J Am Oil Chem Soc (2011) 88:1285–1296
DOI 10.1007/s11746-011-1795-y

ORIGINAL PAPER

Determination of Polyunsaturated Fatty Esters (PUFA) in Biodiesel by GC/GC–MS and ^{1}H-NMR Techniques

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Received: 22 May 2010 / Revised: 22 February 2011 / Accepted: 24 February 2011 / Published online: 26 March 2011

Abstract In the present work, methods based on Gas Chromatography (GC), Gas Chromatography–Mass Spectrometry (GC–MS) and ^{1}H-NMR techniques was developed for the characterization and estimation of Polyunsaturated Fatty Esters (PUFA) in biodiesel. The GC method enables the separation, identification and estimation of these polyunsaturated fatty acid methyl esters to be carried out using a highly polar capillary column (100% cyanopropyl silicon). GC–MS was utilized for unambiguous identification and estimation of esters and isomers present in biodiesel. The estimation of PUFA content is important because PUFA content is a part of the EN 14214 specifications of biodiesel. There is no existing standard method for the estimation of total PUFA content in biodiesel. The developed GC method also quantifies EPA and DHA (C20:5 and C22:6) fatty acid methyl esters which can be used as markers for the estimation of fish oil biodiesel. The presence of EPA and DHA indicates the contamination of fish oil in biodiesel. Further, the ^{1}H-NMR technique has also been employed to identify and estimate PUFA containing $\geq$3 double bonds. The PUFA content was estimated from the integral intensities of the chemical shift region corresponding to all type of double bonds in the ^{1}H-NMR spectra. Based on the developed method, GC fingerprinting of various biodiesel samples was carried out to estimate a PUFA content as low as 1,000 ppm.

Keywords Biodiesel · Unsaturation · Polyunsaturated fatty acids · GC · Mass spectrometry · Polyunsaturated fatty esters

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Introduction

Biodiesel, defined as the monoalkyl esters of vegetable oils or animal fats, is steadily gaining interest and significance in light of recent developments such as the upsurge in petroleum prices and the implementations of financial incentives for its use. With the increasing interest and use, the assurance of fuel properties and quality has become of paramount interest for the successful commercialization and market acceptance of biodiesel. Accordingly, biodiesel standards was developed in various countries of the world, such as the United States of America (ASTM D 6751), Europe (EN 14214), Austria (OMC 1191), Italy (UNI 10635), Sweden (SSI 55436 l) and Indian Standards (IS 15607). In order to account for the nature of biodiesel, many different specifications related to the biodiesel purity was introduced into the standards.

The potential contaminants of biodiesel arise during the trans-esterification reaction such as the starting material triacylglycerol (TAG), the intermediates monoacylglycerol and diacylglycerol (MAG & DAG), residual alcohol, glycerol etc. The limits for these components have already been included in ASTM D 6751 specifications. Some other components have also been included in European specifications such as the linolenic acid methyl ester and FAME (fatty acid methyl esters) with $\geq$3 double bonds (PUFA). Biodiesel primarily contains methyl esters of C14 to C24 carbon range fatty acids with one, two and three double bonds. The content of methyl linolenate is restricted by EN 14214 because of its propensity to oxidize. The maximum limit (12%) was set so as to include high-oleic rapeseed oil, the major biodiesel feed stock in Europe. Similarly, the fish oil fatty acids are even more prone to oxidation than linolenic acid and its esters. In order to eliminate the use of fish oil as biodiesel feedstock, the FAME content with $\geq$3

double bonds (widely known as PUFA, polyunsaturated fatty esters) has also been included in EN 14214 specifications. The PUFA content was limited to 1% max as per the EN 14214 specifications [1].

Marine oils/fish oils are rich in highly unsaturated n-3 fatty acids, also termed as ω–3 fatty acids, i.e., eicosopentaenoic acid [EPA, 20:5(n-3)] and docosahexaenoic acid [DHA, 22:6(n-3)] [2]. In view of their high degree of unsaturation, both EPA and DHA are particularly susceptible to oxidative deterioration, a process generating conjugated dienes and subsequently aldehydic products. Owing to the therapeutic applications of EPA and DHA in various chronic ailments such as heart diseases, AIDS, diabetes and Alzheimer's disease, a variety of chromatographic and spectroscopic methods have been developed for their estimation in vegetable and animal fats [3–6]. However, there is no method for the estimation of EPA, DHA and PUFA content in biodiesel.

Gas chromatography offers a wide range of column chemistries for the analysis of fatty acid composition in vegetable and animal fats. The standard procedure for determining the composition of fats is by the ASTM D 1983 method which is applicable to fats containing C-8 to 24 carbon atoms. The method makes use of a moderately polar DEGS column for separating the saponified fatty acids (fatty acid methyl esters) and the esters appear in the increasing order of carbon number and of the increasing order of unsaturation for the same number of carbon atoms. Carbowax 20 M PEG column is also used for similar kind of separation but in both DEGS and PEG columns, there is a coelution of C24:0 and C22:6n3 (DHA). Similarly, there is a coelution of C22:0 and C 20:5n3. In a nutshell, the highly unsaturated fatty acid methyl esters overlap with lower saturated fatty esters having very close boiling points. Consequently, complete separation and quantification of PUFA is not possible with the existing methods.

Mass Spectrometry coupled with chromatographic techniques has also been used widely for the characterization and estimation of methyl esters of fatty acids (Biodiesel). Holcapek et al. [7] have developed an HPLC method for the separation and identification of TG, DG, MG and ME obtained during the production of rapeseed oil biodiesel according to their equivalent carbon number and compared three detection modes, viz., UV (205 nm), evaporative light scattering detection (ELSD) and mass spectrometric detection. HPLC–MS with an APCI probe was used to determine the Isobaric positional isomers on the basis of different relative abundances of fragment ions formed from the preferred loss of fatty acids by different reaction mechanisms. Ingvar Eide et al. [8] demonstrated a method based on ESI–MS and chemometrics which can be used to discriminate between biodiesel from different feedstocks and manufacturers, to identify fatty acid methyl esters (FAME) and free fatty acids, and to identify and quantify blend composition. Positive and negative ionization was used complementarily to specifically identify FAME or free fatty acids, respectively. A method for determination of fatty acid methyl esters in biodiesel—aviation turbine fuel using GC–MS in selection ion monitoring mode was developed by Energy Institute, UK [9].

