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Article

Determination of Free Fatty Acids and Triglycerides by Gas Chromatography Using Selective Esterification Reactions

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A method for selectively determining both free fatty acids (FFA) and triacylglycerides (TAGs) in biological oils was investigated and optimized using gas chromatography after esterification of the target species to their corresponding fatty acid methyl esters (FAMEs). The method used acid catalyzed esterification in methanolic solutions under conditions of varying severity to achieve complete conversion of more reactive FFAs while preserving the concentration of TAGs. Complete conversion of both free acids and glycerides to corresponding FAMEs was found to require more rigorous reaction conditions involving heating to 120°C for up to 2 h. Method validation was provided using gas chromatography–flame ionization detection, gas chromatography–mass spectrometry, and liquid chromatography–mass spectrometry. The method improves on existing methods because it allows the total esterified lipid to be broken down by FAMEs contributed by FFA compared to FAMEs from both FFA and TAGs. Single and mixed-component solutions of pure fatty acids and triglycerides, as well as a sesame oil sample to simulate a complex biological oil, were used to optimize the methodologies. Key parameters that were investigated included: HCl-to-oil ratio, temperature and reaction time. Pure free fatty acids were found to esterify under reasonably mild conditions (10 min at 50°C with a 2.1:1 HCl to fatty acid ratio) with 97.6 $\pm$ 2.3% recovery as FAMEs, while triglycerides were largely unaffected under these reaction conditions. The optimized protocol demonstrated that it is possible to use esterification reactions to selectively determine the free acid content, total lipid content, and hence, glyceride content in biological oils. This protocol also allows gas chromatography analysis of FAMEs as a more ideal analyte than glyceride species in their native state.

Introduction

The critical importance of identifying sources for renewable fuel has led to the pursuit of oil-producing crops and feedstocks, with increasing pressure to trend away from food-based materials and toward non-edible sources. As such, interest in non-edible seed crops such as camelina, as well as non-terrestrial photosynthetic organisms such as algae, have been increasing. In most cases, the lipid or oil yield from the material is among the most important deciding factors for candidacy as a potential fuel feedstock. Few, if any, standard methods exist for determining algae lipid content. Most commonly, gravimetric methods, whereby the biomass is extracted using non-selective solvent-based methods and the extracted material is subsequently dried and weighed, are used to determine the total lipid content based on the mass of the material extracted (1–3). The primary drawback to gravimetric methods is the tendency to include co-extracted species that can artificially inflate the lipid quantity. It may be possible to provide a more lipid-specific quantity in the extract using chromatographic methods to directly analyze the extract; however, the multitude of chemical species in the lipid fraction may include free acids, mono- di- and tri-glycerides, sterols and alkanes, each with their own specific response factors when flame ionization detection (FID) is used, which makes calibration of the instrument response an arduous task. For example, in this study, gas chromatography (GC)–FID was over 13 times more sensitive to fatty acid methyl esters (FAMEs) than either triacylglycerides (TAG) or free fatty acids (FFA). Also, the high temperatures that are required to elute and detect high-boiling species can lead to analyte degradation before detection (4).

In many cases, the more preferred method for chromatographic determination of the lipid content is through transesterification of the extracted material (5–7). The transesterification process creates FAMEs, which behave more ideally in GC analyses at lower temperatures than their parent species, and also allow for surrogate calibration species to be used. The reactions typically employed to obtain total lipid by esterification of the lipid components into their corresponding FAMEs are not capable of discerning the FAMEs produced from FFAs in the sample from FAMEs produced from TAGs in the sample. Standard methods such as AOCS Official Method Ce 2-66 (8) are excellent for small-scale analytical determinations for common fats and oils, but these methods are also unable to separate the individual chemical species that form FAMEs.

In both the large-scale production of biodiesel and in the analytical preparation of FAMEs, FFA content is an important technical criterion that is critical to selecting the proper processing method. One example of this can be found in base catalyzed methods, in which the risk of loss of material with high FFA content by saponification reactions is possible unless the catalyst and conditions are satisfactorily controlled (9–11). Indeed, the base-catalyzed reactions perform best on oils with low free fatty acid content, necessitating that the quantity of free fatty acids be determined so that the amount of base catalyst required can be adjusted. Unfortunately, standard methods such as AOCS Official Method Ca 5a-40 (12)are not suitable for small-scale analytical lipid determinations, because they require significant quantities of starting material and conversion reagents, and because they are not capable of discerning the free acids from triglycerides in the sample. Therefore, an alternative sample preparation methodology that allows the

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selective chromatographic determination of the amount of free acid in biological oils would be beneficial to the analytical and fuel communities so that proper choice of large-scale fuel production pathways can be made.

