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Microplate assay for quantitation of neutral lipids in extracts from microalgae

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Abstract

Lipid quantitation is widespread in the algae literature but popular methods including gravimetry, GC-MS, and Nile red cell staining suffer drawbacks including poor quantitation of neutral lipids, expensive equipment, and variable results among algae species, respectively. A high-throughput microplate assay was developed that utilizes Nile red dye to quantify neutral lipids that have been extracted from algae cells. Because the algal extracts contained pigments that quenched Nile red fluorescence, a mild bleach solution was used to destroy pigments, resulting in a nearly linear response for lipid quantities in the range of 0.75-40 μg. Corn oil was used as a standard for quantitation, although other vegetable oils displayed a similar response. The assay was tested on lipids extracted from three species of *Chlorella* and resulted in close agreement to TAG levels determined by thin layer chromatography. The assay was found to more accurately measure algal lipids conducive to biodiesel production and nutrition applications than the widely-used gravimetric assay. Assay response was also consistent among different species, in contrast to Nile red cell staining procedures.

Key Words

Neutral lipid assay, Nile red, *Chlorella spp*., GC-MS, gravimetric, TLC, bleach

Introduction

Microalgae have attracted interest as a biofuel feedstock due in part to their ability to grow rapidly and accumulate neutral lipids which can be converted to biodiesel [1]. Algal lipids may also prove valuable as nutrition supplements due to the high omega-3 fatty acid content of many species [2]. Moreover, triglycerides (TAG) can form the building blocks of biodegradable polymeric biomaterials [3]. Accurate quantitation of algal neutral lipids is therefore of great interest for several industrial applications.

Multiple methods have been used to quantify microalgal lipids, including gravimetric methods, thin layer chromatography (TLC), the sulfo-phospho-vanillin (SPV) assay, gas chromatography and mass spectrometry (GC-MS), and Nile red (NR) cell staining. Among these, the gravimetric method is perhaps the most widely used for algal lipid quantitation and is frequently used as a benchmark when validating alternative methods [4; 5; 6]. In this method, lipids are extracted by a solvent mixture (typically chloroform and methanol) [7; 8], the solvent is evaporated, and the residual crude extract is weighed. This method requires a fairly large sample size (~20 mg algae dry weight) and can be time consuming. Furthermore, the assay measures all material extracted by the organic solvent, not just neutral lipids. Polar lipids can comprise 14% to 98% of total lipids in microalgae depending on the strain and growth conditions [9; 10; 11] indicating that neutral lipid content cannot be inferred from total lipid measurements.

Thin layer chromatography can provide additional information about lipid classes (e.g. neutral, polar) present in algal lipid extracts [12]. For example, Vieler et al. determined relative abundance of various lipid classes present in *Chlamydomonas reinhardtii* and *Cyclotella meneghiniana* using TLC [13]. The method can be quantitative when combined with image analysis software and standards. Zhang et al. used TLC to

quantify neutral lipids in *Chlorella sorokiniana* C3 using glycerol trioleate as the standard [14]. They found that lipid content based on TLC corresponded to an increase in intracellular neutral lipid bodies based on fluorescence microscopy and flow cytometry using bodipy staining. In contrast to microplate assays their method was not high-throughput given limited TLC lane space, however.

The sulfo-phospho-vanillin lipid assay has been used to quantify lipids in a variety of samples [15] and was adapted for quantitation of microalgal lipids by Cheng et al. [4]. This method can be used to quantify lipid samples in the range of zero to 60 μg and is compatible with the high-throughput microplate format. This assay is sensitive to the degree of oil saturation, with higher degrees of unsaturation corresponding to a stronger signal [4; 16]. Thus, the SPV assay can give misleading results when the degree of oil saturation changes based on experimental treatments. Incubation time, heating and cooling also appear to have a strong impact on assay results [4].