With the increasing availability of modern instrumentation in research institutions, nuclear magnetic resonance (NMR) spectroscopy has become popular in the study of biodiesel. NMR offers the opportunity to study biodiesel, oils and fats non-invasively and nondestructively. $^{1}H/^{13}C$ NMR has proven useful in determining the distribution of triacylglycerols and the position of the fatty acids on the glycerol backbone [10–15]. The technique was used to obtain qualitative and quantitative information about the fatty acid composition of vegetable oils, vegetable seeds [16] and other intact tissues [17]. Based on 1H-NMR spectroscopy, a rapid and structure-specific method for the determination of ω-3 polyunsaturated fatty acids (PUFA) in fish lipids was provided by Sacchi et al. [18].

Aim of the Present Work

In the present work, Gas Chromatography (GC), GC–MS and ^{1}H-NMR techniques was used for the identification, characterization and estimation of polyunsaturated fatty esters (PUFA) content in biodiesel. The GC and GC–MS method provides the separation, identification and estimation of these polyunsaturated fatty acid methyl esters (PUFA) using a highly polar capillary column (100% cyanopropyl silicon) which allows the separation of fatty acid methyl esters having very close boiling points, cis/trans isomers and olefinic positional isomers. This GC column not only separates EPA and DHA from the corresponding saturated fatty acid methyl esters but also has the ability to separate various isomers of C18 unsaturated fatty acids present in fish oil. Identification of individual PUFA/PUFA components was carried out by running standard reference compounds. Further, ^{1}H-NMR technique was also employed to identify and estimate PUFA containing $\geq$3 double bonds for validation of the GC method.

Experimental Methods

Gas Chromatography

Gas chromatograph and column: Clarus 500 GC from M/s Perkin Elmer equipped with split/splitless injector and flame ionization detector was used for the analysis. Data were recorded and processed on a PC using a Total Chrom

workstation. A polar column CPSil 88 (50 M × 0.25 mm × 0.20 μm thickness) procured from Chrompack was used for the analysis with carrier gas as helium. The analysis was carried out at a split ratio of 1:10, with detector and injector temperature of 320 °C. The carrier gas used was UHP grade helium. An injection volume of 0.2 μl was injected for the analysis.

Oven Programming

The oven temperature was initially set at 150 °C and was increased at a rate of 4 °C up to 200 °C in the first ramp. In the second ramp, the temperature was increased at a rate of 2 °C/minute from 200 °C up to 270 °C (held for 20 min).

A split ratio of 1:10 was chosen since the sensitivity of the EPA peak at 19.0 min decreases and is not detected if we increase the split ratio to 1:50 or 1:100. The injector and detector temperatures were kept at 320 °C since the maximum permissible temperature of CPSIL 88 GC column is 275 °C. The injector and detector temperatures were kept above the maximum temperature limit of the column. A high purity helium carrier was used to avoid any noise level in the baseline. A 0.2-μl injection quantity was chosen to avoid column overloading.

Gas Chromatography–Mass Spectrometry

An Autospec Ultima High resolution mass spectrometer coupled with Agilent 6890 N Gas Chromatograph was used to carry out the analysis of biodiesel samples. The mass spectrometer was tuned for low resolution in the mass range of 50–400 Da. Perfluorokerosene (PFK) was used as reference for calibration of masses. The magnet scan was set at 0.5 s/decade with interscan delay of 0.1 s. The source was maintained at high vacuum of 10^{-8} bar and the temperature of source unit was maintained at 220 °C. Data acquisition and processing were carried out using OPUS 3.6 V software and the NIST mass spectral library was used for identification of components in the samples.

Field Desorption Mass Spectrometry (FDMS)

The analysis of biodiesel samples was also carried out on a Micro mass Auto spec Ultima mass spectrometer in the field desorption (FD) mode to characterize individual FAME from their molecular ion peaks. The mass spectrometer was tuned to a resolution of 1,000 using acetone in the mass range from 50 to 500 Da. Magnet scanning for this mass range was done at 10 s/decade for positive ions. Polyethylene glycols (PEG) were used as reference for mass calibration in the experimental mass range. The temperature of the probe was programmed to increased the heater current gradually up to 60 mA to ensure complete desorption of sample and 1 μl of 2% solution was applied on the emitter for generation of the spectra.

Reagents and Chemicals

The reagents and chemicals (methyl esters of long chain fatty acids; C-10 to C-24, EPA and DHA), standard mixture of C20:1, C20:2, C20:4 and C20:5 fatty acid methyl esters were of high purity (>99.5%) and were procured from M/s Sigma–Aldrich.

NMR Spectroscopic Studies

All the ^{1}H-NMR spectra were recorded on a Bruker ACP-300 MHz NMR spectrometer. The concentration of the sample used was 5–10% w/w in $CDCl_3$ for ^{1}H NMR containing tetramethylsilane (TMS) as the internal reference under the following experimental conditions: spectral width = 5,000 Hz (0.0–12.0 ppm), spectral size = 16 K, digital resolution = 0.49 Hz/point, 90° pulse = 18 μs, relaxation delay = 10 s and number of scans = 64. All the ^{1}H-NMR spectra were integrated three times after base line correction and a mean of three integration values was taken for each calculation.

Statistical Comparison

Table 4 shows the PUFA content estimated by GC/GC–MS and ^{1}H-NMR techniques by the developed methods. A good correlation between GC/GC–MS and ^{1}H-NMR methods was obtained (Pearson Correlation Coefficient $R^2 = 0.9954$, Fig. 1). Similarly, the correlation coefficient between actual BD-2 concentration and BD-2 concentration determined by GC/GC–MS was found to be 0.998 (Fig. 2). The results obtained by ANOVA analysis indicate that there is no significant difference obtained between GC/GC–MS and ^{1}H-NMR methods. A Student's *t* test was performed on both the calibration curves depicted in Figs. 1 and 2. The value obtained on data sets corresponding to Fig. 1, i.e., correlation of GC/GC–MS and NMR methods, was 0.107 whereas for data corresponding to Fig. 2 (correlation between actual BD-2 concentration and GC/GC–MS results, the value of Student's *t* test was 0.388. In the *t* test table, for n-1 degrees of freedom, i.e., 4, at 95% level of confidence the *t* value is 2.132. Therefore, the appreciably lower values of the two data sets, i.e., 0.107 and 0.388 indicate that there is no significant difference between the two normally distributed populations.

