Quantitative Determination of Lipid Analysis Using Nile Red Fluorometry

Xiaozhou Liu

Thesis submitted to the

Faculty of Graduate and Postdoctoral Studies

in partial fulfillment of the requirements for the degree of

Master of Applied Science

Under the auspices of the Chemical & Biological Engineering

University of Ottawa

Ottawa, Ontario, Canada

July, 2014

Abstract

An assay based on Nile red fluorescence was developed for quantitative analysis of triglycerides, a common cellular component with important biological functions and is routinely analyzed for diagnosis of metabolic disorders and as an important feedstock of food industry and biodiesel production. Based on studies on the Nile red fluorescence of pure, binary, and ternary systems of triglycerides, ethanol, and water, 20% ethanol aqueous solution was determined to be the most suitable solvent for lipid fluorescence measurement. Excellent linearity was established for lipid samples in the range of 0.1-0.5 mg/ml with several different lipid standards and vegetable oils. Results also suggest that the fluorescence of triglycerides was not sensitive to the fatty acid composition of lipids. This finding is important since it implies that the assay could potentially be used for the measurement of triglyceride content of different oil crops without causing significant variations. The results of this method were then verified by comparing with the results of the conventional gravimetric methods. The results of the fluorescence assay were consistently lower than that of the gravimetric method by approximately 10%. This phenomenon was tentatively attributed to the fact that the gravimetric method measures the total amount of lipophilic materials in samples while the fluorescence assay is selective to glycerides. Attempts were also made to apply this assay to estimate the lipid content of green alga *Neochloris oleoabundans*. However, the results were less than ideal due to the existence of interfering components in the extract of microalga samples that could significantly repress the fluorescence of lipids.

Keywords: triglyceride; lipid content analysis; Nile red fluorescence

Résumé

Un essai basé sur la Fluorescence du nil rouge a été développé pour l'analyse quantitative des triglycérides, un élément cellulaire commun portant des fonctions biologiques importantes qui sont analysées régulièrement pour la détermination de déficiences métaboliques et en tant qu'importante charge dans l'industrie alimentaire et dans la production de diesel biologique. En résultat des études sur la Fluorescence du nil rouge des systèmes purs, binaires et ternaire de triglycerides, d'éthanol et d'eau, il a été déterminé qu'une solution aqueuse à 20% d'éthanol serait le solvent approprié pour la mesure de la fluorescence des lipides. Une excellente linéarité a été établie pour des échantillons liquides dans la marge de 0.1-0.5 mg/ml, avec plusieurs différents lipides standards et huiles végétales. Les résultats suggèrent aussi que la fluorescence des triglycerides n'était pas sensible à la composition acide grasse des lipides. Cette conclusion est importante puisqu'elle implique que l'essai pourrait potentiellement être utilisé dans la mesuration du contenu de triglycérides dans de différentes récoltes d'huile sans causer de variations significatives. Les résultats obtenus de cette méthode ont été vérifiées en comparant les résultats des méthodes gravimétriques. Les résultats de l'essai sur la fluorescence étaient systématiquement moins élevés que ceux de la méthode gravimetric pour environ 10%. Ce phénomène a été attribué, tentativement, au fait que la méthode gravimetrique mesure le montant total de matériaux lipophiliques dans les échantillons, tandis que l'essai sur la fluorescence est sélectif aux triglycerides. L'application de cet essai a aussi été essayée pour estimer le contenu lipide de l'algue verte *Neochloris oleoabundans* . Néanmoins, les résultats se sont démontrés moins

qu’idéals en raison de l’existence des composantes intervenantes dans l’échantillon d’extrait de microalgues qui pourrait réprimer la fluorescence des lipides de manière significative.

Mots-clés: triglycérides; analyse de contenu lipide; Fluorescence du nil rouge

Acknowledgements

I would like first to acknowledge my supervisor, Dr. Christopher Q. Lan, for providing the opportunity to work in the field of lipids measurement by Nile red fluorometry. His support and guidance was of great help during the past years.

I would also like to acknowledge Dr. Basak from Ottawa Hospital Research Institute for co-supervising the thesis, in particular for providing the key instrument, a fluorometer, for the studies.

I would like to acknowledge lab members: Albert Parisien and Licheng Peng for the help their support, encouragement, and friendship.

I would like to acknowledge the department staff, Louis Tremblay and Franco Ziroldo for the technical assistance as well as Sylvie Saindon and Francine Petrin for administrative assistance.

Table of Contents

List of Figures

List of Tables

List of Abbreviation

1. HPLC, High-performance liquid chromatography
2. TLC, Thin-layer chromatography
3. TD-NMR, Time-domain Nuclear magnetic resonance
4. SPV, Sulfo-phospho-vanillin colorimetric method
5. TAGs, Triglyceides
6. UAE, Ultrasound-assisted extraction
7. ASE, Accelerated solvent extraction
8. PSO, Pomegranate seed oil
9. DMSO, Dimethyl sulfoxide
10. FIV, Fluorescence intensity value
11. GC, Gas chromatography

Chapter 1: Introduction

Lipids are an important group of compounds that provide several biological functions such as signaling, energy storage and cell membrane structure for cells (1). Furthermore, lipids are the major component of biodiesel, which has been established as one of the most promising renewable biofuels in recent years (2, 3). Several crops and microorganisms have long been known as promising produces of lipids owing to their good biomass productivity and the high lipid content in cells (4, 5). In order to select the best species or strain and to optimize the conditions for lipid production, development of efficient and accurate methods for lipid content analysis is of critical importance (6-9). Furthermore, lipid analysis has found important applications in diagnosis of some metabolism related diseases (10).

A large variety of different approaches have been developed for lipid content analysis, including gravimetric quantification method, HPLC/TLC and TD-NMR, Sulfo-phospho-vanillin (SPV) and Nile red fluorescence. However, these methods either require large amounts of samples (e.g., gravimetric), require a complex process for pretreatment (e.g., HPLC/TLC and TD-NMR (9, 11-14)), or are not reliable at its present stage (e.g., Nile red fluorometry and Sulfo-phospho-vanillin (SPV) colorimetric method). For instance, autophototrophic cultures of microalgae typically have a biomass concentration in the range of 3.0 g/L or less (15) and a typical bench-top bioreactor has a working volume in the range of 5.0 L or less. Consequently, one measurement of lipid content by gravimetric method, which requires approximately

0.5 g dry biomass (15), would take one third of a folly-grown microalgal culture in a bioreactor with a working volume of 5 L. This is not practical for kinetic studies, which requires multiple lipid measurements during a cultivation period.

Of particular relevance, Nile red fluorescence in lipids has been exploited for qualitative or quantitative measurement of lipids in cells since the 1980s (16-18). These methods may involve enhancement by microwave or other pretreatment procedures (17, 19, 20). However, its application has been hindered by the fact that the results are not repeatable at the present stage. Systematic studies aiming to improve quantitative measurement of lipid content of biomass samples using the Nile red fluorometric method is therefore warranted.

The objective of this research was to develop a reliable Nile red fluorescence method for quantitative determination of the lipid content of biomass. A mixture of a nonpolar organic solvent, n-hexane, and a polar solvent, ethanol, which has been widely used as the solvents in analysis of cell lipid content, was used to extract cell lipids. Aqueous ethanol solution of 20% (v/v) was determined to be a suitable solvent for measurement of lipid Nile red fluorescence.

The rest of the thesis is structured as follows:

Chapter 2 is a literature review surveying the recent developments in related areas including determination of lipid content of biomass samples, cell disruption, and lipid

extraction.

Chapter 3 presents the experimental methodologies, results and discussion of this study. It starts with selecting the optimum solvent conditions for Nile red fluorescence method for lipid content analysis. In this process, based on analysis of the pure, binary and ternary systems of oil, ethanol, and/or water, the optimum solvent condition was established and used further assays.

The linearity range of Nile red fluorescence at the optimal conditions was determined. Furthermore, the conversion factors of several lipid standards such as olive oil, glycerol trioleate and glyceryl tripalmitoleate were determined in the linear range. Based on the linearity range and the optimum solvent (20% (v/v) aqueous ethanol solution) and the conversion factor determined, the lipid content of several crop seeds and microalgae were measured and the results were compared with the gravimetric method.

Chapter 4 summarizes the conclusions of this work. Several recommendations for reducing the influence of factors which impact the accuracy of Nile red fluorescence method of lipid content determination were also proposed.

1.1 References

1. Cheng, Y. -., Y. Zheng, and J. S. VanderGheynst. 2011. Rapid quantitative analysis of lipids using a colorimetric method in a microplate format. Lipids. 46:95-103.

2. Townshen, A. 1983. Principles of Instrumental Analysis : Douglas A. Skoog and Donald M. West, 2nd edn., Saunders College, Philadelphia, 1980, xi + 769 pp., price U.S. $25. Anal. Chim. Acta. 152:314. doi: http://dx.doi.org/10.1016/S0003-2670(00)84936-3.

3. Chisti, Y. 2007. Biodiesel from microalgae. Biotechnol. Adv. 25:294-306.

4. Han, Y., Q. Wen, Z. Chen, and P. Li. 2011. Review of methods used for microalgal lipid-content analysis, 12: p. 944-950. In Anonymous Energy Procedia.

5. Hu, Q., M. Sommerfeld, E. Jarvis, M. Ghirardi, M. Posewitz, M. Seibert, and A. Darzins. 2008. Microalgal triacylglycerols as feedstocks for biofuel production: Perspectives and advances. Plant Journal. 54:621-639.

6. Demirbaş, A. 2001. Biomass resource facilities and biomass conversion processing for fuels and chemicals. Energy Conversion and Management. 42:1357-1378.

7. Scragg, A. H., A. M. Illman, A. Carden, and S. W. Shales. 2002. Growth of microalgae with increased calorific values in a tubular bioreactor. Biomass Bioenergy. 23:67-73.

8. Brennan, L., and P. Owende. 2010. Biofuels from microalgae-A review of technologies for production, processing, and extractions of biofuels and co-products. Renewable and Sustainable Energy Reviews. 14:557-577.

9. Greenspan, P., E. P. Mayer, and S. D. Fowler. 1985. Nile red: A selective fluorescent stain for intracellular lipid droplets. J. Cell Biol. 100:965-973.

10. Wymann, M. P., and R. Schneiter. 2008. Lipid signalling in disease. Nature Reviews Molecular Cell Biology. 9:162-176.

11. Jones, J., S. Manning, M. Montoya, K. Keller, and M. Poenie. 2012. Extraction of algal lipids and their analysis by HPLC and mass spectrometry. JAOCS, Journal of the American Oil Chemists' Society. 89:1371-1381.

12. Cooksey, K. E., J. B. Guckert, S. A. Williams, and P. R. Callis. 1987. Fluorometric determination of the neutral lipid content of microalgal cells using Nile Red. J. Microbiol. Methods. 6:333-345.

13. BLIGH, E. G., and W. J. DYER. 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology. 37:911-917.

14. Ando, S., and M. Saito. Chapter 9 TLC and HPTLC of Phospholipids and Glycolipids in Health and Disease, p. 266-310. In Anonymous Journal of Chromatography Libraryvol. Volume 37. Elsevier.

15. Chisti, Y. 2007. Biodiesel from microalgae. Biotechnol. Adv. 25:294-306.

16. Zuriani, R., S. Vigneswari, M. N. M. Azizan, M. I. A. Majid, and A. A. Amirul. 2013. A high throughput nile red fluorescence method for rapid quantification of intracellular bacterial polyhydroxyalkanoates. Biotechnology and Bioprocess Engineering. 18: pp 472-478

17. Chen, W., C. Zhang, L. Song, M. Sommerfeld, and Q. Hu. 2009. A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. J. Microbiol. Methods. 77:41-47.

18. Sitepu, I. R., L. Ignatia, A. K. Franz, D. M. Wong, S. A. Faulina, M. Tsui, A. Kanti, and K. Boundy-Mills. 2012. An improved high-throughput Nile red fluorescence assay for estimating intracellular lipids in a variety of yeast species. J. Microbiol. Methods. 91:321-328.

19. Chen, W., M. Sommerfeld, and Q. Hu. 2011. Microwave-assisted Nile red method for in vivo quantification of neutral lipids in microalgae. Bioresour. Technol. 102:135-141.

20. Fedosov, S. N., and X. Xu. 2011. Enzymatic synthesis of biodiesel from fatty acids. Kinetics of the reaction measured by fluorescent response of Nile Red. Biochemical Engineering Journal. 56:172-183. doi: 10.1016/j.bej.2011.06.005.

Chapter 2: Literature review

2.1 Introduction

Due to the increasing emission of carbon dioxide through combustion of fossil fuels, global warming and air pollution have become significant environmental threats worldwide (1). There is a great demand on finding renewable energy sources for sustainable development.

Triglycerides (TAG) are the major feedstock for production of biodiesel which is gaining increasing significance as one of the biofuels (2). TAGs are either esterified or transesterified, or both, depending on the acid value of the feedstock and the type of catalyst used for its synthesis to produce biodiesel. Biodiesel has lower carbon dioxide emissions and has a comparable combustion performance in comparison to fossil diesel (3, 4). As a result of its renewability and sustainability, the production of biodiesel has rapidly increased in recent years (5).

Several agricultural crops and microorganisms have been recognized as promising sources of TAGs, because of their good biomass productivity and high lipid content in cells (6, 7). In order to identify productive strains and to optimize the conditions for cultivation, we need to develop rapid and simplistic methods for lipid analysis (8-11). For different kinds of strains, different techniques may need to be utilized for lipid analysis.

Lipids measurement processes includes several steps: cell disruption; lipid extraction and lipid measurement. Among these steps, the most important one is lipid measurement. Conventional methods (e.g. gravimetric method and HPLC (12, 13)) for analyzing the content of lipid samples are the main the traditional ways. However, disadvantages of traditional ways are obvious. The gravimetric method uses organic solvent to extract lipids and then lipids are quantified gravimetrically. Disadvantages of this method are that it is time-consuming, labor intensive, and requires large volumes of sample.

Besides traditional methods, several staining methods have been developed for lipids analysis in recent decades, such as colorimetric sulfo-phospho-vanillin (SPV) method, BODIPY (boron-dipyrromethene) and DAPI (4', 6-diamidino-2-phenylindole) staining (14, 15). BODIPY and DAPI staining methods were used for qualitative lipids analysis, and SPV method was used for quantitative lipids measurement. SPV method uses organic solvent to dilute extracted samples and then add vanillin-phosphoric acid reagent for color reaction for absorbance measurement. However, the response highly depends on the source of lipid and is very sensitive to the composition of the diluted solvent (16).

In staining methods, Nile red (9-diethylamino-5H-benzo[a]phenoxazine-5-one) fluorescence method is commonly used. Nile red fluorescence was first proposed for staining of lipid droplets in cells 30 years ago (17). Then, quantitative Nile red

fluorometric analysis of lipids was developed by Greenspan et al. in 1985 (12). In recent decades of evolution and improvement, the convenience level of the experimental operation has been increased. Many methods have been developed. Chen (2009) proposed a new method assisted with ultrasonic treatment to measure the lipid concentration using Nile red fluorescence in cells quickly (18). However, most of the Nile red fluorescence methods for lipid measurement have their disadvantages which depend on their different experimental conditions.

This review will give an overview of the recent techniques as well as the common ones which were used for the cell disruption, intracellular lipid extraction and lipid content analysis, with an aim to determine a better method for lipid content analysis.

2.2 Cell disruption

Cell disruption is a method or process for releasing biological molecules from inside a cell. The target of cell disruption is to enhance the physical disruption of cells and therefore increase the efficiency of transfer of intracellular lipids into a solvent.
It is a particularly important step for accurate measurement of lipid cell content if lipid extraction is involved for a particular approach.

Grinding is a common laboratory-scale mechanical method for cell disruption. This method includes using mortar and pestle, small glass, ceramic or steel beads mixed

with dry samples or samples suspended in aqueous media. Grinding method has been widely used in cell disruption, such as leaf, plant seeds and several microbial cells (19). Zheng et al. (19) compared the efficiency of several approaches for green alga *Chlorella vulgaris* and demonstrated that grinding biomass samples gave a higher percentage of oil recovery than sonication, microwave and enzymatic pretreatment methods.

The sonication method applies ultrasound (20-50 kHz) to break up cells. In principle, the high-frequency is generated electronically and the mechanical energy is transferred to samples through a medium (e.g. water, air) that oscillates with high frequency. The cell-containing sample was placed in a medium and the high frequency oscillation causes localized low/high pressure regions which result in alternating cavitation and impaction respectively, to break up cells. Liu et al. (20) compared different sonication systems and reported that the bath-type sonicator was less effective for yeast cell disruption and protein release than horn-type sonicator under the tested conditions.

Microwaves are also employed for cell disruption. The mechanism is that the intra-cellular temperature is higher than the surrounding water, combined with the discontinuous energy delivery with the microwave duty cycle. At this condition, the cell walls are exposed to a fatigue effect, which causes cell disruption by the thermally induced pressure, leading to cell disruption as they fail to contain their

increased internal pressure (21). When the sonication and microwave methods were compared in microalgae cell disruption, at the same treatment time, the efficiency of the microwave method for cells disruption was higher than sonication (21).

Bead milling is another method for cell disruption. Cells are trapped and stressed between moving beads, which are accelerated by the agitator and collide or rub against each other. These strong collisions and robbing make cells disrupt. Zheng et al. (22) studied *Chlorella vulgaris* cell disruption using bead milling. The microalgal suspension was poured into the bead-beater chamber with cooled glass beads (size ranging from 0.40 to 0.60 mm) for 20 min at a rotational speed of 1,500 rpm. Compared with manual grinding, sonication and microwave treatment, bead milling had a lower efficiency for cell disruption. The same result was shown in research with *Botryococcus sp.*, which was carried out by Zheng et al. (22).

In several cell disruption processes, the grinding method was used in combination with the microwave and sonication treatment. After the grinding cell disruption, the results showed the lipid extraction yield was higher than without cell disruption. In addition, the microwave and sonication methods also improved the lipid yield compared to the grinding method (21).

Furthermore, enzymatic methods were used for removing cell walls in the well-established pretreatment for cell disruption. Generally, the enzymes are commercially

available and, in most cases, were originally isolated from biological sources (23, 24, 25, 26). Since the composition of the cell wall in different for different cells, various enzymes were used in researches in Table 2-1.

Table 2-1 Lists some enzymes commonly used for cell disruption

Cell	Composition of cell wall	Enzymes for cell disruption	Reference
Yeast	glucan, mannoprotein and chitin	the β-1, 3 glucanase, protease, proteases	(23)
Bacteria	peptidoglycan	β-d-galactosidase, mannase, lysostaphin, mutanolysin，zymolase	(24, 25)
Microalgae	cellulose	Cellulose, lysozyme, snailase	(22)

Krishnamoorthy (26) reported the enzyme (β-d-galactosidase) used for the disruption of bacterial cell wall was responsible for bacterial toxicity (26). Mariano et al. (23) used the β-1, 3 glucanase and protease for cells wall lysis in yeast P. rhodozyma. For *C. vulgaris* cells, lysozyme and cellulose were used for enzymatic lysis. And lysozyme and cellulose enzymatic methods are better than enzymatic lysis used snailase.

Therefore, sonication, microwave and enzymatic methods were used for cells disruption during the lipid analysis process, but grinding still was a relative easy and accurate method and suited for most kinds of samples.

2.3 Lipid Extraction

The extraction process is one of the key procedures of most lipid content analysis approaches, although some attempts have been made to avoid this tedious exercise. Ideally, a lipid extraction method to be used for lipid content analysis should be able to achieve complete lipid recovery with high selectivity. This has been so far proven to be an extremely difficult task to accomplish.

Organic solvent extraction is the most common method used for lipid extraction, because of its economic and technical advantages, such as its high selectivity and solubility toward lipids, and the low cost of solvents.

Lipid extraction is in general based on the fact that lipids are not soluble in water but are soluble in organic solvents (27) and selection of solvents is therefore one of the most important tasks towards the development of an efficient lipid extraction method. Not surprisingly, a large variety of different solvents have been proposed for extraction of lipids from different samples.

2.3.1 Solvents commonly used for lipid extraction

Many solvents or mixtures were used for lipid extraction from crop seeds, mammalian cells and microorganisms. The type of solvent plays a critical role in lipid extraction. For the specificity of different microorganism strains, choosing a suitable solvent depends on various conditions. Several solvent or mixtures used for crop lipid

extraction are listed in Table 2-2.

Table 2-2 Solvents used for lipid extraction

Classification	Solvents	Subjects	References
Plant seeds	Diethyl ether	Soybean	(28)
	Ethanol- Benzene (1:4, v/v)	Soybean	(29)
	Organic solvent	Sunflower seeds	(30)
	Hexane-Methanol (1∶1, v/v)	Leaf tissue	(31)
	Hexane	Amaranth seeds	(32)
	Hexane	Grape seeds	(33)
	Hexane	Rice bran	(34)
	n-hexane, diethyl ether, ethyl acetate, acetone, and isopropanol	Pomegranate seed oil	(35)
	Hexane- acetone (30:70, v/v), ethyl acetate	Apple seeds	(36)
Mammal cells	Methanol- Chloroform (1:2, v/v)	Tissues	(37, 38)
	Chloroform-isopropanol (7∶11)	Erythrocytes	(39)
	Chloroform-isobutanol	Erythrocytes	(39)
	Methanol- Dichloromethane (1:2, v/v)	Plasma	(40)
	Hexane-Isopropanol(2:3, v/v)	Tissue	(41)
	Isopropanol-hexane (20∶78, v/v)	Tissue	(42)
	Butanol -1- diisopropyl ether (2∶3, v/v)	Plasma	(43)
	Methanol/Methylene chloride	Tissue	(44)
	Methyl-tert-butyl ether (MTBE)	Plasma	(45)
	Acetone and chloroform-methanol (2:1, v/v)	Aortic tissue	(46)
Microorganism	Dimethyl sulfoxide	Red yeast	(47)
	Propanol -2- -benzene-water (2∶2∶1, v/v)	*Stephanurus dentatus*	(48)
	10% NaCl solution and chloroform-methanol (1∶1, v/v)	Microalgae	(49)
	Hot acidic solution and petroleum ether-ether mixtures or chloroform-methanol	*Trichosporon fermentans*	(50)
	Methanol and benzene-methanol (1∶1, v/v)	Intact yeast cells	(51)
	Methanol-chloroform (1∶1, v/v)	Microalgae	(52)
	Methanol-Chloroform (2∶1, v/v)	*Nannochloris oculata*	(38)
	Anhydrous Ethanol (90%)	Microalgae	(53)

One important consideration in the selection of solvent for lipid extraction is the polarity of solvent, because extraction yield highly depends on the polarity of the solvents used (54).

As shown in Table 2-2, for lipids extraction from microbial, plant and animal cells, polar organic solvents are commonly used in combination with non-polar solvents. However, for several crop seeds, nonpolar solvents were used. The difference in the choice of solvent systems was due to the different accessibility of lipids in these samples. For microbial, plant, or animal cells, the lipids are enclosed in the cytoplasmic membrane, which is a bilayer membrane composed primarily of phospholipids, which have a polar head and two nonpolar tails (fatty acid residuals) with the former facing the hydrophilic environment and the latter facing each other to form a hydrophobic barrier. When lipids are extracted by solvent, the solvents must first penetrate the cytoplasmic membrane. Therefore, polar solvents are needed. On the other hand, polar solvents have relatively low solubility of neutral lipids and they also have low selectivity towards neutral lipids over polar ones. Therefore, nonpolar solvents are necessary to increase both the selectivity and efficiency of lipid extraction from cells, no matter if they are microbial, plant, or animal cells. In contrast, the structure of seeds is different from cells as shown in Figure 2-1. When the seed coat is broken by grinding, the lipids in the endosperm are exposed and can be accessed by solvents. Therefore, non-polar solvents are usually directly used for extracting lipids from plant seeds (55).

