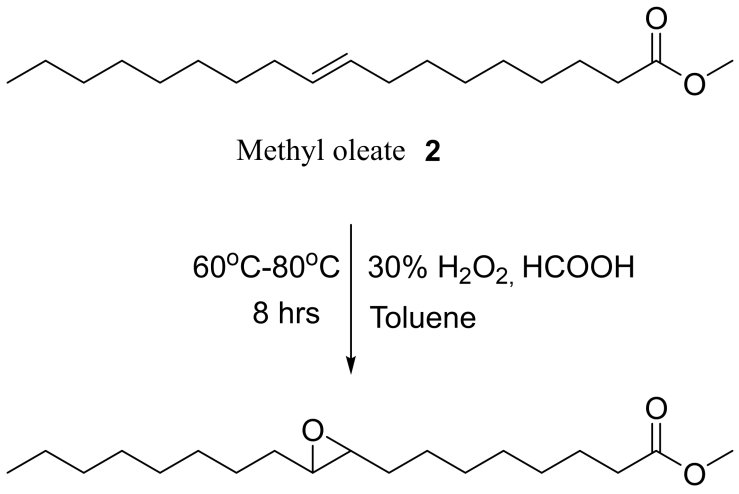
Epoxidation was conducted in a batch mode. and was carried out by two processess namely:

1. In-situ peracid epoxidation
2. Ex-situ peracid epoxidation

1. *In-situ* peracid epoxidation process

Methyl Oleate **2** (15g, 50.59 mmol) in toluene was taken in a 200 ml round bottomed flask and to above reaction mixture hydrogen peroxide (H_2O_2, 6.20 ml, 202.364 mmol) and formic acid (HCOOH, 1.90 ml, 50.59 mmol) were added sequentially as per selected molar ratio (Methyl oleate:H_2O_2:HCOOH :: 1:1:4). Initially, the reaction mixture was stirred at 0°C to control exothermicity. After 15 min the reaction temperature was increased to 60°- 80°C with vigorous stirring till the completion of the reaction (reaction time 8 hrs) as recorded by TLC (hexane:ethyl acetate::9:1 as developing solvent). After the completion of the reaction, the mixture was allowed to cool and extracted with ethyl acetate. The acidic organic layer was washed with

saturated solution of sodium bicarbonate, dried over anhydrous sodium sulphate (Na_2SO_4) and residual solvent was removed using rotatory evaporator to get the epoxy methyl oleate (MO) **3.** MO thus obtained was further purified by silica gel column chromatography (ethyl acetate:hexane :: 95:05 (v/v)) to obtain **3** as a colourless liquid.



Methyl oleate **2**

methyl 8-(3-octyloxiran-2-yl)octanoate **3**

Scheme 2 : Synthesis of Epoxidation formation reaction by using peracid as catalyst.

2. *Ex-situ* peracid epoxidation process

Initially, mixture of formic acid and conc. H_2SO_4 was stirred for 30 mins and resultant peracid was added to the solution of methyl oleate in toluene. The reactor setup and subsequent process was same as that discussed in *in-situ* batch peracid epoxidation.

4.4 Identification of methyl oleate

4.4.1 Method 1

Transformation of oleic acid **1** into methyl oleate **2** be observed by thin layer chromatography (hexane:ethyl acetate :: 90:10 (v/v) at different time intervals. The samples were diluted with appropriate solvent (DCM or ethyl acetate). A yellow coloured spot (observed under iodine chamber) of fatty acid methyl ester which was marked for the determination of Retention factor (R_f) value.

$$R_f = \frac{\text{the distance moved by the solute (the dye to be tested)}}{\text{the distance moved by the solvent (solvent front)}}$$

4.4.2 Method 2

Analysis and quantification of the esterification reaction was carried out using ^1H NMR (JEOL ECS-400, 400 MHz), and $CDCl_3$ as a solvent. TMS (Tetra methyl silane) was the internal standard and chemical shifts were expressed in parts per million (ppm). NMR spectra was recorded with 6.18 seconds pulse period with four seconds and sixteen scans. The presence of of FAME was confirmed by the appearence of singlet at δ 3.6ppm.

4.5 Quantification of methyl oleate

Methyl oleate conversion was calculated by ^1H nuclear magnetic resonance [35].

$$\% \text{ FAME} = \frac{2 \text{ x } I_{(\text{methoxy})}}{3 \text{ x } I_{(\text{methylene})}} \times 100$$

Where, **FAME** = Fatty acid methyl ester

$\mathbf{I_{(Methoxy)}}$ = Integration value of methoxy protons at (δ 3.6 ppm) and

$\mathbf{I_{(Methylene)}}$ = Integration value of methylene protons at (δ 2.33 ppm) in the ^1H-NMR spectrum

4.6 Identification of epoxy methyl oleate

4.6.1 By thin layer chromatography

Conversion of methyl oleate into epoxy methyl oleate (EMO) was observed by thin layer chromatography (TLC) at different intervals of time as discussed earlier in section **4.4.1**. As the epoxy methyl oleate was aliphatic, it is UV inactive and for identification TLC plate was stained in the prepared anisaldehyde solution. A single spot below the methyl oleate (less polar) confirms the epoxidised product (more polar).

