Appl Biochem Biotechnol (2011) 165:155–177
DOI 10.1007/s12010-011-9241-z

Identification and Quantitation of Reaction Intermediates and Residuals in Lipase-Catalyzed Transesterified Oils by HPLC

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Received: 11 January 2011 / Accepted: 4 April 2011 /
Published online: 27 April 2011

Abstract A high-performance liquid chromatography (HPLC) unit equipped with size exclusion column and a refractive index detector was used for simultaneous monitoring, identification, and quantitation of the reaction components from lipase-catalyzed transesterification of three oils. The procedure simultaneously separated and detected the unreacted triacylglycerols (TAG), diacyl-, and monoacyl-glycerol (DAG and MAG) co-products, residual alcohol as well as free fatty acid (FFA) based on retention times. The chromatograms showed well separated and resolved peaks. The elution of the components from the transesterification reaction in increasing order was: TAG<DAG<FFA<MAG. Generally, higher alcohol ratios decreased the conversion of TAG in all the oils studied with between 14% and 94% of TAG remaining at all the treatment combinations. Higher amount of salmon skin oil (SSO) TAG was generally converted to DAG than Rothsay composite (RC) and olive oil (OO) TAG. Relatively higher amount of OO DAG was converted to MAG than SSO and RC with only 5–14% DAG remaining in OO. RC and OO generally accumulated less MAG, and this was reflected as lower MAG levels in RC (<6%) and OO (<14%) compared with SSO (<27%). For the various treatment combinations and the three oils used in this study, the least amount of FFA was recorded in transesterified OO with a maximum of approximately 4%. This HPLC method can be used as a simple and fast technique to analyze the reaction components and products of transesterification reactions without the need for additional derivatization steps.

Keywords Acylglycerols · Lipase · Isocratic elution · Refractive index detection · HPLC

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Introduction

There has been considerable progress in the development of biodiesel (BD) production technologies over the past two decades [1, 2]. While the characterization of the feedstock is essential to accord the best pretreatment regimes for the transesterification reaction, it is equally important to have in place accurate and rapid identification and quantitation techniques for the reaction products and co-products to assure better and continuous quality control [3–5].

When the process of transesterification and/or purification post-synthesis is incomplete, significant amounts of residual mono-, di-, and triacylglycerols, free glycerol, catalyst, and other minor components would remain in the fuel thereby contaminating it. These contaminants can affect fuel quality leading to engine problems and hazardous emissions [5–7]. Standards for BD quality have been established, and these include test for free and total glycerol content, flash point, and acid value in Europe, North America, and elsewhere [4, 5, 7, 8, 9] to monitor the completeness of the transesterification reaction and/or post-synthesis purification.

Although the transesterification reaction process is relatively straightforward, feedstock and product quality testing can be time consuming. Several separation and analytical techniques such as gas chromatography (GC), thin-layer chromatography, high-performance liquid chromatography (HPLC), and infrared techniques [3, 4, 10] have been simplified, automated, and used to identify and measure fuel quality indices. According to Plattner [11] and other workers [12, 13], the commonly used GC method for BD quantitation is not a convenient or direct analytical method for the detection and quantitation of the contaminants (triacylglycerols (TAG), diacylgylcerol (DAG), monoacylglycerol (MAG), free fatty acid (FFA), glycerol, and unreacted alcohol) in the products. They further indicated that the commonly used GC methods require carefully controlled sample derivatization (trimethylsilylation or acetylation) prior to the GC analysis [3, 13, 14]. In addition, an inert, short capillary column with stable and high column temperature ($\geq$350°C) as well as elaborate pretreatment steps to separate the glycerol from the BD is usually required [11, 15].

An alternative to GC and other techniques mentioned above is the HPLC technique, which is fairly sensitive, reproducible, and rapid [11, 15, 16]. Additional advantages of the HPLC method include direct BD analyses, identification, and quantitation of the products and co-products of the reaction [10, 14, 17], thus minimizing the exposure to reagents and the extra cost associated with reagents and time. The HPLC system has been used with various detectors with different selectivities and sensitivities [10, 18, 19]. The HPLC-UV system is commonly used because of its high sensitivity and adaptability to the frequently used gradient elution technique [11, 18, 19]. However, it presents some constraints with respect to the monitoring of fuel contaminants such as TAG, DAG, or MAG due to the unavailability of chromophores above 200 nm. Another limitation of the HPLC-UV system is its incompatibility with some commonly used solvents in lipid analysis such as chloroform and tetrahydrofuran; these solvents absorb strongly in the region of 190–220 nm, where most lipids absorb [11, 20, 21]. In contrast, the refractive index (RI) detector is a robust instrument for liquid chromatographic studies [12, 17, 22] that can be used with most solvent systems compared to the UV detector [11]. In spite of this, the HPLC-RI system has not been widely used in quantifying the products and co-products of transesterification reactions [15, 21, 22].

In this study, the co-products (TAG, DAG, MAG, and FFA) formed from the production of BD from salmon oil, olive oil, and a commercial fat-oil sample obtained from Rothsay®

Biodiesel Company (referred to as Rothsay Composite) were simultaneously verified and quantitated using an HPLC system equipped with a RI detector. While previous papers have mostly described quantitation of these components in methanol-based BD (methyl esters) [15, 17, 22], the present study was on ethanol-based BD (ethyl esters).

Materials and Methods

Materials

A commercial mixture of rendered animal fat and used frying oil (Rothsay composite, RC) was provided by Rothsay® Biodiesel Company (Ste. Catherine, QC); salmon skin oil (SSO) was obtained from smoked salmon skin provided by Atkins et Frères Inc. (Mont-Louis, Gaspé, QC); olive oil and immobilized lipase (Lipozyme-IM®; 86.8 U/g) were purchased from Sigma–Aldrich (Oakville, ON). One unit of immobilized lipase activity is defined as the amount producing 1 μmol of stearic acid per minute from the hydrolysis of tristearin at pH 8.0 and 70°C; anhydrous ethanol (EtOH) was purchased from Commercial Alcohols (Boucherville, QC); toluene and acetic acid were obtained from Fisher Scientific (Whitby, ON); fatty acid ethyl ester (FAEE), TAG, DAG, MAG, and FFA standards were purchased from Nu-Chek Prep Inc. (Elysian, MN).

Methodologies

Oil Characterization

SSO was obtained from salmon skin by solvent extraction according to the method previously described by Aryee and Simpson [23]. The FFA content, moisture content, and the fatty acid composition of the starting oils were determined in duplicates according to the AOCS method (Ca 5a–40), AOCS method (Ca 2c–25) [24], and with GC, respectively.

Time-Course Transesterification Reaction

Transesterification of the test samples (5 g of SSO, RC, or OO) was carried out in 30-ml stoppered vials using a fixed load of Lipozyme®-IM [(Enz 21.70 U)], oil to alcohol molar ratio (AlcoR; ranging from 1:1 to 1:6), at five different temperatures (25, 35, 45, 55, and 65°C) and with uniform shaking in a water bath (Precision Scientific Shaking Water bath 25, Chicago, IL, USA) at 60 shaker rate per min. Aliquots were withdrawn from the vial after reaction times ranging from 8 to 120 h and dissolved in toluene prior to injection into the HPLC-RI unit.

HPLC Identification and Quantitation Analysis

Calibration curves were developed for MAG, DAG, TAG, FAEE, FFA, and EtOH for quantitation of the components produced during the transesterification reaction. Standard solutions of the each of the standard at five different concentration levels (0.3125–5 mg/ml) were prepared in toluene and injected and analyzed by the HPLC-RI system. The data obtained were fitted by linear regression and the corresponding equations generated for quantitation.

The standards and reaction mixtures from the transesterification were analyzed using an HPLC system equipped with a 5-μm Phenogel™ 300×7.8 mm ID size exclusion column (Phenomenex, Torrance, CA, USA) with a 50×7.8-mm guard column and a RI detector. Elution was carried out in isocratic mode using 0.25% (*v*/*v*) acetic acid in toluene at a flow rate of 1.0 ml min^{-1}. An auto sampler and injector were used to inject 25 μl of the standard or test sample into the HPLC system. The peak areas and response factors of the samples were recorded under the same conditions as the standards. A Varian Galaxie™ (Varian, Palo Alto, CA, USA) software was used for data acquisition.

The criteria for identification of the compounds in the samples were established based on comparisons with the retention times and chromatograms of the standards. The peaks in the chromatograms were automatically integrated to generate the data for quantitation. The % yield of TAG, DAG, MAG, and FFA was determined using the integrated data of the corresponding peak and interpolated from calibration curve constructed.

Statistical Analysis

The data obtained from the studies were analyzed using PROC MIXED (Statistical Analysis Systems, Version 9.2, SAS Institute Inc., Cary, NC, USA) with temperature and alcohol ratio as fixed effects, vial as a random effect, and reaction time (8, 12, 24, 36, 48, 60, 72, 96, and 120 h) as repeated measures. The autoregressive 1 was selected among all competing variance–covariance structures to evaluate the correlation between measurements [25, 26].

