Journal of Visualized Experiments

Experimental Protocol for Biodiesel Production with Isolation of Alkenones as Coproducts from Commercial Isochrysis Algal Biomass.

--Manuscript Draft--

Manuscript Number:	JoVE54189R4
Full Title:	Experimental Protocol for Biodiesel Production with Isolation of Alkenones as Coproducts from Commercial Isochrysis Algal Biomass.
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	Algal biofuels; biodiesel; fatty esters; lipids; coproducts; alkenones; Isochrysis.
Manuscript Classifications:	10.1: Technology, Industry, and Agriculture; 4.10: Lipids; 4.27: Chemical Actions and Uses; 92.28: Propellants and Fuels
Corresponding Author:	Gregory W O'Neil, Ph.D Western Washington University Bellingham, Washington UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	oneilg@chem.wwu.edu
Corresponding Author's Institution:	Western Washington University
Corresponding Author's Secondary Institution:	
First Author:	Gregory W O'Neil
First Author Secondary Information:	
Other Authors:	John R Williams Julia Wilson-Peltier Gerhard Knothe Christopher M Reddy
Order of Authors Secondary Information:	

Abstract:

The need to replace petroleum fuels with alternatives from renewable and more environmentally sustainable sources is of growing importance. Biomass-derived biofuels have gained considerable attention in this regard, however first generation biofuels from edible crops like corn ethanol or soybean biodiesel have generally fallen out of favor. There is thus great interest in the development of methods for the production of liquid fuels from domestic and superior non-edible sources. Here we describe a detailed procedure for the production of a purified biodiesel from the marine microalgae Isochrysis. Additionally, a unique suite of lipids known as polyunsaturated long-chain alkenones are isolated in parallel as potentially valuable coproducts to offset the cost of biodiesel production. Multi-kilogram quantities of Isochrysis are purchased from two commercial sources, one as a wet paste (80% water) that is first dried prior to processing, and the other a dry milled powder (95% dry). Lipids are extracted with hexanes in a Soxhlet apparatus to produce an algal oil ("hexane algal oil") containing both traditional fats (i.e. triglycerides, 46-60% w/w) and alkenones (16-25% w/w). Saponification of the triglycerides in the algal oil allows for separation of the resulting free fatty acids (FFAs) from alkenone-containing neutral lipids. FFAs are then converted to biodiesel (i.e. fatty acid methyl esters, FAMEs) by acid-catalyzed esterification while alkenones are isolated and purified from the neutral lipids by crystallization. We demonstrate that biodiesel from both commercial Isochrysis biomasses have similar but not identical FAME profiles, characterized by elevated polyunsaturated fatty acid contents (approximately 40% w/w). Yields of biodiesel were consistently higher when starting from the Isochrysis wet paste (12% w/w vs. 7% w/w)

	which can be traced to lower amounts of hexane algal oil obtained from the powdered Isochrysis product.
Author Comments:	
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	

Cover Letter

WESTERN
WASHINGTON UNIVERSITY
CHEMISTRY DEPARTMENT

Active Minds Changing Lives

516 High Street
Bellingham, Washington 98225-9150
(360) 650-3070 □ Fax (360) 650-2826

October 1, 2015

We would like to submit the following manuscript as an article to JOVE.

Experimental Protocol for Biodiesel Production with Isolation of Alkenones as Coproducts from Commercial *Isochrysis* Algal Biomass

Recently our group has been investigating biofuel production from a marine microalgae *Isochrysis*. This work has focused on a unique class of algal lipids known as long-chain alkenones that have been largely unexplored as a potential fuel source (O'Neil et al., *Energy Fuels* **2012**, 2434; *Energy Fuels* **2014**, 2677; *Energy Fuels* **2015**, 922). Alkenones are biosynthesized by a select number of microalgae that includes *Isochrysis* sp. *Isochrysis* is also one of only a few algae currently grown industrially, harvested for purposes of mariculture.

For all of our previous work we have used *Isochrysis* purchased from Reed Mariculture (San Jose, CA), who supplies a wet (80% water) paste that is dried prior to processing. In this manuscript we compare processing and results from Reed to another commercial source of *Isochrysis*, Necton S.A. (Olhão, Portugal). Multi-kilogram quantities of *Isochrysis* can also be purchased from Necton, but in this case as a dry (5% water) fine milled powder. The color of the algae from Necton is also strikingly different compared to Reed (yellow-brown vs. dark-green near black from Reed) yet all final products are similar in appearance. In addition to yields, we also compare fatty acid profiles which is of importance to the fuel properties of a corresponding biodiesel.

The availability of the algae utilized and standard methods employed, make the protocol presented widely accessible to other groups. We hope that you will find this manuscript worthy of publication and look forward to the opportunity to create a video reference that will encourage further investigations into important lines of research that emerged from the results we describe.

Sincerely,

Dr. Gregory W. O'Neil
Associate Professor of Chemistry
Western Washington University
516 High Street, MS 9150
Bellingham, WA, 98225-9150
Tel. +01.360.650.6283
Fax +01.360.650.2826
oneil@chem.wwu.edu

TITLE:

Experimental Protocol for Biodiesel Production with Isolation of Alkenones as Coproducts from Commercial *Isochrysis* Algal Biomass

AUTHORS:

O'Neil, Gregory W.
Department of Chemistry
Western Washington University
Bellingham, WA, USA
oneil@chem.wwu.edu

Williams, John R.
Department of Chemistry
Western Washington University
Bellingham, WA, USA
willi310@students.wwu.edu

Wilson-Peltier, Julia
Department of Chemistry
Western Washington University
Bellingham, WA, USA
wilso227@students.wwu.edu

Gerhard Knothe
Agricultural Research Service
United States Department of Agriculture
Peoria, IL, USA
gerhard.knothe@ars.usda.gov

Reddy, Christopher M.
Department of Marine Chemistry and Geochemistry
Woods Hole Oceanographic Institution
Woods Hole, MA, USA
creddy@whoi.edu

CORRESPONDING AUTHOR:

Gregory W. O'Neil

KEYWORDS:

Algal biofuels; biodiesel; fatty esters; lipids; coproducts; alkenones; *Isochrysis*

SHORT ABSTRACT:

Detailed methods are presented for the production of biodiesel along with the co-isolation of alkenones as valuable coproducts from commercial *Isochrysis* microalgae.

