A Review on Methods Used in Analysis of Microalgae Lipid Composition

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The present paper provides a brief overview of the most recent techniques for microalgae lipid analysis such as high performance liquid chromatography (HPLC), gas chromatography (GC), mass spectrometry (MS) and nuclear magnetic resonance (NMR). The application of HPLC technique is to break down lipid into smaller fractions such as neutral lipid and polar lipid. MS technique is known as less sensitive than GC technique thus requires coupling with other techniques in order to analyze the microalgae lipid. On the other hand, NMR technique provides comprehensive information on the molecular structure of microalgae lipid but it has disadvantage as the signals may overlap in the spectrum. Notably, GC coupled with flame ionization detector (FID) is the fundamental method which is fast with high accuracy when analyzing microalgae lipid.

Key Words
Microalgae, Lipid, Analysis

1. Introduction

Due to the constraint of resources in fossil fuels and greenhouse gas (GHG) emissions, biofuel has been recognized as an alternative renewable energy which enhances the quality of our environment and human health1,2. Microalgae biomass is an attractive feedstock for biofuel production due to its high growth rate, its ability in bio-fixation of CO₂ from the atmosphere and it could be cultivated in wastewater for nutrients removal3-5. Besides, microalgae biomass has high lipid contents which subsequently led on producing energy-rich biofuel. Many investigations have been carried out across different species of microalgae cultivated under the fixed parameter, but the lipid content reported is different from one another6. Thus, to ensure the success of microalgae biofuel industry, the first priority in microalgae selection is to aim for the highest yield in lipid content to ensure the highest yield will be achieved during biodiesel production7. Furthermore, cultivation criteria such as temperature, irradiance and nutrient availability would affect the microalgae lipid content and its lipid composition8-10. The quality of microalgae lipid and composition must comply with the existing international standard, such as American Society for Testing Materials ASTM Biodiesel Standard 6751 (United States) as well as European Standards EN 14214 and EN 14213 (European Union)11. Hence, in analyzing microalgae lipid, advance lipid profiling technique is used to identify suitable strain and cultivation conditions for optimum biodiesel production12.

For non-algae lipid analysis, thin layer chromatography and liquid chromatography are the suitable old school techniques in profiling lipid composition. Most of the conventional techniques are less precise and require longer time for analysis than the modern techniques. After several decades, more recent techniques and accurate technologies have been invented for lipid composition analysis. The aim of this paper is to give an insight on the advantages and disadvantages of the most recent modern techniques for microalgae lipid analysis.

2. Lipid profiling methods

2.1 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) gave the greatest value during the analysis of volatile components such as short chain fatty acids. In mobile phase, detectors with different principles will sense the solute
as they are being eluted from the column during HPLC analysis. Coupling of HPLC with ultraviolet detection (UV) and refractive index detector (RID) have been reported to be a rapid method to analyze short chain fatty acids. Previous study also shows that reversed phase HPLC with evaporative light scattering detection (ELSD) is the most efficient method to purify and analyze polyunsaturated fatty acids (PUFAs).

The application of ELSD is wide as the detector will react with any solute which does not evaporate before passing through the light beam. Furthermore, the detector is user friendly and offers high flexibility. In addition, when columns and mobile phases are changed, the detector requires only minimal time to stabilize the baseline and no adjustment is required during operation even though complex gradients are being analyzed. ELSD is also reported to be more sensitive than that of transport flame ionization detector (TFID) and UV spectrometry detector which are operated at low wavelengths.

Another advantage of ELSD is that the changes in ambient temperature or small variations in the flowrate of the mobile phase will not have significant effect on the operation of ELSD. The techniques to analyze neutral lipids from microalgae lipid extracted using HPLC normal phase coupled to ELSD have been improved and applied extensively.

HPLC coupled with mass spectrometry (MS) would make an impressive and applicable technology for high accuracy data reading. Its wide range applications includes separation, general detection and potential identification of chemicals in the complex mixture. With HPLC-MS systems, most structural information could be obtained even for low molecular weight or low volatility compounds. However, it is difficult to perform quantitative analysis using absolute MS responses due to a large number of factors involved in the operation such as the cleanliness of the ion source, ion suppression, ion source flow rates, ion optics, the collision cell, collision cell pressure and the ultimate MS vacuum. HPLC-MS method has been used particularly to analyze triacylglyceride (TAG) of various lipid from different microalgae strains for biodiesel production.