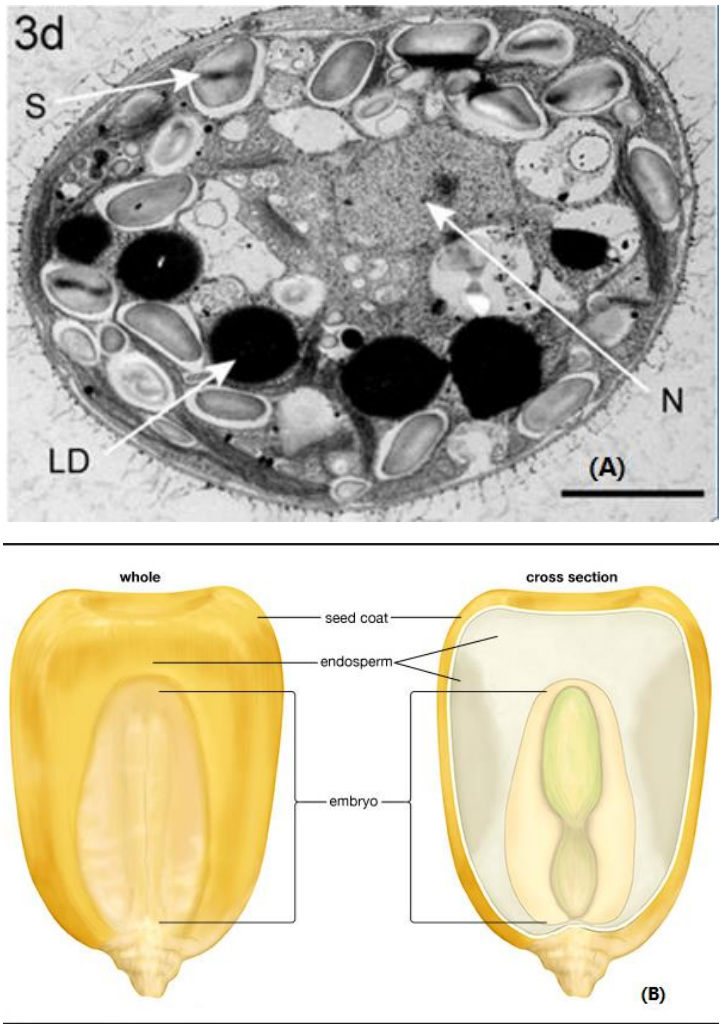


Figure 2-1 (A) Ultrastructure of *Chlamydomonas reinhardtii* cell, LD, lipid droplets; N, nucleus; S, starch (56), (B) Structure of corn kernel

Changing the viscosity of solvents will also affect the solubility which depends on solvent polarity (57).

It is worthwhile to mention that microalgae have an outstanding biotechnological potential since they produce natural substances and biomaterials such as lipids as feedstock for biodiesel production, besides being an alternative source of value-added products conventionally derived from non-renewable sources. (8, 58-64) Microalgal

lipid is stored in subcellular compartments called lipid bodies, lipid droplets or oleosomes. There is a thick cell wall surrounding microalgae, which need to be disrupted, fully or partially, to access these lipid droplets

Components of microalgae cell walls are cellulose, hemicelluloses, and saccharides. There are several methods that have been developed at the laboratory bench scale to achieve oil extraction of microalgae, in order to decrease the solvent consumption and pollution, increase extraction yield and quality of final products, and shorten the extraction time, among other properties. Until now several laboratory lipid extraction methods have been published, which include chemical solvent extraction, subcritical water extraction, supercritical fluid extraction and aqueous extraction processing (54, 65, 66).

The solvent mixture of chloroform and methanol has been used for long time in many researches. In 1959, Bligh and Dyer (67) used a mixed organic solvent (chloroform/methanol 1:1 v/v) to extract lipids from soybean seeds. Moreover, chloroform/methanol also was used as the solvent mixture for microalgae lipid extraction due to its fast and quantitative extraction. In 2011, according to Zheng et al. (68), a slightly modified version of the Bligh and Dyer method (chloroform/methanol 1:1 v/v) also was used in lipid extraction and the results showed the highest lipid concentration for *Chlorella vulgaris*. In addition, in the work carried out by Prabakaran and Ravindran (69), with *Chlorella sp.*, the highest lipid extraction efficiency was obtained with using a modified method of Bligh and Dyer,

chloroform/methanol (2:1). Besides changing the ratio of chloroform and methanol, adding hexane can also increase the efficiency of lipid extraction. Rosenthal et al. (66) reported that for *Nannochloropsis*, the best lipid extraction option was with methanol as solvent, showing the highest extracted oil amount (47%). Other solvents used in this study with less effectiveness were chloroform/methanol and hexane.

However, the high toxicity of the chloroform requires special protection for laboratory personnel. The toxicity of hexane is much lower than chloroform, and the characteristics of hexane make it possible to recover the solvent by distillation and use it again (70). Therefore hexane has become a welcome organic solvent which has been applied at large scale and laboratory operations (71).

Ethanol or ethanol mixture solvent as another low toxicity solvent for the extraction of lipids has been used for many years. For the extraction of lipids from *Nannochloropsis gaditana*, an omega-3 LC-PUFA-rich microalga, dichloromethane/ethanol (1:1) was enriched in neutral lipids and depleted in polar lipids, when compared to the total lipid extract (chloroform/methanol 1:1) (72). Besides microalgae cells, in rapeseed oil, *Peperomia pellucida* (*P. pellucida*) and *Marsilea quadrifolia* (*M. quadrifolia*) extraction, ethanol has been also used as the extraction solvent (73, 74). Ethanol was also demonstrated to be an efficient solvent for sesame seed oil extraction (75). Beyond these crops and microalgae lipid extraction using ethanol as the solvent, corn (76), rice bran, tallow tree (77) and

soybean (78). Lipids were also extracted by ethanol. Therefore, ethanol was widely used in lipid extraction process.

Ethanol and hexane mixture has been demonstrated by many researchers to be more efficient in the extraction of lipids than the other solvents (79). A mixture of ethanol-hexane (5:1, v/v) in solvent lipid extraction reduced the solvent requirement for the same amount of biomass, approximately 10 times compared with the methanol/chloroform extraction processes (80). In addition, Ramalingam et al. (81) discovered that the different ratios of ethanol and hexane also can influence the lipid extraction efficiency. Vegetable oil had a higher solubility in ethanol /n-hexane mixture (v/v: 4:1) at 40°C than at other ratios. Therefore, the mixture of ethanol and hexane may be a better solvent for lipid extraction.

2.3.2 Lipid extraction devices

According to different purposes of lipids extraction (i.e. for industrial applications, food and pharmaceutical production, or lipid analysis), many devices have been developed. Among lipid extraction devices for lipid analysis, the most used one is Soxhlet extraction, which was believed a standard method for lipid extraction for analysis and has been commonly used as the basis for comparison in the development of new extraction methods. The Soxhlet extraction technique was first invented by Franz von Soxhlet in 1879 dealing with the determination of fat content in milk (Soxhlet F, 1879) (55, 82). This “solid-liquid extraction” not only can remove and

separate compounds of interest from insoluble high-molecular-weight fractions, but also from other compounds which may interfere with subsequent steps of the analytical process (83).

As shown in Figure 2-2, a Soxhlet extractor is glassware composed of a boiling flask, a thimble with a siphon arm, an extraction chamber, and a condenser. Soxhlet extraction has three basic steps: extraction, rinsing and concentration (84). Normally dry powder samples, which are covered by filter paper, are placed inside the thimble, which is the main chamber of Soxhlet extractor. The boiling flask, which holds the liquid extracting solvent, is placed under the thimble. And at the top part of Soxhlet extractor, a condenser is placed onto the chamber.

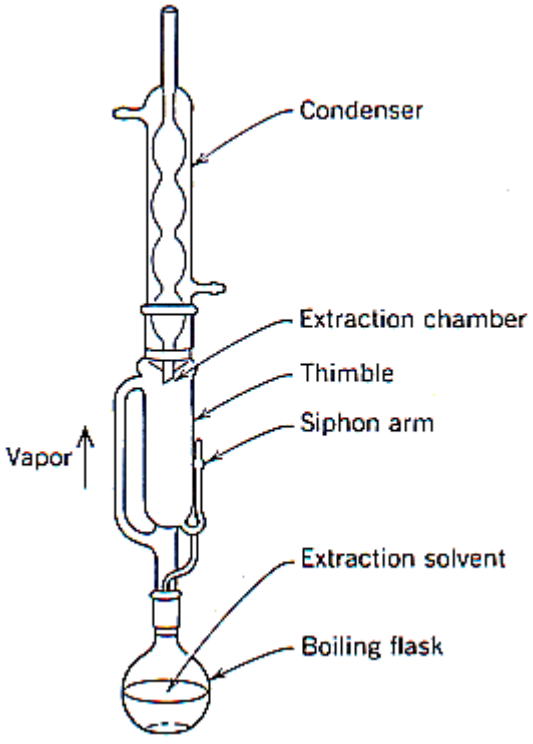


Figure 2-2, A schematic representation of a Soxhlet extractor

During extraction, the liquid solvent is heated in the boiling flask for reflux. Then the solvent vapor rises up to the thimble through a distillation arm. The condenser cools the solvent vapor and the lipid solvent drips back down into the chamber. As the extraction proceeds, the chamber containing the dry samples is slowly filled with warm liquid solvent to extract lipids from the samples. After 8-10 min, the Soxhlet chamber is full, the chamber is emptied by a siphon arm automatically and the warm solvent runs back down to the boiling flask. According to the different desired compounds, the extraction cycle can be repeated multiple times, over a period of hours or even days.

During each cycle, lipids are dissolved in the warm solvent. After several cycles, lipids are concentrated in the boiling flask. Finally, the remaining solvent in the boiling flask is removed by heat and the lipid remains.

As discussed before, the composition of the solvent for Soxhlet extraction is another important factor which can directly influence the extraction of lipid. Increasing the polarity of solvents may increase the productivity of lipids extraction (85). Some common solvents such as dichloromethane, pentane, acetone, chloroform and n-hexane, applied in Soxhlet, can be divided into two groups: non-polar and polar solvent. Mixture of a polar and non-polar solvent is also a popular choice in Soxhlet extraction, but the proportion of each compound is important as it can influence the

length of extraction time as well as the yields. Less polar solvents may increase the extraction time (83, 85).

The attractive advantages of standard Soxhlet extraction are that the sample is repeatedly introduced into contact with fresh extractants, and this is a simple and low-cost method (83). Although the Soxhlet extraction has been applied widely, the shortcomings of Soxhlet method is that it is very time consuming, of lower efficiency, and its results are time-dependent. For instance, an experiment done by de Boer et al. (85) showed that a better yield was achieved after 6 hours than 3 hours on pentane extraction of roach. As a result, it not only raises cost concerns but also uncertainty of results when applied for lipid content analysis, especially if a new type of sample is to be analyzed.

2.3.3 Enhanced lipids extraction

Solvent extraction of lipid can be enhanced by different methods including mechanical pretreatment, elevated pressure and temperature in extraction, ultrasound-assisted extraction and extraction using supercritical fluid.

Kraujalis et al. (86) reported that accelerated solvent extraction (ASE) and standard Soxhlet extraction methods were compared for extraction of lipids from different particle size fractions of milled amaranth seeds. Accelerated solvent extraction (ASE) is oil extraction by organic solvent under high pressure. Although the maximized

extracted lipid yield from two different methods were similar, the extraction time of ASE was shorter than Soxhlet extraction. Ultrasound-assisted extraction (UAE) using hexane was investigated for extracting lipids from grape seeds by Da Porto (87). The results indicated that UAE for grape seeds was more efficient than conventional extraction to obtain a high oil yield, lowering solvent consumption and shortening the extraction time. In addition, the efficiency of ultrasonic-assisted extraction (UAE) of pomegranate seed oil (PSO) was evaluated using a variety of solvents. Compared with n-hexane, ethyl acetate, diethyl ether, acetone, and isopropanol, petroleum ether was the most effective for oil extraction. The PSO yield extracted by UAE was significantly higher than by using Soxhlet extraction (35). Enzymatic hydrolysis also can be used for increasing the lipid extraction efficiency and hexane was used as a solvent after enzymatic pre-treatment (34).

2.4 Methods for Lipid Content Analysis

2.4.1 Gravimetric quantification methods

The most common approach of lipid content analysis is the gravimetric method. This approach involves lipid extraction from a sample using organic solvent, which is then dried before the lipids are quantified gravimetrically (6). Bligh and Dyer (88) were the first to report the gravimetric quantification method in their studies. Lipid content of fish tissue was analyzed in their experiments. A chloroform/methanol/water solvent mixture was used in the extraction process. A sample of fish tissue was homogenized in a chloroform/methanol/water solvent mixture. Then the homogenate was filtered by

a paper filter in a funnel. The filtrate was transferred to a column, and then chloroform solvent separated from methanol solvent naturally by standing. The chloroform layer, which is at the bottom, contained the lipid content. The top layer was removed with aspiration, a thin layer of the chloroform solvent was also removed in order to ensure complete removal of the top layer. The lipid extract was evaporated to dryness, and the retained lipid was measured. Folch et al. (89) simplified the previous method for lipid extraction from brain cells. The modification from the previously discussed experiment to this one is the washing procedure. In Bligh and Dyer's experiment, a small quantity of chloroform solvent was removed to ensure the pureness of the lower layer (88). In Folch's experiment, small amounts of upper phase pure solvents were used to wash the interface of the two layers after most of the top phase was removed by siphoning (89).

Gravimetric quantification methods are simple and widely used. Bligh and Dyer's method (88) is one of the most recommended methods used for lipid quantification (90), and is the standard method for lipid determination in many samples such as of marine fish (27, 90-95), milks (96, 97), and algae (98, 99). However, these methods are time-consuming, labor intensive, and require large volumes of sample (18). In addition, the accuracy relies on the completeness of lipid extraction.

2.4.2 TD-NMR method

Nuclear magnetic resonance (NMR) has been widely used in chemical analysis as well as structural research since it was introduced in the late 1970s (100). This

technology was further developed by Todt et al. (101). A small table-top time-domain nuclear magnetic resonance (TD-NMR) analyzer was built for the solid-to-liquid ratio analysis of fat composition. It now has attracted a lot of attention in its application to quantifying lipid content with the advantages of being less time consuming, minimum requirement of sample and higher accuracy. This application has been recognized as an International Standard Method (6, 101).

In food science and industry, classic TD-NMR is used to quantify the content of fats and waters (102). In fact, this approach has been used to measure the oil and moisture contents of almost all types of food such as seeds, olives, nuts and milk (101). For instance, Pereira et al. (103) determined the moisture content in beef using TD-NMR and Castell-Palou et al. (104) determined the moisture profiles in cheese. Rudi et al. (102) determined the fat and water content in caramel employing TD-NMR. It is worth mentioning that, since lipid extraction of microbial cells is more difficult than crops, therefore the use of TD-NMR for determining lipid contents in microalgae has only generated little attention (105).

2.4.3 TLC/HPLC method

High-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) are two of the other techniques available for lipid analysis. HPLC is a technique widely used in biochemical and analytical fields to separate, identify, purify and quantify individual components from mixtures (6, 104). HPLC is an effective

method to determine TAG content in biomass. Moreover, HPLC potentially presents a method that can analyze various classes of lipids in a crude lipid extract (106).

Thin-layer chromatography is also a traditional method for measuring the lipid composition of bio-samples. It is important to remember that pigments in samples such as chlorophyll in algae, should be removed by column chromatography in order to eliminate interference with the component of interest (107). This means that a preparation of the sample must be done before executing the experiment because a poor sample generally causes unsatisfactory results. A method called solid-phase extraction (SPE) is usually applied in the primary preparation and SPE columns are effective in separating lipid classes according to different polarity (108).

These two methods have their own benefits and can both serve as versatile and sensitive tools in lipid analysis. TLC can achieve excellent separation compared to the traditional chromatography technique such as column chromatography, planar chromatography and paper chromatography. Using TLC, different compounds with different polarities can be separated on one plate. Compounds separated on a plate can be recovered and quantified in situ by densitometric scanning, which is a simple and time-saving process (109). Disadvantages of the TLC method include that spots are often faint, and the results are difficult to reproduce, due to uneven advance of solvent, front streaking and spotting.

The process of HPLC is automatically and it only takes a few minutes to produce results, therefore, HPLC has a marked difference with liquid chromatography which uses gravity instead of a high speed pump to force compounds through the densely packed tubing. The produced results of HPLC are easy to read and have the high resolution, and the assays are easily produced and repeated by the automated process. In addition, HPLC can separate many common polar and neutral lipids, however, there are still problems with the separation of some lipid classes, and consequently additional TLC separation of poorly resolved peaks still has to be performed (13). Furthermore, the HPLC method needs costly equipment and expert handling, and has low sensitivity to some compounds.

2.4.4 Sulfo-phospho-vanillin (SPV) method

The colorimetric sulfo-phospho-vanillin (SPV) method developed by Chabrol et al. (110) is another attractive alternative for lipid measurement because of its fast response. Cheng et al. (16) applied this approach for the measurement of vegetable oil, animal lipid, or purified microalga extract samples. Briefly, to determine the lipid content of a sample using SPV, the lipophilic samples (lipids or lipophilic extracts) are first diluted using an appropriate solvent (e.g., chloroform or the mixture of chloroform and methanol for plant oils and animal lipids and methanol for purified microalga extracts) and then loaded into microplates of a spectrophotometer. After solvent evaporation at 90°C for 10 min, sulfuric acid is added and the mixture is incubated at 90°C for 20 min. Then, after the samples are cooled down to room temperature, the background absorbance is measured at 540 nm. Then, vanillin-

phosphoric acid reagent was added for the color reaction. Finally, the absorbance of the sample is measured at 540 nm. This method requires a small amount of sample, short time and little labor per sample when a large number of samples are analyzed. The coloration developed during the reaction can be read and compared very easily. These advantages make the SPV (Sulfo-Phospho-Vanillin) method potentially applicable for a wide range of applications such as the determination of total lipids in food, serum and ecological samples (17, 105, 111-113). It is also an attractive alternative for lipid analysis of plant seeds and microalga cells.

One major disadvantage of the SPV method is that the response highly depends on the source of oils. It was reported that the mean absorbance/lipid ratio was 0.00755 a.u./μg and 0.00554 a.u./μg for canola oil and cod liver oil, respectively. These results indicate a high dependence of response on the fatty acid composition of lipids. In addition, results are very sensitive to the composition of diluted solvent. Only the samples prepared in a chloroform: methanol ratio of 2:1 had clear results.

2.4.5 Nile red fluorescence methods

Staining cells with fluorescent lipophilic dyes is another approach used for lipid content analysis (114). Nile red, a lipid soluble fluorescent dye, has been frequently used to evaluate the lipid content of microorganisms or animal cells, such as microalgae, yeasts, bacteria and mammalian cells (115-118). It is one of the most common dyes applied in these methods because its fluorescence at defined wavelengths depending upon the polarity of the surrounding medium (112). A

spectrofluorometer is usually used for reading the samples obtained both before and after staining (6).

The fluorescent Nile red dye was originally proposed by Greenspan et al. (12) as a selective dye for visualizing lipid droplets in microalgal cells. Then, an optical microscope and a flow cytometer were used for quantitative but rather rough estimations of the lipid content of cells by visual determination of the size of lipid droplets inside cells and the ratio of cells containing lipid droplets, respectively (98). In addition, several cultured cells were stained with a dilute aqueous solution of Nile red and cytoplasmic components were produced an intense fluorescent (Figure 2-3). As shown in Figure 2-3 (17), there is no apparent difference in distribution or intensity of fluorescence and the appearance of the stained cells viewed for yellow-gold fluorescence (excitation wavelength 450-500 nm, emission wavelength more than 528 nm) and for red fluorescence (excitation wavelength 515-560 nm, emission wavelength greater than 590 nm), no matter cells are fixed or not when the staining process carried out.

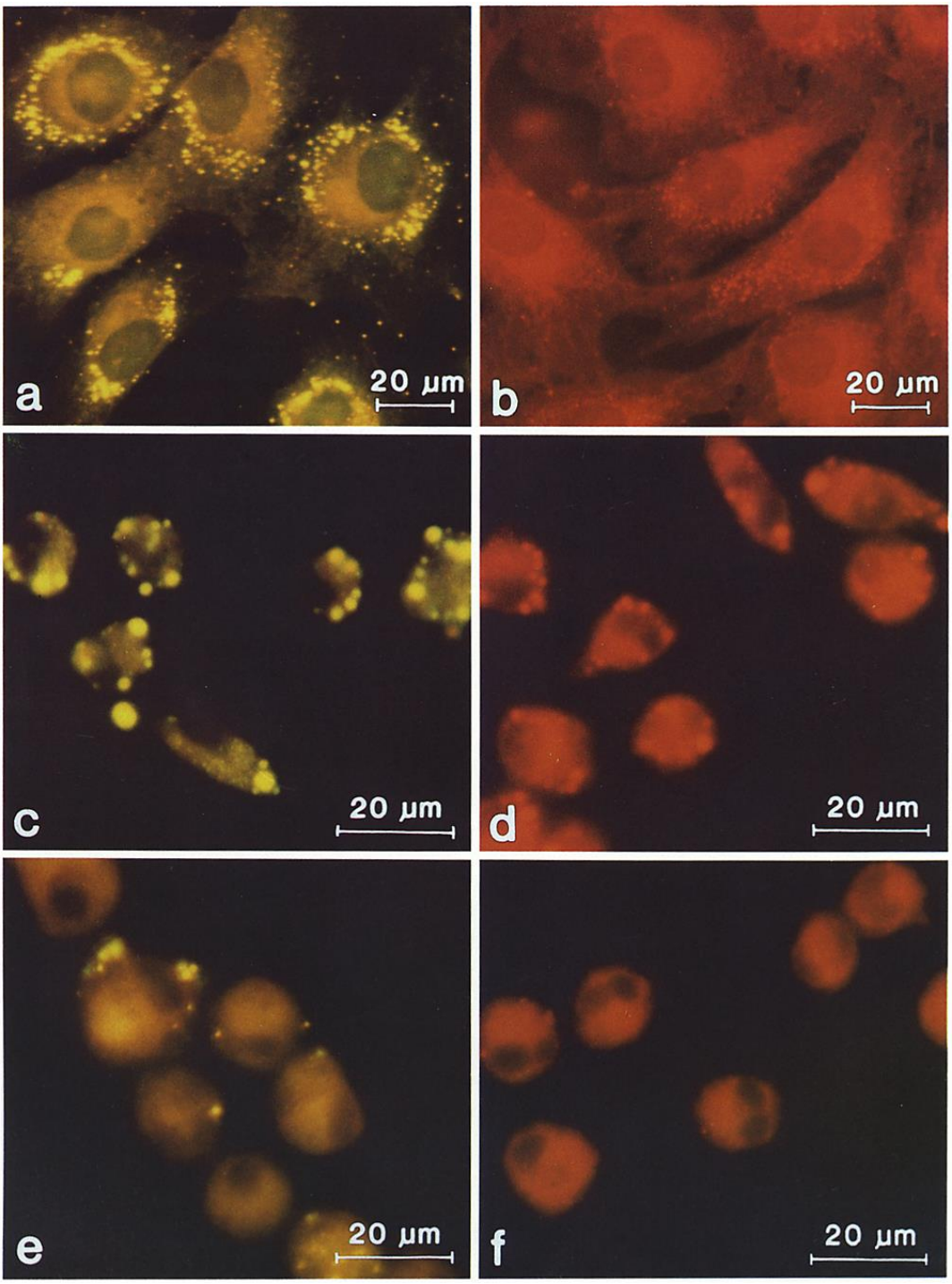


Figure 2-3 Nile red fluorescence of cultured monkey aortic smooth muscle cells and mouse peritoneal macrophages (a and b). Fluorescence of aortic smooth muscle cells treated with Nile red (c and d). Fluorescence of Nile red-stained peritoneal macrophages previously incubated 40 h with acetylated low density lipoprotein (e and f) (17).