LONG ABSTRACT:

The need to replace petroleum fuels with alternatives from renewable and more environmentally sustainable sources is of growing importance. Biomass-derived biofuels have gained considerable attention in this regard, however first generation biofuels from edible crops like corn ethanol or soybean biodiesel have generally fallen out of favor. There is thus great interest in the development of methods for the production of liquid fuels from domestic and superior non-edible sources. Here we describe a detailed procedure for the production of a purified biodiesel from the marine microalgae *Isochrysis*. Additionally, a unique suite of lipids known as polyunsaturated long-chain alkenones are isolated in parallel as potentially valuable coproducts to offset the cost of biodiesel production. Multi-kilogram quantities of *Isochrysis* are purchased from two commercial sources, one as a wet paste (80% water) that is first dried prior to processing, and the other a dry milled powder (95% dry). Lipids are extracted with hexanes in a Soxhlet apparatus to produce an algal oil ("hexane algal oil") containing both traditional fats (i.e. triglycerides, 46-60% w/w) and alkenones (16-25% w/w). Saponification of the triglycerides in the algal oil allows for separation of the resulting free fatty acids (FFAs) from alkenone-containing neutral lipids. FFAs are then converted to biodiesel (i.e. fatty acid methyl esters, FAMEs) by acid-catalyzed esterification while alkenones are isolated and purified from the neutral lipids by crystallization. We demonstrate that biodiesel from both commercial *Isochrysis* biomasses have similar but not identical FAME profiles, characterized by elevated polyunsaturated fatty acid contents (approximately 40% w/w). Yields of biodiesel were consistently higher when starting from the *Isochrysis* wet paste (12% w/w vs. 7% w/w), which can be traced to lower amounts of hexane algal oil obtained from the powdered *Isochrysis* product.

INTRODUCTION:

There has recently been a great resurgence of interest in biofuels from algae, particularly for the production of liquid fuels such as biodiesel[1] and other biomass-derived oils.[2] Proposed benefits include the avoidance of certain food vs. fuel controversies[3] and reportedly higher productivities and CO_2 mitigation capabilities than traditional agricultural crops.[4] This follows the nearly 20 year United States Department of Energy's Aquatic Species Program (ASP) started in 1978 for the purpose of investigating transportation fuel from algae. As outlined in Sheehan's report,[5] the program ended in 1996 primarily because projected costs were not competitive with crude petroleum at that time ($18.46 per barrel (159 L)). While the cost of petroleum has increased dramatically since then ($87.39 per barrel in 2014)[6], which is connected to the renaissance in algal biofuel research, some have argued that nonetheless algal biofuels will prove too costly.[7] As one strategy to offset biofuel production costs, the notion of value-added coproducts has emerged among both critics[7,8] and proponents[9,10] and features as one of the key reasons for pursuing algal biofuels in the United States Department of Energy (DOE) "National Algal Biofuels Technology Roadmap".[11]

Here we describe a method for the coproduction of two separate fuel streams from commercial *Isochrysis* microalgae. We have focused on *Isochrysis* in part because it is already produced industrially, harvested for purposes of mariculture, and also because *Isochrysis* is one of only a few species of algae that in addition to traditional lipids (i.e. fatty acids) biosynthesize a unique class of compounds known as polyunsaturated long-chain alkenones.[12] Alkenone structures are characterized by very long hydrocarbon chains (36-40 carbons), two to four non-methylene interrupted *trans*-double bonds, and a methyl or ethyl ketone (Figure 1). Alkenone unsaturation is sensitive to the algae growing temperature,[13,14] such that the proportion of the diunsaturated C37 methyl alkenone (the so-called "unsaturation index") can be used as a proxy for past sea surface temperatures.[15–20] Alkenones are thought to reside in cytoplasmic lipid bodies and can be more abundant than triglycerides (TAGs).[21,22] Under nitrogen or phosphorous limitation, up to 10−20% of cell carbon in the stationary phase is accumulated as alkenones.[23,24] From an evolutionary standpoint, alkenones may have been favored over TAGs because their *trans*-double bond geometry provides a more stable form of energy storage.[21]

[Place Figure 1 here]

We argue that alkenones represent a promising renewable carbon feedstock from a common algae with a history of industrial cultivation.[25] Biodiesel produced directly from the total lipid extract of *Isochrysis* contains a significant amount (10-15% w/w) of alkenones and contamination by these high-melting compounds results in poor cold flow fuel properties. However, using the saponification/extraction techniques described here, alkenones can be removed and recovered thereby improving the biodiesel quality while producing a secondary product stream. Recently we demonstrated the conversion of alkenones to a liquid fuel by cross-metathesis with 2-butene (butenolysis).[26] The butenolysis reaction employs a commercial ruthenium metathesis-initiator, occurs rapidly at low temperature, and cleanly delivers a predictable mixture of jet fuel range hydrocarbons. This reaction is performed in parallel with biodiesel synthesis from fatty acids, representing the first steps toward a "biorefinery" approach[27] for commercially viable *Isochrysis* biofuel production.