Gas chromatography and mass spectrometry of fatty acid methyl esters (FAME) derived from lipids is a widely used method for algal lipid analysis [17; 18; 19]. With the use of an internal standard, this method can provide accurate quantitation of fatty acids present in a sample. However, this level of detail may not be necessary when the goal is neutral lipid quantitation in algae. Furthermore, GC-MS methods can be time-consuming and expensive since they require derivatization, analytical standards, and expensive instrumentation [20]. Likewise, time-domain nuclear magnetic resonance has been used to quantify lipid content in *Chlorella protothecoides* [21], but equipment requirements could prohibit wide-spread use.

Nile red is a lipophilic dye that has frequently been used to assess relative lipid levels in various strains of algae, yeast, fungi, and mammalian cells [6; 22]. The method involves incubating live cells in the dye (often in conjunction with a solvent) and then reading fluorescence with a spectrophotometer [5; 23]. The dye must first penetrate the cell structure and diffuse into lipid droplets where it fluoresces in the non-polar environment [24]. This method is rapid and can be integrated into routine measurements of optical density, thereby tracking both growth and lipid levels through different stages of cultivation. Nile red can also be used to visualize lipid droplets using fluorescence microscopy. The key shortcoming of Nile red cell staining is the variable dye affinity among algae strains [25]. Cell wall composition can vary significantly among algae species, even within the genus *Chlorella* [26], and may play a role in variable dye penetration [23]. Chen et al. attempted to overcome the challenge of variable dye penetration in microalgae by adding DMSO as a solvent followed by heating of the assay [5]. They successfully demonstrated improved dye penetration but validation of the method was only reported for a single algae species: *Chlorella vulgaris*. Due to these shortcomings, absolute quantitation of lipid and comparison among algae species cannot be accomplished without a pre-established correlation between fluorescence and lipid levels. An assay capable of absolute lipid quantitation is therefore needed to establish this relationship *a priori.*

In addition to live algae cells, Nile red has been used to stain other types of lipid samples. Fowler et al. used Nile red to stain lipid bands obtained through TLC [27]. Nile red has also been used to determine lipid content in wheat germ and commercial

vegetable oils [28]. However, to date there are no reports in which Nile red dye has been used to quantify lipids extracted from algae.

A method of lipid quantitation was developed in which Nile red dye is used to quantify microgram quantities of neutral lipid present in crude algal lipid extracts. By assaying lipids after extraction, differences in cell structure do not affect dye penetration into lipid droplets. The method was tested on extracts from three algae strains: *Chlorella minutissima* (UTEX 2341), *Chlorella protothecoides* (CCAP 211/10C), and *Chlorella sorokiniana* (UTEX 2805) under a variety of culture conditions. The method was then compared to lipid levels determined by gravimetric, GC-MS, and TLC analyses and found to be a viable approach for quantifying neutral lipids in algal extracts. In contrast to total crude lipid, neutral lipids are of particular interest for biodiesel production and nutrition applications [10].

Materials and Methods

Algae cultivation

Algae were cultivated in Pyrex bottles, mixed by magnetic stir bar. Culture volumes were in the range of 400-600 ml; cultures were supplied with an airflow rate of half the culture volume per minute. Two percent (v/v) CO_2 was mixed with the ambient air. Cultures were grown on N8-NH_4 medium (see supplement) with or without ammonium chloride in order to instigate nitrogen stress responses. Lipid extracts from mixotrophic co-cultures of *C. minutissima* and *E. coli* were also analyzed since these cultures can exhibit very high lipid contents [29]. The co-cultures were grown on N8-NH_4 medium supplemented with 10 g/L glucose under varying levels of CO_2 (0.4% and 2%) and ammonium chloride (200 mg N/L and 400 mg N/L). Illumination (10,000 lux) was provided by T5 growth

lamps operating on a 16:8 hour light-dark cycle. Cultures were harvested by centrifugation at 5,000 g and the pellet was washed three times with dH_2O to remove residual salts. Harvested algae were freeze-dried at -45 °C (Freezone4.5, Labconco, Kansas City, MO).