In the past three decades, this method has been modified for quantification of lipids. In 1987, an experiment was conducted by Cooksey et al. (98) using this staining method with algal cells. Nile red acetone solution was added to a suspension of cells in growth medium. The mixture was vigorously shaken on a vortex mixer. The resulting samples were immediately tested with a spectrofluorometer and results showed that cellular fluorescence of stained cells and gravimetrically or chromatographically determined lipid were linearly correlated when Nile red was excited at 488 - 525 nm and the fluorescent emission measured at 570-600 nm.

In 1998, Lee et al. (119) reported that for *Botryococcus braunii*, there was a linear relationship between in vivo fluorescence of cells stained with Nile red and lipid content in *B. braunii* determined gravimetrically. This suggested that Nile red staining as a rapid method was as good as the gravimetric method. In addition, in 2013 Liu et al. (120) reported that ultrasonic pretreatment of *B. braunii* cells can be used before Nile red staining to increase the efficiency of lipid content analysis.

Besides microalgae, the Nile red fluorescence method has also been used for oleaginous fungi and yeasts. Kimura et al. (121) reported that cells after buffer dilution and then were stained with 0.24 –0.47 μg/ml of Nile red for 5 min, and before and after Nile red adding, the fluorescence emission spectra were acquired in the wavelength range of 400 -700 nm at excitation wavelength 488 nm. In addition, the fluorescence intensity value corresponding to the amount of intracellular lipid was determined at the maximum of the corrected spectrum. The FIV have a linear

relationship with the lipid content of different oleaginous fungi and yeasts measured by the gravimetric method.

Zheng et al. reported (122) that the lipid content of *Chlamydomonas reinhardtii* could be measured using the Nile red fluorescence method. This method required 5 % DMSO (dimethyl sulfoxide) with 1 μg/mL Nile red and 5-15 min incubation time for samples. The optimum for measuring lipid content was in the range of 1- 8×10^6 cells/mL. The fluorescence is determined by using excitation at 528 nm and emission at 576 nm. The quantification of neutral lipids can be achieved by gravimetric method.

A two-step microwave-assisted staining method for in vivo quantification of lipids which was an improvement of this method was described by Chen et al. (97). The mixture of algal cells and DMSO was pretreated using a microwave oven for 1 min. The algae solvent mixture was placed in a microwave oven for 1 min before staining took place. The microwave power was set at 1200 W for both processes. The new method provided results that matched well with those obtained with gravimetric methods, while it was more rapid and required much smaller samples. However, this method was proven hard to repeat in our laboratory.

These Nile red fluorescence methods were used for determination of intra-cellular lipids, not for extracted lipids. Since the cell wall is thick, the accuracy of results

could be compromised by light scattering and absorbance of the cell envelope surrounding the lipid droplets.

In 1987, Fowler et al. (123) reported the first attempt using Nile red for quantifying extracted lipids, in which Nile red was used as a general purpose reagent for rapid lipid detection and quantitation after lipids were separated by thin-layer chromatography. Nile red solution (8 μg/ml of methanol-water 80:20, v/v) was added in samples, which were subsequently applied to silica gel plates for chromatography. A dilute aqueous bleach solution was dipped into the silica gel plates which absorbed background fluorescence and then the fluorescence was destroyed by the bleach. After drying, in situ quantitative analysis of the fluorescence of the lipids on the Nile red-stained plate was visualized under visible light using a fluorometer under excitation wavelength 580 nm and emission wavelength 640 nm. This Nile red method relied on thin-layer chromatography and the detection limit of the assay was 25-100 mg for triacylglycerols. In this method, lipids were separated by thin-layer chromatography (TLC) before quantification by Nile red fluorometry. As a result, the disadvantages of TLC as discussed previous are carried into this approach.

2.5 Fluorescence spectroscopy

Fluorescence spectroscopy, which also known as using a spectrofluorometer or fluorometer, is a type of electromagnetic spectroscopy for analyzing the fluorescence intensity of samples. It is a key instrument in lipid determination using Nile red fluorometery.

According to different energy levels, molecules have various states. Fluorescence spectroscopy is primarily related to the electron motion among the vibrational and electronic states of molecules. The ground electronic state of atoms has a low energy level and the excited electronic state has a higher energy level. Furthermore, there are various vibrational states within each of these ground and excited electronic states (124). In fluorescence spectroscopy, when molecules are excited by absorbing a photon, their electrons were moved from the ground electronic state to one of the various vibrational states in the excited electronic state. Then the excited molecule loses vibrational energy and reaches the lowest vibrational state of the excited electronic state because of collisions between each other's molecules. Finally, the molecule drops down back to one of the various vibrational levels of the ground electronic state again, emitting a photon during the process (124). Since molecules may drop back down into different vibrational levels in the ground state, therefore, the emitted photons may have various energy levels, and thus frequencies.

To measure the emission spectra a fluorometer is used. The excitation light is held at a constant wavelength and the emission spectra of samples are scanned within a pre-selected range of wavelengths of the fluorescent light emitted. Similarly, an excitation spectrum can be determined by holding the emission light at a constant wavelength and scanning the excitation light through a range of different wavelengths.

Filter fluorometer and spectrofluorometer are two general types of fluorescence intensity or spectral analysis instruments. Filter fluorometer can be used to filter the incoming and fluorescence light separation. Spectrofluorometer can be used for diffraction grating the incoming and fluorescence light isolation.

There is the mechanism of both two types of fluorometers as follow:

An excitation light source passes through a monochrometer which is an optical device that can selected narrow band of wavelengths of light from a wider range of input wavelengths After this light transmits through the monochrometer, it is fixed at a pre-setting wavelength and absorbed by samples. Then the fluorescence light is emitted in all directions. Some of this emission light can pass through a second monochrometer and the reaches a detector placed at 90° the incoming light. The second monochrometer can minimize the risk of incoming light transmission and reflection for reaching the detector (Figure 2-4).

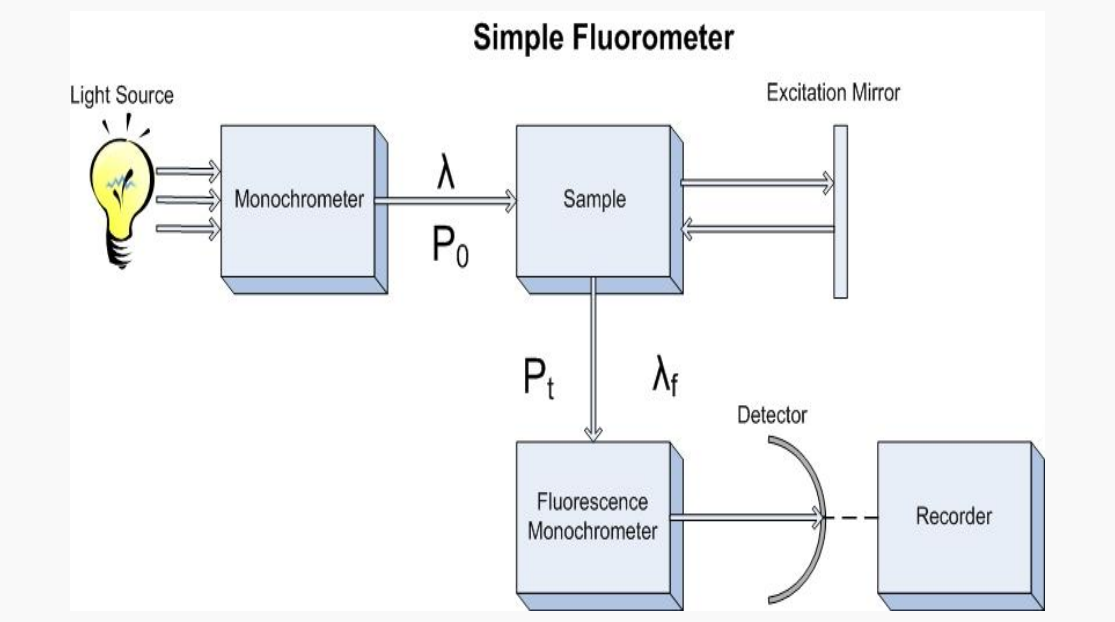


Figure 2-4 A simplistic design of the components of a fluorometer

Both continuous excitation and emission spectra can be measured by versatile fluorometers with dual monochrometers. When fluorescence emission spectra are measured in a range, the wavelength of the excitation light is kept constant. For measuring excitation spectra, the wavelength is kept constant passing though the emission monochrometer while the excitation spectrum is scanned (125).

Several factors influence the spectra or fluorescence intensity, and it is necessary to correct them to reach a relative “true” (i.e. machine-independent) spectrum. Both instrument- and sample-related influence will produce the different types of distortions: 1) Light source intensity and wavelength characteristics change over time during and between each experiment; 2) No lamp has a constant intensity at all wavelengths. 3) The concentration of the fluorophore will influence

the fluorescence intensity at low concentrations. In order to correct this, after the excitation monochromator, a beam splitter can be applied or a filter to direct a portion of the light to reference detectors.

2.6 Influence of different parameters on Nile red fluorescence

Nile red is a benzophenoxazone dye that dissolves in a wide range of organic solvents including xylene, chloroform, acetone, heptane and alkanes, but dissolves negligibly in water. Several physical properties of Nile red have been listed in Table 2-3.

Table 2-3 Physical properties of Nile red (12)

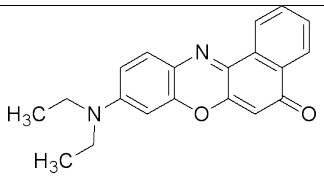
Molecular formula	**$C_{20}H_{18}N_2O_2$, mw = 318**
Structure	
Melting point	192-193ºC
Solubility	1 mg/ml acetone 62 µg/ml n-heptane <1 µg/ml water
Partition coefficient (4ºC)	xylene/water 210 chloroform/water 196 amylacetate/water 198 n-heptane/water 58
Absorption maxima ($HCCl_3$, solvent)	ultraviolet, 264 nm; visible, 538 nm
Infrared maxima ($HCCl_3$ solvent)	1000, 1105, 1265, 1305, 1550, 1580, 1612 (C=O), 1710 cm^{-1}
Fluorescence intensity relative to rhodamine B (HCCl, solvent)	0.36

Table 2-4 summarizes Nile red fluorescence in several common solvents, which highly depends on the polarity of the surrounding environment.

Table 2-4 Fluorescence properties of Nile red in various solvents

Solvent	Dielectric constant(ε)	Peak wavelength (nm)		Reference
		Excitation Maximum(nm)	Emission Maximum(nm)	
Water	78.5	591	657 or 663	(12, 126)
DMSO	47.2	553	637	(127)
Glycerol	46.5	582	656	(127)
EG	41.4	571	654	(127)
DMF	38.3	546	626	(127)
EtOH	25.3	549	637	(127)
Ethanol	24.3	559	629	(12)
Acetone	20.7	536	608	(12)
Chloroform	4.8	534	595	(12)
iso-Amylacetate	-	517	584	(12)
Xylene	2.4	523	565	(12)
Toluene	2.4	524	570	(127)
n-Dodecane	2.0	492	531	(12)
n-Heptane	-	484	529	(12)

Note: Nile red concentration was 1 μg/ml in all samples. Excitation and emission maxima were determined in each solvent was measured at the corresponding excitation and emission maxima

The data listed in Table 2-4 demonstrate a general trend of a red shift in the emission for an increase in solvent dielectric constant (127), which was confirmed by the results of many other researchers with different solvent systems. Zhang et al. (126) reported the emission spectra of Nile red in different solvents, i.e., n-hexane, ethyl acetate, methanol, and water (Figure 2-5), which were scanned using a laboratory constructed fluorescence spectrophotometer. The emission maximum wavelengths of water, methanol, ethyl acetate and n-hexane were 663, 633, 602 and 526 nm respectively. It should be mentioned that while the peak emission of Nile red

fluorescence in water was reported to be 663 nm by Zhang et al. (126), it was reported to be 657 nm by Greenspan et al. (12), which is listed in Table 2-4. Although the results observed by different researchers were very close to each other, it does suggest that Nile red fluorescence in the same solvent may vary when other conditions are different. Besides the red shift of spectra, the dielectric constant (polarity) of solvents also influences the fluorescence intensity and blue shift. Figure 2-6 shows the blue shift and intensity increase of Nile Red fluorescence emission in methanol, ethanol and DMSO compared to water (128).

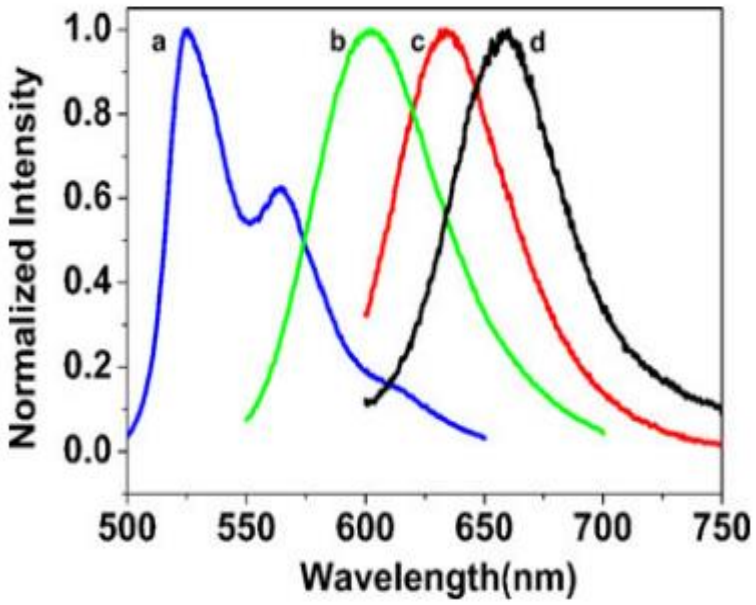


Figure 2-5 Fluorescence emission spectra of Nile red in different solvents: (a) n-hexane, (b) ethyl acetate, (c) methanol, (d) and water (126)

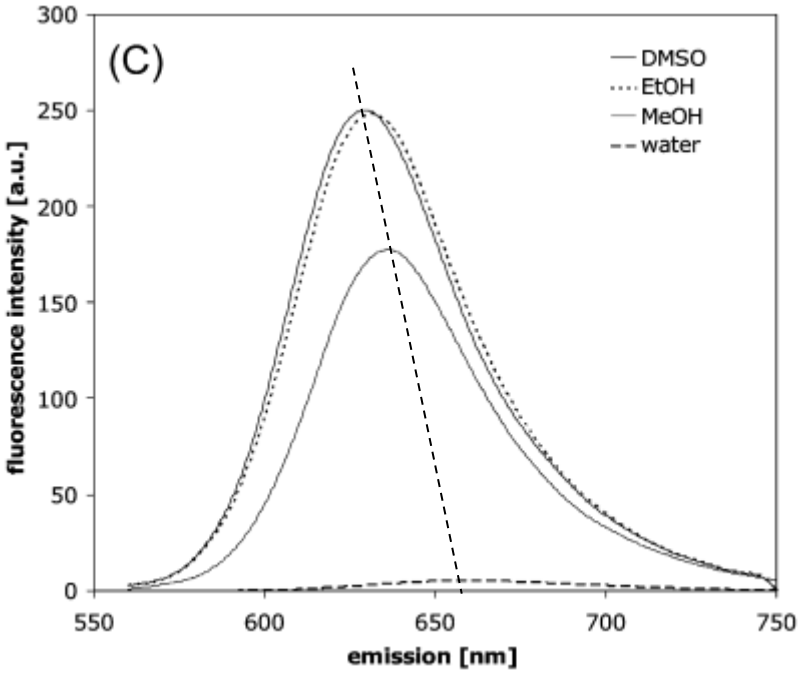


Figure 2-6 1 μM Nile Red excited at 550 nm in different solvents (128)

Moreover, Dutta et al. (129) studied Nile red fluorescence in several binary systems including methanol-water, acetonitrile-water, acetone-water and ethanol-water binaries. The results showed the absorption and emission spectra positions of Nile red shift towards the red with increaing volume fraction (Δf) of water in the binary solvent- water mixtures. Δf were calculated by equation (2-1):

$$\Delta f = \frac{\varepsilon - 1}{2\varepsilon + 1} - \frac{n^2 - 1}{2n^2 + 1} \qquad (2\text{-}1)$$

This volume fraction was calculated from the dielectric contant (ε) and refractive indices (n) of the different volume compositions of the solvent-water mixtures. Similarly, the red shifts observed in pure solvents also increased with the values of the volume fraction. Moreover, increasing the volume fraction of water in the binary mixture led to a decrease of the intensity ratio of the spectra.

The emitted light of Nile red fluorescence is in general at wavelengths that are longer than that of the excitation light due to the loss of vibrational energy of excited electrons. This loss of vibrational energy is rapidly transferred to the solvent. The solvent effect in turn causes the shift of emission to a still lower energy level due to stabilization of the excited state by the polar solvent molecules. Therefore, as the solvent polarity is increased, this effect becomes larger, resulting in emission at lower energy level or, in other words, at longer wavelengths (125). This explains the red shift of Nile red fluorescence observed with the increase of dielectric constant of solvents.

Moreover, the fluorescence intensity may be influenced by the concentration of Nile red. For instance, it was reported by Dutta et al. (129) that, with increasing concentration of Nile red in different solvents, the emission intensity increased in low cencentration. In addition, Chen et al. (18) reported fluorescence of the green algae *C. vulgaris* when the cells were stained by different concentration of Nile red acetone solution (i.e. 0.1, 0.25, 0.5, 1, 2, 5, 10 μg/mL). The highest fluorescense intensity occurred at 0.5 μg/mL and there was a significant decrease in fluorescence intensities observed when Nile red concentration was higher than 5 μg/mL or below 0.1 μg /mL.

2.7 Conclusion

Aanalytical methods for lipid content have important applications in many fields and may affect, for instance, the development of cost-effective biofuel production in many ways. These methods could be generally divided into two categories, those involving extraction of lipids from samples or determination of lipid content with intact cells. From the above literature, we know that most of the traditional methods for lipid measurement are either time consuming and costly or not reliable in their current form, warranting future studies on both fundamental principles and protocol optimization.

A category of attractive alternative lipid measurement methods of Nile red can also be divided to these two groups. For Nile red fluorescence methods measuring lipid content of intact cells, although many approaches to enhance the efficiency and accuracy, the results still were repeated hardly or had same linear range. And the Nile red method involving extracted lipids from cells is still in a primitive form that depends on separation of lipids using highly unreliable TLC. Because of these reasons, studies aiming at development of Nile red fluorescence methods for the measurement of lipid content of different oil crops or cells are warranted.

2.8 Reference

1. **Chisti, Y.** 2007. Biodiesel from microalgae. Biotechnol. Adv. **25:**294-306.

2. **Balat, M.** 2011. Challenges and opportunities for large-scale production of biodiesel. Energy Education Science and Technology Part A: Energy Science and Research. **27:**427-434.

3. **Sun, J., J. A. Caton, and T. J. Jacobs.** 2010. Oxides of nitrogen emissions from biodiesel-fuelled diesel engines. Progress in Energy and Combustion Science. **36:**677-695.

4. **Fahmi, I., and S. Cremaschi.** 2012. Process synthesis of biodiesel production plant using artificial neural networks as the surrogate models. Computers and Chemical Engineering. **46:**105-123.

5. **Dürre, P., and T. Richard.** 2011. Microbial Energy Conversion revisited. Curr. Opin. Biotechnol. **22:**309-311.

6. **Han, Y., Q. Wen, Z. Chen, and P. Li.** 2011. Review of methods used for microalgal lipid-content analysis,12: p. 944-950. *In* Anonymous Energy Procedia.

7. **Hu, Q., M. Sommerfeld, E. Jarvis, M. Ghirardi, M. Posewitz, M. Seibert, and A. Darzins.** 2008. Microalgal triacylglycerols as feedstocks for biofuel production: Perspectives and advances. Plant Journal. **54:**621-639.

8. **Demirbaş, A.** 2001. Biomass resource facilities and biomass conversion processing for fuels and chemicals. Energy Conversion and Management. **42:**1357-1378.

9. **Scragg, A. H., A. M. Illman, A. Carden, and S. W. Shales.** 2002. Growth of microalgae with increased calorific values in a tubular bioreactor. Biomass Bioenergy. **23:**67-73.

10. **Kirrolia, A., N. R. Bishnoi, and R. Singh.** 2013. Microalgae as a boon for sustainable energy production and its future research & development aspects. Renewable and Sustainable Energy Reviews. **20:**642-656.

11. **Brennan, L., and P. Owende.** 2010. Biofuels from microalgae-A review of technologies for production, processing, and extractions of biofuels and co-products. Renewable and Sustainable Energy Reviews. **14:**557-577.

12. **Greenspan, P., and S. D. Fowler.** 1985. Spectrofluorometric studies of the lipid probe, nile red. J. Lipid Res. **26:**781-789.

13. **Olsen, R. E., and R. J. Henderson.** 1989. The rapid analysis of neutral and polar marine lipids using double-development HPTLC and scanning densitometry. J. Exp. Mar. Biol. Ecol. **129:**189-197. doi: http://dx.doi.org/10.1016/0022-0981(89)90056-7.

14. **Govender, T., L. Ramanna, I. Rawat, and F. Bux.** 2012. BODIPY staining, an alternative to the Nile Red fluorescence method for the evaluation of intracellular lipids in microalgae. Bioresour. Technol. **114:**507-511.

15. **Kuroiwa, T., M. Ohnuma, Y. Imoto, O. Misumi, T. Fujiwara, S. -. Miyagishima, N. Sumiya, and H. Kuroiwa.** 2012. Lipid droplets of bacteria, algae and fungi and a relationship between their contents and genome sizes as revealed by BODIPY and DAPI staining. Cytologia. **77:**289-299.

16. **Cheng, Y. -., Y. Zheng, and J. S. VanderGheynst.** 2011. Rapid quantitative analysis of lipids using a colorimetric method in a microplate format. Lipids. **46:**95-103.

17. **Greenspan, P., E. P. Mayer, and S. D. Fowler.** 1985. Nile red: A selective fluorescent stain for intracellular lipid droplets. J. Cell Biol. **100:**965-973.

18. **Chen, W., C. Zhang, L. Song, M. Sommerfeld, and Q. Hu.** 2009. A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. J. Microbiol. Methods. **77:**41-47.

19. **Zheng, H., J. Yin, Z. Gao, H. Huang, X. Ji, and C. Dou.** 2011. Disruption of chlorella vulgaris cells for the release of biodiesel-producing lipids: A comparison of grinding, ultrasonication, bead milling, enzymatic lysis, and microwaves. Appl. Biochem. Biotechnol. **164:**1215-1224.