Results and Discussion

Figure 3 shows a typical chromatogram of biodiesel (BD-1) derived from Jatropha oil on a diethylene glycol

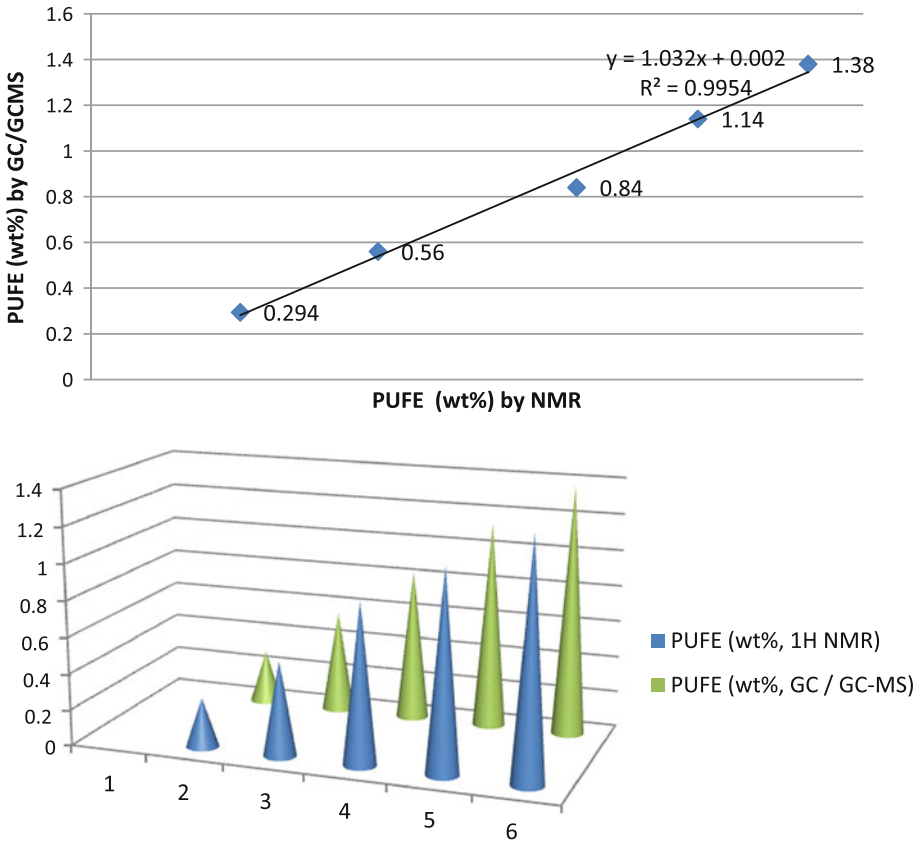


Fig. 1 Correlation of NMR and GC/GC–MS results

succinate (DEGS) column as per ASTM D 1983 procedure. The biodiesel sample usually contains methyl esters of C-12 to C-24 fatty acids having quite appreciable quantities of unsaturated (C18:1 and C18:2) fatty acids. The composition of these fatty acids, however, varies from source to source (Table 1). These conventional biodiesel samples seldom contain fatty acids higher than C-20 carbon atoms barring rapeseed oil. Figure 4 shows the chromatogram of biodiesel derived from fish oil (BD-2) on DEGS column. The major PUFA components of fish oil like EPA and DHA (as discussed above) were not found to elute from the DEGS column at a maximum oven temperature of 200 °C. In addition, lots of unresolved unsaturated fatty acid methyl esters were observed between C-16 and C-20 fatty acids. The NMR analysis of this sample showed the presence of unsaturated fatty esters having three and more than three double bonds besides the presence of oleic/linoleic esters. The higher iodine value of 160 estimated by NMR method showed the presence of PUFA. DEGS column was therefore found unsuitable for the analysis of biodiesel derived from fish oil. In order to establish the complete resolution of higher unsaturated fatty acids, a standard of C20:1, C20:2, C20:4 and C20:5 fatty esters was analyzed on DEGS column. The DEGS column could also not separate a mixture of C20:1, C20:2, C20:4 and C20:5 fatty esters. Instead of four peaks, two broad humps were obtained. This mixture was then analyzed using highly polar CPSIL-88 (cyanopropyl silica) column using the oven program discussed in experimental section. Figure 5 shows the chromatogram of C-12 to C-24 standards overlaid with C20:1, C20:2, C20:4 and C20:5 standards. The column was found to show excellent resolution between saturated as well as unsaturated fatty acid methyl esters and a considerable improvement in the peak shape. Subsequently, a mixture of saturated as well as unsaturated FAME and biodiesel derived from fish oil (BD-2) were analysed on CPSIL 88 column using the oven programming as described in the experimental section. The analysis results showed 80.6% unsaturated FAME and 28% PUFA content with all the components up to DHA (22:6) present (Fig. 6). The EPA and DHA contents (retention times 19.0 and 21.8 min) were estimated as 14.5 and 8% respectively. The quantitation was carried out using area percentage of the fatty acid methyl esters, considering uniform response factors of all the FAME components. In a similar manner, eight more samples of biodiesel derived from fish oil (BD-3 to BD-10) were analysed by the developed method and the data is given in Table 2. The results showed that the total PUFA content varied between 26 and 29% with an average value of 28%. The proper choice of the highly polar CPSIL 88 GC column instead of DEGS column and a multiple ramp GC oven program, enabled the separation of