Lipid extraction and gravimetric analysis

Approximately 20 mg of freeze-dried algae were suspended in 1.5 ml Folch solvent (2:1 chloroform-methanol) and disrupted by bead beating [7]. Each sample was disrupted over the course of six 20-second beating intervals at 6 m/s using a FastPrep FP120 (Savant Instruments, Holbrook, NY) with 0.5 mm zirconia/silica beads (BioSpec Products, Bartlesville, OK). Beads were removed by filtration through wire mesh and washed with an additional 4.5 ml Folch solvent. Phase separation was achieved by addition of 1.2 ml 0.9% NaCl solution, centrifuged at 6,000 g, and the volume of the bottom (chloroform) phase was recorded. The bottom phase was transferred to a glass vial for storage at -20 °C.

Aliquots of the extract were transferred to pre-weighed 1.5 ml polypropylene micro-centrifuge tubes, dried under a stream of filtered air for several hours, and weighed twice on a five-digit analytical balance (Denver Instruments, Bohemia, NY). Tubes were re-weighed at a later time point to verify complete solvent evaporation. The analysis was repeated for eight samples in which aluminum weighing dishes were used in place of plastic tubes and samples were evaporated overnight without supplemental air-flow. The two methods resulted in a mean difference of 1.3% of the lipid mass ($p = 0.384$, t-test) and were treated as statistically equivalent.

Transesterification and GC-MS

A subset of the lipid extracts was transesterified and analyzed by GC-MS using a modified version of the procedure by Xu et al. [30]. 250 μl of extract were transferred to an 8 ml glass tube with a Teflon-lined lid. 50 μl of 1 mg/ml nonadecanoic acid in chloroform was added to each tube as an internal standard. The chloroform was evaporated under a stream of filtered air and the crude lipid was re-suspended in 500 μl hexane. Analytical corn oil (Sigma Aldrich) and commercial-grade canola, corn, olive, and coconut oils were prepared using the same procedure.

Two ml of 1 M methanolic HCl (Sigma Aldrich, St. Louis, MO) were added to each sample and vortexed to mix. The reaction was incubated for 24 hours at room temperature in the dark. The reaction was stopped by the addition of 2 ml of 6 g/L sodium carbonate solution. Vials were swirled by hand and allowed to sit for ~5 minutes until phase separation was achieved. The hexane layer was transferred to a separate glass vial and stored at -20 °C until GC analysis. An additional quantitative mixed FAME standard (Sigma Aldrich) was run alongside the samples in order to calculate response factors for key fatty acids (palmitic, stearic, oleic, linolenic, and arachidic).

An HP 6890 GC-MS equipped with a J&W DB-23 column was used to analyze fatty acid composition and quantity. Helium was used as the carrier gas with a column flow rate of 1.0 ml/min. One μl injection volume was used with splitless injection. The inlet temperature was 250 °C and the column oven was programmed as follows: 120 °C for 4 minutes, ramp to 198 °C at 1.5 °C/min and hold at 198 °C for 4 minutes.

Thin layer chromatography

Thin layer chromatography (TLC) was performed on algal crude lipid extracts using silica gel plates (Analtech, Sigma Aldrich, Saint Louis, MO) based on the procedure by Liu et al. with modifications [31]. Corn oil dissolved in chloroform was used as a standard. A solvent mixture of 80:20:1 hexane/diethyl ether/acetic acid was used for lipid separation. After drying, the plate was sprayed with 0.05% primulin dye dissolved in 80:20 acetone/water [32]. The plate was illuminated with UV light and photographed using an Alpha Imager 2200 (Alpha Innotech Corp., San Leandro, CA). ImageJ (NIH) was used to integrate signal intensity in the bands that corresponded to triacylglycerol.

Nile red lipid assay

Technical grade Nile red dye was purchased from Sigma Aldrich and dissolved in ACS grade DMSO (Sigma Aldrich) to achieve a concentration of 200 μg/ml. This stock solution was then diluted in dH_2O prior to each assay to achieve the desired concentration. Commercial grade bleach (6% sodium hypochlorite, The Clorox Company, Oakland, CA) was obtained from local markets. Polypropylene (PP) 96-well flat-bottom microplates were purchased from Greiner Bio-One.