Results

Oil Characterization

Two of the important quality indices used to monitor the transesterification reaction are acidity as measured by FFA content and moisture content of the feedstock. The FFA content of SSO and RC were relatively higher (4–6%) than that of OO (~0.2% FFA). The corresponding moisture contents of these oils (SSO, RC, and OO) were estimated as 0.02%, 0.03%, and 0.01%, respectively. As shown in Table 1, the predominant FAs were C_{18}-fatty acids (42–82%); furthermore, there were approximately equivalent amounts of saturated and monosaturated FAs in the RC test sample (Table 1).

Characterization of Samples by HPLC

The transesterification reaction occurs in three sequential steps. TAG reacts with ethanol to produce DAG, MAG, and glycerol sequentially, and each step produces a molecule of FAEE. Under optimum reaction conditions, to attain maximum FAEE yield, the TAG, DAG, and MAG components would be expected to be nil or negligible. Characterization of BD produced by the transesterification of lipids for quality assurance is useful to avert the engine and fuel injection problems associated with using poor-quality fuels. For accurate and complete quantitative analysis, complete separation of all the reaction components is required to ensure unequivocal assignment of the peaks in the chromatographic spectra.

Table 1 Fatty acid composition of salmon skin oil, Rothsay composite, and olive oil

Fatty acid	Salmon skin oil	Rothsay composite	Olive oil
Lauric (12:0)	0.14	0.00	0.00
Myristic (14:0)	5.77	1.47	0.00
Palmitic (16:0)	16.94	27.11	14.38
Palmitioleic (16:1, *n*−7)	5.42	2.29	0.13
Steric (18:0)	4.31	12.79	3.75
Oleic (18:1, *n*−9)	19.20	39.59	67.75
Linoleic (18:2, *n*−6)	16.05	15.82	10.78
Linolenic (18:3, *n*−3)	2.82	0.81	0.68
Eicosapentaenoic (20:5, *n*−3)	15.55	0.00	0.00
Docosapentaenoic (22:5, *n*−3)	2.45	0.13	0.00
Docosahexaenoic (22:6, *n*−6)	11.36	0.00	2.58
∑ Saturated fatty acid (SFA)	27.16	41.37	18.13
∑ Monounsaturated fatty acid (MUFA)	24.62	41.88	67.88
∑ Polyunsaturated fatty acid (PUFA)	48.23	16.76	14.04
∑ ω-3 fatty acid	20.28	0.94	0.68
∑ ω-6 fatty acid	27.41	15.82	13.36
PUFA/SUFA	1.78	0.41	0.77
ω-3/ω-6	0.74	0.06	0.05

Values are percentage of the total fatty acid

The components in BD produced from the various oils (SSO, RC, and OO) investigated in this study were verified using an HPLC method based on size exclusion with RI detection in a single 25-min runtime. The RI detector response was linear over the concentration range of 0.3125–5 mg/ml (Fig. 1), as indicated by the high correlation coefficients (R^2>0.99). TAG and DAG eluted ahead of MAG and FFA (Fig. 2).

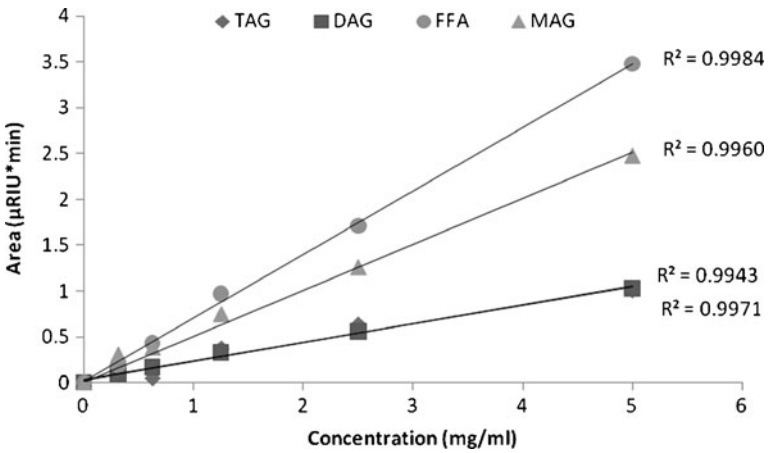


Fig. 1 Calibration curves of *TAG*, *DAG*, *MAG*, and *FFA*

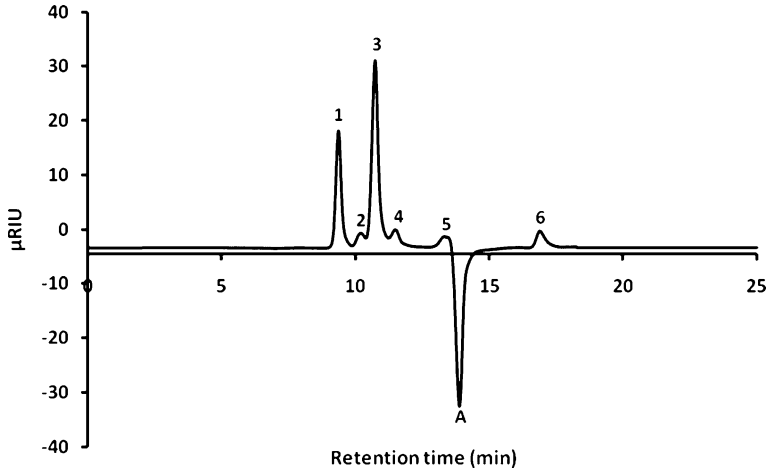


Fig. 2 HPLC chromatogram of transesterification components monitored with RI (isocratic elution mode, 0.25% (v/v) acetic acid in toluene in 25 min). Peaks correspond to *1* TAG, *2* DAG, *3* FAEE/BD, *4* FFA, *5* MAG, and *6* ethanol (EtOH) and *A* internal flow marker

Quantitation of the Reaction Components

The transesterification reactions were performed at 25–65°C using various AlcoR for SSO, RC, and OO, respectively, to evaluate the effects of the reaction parameters on the yield of co-products from the transesterification reaction.

The data for TAG, DAG, MAG, and FFA contents obtained from the transesterification of SSO, RC, and OO at 35°C, AlcoR of 1:4 and at different reaction times are shown in Fig. 3. The TAG content steadily decreased from ~85% (8 h) to 20% (120 h) while MAG yield increased. The highest level of DAG (11%), MAG (24%), and FFA (4%) were attained at 72 h (Fig. 3a). In the RC sample, TAG content decreased from ~81% (8 h) to 54% (120 h) with variable amounts of MAG. The highest amount of DAG (19%), MAG (5%), and FFA (6%) were recorded at 36, 24, and 72 h, respectively (Fig. 3b). The TAG in transesterified OO decreased from ~88% (8 h) to 78% (120 h) with variable MAG contents. The highest amount of DAG (10%), MAG (6%), and FFA (0.6%) were, respectively, recorded at 8, 96, and 60 h (Fig. 3c). The interactions of all the factors at the Type 3 tests of fixed effects were significant ($p<0.05$).

For the purpose of illustration and space limitation, the yields of TAG, DAG, MAG, and FFA for SSO for the various treatment combinations are presented graphically in Figs. 4, 5, 6, and 7 while those for RC and OO are tabulated in Tables 2, 3, 4, and 5. The data presented in Figs. 4, 5, 6, and 7 and Tables 2, 3, 4, and 5 show that ≥14% TAG content was recorded in all the three oils for all the treatments (temperature and oil/alcohol ratio) investigated. In general, higher oil/alcohol ratio decreased the conversion of TAG to BD with various amount of the other co-products (Figs. 4, 5, 6, and 7 and Tables 2, 3, 4, and 5).

SSO Transesterification Residuals and Co-products

At 25°C, there was substantial decrease in TAG content in the SSO sample at all the AlcoRs except at AlcoR 1:6 (Fig. 4a). While all four AlcoRs showed approximately >64%

