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METHODS FOR QUANTITATIVE DETERMINATION OF MICROALGAL LIPID AND FATTY ACIDS CONTENT

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microalgae, lipids, lipid quantification, biodiesel production, biomass

ABSTRACT
Microalgae represent a promising feedstock for sustainable biofuel production and high-value lipid-based bioproducts due to their high lipid productivity and rapid growth rates. Accurate and reproducible lipid quantification is essential for strain selection, process optimization, and industrial scaling. This review presents a comprehensive and critical evaluation of contemporary lipid quantification methods applied to microalgae. The methodologies are categorized into screening, quantitative, and profiling approaches, encompassing techniques such as solvent extraction, *in situ* and direct transesterification, colorimetric assays, spectroscopic tools (NIR, FTIR), and chromatographic techniques (GC, LC–MS/MS). Each method is evaluated across multiple performance axes, including analytical accuracy, throughput, requirement to the sample, technical complexity, and standardization potential. Results are synthesized using the comparative tables. While high-throughput screening tools (e. g., Nile Red, SPV) offer speed and easiness of using, they exhibit limitations in accuracy and reproducibility. Quantitative methods such as acid-catalyzed *in situ* transesterification coupled with gas chromatography demonstrate a strong balance between precision and scalability. Profiling methods, including LC–MS/MS, provide the highest molecular resolution but are cost- and labor-intensive. The review highlights the need for methodological harmonization and discusses the trade-offs associated with analytical choices in research and industry. Practical recommendations are proposed for selecting the appropriate techniques depending on application context — from early-stage screening to advanced lipidomic profiling.

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МЕТОДЫ КОЛИЧЕСТВЕННОГО ОПРЕДЕЛЕНИЯ СОДЕРЖАНИЯ ЛИПИДОВ И ЖИРНЫХ КИСЛОТ В МИКРОВОДОРОСЛЯХ

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КЛЮЧЕВЫЕ СЛОВА:
микроводоросли, липиды, количественное определение липидов, производство биодизельного топлива, биомасса

АННОТАЦИЯ
Микроводоросли представляют собой перспективное сырьё для устойчивого производства биотоплива и ценных биопродуктов благодаря высокой липидной продуктивности и быстрому темпу роста микроводорослей. Точное и воспроизводимое количественное определение липидов имеет решающее значение для отбора штаммов, оптимизации процессов и масштабирования производства. Настоящий обзор представляет собой всестороннюю и критическую оценку современных методов количественного анализа липидов, применяемых к микроводорослям. Рассмотренные методики классифицируются по типу применения: скрининговые, количественные и профилирующие подходы, включая такие технологии, как экстракция растворителями, *in situ* и прямая этерификация, колориметрические тесты, спектроскопические методы (NIR, FTIR), а также хроматографические техники (ГХ, ВЭЖХ–МС/МС). Каждый метод оценивается по нескольким критериям, включая аналитическую точность, пропускную способность, требования к образцам, техническую сложность и потенциал стандартизации. Результаты обобщаются в виде сравнительных таблиц. Несмотря на высокую скорость и простоту применения, скрининговые инструменты (например, Nile Red, SPV) недостаточно точны и воспроизводимы. Количественные методы, такие как кислотно-катализируемая *in situ* этерификация в сочетании с газовой хроматографией, демонстрируют оптимальное соотношение точности и масштабируемости применения. Методы профилирования, включая ВЭЖХ–МС/МС, обеспечивают наивысшее молекулярное разрешение, но требуют значительных экономических и трудовых затрат. Обзор подчёркивает необходимость гармонизации методик и обсуждает компромиссы, связанные с выбором аналитического подхода в научных и прикладных целях. Предлагаются практические рекомендации по выбору наиболее подходящих методов в зависимости от контекста применения — от раннего скрининга до продвинутого липидомного профилирования.