4.6.2 D_2O Exchange and NMR Quantification

NMR spectrum of prepared epoxy methyl oleate sample was taken in D_2O. In this experiment, free hydroxy protons exchange with the deuterium and disappear from the spectrum. The D_2O should not have be miscible with the solvent. Appearence of peak at δ 2.9 - 3.2 ppm and disappearence of peak at δ 5.31 ppm into the ^1H-NMR

confirmed the formation of epoxy methyl oleate. In the ^{13}C- NMR the epoxy carbons were found to be at δ 55-60 ppm which further confirmed the formation of epoxy ring.

4.7 Quantification of epoxy methyl oleate (EMO)

Following NMR based derivations were proposed and applied for the quantification of EMO

$$\%EMO = \frac{3xI_{(Ep)}}{2x\ I_{(MO)}} X\ 100 \quad \text{..... (1)}$$

Where, EMO = epoxidised methyl oleate

$\mathbf{I_{(Ep)}}$ = Integration value of epoxy protons

$\mathbf{I_{(MO)}}$ = Integration value of methoxy protons

Another proposed formula for quantification is :

$$\%EMO = 100 - \frac{3xI_{(Ap)}}{2x\ I_{(MO)}} X\ 100 \quad \text{..... (2)}$$

Where, $\mathbf{I_{(Ap)}}$ = Integration value of Alkene protons

$\mathbf{I_{(MO)}}$ = Integration value of methoxy protons

To testify these equations and reproducibility of qHNMR technique, different samples were prepared by mixing known amount of methyl oleate and epoxidised methyl oleate in different molar ratios varying from 0.9:0.1 to 0.1 to 0.9 as described in Table 1 (eqn 1) and Table 2 (eqn 2).

Correlation was drawn between the concentrations supplemented *versus* those predicted by equation.

Chapter 5

Results and Discussions

5.1 Characterization of Methyl oleate

5.1.1 Examination of Methyl oleate by TLC

Thin Layer Chromatography is the quickest way to analyze the progress of the reaction towards product as shown in **Figure 1**. Retention factor (R_f) value tells the degree of retention of component retardation factor. It is always less than 1. R_f value of 0.92 indicates the formation of methyl oleate [36] while greater value of R_f indicates higher affinity of methyl oleate towards hexane, i.e. non- polar [37].

Figure 1 : Progress of the esterification reaction where **R** represents reactant i.e. Oleic acid, **C** is Co-product i.e mixture of oleic acid and the product formed and **P** is the Final product i.e. Methyl Oleate

5.1.2 Examination of Methyl oleate by ^{1}HNMR

The methyl oleate was characterized by ^{1}HNMR (400 MHz, $CDCl_3$) δ (ppm): 5.2-5.3 (m, -C**H**=C**H**-), 3.63 (s, -OC**H**$_3$), 2.71-2.77 (m, -C**H**$_2$-CO-), 2.25-2.30 (m, -C**H**$_2$CH=CHC**H**$_2$-), 1.22-2.0 (m, -(C**H**$_2$)$_n$-), 0.86 (m, -CH$_2$-C**H**$_3$-) as shown in **Figure 2a**