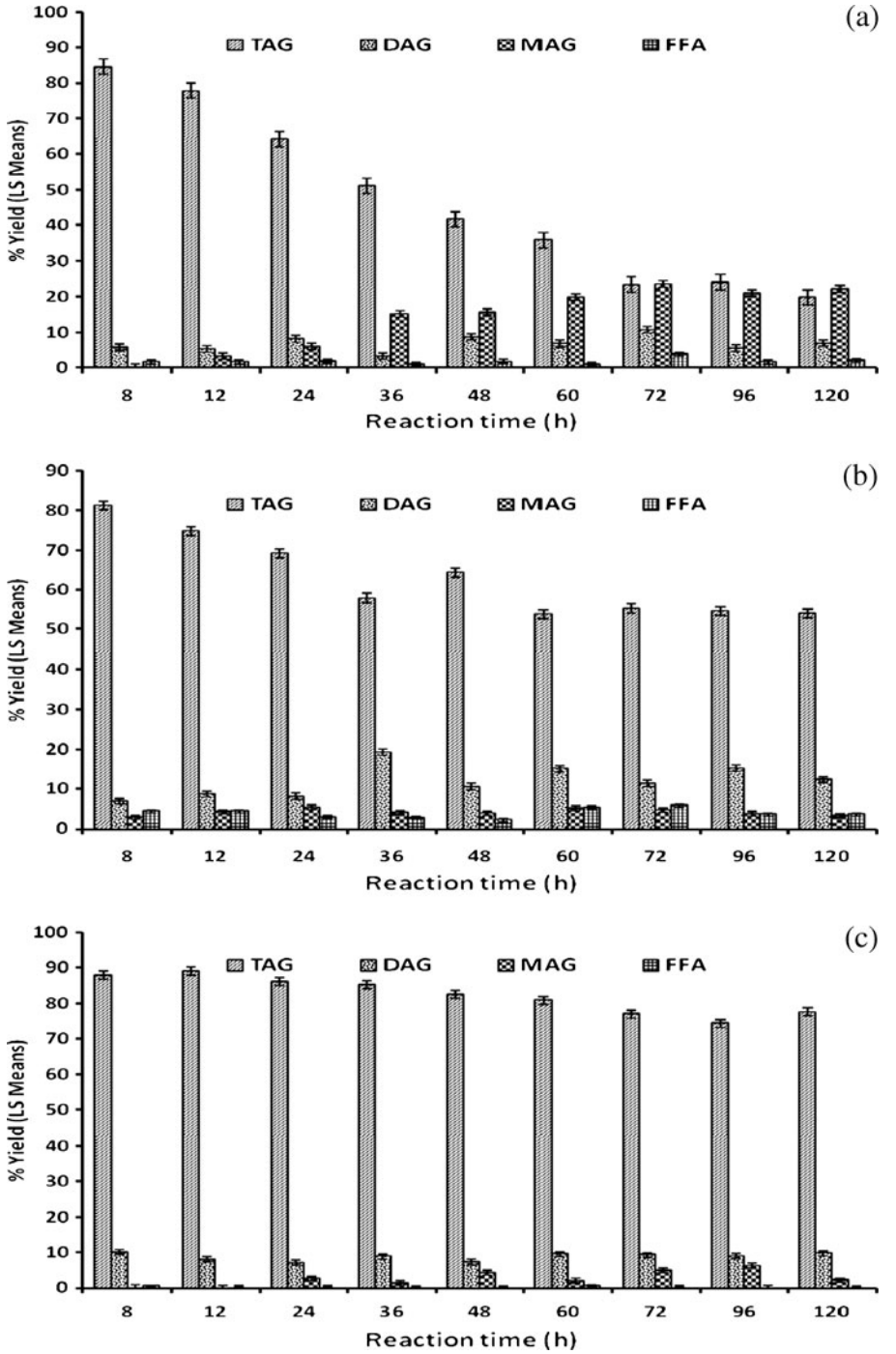
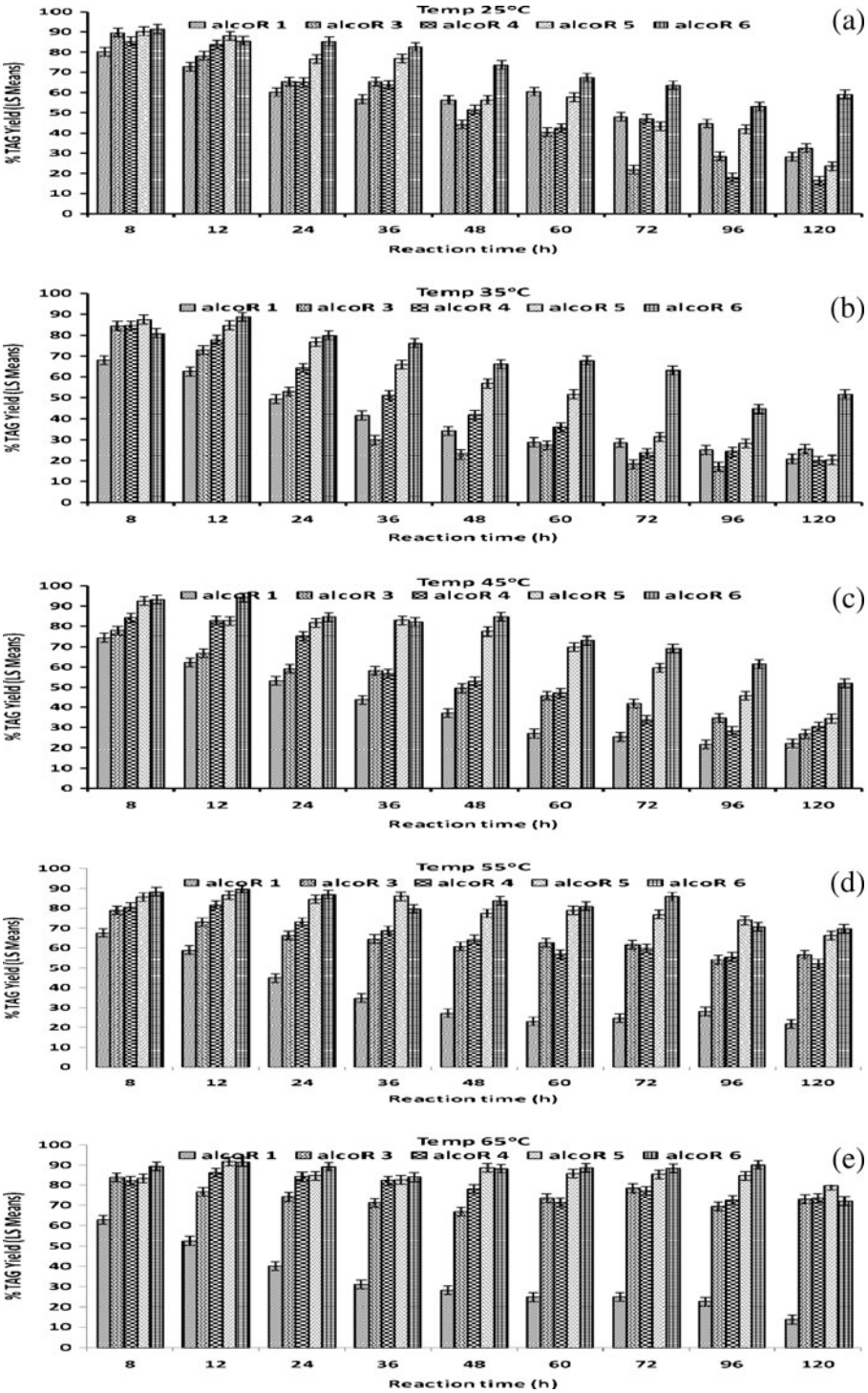


Fig. 3 Remaining and residual components during the transesterification of **a** SSO, **b** RC, and **c** OO (reaction conditions, oil: alcohol ratio 1:4, temperature 35°C)


Temp 25°C
alcoR 1
alcoR 3
alcoR 4
alcoR 5
alcoR 6
(a)
% TAG Yield (LS Means)
Reaction time (h)
Temp 35°C
(b)
Temp 45°C
(c)
Temp 55°C
(d)
Temp 65°C
(e)

◀ **Fig. 4** Least Square means (LS Means)±standard error of treatment × time of TAG (% TAG yield) of transesterified SSO at **a** 25°C, **b** 35°C, **c** 45°C, **d** 55°C, and **e** 65°C

decrease in TAG content between 8 and 120 h, AlcoR 1:6 showed only 35% decrease in TAG content, and these were all significant ($p<0.05$).

The DAG content was fairly moderate (≤11%) at 25°C (Fig. 5a) at all the AlcoRs studied up until 48 h, beyond which DAG content increased to between 1% and 20% at all the AlcoRs for the remainder of the experimental period. There was slow conversion of DAG to MAG and low accumulation of it during the initial period of the experiment at all the treatment combinations (Figs. 6a–e). Higher amounts ($p<0.05$) of MAG were accumulated at AlcoRs 1:3, 1:4, and 1:5 beyond 72 h at 25°C (Fig. 6a). With the exception of AlcoR, 1:1<7% of FFA was recorded at all the other treatment combination at 25°C in transesterified SSO (Fig. 7a).

Similar pattern in TAG content was observed at 35°C in transesterified SSO (Fig. 4b). DAG content increased at all the reaction times studied at 35°C and AlcoR 1:1 (Fig. 5b); it, however, maintained a fairly constant though significant ($p<0.05$) DAG content through the experimental period at the other AlcoRs. MAG content at 35°C followed similar trend as 25°C as evident by its appreciable increase with time at AlcoRs 1:3, 1:4, and 1:5 (Fig. 6b). FFA content was generally low at all the treatment combinations at 35°C with ≤6% FFA at all the AlcoRs except AlcoR 1:1 (Fig. 7b).