1. Introduction

Microalgae are a diverse group of photosynthetic microorganisms with immense biotechnological potential due to their rapid growth rates, high photosynthetic efficiency, and ability to accumulate substantial amounts of lipids under specific environmental conditions. These unicellular organisms can synthesize a wide variety of lipids, including triacylglycerols (TAGs), phospholipids, glycolipids, and free fatty acids, thus making them a valuable raw material for biofuel production, nutraceuticals, cosmetics, and pharmaceuticals [1]. The increasing demand for sustainable energy sources and environmentally friendly industrial raw materials has spurred significant interest in the development of efficient methods for analysis of lipid content in microalgae, which is critical for optimizing cultivation strategies and lipid extraction protocols [2].

Lipid content and composition in microalgae can vary significantly depending on species, cultivation conditions, and the physiological state of the cells [3]. Hence, accurate, reliable, and reproducible analytical methods are essential for the qualitative and quantitative characterization of lipid fractions. Over the past decades, a broad range of methodologies has been developed and refined to analyze lipids in microalgae. These methods differ in terms of sensitivity, specificity, throughput, complexity, and suitability for different types of samples or analytical purposes. The choice of an appropriate method is often guided by the goals of the study, available equipment, and the desired accuracy [4].

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Traditional gravimetric methods, such as those proposed by Folch or Bligh and Dyer, remain widely used due to their simplicity and applicability for total lipid quantification [5]. However, these methods often lack specificity and may underestimate or overestimate lipid content due to co-extraction of non-lipid components or incomplete extraction. More advanced techniques, including chromatographic and spectroscopic methods, provide detailed compositional data and improved sensitivity but may require sophisticated tools, instrumentation and technical expertise [6].

In recent years, the development of fluorescence-based and spectrophotometric assays has provided faster and more accessible alternatives for high-throughput screening of lipid content, especially in strain selection and metabolic engineering studies [7]. Furthermore, non-destructive techniques such as nuclear magnetic resonance (NMR), Fourier-transform infrared (FTIR), and Raman spectroscopy have been explored as tools for real-time monitoring of lipid accumulation *in vivo* conditions [8].

Despite the availability of numerous analytical methods, there is still a lack of consensus regarding standardized protocols for lipid quantification in microalgae. Variations in extraction procedures, calibration standards, and sample pretreatment can lead to inconsistent results across the studies, thus hindering the comparison and interpretation of data. Therefore, a comprehensive review of the existing methodologies, their principles, advantages, limitations, and applications is essential for guiding the researchers in selecting the appropriate analytical strategies tailored to the specific research goals [9].

This review aims to provide an in-depth overview of the current state-of-the-art methods used for lipid analysis in microalgae, categorized into gravimetric, spectrophotometric/fluorometric, chromatographic, and spectroscopic techniques. Special emphasis is placed on methodological considerations, accuracy, reproducibility, and practical applicability in the context of microalgal biotechnology. By summarizing the strengths and weaknesses of each approach, we intend to support the informed decision-making in the design of experimental workflows for lipid profiling and quantification.

2. Objects and methods

2.1. Study objective

The primary objective of this review was to conduct a comparative analysis of the most prevalent quantitative methods used for lipid analysis in microalgae. This involved evaluating the accuracy, technical requirements, and methodological performance of various extraction, transesterification, spectroscopic, colorimetric, and chromatographic approaches. A total of ten studies meeting specific inclusion criteria were selected for in-depth analysis, with the goal of elucidating methodological trade-offs and supporting the development of the best practices for lipid quantification in algal cultivation biotechnology.

2.2. Literature search strategy

To conduct a comprehensive review of analytical methods for lipid quantification in microalgae, a systematic literature research was performed using two primary scientific databases: Scopus and Web of Science. These databases were selected due to their extensive coverage of peer-reviewed scientific literature and their relevance to the fields of biotechnology and analytical chemistry.

The search strategy involved the use of specific keywords and Boolean operators to identify relevant publications. The keywords included "microalgae," "lipid analysis," "lipid quantification," "gravimetric," "spectrophotometric," "chromatographic," and "spectroscopic." The search was conducted within the titles, abstracts, and keywords of the articles to ensure a specifically focused and relevant selection of studies.