This study was designed to establish a set of reaction parameters and protocol aimed at the selective conversion and determination of free fatty acid in oil mixtures, while allowing the popular and accurate use of GC–FID for analysis and quantitation of FAMEs. FID was chosen because of its ubiquitous nature in chromatographic quantitative analyses, as well as its rugged and less expensive nature when compared with other detectors such as mass spectrometry (MS). Some key parameters for the selective esterification reaction pathway that have been identified in the literature (13)and were investigated in this work include reaction temperature, mass of substrate, catalyst load and reaction time. In addition, this study explored the completeness of conversion of the entire lipid matrix into corresponding FAMEs.

Experimental

Chemicals and reagents

The following chemicals and their respective grades were purchased from commercial sources and used as received: trimyristin (Fluka Chemicals 97% GC), myristic acid (Alltech Associates 99%), methanol (Sigma–Aldrich 99.93% ACS HPLC grade), ethanol (Sigma–Aldrich 95%), toluene (Sigma–Aldrich 99.8% HPLC grade), hexane (Sigma–Aldrich 95 + % anhydrous) and calcium chloride (EM Science, anhydrous powder). Hydrochloric acid (trace metal grade) was purchased from Fisher Scientific and used as received. Triglycerides with the following chain length were purchased from Sigma–Aldrich and used as received: C14:0, C16:0, C17:0, C18:0 and C18:1 (n-9), all at 98 + % purity. Likewise, the diglycerides and monoglycerides C14:0, C16:0, C18:0 and fatty acids C20:5 (n-3) and methyl esters C13:0, C16:1 (n-7), C16:0, C17:0, C18:0, C18:1 (n-9) C18:2 (n-6), C18:3 (n-6) were purchased from Sigma–Aldrich at ~98 + % purity and used as received. Hydrochloric acid solutions were prepared in methanol at 0.25, 0.5, 1, 2, 5, 10, 15 and 20% (v/v) concentrations and the weight percent of acid in each alcohol solution was determined by titration with a 0.1M KOH solution that was standardized according to AOCS Official Method H 12-52 (14). Ratios specified in the current study refer to the mass of HCl in a selected solution of HCl–MeOH relative to the mass of reactive substrate (TAG or FFA). The mass of HCl was determined by titration of the acid solution, and not based on the assumption that HCl concentration is 32% HCl by weight. A 5% (w/w) solution of calcium chloride in deionized water was prepared as needed.

Pure component experimental procedures

To investigate key parameters for conversion reactions, solutions of pure FFAs and pure TAGs, as well as mixtures of the two, were used. Stock solutions were prepared at ~4,000 μg/mL in toluene. Reactions were conducted from 40 to 80°C in a water or glycerol bath on a hotplate over reaction times ranging from 10 min to 2 h. A typical reaction for pure component testing is outlined as follows: 500 μL (~2 mg substrate) of the desired stock solution was added to an autoclaveable amber glass vial. Next, 1.25 mL of the appropriate acid/alcohol solution was added. The vial was capped and placed in a pre-equilibrated bath at the desired temperature for a known amount of time. After the desired reaction time was complete, the vials were cooled to room temperature and 1.25 mL of a 5% calcium chloride solution (w/w) was added to aid in phase separation. The aqueous phase was extracted four times with 1.0-mL aliquots of hexane and the extracts were combined. The hexane extracts were dried over fresh anhydrous calcium chloride, filtered via a 0.45-μm syringe filter and evaporated to dryness under a gentle stream of nitrogen. The residue was redissolved in 500 μL of toluene and analyzed by GC with either mass spectrometric or flame ionization detectors. For the mixed component studies, portions of pure stock solutions were combined to produce a ~10:1 solution of TAG–FFA, which was further diluted in toluene to produce the desired concentrations. This sample was then reacted in the same manner as the pure component samples, as outlined previously.