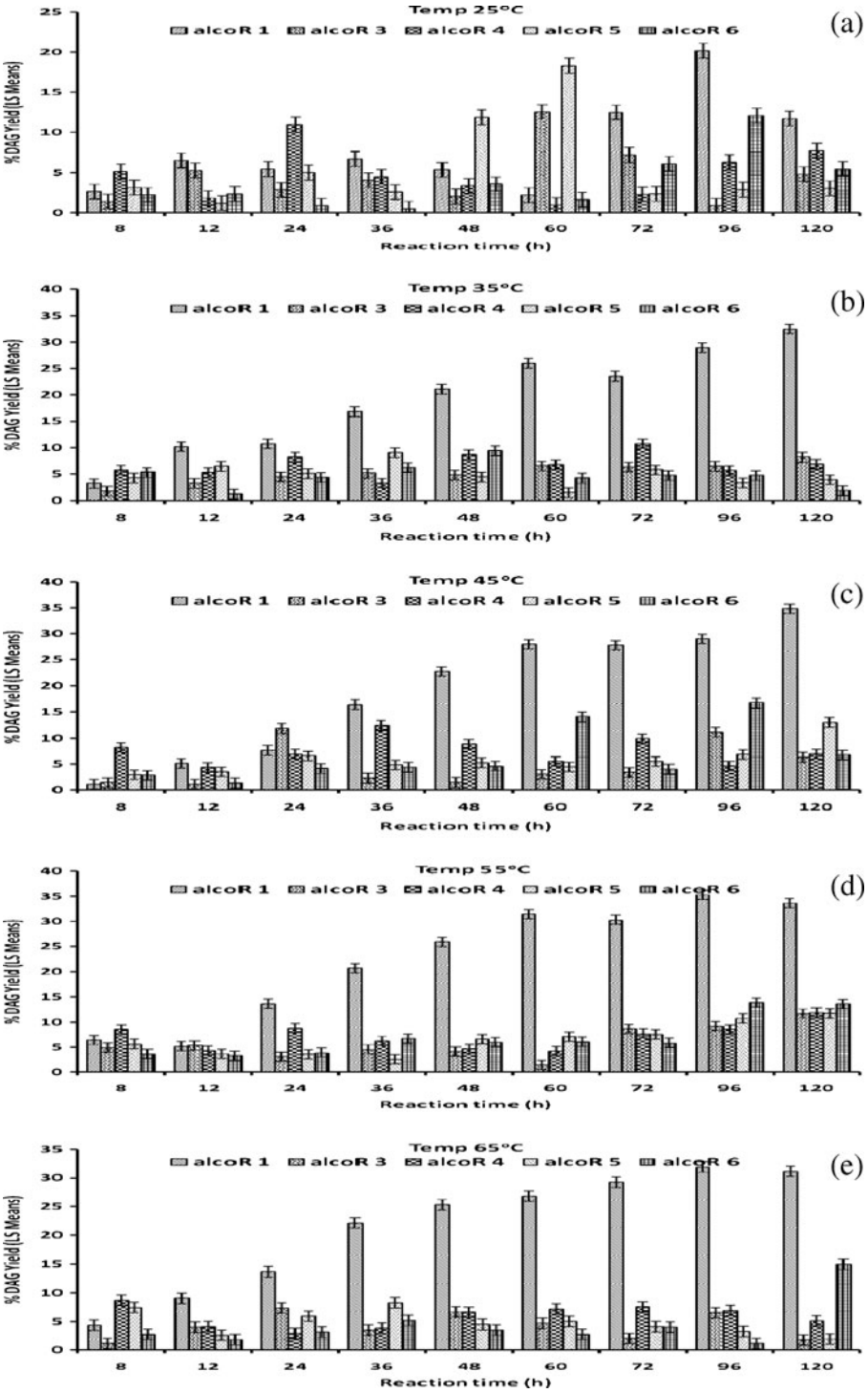
At 45°C, there was consistent and substantial decrease in TAG content at AlcoRs 1:1, 1:3, and 1:4 at all the reaction times studied, but comparably high amount of TAG remained at AlcoRs 1:5 and 1:6 during the same period in transesterified SSO (Fig. 4c). A similar DAG pattern as 35°C was observed at 45°C but with occasional increases at some of the AlcoRs studied (Fig. 5c). The trend in MAG content at 45°C (Fig. 6c) was similar to 25 and 35°C. FFA content was also low at 45°C at all the treatment combinations but >15% at AlcoRs 1:5 and 1:6 at 120 h.

Except for AlcoR 1:1, all the other AlcoRs at 55°C (Fig. 4d) and 65°C (Fig. 4e) showed only marginal decreases in TAG content in the course of the reaction in transesterified SSO.

The DAG content at 55°C (Fig. 5d) and 65°C (Fig. 5e) also followed similar trends as the DAG content at 35 and 45°C. At 55°C, AlcoRs 1:1, 1:3, and 1:4 showed clear increase in MAG content with time (Fig. 6d) up to 60 h, and all these were significant ($p<0.05$), while AlcoR 1:1 maintained the increase to the end of the experimental period. Similar to 55°C, the reaction at 65°C showed clear increases in MAG contents with time (Fig. 6e) at AlcoR 1:1, and all these were significant ($p<0.05$). Reaction at 55 and 65°C revealed a generally constant FFA content over time at all the AlcoRs studied and an equally higher FFA content at AlcoR 1:1 in transesterified SSO (Figs. 7d, e).

RC Transesterification Residuals and Co-products

The remaining TAG content in the RC sample showed a decreasing trend at AlcoR 1:1 at 25°C throughout the course of the reaction from ~84% at 8 h to 48% at 120 h (Table 2) with concomitant increase in DAG, MAG, and FFA (Tables 3, 4, and 5). Similar TAG, DAG, MAG, and FFA patterns were observed with AlcoRs of 1:3 and 1:4 (Tables 2, 3, 4, and 5). However, the decreases in TAG at AlcoRs 1:5 and 1:6 were comparably lower during the first 24 h (Table 2) while accumulation of DAG, MAG, and remaining FFA (Tables 3, 4, and 5) followed similar trends as described for AlcoR 1:1.


Temp 25°C
alcoR 1
alcoR 3
alcoR 4
alcoR 5
alcoR 6
(a)
% DAG Yield (LS Means)
Reaction time (h)
Temp 35°C
(b)
Temp 45°C
(c)
Temp 55°C
(d)
Temp 65°C
(e)

◀ **Fig. 5** Least Square means (LS Means)±standard error of treatment × time of DAG (% DAG yield) of transesterified SSO at **a** 25°C, **b** 35°C, **c** 45°C, **d** 55°C, and **e** 65°C

At 35°C and AlcoR 1:1, there was approximately 41–80% TAG remaining between 8 and 120 h (Table 2). Similar amounts of TAG remained at 35°C at higher AlcoRs (1:5 and 1:6) as 25°C.

A general increase in DAG and MAG content with time was observed at all the AlcoRs studied at 35°C ($p<0.05$; Tables 3 and 4). Similar to transesterified SSO and RC at 25°C, <7% FFA was recorded over time at all the AlcoRs at 35°C in transesterified RC (Table 5).

Almost similar patterns in TAG content were observed at all the AlcoRs studied at 45, 55, and 65°C (Table 2). For instance, TAG content decreased significantly ($p<0.05$) from 71% to 34%, 68% to 36%, and 65 to 38% between 8 and 120 h at 45, 55, and 65°C, respectively. At 55 and 65°C, AlcoR 1:1 showed higher MAG content than the other AlcoRs studied ($p<0.05$) but followed similar patterns as 25 and 35°C at all the other temperatures and reaction times studied in transesterified RC (Tables 3 and 4). Beyond 35°C, lower levels of FFA were generally observed at >1:1 AlcoR in all the other treatment combinations ($p<0.05$; Table 5).

OO Transesterification Residuals and Co-products

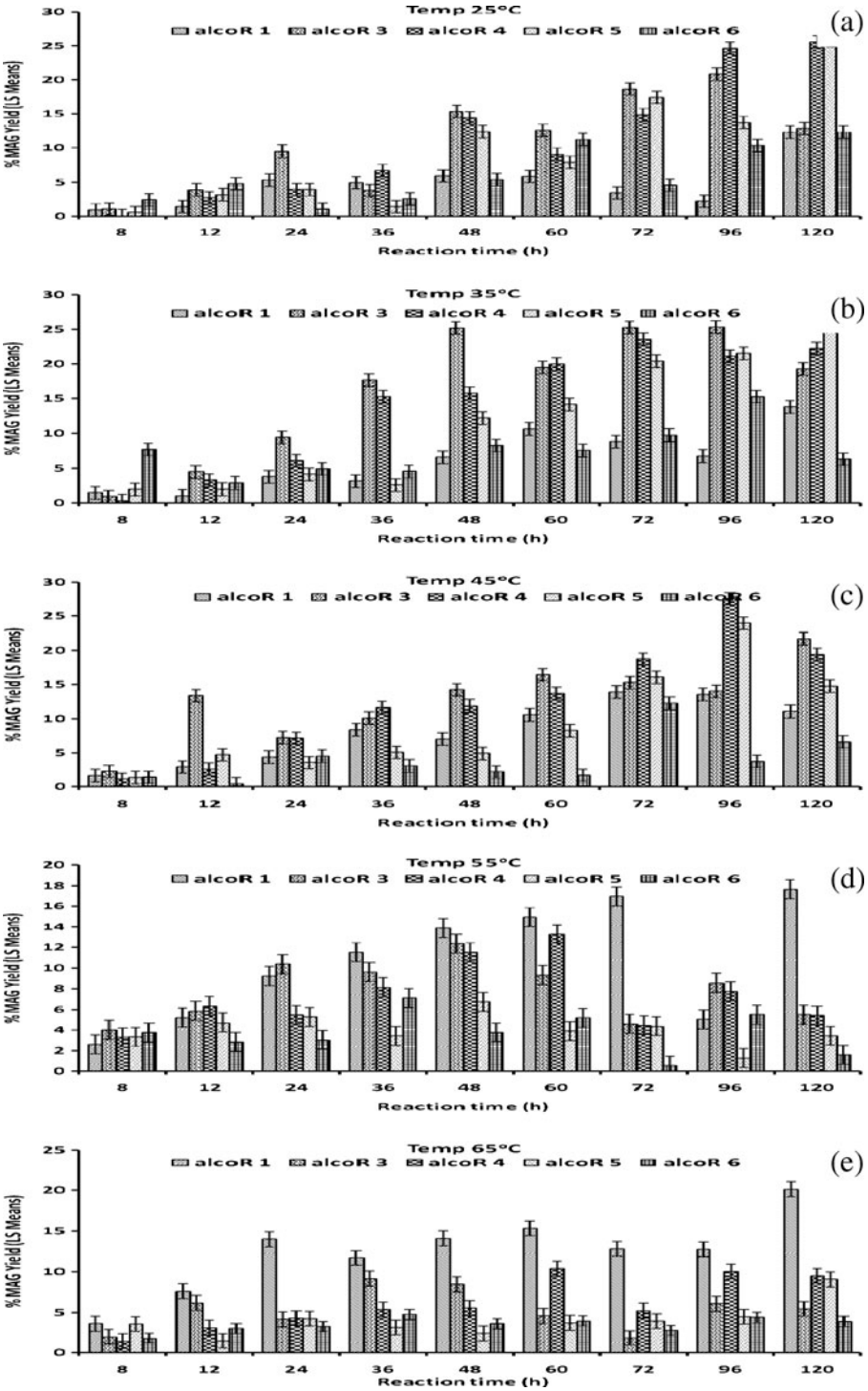
An AlcoR of 1:3 was found to decrease TAG content at 25°C more than the other AlcoRs studied with transesterified OO (Table 2). At 35°C, AlcoRs 1:1 and 1:3 showed <79% TAG content at the end of the reaction cycle (120 h), while >74% TAG remained at AlcoRs 1:4, 1:5, and 1:6 (Table 2). The results at 45, 55, and 65°C followed similar trends with substantial decrease in TAG at AlcoR of 1:1 and only minor decreases in TAG contents for the other AlcoRs studied.