20. **Liu, D., X. -. Zeng, D. -. Sun, and Z. Han.** 2013. Disruption and protein release by ultrasonication of yeast cells. Innovative Food Science and Emerging Technologies. **18:**132-137.

21. **McMillan, J. R., I. A. Watson, M. Ali, and W. Jaafar.** 2013. Evaluation and comparison of algal cell disruption methods: Microwave, waterbath, blender, ultrasonic and laser treatment. Appl. Energy. **103:**128-134.

22. **Zheng, H., J. Yin, Z. Gao, H. Huang, X. Ji, and C. Dou.** 2011. Disruption of chlorella vulgaris cells for the release of biodiesel-producing lipids: A comparison of grinding, ultrasonication, bead milling, enzymatic lysis, and microwaves. Appl. Biochem. Biotechnol. **164:**1215-1224.

23. **Michelon, M., d. B. de Matos, S. R. da, C. A. V. Burkert, and M. B. de.** 2012. Extraction of carotenoids from Phaffia rhodozyma: A comparison between different techniques of cell disruption. Food Science and Biotechnology. **21:**1-8.

24. **Sawicki, R., M. Stankeviç, and G. Ginalska.** 2009. Optimization of total RNA extraction from Gram-positive bacteria (coagulase-negative staphylococci) for expression studies. Annales Universitatis Mariae Curie-Sklodowska, Sectio DDD: Pharmacia. **22:**35-38.

25. **Sadler, D. F., J. W. Ezzell Jr., K. F. Keller, and R. J. Doyle.** 1984. Glycosidase activities of Bacillus anthracis. J. Clin. Microbiol. **19:**594-598.

26. **Krishnamoorthy, K., M. Veerapandian, K. Yun, and S. J. Kim.** 2013. New function of molybdenum trioxide nanoplates: Toxicity towards pathogenic bacteria through membrane stress. Colloids and Surfaces B: Biointerfaces. 112: p 521 – 524.

27. **Christie, W. W.** - Lipid Analysis: Isolation, Separation, Identification, and Structural Analysis of Lipids. 1974, 101: p 248

28. **Harvey, D. M. R.** 1982. Freeze-substitution. J. Microsc. **127:**209-221.

29. **Melton, S. L., R. E. Moyers, and C. G. Playford.** 1979. Lipids extracted from soy products by different procedures. J. Am. Oil Chem. Soc. **56:**489-493.

30. **Amalia Kartika, I., P. Y. Pontalier, and L. Rigal.** 2006. Extraction of sunflower oil by twin screw extruder: Screw configuration and operating condition effects. Bioresour. Technol. **97:**2302-2310.

31. **Somersalo, S., P. Karunen, and E. M. Aro.** 1986. The acyl lipid composition of wheat leaves and moss protonemata using a new, non-carcinogenic extraction solvent system. Physiol. Plantarum. **68:**467-470.

32. **Kobayashi, N., E. A. Noel, A. Barnes, J. Rosenberg, C. Dirusso, P. Black, and G. A. Oyler.** 2013. Rapid detection and quantification of triacylglycerol by HPLC-ELSD in chlamydomonas reinhardtii and chlorella strains. Lipids. **48:**1035-1049.

33. **Da Porto, C., D. Decorti, and A. Natolino.** 2013. Effect of commercial enzymatic preparation with pectolytic activities on conventional extraction and ultrasound-assisted extraction of oil from grape seed (Vitis vinifera L.). International Journal of Food Science and Technology. **48:**2127-2132.

34. **Revilla, E., C. S. Maria, E. Miramontes, J. Bautista, A. García-Martínez, O. Cremades, R. Cert, and J. Parrado.** 2009. Nutraceutical composition, antioxidant activity and hypocholesterolemic effect of a water-soluble enzymatic extract from rice bran. Food Res. Int. **42:**387-393.

35. **Tian, Y., Z. Xu, B. Zheng, and Y. Martin Lo.** 2013. Optimization of ultrasonic-assisted extraction of pomegranate (Punica granatum L.) seed oil. Ultrason. Sonochem. **20:**202-208.

36. **Fromm, M., S. Bayha, R. Carle, and D. R. Kammerer.** 2012. Characterization and quantitation of low and high molecular weight phenolic compounds in apple seeds. J. Agric. Food Chem. **60:**1232-1242.

37. **Tybulewicz, V. L. J., M. L. Tremblay, M. E. LaMarca, R. Willemsen, B. K. Stubblefield, S. Winfield, B. Zablocka, E. Sidransky, B. M. Martin, S. P. Huang, K. A. Mintzer, H. Westphal, R. C. Mulligan, and E. I. Ginns.** 1992. Animal model of Gaucher's disease from targeted disruption of the mouse glucocerebrosidase gene. Nature. **357:**407-410.

38. **Park, S. -., Y. -. Choi, E. J. Kim, W. -. Park, C. W. Kim, and J. -. Yang.** 2012. Serial optimization of biomass production using microalga Nannochloris oculata and corresponding lipid biosynthesis. Bioprocess and Biosystems Engineering. **35:**3-9.

39. **ROSE, H. G., and M. OKLANDER.** 1965. IMPROVED PROCEDURE FOR THE EXTRACTION OF LIPIDS FROM HUMAN ERYTHROCYTES. J. Lipid Res. **6:**428-431.

40. **Carlson, L. A.** 1985. Extraction of lipids from human whole serum and lipoproteins and from rat liver tissue with methylene chloride-methanol: A comparison with extraction with chloroform-methanol. Clinica Chimica Acta. **149:**89-93.

41. **Parkin, K. L., and S. J. Kuo.** 1989. Chilling-induced lipid degradation in cucumber (Cucumis sativa L. cv Hybrid C) fruit. Plant Physiol. **90:**1049-1056.

42. **Yahara, S., N. Kawamura, Y. Kishimoto, T. Saida, and W. W. Tourtellotte.** 1982. A change in the cerebrosides and sulfatides in a demyelinating nervous system. Development of the methodology and study of multiple sclerosis and Wallerian degeneration. J. Neurol. Sci. **54:**303-315.

43. **Cham, B. E., and B. R. Knowles.** 1976. A solvent system for delipidation of plasma or serum without protein precipitation. J. Lipid Res. **17:**176-181.

44. **Martins, S. V., P. A. Lopes, C. M. Alfaia, P. O. Rodrigues, S. P. Alves, R. M. A. Pinto, M. F. Castro, R. J. B. Bessa, and J. A. M. Prates.** 2010. Serum adipokine profile and fatty acid composition of adipose tissues are affected by conjugated linoleic acid and saturated fat diets in obese Zucker rats. Br. J. Nutr. **103:**869-878.

45. **Matyash, V., G. Liebisch, T. V. Kurzchalia, A. Shevchenko, and D. Schwudke.** 2008. Lipid extraction by methyl-terf-butyl ether for high-throughput lipidomics. J. Lipid Res. **49:**1137-1146.

46. **McGrath, L. T., and R. J. Elliott.** 1990. Lipid analysis and fatty acid profiles of individual arterial atherosclerotic plaques. Anal. Biochem. **187:**273-276.

47. **Kim, B. K., P. K. Park, H. J. Chae, and E. Y. Kim.** 2004. Effect of phenol on ß-carotene content in total carotenoids production in cultivation of Rhodotorula glutinis. Korean Journal of Chemical Engineering. **21:**689-692.

48. **Allen, P. C.** 1972. New extraction method for nematode lipids. Anal. Biochem. **45:**253-259.

49. **Lee, J. -., C. Yoo, S. -. Jun, C. -. Ahn, and H. -. Oh.** 2010. Comparison of several methods for effective lipid extraction from microalgae. Bioresour. Technol. **101:**S75-S77.

50. **Du, K., X. L. Sun, Y. M. Sun, and L. Chen.** 2010. Selection of carbon source and nitrogen source for microbial lipid production by fermentation with Trichosporon fermentans. China Oils and Fats. **35:**35-38.

51. **Sobus, M. T., and C. E. Holmlund.** 1976. Extraction of lipids from yeast. Lipids. **11:**341-348.

52. **Lee, J., C. Yoo, S. Jun, C. Ahn, and H. Oh.** 2010. Comparison of several methods for effective lipid extraction from microalgae. Bioresour. Technol. **101:**S75-S77. doi: 10.1016/j.biortech.2009.03.058.

53. **Gerde, J. A., M. Montalbo-Lomboy, L. Yao, D. Grewell, and T. Wang.** 2012. Evaluation of microalgae cell disruption by ultrasonic treatment. Bioresour. Technol. **125:**175-181.

54. **Sheng, J., R. Vannela, and B. E. Rittmann.** 2011. Evaluation of methods to extract and quantify lipids from Synechocystis PCC 6803. Bioresour. Technol. **102:**1697-1703.

55. **Shin, J., Y. Hwang, O. Tu, H. Jo, J. Kim, Y. Chae, K. Rhu, and S. Park.** 2013. Comparison of different methods to quantify fat classes in bakery products. Food Chem. **136:**703-709. doi: 10.1016/j.foodchem.2012.08.033.

56. **Moellering, E. R., and C. Benning.** 2010. RNA interference silencing of a major lipid droplet protein affects lipid droplet size in Chlamydomonas reinhardtii. Eukaryotic Cell. **9:**97-106.

57. **Burke, J.** 1984. Solubility Parameters: Theory and Application. 3: p 03-04.

58. **Pulz, O., and W. Gross.** 2004. Valuable products from biotechnology of microalgae. Appl. Microbiol. Biotechnol. **65:**635-648.

59. **Octave, S., and D. Thomas.** 2009. Biorefinery: Toward an industrial metabolism. Biochimie. **91:**659-664. doi: 10.1016/j.biochi.2009.03.015.

60. **Mata, T. M., A. A. Martins, and N. S. Caetano.** 2010. Microalgae for biodiesel production and other applications: A review. Renewable and Sustainable Energy Reviews. **14:**217-232. doi: 10.1016/j.rser.2009.07.020.

61. **Plaza, M., S. Santoyo, L. Jaime, G. García-Blairsy Reina, M. Herrero, F. J. Señoráns, and E. Ibáñez.** 2010. Screening for bioactive compounds from algae. J. Pharm. Biomed. Anal. **51:**450-455. doi: 10.1016/j.jpba.2009.03.016.

62. **Cheng, J. J., and G. R. Timilsina.** 2011. Status and barriers of advanced biofuel technologies: A review. Renewable Energy. **36:**3541-3549. doi: 10.1016/j.renene.2011.04.031.

63. **Duong, V. T., Y. Li, E. Nowak, and P. M. Schenk.** 2012. Microalgae isolation and selection for prospective biodiesel production. Energies. **5:**1835-1849.

64. **Andrich, G., A. Zinnai, U. Nesti, F. Venturi, and R. Fiorentini.** 2006. Supercritical fluid extraction of oil from microalga Spirulina (Arthrospira) platensis. Acta Aliment. **35:**195-203.

65. **Herrero, M., A. Cifuentes, and E. Ibañez.** 2006. Sub- and supercritical fluid extraction of functional ingredients from different natural sources: Plants, food-by-products, algae and microalgae: A review. Food Chem. **98:**136-148. doi: 10.1016/j.foodchem.2005.05.058.

66. **Rosenthal, A., D. L. Pyle, and K. Niranjan.** 1996. Aqueous and enzymatic processes for edible oil extraction. Enzyme Microb. Technol. **19:**402-420. doi: 10.1016/S0141-0229(96)80004-F.

67. **BLIGH, E. G., and W. J. DYER.** 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology. **37:**911-917.

68. **Zheng, H., Z. Gao, F. Yin, X. Ji, and H. Huang.** 2012. Lipid production of Chlorella vulgaris from lipid-extracted microalgal biomass residues through two-step enzymatic hydrolysis. Bioresour. Technol. **117:**1-6. doi: 10.1016/j.biortech.2012.04.007.

69. **Prabakaran, P., and A. D. Ravindran.** 2011. A comparative study on effective cell disruption methods for lipid extraction from microalgae. Lett. Appl. Microbiol. **53:**150-154.

70. **Lee, J., C. Yoo, S. Jun, C. Ahn, and H. Oh.** 2010. Comparison of several methods for effective lipid extraction from microalgae. Bioresour. Technol. **101:**S75-S77. doi: 10.1016/j.biortech.2009.03.058.

71. **Mercer, P., and R. E. Armenta.** 2011. Developments in oil extraction from microalgae. European Journal of Lipid Science and Technology. **113:**539-547.

72. **Ryckebosch, E., S. P. C. Bermúdez, R. Termote-Verhalle, C. Bruneel, K. Muylaert, R. Parra-Saldivar, and I. Foubert.** 2013. Influence of extraction solvent system on the extractability of lipid components from the biomass of Nannochloropsis gaditana. J. Appl. Phycol. 26: pp 1501-1510.

73. **Li, Y., F. Fine, A. -. Fabiano-Tixier, M. Abert-Vian, P. Carre, X. Pages, and F. Chemat.** 2013. Evaluation of alternative solvents for improvement of oil extraction from rapeseeds. Comptes Rendus Chimie. 17: p 242 – 251.

74. **Gini, T. G., and G. J. Jothi.** 2013. Preliminary phytochemical screening of whole plant extracts of peperomia pellucida (linn.) HBK (piperaceae) and marsilea quadrifolia linn. (marsileaceae). International Journal of Pharmacognosy and Phytochemical Research. **5:**200-214.

75. **Tir, R., P. C. Dutta, and A. Y. Badjah-Hadj-Ahmed.** 2012. Effect of the extraction solvent polarity on the sesame seeds oil composition. European Journal of Lipid Science and Technology. **114:**1427-1438.

76. **Zhang, L., Y. Wan, P. Chen, and R. Ruan.** 2009. A high oil corn and its biorefining model for biofuel production, 8: p. 5229-5240. *In* Anonymous American Society of Agricultural and Biological Engineers Annual International Meeting 2009.

77. **Boldor, D., A. Kanitkar, C. Sabliov, and S. Balasubramanian.** 2009. Lipid extraction from rice bran and Chinese tallow tree (CTT) using microwaves, p. 3702-3705. *In* Anonymous American Society of Agricultural and Biological Engineers Annual International Meeting 2009.

78. **Li, J., S. Matsumoto, A. Nakamura, H. Maeda, and Y. Matsumura.** 2009. Characterization and functional properties of sub-fractions of soluble soybean polysaccharides. Bioscience, Biotechnology and Biochemistry. **73:**2568-2575.

79. **Ramluckan, K., K. G. Moodley, and F. Bux.** 2014. An evaluation of the efficacy of using selected solvents for the extraction of lipids from algal biomass by the soxhlet extraction method. Fuel. **116:**103-108. doi: http://dx.doi.org/10.1016/j.fuel.2013.07.118.

80. **Fajardo, A. R., L. E. Cerdán, A. R. Medina, F. G. A. Fernández, P. A. G. Moreno, and E. M. Grima.** 2007. Lipid extraction from the microalga Phaeodactylum tricornutum. European Journal of Lipid Science and Technology. **109:**120-126.

81. **Kaparthi, R., and K. S. Chari.** 1959. Solubilities of vegetable oils in aqueous ethanol and ethanol-hexane mixtures. J. Am. Oil Chem. Soc. **36:**77-80.

82. **Virot, M., V. Tomao, G. Colnagui, F. Visinoni, and F. Chemat.** 2007. New microwave-integrated Soxhlet extraction: An advantageous tool for the extraction of lipids from food products. Journal of Chromatography A. **1174:**138-144. doi: 10.1016/j.chroma.2007.09.067.

83. **Luque de Castro, M. D., and F. Priego-Capote.** 2010. Soxhlet extraction: Past and present panacea. Journal of Chromatography A. **1217:**2383-2389. doi: 10.1016/j.chroma.2009.11.027.

84. **Manirakiza, P., A. Covaci, and P. Schepens.** 2001. Comparative Study on Total Lipid Determination using Soxhlet, Roese-Gottlieb, Bligh & Dyer, and Modified Bligh & Dyer Extraction Methods. Journal of Food Composition and Analysis. **14:**93-100. doi: 10.1006/jfca.2000.0972.

85. **de Boer, J.** 1988. Chlorobiphenyls in bound and non-bound lipids of fishes; comparison of different extraction methods. Chemosphere. **17:**1803-1810. doi: 10.1016/0045-6535(88)90108-7.

86. **Kraujalis, P., P. R. Venskutonis, A. Pukalskas, and R. Kazernaviciute.** 2013. Accelerated solvent extraction of lipids from Amaranthus spp. seeds and characterization of their composition. LWT - Food Science and Technology. **54:**528-534.

87. **Da Porto, C., D. Decorti, and A. Natolino.** 2013. Effect of commercial enzymatic preparation with pectolytic activities on conventional extraction and ultrasound-assisted extraction of oil from grape seed (Vitis vinifera L.). International Journal of Food Science and Technology. **48:**2127-2132.

88. **BLIGH, E. G., and W. J. DYER.** 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology. **37:**911-917.

89. **FOLCH, J., M. LEES, and G. H. SLOANE STANLEY.** 1957. A simple method for the isolation and purification of total lipides from animal tissues. The Journal of Biological Chemistry. **226:**497-509.

90. **Smedes, F., and T. K. Askland.** 1999. Revisiting the development of the Bligh and Dyer total lipid determination method. Mar. Pollut. Bull. **38:**193-201.

91. **Iverson, S. J., S. L. C. Lang, and M. H. Cooper.** 2001. Comparison of the bligh and dyer and folch methods for total lipid determination in a broad range of marine tissue. Lipids. **36:**1283-1287.

92. **Roose, P., and F. Smedes.** 1996. Evaluation of the results of the QUASIMEME lipid intercomparison: The Bligh and Dyer total lipid extraction method. Mar. Pollut. Bull. **32:**674-680.

93. **Iverson, S. J., K. J. Frost, and L. F. Lowry.** 1997. Fatty acid signatures reveal fine scale structure of foraging distribution of harbor seals and their prey in Prince William Sound, Alaska. Mar. Ecol. Prog. Ser. **151:**255-271.

94. **Payne, S. A., B. A. Johnson, and R. S. Otto.** 1999. Proximate composition of some north-eastern pacific forage fish species. Fish. Oceanogr. **8:**159-177.

95. **Linko, R. R., J. K. Kaitaranta, and R. Vuorela.** 1985. Comparison of the fatty acids in Baltic herring and available plankton feed. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry. **82:**699-705. doi: http://dx.doi.org/10.1016/0305-0491(85)90511-5.

96. **Wamberg, S., C. R. Olesen, and H. O. Hansen.** 1992. Influence of dietary sources of fat on lipid synthesis in mink (Mustela vison) mammary tissue. Comparative Biochemistry and Physiology - A Physiology. **103:**199-204.

97. **Arnould, J. P., I. L. Boyd, and A. Clarke.** 1995. A simplified method for determining the gross chemical composition of pinniped milk samples. Can. J. Zool. **73:**404-410.

98. **Cooksey, K. E., J. B. Guckert, S. A. Williams, and P. R. Callis.** 1987. Fluorometric determination of the neutral lipid content of microalgal cells using Nile Red. J. Microbiol. Methods. **6:**333-345.

99. **Feng, G. -., F. Zhang, L. -. Cheng, X. -. Xu, L. Zhang, and H. -. Chen.** 2013. Evaluation of FT-IR and Nile Red methods for microalgal lipid characterization and biomass composition determination. Bioresour. Technol. **128:**107-112.

100. **Wasson, J. R.** 1978. Nuclear magnetic resonance spectrometry. Anal. Chem. **50:**121R-130R.

101. **Todt, H., W. Burk, G. Guthausen, A. Guthausen, A. Kamlowski, and D. Schmalbein.** 2001. Quality control with time-domain NMR. European Journal of Lipid Science and Technology. **103:**835-840.

102. **Rudi, T., G. Guthausen, W. Burk, C. T. Reh, and H. -. Isengard.** 2008. Simultaneous determination of fat and water content in caramel using time domain NMR. Food Chem. **106:**1375-1378. doi: 10.1016/j.foodchem.2007.04.079.

103. **Pereira, F. M. V., and L. A. Colnago.** 2012. Determination of the Moisture Content in Beef Without Weighing Using Benchtop Time-Domain Nuclear Magnetic Resonance Spectrometer and Chemometrics. Food Analytical Methods. **5:**1349-1353.

104. **Castell-Palou, A., C. Rosselló, A. Femenia, J. Bon, and S. Simal.** 2011. Moisture profiles in cheese drying determined by TD-NMR: Mathematical modeling of mass transfer. J. Food Eng. **104:**525-531. doi: 10.1016/j.jfoodeng.2011.01.010.

105. **Gao, C., W. Xiong, Y. Zhang, W. Yuan, and Q. Wu.** 2008. Rapid quantitation of lipid in microalgae by time-domain nuclear magnetic resonance. J. Microbiol. Methods. **75:**437-440.

106. **Jones, J., S. Manning, M. Montoya, K. Keller, and M. Poenie.** 2012. Extraction of algal lipids and their analysis by HPLC and mass spectrometry. JAOCS, Journal of the American Oil Chemists' Society. **89:**1371-1381.

107. **Vieler, A., C. Wilhelm, R. Goss, R. Süß, and J. Schiller.** 2007. The lipid composition of the unicellular green alga Chlamydomonas reinhardtii and the diatom Cyclotella meneghiniana investigated by MALDI-TOF MS and TLC. Chem. Phys. Lipids. **150:**143-155. doi: 10.1016/j.chemphyslip.2007.06.224.

108. **Touchstone, J. C.** 1995. Thin-layer chromatographic procedures for lipid separation. Journal of Chromatography B: Biomedical Sciences and Applications. **671:**169-195. doi: 10.1016/0378-4347(95)00232-8.

109. **Ando, S., and M. Saito.** Chapter 9 TLC and HPTLC of Phospholipids and Glycolipids in Health and Disease, p. 266-310. *In* Anonymous Journal of Chromatography Libraryvol. Volume 37. Elsevier.

110. **Johnson, K. R., G. Ellis, and C. Toothill.** 1977. The sulfophosphovanillin reaction for serum lipids: a reappraisal. Clin. Chem. **23:**1669-1678.

111. **Laurens, L. M. L., M. Quinn, S. Van Wychen, D. W. Templeton, and E. J. Wolfrum.** 2012. Accurate and reliable quantification of total microalgal fuel potential as fatty acid methyl esters by in situ transesterification. Analytical and Bioanalytical Chemistry. **403:**167-178.

112. **Elsey, D., D. Jameson, B. Raleigh, and M. J. Cooney.** 2007. Fluorescent measurement of microalgal neutral lipids. J. Microbiol. Methods. **68:**639-642.

113. **Laurens, L. M. L., and E. J. Wolfrum.** 2011. Feasibility of spectroscopic characterization of algal lipids: Chemometric correlation of NIR and FTIR Spectra with exogenous lipids in algal biomass. Bioenergy Research. **4:**22-35.

114. **Han, Y., Q. Wen, Z. Chen, and P. Li.** 2011. Review of Methods Used for Microalgal Lipid-Content Analysis. Energy Procedia. **12:**944-950. doi: 10.1016/j.egypro.2011.10.124.

115. **Sitepu, I. R., L. Ignatia, A. K. Franz, D. M. Wong, S. A. Faulina, M. Tsui, A. Kanti, and K. Boundy-Mills.** 2012. An improved high-throughput Nile red fluorescence assay for estimating intracellular lipids in a variety of yeast species. J. Microbiol. Methods. **91:**321-328.