Sesame oil experimental procedures

A sesame oil sample was used to demonstrate the effectiveness of the method on a high-oil material. The weight percent of FFAs in the sesame oil was determined by titration, as described in AOCS Official Method Ca 5a-40 (12). A unique double-spike methodology was also employed in which oil samples were spiked with a TAG conversion standard (glyceryl triheptadecanoate) and a FAME extraction recovery standard (methyltridecanoate). This was done to assure that any observed FAME recovery differences were a result of incomplete conversion and not due to incomplete hexane extraction, with glyceryl triheptadecanoate used to monitor the completeness of both the esterification reaction and the ease of extraction of the resulting FAMEs, while the methyltridecanoate was used to monitor only the effectiveness of the hexane extraction step for FAME recovery. To our knowledge, this is the first instance of a double-spike experimental protocol being used to separately evaluate both the conversion reaction and the extraction step, while at the same time quantifying both using FAMEs.

For complete esterification reactions of the FFAs and TAGs in sesame oil, ~3.5 mg of oil was placed into an autoclavable amber glass vial along with ~100 μg of glyceryl triheptadecanoate spike and 1.25 mL of acidic methanol. The vial was sealed and reacted at 120°C for 120 min to ensure complete conversion of the large percentage of oils in the sample. After reaction, the solution was cooled to room temperature, ~100 μg of methyl tridecanoate was added and the vial was vigorously mixed for 1–2 min. Next, 1.25 mL of a 5% calcium chloride solution (w/w) was added. The aqueous phase was extracted four times with 1.0 mL of hexane and the extracts were combined. The hexane extracts were dried over fresh anhydrous calcium chloride, filtered via a 0.45-μm syringe filter and evaporated to dryness under a gentle stream of nitrogen gas. The residue was redissolved in 100 μL of toluene and immediately analyzed. Selective reactions of sesame oil required ~20 mg of oil for reaction due to the small amounts of FFA typical for food grade oils. A subsample of the oil was spiked at 0.5% (w/w) with a pure oleic acid standard to verify spike recovery. The FFA content of both the oleic acid spiked and unspiked samples was also determined by titration using AOCS Official Method Ca 5a-40 (12). Liquid chromatography with

electrospray ionization in the negative mode (LC–ESI-MS) was used to verify the results of the total FFA content that was determined by the selective esterification method and by the Official AOCS method Ca 5a-40 (15). An external calibration curve was prepared from pure component FFA calibration standards whose concentrations ranged from 0.05–10 μg/mL in acetonitrile and the response was found to be linear over this range. The analytical samples were prepared as 5,000 μg/mL solutions of the oil, or spiked oil, in toluene followed by a 1:10 dilution in acetonitrile.

Chromatographic methods

GC–MS analysis was performed to confirm peak identities and identify possible degradation products using an Agilent 6890 GC-Agilent 5973 MSD system. Separation was provided by a Zebron, ZD-1HT Inferno (Phenomenex; Torrance, CA) capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness). GC–FID analysis was conducted using an Agilent 6890 GC equipped with a flame ionization detector and an identical Zebron ZD-1HT Inferno column. The chromatographic parameters are described in the following. For GC–FID, inlet: 1 μL injection volume at 20:1 split, at a temperature of 360°C in constant flow mode at 1.2 mL/min, oven: 40°C for 0.5 min, 50°C/min to 150°C, 8°C/min to 310°C, 4°C/min to 360°C, held 360°C for 30 min; detector temperature was 380°C. For GC–MS, inlet: 1 μL injection volume at 20:1 split, at a temperature of 360°C in constant flow mode at 0.8 mL/min, oven: 40°C for 0.5 min, 50°C/min to 150°C, 8°C/min to 310°C, 4°C/min to 360°C, held 360°C for 30 min; MS detector transfer-line temperature was 360°C. Injections for both GC–FID and GC–MS analyses were provided by autosampler. These parameters were modified from a previously reported study using an on-column injection method for the separation and analysis of total lipids (16).

External standards were prepared in toluene from ~50 to 2,000 μg/mL for FFAs and 10 to 2,000 μg/mL for esters, monotriglycerides, di-triglycerides and triglycerides. The concentrations of pure components and their reaction products were taken directly from calibration curves. In the case of sesame oil and its reaction products, the unknown peaks were identified by class [monoacylglyceride (MAG), diacylglyceride (DAG), and TAG] by a combination of retention time matching to known standards and mass spectrometry spectral library matching.