The Nile red lipid assay was performed on algal lipid extracts suspended in chloroform. The general steps of the assay were to 1) add extracts to a microplate and evaporate the solvent, 2) re-suspend lipids in 25 μl isopropyl alcohol (IPA), 3) add 200 μl Nile red solution (diluted in dH_2O) to the suspensions, 4) add 20 μl bleach solution to destroy pigments, 5) incubate the assay, and 6) read fluorescence. The effects of a number of variables on the assay were assessed. The concentrations of Nile red and bleach used in the assay were varied in an effort to achieve maximum fluorescence. Nile

red concentrations were tested in the range of 0.25-8 μg/ml. The addition of 20 μl bleach solution was tested at 1.5%, 3%, and 6% (w/v) sodium hypochlorite concentration. The order of addition of Nile red and bleach was also examined. Direct addition of chloroform extract to the microplate was compared to extracts that were first diluted with two parts methanol. Finally, the effect of incubation time on the fluorescence signal was determined. In addition to lipid extracts from three algae species, the assay was tested on corn, canola, olive and coconut oils. It was also tested using a phospholipid mixture (Avanti Polar Lipids, Alabaster, AL) and a cholesterol standard (MP Biomedicals, Solon, OH). Fluorescence wavelength selection was also investigated in order to ensure measurement of neutral lipids.

Optimized Nile red lipid assay procedure

Algal extracts, suspended in chloroform, were diluted with two parts ACS grade methanol (Fisher) and mixed by pipetting up and down. Dilutions were prepared in 1.5 ml polypropylene micro-centrifuge tubes and 80 μl of each diluted sample was applied to the microplate in quadruplicate. Commercial grade corn oil was dissolved in 2:1 methanol/chloroform and applied to the microplate to achieve a range of 0-40 μg oil per well.

The plate was incubated for 30 minutes on a dry heating block (Isotemp 125D, Thermo Fisher Scientific Inc.) set to 55 °C to evaporate the solvent. The plate was allowed to cool to room temperature prior to addition of 25 μl of IPA to re-dissolve lipids. Then, 200 μl of 0.5 μg/ml Nile red solution in dH_2O was added. Because the stock Nile red solution was dissolved in 100% DMSO, the final Nile red solution contained 0.25% (v/v) DMSO. The well contents were mixed by pipetting up and down and the

plate was incubated at room temperature for 5 minutes. Bleach (6% sodium hypochlorite) was diluted 1:1 with dH_2O and 20 μl of the resulting solution was applied to each well (including corn oil standards). Contents again were mixed by pipetting up and down and the microplate was incubated at room temperature for ~30 minutes.

Fluorescence was determined using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA) with excitation set to 530 nm and emission to 575 nm with a 570 nm cutoff. The plate was read every 5-10 minutes for ~20 minutes or until a peak in fluorescence was observed in wells containing algal samples. The first reading after the fluorescence peak was used for quantitation.

Nile red cell assay

Fresh algae cells were also stained with Nile red solution in order to provide comparison with the other lipid methods. Fresh algae culture (200 μl, OD550 in range of 0.35-1.0 AU) was added to a polystyrene microplate followed by 50 μl of 15 μg/ml Nile red solution. The 15 μg/ml Nile red solution was prepared by diluting 200 μg/ml Nile red (in DMSO) with dH_2O. The microplate was incubated at room temperature for 10 minutes and then fluorescence was read at 530 nm excitation, 575 nm emission with a 570 nm cutoff [5]. The fluorescence readings were normalized by dividing by the optical density at 550 nm. This procedure was established after some optimization in which Nile red concentration was varied. Efforts were made to improve dye penetration into algal cells by addition of DMSO and heating, as described by Chen et al. [5]. However, these variations did not lead to any change in fluorescence signal for *C. minutissima* or *C. sorokiniana* and were not employed in the final method.