116. **Zuriani, R., S. Vigneswari, M. N. M. Azizan, M. I. A. Majid, and A. A. Amirul.** 2013. A high throughput nile red fluorescence method for rapid quantification of intracellular bacterial polyhydroxyalkanoates. Biotechnology and Bioprocess Engineering. **18**: pp 472-478

117. **Chen, W., M. Sommerfeld, and Q. Hu.** 2011. Microwave-assisted Nile red method for in vivo quantification of neutral lipids in microalgae. Bioresour. Technol. **102:**135-141.

118. **Genicot, G., J. L. M. R. Leroy, A. Van Soom, and I. Donnay.** 2005. The use of a fluorescent dye, Nile red, to evaluate the lipid content of single mammalian oocytes. Theriogenology. **63:**1181-1194.

119. **Lee, S. J., B. -. Yoon, and H. -. Oh.** 1998. Rapid method for the determination of lipid from the green alga Botryococcus braunii. Biotechnol. Tech. **12:**553-556.

120. **Liu, X., Z. Wang, J. Yu, B. Lü, L. Ma, and Z. Chen.** 2013. Improved fluorescence spectrometric determination of lipid content in Botryococcus braunii. Shengwu Gongcheng Xuebao/Chinese Journal of Biotechnology. **29:**382-391.

121. **Kimura, K., M. Yamaoka, and Y. Kamisaka.** 2004. Rapid estimation of lipids in oleaginous fungi and yeasts using Nile red fluorescence. J. Microbiol. Methods. **56:**331-338.

122. **Kou, Z., S. Bei, J. Sun, and J. Pan.** 2013. Fluorescent measurement of lipid content in the model organism Chlamydomonas reinhardtii. J. Appl. Phycol. **25:**1633-1641.

123. **Fowler, S. D., W. J. Brown, J. Warfel, and P. Greenspan.** 1987. Use of nile red for the rapid in situ quantitation of lipids on thin-layer chromatograms. J. Lipid Res. **28:**1225-1232.

124. **Croney, J. C., D. M. Jameson, and R. P. Learmonth.** 2001. Fluorescence spectroscopy in biochemistry: teaching basic principles with visual demonstrations. Biochemistry and Molecular Biology Education. **29:**60-65. doi: http://dx.doi.org/10.1016/S1470-8175(01)00019-4.

125. **Ricchelli, F.** 1989. Principles of fluorescence spectroscopy : by Joseph R. Lakowicz; published by Plenum, New York, 1986, 3rd edn.; 496 pp.; price, U.S. $40; ISBN 0-306-41285-3. Journal of Photochemistry and Photobiology B: Biology. **3:**289-290. doi: http://dx.doi.org/10.1016/1011-1344(89)80070-3.

126. **Zhang, L. -., X. -. Cao, W. -. Cai, and Y. -. Li.** 2011. Observations of the effect of confined space on fluorescence and diffusion properties of molecules in single conical nanopore channels. J. Fluoresc. **21:**1865-1870.

127. **Hungerford, G., and J. A. Ferreira.** 2001. The effect of the nature of retained solvent on the fluorescence of Nile Red incorporated in sol-gel-derived matrices. J Lumin. **93:**155-165.

128. **Hawe, A., M. Sutter, and W. Jiskoot.** 2008. Extrinsic fluorescent dyes as tools for protein characterization. Pharm. Res. **25:**1487-1499.

129. **Dutta, A. K., K. Kamada, and K. Ohta.** 1996. Spectroscopic studies of nile red in organic solvents and polymers. J. Photochem. Photobiol. A. **93:**57-64.

Chapter 3: Quantitative determination of lipid content using Nile red fluorometry

Abstract

The spectra of Nile red fluorescence of pure, binary and ternary systems of oil, ethanol and water were measured to elucidate the fluorescence of these systems at different ratios. It was also determined that the optimal ethanol/water ratio as solvent for determination of lipid content using Nile red fluorometry was 20% (v/v) and the linear range was 0.1 to 0.5 mg oil/mL in 20% ethanol aqueous solution. Based on these understandings, a Nile red fluorescence method was proposed for high throughput analysis of triglycerides, which is the main composition of lipids produced by oil crops such as plants and microalgae. The new assay was found to produce results that matched well with the lipid content determined by gravimetric determination for plant seeds. It offers some important advantages over conventional methods of lipid measurement such as: (1) requiring a small amount of biomass samples; (2) easy, time-saving and cost-effective; (3) high throughput, allowing the measurement of a large number of samples simultaneously; (4) not sensitive to the structure of triglycerides and therefore applicable for determination of lipid contents of different biomass samples; and (5) having a reasonably large linear range and repeatable results.

Keywords: Nile red, fluorometry, lipid content, lipid analysis

3.1 Introduction

Lipids are an important group of cellular compounds which provide several biological functions such as energy storage, signaling and cell membrane structure (1-3). For these reasons, lipid measurement and analytical methods are required in many different areas. For example, the health conditions of cells growth and culture can be monitored by the analysis of cell lipid content (4, 5). In addition, rapid lipid and high throughput measurement methods are needed for screening oleaginous organisms and optimizing cultivation conditions for cost-effective production of lipids as feedstock for biofuels, foods, nutraceuticals and other novel and bulk products (6-8).

Several methods have been developed to quantify lipid content. For instance, the most commonly used conventional method, the gravimetric method, involves drying, grinding, solvent extraction of lipids from dry samples and gravimetric determination of lipid content, which takes a long time and requires a large amount of sample (9). Furthermore, the results of the gravimetric method represent the total content of all lipophilic materials contained in a sample. Some more sophisticated methods for the quantification of neutral lipids require the separation of crude extracts and quantification of lipid fractions by thin-layer chromatography (TLC), HPLC and gas chromatography (GC) (10-12). They require relatively small quantities of samples but require tedious sample preparation procedures. As a result, increasing attention has focused on developing relatively easy measurements of lipid content (13, 14).

The colorimetric sulfo-phospho-vanillin (SPV) method developed by Chabrol et al. (15) is an attractive alternative for lipid measurement as it is a rapid method requiring a small amount of sample. However, Cheng et al. (1), when they applied this approach for the measurement of vegetable oil, animal lipid, or purified microalga extract samples, reported that the response of the SPV approach was highly dependent on the source of the oil. For instance, it was reported that the mean absorbance/lipid ratio, and therefore the conversion factor, was 0.00755 a.u./μg and 0.00554 a.u./μg for canola oil and cod liver oil, respectively, representing a difference of 36.3% between vegetable oils from the two different plant seeds, probably due to a high degree of dependence of the SPV response on the fatty acid composition of lipids. This could be problematic since the fatty acid composition of triglycerides may vary not only between different source organisms but also the same organism produced under different environments such as seasons, climates, and physiological states. As a result, the results of the SPV approach may become very uncertain. In addition, it was also noticed by Cheng et al. (1) that the SPV response was very sensitive to the composition of the diluting solvent. In fact, it was observed by the same authors (1) that only the samples prepared in a chloroform: methanol mixture of 2:1 showed detectable change of fluorescent responses corresponding to oil concentration gradient. In other words, a small change in the solvent ratio, which is almost inevitable in practical measurements, may result in significantly different measurement results. These observations indicate that there are still significant technical hurdles to overcome before the SPV method matures as a lipid analytic

method in practical applications.

Nile red, a lipid soluble fluorescent dye, has also been proposed for evaluating the lipid content of microorganisms or animal cells, such as microalgae, yeasts, bacteria and mammalian cells (16-19). The fluorescence of Nile red was first suggested by Greenspan et al. (20) for staining and visualization of lipid droplets in cells, a qualitative approach for cell lipid content evaluation. In the past several years, this method has been further modified for quantification of lipids (21, 22).

Most Nile red fluorescence methods for quantitative determination of lipid cell content that have been reported to date involve the staining of intact cells, which may be enhanced by different mechanisms such as heating (23), sonication (24), or microwaving (18). However, these approaches are compromised to different extents by the fact that cells and other solids in cell suspension may scatter and absorb both excitation and emission light. Furthermore, the thick and rigid cell walls commonly found in many kinds of cells and the cytoplasmic membrane, which is a phospholipid bilayer, may block the penetration of Nile red dye across the cell envelope to reach the intracellular lipid droplets, causing potential inaccuracy in fluorescence intensity measurement. As a result, these methods commonly encounter problems such as small linear range, organism-dependency, and poor repeatability.

In 1987, Fowler et al (25) reported an attempt using Nile red fluorometry for

quantifying extracted lipids. In this case, Nile red was used as a general purpose reagent for rapid lipid detection and quantitation after lipids were separated by thin-layer chromatography (TLC). The detection limit of the assay was 25-100 μg/mL for triacylglycerols. Since lipids were separated by thin-layer chromatography (TLC) before quantification, the disadvantages of TLC were significant such as spots are often faint, and results that are difficult to reproduce, due to uneven advance of solvent, front streaking and spotting.

The present work explores the possibility of using Nile red fluorescence for quantification of lipids in different biomass samples. To avoid the interference of cellular materials, lipids were extracted before measurement. A high throughput microplate fluorometer was used to enable faster measurement of multiple samples. Since solvent has significant impacts on Nile red fluorescence, efforts were also made to study the Nile red fluorescence of ethanol and ethanol aqueous solution of different concentrations containing or not containing lipids as ethanol has been a commonly used solvent in the measurement of lipid Nile red fluorescence (24) .

3.2 Materials and Methods

3.2.1 Nile red fluorescence in pure ethanol, water, and triglycerides

Anhydrous ethanol (Fisher Scientific, Canada), deionized water, and sunflower oil (>99%, Messina, Canada) were used for the test of Nile red fluorescence in pure ethanol, water, and triglycerides, respectively. In these experiments, 50 μL of 250

ppm Nile red (Sigma, Canada) acetone solution was added to 2.5 mL of anhydrous ethanol, deionized water, or sunflower oil. After mixing by means of vigorous hand shaking, 50 μL of the mixture was transferred to a 100 μL black 96-well microplate. The fluorescence intensity values (FIV) of samples were scanned immediately over an emission wavelength range of 540-700 nm with an interval of 10 nm at a fixed excitation wavelength of 490 nm. All Nile red fluorescence experiments were carried out at room temperature with a fluorometer (Westinghouse, Canada).

3.2.2 Binary systems

Nile red fluorescence in three different binary systems, i.e., water/ethanol, oil/ ethanol, and oil/ water systems, was tested at different concentrations. The oil/ethanol binary systems of different oil concentration in ethanol, which was in the range of 0.04 -8.0 mg/mL, were prepared by adding appropriate amount of oil into anhydrous ethanol. Similarly, ethanol/water binary systems in the range of 5%- 50% (v/v) were prepared by adding an appropriate volume of anhydrous ethanol into deionized water. Different from the oil/ethanol and ethanol/water systems, which were miscible in the corresponding concentration ranges, the oil/water binary systems of different oil concentration in water, which was in the range of 2.0-8.0 mg/mL, were prepared by adding appropriate amount of oil into deionized water. In these experiments, after binary systems preparation, 50 μL of 250 ppm Nile red acetone solution was added into 2.5 mL binary mixtures which were then homogenized using an ultrasonic bath (FS140, Fisher Scientific, Canada) at 42 KHz and 135 W for 30 min. Finally, 50 μL of

the homogenized mixture was transferred to a 100 μL black 96-well microplate, in which the FIV of samples were scanned immediately over an emission wavelength range of 540-700 nm with an interval of 10 nm at a fixed excitation wavelength of 490 nm. All Nile red fluorescence experiments were carried out at room temperature with a fluorometer.

3.2.3 Ternary systems

The ternary system was composed of oil, ethanol and water. The oil content in these ternaries was always kept at 0.4 mg/mL, while the ratio of ethanol and water varied. To prepare ternaries, ethanol aqueous solutions of 10%, 20%, 30%, 40%, and 50% (v/v) were prepared before 0.4 mg/mL sunflower oil was added to make the ternaries have the same oil concentration (i.e., 0.4 mg/mL) but different ethanol and water ratios. Then, 2.5 mL of a ternary was mixed with 50 μL 250 ppm Nile red acetone solution. After mixing by means of 30 min ultrasonic treatment, 50 μL of the mixture was transferred to a 100 μL black 96-well microplate. The FIV of samples were scanned immediately over an emission wavelength range of 540-700 nm with an interval of 10 nm at a fixed excitation wavelength of 490 nm or over an excitation wavelength range of 550-700 nm with an interval of 10 nm at a fixed emission wavelength of 590 nm. All Nile red fluorescence experiments were carried out at room temperature with the fluorometer specified in Section 3.2.1.

3.2.4 Nile red fluorescence of triglycerides/water/ethanol ternary systems at fixed excitation and emission frequencies

Sunflower oil and olive oil (Loblaws lnc, Toronto, ON, Produce of USA), and three analytic grade lipid standards, i.e., glyceryl trioleate T7140-500MG, and T2630 - glyceryl tripalmitoleate (Sigma, Canada) were used for measurement the Nile red fluorescence of ternary systems containing one of these lipid samples at fixed excitation (490 nm) and emission (590 nm) wavelengths. To prepare these ternary systems, appropriate amount of lipid samples were added into pre-prepared ethanol aqueous solution of 20% (v/v) to make ternary sytesms with lipid concentration in the range of 0.1-0.6 mg/mL. Then, 50 μL of 250 ppm Nile red acetone solution was added into 2.5 mL of the ternary mixture, which was then homogenized using ultrasonic treatment for 30 min. Finally, 50 μL of the mixture was transferred to a 100 μL black 96-well microplate, which was then placed in the fluorometer for reading FIV of samples at an emission wavelength of 590 nm and excitation wavelength of 490 nm.

3.2.5 Microalgal strain and cultivation conditions

N. oleoabundans UTEX 1185, a green microalga strain purchased from the microalgae culture collection at the University of Texas in Austin, was cultivated at 30±3°C with continuous illumination at a photosynthetic photon flux density (PPFD) of 360 μmol/($m^2 \cdot s^1$) in a modified soil extract (SE) medium, which was composed of (g/L) $MgSO_4$ (37), $FeCl_3$ (3), $CaCl_2$·2H2O (25), NaCl (0.025), $NaNO_3$ (450), KH_2PO_4 (75), K_2HPO_4 (175), EDTA-Fe (1.642), H_3BO_3 (2.860), $MnCl_2 \cdot 4H_2O$ (1.810),

$ZnSO_4 \cdot 7H_2O$ (0.220), $CuSO_4 \cdot 5H_2O$ (0.079), and $(NH_4)_6M_{O7}O_{24} \cdot 4H_2O$ (0.039) (26). The cultivation bottles were 1,000-mL glass columniform flasks with a height of approximately 18 cm and an inner diameter of approximately 8.5 cm. The volume of working was approximately 750 mL. An enriched air stream containing 5% CO_2 was saturated with distilled water firstly by passing through a glass 500-mL water bottle and then was bubbled into the cultivation bottle from the bottom at a flow rate of 0.5 vvm using a 0.47 μm microfiltration cartridge. The cultivation bottles were located inside a cubic incubator box, which was installed with 16 fluorescent lamps (tubes). Temperature inside the incubator was controlled by forced circulation of ambient air using two fans fixed on two sides of the box. Agitation was achieved by combined a magnetic stirrer and bubbling. The samples were taken from microalgae cultures after 10 days.

3.2.6 Gravimetric determination of lipid content using Soxhlet extraction

Lipids of approximately 1 g dry samples of different oil crops including sesame seeds (Easy & Busy Ltd. Product of India), peanuts (imported for Uncle T Food., B.C. Canada. Product of China), and sunflower seeds (Wing On New Group Canada Inc., Product of China) and microalgal biomass (*N. oleoabundans* UTEX 1185) were extracted with 300 mL ethyl ether in a Soxhlet extractor. The samples were first dried in an oven at 95ºC overnight. The dried seeds or microalgal biomass was then ground to powder using a pestle and mortar and put in the thimble of the Soxhlet extractor for extraction with reflux at 65ºC for 8-16 h. At the end of the extraction, the extractant

was distilled at 50ºC to evaporate ethyl ether, followed by drying the residue at 80ºC for 2h and weighing after cooling to room temperature in a desiccator. Lipid content was calculated by dividing the residue weight with the dry samples weight as follows (27):

C_{lipid}= RW/DCW*% (3-1)

Where C_{lipid} is lipid content, RW is residue weight and DCW is dry samples weight.

3.2.7 Nile red fluorescence for measurement of lipid content of different oil crops

As illustrated in Figure 3-1, 10 mg dry sample was ground to powder, which was then mixed with 8 mL anhydrous ethanol and 2 mL hexane. Then the mixture was gently shaken for 30 min at 40ºC on a shaker (Fisher Scientific, Canada) (28) at 200 rpm. The extract was centrifuged at 14,000 g for 30 min and the supernatant was transferred to a beaker. The cell debris was then washed three times using 10 mL of 1:4 hexane: ethanol mixture each. Supernatants were combined and evaporated overnight in an incubator at 55ºC until the solvent evaporated completely. Ethanol aqueous solution of 20% (v/v) was then added to the lipid extracts to a total volume of 20 mL and homogenized by vortexing. A volume of 10 mL of the mixture was mixed with 200 µL of 250 ppm Nile red acetone solution in a 15-mL round bottom polypropylene centrifuge tube (Fisher scientific, Canada). The mixture was ultrasonicated (135 W, 40 kHz) for 30min, before transferring 50 µL of the mixture immediately to a cell on a 96-well black Varian® microplate. Fluorescence was read using medium scan control and high PMT detector voltage mode as described by

Chen et al. (23) in the fluorometer at 490 nm excitation wavelength and 590 nm emission wavelength at room temperature (24).

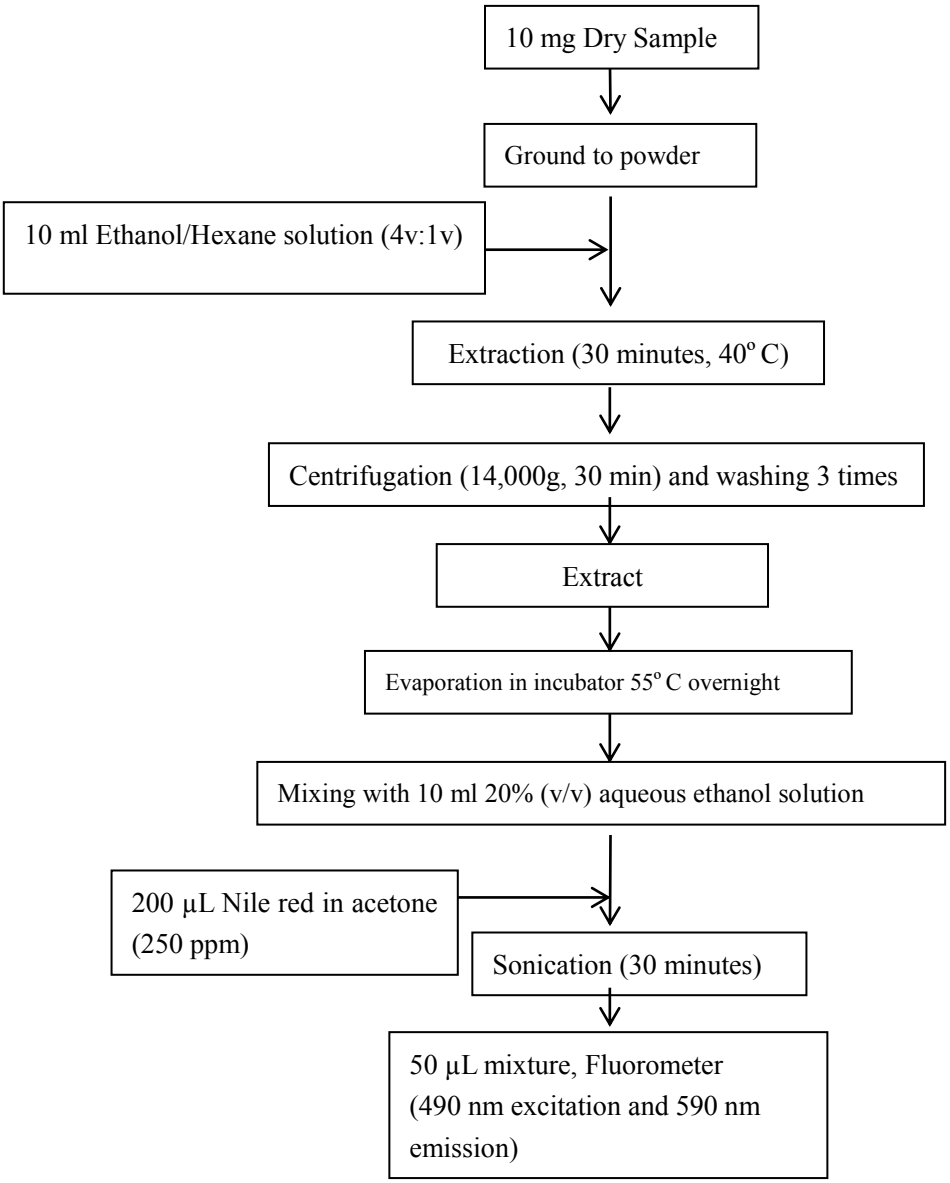


Figure 3-1 Evaluation of lipids content for dry samples using Nile red fluorescence method

The lipid content of samples was calculated by the following equation:

$$C_{Lipid}\,(g/g) = \frac{(FIV + intercept) * V\ (ml)}{\text{Conversion factor (a.u.}-\text{mL/mg}) * M0(mg)} \qquad (3\text{-}2)$$

Where C_{Lipid} (g/g) is neutral lipid content, FIV (absorbance unit, a.u.) is the value of

fluorescence intensity of the Nile red stained at excitation and emission wavelengths of 490nm and 590 nm, respectively. V is the volume of 20% aqueous ethanol (10.2 mL) used as solvent, and the conversion factor was 2581.0 a.u.-mL/mg, which was pre-determined as the slope of the linear FIV vs lipid concentration curve generated with standard lipid samples (Figure 3-13) and the intercept was 92.4. M_0 is the dry sample weight for the extraction (mg).

3.3 Result and Discussion

3.3.1 Nile red fluorescence in oil/ethanol binary systems

The emission spectra of Nile red in anhydrous ethanol and several different concentrations of oil/ethanol binary systems are shown in Figure 3-2. It is clear that all the emission spectra of oil/binary systems with oil contents ranging from 0.04 mg/mL to 8.0 mg/mL were similar to that of the spectrum of the pure anhydrous ethanol, with a maximum fluorescence intensity of approximately 3000 a.u. at around 635 nm. These results suggest that the addition of oil of up to 8 mg/mL into ethanol did not have an impact on the Nile red fluorescence of ethanol. In other words, the lack of the characteristic peak of lipid fluorescence at 590 nm suggests the repression of it by ethanol in the binaries. Apparently, using anhydrous ethanol as solvent would not allow accurate measurement of oil concentration in samples.