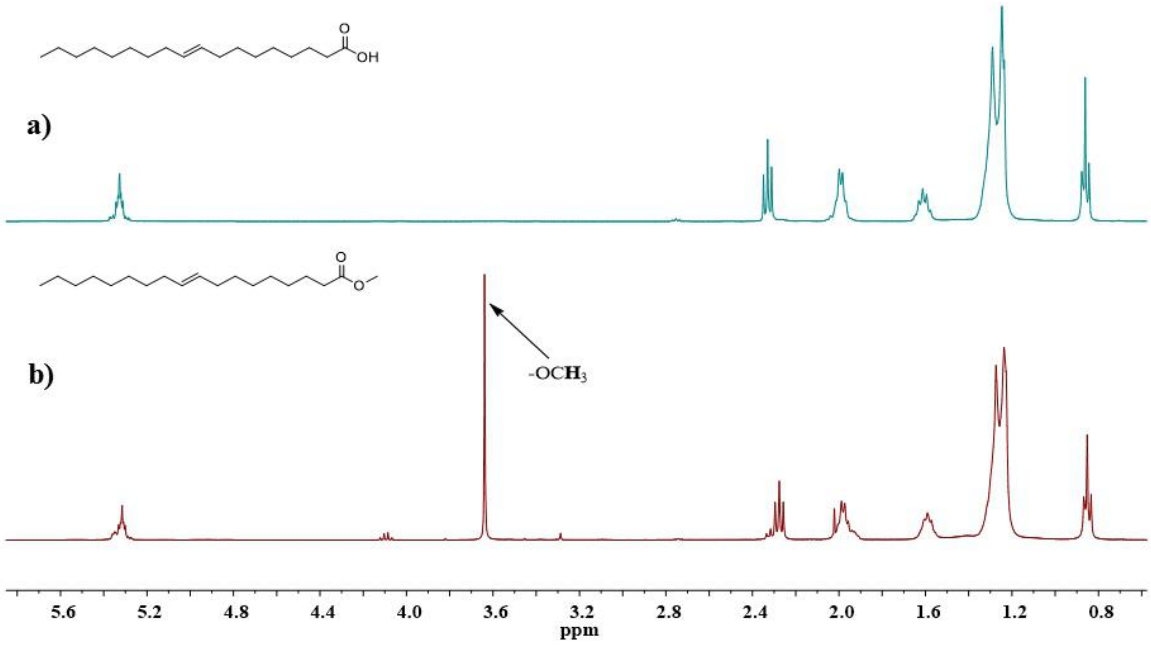


Figure 2 : Comparison of ^{1}HNMR between (a) Oleic acid and (b) methyl oleate

Non-edible oils and fats often have considerble amounts of phospholipids and other impurities beside the free fatty acids [38]. Deshielding effect of carboxylic acid is more as compared with methyl ester group which lead to difference in chemical shifts [39]. The signals at δ 2.71 ppm shows triplet due to (-C**H**$_2$CO-) carbon next to carbonyl carbon. The peaks of the aliphatic region observed the the region of δ 0.85-1.7 ppm. In esterification of oleic acid to methyl, oeate via acid catalysts, the oil content decreases with time showing a quartet pattern [40].

As reported by Satyarthi *et. al.*, 2009, the signals at δ 0.87-0.88 ppm and δ 0.8-0.99 ppm resulted from terminal methyl group (-C**H**$_3$-CH$_2$-) of oil and methyl ester respectively. An intense peak at δ 3.63 ppm shows the formation of ester via acid catalysts esterification [39].

The extent of conversion of esterification was calculated to be 94 % using the integration values observed by glyceridic and methyl ester protons in 1H-NMR using equation proposed by Sharma et. al. (2000)

5.2 Characterization of Methyl oleate epoxide

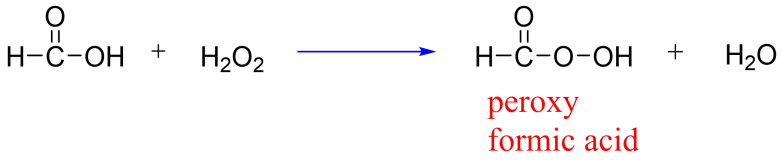
5.2.1 Examination of Methyl oleate epoxide by TLC

Purity and nature of compound was detected by the TLC. Using hexane:ethyl acetate (90:10 v/v) solvent system, the obtained R_f value 0.82 was the sign of formation of the epoxidised methyl oleate as shown in **Figure 3** [41]. It also. There is a decrease in R_f value as compared to methyl oleate (R_f = 0.82) clearly indicated that the product was more polar. Smaller the R_f value of product, more is the polar character and Higher the value the R_f of product, more is the non-polar character [42].

Figure 3 : Progression of the epoxidation reaction , where **R** represents methyl oleate, **C** is mixture of R and P, **P** is epoxy methyl oleate

Mechanism of Epoxidation Process

Step 1: Formation of peracid.



Step 2: Formation of epoxy ring

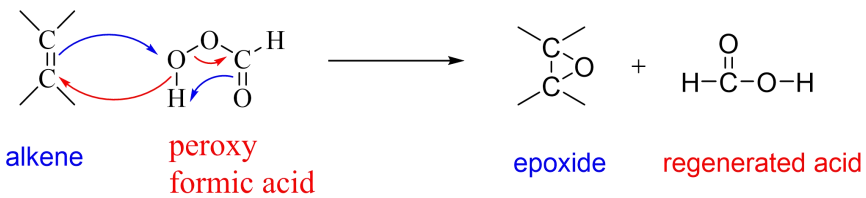


Figure 4 : Mechanism showing formation of epoxide [15].