Data analysis

Linear regression and t-tests were performed using Microsoft Excel and differences were considered significant at $p<0.05$. ImageJ was used to outline TLC bands and determine white pixel intensity. Background intensity was subtracted from an area adjacent to the band. Band area was then multiplied by the corrected pixel intensity to give a raw score that could be correlated to TAG levels. The resulting standard curve for varying corn oil levels is provided in Figure S1 and exhibits a strong correlation ($R^2 > 0.99$) using a quadratic fit.

Results and Discussion

Development of assay concept

Vegetable oils and algal lipid extracts, dissolved in chloroform, were applied to a PP microplate and the solvent was evaporated. Addition of 200 μl of 0.5 μg/ml Nile red solution to corn oil resulted in strong fluorescence. When Nile red solution was applied to algal lipid extracts after solvent evaporation, no fluorescent signal was detected. This implied that something present in the algal extracts was either preventing the formation of lipid droplets or quenching the signal. This was confirmed when 20 μl of algal lipid extract was added to known quantities of corn oil and resulted in no signal (Figure 1a).

To determine if pigment quenching interfered with Nile red fluorescence, green food coloring (tartrazine and brilliant blue FCF) was added to corn oil suspended in 2:1 methanol-chloroform, the solvent was evaporated, and the Nile red solution added. As with the algal extracts, the food coloring quenched the fluorescent signal unless bleach was added. Nearly identical fluorescence was detected for corn oil with and without green food coloring so long as bleach was present (Figure 1b)

Pigments present in algal lipid extracts were likewise found to quench fluorescence. Addition of 20 μl of 3% sodium hypochlorite bleach solution after the addition of Nile red solution enhanced fluorescence which increased over time up to approximately 30-40 minutes. Moreover, the resulting signal was linear with respect to quantity of extract added to the microplate well (Figure 2). Visually, the green hue of the algal lipid extract turned a pale yellow that faded over the course of 30-40 minutes. The order of Nile red and bleach addition was also important for the assay. Addition of bleach before Nile red resulted in a non-linear response and lower signal intensity (<100 FU for 40 μg of corn oil per well) than the >800 FU observed in the optimized assay. This suggested that Nile red must first penetrate the lipid droplets prior to bleach addition.

Wavelength selection

Excitation at 530 nm and emission at 575 nm were selected based on testing of corn oil, algal lipid, and polar lipid samples after Nile red and bleach addition. Corn oil, which is composed primarily of TAG, exhibited peak fluorescence at 580 nm whereas the polar lipid standard had a fluorescence peak at 620 nm (Figure 3). These results are comparable to De la Hoz Siegler et al. who observed 590 and 645 nm for neutral and polar algal lipids, respectively [33]. The emission peak for extract from *C. minutissima* was close to that of corn oil.

Solvent selection and evaporation method

Initially, algal extracts in chloroform were applied directly to the microplate at 0, 10, 20, 30, and 40 μl to assess assay linearity. Chloroform blanks were included to control for background fluorescence and subtracted from the sample wells. These blank wells also yielded a linear response that increased with the volume of chloroform added (Figure S2).

This result suggested that chloroform was interacting with the microplate during evaporation; perhaps allowing Nile red to more easily penetrate the plastic. To reduce background fluorescence, extracts were diluted in methanol to achieve a 2:1 methanol/chloroform ratio. The volume of extract added to wells was increased three-fold to maintain the same quantity of lipid per well that had been used in previous tests. The result was a decline in background fluorescence of up to 57% (Figure S2).

Solvent evaporation was performed on a heating block set to 55 °C, below the boiling points of methanol and chloroform in order to avoid splattering of lipids onto the walls of the microplate wells. The plate was cooled to room temperature and 25 μl of IPA was added to re-dissolve lipids prior to addition of Nile red solution. Use of IPA resulted in a fluorescent signal that was more than double that achieved without IPA addition (Figure S3).