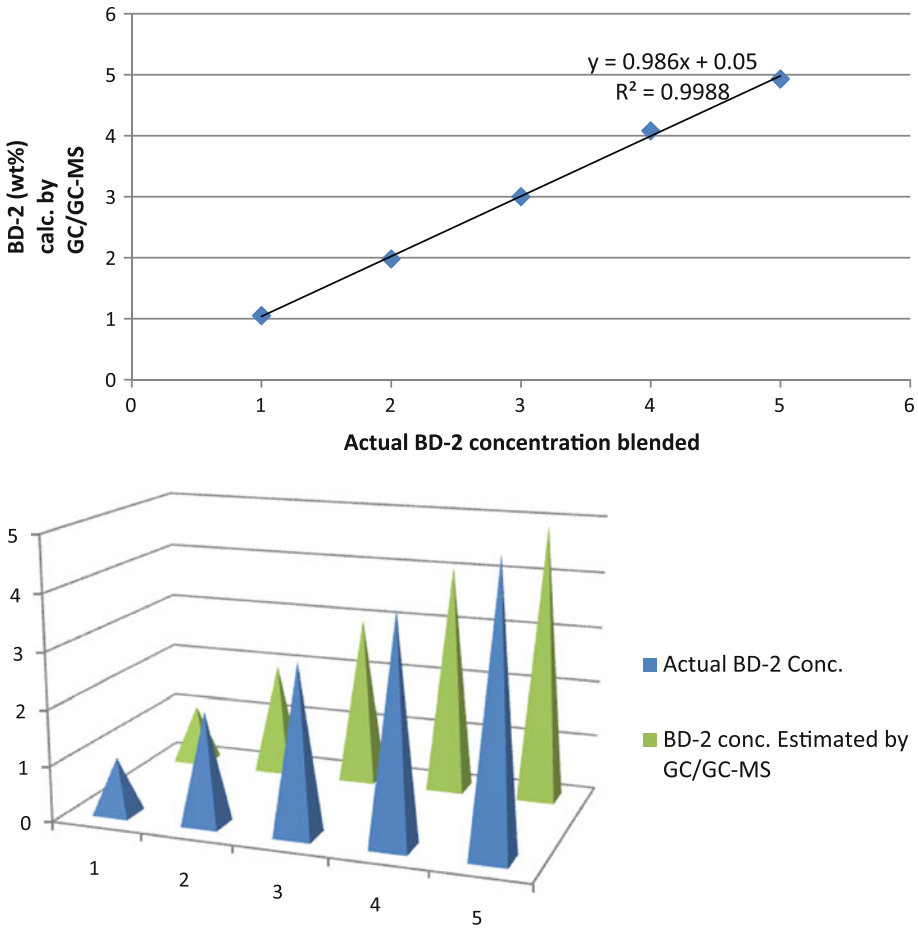


Fig. 2 Correlation between Actual BD-2 concentration blended and BD-2 (wt%) calculated

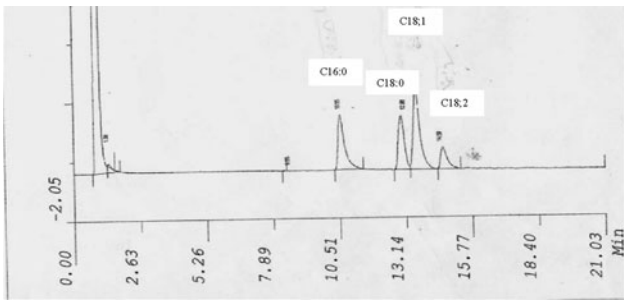


Fig. 3 GC chromatogram of Jatropha biodiesel on a DEGS column

EPA (20:5) and DHA (22:6) in fish oil biodiesel. The resolution of EPA and DHA in fish oil biodiesel was most important since the peak area corresponding to the EPA peak at 19.0 min in the GC chromatogram was used in this work for the estimation of PUFA content in biodiesel.

To confirm the identification of FAME in the biodiesel samples, BD-2 was subjected to GC–MS analysis using a CPSIL 88 GC column. The 70 eV EI mass spectrum of the separated components was identified based on the fragmentation pattern. The separated components were identified by comparing the mass spectrum of the component with spectra in the NIST mass spectral library. In this manner about 33 compounds were identified in BD-2 (Table 3). Quantitation of the components in the fish oil by GC–MS analysis was done by area normalization in the TIC chromatogram and the results were found to correlate well with GC data. Some additional fatty acids (isomers and branched fatty acids) were identified by GC–MS (GC identified 22 components) which are present in traces. Total PUFA content in BD-2 by GC–MS technique was found to be 28.6%.

In order to complement the GC–MS analysis of BD-2, FDMS analysis was carried out to characterize the components present in the sample from the molecular ion analysis. To optimize the condition of FDMS analysis, reference standard mixture containing C20:1, C20:2, C20:4 and C20:5 fatty esters was used. Figure 7 shows the FDMS spectrum of the reference sample. Figure 8 shows the FDMS spectrum of BD-2. Relative ratio of the molecular ions represents the ratio of the components in the sample. FDMS analysis of BD-2 shows the presence of molecular ion corresponding to methyl ester of C20:5 at *m/z* 316 and molecular ion corresponding to methyl ester of C22:6 at *m/z* 344 along with the presence of C16 and C18 fatty acid

Table 1 Fatty acid composition (wt%) of biodiesel derived from various sources

S. no.	Fatty acid	Fish oil[b]	Jatropha oil[a]	Karanjia oil[a]	Rape seed oil[a]	Palm oil[a]
1	C8:0	–	–		4.9	–
2	C10:0	–	–		0.9	–
3	C12:0	–	–		1.7	0.2
4.	C14:0	5.0	0.5–1.4	–	0.3	0.4
5	C16:0	10.0	12.0–17.0	3.7–7.9	5.0–14.8	40.2–44.0
6	C16:1	22	–	–	–	–
7	C16:2	7.0	–	–	–	–
8	C16:3	1.3	–	–	–	–
9	C18:0	1.9	5.0–11.0	2.4–8.9	1.0–3.2	2.7–4.6
10	C18:1	16	37.0–63.0	44.5–71.3	26.0–28.8	38.7–43.4
11	C18:2	4.1	19.0–41.0	10.8–18.3	13.0–22.1	10.5–12.7
12	C20:0	2.0	–	–	1.0–2.6	–
13	C18:3	0.3	–	–	4.6–6.2	–
14	C20:1	2.1	–	–	–	–
15	C22:0	0.6	–	–	0.5–2.9	–
16	C20:4	1.9	–	–	–	–
17	C20:5	14.5	–	–	–	–
18	C22:1	–	–	–	14.0–40.0	–
20	C24:1	1.4	–	–	–	–
21	C22:5	2.0	–	–	–	–
22	C22:6	8.0	–	–	–	–
23	Unidentified	Traces	–	–	–	–
	Total PUFA content (≥3 double bond)	28%	Nil	Nil	Nil	Nil