Liquid chromatography with mass spectral detection (LC–MS) was performed on a Shimadzu model 2020 liquid chromatograph equipped with an electrospray ionization detector operating in the negative ion mode. The FFAs were eluted from a Shimadzu Shim Pak C18 column (2 × 75 mm; 2 μm) at a temperature of 60°C, with a mobile phase consisting of a 72:28 mixture of acetonitrile–water in a gradient to 100% acetonitrile over 20 min, then to 72% acetonitrile for 3 min, followed by a hold for 2 min, at a flow rate of 0.3 mL/min. The mass spectral detector parameters were as follows: interface 350°C, desolvation line 240°C, nebulizer gas 1.5 L/min, heater block 240°C, drying gas 15 L/min and detector voltage 1.3 kV.

Data analysis

One-way analysis of variance (ANOVA) analysis was employed in sesame oil reactions to determine the significance of any differences in the methyl tridecanoate and methyl heptadecanoate spike recoveries (for evaluating triglyceride conversion and FAME extraction efficiency) under optimized reaction conditions and under the AOCS reaction conditions. In all cases, the data were examined at the 99% confidence level with an F_{crit} (1, 6) value of 13.74. One-way ANOVA of the selective esterification, AOCS titrations and LC–MS data was examined at the 99% confidence level with an F_{crit} (1, 4) value of 21.20.

Results and Discussion

Pure component selective reactivity

Figures 1 and 2 illustrate the effect of several reaction parameters on the overall conversion of FFAs and TAGs to FAMEs. The most important parameters governing the conversion are temperature and time. Figure 1A shows that conversion of FFA is highly dependent on temperature, with reactions conducted at as low as 50°C achieving complete conversion of the reactants into their corresponding FAMEs, whereas the same reaction conducted at 40°C yielded incomplete conversion. The sensitivity of the FFA esterification reaction to temperature is significant, because a 10°C increase in temperature raised the conversion from 70% to nearly 100% (97.6 ± 2.3%) with a reaction time constant at 120 min and HCl–FFA ratio constant at 2:1. It appears that reactions above a temperature of 50°C did not improve the conversion of FFAs, and so it was determined that a reasonably mild temperature could be used to convert FFAs to FAMEs. The role of temperature in TAG conversion is shown in Figure 2A. It was determined that temperature had a significant impact on the completeness of TAG conversion, with a temperature of 80°C yielding over 90% conversion to ester, while a temperature of 40°C limited the conversion to approximately 10%. Clearly, when trying to minimize TAG conversion, a lower reaction temperature should be used.

Using the conditions of 60 min at 50°C, the next variable that was explored was the ratio of HCl to substrate. HCl was chosen as acid catalyst to provide a simple monoprotic acid as opposed to other potential acid catalysts such as sulfuric acid and phosphoric acid. As shown in Figure 1B, when the ratio of HCl to FFA substrate was varied over a range from 0.5 to 10.7 (w/w), very little impact was observed upon the FAME production from the pure FFA. A ratio of 0.5 still gave 97% conversion, and ratios above 2 allowed conversion to approach 100%. However, ester production from TAG conversion ranged from a maximum of 30% to a minimum of 2.5%, as shown in Figure 2B. This demonstrates that lower HCl–oil ratios minimize the TAG conversion, while the FFA conversion remains unaffected and is essentially complete regardless of the ratio. Again, to limit ester production from TAG conversion, a lower ratio of HCl to substrate is required. The trend observed for TAG reactivity indicates that lowering the acid catalyst–substrate ratio below 2 is effective for minimizing the degree of conversion to FAME.

Finally, when reaction temperature and substrate–HCl ratio were held constant at 50°C and 1.5:1, respectively, the effects of reaction time was explored. As Figure 1C shows, a series of reaction times from 5 min through 1 h showed little increase in the FAME production from FFA conversion. The trend for TAG conversion, shown in Figure 2C, is that longer reaction times