5.2.2 Examination of Methyl oleate epoxide by ^{1}HNMR

The methyl oleate epoxide was characterized by ^{1}HNMR (400 MHz, $CDCl_3$) δ (ppm): 3.63 (s, -OC**H**$_3$), 2.87 (m,-C**H**OC**H**-), 2.71-2.77 (m, -C**H**$_2$-CO-), 2.25-2.30(m, 4H, -C**H**$_2$CH=CHC**H**$_2$-), 1.22-2.0 (m, -(C**H**$_2$)$_n$-), 0.86 (m, -CH$_2$-C**H**$_3$-) as shown in **Figure 5.**

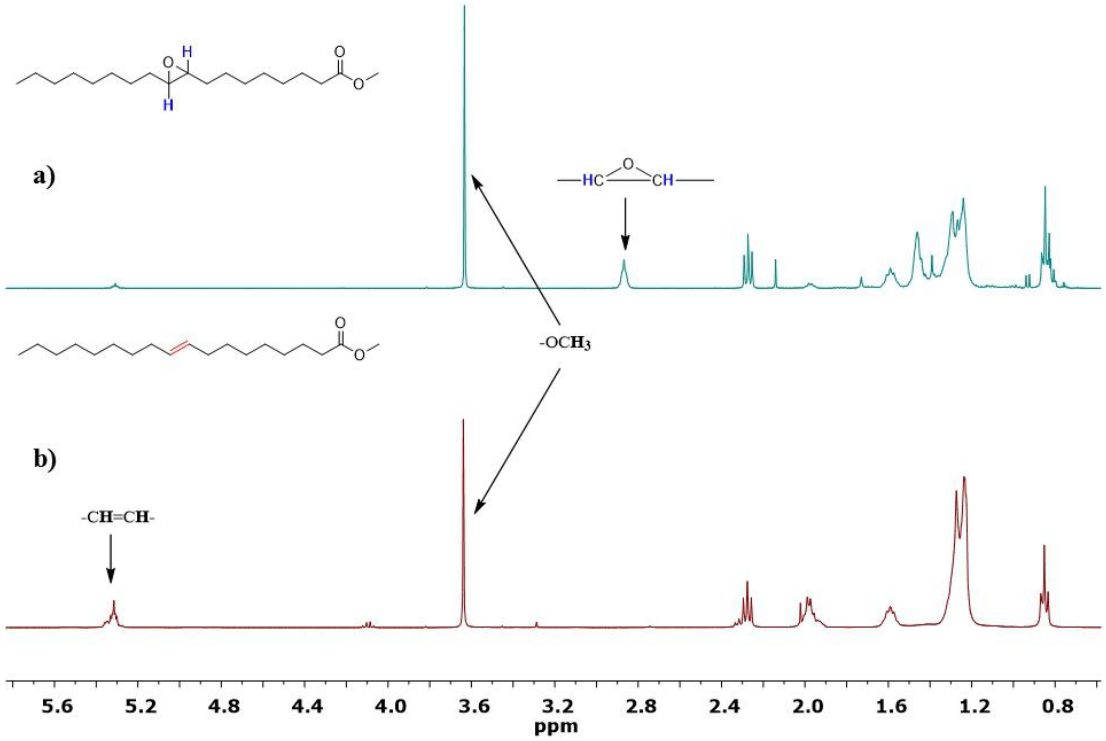


Figure 5 : Comparison of ^{1}HNMR between (a) epoxy methyl oleate and (b) methyl oleate

To demonstrate the epoxidation of methyl oleate with perocyacetic acid, ^{1}HNMR analytical technique was used. The steps forward the epoxidation of methyl oleate with the decrease of double bonds and the emergence of epoxide peak between the region δ 2.8-3.2 ppm signifies the formation of epoxides and the residual double bonds show signals at δ 5.33 ppm [43]. However, their chemical shifts remains same. The aliphatic region signals shows between δ 1.2-1.7 ppm. After epoxidation, there was no change in the chemical shift value of the methyl group (-C**H**$_3$-C) near δ 0.8-0.9 ppm indicates that the epoxy group is far distant from terminal methyl group with no downfield effect and acys as a internal standard in both spectra. The appearence of signals at δ 2.87 ppm confirms the epoxy methyl oleate [44].

