

Diffusion Coefficient–Formula Weight Correlation Analysis via Diffusion-Ordered Nuclear Magnetic Resonance Spectroscopy (DOSY NMR) To Examine Acylglycerol Mixtures and Biodiesel Production

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Biodiesel, fatty acid methyl esters derived from vegetable oils, is a well-established alternative to petroleum diesel. We have developed a rapid ¹H diffusion-ordered nuclear magnetic resonance spectroscopy (DOSY NMR) method to resolve mixtures of mono-, di-, and triacylglycerols along with their methyl esters. Because of the differences in diffusion coefficients between the starting materials (triglycerides), intermediates (mono- and diglycerols), and products (methyl esters), we were able to accurately predict the formula weights of these species in solution. This technique was used to monitor transesterification reactions of virgin and waste vegetable oils. In addition to proving its utility to assess conversion of starting materials, we found that 1,3-diacylglycerol is the major intermediate formed during alkali-catalyzed biodiesel production.

Introduction

At the current rate of oil consumption, it is estimated that worldwide reserves will be exhausted by 2050.¹ This prediction, alongside a dramatic rise in global greenhouse gas emissions, has made biomass an attractive fuel option. Biodiesel, derived from oils and fats of plants and animals, is a renewable and biodegradable alternative to petroleum diesel. In comparison to bioethanol, biodiesel provides nearly 4 times more energy return, and the production of biodiesel yields approximately 90% less agricultural pollution.² Because of its higher oxygen content, biodiesel provides enhanced engine lubricity³ and can be used in compression-ignition engines without modification.⁴ Industrial production of biodiesel largely relies upon the alkali-catalyzed transesterification of virgin plant oils (soy, canola, and palm) and recycled cooking oil to produce methyl esters and glycerol (Figure 1).

There is considerable interest in monitoring the conversion of triacylglycerols into biodiesel, and the standard tools of analytical chemistry can be used to analyze methyl ester production. Mono- and di-substituted intermediates of the transesterification reaction can be monitored by liquid chromatography (LC)– or gas chromatography–mass spectrometry (GC–MS), but because of the heterogeneous nature of vegetable oil feedstock, these methods give complex chromatograms, requiring numerous analytical standards for proper interpretation. ¹H nuclear magnetic resonance (NMR)

spectroscopy has also been used for mechanistic⁵ and kinetic⁶ studies and to quantify methyl ester production from virgin⁷ and waste^{8,9} oil. The primary drawback to using one-dimensional (1D) ¹H NMR is that the spectral complexity can preclude unambiguous interpretation. Furthermore, when used to measure biodiesel reaction intermediates and side products¹⁰ or extracts of oil-producing microorganisms,¹¹ 1D NMR methods are confounded by signal overlap and these analytes often require derivitization prior to analysis.

We proposed that the analytical limitations of 1D NMR in the context of biodiesel could be overcome using diffusion-ordered NMR spectroscopy (DOSY). This technique has been used to analyze a variety of biologically important nuclei (¹H, ¹³C, and ³¹P) by providing rapid visual confirmation of the relative sizes of compounds in solution.^{12–18} The chemical basis of DOSY is that higher molecular-weight compounds

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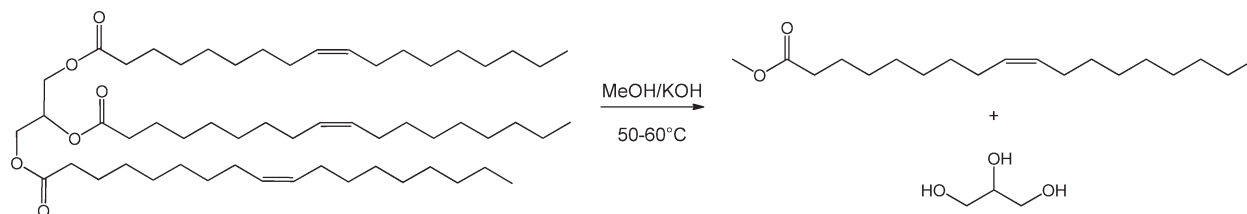


Figure 1. Base-catalyzed transesterification of an abundant triacylglycerol (triolein) to its fatty acid methyl ester (biodiesel).