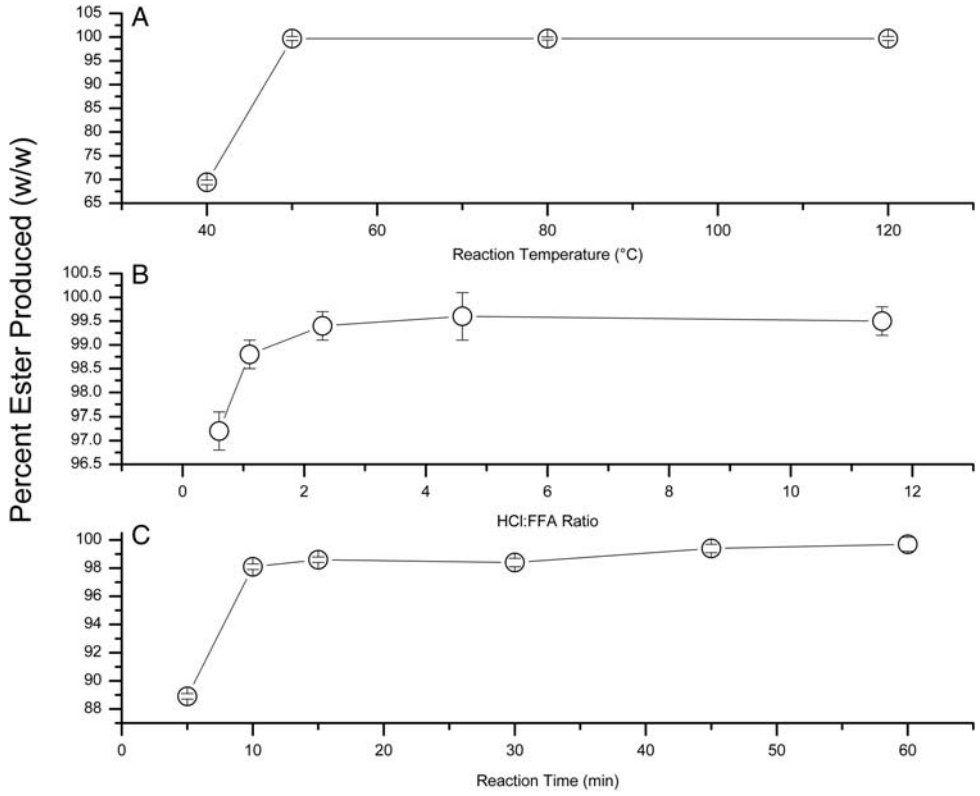

Figure 1. Ester production from pure FFA as a function of temperature with time constant at 2 h and HCl–FFA ratio of 2:1 (A); as a function of HCl–FFA ratio with temperature constant at 50°C and time constant at 15 min (B); as a function of reaction time with temperature constant at 50°C and HCl–FFA constant at 1.5:1 (C).