The rate of DAG conversion to MAG was greater than the rate of DAG production from TAG in transesterified OO.

The DAG contents were fairly constant throughout the course of the reaction and were comparably low, ranging between 5% and 14% at all the treatment combinations, and these were significant ($p<0.05$; Table 3). It was between 1.4 and 39 and 0.5–35% for RC and SSO, respectively.

Like RC, higher amounts of MAG were accumulated at 55 and 65°C and at AlcoR 1:1 ($p<0.05$; Table 4). At 25, 35, and 45°C, more MAG accumulated over time at AlcoR 1:3 ($p<0.05$). The transesterification of MAG was slow in the presence of high amounts of TAG. Overall, RC and OO accumulated less MAG (6% and 14%, respectively) over time at all the AlcoR and temperatures studied compared with SSO (27%). The least amount of FFA was recorded in transesterified OO with a maximum of ≤4% FFA at all the treatment combinations ($p<0.05$; Table 5). FFA content remained fairly constant with AlcoR 1:1 giving higher FFA values compared to the other AlcoRs ($p<0.05$) studied (Table 5).

This study revealed that rate of transesterification is dependent on temperature, reaction time, oil/alcohol molar ratio, and thermal stability of the lipase and that there were significant ($p<0.05$) differences in residual yield between temperature, alcohol ratio, and reaction time as assessed during the transesterification reaction.


Temp 25°C
alcoR 1
alcoR 3
alcoR 4
alcoR 5
alcoR 6
(a)
% MAG Yield (LS Means)
Reaction time (h)
Temp 35°C
(b)
Temp 45°C
(c)
Temp 55°C
(d)
Temp 65°C
(e)

◀ **Fig. 6** Least Square means (LS Means)±standard error of treatment × time of MAG (% MAG yield) of transesterified SSO at **a** 25°C, **b** 35°C, **c** 45°C, **d** 55°C, and **e** 65°C

Discussion

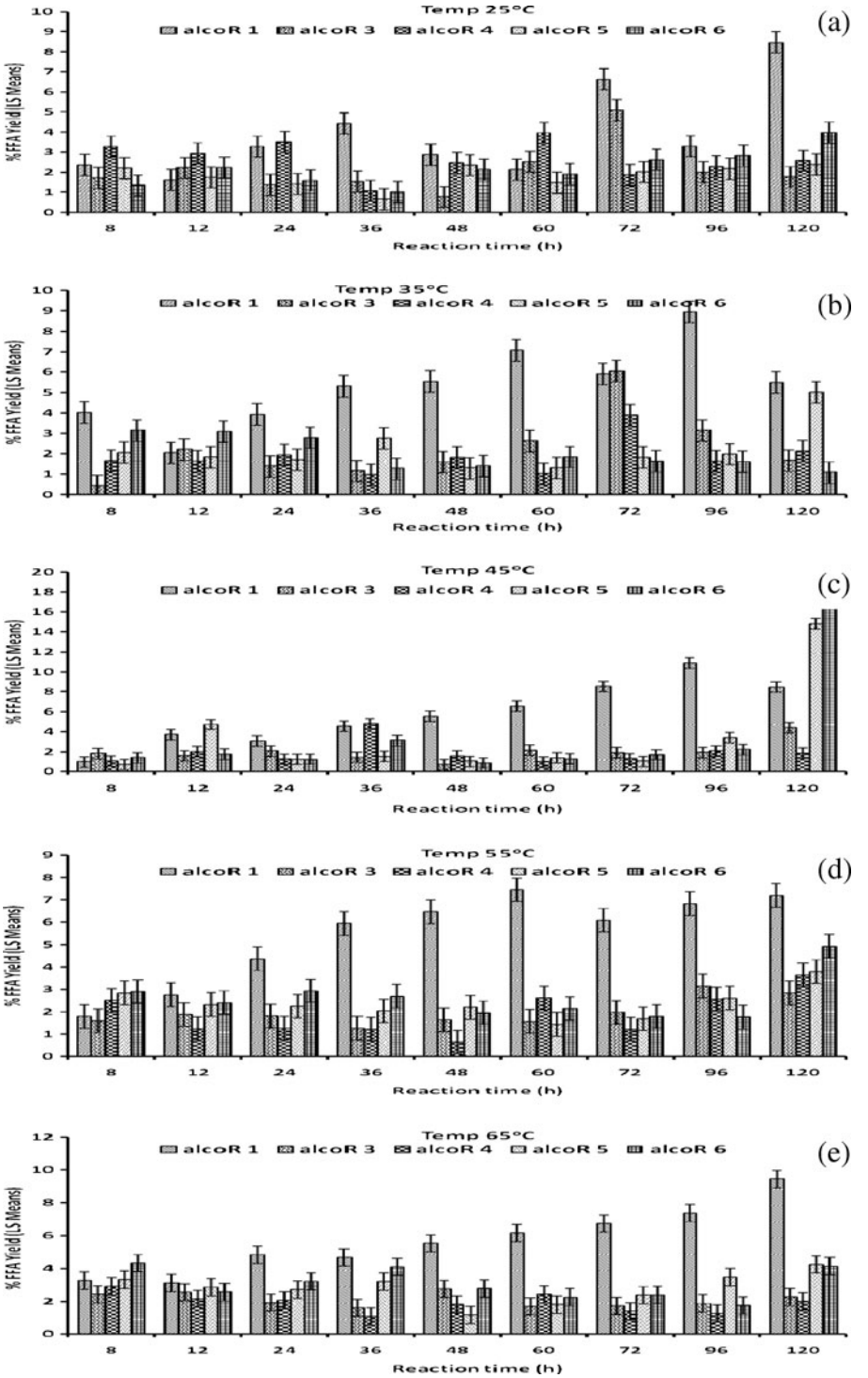
The Effects of Reaction Parameters on the Residual Yields as Elucidated by Stoichiometry, Polarity, and Thermal Stability

Increasing oil: alcohol ratio seemed to have an initial inhibitory effect on the transesterification reaction in all the three oils studied. The initial low conversion of the TAG and relative high amount of unreacted TAG quantified in the transesterified oils during the initial segment of the reaction may be due to a myriad of factors, such as initial delay in the transesterification reaction and may be due to minimal mixing and dispersion of the alcohol with oil, poor solvation, or low miscibility of TAG in the alcohol creating a two-phase/heterogeneous system, the deactivation effect of the polar ethanol on the enzyme, interfacial action of lipase, and the nature of lipase catalysis in non-aqueous media [27–32].

Polar alcohols such as ethanol possess the ability of distorting the ordered layer of water molecules that surround the enzyme needed for conformational integrity and stability [16, 33]. Beyond the stoichiometric (AlcoR 1:3) amount, this effect appeared to be more pronounced (Figs. 4, 5, 6, and 7 and Tables 2, 3, 4, and 5) and consistent with previous reports [30, 32, 34]. In the presence of polar alcohols, the oil exists in a two-phase system with very minimal dispersion [34–36] creating diffusion limitations and a low concentration of oil in the alcohol phase. Lipases are easily deactivated when in contact with the insoluble alcohol [37]. In addition to this is another limitation from the immobilized lipase creating a three-phase system [34, 38]. For efficient catalysis, both the external diffusion limitation, i.e., substrate from the bulk solvent through the boundary layer to the surface of enzyme and the internal diffusion limitation of substrate to the active site of the enzyme must be overcome (e.g., through effective mixing).