[a] Literature data

[b] Experimentally determined values

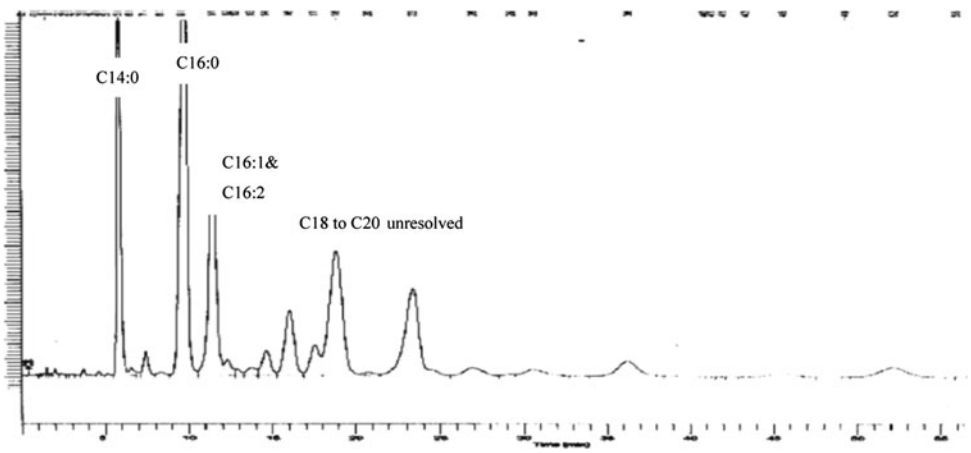


Fig. 4 GC Chromatogram of BD-2 on DEGS column

methyl esters. FDMS analysis therefore compliments the findings of the GC–MS analysis by providing intense molecular ion of the species present. The developed GC/GC–MS method could be used for estimating the quantities of EPA and DHA (C20:5 and C22:6) fatty acid methyl esters which are the markers of fish oil and to estimate the PUFA content in biodiesel. PUFA content, in other words, is an indication of contamination of fish oil in biodiesel.

PUFA content was also estimated from the ^{1}H-NMR spectral features of biodiesel derived from fish oil (BD-3 to BD-10) (Fig. 9). From the ^{1}H-NMR spectral features, one can clearly differentiate among the 1, 2 or ≥3 double bonds along the fatty ester chain. The ^{1}H-NMR spectra of biodiesel samples derived from different sources display characteristic signals due to unsaturated protons at 5.1–5.4 (multiplets), $-CH_2-CH=CH$(triplets) protons at 2.2–2.37 ppm and

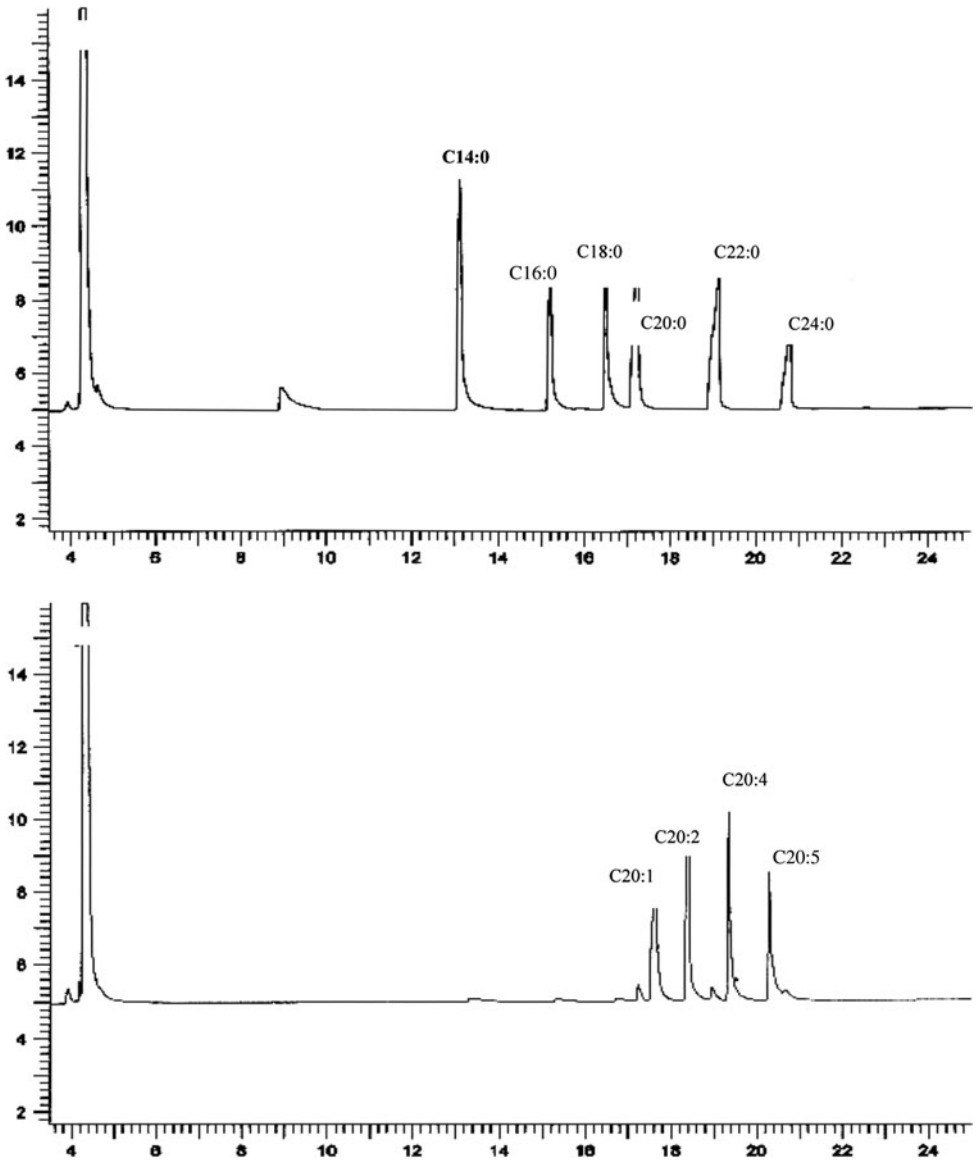


Fig. 5 Overlaid chromatogram of C-12 to C-24 standard and C20:1, C20:2, C20:4 and C20:5 standard