2.2 Gas Chromatography

Gas chromatography (GC) has been recognized as one of the most widely applied analysis techniques. The advantages of GC are highly sensitive, rapid, accurate and excellent reproducibility. These advantages of GC have led to the relatively specific analysis of fatty acids (FAs). The polarity of the stationary phase, column length and type of detector would affect the resolutions of the GC. Thus, GC requires a packed column instead of capillary column, more polarizable stationary phase and a long column. Due to its extensive applications, GC has been used for determination of FAs compositions of lipid from different microalgae strains.

GC-MS is a valuable method which combines the functions of GC and MS. GC-MS is being used in broad applications due to its improved sensitivity in sample identification, enhanced molecular ion, extended range of thermally labile and low volatility sample for analysis. GC-MS commonly uses electron ionization (EI) and chemical ionization (CI) techniques. GC-MS has been used for analysis of fatty acids from various microalgae strains including Scenedesmus obliquus and Phaeodactylum tricornutum.

Flame ionization detector (FID) is a favorite tool for GC routine analysis of complex biological samples since 1958 since FID responds practically to all organic compounds. FID is also resistant to small fluctuations of the gas flow and is insensitive to gas impurities. On top of that, the FID response is predictable since it follows the rule of equal carbon response.

FID is a common detector which is coupled with many current analysis techniques for identification of FAs from microalgae. Overall, 76 different fatty acids and 10 other lipophilic substances were successfully identified and quantified by Göttingen University from 2076 microalgae species. GC-FID provides the strongest detection sensitivity to analyze FAs in lipid extracts from Chlorella vulgaris, Ankistrodesmus gracilis and Scenedesmus quadricauda. GC-FID is commonly used for screening of FAs composition in marine microalgae species including Diophyceae, Bacillariophyceae, Chlorophyceae, Haptophyceae and Raphidophyceae species.

2.3 Spectrometry

The MS-based method is the best method in terms of high sensitivity and specificity, high throughput and high accuracy.

2.3.1 Electron Ionization (EI) and Chemical Ionization (CI)

EI detection is based on high energy electrons interact with gas phase atoms or molecules to produce ions. EI-MS has been used in determination of sterol, cholesterol and FAs. However, it should be noted that esterification is required for FAs analysis using EI-MS. EI-MS has weak molecular ion signal because of the high energy collision. Thus, CI was developed to produce an intact molecular ion species using lower energy collision of the analyte and ions of a reagent gas. The intact signal of the molecular ion for FAs was successfully
detected by using pentafluorobenzyl bromide and negative chemical ionization (NCI) \(^43\). Nevertheless, the EI/CI MS-based method for lipid analysis is limited because of the unpleasant derivative steps and lower sensitivity.

2.3.2 Matrix assisted laser desorption ionization (MALDI)

MALDI-MS is broadly used in the analyses of organic compounds. The lipid extracts from a biological sample are complex which have interferences of several molecules and resulted in difficulty in analysis. The critical point for a successful MALDI-MS analysis is to choose the proper matrix \(^44\). MALDI-MS technique was applied to determine the TAG composition of different saltwater microalgal strains such as \textit{Phaeodactylum tricornutum}, \textit{Nannochloropsis salina}, \textit{Nannochloropsis oculata}, and \textit{Tetraselmis suecica} \(^45\) and it was proven as particularly useful for identifying microalgae TAG profiles with the desirable fatty acid composition for biofuel purposes.

2.3.3 Electrospray Ionization (ESI)

ESI is the main ionization method in MS for lipid analysis. Numerous reports have been published on usage of ESI-MS for direct lipid analysis without pre-separation by LC \(^46\), profiling of glycosylphospholipids and phospholipids of microalgal lipid \(^47\) and structural characterization of TAG from microalgal lipid \(^48\). Recently, atmospheric pressure chemical ionization (APCI), atmospheric pressure photoionization (APPI) and desorption electrospray ionization (DESI) were also developed for lipid analysis.

Nuclear magnetic resonance (NMR) method is based on the \(^1\)H, \(^13\)C, \(^31\)P and heteronuclear single quantum coherence (HSQC) spectral analysis to identify and quantify chemical compositions in biomass including microalgal lipid. In this method, the area per proton (determined by integration) is calculated to derive the equations to determine the amount of the unsaturated fatty acids. Although NMR analysis provides more molecule structure information than other techniques, its use for lipid analysis is not popular due to relatively low sensitivity and overlapping of signal in the spectrum \(^49\). In addition, NMR-based lipid analysis methods are less sensitive than MS-based methods.