PROTOCOL:

1. Microalgae and biomass preparation.

Note: The marine microalgae *Isochrysis* sp. "T-iso" used in the present study can be purchased (see Table of Materials). Multi-kilogram quantities of *Isochrysis* can be purchased as a frozen wet paste (*Iso*-paste) containing approximately 80% water and 20% biomass, and is dark green/near black in color with a pungent odor smelling of the sea. *Isochrysis* can also be purchased as a dry (95% dry) yellow-brown powder (*Iso*-powder) with a similar odor.

1.1) In order to dry the *Isochrysis* paste, open a 1 kg package by cutting a 1 – 2 inch hole in the corner of the plastic packaging using scissors.

1.2) Squeeze approximately 300 g of this *Isochrysis* paste through the hole into a 150 x 75 mm crystallizing dish to create a thin layer (~20 mm).

1.3) Leave the paste to air-dry at room temperature until it becomes dry and flaky (typically, 48 – 96 hours).

Note: Actual drying times may vary and are dependent on temperature. However, no difference was noticed in the yields or product quality with even longer drying times (up to two weeks). The drying process may also be made more uniform and/or accelerated by placing the crystallizing dish on a warm plate (30 – 40 ^{o}C).

1.4) Scrape the dry biomass from the crystallizing dish using a spatula and collect into a cellulose extraction thimble (Length: 123 mm, 43 mm ID). Record the weight of the dry *Isochrysis* biomass.

2. Soxhlet extraction of dry *Isochrysis* biomass

2.1) Load an *Isochrysis*-containing cellulose extraction thimble (typically 50 - 60 g dry biomass) into a Soxhlet extraction apparatus.

2.2) Fill the Soxhlet flask with hexanes (400 mL), turn on the condenser water and heat source, and allow the Soxhlet to cycle for 24 - 48 hours (until the color of the solvent has gone from dark green to a faint yellow).

2.3) Turn off the heat and allow the apparatus to cool to room temperature, then disconnect the flask from the Soxhlet extractor.

2.4) Remove the hexanes using a rotary evaporator and record the weight of the hexanes-extractable material (“hexane algal oil” (h-AO)).

3. Saponification of the algal oil and separation of fatty acids and neutral lipids.

3.1) Redissolve the h-AO in the same round bottom flask from Step 2.4 above with methanol:dichloromethane (2:1, volume = 10 x mass of algal oil).

3.2) Add a stir bar and attach a reflux condenser (Coil: 500 mm length).

3.3) Add H_2O (volume = 2.67 x mass of algal oil) and KOH (50% w/w algal oil) and heat the contents with stirring to 60 °C for 3 h.

3.4) After cooling to room temperature, remove the organic solvents (methanol and dichloromethane) on a rotary evaporator.

3.5) Transfer the remaining aqueous mixture by pouring into a 1-L separatory funnel. Add hexanes (equivalent in volume to the aqueous solution), shake the separatory funnel, and allow the layers to separate.

3.6) Drain the lower aqueous layer into an Erlenmeyer flask, and pour off the top organic phase into a separate Erlenmeyer.

3.7) Repeat steps 3.5 and 3.6 until the organic layer is colorless (typically 1-2 more times).

3.8) Concentrate the combined organic extracts on a rotary evaporator to isolate the neutral lipids as a greenish solid (mp. ≈ 60 – 70 °C).

3.9) Acidify the aqueous phase with HCl (6 M, until pH ~ 2 as indicated by pH paper).

3.10) Extract the free fatty acids (FFAs) from the acidified aqueous phase with hexanes (equivolume to the aqueous phase) using a 1 L separatory funnel as described in steps 3.5 and 3.6.

3.11) Remove the hexanes on a rotary evaporator to obtain the FFAs as a dark green near-black oily residue (liquid at temperatures >30 °C).

4. Acid-catalyzed esterification of free fatty acids and production of a green biodiesel.

4.1) Transfer the FFAs using methanol:chloroform (1:1, 6 x volume of algal oil) to first dissolve the FFAs and then by pouring into a thick-walled high pressure reaction flask equipped with a stir bar.

4.2) Add concentrated H_2SO_4 (20% w/w algal oil), seal the flask, and heat the mixture to 90 °C while stirring for 1 h.

4.3) After cooling to room temperature, transfer the mixture by pouring into a separatory funnel.

4.4) Add H_2O (2 x volume of algal oil), shake the separatory funnel, and allow the phases to separate.

4.5) Drain the bottom layer into a pre-weighed round bottom flask and concentrate on a rotary evaporator. Record the mass of the resulting biodiesel.

4.6) Analyze the fatty acid profile by gas chromatography with flame-ionization detector (GC-FID)[28] (gas chromatograph equipped with a DB–88 [(88 % cyanopropyl) methylarylpolysiloxane] column (30 m x 0.25 mm ID x 0.20 µm film thickness).

Note: Common fatty acid methyl esters are verified by retention time comparison with authentic samples obtained commercially. Additionally, gas chromatography-mass spectrometry (GC-MS; gas chromatograph coupled to a mass selective detector) is performed under identical conditions of temperature program and column to analyze components such as C18:4 for which no authentic standards are available with the results applied to GC quantitation.

5. Biodiesel decolorization.

5.1) Heat the dark green colored biodiesel to 60 °C in a round bottom flask equipped with a stir bar.

5.2) Add montmorillonite K 10 (MK10) powder (10-20% w/w of the biodiesel) and stir for 1 hr.

5.3) Remove the round bottom from the heat and allow the solution to cool to room temperature.

5.4) Prepare a filtration apparatus consisting of a round bottom flask and filter funnel containing a cellulose filter paper (Ash 0.007%).

5.5) Pour the cooled decolorized biodiesel through the filter funnel using a minimal amount of hexanes to rinse the round bottom flask.

5.6) Disconnect the filter funnel from the round bottom flask (this now contains a hexanes solution of the decolorized biodiesel) and remove the hexanes with a rotary evaporator to afford an orange/red biodiesel.