The initial search yielded a total of 1,339 articles published between 1990 and November 2025 in the Scopus database. A similar search in the Web of Science database provided a comparable number of publications, indicating a substantial body of research in this area. To refine the selection, the following inclusion criteria were applied:

Publications must be peer-reviewed articles written in English.

Studies must focus on methods for lipid quantification in microalgae.

Articles must provide experimental data or comprehensive reviews of analytical techniques.

2.3. Exclusion and screening criteria

To ensure the methodological relevance and scientific rigor of the included publications, a set of predefined screening criteria was applied. Each candidate paper was assessed for the following:

- Study Subject — Inclusion was limited to studies analyzing lipid content specifically in microalgae species.
- Quantitative Analytical Method — Only the studies that described and implemented at least one quantitative analytical method for lipid measurement were considered.
- Comparative Analysis — Studies were required to compare at least two different methods for lipid quantification, allowing for relative performance assessment.
- Validation Data — Only studies that included validation data or reported performance metrics (e. g., precision, R^2 values, standard deviations) were chosen.
- Methodological Detail — Sufficient methodological descriptions were necessary to permit replication or technical assessment.
- Quantitative Outcomes — Studies needed to report quantitative lipid measurements (e. g., mg/g biomass, percentage content).
- Study Type — The analysis was restricted to primary research articles and systematic reviews explicitly focused on analytical method evaluation.

Papers, that did not simultaneously meet all criteria, were excluded after a holistic assessment.

2.4. Data extraction procedure

Following screening, a structured data extraction protocol was used using a large language model to ensure consistency and accuracy in data collection. The following key data fields were extracted from each included publication:

- Lipid Quantification Method: Identification of the primary analytical technique(s), including standard methods (e. g., Bligh and Dyer, Folch), their modifications, and associated tooling and instrumentation (e. g., GC–MS, LC–MS/MS).
- Microalgae Species and Sample Characteristics: Information regarding species identity, number of strains examined, sample type (e. g., fresh, freeze-dried), and cell preparation procedures (e. g., disruption methods).
- Analytical Techniques and Instruments: Detailed listing of analytical platforms (e. g., chromatography, spectrophotometry), catalysts used (e. g., HCl, methanolic KOH), and specific quantification protocols.
- Key Results: Quantitative outcomes such as lipid yield, accuracy (e. g., correlation coefficients), relative standard deviation, and method comparison outcomes (e. g., method A yielded 15 % higher lipid values than method B).
- Limitations and Challenges: Method-specific critiques, reported analytical difficulties, limitations in sensitivity or specificity, and any author-recommended improvements.

Where specific values or techniques were not fully described in the text, "Partial information available" or "Not reported" annotations were included for transparency.

For data synthesis, methods were thematically grouped and quantitatively compared based on shared criteria such as lipid recovery efficiency, sample requirements, instrumentation, and validation protocols. Categorization enabled structured comparisons across the techniques — the ranging from traditional extraction to advanced LC–MS/MS and NIR spectroscopy. These efforts provided a reliable basis for evaluating analytical trade-offs in lipid quantification strategies.

3. Results and discussion

Lipid quantification in microalgae is a multifactorial analytical challenge due to cellular diversity, lipid heterogeneity, and environmental influences on biosynthesis. Among the peer-reviewed studies analyzed in this review, nine unique method categories were identified, each aligned with certain technical principles, ranging from gravimetric solvent extraction to real-time spectroscopic assessment. These methods were classified in the Table 1 based on technical approach, degree of quantification, and methodological complexity.

Gravimetric methods, particularly the classical Bligh and Dyer method, and Folch method of extraction, remain popular due to their simplicity and ability to provide total lipid content measuring. However, these methods lack specificity, require toxic solvents, and are labor-intensive [5,10].

Spectrophotometric methods such as the sulfo-phospho-vanillin (SPV) assay are used for their simplicity and relative cost-effectiveness. However, the presence of interfering compounds such as pigments and carbohydrates can influence the accuracy of measurements [11].

Fluorimetric methods using dyes like Nile Red and BODIPY505/515 are increasingly favored for high-throughput and *in vivo* applications usability. These dyes bind selectively to neutral lipids and fluoresce, allowing rapid quantification. However, their efficiency depends on algal cell wall composition, thus leading to inconsistencies among the species [12,13].