Table 1 shows the comparison of HPLC, GC and spectrometry analysis method of microalgae lipid.

### Table 1  Comparison of several analysis methods of microalgae lipid

| Detector    | Principle                                           | Advantages                                                                 | Disadvantages                                                                 | Limit of detection |
|-------------|-----------------------------------------------------|----------------------------------------------------------------------------|-------------------------------------------------------------------------------|-------------------|
| HPLC        | UV-VIS Detection base on the sample’s absorption of light at different wavelengths. | Wide light range from 190-700nm widely used for compounds detection, especially in separating phospholipid \(^50\). | Sensitivity depends on the component and its concentration. Many solvents absorb strongly from 200-210nm while most microalgae lipids exhibit a weak absorbance at such wavelength \(^51\). | 0.15 µg \(^52\) |
| Refractive Index (RID) | Involves measuring of the changes in refractive index of the column effluent passing through the flow cell. | Any component that differs in refractive index from the eluate can be detected, despite its low sensitivity. | Cannot be used to perform gradient analysis. | 10 ng \(^53\) |
| Evaporative light scattering (ELSD) | Detection is based on the scattering of a beam of light by particles of compound remaining after evaporation of the mobile phase. | Non-UV absorbing compounds can be detected selectively with high sensitivity. | ELSD is closest to become a universal detector for HPLC that can be used for almost every component. | 3 ng \(^54\) |
| Mass spectrometry (MS) | The detection principle is using the mass fragments that resulted from ionization of the molecules. | Component can be detected with essential information: the molecular weight, the empirical formula and often the complete structure with the amount present. | It is difficult to remove the solvent prior to ionization. | 100 pg - 1 ng |

3. Conclusion

Each analytical method has its own advantages and disadvantages during analysis of microalgal lipid from different species. Hence, combined techniques are usually used to attain high accuracy results. The most common method used is the GC-FID which is the most...
### GC Mass spectrometry
(MS)

An inert gas will carry the heated gasses which are separated into individual substances through a column then flow to the MS. Analyte molecule is identified by the mass.

GC-MS can separate and identify individual compounds with superior sensitivity and selectivity of analytes. Fatty acids with labile functional groups are difficult to recognize its molecular ion because of excessive fragmentation. Furthermore, it is impossible to archive the quantitative accuracy because of difficulties to calibrate the standards.

| Analyte | Mass Range |
|---------|------------|
| 100 ng - 1 µg |

### Flame ionization detector (FID)

Combustion form ions and free electrons of organic compound in a synthetic air flame. The generation of these ions is proportional to the concentration of organic species in the sample gas stream.

GC-FID can detect virtually all organic compounds with high sensitivity and stability.

Hydrocarbon is oxidized to carbon dioxide and water which is resulted in sample destruction.

| Analyte | Mass Range |
|---------|------------|
| 0.3 - 0.9 ng |

### MS Electron Impact
(EI)

In EI, molecules are ionized by collision with electrons produced by a heated filament in order to produce a positive charge.

Molecular weight of analyte is determined easily by EI-MS and CI-MS with simple mass spectra.

The sample must be thermally volatile and stable.

### Chemical Ionization
(CI)

In CI, the collision of the analyte with ions of a reagent gas in ion source forms analyte ions.

CI-MS is less fragmented than EI-MS, fragment patterns not informative enough for library search.

| Analyte | Mass Range |
|---------|------------|
| 22.5 fmol |

### Matrix-assisted laser desorption ionization (MALDI)

The analyte is dissolved in a solution containing an excess of a matrix that has a chromophore that absorbs at the laser wavelength. A small amount of this solution is placed on the laser target. The matrix absorbs the energy from the laser pulse and produces a plasma that results in vaporization and ionization of the analyte.

High molecular weight analyte can be ionized and sub-picomole sensitivity easy to obtain.

Pulsed nature source limits compatibility with many mass analyzers.

| Analyte | Mass Range |
|---------|------------|
| 25 ng |

### ESI

Ions from solution can be transferred into the gaseous phase by the assistance of ESI electrical energy. Neutral compounds after converting to ionic form then into gaseous phase and ions species can be analyzed by MS.

ESI-MS is feasible to separate different lipid classes and analyze individual compounds without chromatography separation.

The mixture should be avoided when using ESI-MS because of attachment of the multiple charges to the molecular ions that can cause spectral data confusion.

| Analyte | Mass Range |
|---------|------------|
| 25 ng |

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