5.7) Store samples at 4 °C during which time some settling of insoluble material (~10% w/w) will occur.

5.8) Remove the insoluble material by decanting or filtration as described in step 5.4 and 5.5 to produce a clear homogeneous biodiesel for analysis.

6. Isolation and purification of alkenones from the neutral lipids.

6.1) Dissolve the neutral lipids (from Step 3.8) in a minimal amount of dichloromethane (approximately 50 mL for 10 g neutral lipids) and add the solution with a pipet to the top of a chromatography column (O.D 60 mm, ID 55 mm, length 18”) containing silica gel (230-400 mesh, 100 g).

6.2) Elute the solution through the silica with pressure (~5 psi) using dichloromethane (approximately 150 mL) as solvent and collect the eluent in a 250 mL round bottom flask.

6.3) Remove the dichloromethane with a rotary evaporator to give an orange-colored solid.

6.4) Recrystallize the solid using hexanes by adding approximately 100 mL of boiling hexanes followed by incremental additional amounts of boiling hexanes until the solution is homogeneous (total volume ~ 150 mL). Then, slowly cool the solution to room temperature to promote crystallization.

6.5) Collect the crystallized alkenones using a filtration apparatus as described in step 5.4 using a small amount of cold (0 °C) hexanes to rinse the flask.

REPRESENTATIVE RESULTS:

Prior to processing, the *Isochrysis* paste (*Iso*-paste) was first dried. This was conveniently accomplished on larger scale by adding the *Iso*-paste to a large crystallizing dish and allowing the material to air-dry at room temperature. During drying, some pooled water forms (generally reddish colored) that can be removed by decanting or pipetting to accelerate the drying process. After approximately 48 - 96 h, the now dry *Isochrysis* could be scraped out of the crystallizing dish and obtained as a black/green flaky material with a seaweed-like smell (Figure 2). Yields of dry biomass were generally 20% w/w of the paste as advertised. By contrast, the powdered *Isochrysis* product (*Iso*-powder) was a yellow-brown, finely milled, dry powder (95% dry) that was used directly without further processing (Figure 2).

[Place Figure 2 here]

Extraction of either the dried *Iso*-paste or *Iso*-powder by Soxhlet with hexanes gave, after removal of the hexanes, algal oils (h-AO) that were similar in appearance as dark-green/near-black solids (mp. ~ 50-60 °C). Yields of h-AO when starting from the paste ("*Iso*-paste-hAO") were typically 20% w/w of the dry *Isochrysis* biomass, consistent with our previous results,[26] whereas yields of h-AO by Soxhlet extraction of the commercial powdered *Isochrysis* ("*Iso*-powder-hAO") were 15% w/w (Table 1).

[Place Table 1 here]

Acylglycerols in the h-AO were converted to the corresponding water-soluble carboxylate salts (i.e. soaps) upon addition of aqueous KOH in methanol/CH_2Cl_2. Neutral lipids including alkenones were then extracted from this aqueous mixture by selective partitioning with hexanes. After removal of the neutral lipids, reacidification of the soaps then produced the corresponding free fatty acids (FFAs) that could be similarly extracted from the aqueous phase with hexanes. The overall mass recoveries for combined FFAs and neutral lipids from either *Iso*-paste-hAO or *Iso*-powder-hAO were consistently near quantitative. However, the ratio of products (i.e. neutral lipids + FFAs) was different. From the *Iso*-paste-hAO we obtained 60% (w/w) FFAs and 40% (w/w) neutral lipids (Table 1). Conversely, *Iso*-powder-hAO proved enriched in neutral lipids (average = 54% neutral lipids + 46% FFAs) as detailed in Table 1.

Esterification of the FFAs with H_2SO_4 and methanol then produced fatty acid methyl esters (FAMEs, i.e. biodiesel) as a dark green near black oily liquid in greater than 90% yield (Figure 3). Decolorization by heating over Montmorillonite K10[29] (MK10) clay then gave a yellow/orange product, similar in appearance to other commercial biodiesel fuels (see Table of Materials) (Figure 3). Results from the FAME analysis of decolorized *Isochrysis* biodiesel fuels are shown in Table 2.

[Place Figure 3 here]

[Place Table 2 here]

Neutral lipids were obtained as a greenish solid mixture at 40% w/w from the *Iso*-paste-hAO and 54% (avg.) from the *Iso*-powder-hAO (Table 1). Filtering the dissolved neutral lipids through silica using DCM gave after removal of the solvent, a reddish/orange solid that could be recrystallized with hexanes to afford analytically pure alkenones as a white solid. This procedure resulted in 16% (w/w) isolated yield of alkenones from *Iso*-paste-hAO and 25% yield from *Iso*-powder-hAO (Table 1).

Figure 1: Structures of polyunsaturated long-chain alkenones. Common alkenone methyl 37:3 isolated from *Isochrysis* exemplifying long hydrocarbon chain lengths (36 – 40 carbons), *trans* non-methylene interrupted double bonds, and terminating in a methyl or ethyl ketone. Nomenclature is similar to fatty acids where #:# refers to number of carbons:number of double bonds.

Figure 2: Comparison of commercial *Isochrysis*. *Isochrysis* paste (80% wet) is spread along the bottom of a crystallizing dish and left to air-dry at room temperature for 48-96 h before processing. The resulting dried *Isochrysis* is obtained as a dark-colored flaky material (right) that is different in appearance than the commercial dry powder *Isochrysis* (left).

Figure 3: Comparison of *Isochrysis* and soybean biodiesel fuels. Green *Isochrysis* biodiesel (middle) is produced by esterification of extracted and purified free fatty acids. Decolorization produces a product (right) with similar properties to commercial biodiesel (left).