Optimization of Nile red and bleach concentrations

Initially, 200 μl of Nile red solution was added to the microplate wells with concentrations ranging from zero to 8 μg/ml. Concentrations above 2 μg/ml resulted in strong background fluorescence. Three promising Nile red concentrations were then tested: 0.25, 0.5, and 1 μg /ml, with 200 μl added per assay. The 0.5 μg/ml Nile red addition produced the strongest fluorescence intensity after subtraction of the solvent background (Figure 2). Twenty microliters of 3% sodium hypochlorite bleach solution was added in all tests. The solvent blanks rarely exceeded 35 fluorescence units when 0.5 μg/ml Nile red was used. Despite significant differences in the assay method, the optimal Nile red concentration was similar to the cell staining procedure developed by Chen et al. [5]. To test the effect of bleach concentration, 20 μl of 1.5%, 3%, or 6% sodium

hypochlorite bleach solution was added after the addition of 0.5 μg/ml Nile red solution. The strongest fluorescence was obtained with 3% sodium hypochlorite (Figure 4).

Incubation time

Incubation of the assay with bleach led to an initial increase in fluorescence in wells with algal extracts. Typically, fluorescence reached a peak between 30 and 40 minutes and declined thereafter. Fluorescence of wells containing corn oil decreased linearly over time after about 10 minutes (Figure 5). After reaching a fluorescence peak, algal samples declined linearly at approximately the same rate as the corn oil standards. These results suggest that the lipid-dye complex was destroyed at the same rate for both corn oil and algal lipids. However, the initial increase in fluorescence observed in algal samples was the result of pigment destruction during the first 30-40 minutes of incubation. This result was also apparent when food coloring was added to corn oil, initially quenching the fluorescence. Once the dye was destroyed by bleach, fluorescence of corn oil samples with and without food coloring declined in unison resulting in the final plot shown in Figure 1b. These results suggest that the optimal time to read fluorescence is after the algal samples reach peak fluorescence.

Effect of oil composition

In addition to corn oil, canola, olive, and coconut oils were tested using the optimized assay procedure. GC-MS analysis of FAME derived from each oil showed that corn and canola oil had similar fatty acid profiles, mostly oleic and linoleic fatty acids. Olive oil contained 74% oleic acid whereas coconut oil was composed of 87% short chain saturated fatty acids (C12:0 and C14:0). Given the differences in their composition, these oils were selected to determine assay response to different fatty acid profiles. For

reference, the algal fatty acid profiles were closest to those of corn and canola oils. All four vegetable oils had nearly identical responses to the assay (Figure 6). This result was expected since all oils were primarily triacylglycerol and therefore should exhibit comparable hydrophobicity. In contrast, a mixture of phospholipids obtained from soy was found to have minimal fluorescence compared to the vegetable oils. These results indicate that the Nile red assay measures primarily neutral lipids and, unlike the SPV assay, is insensitive to degree of oil saturation. The assay was also found to respond linearly to cholesterol concentration but with approximately 80% lower fluorescence intensity than the vegetable oils (Figure 6). This indicates that in addition to TAG, the assay likely provides limited detection of sterol species.

Comparison to fatty acid levels

The optimized Nile red procedure (Figure 7) was compared to GC-MS analysis of fatty acids using a common set of extracts from three *Chlorella* strains: *C. minutissima*, *C. sorokiniana*, and *C. protothecoides*. Fatty acids are the foundation of biodiesel molecules but also have value for nutrition and biomaterial synthesis, as previously mentioned. The Nile red lipid assay generally underestimated fatty acid content (negative intercept) but had a fairly strong correlation ($R^2 = 0.81$) to fatty acid content. However, the slope of the line was close to unity indicating that the Nile red assay had good sensitivity to changes in fatty acid levels. The method used for derivatization is known to esterify fatty acids from both polar lipids and neutral lipids [34]. Because the Nile red lipid assay is insensitive to polar lipids, this likely accounts for the optimized assay's underestimation of total fatty acid content.

In comparison, gravimetric assays consistently overestimated lipid content, in some cases by more than 100%, compared to fatty acid levels measured by GC-MS. This was expected because the gravimetric method quantifies all material soluble in chloroform including pigments and other moderately hydrophobic molecules. Furthermore, the gravimetric assay exhibited limited sensitivity to changes in fatty acid content as measured by GC-MS. This was apparent from the shallow slope (<0.64) of the fit line (Figure 8).