5.2.3 Examination of Methyl oleate epoxide by ^{13}CNMR

The methyl oleate epoxide was quantified and characterized by ^{13}CNMR (100 MHz, $CDCl_3$) δ (ppm): 174 (-CH_2**C**O-), 57.1 (-**C**HO**C**H-), 51.4 (-O**C**H_3), 34.0 (-**C**H_2CO-), 31.8 (-CH_3CH_2**C**H_2), 29.6 (-(**C**H_3)$_2$(CH_2)$_2$CHOCH(CH_2)$_2$(**C**H_2)$_3$), 27.8 (-(**C**H_3)$_2$(CH_2)$_2$CHOCH(CH_2)$_2$(**C**H_2)$_3$), 27.2 (-**C**H_2CHOCH**C**H_2), 26.6 (-**C**H_2CH_2CHOCHCH_2**C**H_2-), 24.9 (-**C**H_2CH_2CO-), 22.6 (-**C**H_2CH_3), 14.1 (-**C**H_3) as shown in F**igure 6.**

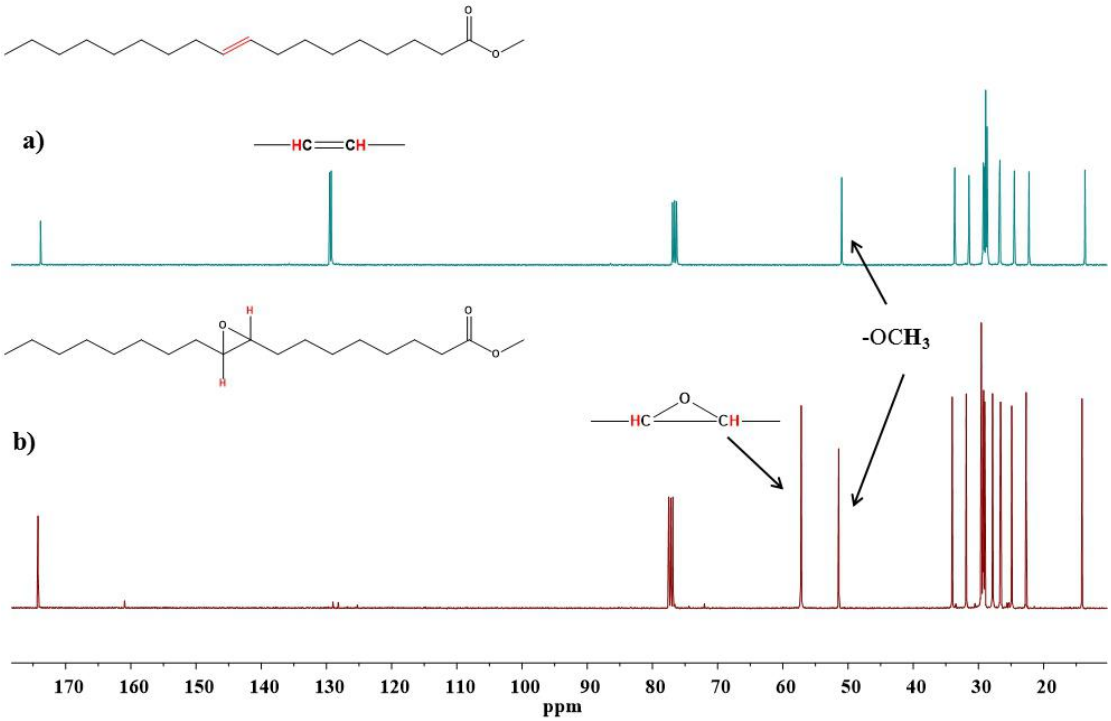


Figure 6 : ^{13}CNMR of (a) methyl oleate and (b) epoxy methyl oleate

The epoxidation process is also analyzed by ^{13}CNMR to confirm the formation of epoxy ring. The signals at δ 57.1 ppm confirms the formation of the epoxy methyl oleate [45]. The formation of diol signal was always appeared at δ 70.0 ppm. The C_4-C_7 signals were appeared at the region of δ 25-34 ppm while thw absence of signals near δ 120-130 ppm showing that there is no alkene left in the reaction confirmed that all the alkene gets converted into their respective epoxide. Various other signals between δ 12-35 ppm shows the aliphatic region signal resembles with the signals of methyl oleate indicates that there is very less change in the structure of

pure methyl oleate in case of reagent using peroxyformic acid for the epoxidation process [44].

5.3 Quantification by ^{1}H-NMR analysis

Along with other determinations, it was necessary to find percent yield of epoxides formed during reaction. We considered to explore ^{1}H-NMR spectroscopy for the quantification of epoxy methyl oleate in the reaction.

The following derived equations (section 4.7, Chapter 4) was quantified by ^{1}H-NMR spectra. The ^{1}H-NMR spectra of the prepared samples were performed at a frequency of 400 MHz on JEOL by the use of 5 mm NMR tube. A set of 9, methyl oleate and epoxidised methyl oleate samples of different known ratios were prepared and analyzed by ^{1}H-NMR. Signals of methoxy protons (δ 3.63 ppm), epoxy protons (δ 2.87 ppm) and ethylene protons (δ 5.3 ppm) were taken as an internal standard for quantification as one of them is reactant and other is product and remained constant during epoxidation. The molar ratios of the methyl oleate (MO) and epoxy methyl oleate (EMO) prepared and analyzed three times and their resulted mean value was taken into account for calculating correlation between supplemented concentrations and observed concentrations for Table 1 (equation **1**) and Table 2 (equation **2**).