DISCUSSION:

Isochrysis is one of only a select number of algal species farmed industrially, harvested as a primary component of shellfish feed, and therefore representative of the scale necessary for biofuel production. The availability of the algae utilized and standard methods employed in this study, make the protocol presented widely accessible to other groups for further investigations. Critical steps include air-drying the algae (as opposed to lyophilizing[33]), solvent extractions, saponification, and esterification. Through these operations one can examine yields of lipids and other coproducts from the various *Isochrysis* available [30]. It is anticipated that these may differ as a result of differing strains and cultivation methods,[31] and may also be impacted by the nature of the product and any additional processing (e.g. drying or freezing) utilized by the supplier. As we demonstrate here, the protocol developed can be successfully applied to different types of *Isochrysis* products, ranging from a wet paste to a dry milled powder. Yields of biodiesel were however lower from the powdered biomass (7% w/w dry biomass vs. 12% w/w from the dried paste), which corresponds with lower amounts of algal oil (h-AO) extracted. This may suggest that an alternative extraction protocol[32] other than a Soxhlet apparatus may be better suited for dry powdered *Isochrysis* products. The *Isochrysis* powder used in this study is advertised as containing 23-25% lipids, which similar to what we have experimentally obtained from dried *Isochrysis* paste.[33,34,26]

Despite the different colors of the starting dry biomass, the *Iso*-paste-hAO and *Iso*-powder-hAO were essentially indistinguishable, both dark green/near black solids with melting points of approximately 50 °C. Interestingly, the ratio of FFAs to neutral lipids within the two hexane extracts was different. After saponification and separation of the neutral lipids, we obtained 60% (w/w) FFAs and 40% (w/w) neutral lipids from the *Iso*-paste-hAO. The *Iso*-powder-hAO produced on average 46% (w/w) FFAs and 54% (w/w) neutral lipids. The results suggest that either the starting powdered biomass may contain higher amounts of neutral lipids relative to FA derivatives than the *Isochrysis* paste, or that Soxhlet extraction of powdered *Isochrysis* is somewhat selective for neutral lipids.

Not only were the yields of products obtained from the two commercial *Isochrysis* biomasses different, but also the fatty acid profiles of the resulting biodiesel. This is important, as the fuel properties of a biodiesel are directly dependent on the nature and contents of individual FAMEs.[35] To be commercialized, all biodiesel must conform to the standards described in the documents ASTM D6751 or EN 14214 in the U.S or Europe respectively. Specifications include ranges for lubricity and kinematic viscosity, and minimum values for cetane number and oxidative stability. Other important recommendations are related to cold flow properties in the form of a cloud point (CP) or cold filter plugging point (CFPP). We have previously reported results from the comprehensive fuel testing of biodiesel prepared from *Iso*-paste.[36] Since the FAME profile of biodiesel produced from the *Iso*-powder in this study is similar to those previously tested, we can predict certain fuel properties to be similar for both biodiesel fuels. For instance, polyunsaturated fatty acids (PuFAMEs, more than two double bonds) account for approximately 40% of both FAME mixtures (35.2% and 39.9%, Table 2). This will result in poor oxidative stability and favorable cold-flow.[35] There are, however, slight differences in the FAME profiles of the two biodiesel fuels. Biodiesel produced from the powdered *Isochrysis* contained higher amounts of 14:0 (19.4 mg/g vs. 16.4 mg/g), 18:3 (13.5 mg/g vs. 8.5 mg/g), and 22:6 (11.0 mg/g vs. 6.9 mg/g) FAMEs, yet lower amounts of 18:4 (10.4 mg/g vs. 19.8 mg/g). The extent of the impact of these differences on the various fuel properties contained in the ASTM standards remains to be investigated.

The initial biodiesel obtained from both commercial *Isochrysis* algae were similarly dark green in color which can be explained by the presence of chlorophylls.[36] Chlorophyll and its derivatives have been reported to have a negative effect on the stability of vegetable oils and their corresponding biodiesel fuels.[36,29] Based on the method of Issariyakul and Dalai for decolorizing greenseed canola oil in connection with biodiesel production,[29] stirring our green biodiesel over 10% (w/w) MK10 at 60 °C for 1 hr resulted in a dramatic reduction in pigment content by visual inspection (*ref.* Figure 2). Mass recoveries from the decolorization process were on average 90%.

Yields of purified alkenones from *Iso*-paste-hAO and *Iso*-powder-hAO neutral lipids were comparable at 40% and 46% w/w respectively (Table 1). Since neutral lipids represent a higher proportion of material contained in the *Iso*-powder-hAO (54% w/w vs. 40% w/w), alkenone yield from the *Iso*-powder-hAO exceeds the *Iso*-paste-hAO alkenone yield by approximately 10% (25% w/w vs. 16% w/w). However, considering that yields of the *Iso*-powder-hAO itself were lower than *Iso*-paste-hAO (15% vs. 20% w/w), overall yields of alkenones from both dry

Isochrysis biomasses are more similar (0.2 x 0.4 x 0.4 = 3.2% w/w from dried *Isochrysis* paste and 0.15 x 0.54 x 0.46 = 3.7% from *Isochrysis* powder).

ACKNOWLEDGMENTS:

This work was supported by the National Science Foundation (CHE-1151492), the Northwest Advanced Renewables Alliance (fellowship to J. Wilson-Peltier), and through a private donation from friends of WHOI. We thank Kevin R. Steidley and Kim Ascherl (USDA / ARS / NCAUR) for excellent technical assistance.