Lipases exhibit maximum activity at the interface of oil and water by interfacial activation [39, 40]. The active site of the lipase, which is covered by a lid, unhinges during interfacial activation for the substrate to reach the active site [41]. In non-aqueous reaction systems, the absence of the oil–water interface keeps the lid in closed position and prevents contact and interaction between the substrate and enzyme. However, an excess amount of water may lead to some unwanted side reactions such as hydrolysis [30, 37, 42]. The amount of water required to either activate the enzyme for transesterification or the unwanted hydrolysis varies, and it is based on several factors such as the lipase itself, the type of oil, and alcohol used in the transesterification reaction [37, 42].

The observation in this study suggests that increasing the temperature or alcohol content did not appreciably decrease TAG content (though statistically significant, $p<0.05$). Increasing reaction temperature is known to alter the limitations of mass transfers by reducing the viscosity of the reaction mixture [34]. This increases solubility and facilitates the movement of the reactants to the catalytic site and the products formed away from the catalytic site of the enzyme and support (if immobilized). The low conversion of TAG with increasing temperature (>45°C) maybe ascribed to thermal instability of the enzyme. The optimum temperature is dependent on other reaction parameters such as the oil/alcohol molar ratio [30, 31] as was observed in this study.


Temp 25°C
alcoR 1
alcoR 3
alcoR 4
alcoR 5
alcoR 6
(a)
% FFA Yield (LS Means)
Reaction time (h)
Temp 35°C
(b)
Temp 45°C
(c)
Temp 55°C
(d)
Temp 65°C
(e)

◀ **Fig. 7** Least Square means (LS Means)±standard error of treatment × time of FFA (% FFA yield) of transesterified SSO at **a** 25°C, **b** 35°C, **c** 45°C, **d** 55°C, and **e** 65°C

DAG, MAG, and FFA in the Transesterification Reaction

Transesterification reaction results in the formation of the intermediates, DAG and MAG. The high accumulation of DAG is consistent with the *sn*-1,3 specificity of Lipozyme® [18, 43] and the synthesis of TAG [44], while the overall low MAG yield compared to DAG at all the treatment combinations for all three oils may be due to faster conversion of the more soluble MAG [21].

The source of FFA in transesterified stock can be diverse, such as: initial starting oil, hydrolysis product by the moisture in the feedstock with lipase, and incomplete transesterification reaction [37, 44–46]. Although volumetric titration is used to measure the FFA content in both ASTM D 664/974 [USA]; EN 14104 [EU], the HPLC method measures the FFA content exclusively, while the former measures the total acid value which will include other acidic components such as MAG and DAG present. Some of the inherent problems associated with ASTM D 664 include variability in the electrodes used in the potentiometric titration [4], and this is overcome by the HPLC method.

Suitability of the HPLC Analysis

The HPLC method was a straightforward approach with no requirement for prior derivatization or chemical modifications of the components, as is the case with GC methods. Each class (TAG, DAG, MAG, and FFA) eluted as a single peak each (Fig. 2) instead of several peaks as often seen with GC analysis. This simplifies the quantitation and avoids overlapping information.

Previous reports of authors using size exclusion chromatography did not simultaneously detect FFA with the other reaction components [3, 15, 21]. Overall, the results showed general agreement with previous size exclusion chromatographic methods despite the differences in sample preparation or equipment [3, 15, 17, 21]. Glycerol could not be detected similar to reports by Kittirattanapiboon and Krisnangkura [17] and can be attributed to its small quantity in the ethyl-ester-rich phase. Authors who detected glycerol had ≥2 columns coupled in series to the detector [3, 15, 21]. However, these authors often reported poor separation and resolution of the other lipids classes (TAG, DAG, and MAG) when only one column is used. Like Arzamendi et al. [21], this method was able to monitor the residual ethanol in the transesterification reaction (Fig. 2). Additionally, since the analysis was performed at room temperature (23°C) with isocratic elution, new analysis could be started immediately after the 25 min since there was no need to change the column temperature or mobile phase.

Conclusions

The need for a reliable and fast technique for both qualitative and quantitative information about the alkyl esters (BD) and reaction components was achieved in a single HPLC-RI run. This paper summarized a simple, rapid, and practical HPLC technique for the unambiguous identification and quantitation of reaction residuals produced during lipase

Table 2 Least Square means of triacylglycerol (% yield) at different temperature, oil: alcohol ratio, and reaction times during the transesterification of Rothsay composite and olive oil

Treatments		Reaction time								
Temperature (°C)	Oil/alcohol ratio	8 h	12 h	24 h	36 h	48 h	60 h	72 h	96 h	120 h
RC										
25	1:1	83.63	78.19	73.29	71.37	62.25	59.71	52.94	48.87	48.30
	1:3	85.81	80.49	74.29	74.04	71.20	69.64	71.32	71.19	68.35
	1:4	92.37	82.70	78.35	72.34	69.22	66.11	59.98	61.37	55.52
	1:5	89.02	85.63	86.43	78.95	74.17	71.63	67.28	63.42	58.23
	1:6	88.75	85.75	82.97	79.87	77.40	74.14	66.14	68.23	59.86
35	1:1	79.53	62.28	51.54	45.58	45.73	41.87	43.11	40.66	40.78
	1:3	79.48	80.04	76.94	77.33	65.02	60.45	62.88	64.08	57.32
	1:4	81.21	74.80	69.12	57.89	64.25	53.80	55.22	54.52	53.92
	1:5	86.06	83.96	76.57	75.67	71.06	68.01	62.38	62.01	58.73
	1:6	87.88	84.11	84.54	78.56	78.45	75.05	71.15	67.30	64.73
45	1:1	70.55	62.37	56.41	45.87	44.76	39.23	34.63	35.21	33.64
	1:3	73.47	73.51	71.62	71.99	69.30	68.13	68.61	71.38	69.91
	1:4	84.70	81.35	75.56	74.98	74.87	75.50	76.38	66.50	76.33
	1:5	88.31	85.31	84.53	82.91	80.96	82.45	79.92	81.36	79.54
	1:6	87.29	88.02	86.04	84.75	84.48	81.53	80.91	80.96	80.30
55	1:1	68.01	58.99	45.55	40.74	37.70	37.26	36.80	35.91	36.03
	1:3	77.87	77.05	76.79	76.60	76.28	76.79	74.10	73.62	71.47
	1:4	86.63	85.85	83.55	82.79	83.65	81.52	78.01	77.78	74.07
	1:5	87.97	88.21	87.26	86.59	85.27	85.53	83.66	84.91	80.88
	1:6	88.14	89.20	88.21	88.12	86.94	86.00	84.19	85.52	79.97
65	1:1	64.94	54.63	45.61	39.72	40.42	39.49	38.38	37.97	38.35
	1:3	82.17	81.56	81.23	79.61	79.75	79.03	80.05	78.25	77.70
	1:4	86.66	88.39	87.84	85.53	84.10	81.86	82.64	81.00	81.74
	1:5	89.76	89.10	87.49	87.18	86.83	88.08	85.89	87.34	86.22
	1:6	87.59	87.49	87.97	89.32	87.41	86.34	85.49	84.12	86.21
OO										
25	1:1	85.26	78.06	72.75	71.66	68.14	66.30	68.82	60.96	61.39
	1:3	84.36	80.98	69.12	62.84	62.34	55.66	55.03	51.15	52.87
	1:4	89.74	90.33	85.33	82.61	82.33	76.20	69.99	63.90	59.96
	1:5	92.07	90.09	89.10	87.94	87.68	86.66	87.38	81.59	80.42
	1:6	91.36	89.36	91.80	88.42	85.37	83.73	87.14	88.01	84.57
35	1:1	78.42	70.17	66.02	67.38	61.34	59.59	61.03	63.92	57.59
	1:3	78.91	73.53	67.83	67.68	65.35	64.18	65.24	64.88	63.19
	1:4	87.87	88.97	86.06	85.19	82.47	80.84	76.98	74.28	77.56
	1:5	90.77	90.55	88.88	90.25	90.25	87.22	88.25	85.14	76.92
	1:6	87.40	87.73	89.72	89.58	89.08	89.27	87.95	87.04	83.80
45	1:1	71.41	66.99	66.78	61.26	62.86	58.08	57.96	54.42	56.21
	1:3	81.28	80.92	77.59	73.39	74.07	71.89	72.94	76.12	71.52
	1:4	91.84	89.39	85.82	85.28	82.89	82.42	80.15	80.35	78.40
	1:5	91.09	90.76	87.70	89.36	86.91	87.71	85.75	84.32	84.08

Table 2 (continued)

Treatments		Reaction time								
Temperature (°C)	Oil/alcohol ratio	8 h	12 h	24 h	36 h	48 h	60 h	72 h	96 h	120 h
	1:6	92.34	90.73	86.29	91.02	88.95	88.66	86.02	87.26	85.92
55	1:1	72.51	68.77	64.29	58.79	52.64	56.79	56.77	57.91	56.03
	1:3	82.55	82.26	81.50	81.11	82.79	81.67	78.55	71.93	73.32
	1:4	89.83	89.21	87.29	86.55	85.28	87.28	82.25	76.31	76.45
	1:5	89.20	90.33	90.18	88.22	90.31	88.90	84.68	81.38	78.28
	1:6	90.96	89.23	90.36	90.34	89.27	89.30	87.83	85.71	79.83
65	1:1	69.19	68.39	62.75	65.22	59.77	61.04	59.34	59.58	60.83
	1:3	85.45	85.38	84.98	82.86	83.83	83.65	83.06	85.03	84.57
	1:4	86.74	86.78	86.40	84.73	85.83	86.20	85.84	84.46	85.67
	1:5	86.94	86.86	88.32	89.70	89.60	89.56	89.92	90.45	90.18
	1:6	86.35	87.68	88.38	91.42	89.36	89.95	90.84	90.46	89.22