Table 1: The definite methyl oleate epoxide concentrations and those predicted by the qHNMR (EQUATION 1)

S. No	Mole % taken		Predicted $\%C_{EMO}$ by NMR	Mean ± SD analysis	1st analysis	2nd analysis	3rd analysis	R^2 value
	MO	EMO						
1	90	**10**	**10.7**	0.6	11	11	10	
2	80	**20**	**20.7**	0.6	21	21	20	
3	70	**30**	**31.0**	0.5	31	31.5	30.5	
4	60	**40**	**41.0**	0.5	41	41.5	40.5	
5	50	**50**	**51.3**	1.2	52	52	50	**0.99937**
6	40	**60**	**61.2**	1.0	62	61.5	60	
7	30	**70**	**71.2**	1.0	71.5	72	70	
8	20	**80**	**81.0**	1.0	82	81	80	
9	10	**90**	**91.8**	1.8	92	93.5	90	

MO = Methyl oleate, EMO = epoxy mehyl oleate, C_{EMO} = % Molar concentration of epoxy methyl oleate.

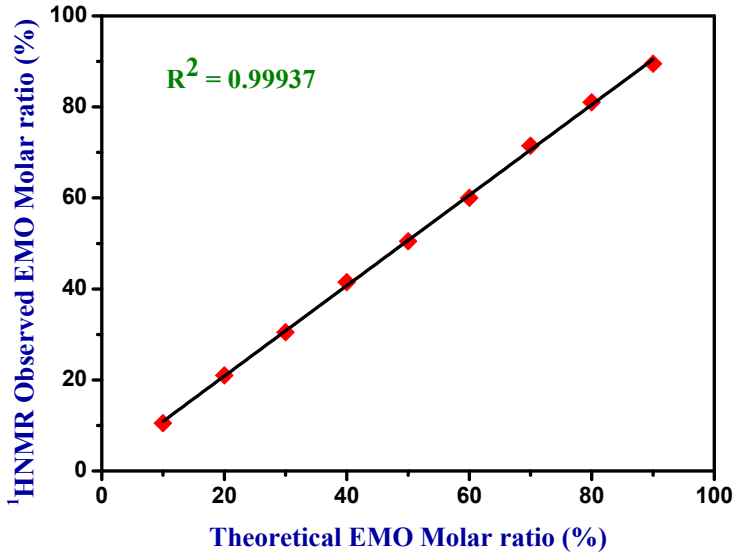


Figure 7 : A correlation line between the concentartion epoxy methyl oleate (EMO) *versus* those predicted by the ^{1}H-NMR of EMO.

Table 2: The definite methyl oleate epoxide concentrations and those predicted by the qHNMR (EQUATION 2)

S. No	Mole % taken		Predicted $\%C_{EMO}$ by NMR	Mean ± SD analysis				
	MO	EMO			1st analysis	2nd analysis	3rd analysis	R^2 value
1	90	**10**	**16.56**	6.56	17.74	15.74	16.21	
2	80	**20**	**25.94**	5.94	26.83	25.19	25.79	
3	70	**30**	**35.62**	5.62	36.45	34.79	35.63	
4	60	**40**	**44.75**	4.75	45.26	44.24	44.76	
5	50	**50**	**54.34**	4.34	54.96	54.13	53.92	**0.99977**
6	40	**60**	**64.36**	4.36	64.71	63.69	64.67	
7	30	**70**	**72.66**	2.66	74.05	71.1	72.83	
8	20	**80**	**82.93**	2.93	83.25	82.6	82.95	
9	10	**90**	**91.48**	1.48	91.68	91.24	91.52	

MO = Methyl oleate, EMO = epoxy mehyl oleate, C_{EMO} = % Molar concentration of epoxy methyl oleate.