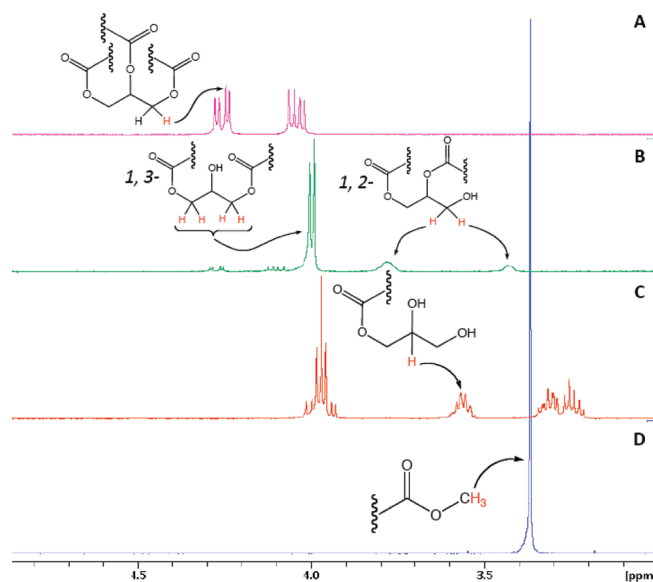


Figure 2. Expanded region (3.0–4.9 ppm) of individual ^1H NMR spectra of (A) triolein, (B) 1,3-dioleoylglycerol and 1,2-dioleoylglycerol, (C) 1-oleoyl-rac-glycerol, and (D) methyl oleate. Resonances used for fw correlation analysis are indicated with arrows.

diffuse more slowly than those of lower molecular weight and can thereby be resolved along the y axis (F1). A correlation between the diffusion coefficients for analytes of interest and those for internal reference standards allows one to deduce the average formula weight (fw) of the analyte. This method is particularly useful to study molecular complexes that form aggregates.^{19,20} In this report, we use DOSY NMR to differentiate the mono-, di-, and triacylglycerols of oleic acid, as well as its methyl ester.

Experimental Section

All analytical-grade reagents (>97%) were purchased from Sigma-Aldrich and used without further purification unless otherwise stated. DOSY NMR analyses were performed in toluene- d_8 (600 μL , Cambridge Isotopes) containing triolein (15 mg, 16.8 μmol), dioleoylglycerols (10.5 mg, 16.8 μmol), 1-oleoyl-rac-glycerol (6 mg, 16.8 μmol), methyl oleate (5 mg, 16.8 μmol), and 4 μL of squalene in benzene (1:1, mol/mol). The dioleoylglycerol standard was determined to be a 2.6:1 mixture of 1,3-dioleoylglycerol/1,2-dioleoylglycerol by ^1H NMR (see the Supporting Information). Transesterification reactions were performed with 200 μL (175 mg) of extra virgin olive oil (Whole Foods, Providence, RI) dissolved in 400 μL of methanol containing 3 mg of KOH. The reaction was heated to 50 $^\circ\text{C}$ for 2 min with stirring in a chemical microwave (Biotage). A small aliquot (18.6 mg) was removed, concentrated *in vacuo*, diluted in 600 μL of toluene- d_8 , and directly analyzed. The esterification reaction of

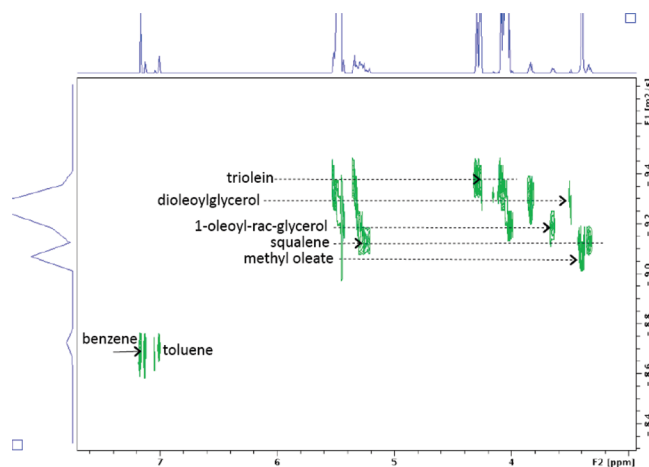


Figure 3. ^1H DOSY NMR spectrum of a mixture of differentially substituted acylglycerols, methyl oleate, and internal standards (benzene and squalene). Resonances used for formula-weight analysis are indicated at the arrowhead. Dashed horizontal lines pass through additional signals for individual compounds in the mixture.