Std err=1.1463 for RC and Std err=1.1241 for OO

Table 3 Least Square means of diacylglycerol (% yield) at different temperature, oil: alcohol ratio, and reaction times during the transesterification of RC and olive oil

Treatments		Reaction time								
Temperature (°C)	Oil/alcohol ratio	8 h	12 h	24 h	36 h	48 h	60 h	72 h	96 h	120 h
RC										
25	1:1	6.13	8.62	10.17	10.92	13.80	14.44	22.39	22.54	24.60
	1:3	6.20	5.72	7.45	7.18	6.15	10.00	5.47	5.36	6.14
	1:4	1.42	6.53	6.84	8.67	9.03	7.53	8.86	8.41	11.01
	1:5	4.67	5.15	2.76	6.05	6.35	5.36	5.50	6.17	8.56
	1:6	4.98	5.76	7.61	6.68	8.71	7.19	10.36	8.02	10.87
35	1:1	7.38	15.69	22.80	28.73	28.76	32.85	32.01	32.01	31.17
	1:3	7.44	4.73	6.47	6.31	14.13	20.75	16.06	12.55	23.28
	1:4	6.77	8.61	8.11	19.19	10.52	15.02	11.34	15.19	12.24
	1:5	6.61	6.64	9.38	8.06	11.15	11.57	12.17	9.97	10.55
	1:6	5.43	7.35	6.12	7.63	6.57	7.54	8.14	10.26	8.50
45	1:1	10.41	13.82	19.15	29.15	30.83	34.28	35.63	38.22	39.59
	1:3	9.35	9.16	12.14	9.81	10.68	10.44	10.34	8.90	10.51
	1:4	7.33	7.08	9.84	10.82	10.82	9.03	9.30	13.43	9.81
	1:5	5.47	7.61	7.38	7.96	8.59	6.82	8.92	7.71	8.60
	1:6	6.15	5.53	6.64	7.25	6.37	8.08	8.64	7.94	8.17
55	1:1	11.84	17.05	29.00	33.72	36.40	37.27	36.69	38.12	37.71
	1:3	8.01	8.08	8.80	9.21	8.64	8.56	11.14	11.97	12.20
	1:4	6.65	7.39	7.97	8.86	7.39	8.78	11.17	10.35	12.53
	1:5	6.04	5.44	6.48	6.58	7.77	6.95	8.55	7.88	9.89
	1:6	5.79	5.35	5.83	5.88	6.12	7.36	8.09	7.64	11.55

Table 3 (continued)

Treatments		Reaction time								
Temperature (°C)	Oil/alcohol ratio	8 h	12 h	24 h	36 h	48 h	60 h	72 h	96 h	120 h
65	1:1	13.50	19.82	29.30	34.32	34.11	36.07	36.32	36.26	36.09
	1:3	8.34	8.80	8.53	9.32	8.77	8.76	8.85	9.87	9.76
	1:4	7.46	6.21	6.41	7.55	8.45	8.76	8.42	7.99	7.42
	1:5	4.85	5.55	6.32	6.81	6.84	6.34	7.34	6.69	7.72
	1:6	6.91	6.94	6.34	5.41	6.14	7.18	8.03	8.85	7.67
OO										
25	1:1	6.49	9.83	9.17	10.77	12.49	11.11	12.18	13.30	13.48
	1:3	8.06	10.88	10.96	9.45	9.31	11.07	8.00	8.77	10.46
	1:4	7.36	7.43	9.53	9.30	7.36	10.12	7.86	11.58	11.98
	1:5	6.82	7.80	7.30	6.96	6.66	7.80	5.69	7.98	8.26
	1:6	6.62	8.21	5.70	8.11	8.93	9.51	6.58	5.67	5.79
35	1:1	9.54	10.65	13.29	10.45	11.65	13.64	13.66	13.48	14.06
	1:3	6.74	10.97	11.36	10.67	9.12	11.63	9.09	10.53	10.99
	1:4	10.08	8.10	7.11	8.81	7.32	9.52	9.28	8.93	9.75
	1:5	8.46	7.71	7.72	7.06	5.60	7.84	6.05	9.24	5.20
	1:6	8.25	9.32	7.78	8.25	6.50	6.98	7.77	6.55	9.25
45	1:1	11.67	12.21	11.45	11.61	12.46	12.94	14.09	14.10	14.34
	1:3	10.29	9.67	10.48	11.76	10.14	10.06	10.44	9.64	10.35
	1:4	6.17	7.08	8.71	8.32	9.11	8.50	9.47	9.30	10.59
	1:5	7.40	8.15	9.50	7.17	8.39	8.85	7.59	9.99	9.49
	1:6	5.02	8.01	11.54	6.71	7.14	8.31	8.79	7.90	9.20
55	1:1	12.71	13.71	12.18	11.36	14.34	13.80	14.24	14.34	13.95
	1:3	9.74	9.85	9.79	9.49	8.94	9.05	10.28	14.31	13.32
	1:4	7.31	8.44	8.90	9.44	9.64	8.48	10.58	13.34	13.38
	1:5	9.50	8.22	7.95	9.37	7.82	8.32	10.50	11.78	13.14
	1:6	8.08	7.97	8.63	8.27	8.10	9.17	9.17	10.33	13.74
65	1:1	13.95	11.61	11.57	12.21	13.94	14.26	14.46	13.57	13.45
	1:3	11.04	10.64	10.20	11.02	11.15	10.05	10.34	8.78	8.66
	1:4	10.11	9.94	9.47	9.42	9.76	8.94	8.57	9.38	8.54
	1:5	10.66	9.75	9.79	8.33	8.52	8.47	8.33	8.00	7.26
	1:6	11.30	10.33	9.76	8.15	9.53	8.80	8.31	8.67	6.81

Std err=0.7725 for RC and Std err=0.6887 for OO

Table 4 Least Square means of monoacylglycerol (% yield) at different temperature, oil: alcohol ratio, and reaction times during the transesterification of Rothsay composite and olive oil

Treatments		Reaction Time								
Temperature (°C)	Oil/ alcohol ratio	8 h	12 h	24 h	36 h	48 h	60 h	72 h	96 h	120 h
RC										
25	1:1	1.86	1.79	1.50	1.16	3.32	1.66	1.82	4.36	4.98
	1:3	1.16	3.52	3.73	4.54	5.87	3.35	2.89	4.34	4.46

Table 4 (continued)

Treatments		Reaction Time								
Temperature (°C)	Oil/ alcohol ratio	8 h	12 h	24 h	36 h	48 h	60 h	72 h	96 h	120 h
	1:4	1.41	2.54	3.51	4.48	5.27	4.95	4.61	5.02	4.48
	1:5	1.26	2.26	2.13	5.27	5.46	5.44	4.22	5.41	5.07
	1:6	1.49	2.31	1.61	2.99	4.25	4.33	4.04	4.05	4.40
35	1:1	1.79	1.94	3.91	3.76	3.68	3.16	3.72	4.55	4.89
	1:3	3.00	2.63	3.31	1.94	4.09	4.68	4.40	4.33	3.24
	1:4	2.78	4.18	5.23	3.96	3.71	5.09	4.52	3.64	3.09
	1:5	1.53	2.02	3.94	4.38	3.10	3.76	4.68	4.86	3.44
	1:6	1.37	1.62	2.29	4.43	3.27	4.47	4.38	3.47	4.82
45	1:1	1.59	2.22	3.23	3.35	3.70	5.23	5.37	4.40	5.10
	1:3	4.53	3.56	2.95	4.41	4.81	5.53	5.12	3.58	3.14
	1:4	1.97	2.74	3.06	2.60	2.33	3.10	1.70	5.37	1.38
	1:5	1.29	1.55	1.39	1.87	1.92	1.95	2.82	2.07	2.90
	1:6	1.29	1.39	1.37	1.90	1.71	2.05	1.36	2.24	1.77
55	1:1	3.14	4.29	4.24	3.96	4.01	3.98	5.50	4.91	5.57
	1:3	3.96	4.34	3.01	2.24	3.22	2.34	2.74	1.72	1.78
	1:4	1.37	1.18	1.77	1.55	1.34	1.49	1.73	1.30	2.47
	1:5	1.40	1.45	1.63	1.52	1.39	1.18	1.58	1.70	1.79
	1:6	1.27	1.26	1.33	1.23	1.47	1.50	1.44	1.54	1.55
65	1:1	2.38	5.22	4.56	5.02	5.47	4.26	4.76	5.58	5.79
	1:3	1.78	1.13	1.56	1.85	1.99	2.68	1.47	1.85	1.70
	1:4	1.37	1.22	1.31	1.63	1.29	1.64	1.36	1.91	2.04
	1:5	1.26	1.22	1.82	1.34	1.72	1.24	1.38	1.72	1.54
	1:6	1.36	1.45	1.44	1.28	1.77	1.57	1.35	1.23	1.22
OO										
25	1:1	1.12	2.50	3.96	0.63	1.51	1.56	1.76	6.12	3.50
	1:3	3.81	2.25	8.33	11.93	10.18	12.54	12.85	14.71	9.84
	1:4	1.02	0.23	0.72	2.68	3.52	3.99	9.38	9.91	9.70
	1:5	0.07	1.03	1.26	2.57	2.22	0.99	2.96	3.88	4.60
	1:6	1.18	0.84	0.52	1.22	2.14	1.96	1.89	0.64	0.90
35	1:1	2.22	4.96	2.90	3.03	6.29	4.72	4.06	3.68	8.02
	1:3	3.77	5.85	7.95	6.08	8.77	7.58	8.82	6.89	7.92
	1:4	0.26	0.13	2.56	1.31	4.24	2.02	4.94	6.25	2.11
	1:5	0.32	0.66	1.55	0.68	1.07	1.81	2.11	0.88	2.67
	1:6	2.60	0.87	0.89	0.34	1.78	0.94	1.40	2.97	1.37
45	1:1	2.09	4.43	3.08	7.01	5.70	8.74	8.12	10.46	9.65
	1:3	2.15	2.54	5.06	4.50	4.55	6.01	4.54	3.96	5.62
	1:4	0.44	0.68	1.85	2.23	2.29	2.63	3.86	1.88	2.79
	1:5	0.64	0.67	1.53	1.36	1.84	1.06	3.10	1.56	0.78
	1:6	0.64	0.25	0.43	0.71	1.95	0.86	2.80	2.30	1.28
55	1:1	3.24	3.74	6.25	10.87	12.88	9.26	9.00	7.04	10.53
	1:3	3.03	2.39	2.72	2.74	1.81	1.97	3.33	2.71	2.93
	1:4	1.14	1.31	1.74	0.86	1.63	0.36	2.04	2.34	2.13