DISCLOSURES:

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

REFERENCES:

1. Ahmad, A.L., Mat Yasin, N.H., Derek, C.J.C., Lim, J.K. Microalgae as a sustainable energy source for biodiesel production: A review. *Renew. Sustain. Energy Rev.* **15**(1), 584–593, DOI: 10.1016/j.rser.2010.09.018 (2011).
2. Vardona, D. R., Sharmab, B.K., Blazinaa, G.,V., Rajagopalanb, K., Strathmann, T.J. Thermochemical conversion of raw and defatted algal biomass via hydrothermal liquefaction and slow pyrolysis. *Bioresour. Technol.* **109**, 178–187, DOI: 10.1016/j.biortech.2012.01.008 (2012).
3. Tenenbaum, D.J. Food vs fuel: Diversion of crops could cause more hunger. *Environ. Health Perspect.* **116**, A254–A257, PMCID: PMC2430252 (2008).
4. Pienkos, D.T., Darzins, A. The promise and challenges of microalgal-derived biofuels. *Biofuels, Bioprod. Biorefin*. **3**(4), 431-440, DOI: 10.1002/bbb.159 (2009).
5. U.S First Crude Oil Purchase Price. *U.S Energy Information Administration*. http://www.eia.gov/dnav/pet/hist/LeafHandler.ashx?n=PET&s=F000000__3&f=A (accessed September 30, 2015).
6. Sheehan, J., Dunahay, T., Sheehan, J., Dunahay, T., Benemann, J., Roessler, P.A. A look back at the U.S. Department of Energy's Aquatic Species Program: Biodiesel from algae; National Renewable Energy Laboratory, report NREL/TP-580-24190; 1996.
7. Petkov, G., Ivanova, A., Iliev, I., Vaseva, I. A critical look at the microalgae biodiesel. *Eur. J. Lipid Sci.* **114**(2), 103-111, DOI: 10.1002/ejlt.201100234 (2012).
8. van Beilen, J.B. Why microalgal biofuels won't save the internal combustion engine. *Biofuels, Bioprod. Biorefin*. **4**, 41-52, DOI: 10.1002/bbb (2010).
9. Chisti, Y. Biodiesel from microalgae. *Biotechnol. Adv.* **25**(3), 294–306, (2007).
10. Wijffles, R.H., Barbosa, M.J. An outlook on microalgal biofuels. *Science* **329**, 796-799, DOI: 10.1126/science.1189003 (2010).
11. Ferrell, J. Sarisky-Reed, V. *National Algal Biofuels Technology Roadmap* (Eds. D. Fishman, R. Majumdar, J. Morello, R. Pate, J. Yang), United States Department of Energy, 2010, http://www1.eere.energy.gov/bioenergy/pdfs/algal_biofuels_roadmap.pdf (accessed August 24, 2015).
12. Patterson, G.W., Tsitsa-Tsardis, E., Wikfors, G.H., Gladu, P.K., Chitwood, D.J., Harrison, D.

Sterols and alkenones of *Isochrysis*. *Phytochem.* **35**(5), 1233-1236, DOI: 10.1016/S0031-9422(00)94826-X (1994).

13. Volkman, J.K., Eglinton, G., Corner, E.D.S. Long-chain alkenes and alkenones in the marine coccolithophorid *Emiliania huxleyi*. *Phytochem.* **19**, 2619-2622, DOI: 10.1016/S0031-9422(00)83930-8 (1980).

14. Conte, M.H., Thompson, A., Lesley, D., Harris, R.P. Genetic and physiological influences on the alkenone/alkenoate versus growth temperature relationship in *Emiliania huxleyi* and *Gephyrocapsa Oceanica*. *Geochim. Cosmochim. Acta* **62**(1), 51-68, DOI: 10.1016/S0016-7037(97)00327-X (1998).

15. Brassell, S.C., Eglinton, G., Marlowe, I.T., Pflaumann, U., Sarnthein, M. Molecular stratigraphy: a new tool for climatic assessment. *Nature* **320**, 129-133, DOI: 10.1038/320129a0 (1986).

16. Marlowe, I.T., Brassell, S.C., Eglinton, G., Green, J.C. Long chain unsaturated ketones and esters in living algae and marine sediments. *Org. Geochem.* **6**, 135-141, DOI: 10.1016/0146-6380(84)90034-2 (1984).

17. Prahl, F.G., Wakeham, S.G. Calibration of unsaturation patterns in long-chain ketone compositions for palaeotemperature assessment. *Nature* **330**, 367-369, DOI: 10.1038/330367a0 (1987).

18. Eglinton, G., Bradshaw, S.A., Rosell, A., Sarnthein, M., Pflaumann, U., Tiedemann, R. Molecular record of secular sea surface temperature changes on 100-year timescales for glacial terminations I, II and IV. *Nature* **356**, 423-426, DOI: 10.1038/356423a0 (1992).

19. Müller, P.J., Kirst, G., Ruhland, G., von Storch, I., Rosell-Melé, A. Calibration of the alkenone paleotemperature index U37K′ based on core-tops from the eastern South Atlantic and the global ocean (60°N-60°S). *Geochim. Cosmochim. Acta* **62**(10), 1757 – 1772, DOI: 10.1016/S0016-7037(98)00097-0 (1998).

20. Volkman, J.K., Barrerr, S.M., Blackburn, S.I., Sikes, E.L. Alkenones in *Gephyrocapsa oceanica*: Implications for studies of paleoclimate. *Geochim. Cosmochim. Acta*. **59**(3), 513-520, DOI: 10.1016/0016-7037(95)00325-T (1995).

21. Eltgroth, M.L., Watwood, R.L., Wolfe, G.V. Production and cellular localization of neutral long-chain lipids in the haptophyte algae *Isochrysis Galbana* and *Emiliania Huxleyi. J. Phycol.* **41**, 1000-1009, DOI: 10.1111/j.1529-8817.2005.00128.x (2005).

22. Volkman, J.K., Everitt, D.A., Allen, D.I. Some analyses of lipid classes in marine organisms, sediments and seawater using thin-layer chromatography—flame ionisation detection. *J. Chromatogr. A.* **356**, 147-162, DOI: 10.1016/S0021-9673(00)91474-2 (1986).