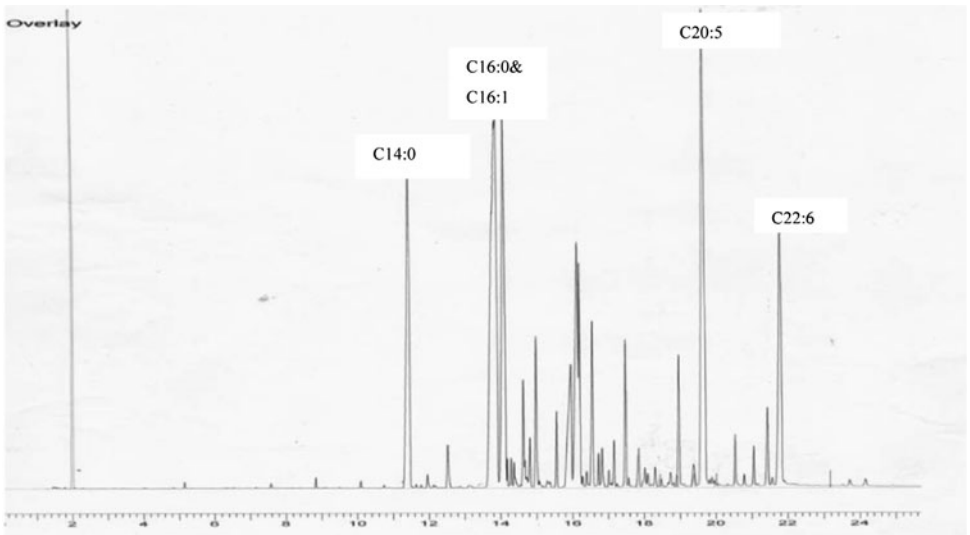


Fig. 6 GC-FID chromatogram of BD-2 on CP-SIL 88 column

Table 2 Fatty acid composition of biodiesel derived from fish oil

S. no.	Fatty acid	BD-3	BD-4	BD-5	BD-6	BD-7	BD-8	BD-9	BD-10	Average value
1	C8:0									
2	C10:0									
3	C12:0			0.5	0.2			0.5		0.4
4	C14:0	7.6	7.0	10.2	10.5	9.5	10.0	9.5	11.0	9.975
5	C16:0	0.9	13.5	5.5	10.5	1.6	1.1	0.5	1.3	4.362
6	C16:1	21.5	10.0	18.8	5.0	10.2	16.2	16.8	21.0	14.93
7	C16:2	11.2	–	1.5	1.5	16.8	10.8	9.8	11.1	8.95
8	C16:3	1.3	–	–	–	0.5	0.0	–	1.4	1.06
9	C18:0	1.5	6.8	2.0	7.0	2.3	5.7	1.5	2.2	3.625
10	C18:1	19.2	20.5	18.1	13.1	15.0	15.0	14.8	13.5	16.15
11	C18:2	3.9	4.0	4.5	4.5	9.5	4.0	4.0	7.3	5.21
12	C20:0	2.2	2.5	7.5	7.0	1.3	2.3	2.0	2.0	3.35
13	C18:3	–	2.5	4.5	5.0	2.0	4.0	4.3	–	3.71
14	C20:1	2.2	2.5	2.0	1.8	2.9	1.9	1.8	2.2	2.16
15	C22:0	0.7	3.0	0.8	1.0	–	1.6	1.7	0.7	1.357
16	C20:4	1.7	1.5	2.0	1.0	0.6	1.1	2.0	2.3	1.525
17	C20:5	14.3	14.0	13.5	14.5	13.8	13.2	13.3	11.5	13.52
18	C22:1	–		–	7.8	–	–	7.3	–	7.55
19	C21:5	0.6	2.5	0.8	0.1	0.2	0.5	–	0.8	0.78
20	C24:1	1.1	–	1.3	2.0	1.5	1.1	0.7	1.3	1.28
21	C22:5	2.6	0.8	2.2	0.5	3.5	1.4	2.4	2.2	1.95
22	C22:6	7.5	8.7	5.3	7.0	8.8	6.1	7.1	8.2	7.33
23	Unidentified	Traces	Traces	Traces	Traces	Traces	Traces	Traces	Traces	
24	Total PUFA content	28.0	28.0	27.5	28.1	29.4	26.3	29.1	26.4	27.85

Average PUFA content: 27.85%

The standard deviation of estimation of fatty acid composition in each of the above seven samples (BD-3 to BD-10) was found to be ±3 to 5%

terminal methyl protons of the long carbon fatty acid chain at 0.8–1.0 ppm. The sandwiched CH_2 protons between unsaturated protons (CH=CH–CH_2–CH=CH) are overlapped and displayed at 2.8 ppm (multiplets). The appearance of these distorted multiplets of unsaturated protons is also indicative of the presence of three or more double bonds in a sample. The signal due to protons of ester group OCH_3 and long alkyl chain (–CH_2)n are indicated at 3.65 ppm(sharp) and 1.27 ppm respectively. The appearance of methyl groups at 0.875, 0.90 and 0.97 ppm are exclusively due to saturated; mono- and di-unsaturated and polyunsaturated fatty acids($\geq$3 double bonds) respectively. Thus low field methyl signals at 0.90–1.0 ppm and other methyl signals between 0.90 and 0.8 ppm can safely be taken for the estimation of PUFA and other fatty acids respectively in a bio diesel sample. On this basis, the methyl signal intensity at 0.97 ppm was taken as a measure of PUFA present in biodiesel. The integral intensities of these regions and total region (0.5–9.0 ppm) were estimated for the quantitative estimation of PUFA and other fatty acids in a sample. The total PUFA content was calculated from the following equation:

$$\text{PUFA (wt \%)} = (I_P/9.2 \times 100) \quad (1)$$

where I_P is the percentage integral intensity of the PUFA region from 0.9 to 1.02 ppm and factor 9.2 is the average of percentage of methyl protons present in methyl esters of unsaturated fatty acids containing three or more double bonds such as linolenic (C18:3), EPA(C20:5), DHA (C22:6) etc. The factor 9.2 was calculated from the NMR spectra of the reference compounds of PUFA. Results by the NMR method was validated by the blend preparation.