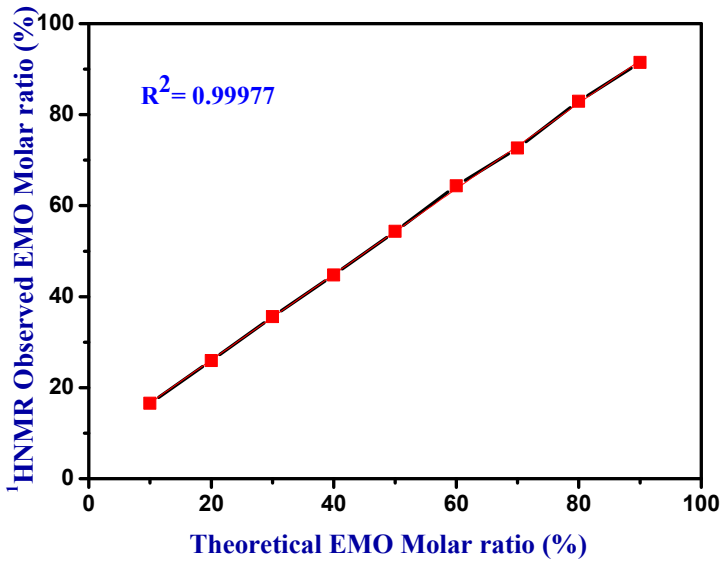


Figure 8 : A correlation line between the concentartion epoxy methyl oleate (EMO) *versus* those predicted by the ^{1}H-NMR of EMO.

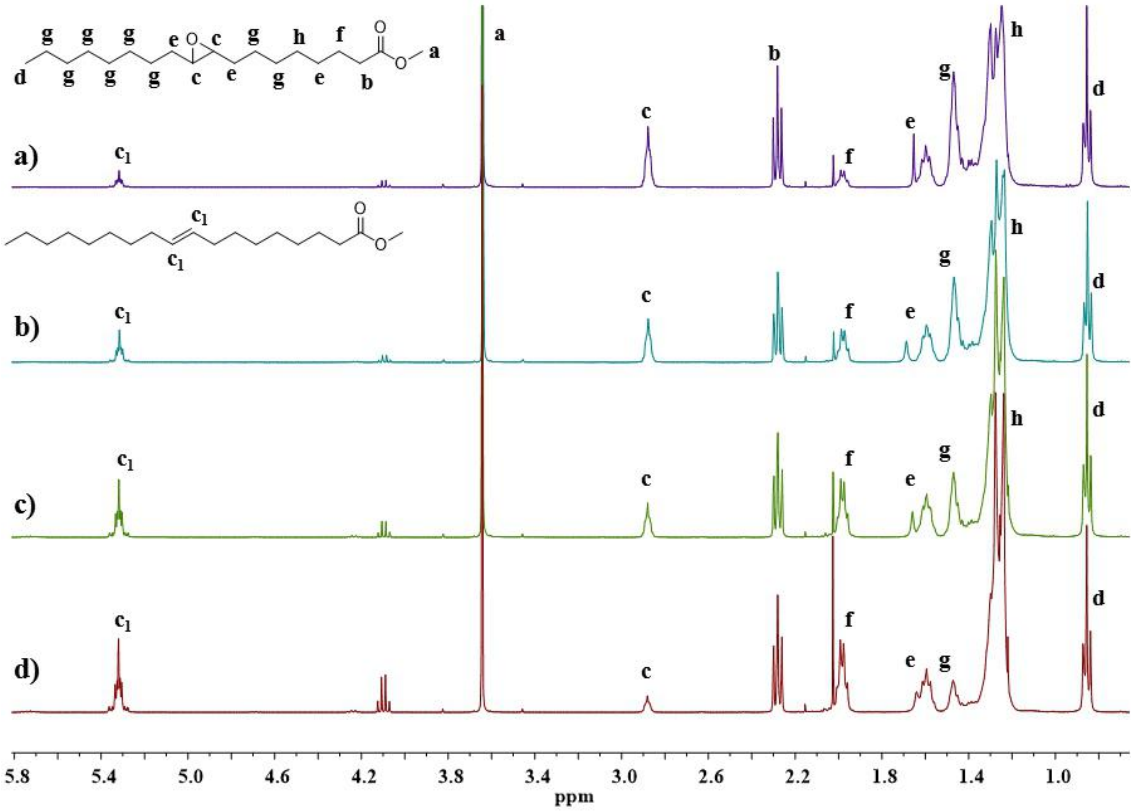


Figure 9 : (a) 80:20 (m/m) mixture of MO and EMO; b) 60:40 (m/m) mixture of MO and EMO; c) 40:60 (m/m) mixture of MO and EMO; d) 20:80 (m/m) mixture of MO and EMO**.**