23. Epstein, B. L.; D'Hondt, S.; Quinn, J. G.; Zhang, J.; Hargraves, P. An effect of dissolved nutrient concentrations on alkenone-based temperature estimates. *Paleoceanography.* **13**(2), 122–126, DOI: 10.1029/97PA03358 (1998).

24. Prahl, F.G., Sparrow, M.A., Wolfe, G.V. Physiological impacts on alkenone paleothermometry. *Paleoceanogaphy* **18**(2), 1025–1031, DOI: 10.1029/2002PA000803 (2003).

25. Sachs, D., Sachs, J.P. Inverse relationship between D/H fractionation in cyanobacterial lipids and salinity in Christmas Island saline ponds. *Geochim. Cosmochim. Acta*. **72**(25), 793-806, DOI: 10.1016/j.gca.2007.11.022 (2008).

26. O'Neil, G.W., Culler, A.R., Williams, J.R., Burlow, N.P., Gilbert, G.J., Carmichael, C.A., Nelson, R.K., Swarthout, R.F., Reddy, C.M. Production of jet fuel range hydrocarbons as a

coproduct of algal biodiesel by butenolysis of long-chain alkenones. *Energy Fuels.* **29**(2), 922-930, DOI: 10.1021/ef502617z (2015).

27. Foley, P.M., Beach, E.S., Zimmerman, J.B. Algae as a source of renewable chemicals: opportunities and challenges. *Green Chem.* **13**, 1399-1405, DOI: 10.1039/C1GC00015B (2011).

28. Razon, L.F., Bacani, FT., Evangelista, R.L., Knothe, G. Fatty acid profile of kenaf seed oil. *J. Am. Oil Chem. Soc*. **90**(6), 835-840, DOI: 10.1007/s11746-013-2230-3 (2013).

29. Issariyakul T., Dalai AK. Biodiesel production from greenseed canola oil. *Energy Fuels* **24**, 4652-4658, DOI: 10.1021/ef901202b (2010).

30. Nalder, T.D., Miller, M.R., Packer, M.A. Changes in lipid class content and composition of *Isochrysis* sp. (T-Iso) grown in batch culture. *Aquacult. Int.* **23**, 1293-1312, DOI: 10.1007/s10499-015-9884-9 (2015).

31. Mercer, P., Armenta, R.E., Developments in oil extraction from microalgae. *Eur. J. Lipid Sci. Technol.* **113**(5), 539-547, DOI: 10.1002/ejlt.201000455 (2011).

32. O'Neil, G.W., Carmichael, C.A., Goepfert, T.J., Fulton, J.M., Knothe, G., Lau, C.P-L., Lindell, S. R., Mohammady, N.G-E., Van Mooy, B.A.S., Reddy, C.M. Beyond fatty acid methyl esters: expanding the renewable carbon profile with alkenones from *Isochrysis* sp. *Energy Fuels*. **26**, 2434-2441, DOI: 10.1021/ef3001828 (2012).

33. O'Neil, G.W., Knothe, G., Williams, J.R., Burlow, N.P., Culler, A.R., Corliss, J.M., Carmichael, C.A., Reddy, C.M. Synthesis and analysis of an alkenone-free biodiesel from *Isochrysis* sp. *Energy Fuels* **28**(4), 2677-2683, DOI: 10.1021/ef500246h (2014).

34. Knothe, G. Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. *Fuel Process. Technol.* **86**, 1059-1070, DOI: 10.1016/j.fuproc.2004.11.002 (2005).

35. Valenzuela-Espinoza E., Millán-Núñez R., Núñez-Cebrero F. Protein, carbohydrate, lipid and chlorophyll a content in Isochrysis aff. galbana (clone T-Iso) cultured with a low cost alternative to the f/2 medium. *Aquacult. Eng.* **25**, 207-216, DOI: 10.1016/S0144-8609(01)00084-X (2002).

36. Kulkarni M.G., Dalai A.K., Bakshi N.N. Utilization of green seed canola oil for biodiesel production. *J. Chem. Technol. Biotechnol.* **81**, 1886–1893, DOI: 10.1002/jctb.1621 (2006).

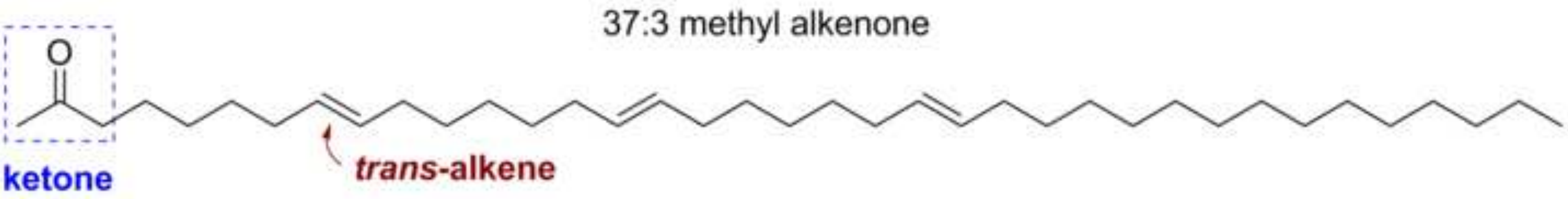
37:3 methyl alkenone
O
ketone
trans-alkene

Figure 3

Table 1. Product yields from commercial *Isochrysis* biomass.

Product (g)	***Iso*- paste**	***Iso*- powder-1**	***Iso*- powder-2**
Dry Biomass	30	20	20
Hexane Algal Oil	5.86	2.87	3.11
FFAs	3.52	1.34	1.38
Neutral lipids	2.34	1.38	1.61
Alkenones	0.94	0.63	0.74

Notes for table: *Iso*- powder-1 and *Iso* -powder-2 represent results from two samples of powdered *Isochrysis* that were processed in parallel. For other reports with yields of these products from *Iso*- paste see references 26, 32, and 33.