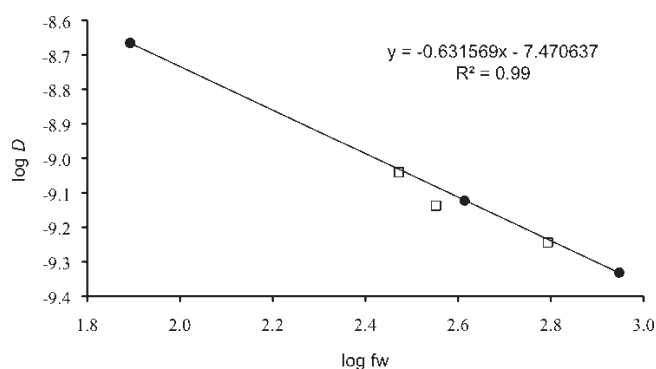


Figure 4. Plot of diffusion coefficient ($\log D$) versus formula weight ($\log fw$) of a mixture of acylglycerol analytes (\square) and internal standards (\bullet).

recycled cooking oil was performed by heating 1 L of 3 day old cooking oil in 200 mL of MeOH containing 5 mL of 12 N H_2SO_4 as the catalyst. The reaction was allowed to stir (1 h, 55 $^\circ\text{C}$), after which point a separatory funnel was used to remove the top layer. The lower layer was then reacted with 200 mL of MeOH and 5 g of NaOH (55 $^\circ\text{C}$, 10 min). An approximately 40 μL sample of the reaction mixture was concentrated *in vacuo* and directly used for DOSY analysis by dissolving in 500 μL of toluene- d_8 containing 2 μL of squalene in benzene (1:1, mol/mol).

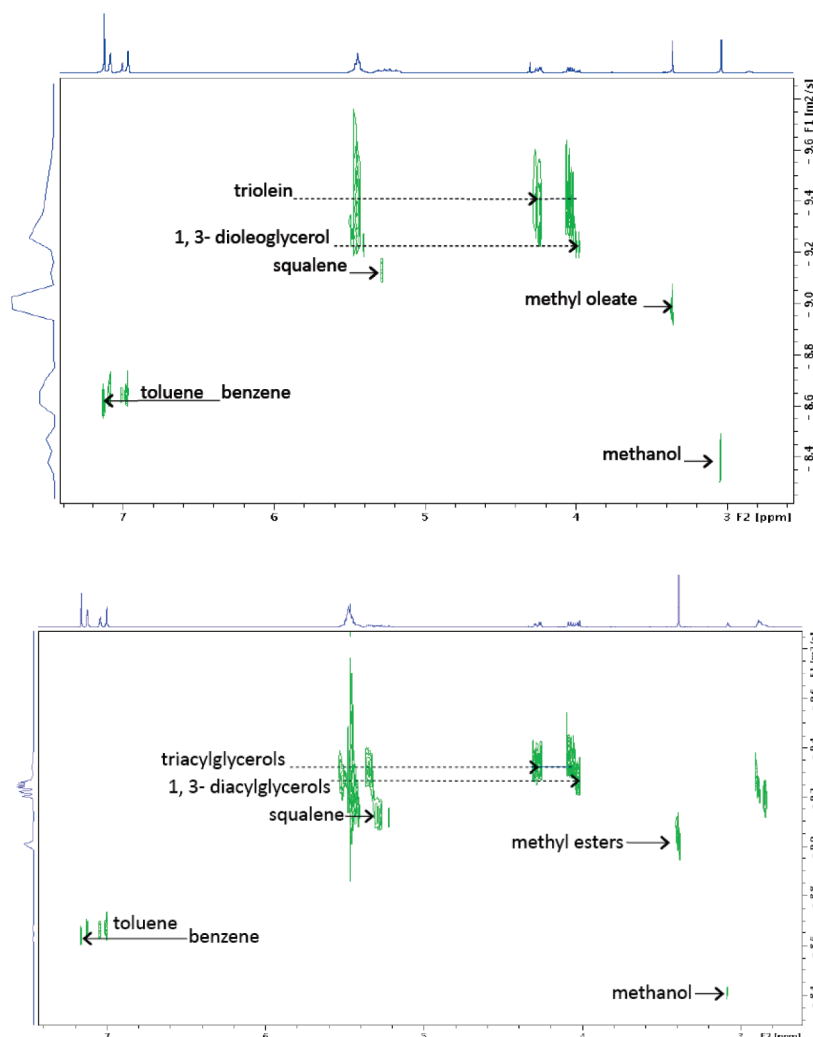
The lower limits of detection for 1,3-dioleoylglycerol, 1,2-dioleoylglycerol, and 1-oleoyl-rac-glycerol were measured by titrating these compounds into a solution of methyl oleate (6.8 μL , 7.8 mg, 26.2 μmol) in 505 μL of toluene- d_8 . Using the 1- and 3-position methylene peaks at δ 4.02, 1,3-dioleoylglycerol was detected at 8 mol % by adding 1.25 mg (2.02 μmol). Using the 3-position methylene peaks at δ 3.78 and δ 3.49, 1,2-dioleoylglycerol was detected at 4 mol % by adding 0.69 mg (1.12 μmol).