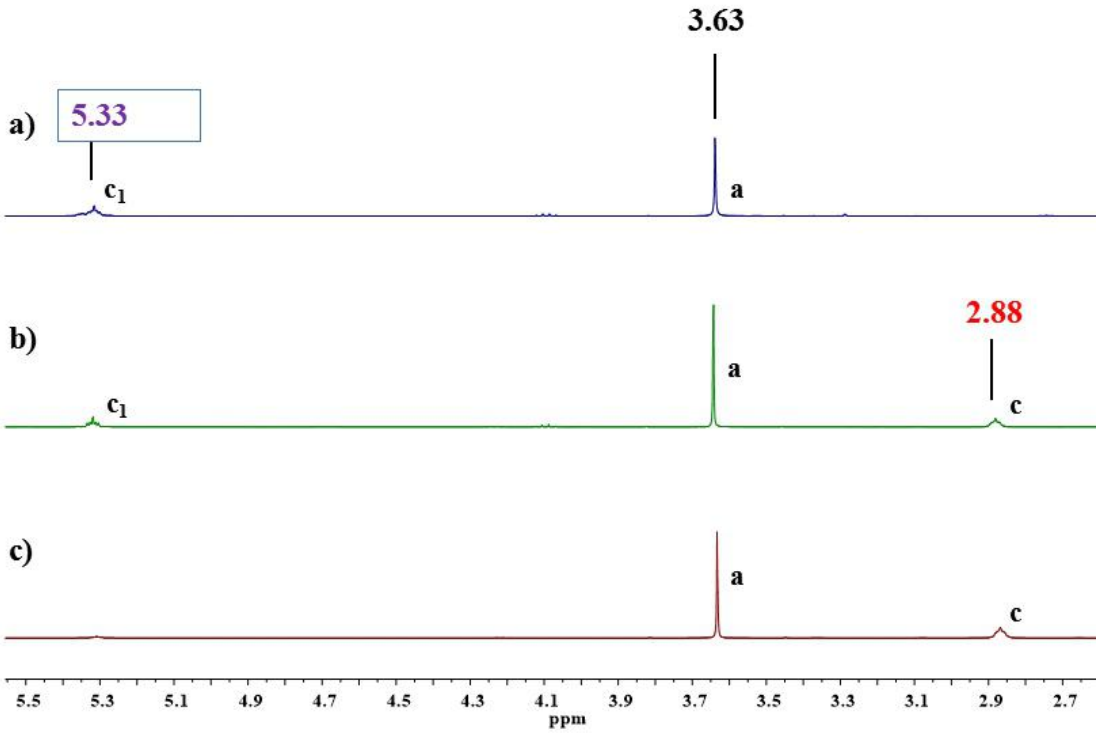


Figure 10 : Enlarged region δ 2.7-5.61 ppm of ^{1}HNMR spectra in $CDCl_3$ a) methyl oleate b) 50/50 (m/m) **2**/**3** mixture and c) epoxy methyl oleate

In the above spectra (**Fig 10**) a broad spectra showing the broad signals of methoxy signals, **a** (δ 3.63 ppm), epoxy signals, **c** (δ 2.88 ppm)and alkene signals, $\mathbf{c_1}$, (5.33 ppm).

From the Fig 9 NMR spectra (**Fig 9**), showing the different NMR spectra at different ratios, To obtain integral values, every ^{1}H-NMR spectrum was normalized by assigning the value in equation **1** and **2,** it would be concluded that the two equations (section 4.7, Chapter 4) are properly satisfied. While obtaining R^2 values by linear fitting the two equations are highly correlated and properly satisfied.

By interprating the values of these two tables, A correlation line between the concentartion epoxy methyl oleate (EMO) *versus* those predicted by the ^{1}H-NMR of EMO. The strength of the relationship between the concentration of the two variables by fitting the linear equation observed data. A correlation between the molar ratios of MO and EMO, line has an equation in the form of $\mathbf{y = a + bx}$, where **x** is explanatory variable, **y** is the dependent variable, **b** is the slope of the line and **a** is the intercept. The observed linear plotting was shown in **Figure 7 & 8.**

Conclusion

Conversion of oleic acid into methyl oleate (Biodiesel) was done by esterification process via acid catalyst where alcohol to oleic acid ratio was 9:1 taken. The % yield for the conversion of oleic acid into methyl oleate was found to be 92.5. Biodiesel (methyl oleate) was converted into EMO using peroxyacetic acid (HCOOOH) as catalyst. The conversion of MO into EMO was found to be 94.5 %. ^{1}HNMR technique has emerged as the best analytical tool for quantification purposes as it is user friendly and need less time as compared to GC and HPLC techniques. Owing to its advantages, ^{1}H NMR technique has been utilised for the first time quantification of the EMO in the mixture of MO and EMO. Two different equations have been purposed for the quantification via ^{1}H-NMR technique. The results predicted from both the equations showed good correlation with that of prepared theoretical mixture of molar ratios, MO and EMO having correlation coffiecient of 0.99937 (equation 1) and 0.99977 (equation 2). The results coming out from the ^{1}H NMR technique showed good correralation with the HPLC technique as well as with (R^2 = 0.996) mixture of molar ratios, MO and EMO. Thus ^{1}HNMR technique could be employed for the quantification of mixture of molar ratios MO and EMO.

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