Comparison to TAG levels

The Nile red lipid assay had a strong correlation to TAG levels measured by TLC ($R^2 = 0.93$). However, the intercept was greater than zero suggesting that the Nile red assay may overestimate TAG (Figure 9). While TAG was the predominant neutral lipid as shown by TLC, additional faint bands were observed in the non-polar (upper) region of the TLC plate (Figure S4). Because of their non-polar nature, these additional lipid species may contribute to Nile red fluorescence resulting in TAG overestimation. Sterol lipids were also shown to contribute to Nile red fluorescence. Lipid levels from all three algae strains fell approximately on the same fit line indicating that the Nile red assay was not sensitive to the algae strain used. In contrast, the gravimetric assay had a poor correlation with TAG levels measured by TLC ($R^2 = 0.10$) and a nearly flat slope indicating limited sensitivity to changes in TAG level. GC-MS had a positive correlation with TAG levels ($R^2 = 0.57$) but had a larger intercept and shallower slope than the Nile red method.

C. sorokiniana presented an interesting case study for comparison of methods because each assay conveyed a different result for how lipid levels responded to nitrogen

stress (Figure 9). Gravimetric analysis showed a decline in crude lipid content under nitrogen stress (from 30% to 23% of dry weight). GC-MS analysis indicated that fatty acid levels declined slightly from 13.3% to 12.3% of dry weight after nitrogen limitation. However, TLC revealed that TAG content increased to a limited extent under nitrogen stress and the Nile red assay was able to detect this neutral lipid increase in *C. sorokiniana* (from 6.1 to 8.6% of dry weight). These trends among assays were also apparent in the scatter plots of Figure 9 where the gravimetric assay had a clear negative correlation with TAG levels in *C. sorokiniana*. The results demonstrate the limitations of gravimetric analysis for quantification of neutral lipids from *Chlorella*.

Cell staining with Nile red

Cell staining with Nile red was also performed on cultures of *C. minutissima* and *C. sorokiniana* under nitrogen replete and depleted conditions. Fluorescence intensity was low in cultures grown on sufficient nitrogen, which corresponded to the low TAG levels measured by TLC. However, the two strains yielded different correlations between TAG levels and fluorescence. This result was not surprising given the different cell structures, and similar findings reported by other researchers [25; 33]. Despite using DMSO as a solvent to aid dye penetration, Sitepu et al. achieved a correlation coefficient of only 0.56 between lipid levels and Nile red fluorescence among various yeast strains suggesting persistent variability in dye affinity [6]. In addition, the non-linear response of *C. minutissima* to the cell staining procedure was also cause for concern and raises the possibility that changes in cell structure resulting from nitrogen limitation could affect dye penetration. Research has shown that substantial changes to algal cell wall composition and morphology can occur in response to nitrogen limitation [35; 36]. These

results indicate clear advantages of assaying algal lipids after extraction as was the case for the Nile red assay method developed in this study

Nile red assay variability and detection limit

A set of twelve algal extracts, with lipid concentrations ranging from 0.49 to 1.70 mg/ml were selected for analysis of assay variability. Some of the samples were obtained from cultures subjected to nitrogen limitation. Four technical replicates of each sample were included within a microplate and the standard errors of these replicates divided by their respective averages resulted in scores of 0.6-2.8%. The entire microplate assay was repeated four times (on different days) and the lipid content of each sample was determined using the corresponding corn oil standard. The standard error across microplates was divided by the average lipid content determination for each sample giving a score range of 0.5-4.5%.

The assay maintained its linearity ($R^2 = 0.993$) throughout the range from 0 to 30 μg corn oil per well but the range could be expanded to 40 μg with a quadratic fit (Figure S5). The limit of detection (3x blank SD) was 0.22 μg and the limit of quantification (10x blank SD) was 0.75 μg of oil per well.