Analysis of Blends of Biodiesel Derived From Jatropha (BD-1) and Biodiesel Derived From Fish Oil (BD-2)

In order to check the contamination of fish oil biodiesel in any other conventional biodiesel such as jatropha oil, palm oil, karanjia oil biodiesel etc., PUFA content can be estimated by taking the EPA or DHA peak into consideration. Assuming that fish oil biodiesel contains 14% EPA and 28% total PUFA as an average value, the EPA concentration determined directly from the GC/GC–MS chromatogram can be either used to estimate the fish oil

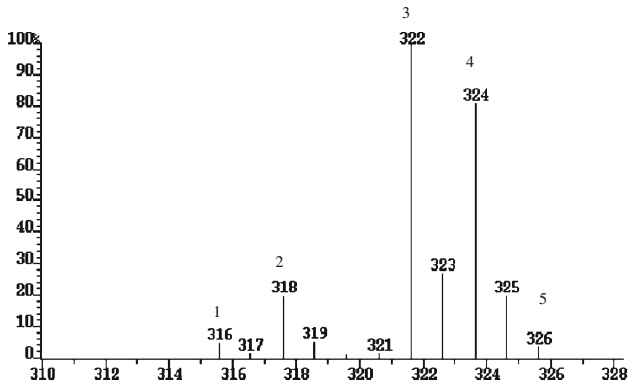
Table 3 Components identified in BD-2 by GC–MS analysis

S. no.	Component	M.F	M.wt
1	Methyl ester of tetradecanoic acid	$C_{15}H_{30}O_2$	242
2	Methyl ester of 9-methyl tetradecanoic acid	$C_{16}H_{32}O_2$	256
3	Methyl ester of pentanoic acid	$C_{16}H_{32}O_2$	256
4	Methyl ester of hexadecatetraenoic acid (16:4) + unidentified	$C_{17}H_{26}O_2$	262
5	Methyl ester of hexadecatrienoic acid (16:3)	$C_{17}H_{28}O_2$	262
6	Methyl ester of hexadecenoic acid	$C_{17}H_{32}O_2$	268
7	Methyl ester of hexadecanoic acid	$C_{17}H_{34}O_2$	270
8	Methyl ester of methyl hexadecenoic acid	$C_{18}H_{36}O_2$	282
9	Methyl ester of methyl hexadecanoic acid	$C_{18}H_{38}O_2$	284
10	Methyl ester of methyl hexadecenoic acid	$C_{18}H_{36}O_2$	282
11	Methyl ester of heptadecanoic acid	$C_{18}H_{34}O_2$	284
12	Methyl ester of octadecatrienoic acid (C18:3)	$C_{19}H_{32}O_2$	292
13	Methyl ester of octadecadienoic acid (isomer)	$C_{19}H_{34}O_2$	294
14	Methyl ester of octadecadienoic acid	$C_{19}H_{34}O_2$	294
15	Methyl ester of octadecenoic acid (isomer)	$C_{19}H_{36}O_2$	296
16	Methyl ester of octadecenoic acid (isomer)	$C_{19}H_{36}O_2$	296
17	Methyl ester of octadecanoic acid	$C_{19}H_{38}O_2$	298
18	Methyl ester of methyl octadecanoic acid	$C_{20}H_{40}O_2$	312
19	Methyl ester of eicosa-5,8,11,14,17-pentaenoate (C20:4)	$C_{21}H_{34}O_2$	318
20	Methyl ester of eicosa-5,8,11,14,17-pentaenoate (C20:5) -isomer	$C_{21}H_{32}O_2$	316
21	Methyl ester of eicosatrienoic acid (C20:3)	$C_{21}H_{36}O_2$	320
22	Methyl ester of eicosadienoic acid	$C_{22}H_{38}O_2$	322
23	Methyl ester of eicosadienoic acid	$C_{22}H_{38}O_2$	322
24	Methyl ester of eicosenoic acid	$C_{21}H_{38}O_2$	324
25	Methyl ester of eicosenoic acid (isomer)	$C_{21}H_{40}O_2$	324
26	Methyl ester of eicosanoic acid	$C_{21}H_{42}O_2$	326
27	Unidentified ester		
28	Methyl ester of docosahexaenoic acid (C22:6)	$C_{23}H_{34}O_2$	342
29	Methyl ester of docosapentaenoic acid (C22:5)	$C_{23}H_{36}O_2$	344
30	Methyl ester of docosenoic acid	$C_{23}H_{44}O_2$	352
31	Methyl ester of docosanoic acid	$C_{23}H_{46}O_2$	354
32	Methyl ester of (C24:1)	$C_{24}H_{48}O_2$	380
33	Methyl ester of tetracosanoic acid	$C_{24}H_{50}O_2$	382

biodiesel content or the PUFA content. The use of the EPA peak as a marker for fish oil biodiesel content or PUFA content, is required in the case of very low concentrations of PUFA, especially 1% or less. The EN 14214 specifications (1% max) demand the determination of PUFA content in the lower concentration range. The well-resolved peak of EPA in the chosen column chemistry and oven program in the GC facilitates the determination of PUFA content at such a low level. Consequently, BD-2 was blended in BD-1 in the concentration range of 1.0–5.0 weight% so that the PUFA concentration lies in the range 0.2–1.50 wt%. These synthetic blends were analysed by the developed GC/GC–MS and ^{1}H-NMR methods. The peak area corresponding to EPA peak at 19.0 min in the GC/GC–MS chromatogram can be used for estimating the fish oil biodiesel content and the PUFA content in biodiesel. The ^{1}H-NMR results were calculated from Eq. 1. Table 4 shows the data obtained by the developed GC/GC–MS and ^{1}H-NMR methods in five blends of conventional biodiesel (BD-1) and fish oil derived biodiesel (BD-2). The results show good correlation between the actual values (blended) and the experimental results.