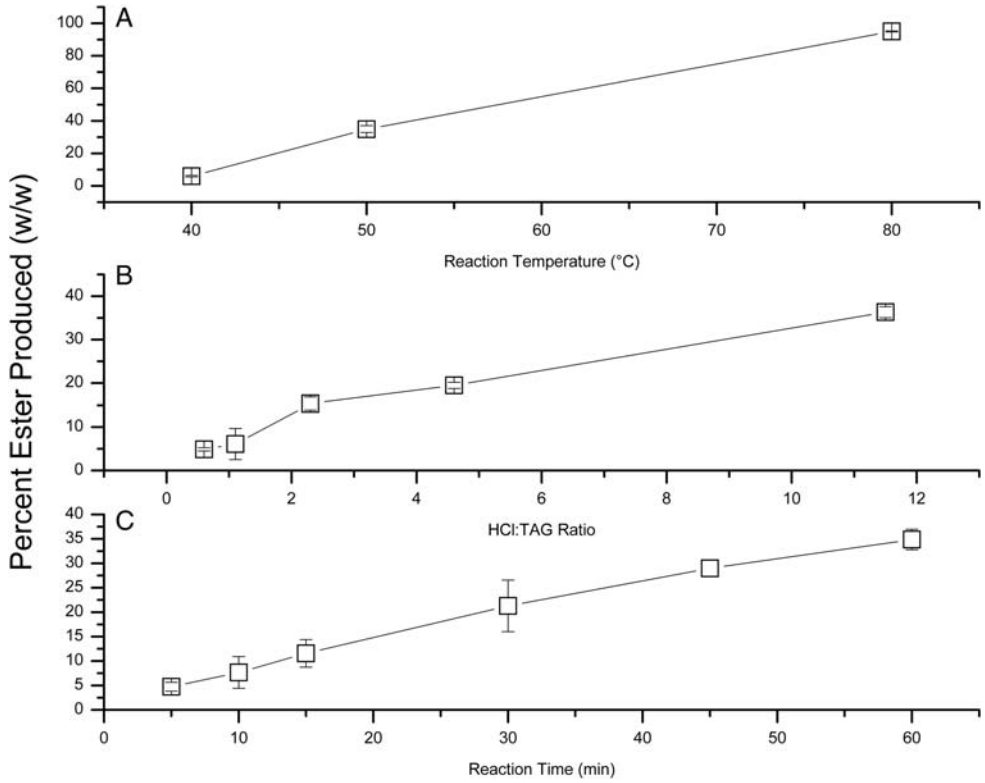

Figure 2. Percent ester production from pure TAG as a function of temperature with time constant at 2 h and HCl–FFA ratio of 2:1 (A); as a function of HCl–FFA ratio with temperature constant at 50°C and time constant at 15 min (B); as a function of reaction time with temperature constant at 50°C and HCl–FFA constant at 1.5:1 (C).

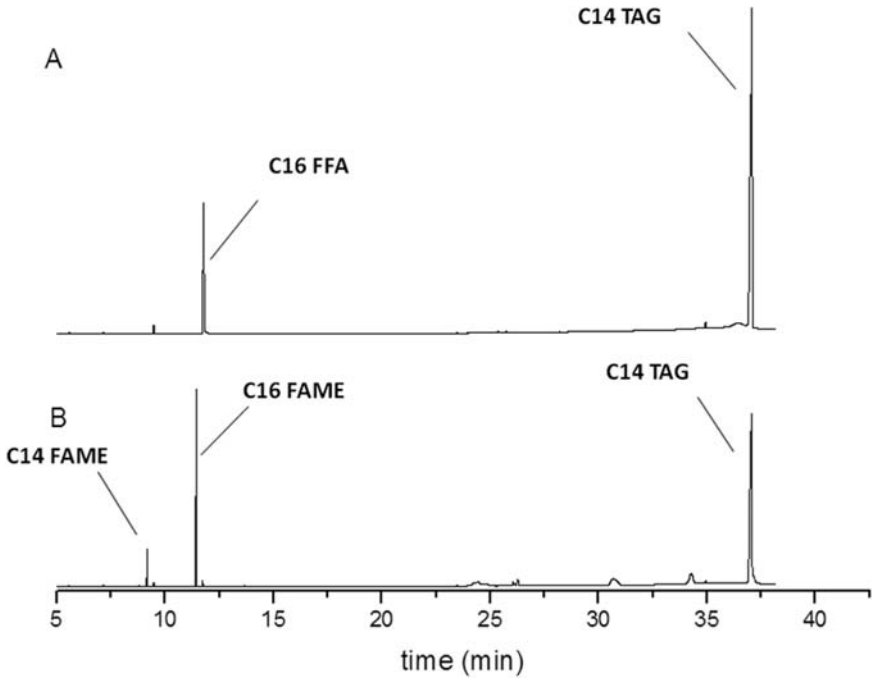

Figure 3. Chromatograms illustrating the selective esterification procedure on a mixture of FFA and TAG: starting material (C16 FFA and C14 TAG) (A); reaction products after selective esterification procedure (B).

favor more conversion, but that it is possible to use short reaction times to minimize FAME production from TAG.

Verification of selective reactivity was obtained by performing the conversion reaction on a mixture of pure C16:0 FFA and pure C14:0 TAG at 50°C (a simulated 12% w/w FFA in TAG) for 10 min in acidic methanol with a 2.1:1 ratio of HCl to total lipid. In this mixture, esterification of the TAG would be identified by the presence of C14 FAMEs, while the C16 FAMES would be the exclusive result of FFA conversion. Figure 3A shows the chromatogram of the starting material, while Figure 3B shows the chromatogram of the product of the selective esterification reaction. As Figure 3B indicates, $97 \pm 4\%$ (w/w) of the C14:0 TAG was recovered as unreacted starting material and 99 ± 2 % (w/w) of the C16:0 FFA was recovered as the C16:0 FAME. Furthermore, only trace amounts of C16:0 FFA and C14:0 FAME were observed in the reaction product. The slight conversion of C14 TAG appears larger than it really is due to the more sensitive response of the FID to FAME species (over 13 times more sensitive in the current study). Taken together, these results confirm that the selective esterification of FFA can be performed with minimal transesterification of TAG, using a 2.1:1 ratio of HCl to substrate, 50°C reaction temperature, and 10-min reaction time.