Table 4 (continued)

Treatments		Reaction Time								
Temperature (°C)	Oil/ alcohol ratio	8 h	12 h	24 h	36 h	48 h	60 h	72 h	96 h	120 h
	1:5	0.62	0.41	1.00	1.24	0.50	1.04	1.30	2.52	2.47
	1:6	0.71	1.37	0.59	0.80	1.26	0.77	1.40	1.24	1.42
65	1:1	4.28	4.51	8.19	4.92	7.86	8.19	8.66	8.70	10.11
	1:3	0.29	0.61	0.93	1.91	1.05	2.06	1.45	1.26	2.21
	1:4	0.53	0.59	1.22	2.94	1.18	1.14	2.06	0.87	1.72
	1:5	0.73	1.63	0.68	0.82	0.77	1.01	0.37	0.60	1.27
	1:6	0.93	0.83	0.74	0.24	0.41	0.75	0.21	0.44	2.05

Std err=0.5493 for RC and Std err=0.6194 for OO

Table 5 Least Square means of free fatty acid (% yield) at different temperature, oil: alcohol ratio, and reaction times during the transesterification of Rothsay composite and olive oil

Treatments		% FFA Yield (RC and OO)								
Temperature (°C)	Oil/alcohol ratio	8 h	12 h	24 h	36 h	48 h	60 h	72 h	96 h	120 h
RC										
25	1:1	3.64	3.45	5.29	2.94	5.70	7.17	5.38	6.49	6.50
	1:3	3.07	3.34	4.26	2.03	3.03	3.69	2.93	2.15	3.63
	1:4	2.54	3.41	2.92	3.55	1.96	2.40	3.35	2.60	2.41
	1:5	3.27	3.17	2.16	1.81	1.87	3.84	3.69	1.92	1.56
	1:6	1.91	2.93	2.39	2.53	1.61	1.37	4.83	0.75	1.00
35	1:1	4.35	5.59	5.59	5.32	5.09	6.20	5.34	6.92	6.55
	1:3	3.72	4.46	3.37	3.35	5.85	2.27	4.44	6.25	2.77
	1:4	4.33	4.33	2.81	2.65	2.07	5.25	5.71	3.52	3.53
	1:5	3.74	4.02	3.59	2.72	1.99	1.69	3.39	1.68	2.98
	1:6	3.68	4.57	2.95	2.84	2.88	1.72	2.69	1.95	2.27
45	1:1	5.00	6.50	4.65	5.13	4.82	5.94	5.76	6.06	5.48
	1:3	2.47	2.35	2.36	2.17	2.79	2.92	2.73	2.13	2.11
	1:4	2.47	2.95	0.85	1.86	1.70	1.65	1.96	3.24	1.31
	1:5	3.73	2.92	2.84	2.42	2.54	1.74	1.61	1.61	1.30
	1:6	3.93	3.39	3.36	2.59	2.36	2.53	2.57	1.76	1.18
55	1:1	4.57	4.35	5.64	5.91	6.30	5.72	5.57	4.92	5.23
	1:3	3.11	2.86	2.86	3.11	2.61	2.77	2.32	2.62	3.86
	1:4	3.43	3.23	2.88	2.29	2.59	2.23	2.13	2.66	2.94
	1:5	3.84	3.92	3.08	3.25	2.91	3.38	2.67	1.75	2.75
	1:6	4.23	3.52	3.30	3.07	3.51	3.10	2.84	2.43	3.10
65	1:1	6.07	5.55	5.84	5.70	5.63	5.38	5.45	5.51	5.40
	1:3	3.39	3.56	3.11	2.94	3.09	2.94	2.56	2.86	3.18

Table 5 (continued)

Treatments		% FFA Yield (RC and OO)								
Temperature (°C)	Oil/alcohol ratio	8 h	12 h	24 h	36 h	48 h	60 h	72 h	96 h	120 h
	1:4	3.69	3.28	3.10	3.22	3.42	3.17	2.73	2.75	2.27
	1:5	3.31	3.65	3.55	3.60	3.52	3.18	3.88	2.80	2.77
	1:6	3.85	3.61	3.81	3.31	3.82	3.88	3.95	4.37	3.65
OO										
25	1:1	0.69	0.75	1.33	0.60	1.37	2.50	1.23	2.49	3.47
	1:3	0.49	0.45	0.44	1.19	0.45	0.93	1.35	0.99	2.42
	1:4	0.57	0.32	0.47	0.26	0.26	1.24	0.83	0.67	0.32
	1:5	0.15	0.09	0.32	0.17	0.26	0.23	0.49	0.22	0.37
	1:6	0.27	0.77	0.39	0.22	0.34	0.61	0.23	0.39	1.11
35	1:1	0.50	0.82	1.39	2.16	2.44	3.68	3.79	2.32	3.26
	1:3	0.65	0.33	0.34	1.19	1.12	0.61	0.68	1.25	0.95
	1:4	0.54	0.49	0.36	0.27	0.26	0.61	0.44	0.32	0.27
	1:5	0.33	0.30	0.36	0.18	0.30	0.18	0.53	0.23	0.35
	1:6	0.33	0.96	0.32	0.18	0.35	0.62	0.30	0.33	1.48
45	1:1	0.84	0.90	1.60	2.44	1.72	2.89	2.45	4.37	2.90
	1:3	0.43	0.12	0.29	0.36	0.48	0.50	0.54	0.61	0.82
	1:4	0.53	0.27	0.44	0.22	0.24	0.50	0.39	0.98	0.35
	1:5	0.43	0.29	0.32	0.17	0.24	0.24	0.50	0.32	1.07
	1:6	0.34	0.88	0.26	0.21	0.59	0.60	0.36	0.44	0.86
55	1:1	1.05	0.80	1.31	3.03	3.91	3.01	2.81	4.05	3.30
	1:3	0.44	0.24	0.34	0.14	0.30	0.42	0.51	2.12	1.79
	1:4	0.72	0.42	0.32	0.27	0.22	0.47	0.39	2.02	1.47
	1:5	0.42	0.34	0.58	0.19	0.26	0.16	0.54	0.27	1.80
	1:6	0.30	0.72	0.20	0.21	0.41	0.35	0.29	0.41	2.02
65	1:1	1.40	2.08	1.74	1.79	2.48	1.66	2.21	2.20	0.31
	1:3	0.53	0.57	0.40	0.58	0.18	0.14	0.38	0.19	0.04
	1:4	0.77	0.48	0.30	0.26	0.30	0.36	0.31	0.51	0.23
	1:5	0.70	0.80	0.38	0.21	0.28	0.22	0.57	0.31	0.65
	1:6	0.54	0.69	0.41	0.18	0.27	0.30	0.32	0.37	1.40

Std err=0.3913 for RC and Std err=0.2453 for OO

catalyzed transesterification reaction of salmon skin oil, Rothsay composite, and olive oil samples to BD. The results revealed various degree of conversion during the reaction period and the interactive effects of reaction parameters on yield. Other lipids with similar transesterification scheme can be analyzed using this approach without any elaborate modifications. The accurate quantitation of components such as partial glycerides, unreacted TAG, FFA, and residual alcohol with tendencies to contaminate the fuel is very important in transesterified oils intended for use as fuel.

Acknowledgments The authors acknowledge the financial support from the Natural Sciences and Engineering Research Council (NSERC-Strategic Program) of Canada, and the provision of research samples by Atkins and Frères Inc. and Rothsay® Biodiesel, as well as the technical assistance with the HPLC from Dr. Veronique Fournier.

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