Calculation of PUFA Content in Biodiesel Samples

In an unknown sample of biodiesel, the area percent of the peak corresponding to EPA at 19.0 min is determined denoted by *X* (area of the peak at 19.0 min/total area in the



Methyl esters identified	Molecular ion (observed)
1. C20:5	316
2. C20:4	318
3. C20:2	322
4. C20:1	324
5. C20:0	326

Fig. 7 FDMS spectrum of reference containing C20:1, C20:2, C20:4 and C20:5 fatty esters

GC chromatogram). The PUFA content (Y) is then estimated by the following equation:

$$\text{PUFA content, } Y = X \times (27.8/14.5)$$

where, 27.8 is the average percent of PUFA content in fish oil biodiesel and 14.5 is the average percent EPA content in fish oil biodiesel.

Conclusions

Both ^{1}H NMR and Gas Chromatography/GC–MS techniques offer a quick and direct method for the estimation of fish oil methyl esters as well as the PUFA content in biodiesel derived from vegetable as well as animal origin. Gas chromatography coupled with Mass Spectrometry was used to identify and separate EPA and DHA from fish oil methyl ester and explored the possibility of using them as markers for the estimation of fish oil methyl ester content in biodiesel. From the ^{1}H-NMR spectral features, we can clearly differentiate and estimate among the 1, 2 or $\geq$3 double bonds along the fatty ester chain while GC can identify and quantify each saturated as well as unsaturated fatty ester. An excellent correlation was obtained between GC/GC–

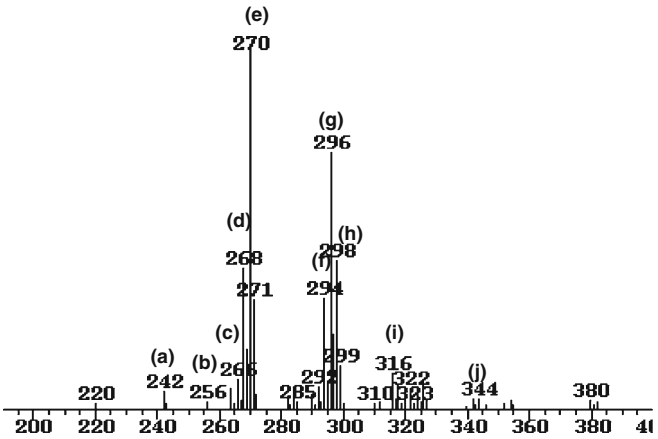


Fig. 8 FDMS spectrum of BD-2 showing molecular ion at m/z 316 and 344 corresponding to EPA and DHA

Components identified	General Formula	Molecular ion (m/z)
(a) Methyl ester of Tetracanoic acid (C14:0)	$C_{15}H_{30}O_2$	242
(b) Methyl ester of 9-methyl Tetradecanoic acid/ Pentanoic acid	$C_{16}H_{32}O_2$	256
(c) Methyl ester of Hexadecadienoic acid	$C_{17}H_{30}O_2$	266
(d) Methyl ester of Hexadecenoic acid $C_{17}H_{32}O_2$	268	
(e) Methyl ester of Hexadecanoic acid	$C_{17}H_{34}O_2$	270
(f) Methyl ester of Octadecadienoic acid	$C_{19}H_{34}O_2$	294
(g) Methyl ester of Octadecenoic acid (isomer)(C18:1) $C_{19}H_{36}O_2$		296
(h) Methyl ester of Octadecanoic acid	$C_{19}H_{38}O_2$	298
(i) Methyl ester of Eicosa-pentaenoate(C20:5)	$C_{21}H_{32}O_2$	314
(j) Methyl ester of Docashexaneoic acid(C22:6)	$C_{23}H_{34}O_2$	342

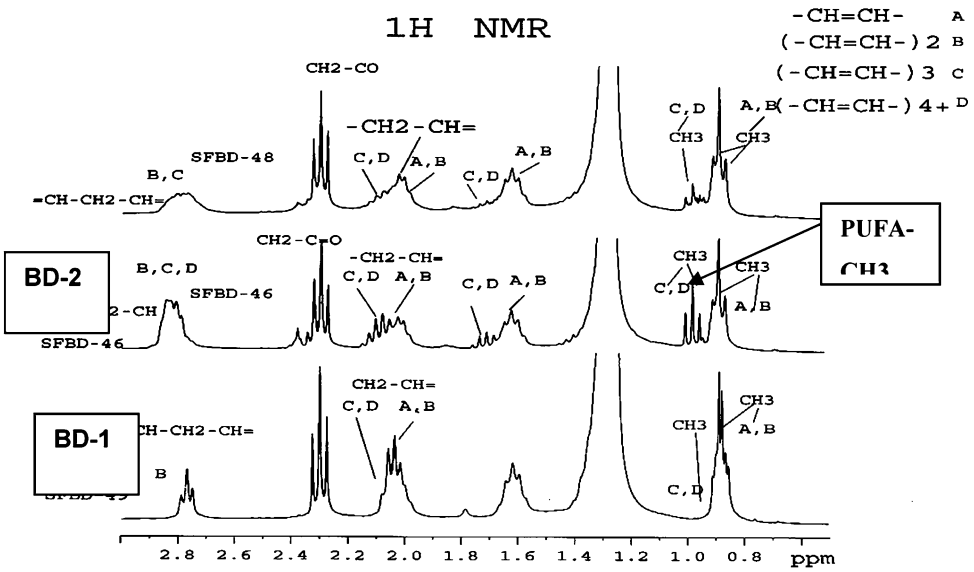


Fig. 9 1H-NMR spectrum of samples containing PUFA and other fatty acids in biodiesel

Table 4 PUFA Content (wt%) of synthetic blends obtained by ^{1}H-NMR and GC techniques

Blend no.	Actual BD-2 conc. blended	BD-2 (wt%, GC/GC–MS)	PUFA (wt%, ^{1}H NMR)	PUFA (wt%, GC/GCMS)
BL-1	1.0	1.05	0.27	0.294
BL-2	2.0	1.98	0.52	0.56
BL-3	3.0	3.0	0.88	0.84
BL-4	4.0	4.08	1.10	1.14
BL-5	5.0	4.93	1.30	1.38

The standard deviation of the estimation of BD-2 content by GC/GC–MS was found to be ±4% and the standard deviation of estimation of PUFA content by ^{1}H NMR was found to be ±2.5% and by GC/GC–MS was also found to be ±2.5%

MS and ^{1}H-NMR results up to a detection limit of 1,000 ppm. The repeatability of the estimation of PUFA content was found to be ±2.5%.

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