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Table 1. Predicted Formula Weights (fw*) from D–fw Correlation Analysis of Acylglycerols and Internal Standards

analyte	δ (ppm)	D ($\times 10^{-10}$, $\text{m}^2 \text{s}^{-1}$)	fw (g mol^{-1})	fw* (g mol^{-1})	percent error (%)
triolein	4.27	4.667	885.43	882	0.4
1, 3- dioleoglycerol	4.04	5.729	620.99	638	-2.7
1, 2- dioleoglycerol	3.49	5.701	620.99	643	-3.5
squalene	5.22	7.538	410.72	413	-0.5
1-oleoyl-rac-glycerol	3.66	7.294	356.54	435	-22.0
methyl oleate	3.40	9.110	296.49	306	-3.2
benzene	7.16	21.60	78.11	78	0.2

**Figure 5.** DOSY spectra of the reaction mixture from virgin oil (top) and waste oil (bottom) transesterification.

Using the 1-position methylene peaks at δ 3.98, 1-oleoyl-rac-glycerol was detected at 7 mol % by adding 0.7 mg (1.96 μmol). ^1H DOSY spectroscopy was performed on a Bruker DRX400 spectrometer equipped with an Accustar z -axis gradient amplifier and an ATMA BBO probe with a z -axis gradient coil. Experiments were conducted in 32 steps using the standard Bruker ledbpgp2s pulse program using stimulated echo and longitudinal eddy delay with bipolar gradients and two spoil gradients. The maximum gradient strength was 0.214 T/m. The longest T1 measured was approximately 0.7 s (1-oleoyl-rac-glycerol). Rectangular gradients were used with a total duration of 2 ms. Gradient recovery delays were 80 ms. Diffusion times were 100 ms. Individual rows of the quasi-2D diffusion databases were phased and baseline-corrected.

Results and Discussion

Individual acylglycerols were first examined by ^1H NMR to identify compound-specific chemical shifts (δ) that could be

distinguished in mixtures. Analytes can be separated in the diffusion dimension (F1) if they have at least one unique resonance within an unresolved multiplet derived from two or more compounds. This additional resolution offers a significant advantage over 1D NMR when analyzing mixtures of related compounds. We first selected a methylene doublet of triolein at δ 4.26 (Figure 2A). The chemically equivalent 1- and 3-position methylenes (δ 4.02) were selected for analysis of 1,3-dioleoglycerol, and this resonance was subsequently observed in reaction mixtures. The diastereotopic 3-position methylene (δ 3.78 and 3.49) was selected for DOSY analysis of 1,2-dioleoglycerol (Figure 2B). The multiplet (δ 3.64) representing 2-position methine of 1-oleoyl-rac-glycerol was selected for analysis of the monoglycerol (Figure 2C). Finally, the prominent methyl singlet at δ 3.35 was used to identify the desired biodiesel product, methyl oleate.