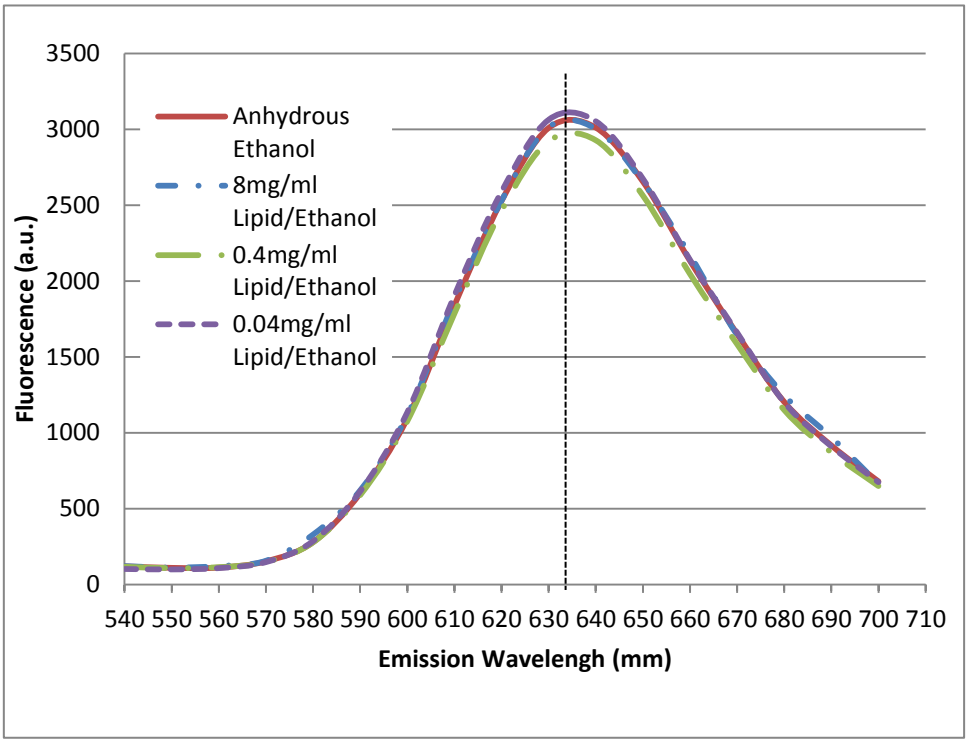


Figure 3-2 Fluorescence emission spectra of Nile red in Oil/Ethanol binaries of different concentrations: (a) Anhydrous Ethanol, (b) 8 mg/mL lipid/ ethanol, (c) 0.4 mg/mL lipid/ ethanol, (d) 0.04 mg/mL lipid/ethanol. The excitation wavelength used was 490nm and the emission wavelength scanned was from 540 nm to 700 nm. Error bars were omitted for clarity since they were small.

3.3.2 Fluorescence of Nile red in ethanol/water binary systems

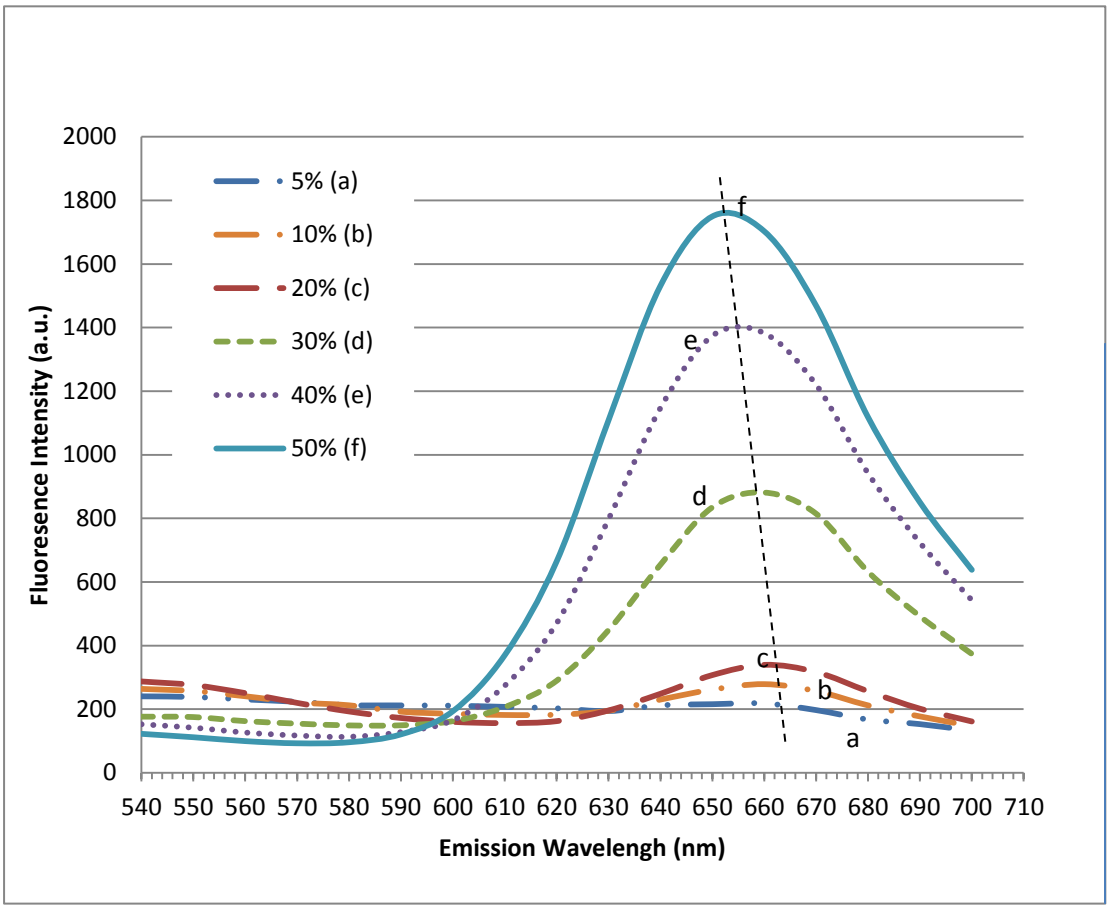


Figure 3-3, Emission spectra of Nile red in ethanol aqueous solution of different concentration: (a) 5% (v/v), (b) 10% (v/v), (c) 20% (v/v), (d) 30% (v/v), (e) 40% (v/v), and (f) 50% (v/v). The excitation wavelength used was 490 nm and the range of emission wavelength scanned was 540 - 700 nm. The results of spectra were mean values of triplicates. Error bars were omitted for clarity since they were small.

To investigate Nile red fluorescence in ethanol/water binary systems, 2.5 mL of ethanol aqueous solutions of five different concentrations ranging from 5% to 50% (v/v) were mixed with 50 µL of 250 ppm Nile red acetone solutions at room temperature and then the fluorescence emission spectra were scanned in the range of 540-700 nm at excitation wavelength of 490 nm. As shown by the dotted line in

Figure 3-3, red shift of emission occurred with the decrease of ethanol concentration. When the ethanol concentration decreased from 50% to 20%, the wavelength of maximum emission changed from approximately 650 nm to 660 nm. The reason of the red shift in association with the dilution of ethanol aqueous solution with water was compatible with results of previous studies, which can be tentatively attributed to the increase of Bielectric constant (29). The Bielectric constants of water and ethanol are 78.5 and 24.3 (20), respectively.

The results confirmed the results of Dutta et al. (29), who studied Nile red fluorescence in several binary systems including methanol- water, acetonitrile-water, acetone-water and ethanol-water binaries. They concluded that the absorption and emission spectra positions of Nile red shift towards the red with increaing volume fraction of water in the binary solvent-water mixtures.

It is also clear that the fluorescence intensity values (FIV) at the scanned range decreased with ethanol concentration. At 50% ethanol concentration, the maximum FIV, which occurred at 650 nm, was approximately 1750 FIV. The maximum value, however, decreased to approximately 380 a.u. when the ethanol concentration was reduced to 20% and the maximum value occurred at approximately 660 nm. Furthermore, there was no visible maximum value of emission when ethanol concentration was 5%.

As shown in Figure 3-4, the maximum FIV had a linear relation with ethanol concentration when it was 20% (v/v) or above. However, when the concentration was lower than 20% (v/v), the FIV became very low and it was not significantly higher than the baseline when ethanol concentration was 5%. This result suggests that Nile red fluorescence in ethanol/water binary system requires a minimum ethanol concentration, which is approximately 20% (v/v). In other words, there exists a lower limit of ethanol concentration, which is in the proximity of 20%, for ethanol aqueous solution to emit fluorescence when dyed with Nile red.

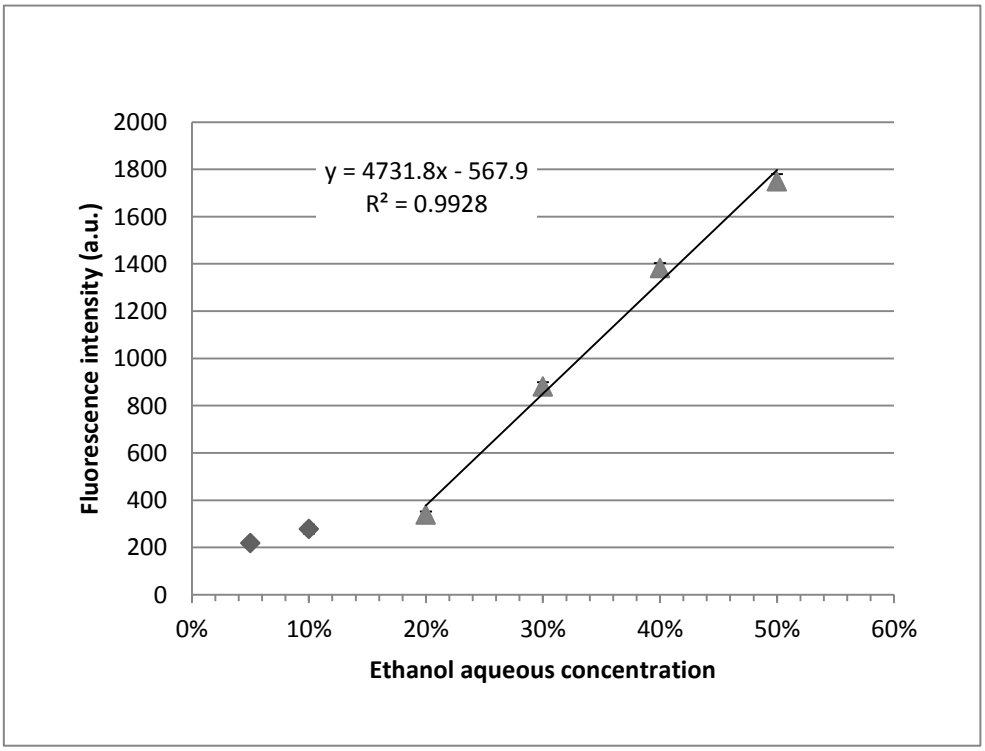


Figure 3-4, Maximum FIV at different ethanol concentrations. The results were mean values of triplicates. Error bars were omitted for clarity since they were small.

3.3.3 Oil/water binary system

The Nile red fluorescence of oil/water binary systems with four different oil/water ratios, i.e., pure oil, 4 mg/mL oil/water, 2 mg/mL oil/water, 1 mg/mL oil/water, 0.4 mg/mL oil/water and 0.04 mg/mL oil/water, were tested. It should be mentioned that, due to the low solubility of oil in water, the oil/water binary system existed as suspensions with oil micro-droplets dispersed in water. As shown in Figure 3-5, the spectra of pure oil showed that the maximum FIV of pure lipids appeared at 580 nm wavelength. The FIV of oil/water system decreased with the oil ratio in the suspension until the responses level off to zero. The FIV of oil/water system with a ratio of 0.04 mg/mL was close to zero throughout the scanned emission range of 540 to 700 nm.

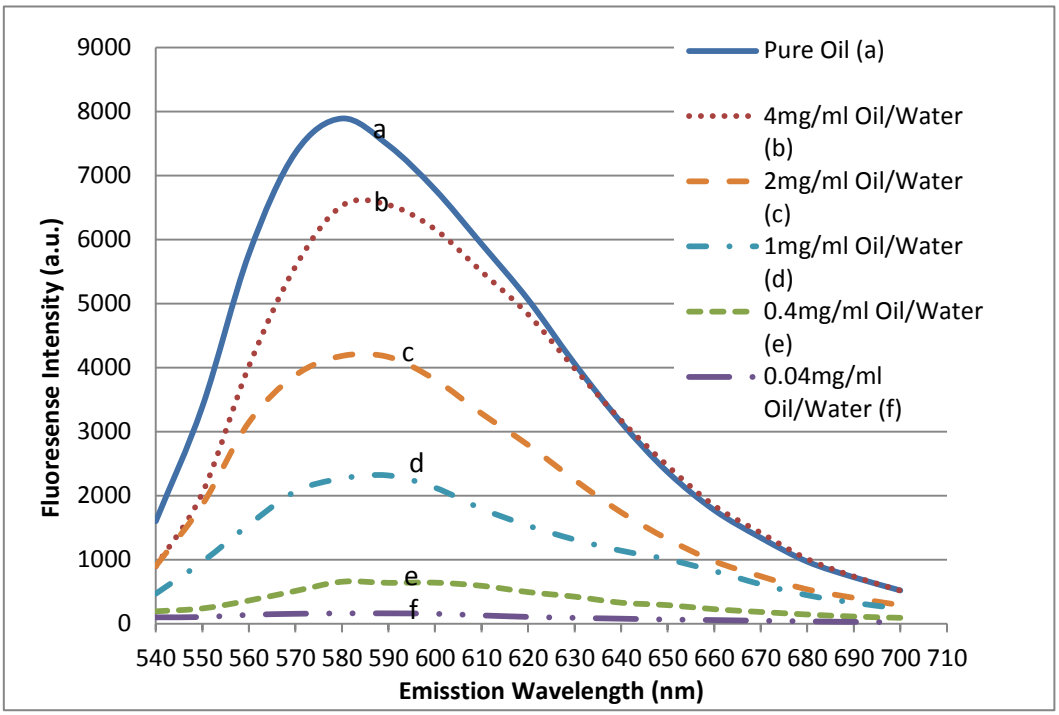


Figure 3-5 Emission spectra of Nile red in Oil/Water binaries of different ratios; (a) Pure Oil, (b) 4.0 mg/mL oil/water, (c) 2.0 mg/mL oil/water, (d) 1.0 mg/mL oil/water (e) 0.4 mg/mL oil/water (f) 0.04 mg/mL oil/water. The excitation wavelength used was 490 nm and the emission wavelength scanned from 540 nm to 700 nm. The results were mean values of triplicates. Error bars were omitted for clarity since they were small.

As shown in Figure 3-6, the maximum FIV (i.e., the peak value) had a linear relation with oil/water concentration in range from 0.04 mg/mL to 4 mg/mL.

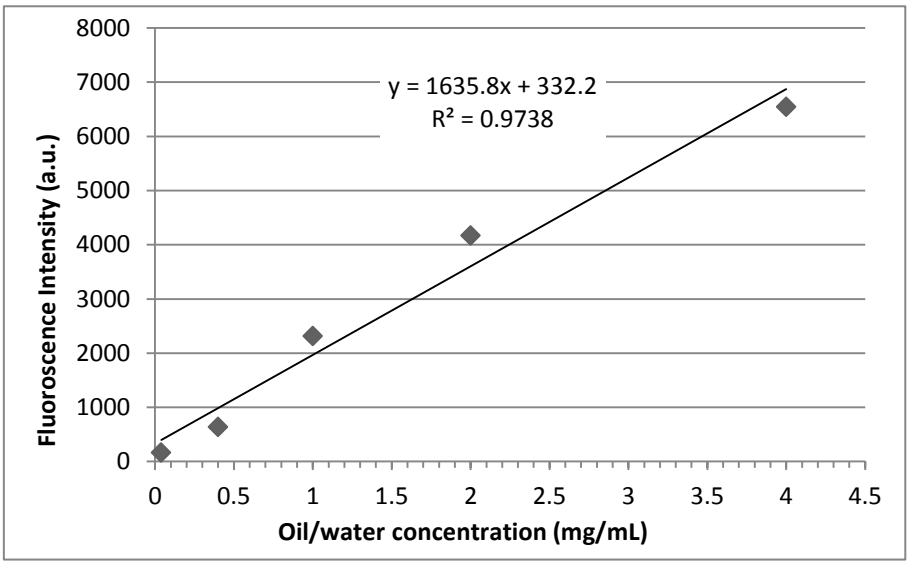


Figure 3-6 Maximum FIV at different oil/water ratios. The results were mean values of triplicates. Error bars were omitted for clarity since they were small.

It is worth mentioning that the oil contents in the oil/water system used in this research were much higher than the oil solubility in water at the experimental temperature (23°C), which was evidenced by the existence of large number of micro-droplets of oil in all the binary suspensions. In other words, the oil concentration in the water phase should be the same regardless of the oil content in the binary system and the rest of the oil existed as micro-droplets of oil dispersed in water. The decrease of FIV and the red shift observed with the decrease of oil content seems to be caused by the decrease of the ratio of oil micro-droplets in water rather than the concentration of dissolved oil in water phase.

3.3.4 Fluorescence of Nile Red in oil/ethanol/water ternary systems

Nile red fluorescence of lipid/ethanol/water ternary system with fixed lipid content of 0.4 mg/mL but varied ethanol concentration in the range of 5%-50% (v/v) was

investigated by scanning the emission in the range of 540-700 nm at fixed excitation wavelength of 490 nm.

Nile red fluorescence was significantly affected by ethanol concentration. As shown in Figure 3-7, the peak FIV wavelength of the ternary was all in the range of 580-590 nm when the ternary containing 0.4 mg/mL oil systems were prepared from ethanol aqueous solution of 20% (v/v) ethanol or less (i.e., 5%, 10%, and 20%). However, when the system was made by adding 0.4 mg/mL sunflower oil into 30% ethanol aqueous solution, a wide plateau appeared in the range of 580-660 nm, which seemed to be the merger of two peaks, the lipid peak in the range of 580-590 nm and the ethanol/water peak in the range of 540-560 nm. When ethanol concentration further increased to 40%, the lipid peak at 550-590 nm disappeared but FIV of approximately 650 a.u. could still be read at 590 nm. Whereas, when ethanol concentration increased to 50%, the FIV reading at 590 nm was very close to that of the baseline, indicating that the fluorescence of lipid was repressed by the high concentration of ethanol. These results were consistent with that from the binaries of oil/ethanol (Figure 3-2) and ethanol/water (Figure 3-3).

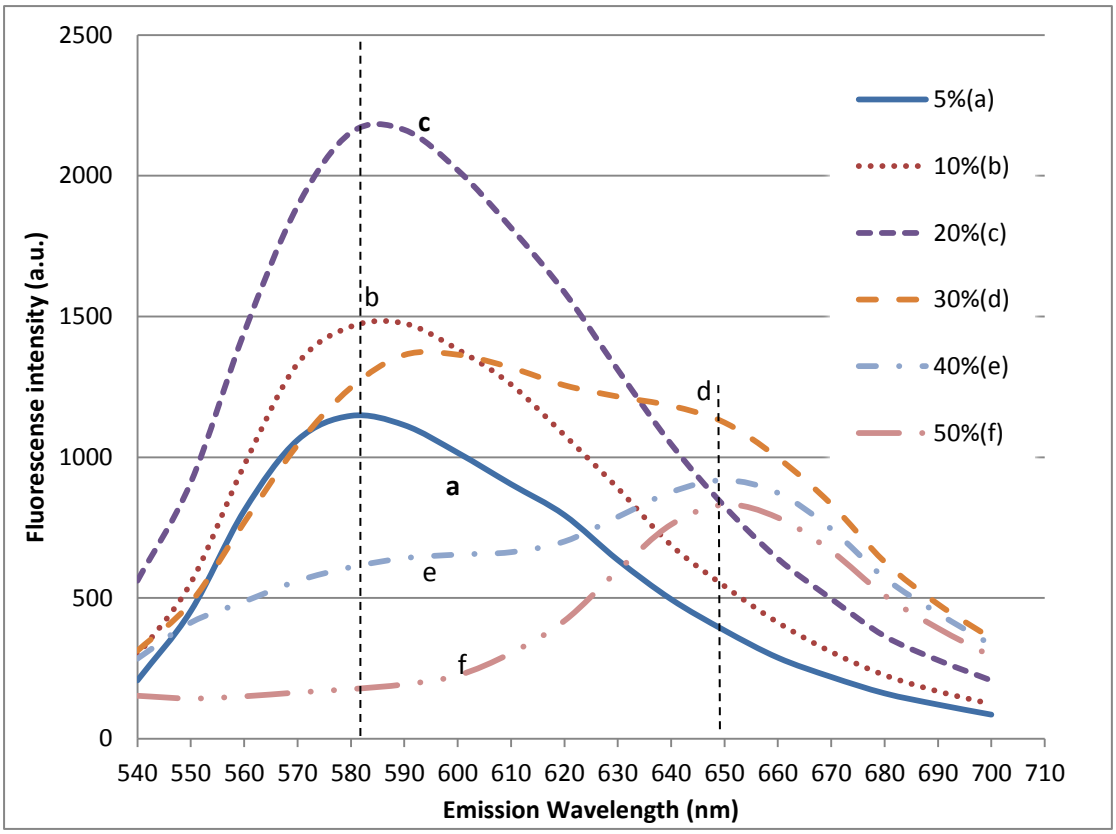


Figure 3-7 Emission spectra of ternaries containing 0.4 mg/mL lipids in several different ethanol aqueous solutions at different concentrations; (a) 5% ethanol/ water(v/v), (b) 10% ethanol/water(v/v) (c) 20% ethanol/ water(v/v), (d) 30% ethanol/ water(v/v), (e) 40% ethanol/water(v/v), (f) 50% ethanol/ water(v/v). The excitation wavelength used was 490nm. The results were mean values of triplicates. Error bars were omitted for clarity since they were small.

As shown in Figure 3-2, all lipid/ethanol binary systems containing 0.04 to 8.0 mg/mL lipids exhibited similar Nile red fluorescence spectra as that of pure ethanol, indicating that the Nile red fluorescence of lipids was completely repressed by ethanol. On the other hand, as shown in Figure 3-5, the emission spectra of lipid/water binary systems exhibited similar Nile red fluorescence spectra as that of pure oil, which has a peak at 580 nm and the FIV decreased with the lipid content in the binary system. Furthermore, Figures 3-3 and 3-4 demonstrated that dilution of ethanol using water would result in two potentially beneficial effects: 1) red shift of the emission

spectra (e.g., peak FIV at 640 nm for anhydrous ethanol and 660 nm for 20% (v/v) ethanol aqueous solution) that drives the peak FIV further away from that of lipid, which is 590 nm; and 2) reducing the peak FIV at the ethanol peak, which was, for instance, about 3000 a.u. for anhydrous ethanol but approximately 340 a.u. for 20% (v/v) ethanol aqueous solution. These results indicate that, by diluting ethanol with water, it might allow us to minimize the interference of ethanol to the Nile red fluorescence of lipid.

Table 3-1 lists the FIV at a wavelength of 590 nm (FIV_{590}), which is characteristic of lipid fluorescence, and at 650 nm (FIV_{650}), which is characteristic of ethanol fluorescence (Figure 3-7). When ethanol concentration was 20% or lower, FIV_{590} increased with ethanol concentration (1113.9 a.u. at 5%, 1475.5 a.u. at 10% and 2163.3 a.u. at 20%). However, this trend was reversed when ethanol concentration was 30% or higher, which were 2136.4 a.u. for 30%, 641.3 a.u. for 40% and 192.8 a.u. for 50%.

Table 3-1 Nile red FIV of lipid/ethanol/water ternaries at the lipid peak (590 nm) and ethanol peak (650 nm) at different ethanol concentrations. The oil content of ternaries was always 0.4 mg/mL.