Selective reactivity of sesame oil components

To demonstrate method effectiveness on a complex biological oil, a well-characterized sesame oil standard was reacted under the optimized selective esterification conditions (2.1:1 ratio of HCl–oil at 50°C for 10 min). The oil was also subjected to a more rigorous esterification reaction conducted at 80°C for over 100 min, which was done to convert the entire FFA and

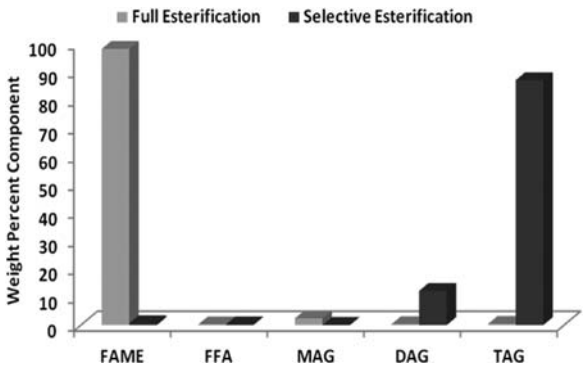

Figure 4. Composition of sesame oil samples reacted under selective esterification conditions and full transesterification conditions.

TAG component to FAMEs. Additional experiments were performed with this sesame oil that was spiked with 0.50% (w/w) FFA as oleic acid. The composition of the selective esterification reaction product was determined by GC–FID and compared to that of the fully esterified reaction product (shown in Figure 4). The results of these spike experiments are shown in Table I. The accuracy of the selective esterification method was established by confirming that the total FFA content of the unspiked oil was lower than the spiked oil by the amount of oleic acid spike. Moreover, by tracking just the concentration of oleic acid itself, the method was able to effectively recover the 0.5% (wt) that was spiked into the oil.

Figure 4 shows that, as expected from an oil having low free acid content, the selective conversion reaction under mild conditions produced less than 1% each of FAME, FFA and MAG, indicating that the TAG content remained largely intact. In an

Table I
Comparison of Results for FFA Determinations Using the Selective Esterification Method, LC–MS Analysis, and Titration*

Method	Non-spiked oil Weight (%) total FFA	Spiked oil† Weight (%) total FFA	$FFA_{spike} - FFA_{nonspike}$ Weight (%) total FFA added
Selective FFA	0.635 ± 0.15	1.24 ± 0.03	0.535 ± 0.05
LC–MS	0.530 ± 0.001	1.03 ± 0.03	0.507 ± 0.04
Method	Weight (%) oleic	Weight (%) oleic	Weight (%) oleic added
Selective FFA	0.22 ± 0.08	0.726 ± 0.1	0.507 ± 0.09
LC–MS	0.16 ± 0.02	0.632 ± 0.03	0.492 ± 0.04

*Note: Uncertainties are stated as ± standard deviation, $n = 3$.
†Spiked oil samples contained 0.50 % (w/w) oleic acid as spiked material.

attempt to provide further verification of the true FFA content of the oil, the sample was analyzed by LC–MS. The LC–MS data are in reasonably good agreement with the selective esterification data for the total amount of FFA in the starting sample (F = 0.29) as well as the total amount of spike added (F = 1.51). The weight percent of free oleic acid in the unspiked sample determined by selective esterification was in excellent agreement with the value determined by LC–MS (F = 0.88), as well as in the observed weight percent of oleic spike (F = 6.69).

Conclusions

The reactivity of FFA and TAG materials under acid-catalyzed esterificaton conditions was found to differ significantly. Unlike other popular methods of total lipid analysis based on conversion of the entire matrix to FAMEs, this study found that it is possible to discern the FFA content from the TAG content in a complex bio-oil mixture using selective esterification. Using mild conditions of 50°C for 10 min, and using a 2.1:1 ratio of HCl–substrate, pure free fatty acids were found to esterify rather easily, with 97.6 ± 2.3% recovery as FAMEs, while triglycerides were largely unaffected. Pure triglycerides were more resilient and required increased reaction times of 120 min at 80°C with a higher ratio of HCl–triglyceride, achieving 99.0 ± 3.3% recovery as FAMEs.

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