^1H DOSY NMR was conducted using a mixture of the aforementioned compounds. This method cleanly differentiated the individual components of the mixture based on their diffusion rate coefficients (D) in solution (Figure 3). Triolein diffuses most slowly ($D = 4.67 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$). 1,3-Dioleoglycerol and 1,2-dioleoglycerol showed identical diffusion rates of $5.70 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$. The monoglycerol, 1-oleoyl-rac-glycerol, was next ($D = 7.29 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$), followed by methyl oleate, the lightest compound in the mixture ($D = 9.11 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$). To perform diffusion coefficient–formula weight (D –fw) correlation analysis of these molecules, two additional compounds, squalene ($D = 7.54 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$; fw = $410.72 \text{ g mol}^{-1}$) and benzene ($D = 21.6 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$; fw = 78.11 g mol^{-1}) were added to the NMR tube. The \log_{10} diffusion coefficients were plotted against the \log_{10} formula weights, and both the analytes and standards showed very good linear correlation (Figure 4). Therefore, interpolating the diffusion coefficients of triolein, dioleoglycerol, and methyl oleate from this plot yields accurate ($<4\%$ error) formula-weight information (Table 1). Although D –fw analysis gave a poor prediction (22% error) for 1-oleo-rac-glycerol, a unique chemical shift from the 2-position methine allowed for its distinction as a mono-substituted glycerol.

On the basis of the success of D –fw analysis for the identification of acylglycerols in complex mixtures, we predicted that ^1H DOSY could be used to monitor the production of biodiesel. Two separate transesterification reactions were performed using either virgin oil or waste cooking oil. The DOSY spectrum of these starting materials prior to any chemistry showed only triacylglycerols. To observe reaction intermediates and assess the ease of product analysis by the DOSY method, the transesterifications were intentionally quenched prior to completion and the resulting mixtures were not allowed to phase separate before sampling. In both reaction mixtures, we clearly resolved unreacted starting material (triolein) from biodiesel product (methyl oleate) and residual methanol (Figure 5). In agreement with the interpretation of 1D NMR spectra by Jin et al.,⁵ the DOSY spectra of our reactions also revealed 1,3-dioleoglycerols as the major intermediate. This product is most likely due to 2–1 acyl migration after methoxide attack at the 1 position. D –fw analysis, analogous to that used in our standard

analytical sample, was then applied to these reaction mixtures. The results showed a high linear correlation between all components ($R^2 = 0.99$) and, thus, accurate fw predictions for both reactions (see the Supporting Information).

Conclusions

^1H DOSY NMR methods were used to examine acylglycerols and fatty acid methyl esters. NMR spectroscopy is a cornerstone of biodiesel analysis,²¹ and ^1H DOSY provides an additional tool for fuel chemists investigating complex sample mixtures. The methylene signal overlap between δ 3.98 and 4.20 ppm often confuses 1D analyses of mono-, di-, and triacylglycerol mixtures. Because these molecules diffuse at different rates, the resonances have been resolved with our method. DOSY is qualitative and does not obviate the need for quantitative techniques at the final stage of biodiesel processing. Because of its ability to separate compounds based on their molecular size, diffusion coefficient–formula weight analysis may also prove useful for monitoring triacylglycerol and/or phospholipid production in the culture media of oil-producing microorganisms. Additionally, mechanistic insight toward next-generation biodiesel catalysts may be obtained with this method.

Contrary to studies performed with phospholipids,¹⁵ our analysis showed no vesicle formation at the concentrations tested. In model transesterification reactions using virgin olive oil and waste cooking oil, DOSY identified unreacted di- and triacylglycerols as well as their methyl ester products and residual methanol. Because washing biodiesel to remove methanol and glycerol is a time-intensive step often requiring several days to complete, it is critical to know when a reaction has reached completion before working up the entire batch. The DOSY method requires minimal sample preparation and allows for direct observation of individual components within a reaction mixture in a single, rapid experiment.

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Supporting Information Available: D –fw analyses and detailed NMR data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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