Conclusion

A high-throughput microplate assay for quantification of neutral lipids present in algal extracts was developed. This method was found to correlate well with TAG levels quantified by TLC, potentially providing a more accurate assessment of lipids conducive to biodiesel production than the gravimetric lipid assay. Unlike the SPV assay, the Nile red assay was insensitive to fatty acid composition. It was also insensitive to the

Chlorella strain used, in contrast to Nile red cell staining methods. The assay can quantify lipids in the range of 0.75 to 40 μg, allowing for analysis of small oil samples, such as those prepared from *Chlorella* spp.

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Figure 1. (A) Corn oil exhibited a strong signal upon Nile red addition, no signal was observed for *C. minutissima* extracts (UTEX 2341). Addition of *C. minutissima* extract to known quantities of corn oil suppressed the signal. (B) Addition of food coloring (FC) to corn oil suppressed the signal but addition of bleach destroyed the pigment and fluorescence was recovered. Each point is the average of three within-plate technical replicates.

Figure 2. A linear response between quantity of lipid extract and fluorescence intensity was obtained after assay was incubated in bleach solution. Optimal Nile red concentration was found to be 0.5 μg/ml. Error bars are SD across three microplate replicates.

Figure 3. Fluorescence spectra for assay after incubation in bleach solution. (A) Optimal excitation for corn oil was 530 nm, emission was 575 nm. A broad peak was observed for *C. minutissima* (UTEX 2341) extracts. (B) Emission spectra upon excitation at 530 nm. Corn oil peak was at 580 nm, polar lipid standard at 620 nm. Note that different curves do not represent the same quantity of oil. Each point is a single measurement.

Figure 4. Optimization of bleach concentration in assay holding Nile red concentration at 0.5 μg/ml. Twenty μl of either 1.5%, 3%, or 6% (w/v) sodium hypochlorite bleach solution was added in all cases. Extracts were tested from (A) *C. sorokiniana* and (B) *C. minutissima.* Error bars are SD across three microplate replicates.

Figure 5. Decline in fluorescence observed during incubation with bleach. *C. minutissima* (UTEX 2341) extracts exhibited peak fluorescence within ~30 minutes and declined at the same rate as corn oil. Each point is the average of four within-plate technical replicates.

Figure 6. Fluorescence of vegetable oils and polar lipid standard when tested with optimized assay procedure. Assay was incubated in bleach solution for 40 minutes prior to reading. Each point is the average of four within-plate technical replicates.

Figure 7. Summary of optimized Nile red lipid assay procedure

Figure 8. Comparison of optimized Nile red lipid assay (top) and gravimetric assay (bottom) with GC-quantified fatty acids for three strains of *Chlorella*. Lipid extracts from co-cultures of *C. minutissima* and *E. coli* grown on 10 g/L glucose under varying nitrogen levels and CO_2 supplentation also are shown. Each point is the average of four within-plate technical replicates using the Nile red assay and a single measurement for GC-MS.

Figure 9. Comparison of Nile red (top), gravimetric (middle), and GC-MS (bottom) assays to TAG levels determined by TLC. The same samples were measured using all three methods. Samples to the right of the dashed vertical line were subjected to nitrogen limitation. Each point is the average of four within-plate technical replicates using the Nile red assay and a single measurement for the other assays.

Figure 10. Cell fluorescence after incubation in Nile red dye compared to TAG content of the biomass. Fluorescence was normalized by dividing by the optical density at 550 nm. Each point is the average of three within-plate technical replicates for the Nile red cell assay and a single measurement of TAG by TLC.

Abbreviations

ACS	American Chemical Society
AU	Absorbance units
CCAP	Culture collection of algae and protozoa
DMSO	Dimethyl sulfoxide
FAME	Fatty acid methyl ester
FU	Fluorescence units
GC-MS	Gas chromatography and mass spectrometry
IPA	Isopropyl alcohol
NR	Nile red
OD	Optical density
SPV	Sulfo-phospho-vanillin
TLC	Thin layer chromatography
UTEX	University of Texas culture collection