FIV	FIV_{590} (a.u.) (ternary)	FIV_{650} (a.u.) (ternary)	$FIV_{590/650}$ Ratio	FIV_{650} (binary)	FIV_{650} ternary/binary Ratio
5% (v/v) ethanol/water	1113.9	384.4	2.9	215.8	1.8
10% (v/v) ethanol/water	1475.5	542.1	2.7	261.5	2.1
20% (v/v) ethanol/water	2163.3	825.4	2.6	307.3	2.7
30% (v/v) ethanol/water	1363.6	1123.3	1.2	834.8	1.4
40% (v/v) ethanol/water	641.3	918.0	0.7	1374.1	0.7
50% (v/v) ethanol/water	192.8	831.1	0.2	1749.6	0.5

Figure 3-8 depicts the dependence of the FIV of oil/ethanol/water ternaries at the characteristic wavelengths of lipid, FIV_{590} (i.e., the lipid fluorescence), and ethanol, FIV_{650} (i.e., the ethanol fluorescence) and the ratio between the two on the concentration of ethanol ($FIV_{590/650}$). It is worth noting that the FIV_{590} increased when ethanol concentration was 20% (v/v) or less but decreased almost linearly with ethanol concentration when it was 20% (v/v) or higher. FIV_{590} had its peak value at ethanol concentration of 20% (v/v). On the other hand, the FIV_{650} increased in the ethanol concentration range of 5-30% (v/v) but was stabilized in the range of 40-50% (v/v). Correspondingly, the FIV_{590}/FIV_{650} ratio was relatively stable when the ethanol concentration was 20% or less (v/v) but decreased almost linearly with ethanol concentration when it was 20% (v/v) or higher. For the purpose of lipid content

analysis, we would like to have reasonably large FIV for a given concentration of lipids and a large FIV_{590}/FIV_{650} ratio so as to minimize the interference of ethanol. Therefore, 20% aqueous ethanol solution was selected to be the solution for determination of lipid content using Nile red fluorometry.

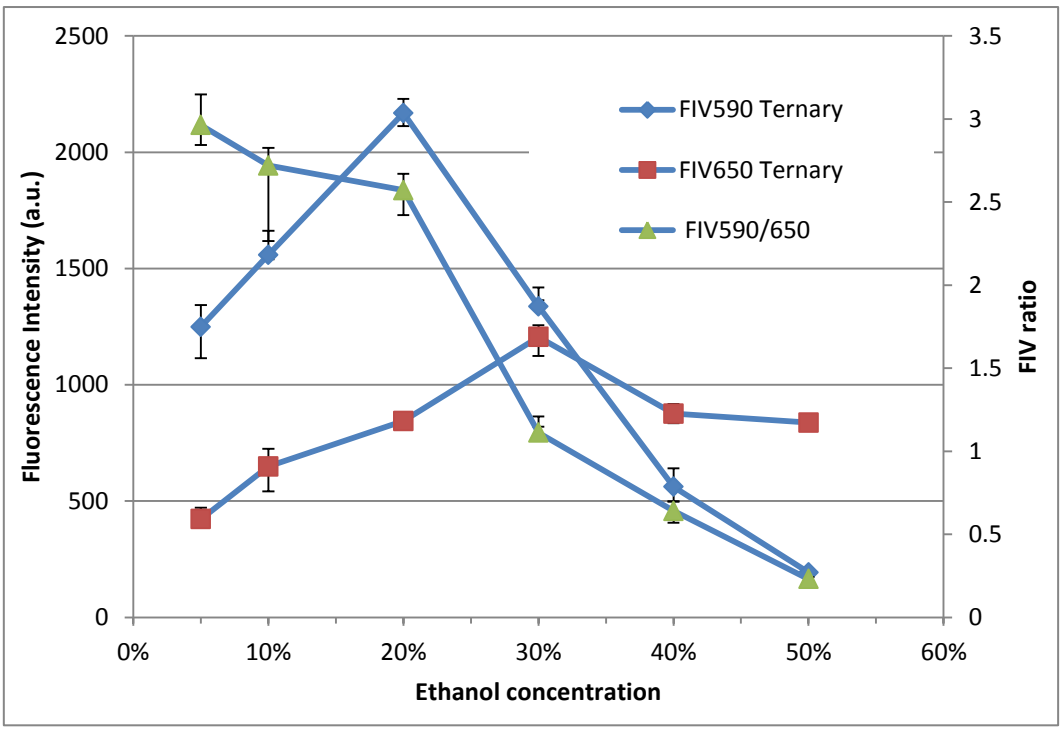


Figure 3-8 Effects of ethanol concentration on Nile red fluorescence of oil/ethanol/water ternaries containing 0.4 mg/mL sunflower oil. The results were mean values of triplicates.

The observation that the increase of ethanol concentration from 5% to 20% (v/v) could significantly increase the fluorescence of the ternary at the lipid peak (i.e., 590 nm) could be explained as follows.

As shown in Figure 3-9, the color of the ternaries containing 0.4 mg/L sunflower oil changed from pink, the characteristic color of oil droplets in water when they are

stained with Nile red and exposed to visible light, to purple, the characteristic color of ethanol aqueous solution stained with Nile red. Visual investigation also revealed that large numbers of oil droplets existed in the ternaries when the ethanol concentration was 25% (v/v) or lower. However, the ternaries became homogeneous solutions when the ethanol concentration was 30% (v/v) or higher.

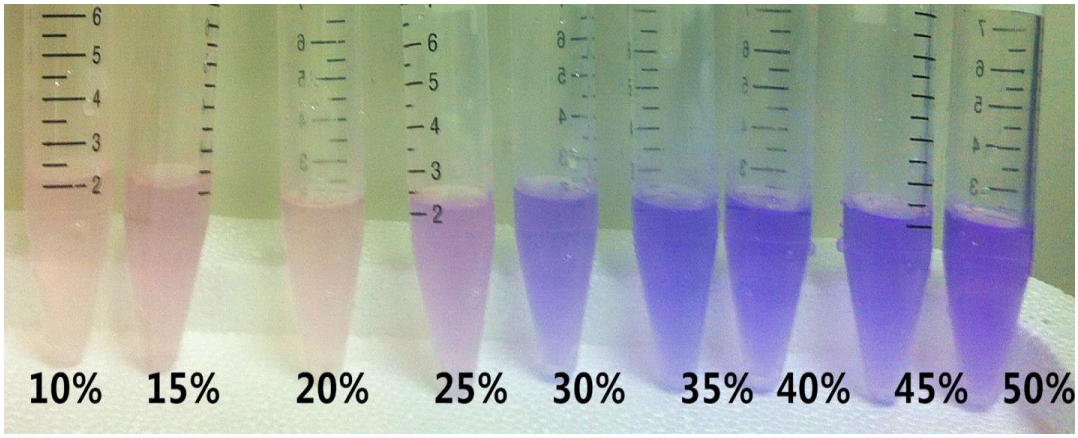


Figure 3-9 Color of oil/ethanol/water ternaries containing 0.4 mg/mL sunflower oil with varied ethanol concentration after mixing with 50 μL 250 ppm Nile red acetone solution in 2.5 mL ternary mixture.

Since ethanol is a hydrophilic compound having a nonpolar group (CH_3CH_2-) and a polar group (-OH). Furthermore, the solubility of aqueous ethanol solution to lipid increases with ethanol concentration. In oil/water and oil/ethanol binary systems of low ethanol concentration of 20% or lower, lipids were not completely dissolved and lipid droplets existed in the ternary system. In the ternary system, oil droplets were surrounded by ethanol molecules with the nonpolar group anchored at the interface of the lipid phase, and the polar group (i.e., -OH) facing the aqueous phase. As a result,

stable micelles of lipid are formed. At low ethanol concentration range (e.g., 20% or less), increase of ethanol concentration may increase the stability of the micelles, decrease the micelle diameter, increase the number of micelles and therefore the surface area of them.

On the other hand, the decrease of lipid fluorescence in ternaries with the increase of ethanol concentration when it was 30% (v/v) ethanol or higher seems to suggest that high concentration ethanol could not only intervene in lipid fluorescence but, furthermore, could repress it when its concentration is high enough. Indeed, as shown in Figure 3-7 and Figure 3-2, the lipid fluorescence decreased when ethanol concentration increased from 20% to 40% and almost completely diminished when ethanol concentration was 50% (Figure 3-7 (f)). Furthermore, in lipid/ethanol binaries, i.e., when anhydrous ethanol was used as the solvent, as shown in Figure 3-2, the lipid fluorescence of lipid was completely repressed regardless of lipid content when the lipid concentration was in the range of 0.04 mg/mL to 8 mg/mL.

As aforementioned, for a ternary containing 0.4 mg/mL lipid, the lipid was complete dissolved when the ethanol concentration was 30% (v/v) or above and a suspension with oil droplet dispersed in the ethanol/water solution was formed when ethanol concentration was 20% (v/v) or less. Another possible explanation is, therefore, the lipid lost its characteristic fluorescence when it was dissolved in a solvent, which was an aqueous ethanol solution in this case. This hypothesis, however, needs to be confirmed with more systematic experiments.

Figure 3-10 compares the FIV of ethanol/water binaries and oil/ethanol/water ternaries containing 0.4 mg/mL sunflower oil at different ethanol concentrations. FIV was measured at 650 nm, the characteristic emission wavelength of ethanol Nile red fluorescence and therefore signifies the effects of lipids on ethanol fluorescence. As shown in Figure 3-10, while the FIV of ethanol/water binaries increased monotonically with the concentration of ethanol, the FIV of lipid/ethanol/water ternaries had a peak at around ethanol concentration of 30% v/v. These results suggest the addition of lipids at a concentration of 0.4 mg/mL into aqueous ethanol has a strong impact on the Nile red fluorescence of ethanol. When ethanol concentration was lower than 30% (v/v), the ternaries including 0.4 mg/mL had a FIV that is higher than the binaries containing zero lipid. On the other hand, when ethanol concentration was higher than 30% (v/v), the ternaries containing 0.4 mg/mL oil had a FIV that is lower than the binaries.

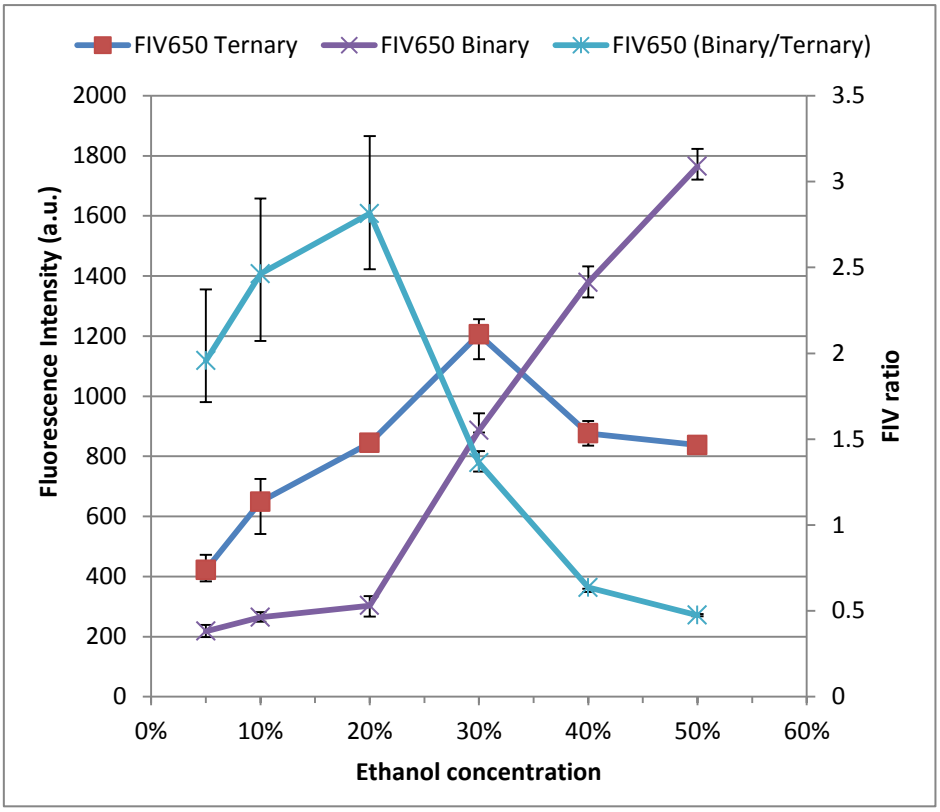


Figure 3-10 Comparison of the ethanol characteristic Nile red fluorescence i.e., FIV_{650}, of ethanol/water binaries and oil/ethanol/water ternaries at different ethanol concentrations. The results were mean values of triplicates.

The above observations seem to suggest that the existence of microdroplets of oil in oil/ethanol/water ternaries could increase the fluorescence of ethanol while dissolved oil in the ternaries could repress the ethanol fluorescence. The mechanism of this phenomenon is unknown at present. One possible reason to explain the enhancement of oil droplets on ethanol fluorescence at low ethanol concentration could be the accumulation of ethanol molecules on the interface of the lipid droplets and the ethanol aqueous phase due to the anchoring of the non-polar CH_3CH- group of ethanol molecules to create an ethanol rich microenvironment surrounding the oil droplets. Since the hydrophobic Nile red molecules have a much larger solubility in

ethanol than in water, it is expected that more Nile red molecules would be distributed to the ethanol-rich interface than the aqueous phase and therefore increase the ethanol fluorescence. This hypothesis, however, needs to be verified by experimental results.

3.3.5 Stability of Nile red fluorescence of lipid/ethanol/water ternary system

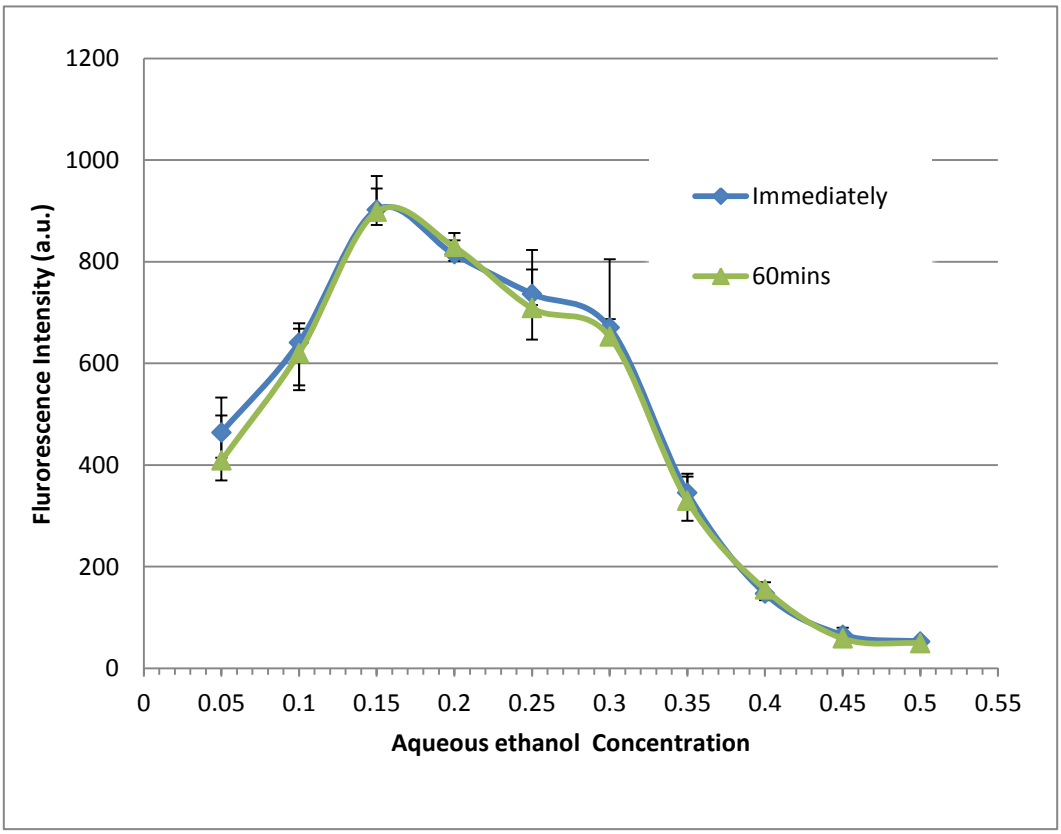


Figure 3-11 Comparison of fluorescence intensity of oil/ethanol/water ternaries containing 0.4mg/mL oil at excitation wavelength at 490 nm emission wavelength of 590 nm, which is characteristic of lipid fluorescence, measured immediately and 60 minutes after sonication. The results were mean values of triplicates.

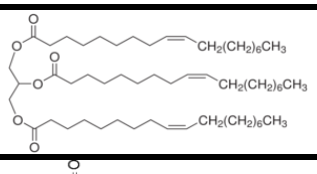
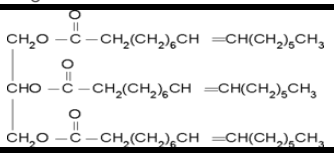
According to the measurement procedure, after adding Nile red into the oil ethanol aqueous mixtures, the sample was treated using an ultrasonic bath for 30 minutes at room temperature, 42Hz. During the ultrasonic treatment, the lipid droplets micelles

were created. Since the micelles are relatively unstable, they will gather to form large droplets as the time goes by. Therefore, after ultrasonic treatment finished, 50μL Nile red stained mixture was transferred into the black 96-well microplate to measure the FIV immediately. Due to the large number of samples, about 5-10 minutes were needed to complete the sample transfers and scan. It is therefore necessary to understand the stability of the micelle suspension. For this purpose, we measured FIV of Nile red stained lipids in aqueous ethanol solution two times, one is measured immediately after sonification and the other is measured 60 minutes later at the same conditions. As shown in Figure 3-11, the response of these two measurements were almost identical, indicating the oil micelles suspended in the oil/ethanol/water ternaries after sonification were stable within 60 minutes. This lends much needed flexibility to this approach since measurement of fluorescence could be easily done within 15 minutes.

3.3.6 Influence of triglyceride structure on Nile red fluorescence

Since triglycerides produced by oil crops such as plants and microalgae have different fatty acid compositions, it is of interest to study the influence of fatty acid composition on Nile red fluorescence of triglycerides in order to apply it to the measurement of lipid content of oil crops. Experiments were carried out with different triglyceride samples, including glyceryl trioleate, glyceryl tripalmitoleate, sunflower oil and olive oil. The fatty acid compositions of these samples are shown in Table 3-2.

Table 3-2 The structure of glyceryl trioleate (C18) and glyceryl tripalmitoleate (C16)

Name of Triglyceride	Structural formula	Fatty acid	Saturated	Unsaturated
glyceryl trioleate		C18:1		√
glyceryl tripalmitoleate		C16:1		√

As shown in Figure 3-12, the FIV of all samples exhibited a linear relationship in the concentration range of 0.1 to 0.5 mg/mL. It was also observed that the FIV of all the samples were very similar when measured at the same concentration. Regression of all the 60 data points generated with the four samples (i.e., glyceryl trioleate, glyceryl tripalmitoleate, sunflower oil and olive oil) at five different concentrations in triplicated gave a linear relationship with a slope of 2402.5 a.u.-mL/mg, the intercept is 17.9 and R^2 value of 0.9668.

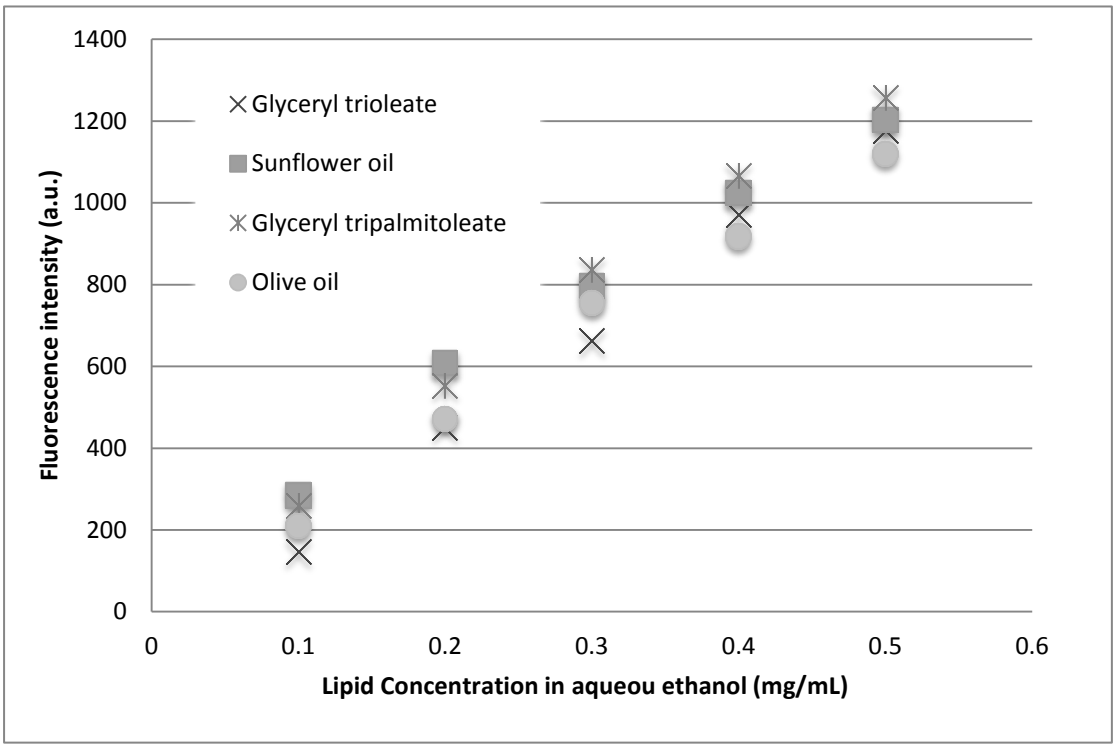
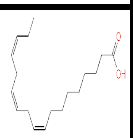
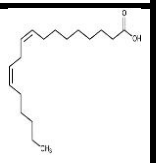


Figure 3-12 The correlation of lipids concentration and fluorescence intensity of lipids samples from different sources: (▪) Sunflower Oil, (ˣ) glyceryl trioleate, (•) Olive Oil, and (ẋ) glyceryl tripalmitoleate. Data were the mean values of replicates. Error bars were omitted for clarity since they were small.

As shown in Table 3-3, the four long-chain triglyceride samples covered a wide range of triglycerides with different fatty acid compositions with a carbon chain length in the range of C16-18. These included pure glyceryl trioleate (C18:1), pure glyceryl tripalmitoleate (C16:1), oils contain different percentage of C18:1 and C18:2. These results seem to suggest that the location and number of unsaturated bonds on the fatty acid carbon chain do not have a significant effect on the Nile red fluorescence under the investigated conditions. C18:1 and C16:1 also didn't show significant difference in the tested condition.

Table 3-3 The fatty acid composition (percentage) of several oils

No.	Name of the oil	Palmitic% (Hexadecanoic) $C_{16:0}$	Stearic%(n-Octadecanoic) $C_{18:0}$	Oleic% ($C_{18:1}$)	Linolenic% ($C_{18:3}$)	Linoleic% ($C_{18:2}$)
	Structure					
1	Sunflower oil	3.0-6.0	1.0-3.0	14.0-35.0	----	44.0-75.0
2	Olive oil	7.0-16.0	1.0-3.0	65.0-80.0	----	4.0-10.0
3	Sesame oil	7.0-12.0	3.5-6.0	35.0-50.0	----	35.0-52.0
4	Peanut oil	9.0-11.0	2.5-4.0	40.0-50.0	----	30.0-40.0

The independence of Nile red fluorescence on the fatty acid composition of triglycerides is a desirable feature since it allows the application of the Nile red fluorometric method to estimation of lipid contents of different samples, including those from unknown sources, sources of unknown fatty acid composition, or sources of complex fatty acid composition, which are the most commonly encountered scenarios in practical applications. This represents a distinctive advantage of this approach in comparison to the aforementioned SPV method, which is very sensitive to the source of lipids (1).