Table 2. FAME composition of biodiesel produced from commercial *Iso* -paste and *Iso-* pow *Isochrysis* biomass.

FAME[A]	***Iso-* paste**	***Iso-* powder**
14:00	16.4	19.4
14:01	-	0.3
15:00	trace	0.3
16:00	10.1	8.8
16:1 Δ9	7.6	5.5
16:02	ND	0.3
16:03	ND	0.5
18:00	Trace	0.2
18:1[B]	12.1	14.3
18:02	8.1	7.1
18:3[C]	8.5	13.5
18:04	19.8	10.4
18:05	ND	3
20:05	ND	-
22:05	ND	2
22:06	6.9	11
Σ[D]	89.8	96.2

Notes for Table: [A]Fatty acid nomenclature is #carbons:#*cis*- double bonds. [B]Combined 18:1 Δ9 + 18:1 Δ11. [C]Combined Δ6,9,12 and Δ9,12,15 isomers. [D]The remaining material is roughly 50:50 other FAMEs and non-FAME components (Total ~95% FAME). ND = Not detected

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Isochrysis	Reed Mariculture	Iso, Raw, Unprocessed, 20%dw	Live culture paste, 20% w/w biomass
Isochrysis	Necton, S.A	AADISS004	PhytoBloom Prof Isochrysis (Freeze-dried)
Hexanes	Fisher Chemical	H291-4	ACS Certified
Methanol	Fisher Chemical	A452-4	HPLC Grade
Dichloromethane	Fisher Chemical	D37-4	Certified/Stabilized
Soxhlet Apparatus	Sigma Aldrich	64826	
Extraction Thimble	Sigma Aldrich	64842	
Büchner Funnel	Chemglass	CG-1406-25	
High Pressure Reaction Vessel	Chemglass	CG-1880-12	
Whatman Filter Paper	GE Life Sciences	1442-042	Grade 42, Ash 0.007%, circle, 42.5 mm
Biodiesel (B100)	Bellingham Shell		The biodiesel (B100) in Figure 3 was purchased at a local filling stati
Isochrysis	Aquacave		In addition to Reed and Necton, *Isochrysis* can also be purchased fr
Isochrysis	Brine Shrimp Direct		*Isochrysis* can also be purchased from Brine Shrimp Direct (Ogden,

›n: Bellingham Shell, Bellingham, WA 98226
›m (Aquacave. (Gurnee, IL) at: www.aquacave.com (accessed September 30, 2015).

JT) at: www.brineshrimpdirect.com (accessed September 30, 2015).

JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Experimental Protocol for Biodiesel Production with Isolation . . .

Author(s): O'Neil, Gregory W. et. al

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via: ☐ Standard Access ☒ Open Access

Item 2 (check one box):

☒ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name: Greg O'Neil
Department: Chemistry
Institution: Western Washington University
Article Title: Experimental Protocol for Biodiesel Production with Isolation...
Signature:
Date: 10/1/2015

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

WESTERN
WASHINGTON UNIVERSITY
CHEMISTRY DEPARTMENT

Active Minds Changing Lives

516 High Street
Bellingham, Washington 98225-9150
(360) 650-3070 □ Fax (360) 650-2826

From: Gregory O'Neil
To: Nam Nguyen, Science Editor, JOVE
Date: January 21, 2016

Editorial and reviewer comments are appreciated and have been addressed in the revised manuscript as outlined below:

<u>Editorial</u>:
1. The document has been thoroughly proofread
2. All abbreviations have been defined at their first use within each section (e.g. FFAs in the Long Abstract, Protocol (Step 3.10), and Results sections)
2.1. Soxhlet specifications in Protocol Step 2.1 have been removed as requested.
2.2. The description of fatty acid quantitation (Protocol Step 4.6) is a note in the references section as requested (reference #29).
3. Protocol Steps 3.5-3.8 and 4.5 are highlighted as suggested.
4.1. Additional detail has been added to Protocol Steps 4.1 and 5.5 to make clear how the solvents are introduced and their purpose.
4.2. A reference to Protocol Step 3.8 has been added to Step 6.1 to remind the reader of when the neutral lipids were obtained.
5. The names Reed and Necton now only appear as a note in the Protocol section.
6. Possibilites for future work using the protocol described are givenin the first paragraph of the Discussion (Lines 325 – 334). These include analyzing yields from various Isochrysis strains and commercial products and other extraction protocols.

<u>Reviewer #1</u>:
1. **Comment**: "It will also be interesting to see a small paragraph to explain what has been done and what was the need for this protocol." **Response**: A few sentences have been added (Lines 102-106) to explain the purpose for separating alkenones and fatty acids with reference to our previous work.
2. **Comment**: Request to "prove the consistency of the results from this protocol." **Response**: Wording has been added with reference to our three previous articles to demonstrate the consistency of yields from Reed *Isochrysis*. Table 1 shows duplicate results obtained from Necton *Isochrysis* (*Iso*-powder-1 and *Iso*-powder-2) and references our prior work as proof of consistency.
3. **Comment**: "Mention the moisture content after 24 h." **Response**: The moisture content after 24 h. varies depending on the actual "room temperature". A reference has been added (#40) to clarify.

We hope that you will find this revised manuscript worthy of publication in your prestigious journal.

Sincerely,

Dr. Gregory W. O'Neil
Associate Professor of Chemistry

Western Washington University
516 High Street, MS 9150
Bellingham, WA, 98225-9150
Tel. +01.360.650.6283
Fax +01.360.650.2826
oneil@chem.wwu.edu