Considering the fact that different triglyceride samples demonstrated very similar quantitative dependence of FIV on lipid concentration in the linear range, i.e., 0.1-0.5 mg/mL, we decided to use glyceryl trioleate (C18), which has a FIV/lipid conversion factor of 2581.0 a.u.-mL/mg and the intercept is -92.4, as the standard for estimation of the lipid content of different plant seeds and microalga cells (Fig.3-13).

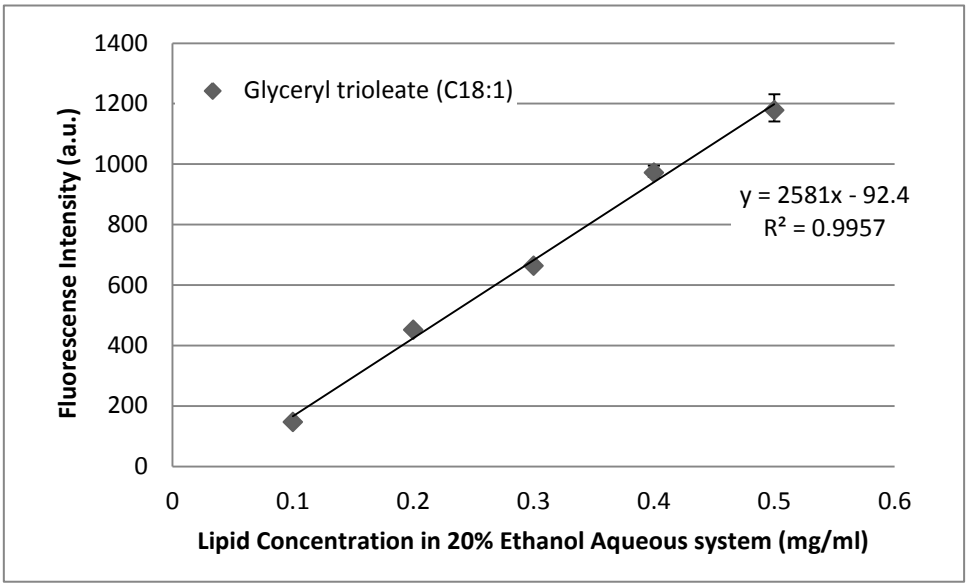


Figure 3-13 The standard curve for lipids measurement using Nile red fluorescence based on analytic grade glyceryl trioleate (C18:1), y=2581.0x- 92.4 and R^2= 0.9957. The results were mean values of triplets.

We used the linear line (Figure 3-13) as the standard curve, y=2581.0x- 92.4. The neutral lipid content (wt%) was calculated usiIg the equation as follows:

$$C_{Lipid}\,(g/g) = \frac{(FIV(a.u.)+92.4)*10.2\ (ml)}{2581.0(\text{a.u.}-\text{mL/mg})*10(mg)} \qquad (3\text{-}3)$$

3.3.7 Comparison between lipid contents of different crops measured using Nile red fluorescence method and gravimetric measurement

The lipid contents of four different biomass samples, i.e., sunflower seeds, peanuts, and sesame seeds were estimated using gravimetric method, which involved Soxhlet extraction and Nile red fluorescence with lipids extracted using an ethanol/hexane (4:1) mixture. The results are shown in Table 3-4.

Table 3-4 Comparison of lipid contents of different crops determined by gravimetric and Nile red fluorescence methods

	Gravimetric	Nile red fluorescence	Difference	Difference (%)
Sesame	58.2%	51.2%	7.0%	12.0%
Peanut	56.8%	50.5%	6.3%	11.1%
Sunflower seeds	56.4%	49.7%	6.7%	11.9%

As shown in Table 3-4, the Nile red fluorescence method for lipid determination provided similar results to the conventional gravimetric method using Soxhlet extraction for samples of sesame, peanut and sunflower seeds. Average sesame neutral lipid contents of 51.4% and 59.20% were obtained by the Nile red fluorescence method and the Soxhlet extraction method, respectively. The differences detected between the results from the two methods for crop lipid measurement were around 11-12%.

It's worth mentioning that neither the Soxhlet extraction nor the gravimetric method are discriminative and the results were the total amount of all lipids, including fatty acids, monoglycerides, diglycerides, triglycerides, phospholipids, and other lipophilic materials. On the other hand, the Nile red fluorescence method is selective to neutral lipids. This explains, at least partially, why the lipid contents of different samples determined by the Nile red fluorometric methods were consistently smaller than that measured using the gravimetric method. In other words, the results of Nile red

fluorometric results could be treated as the content of triglycerides while the gravimetric results are the total lipid content of samples. Nonetheless, it is worth mentioning that the pigments and waxes in the extracted oil also might have influenced the results of Nile red fluorescence method.

3.3.8 Challenges in applying the modified Nile red fluorescence method for measurement of lipid content of microalgal biomass

The average Nile red fluorescence of microalgal extract was 20.6 a.u., which was too low and out of the linear range of this new assay, therefore cannot be measured by the Nile red method. However, the lipid content of microalgal cells can be measured by Soxhlet extraction and the result was 26.5%, suggesting that the lipid Nile red fluorescence was repressed by some impurities in the microalgal extract.

As shown in Fig 3-14, significant light absorption of chlorophyll a and b was observed in the range of 390-500 nm (32), which coincidences with the peak excitation wavelength of Nile red fluorescence at approximately 490 nm. On the other hand, it was reported that chlorophylls were extracted together with lipids when the ethanol/hexane mixture solvent system was used to extract lipids from microalgal cells (30, 31). We therefore hypothesized that the intervention of chlorophyll a and b, which are abundant in microalga cells (26), might be the major contributor to the low response of microalgal extract to Nile red fluorescence. To test this hypothesis, microalgal extract was mixed with pure sunflower oil at varied ratios and tested for

Nile red fluorescence at an excitation wavelength of 490 nm and emission wavelength of 590 nm. The total raw lipid content, i.e., microalgal extract plus sunflower oil, was kept constant at 0.4 mg/mL while the ratio of sunflower oil: microalgal extract was varied as 0:1, 4:1, 9:1, 19:1, or 99:1. Ethanol aqueous solution of 20% (v/v) was used as the solvent in the measurements. The results are shown in Figure 3-15.

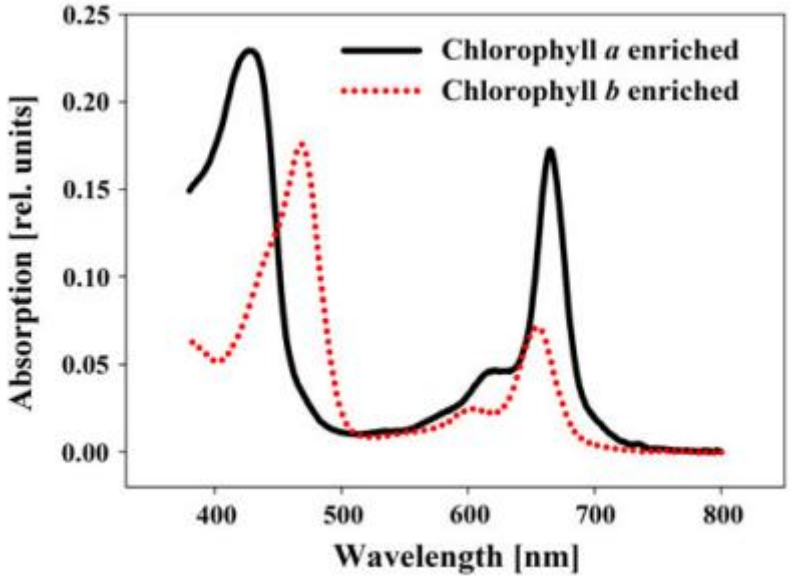


Figure 3-14 Absorption spectra of chlorophyll a and chlorophyll b fractions of *Liriodendron tulipifera* methanol extracts separated by thin-layer chromatography (32).

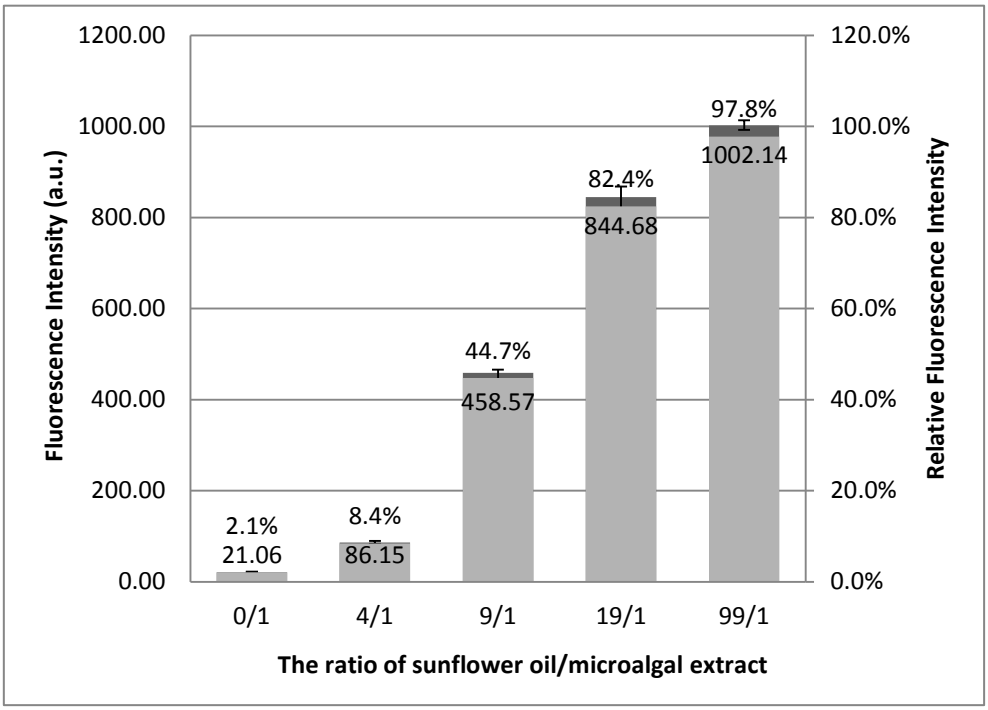


Figure 3-15 FIV of microalgal extract and sunflower oil mixtures at oil: microalgal extract ratios of 0:1, 4:1, 9:1, 19:1, and 99:1. The excitation and emission wavelengths used for fluorescence determination were 490 nm and 590 nm, respectively.

As shown in Figure 3-15, the FIV of pure microalgal extract was 21 a.u., which increased to 86.1 a.u. when it was diluted 5 times by sunflower oil (o/e ratio 4:1), to 458.6 a.u. when it was diluted 10 times by sunflower oil (o/e ratio 9:1), and to 844.7 a.u when it was diluted 20 times by sunflower oil (o/e ratio 19:1), representing an almost linear relationship between the proportion of the microalgal extract in the oil/ethanol mixture. Further diluting microalgal extract to 100 times by sunflower oil, however, resulted in a mild increase of FIV to 1002.1 a.u. It is worth mentioning that the FIV of sunflower oil, glyceryl tripalmitoleate and glyceryl trioleate were 1025.1 a.u., 1066.1 a.u., and 970.8 a.u., respectively (Table 3-3), when the oil content in 20% ethanol aqueous solution was 0.4 mg/mL. They are very close to the FIV of microalgal extract diluted 100 times by sunflower oil, indicating the repressive effects

of microalgal extract to the Nile red fluorescence of sunflower oil was eliminated at this dilution ratio. Since the FIV at 590 nm was 1000 a.u. at oil/ethanol ratio of 99:1 was not significantly larger than that at the oil/ethanol ratio of 19:1, it is reasonable to suggest that the concentration of the color material in microalgal extract was close to the threshold when it was diluted by 20 times so that it did not have a significant influence on FIV. The demonstrated significant repression of microalgal extract to the Nile red fluorescence of sunflower oil suggests the existence of repressive components in the microalgal extract. Therefore, if we can successfully develop a selective extraction process, either by selecting suitable solvent system or by optimizing extraction conditions, that could reduce the concentration of the repressive materials in extract by 20 times or more, then, the Nile red fluorescence could likely be applied for lipid content measurement of microalgal cell samples.

3.4 Conclusion

In summary, the present work reports on the emission spectra of Nile red fluorescence of pure, binary and ternary systems of oil, ethanol and water were measured at different ratios. It was determined that the optimal ethanol/water ratio as solvent for determination of lipid content using Nile red fluorometry was 20% (v/v) and the linear range was 0.1 to 0.5 mg oil/mL in 20% ethanol aqueous solution. According to these results, Nile red fluorescence method was developed for high throughput analysis of triglycerides, which are extracted from oil crops and microalgae. Results of this new method are comparable with that obtained with a gravimetric

determination for plant seeds. There are several important advantages over conventional methods of lipid measurement. This new Nile red method requires a small amount of biomass sample and allows the measurement of a large number of samples. In addition, it is easy, time-saving, cost-effective and results are repeatable and not sensitive to the structure of triglycerides. Therefore, this method is applicable for determination of lipid contents of different biomass samples in a reasonably large linear range.

3.5 References

1. **Cheng, Y. -., Y. Zheng, and J. S. VanderGheynst.** 2011. Rapid quantitative analysis of lipids using a colorimetric method in a microplate format. Lipids. **46:**95-103.

2. **Wymann, M. P., and R. Schneiter.** 2008. Lipid signalling in disease. Nature Reviews Molecular Cell Biology. **9:**162-176.

3. **Wang, X.** 2004. Lipid signaling. Curr. Opin. Plant Biol. **7:**329-336.

4. **John R. Post and E. A. Parkinson** - ENERGY ALLOCATION STRATEGY IN YOUNG FISH: ALLOMETRY AND SURVIVAL. - Ecology. - 82: 1040-1051. doi: - 10.1890/0012-9658(2001)082[1040:EASIYF]2.0.CO;2.

5. **Rinchard, J., S. Czesny, and K. Dabrowski.** 2007. Influence of lipid class and fatty acid deficiency on survival, growth, and fatty acid composition in rainbow trout juveniles. Aquaculture. **264:**363-371. doi: http://dx.doi.org/10.1016/j.aquaculture.2006.11.024.

6. **Ratledge, C.** 2002. Regulation of lipid accumulation in oleaginous micro-organisms. Biochem. Soc. Trans. **30:**1047-1050.

7. **Ratledge, C., and Z. Cohen.** 2008. Microbial and algal oils: Do they have a future for biodiesel or as commodity oils? Lipid Technol. **20:**155-160.

8. **Papanikolaou, S., M. Komaitis, and G. Aggelis.** 2004. Single cell oil (SCO) production by Mortierella isabellina grown on high-sugar content media. Bioresour. Technol. **95:**287-291. doi: http://dx.doi.org/10.1016/j.biortech.2004.02.016.

9. **BLIGH, E. G., and W. J. DYER.** 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology. **37:**911-917.

10. **Vieler, A., C. Wilhelm, R. Goss, R. Süß, and J. Schiller.** 2007. The lipid composition of the unicellular green alga Chlamydomonas reinhardtii and the diatom Cyclotella meneghiniana investigated by MALDI-TOF MS and TLC. Chem. Phys. Lipids. **150:**143-155. doi: 10.1016/j.chemphyslip.2007.06.224.

11. **Jones, J., S. Manning, M. Montoya, K. Keller, and M. Poenie.** 2012. Extraction of algal lipids and their analysis by HPLC and mass spectrometry. JAOCS, Journal of the American Oil Chemists' Society. **89:**1371-1381.

12. **Eltgroth, M. L., R. L. Watwood, and G. V. Wolfe.** 2005. Production and cellular localization of neutral long-chain lipids in the haptophyte algae Isochrysis galbana and Emiliania huxleyi. J. Phycol. **41:**1000-1009.

13. **Cooksey, K. E., J. B. Guckert, S. A. Williams, and P. R. Callis.** 1987. Fluorometric determination of the neutral lipid content of microalgal cells using Nile Red. J. Microbiol. Methods. **6:**333-345.

14. **Izard, J., and R. J. Limberger.** 2003. Rapid screening method for quantitation of bacterial cell lipids from whole cells. J. Microbiol. Methods. **55:**411-418.

15. **Chabrol, E., R. Charonnat, K. R. Johnson, G. Ellis, and C. Toothill.** 1977. Determination of total lipids; The sulfophosphovanillin reaction for serum lipids: a reappraisal. Clin. Chem. **45; 23:**1713; 1669-1720; 1678.

16. **Sitepu, I. R., L. Ignatia, A. K. Franz, D. M. Wong, S. A. Faulina, M. Tsui, A. Kanti, and K. Boundy-Mills.** 2012. An improved high-throughput Nile red fluorescence assay for estimating intracellular lipids in a variety of yeast species. J. Microbiol. Methods. **91:**321-328.

17. **Zuriani, R., S. Vigneswari, M. N. M. Azizan, M. I. A. Majid, and A. A. Amirul.** 2013. A high throughput nile red fluorescence method for rapid quantification of intracellular bacterial polyhydroxyalkanoates. Biotechnology and Bioprocess Engineering **18**: pp 472-478.

18. **Chen, W., M. Sommerfeld, and Q. Hu.** 2011. Microwave-assisted Nile red method for in vivo quantification of neutral lipids in microalgae. Bioresour. Technol. **102:**135-141.

19. **Genicot, G., J. L. M. R. Leroy, A. Van Soom, and I. Donnay.** 2005. The use of a fluorescent dye, Nile red, to evaluate the lipid content of single mammalian oocytes. Theriogenology. **63:**1181-1194.

20. **Greenspan, P., and S. D. Fowler.** 1985. Spectrofluorometric studies of the lipid probe, nile red. J. Lipid Res. **26:**781-789.

21. **Ge, Y., J. A. Thomasson, and M. Korte.** 2012. Algal lipid quantification in-situ with Nile red fluorescence, **3**: p. 2501-2506. *In* Anonymous American Society of Agricultural and Biological Engineers Annual International Meeting 2012.

22. **Chisti, Y.** 2007. Biodiesel from microalgae. Biotechnol. Adv. **25:**294-306.

23. **Chen, W., C. Zhang, L. Song, M. Sommerfeld, and Q. Hu.** 2009. A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. J. Microbiol. Methods. **77:**41-47.

24. **Gerde, J. A., M. Montalbo-Lomboy, L. Yao, D. Grewell, and T. Wang.** 2012. Evaluation of microalgae cell disruption by ultrasonic treatment. Bioresour. Technol. **125:**175-181.

25. **Fowler, S. D., W. J. Brown, J. Warfel, and P. Greenspan.** 1987. Use of nile red for the rapid in situ quantitation of lipids on thin-layer chromatograms. J. Lipid Res. **28:**1225-1232.

26. **Li, Y., M. Horsman, B. Wang, N. Wu, and C. Q. Lan.** 2008. Effects of nitrogen sources on cell growth and lipid accumulation of green alga Neochloris oleoabundans. Appl. Microbiol. Biotechnol. **81:**629-636.

27. **Li, Y., M. Horsman, B. Wang, N. Wu, and C. Q. Lan.** 2008. Effects of nitrogen sources on cell growth and lipid accumulation of green alga Neochloris oleoabundans. Appl. Microbiol. Biotechnol. **81:**629-636.

28. **Kaparthi, R., and K. S. Chari.** 1959. Solubilities of vegetable oils in aqueous ethanol and ethanol-hexane mixtures. J. Am. Oil Chem. Soc. **36:**77-80.

29. **Dutta, A. K., K. Kamada, and K. Ohta.** 1996. Spectroscopic studies of nile red in organic solvents and polymers. J. Photochem. Photobiol. A. **93:**57-64.

30. **Lamb, J. J., J. J. Eaton-Rye, and M. F. Hohmann-Marriott.** 2012. An LED-based fluorometer for chlorophyll quantification in the laboratory and in the field. Photosynthesis Res. **114:**59-68.

31. **Ferreira, D. S., O. F. Galão, J. A. L. Pallone, and R. J. Poppi.** 2014. Comparison and application of near-infrared (NIR) and mid-infrared (MIR) spectroscopy for determination of quality parameters in soybean samples. Food Control. **35:**227-232. doi: http://dx.doi.org/10.1016/j.foodcont.2013.07.010.

32. **Ramluckan, K., K. G. Moodley, and F. Bux.** 2014. An evaluation of the efficacy of using selected solvents for the extraction of lipids from algal biomass by the soxhlet extraction method. Fuel. **116:**103-108. doi: http://dx.doi.org/10.1016/j.fuel.2013.07.118.

Chapter 4: Conclusion and recommendations

4.1 Conclusion

A method for quantitative analysis of triglycerides based on Nile red fluorescence was developed in this study. In comparison to conventional methods, Nile red fluorescence does not require tedious pre-treatment, requires small quantities of samples, and is not sensitive to the fatty acid composition of lipids.

The developed Nile red fluorescence assay includes extraction of lipids from dried samples and is therefore not subjected to the interference of cellular materials such as cell wall, cytoplasmic membrane, and the structure and size of lipid droplets in cells. The Nile red fluorescence of pure, binary, and ternary systems of sunflower oil, ethanol, and water were studied systematically. It was found that the concentration of ethanol had strong impact on the fluorescence of lipid. A hypothesis was proposed to explain the interaction between lipids and ethanol in corresponding binary and ternary systems.

According to the results, 20% (v/v) ethanol aqueous solution was determined to be the suitable solvent for the Nile red fluorescence lipid assay. Excellent linearity was established for dependency of fluorescence on lipid content of samples in the range of 0.1- 0.5 mg/ml with several lipid samples including olive oil, sunflower seed oil,

glyceryl trioleate (C18), and glyceryl tripalmitoleate (C16). Furthermore, results of studies also suggest that there is no significant Nile red fluorescence difference in triglycerides with different fatty acid compositions.

The results of the fluorescence assay were verified with that of the conventional gravimetric assay, using seeds of different oil crops including peanuts, sunflower, and sesame, and green microalgae *Neochloris oleoabundans* as samples. It was observed that the results of the fluorescence assay were consistently less than the gravimetric results by about 10%, an observation that is tentatively attributed to the fact that the gravimetric method measures the total lipid content while the fluorescence assay is selective to triglycerides.

This method has several important advantages over conventional methods of lipid measurement such as: (1) requiring small amount of biomass samples; (2) easy, time-saving and cost-effective; (3) high throughput, allowing the measurement of a large number of samples simultaneously; and (4) not sensitive to the structure of triglycerides and therefore applicable for determination of lipid contents of different biomass samples; (5) having a reasonably large linear range and repeatable results.

Nevertheless, the attempts to apply this assay for determination of lipid contents of microalga biomass revealed the existence of factors in the microalga extract that could

strongly inhibit the Nile red fluorescence of triglycerides.

4.2 Recommendation

Based on the results obtained from the failed attempts of applying the Nile red fluorescence method for determination of microalgae lipid contents, it seems to be logical to suggest the development of an effective means for selective lipid extraction to exclude the fluorescence repressing factor(s) from the extract. This may include the selection and optimization of solvent systems, design of lipid extraction apparatus, and optimization of extraction conditions.

Furthermore, a systematic comparison between the compositions of the extract obtained through Soxhlet extraction and the ethanol/hexane mixture would help determine more precisely the causes of the consistently lower Nile fluorescence assay results in comparison to the gravimetric assay results and therefore improve the accuracy of the assay.

Finally, systematic optimization on lipid extraction would be valuable since it may lead to more complete and selective extraction of triglycerides from the samples and therefore warrants more accurate results.