Chromatographic methods-such as gas chromatography with flame ionization detection (GC-FID) or high-performance liquid chromatography (HPLC)-are considered the gold standards for lipid profiling due to

Table 1. **Summary of analytical methods for lipid quantification in microalgae**

Таблица 1. **Обобщенное изложение результатов аналитических методов количественной оценки объема липидов в микроводорослях**

Method category	Specific techniques	Sensitivity	Specificity	Throughput	Major advantages	Limitations	Reference
Gravimetric	Bligh and Dyer, Folch, Soxhlet	Moderate	Low	Low	Simple; provides total lipid content	Time-consuming; low selectivity; use of hazardous solvents	[5,10]
Spectrophotometric	Sulfo-phospho-vanillin (SPV)	Moderate	Low	Moderate	Inexpensive; relatively quick	Interference from pigments and proteins	[11]
Fluorimetric	Nile Red, BODIPY505/515	High	Moderate	High	Non-destructive; rapid; high-throughput screening	Variability in dye uptake; affected by cell wall permeability	[12,13]
Chromatographic	GC-FID, HPLC, TLC	High	High	Low–Medium	High accuracy; allows fatty acid profiling	Requires derivatization; expensive; complex sample preparation	[14,15]
Spectroscopic	FTIR, Raman, NMR, Near-IR	Moderate	Moderate	Moderate	Minimal sample prep; potential for real-time monitoring	Requires calibration; lower accuracy in absolute quantification	[16,17]

their high sensitivity and ability to distinguish between lipid classes and fatty acid chains. However, they require complex instrumentation and derivatization procedures [14,15].

Spectroscopic techniques such as Fourier-transform infrared (FTIR) technique, nuclear magnetic resonance (NMR) method, and Raman spectroscopy are gaining dominance for non-destructive analysis. Though less accurate for absolute lipid quantification, they are useful for monitoring trends in lipid accumulation and require minimal sample preparation [16,17].

Figure 1 provides a comprehensive comparative overview of the principal lipid quantification methods applied in microalgae research. The figure categorizes these techniques into four main groups: extraction methods, fluorometric/colorimetric methods, spectroscopic methods, and chromatographic methods. Under extraction methods, both solvent extraction and direct transesterification are presented, highlighting their operational peculiar characteristics such as efficiency, accuracy, and susceptibility to the sample composition. Fluorometric and colorimetric approaches, including Nile Red fluorescence and the sulfo-phospho-vanillin (SPV) assay, are noted for their rapidity and high accuracy, although limitations like cell wall permeability can affect performance. Spectroscopic techniques, such as Fourier-transform infrared (FTIR) spectroscopy and near-infrared (NIR) spectroscopy, offer non-destructive, rapid analysis, with NIR achieving superior accuracy across the species. Chromatographic techniques, encompassing supercritical CO_2 extraction and liquid chromatography-mass spectrometry (LC–MS/MS), demonstrate high resolution in lipid profiling but require expensive tooling, instrumentation and longer processing times. This integrative visual summary (Figure 1) facilitates an at-a-glance understanding of the methodological landscape, thus enabling researchers to evaluate trade-offs between precision, throughput, and resource demands when selecting the appropriate lipid analysis techniques for microalgae.

3.1. Extraction methods

Lipid extraction remains a foundational step in the quantitative and qualitative analysis of lipid content in microalgal biomass. Among the various approaches, solvent-based extraction and direct transesterification are the most widely utilized due to their relatively high reliability, albeit with certain limitations that have prompted continuous methodological refinements.

3.1.1. Solvent-based extraction methods

The most commonly cited classical methods include the Bligh and Dyer method and the Folch method. These are based on the partitioning of lipids into a mixture of polar and nonpolar solvents, typically chloroform-methanol-water systems [5].

The Bligh and Dyer Method (1959) is one of the most extensively applied protocols, particularly suited for the wet samples. This method utilizes a chloroform-methanol (1:2, v/v) solution followed by water addition to induce phase separation. However, while efficient for samples with lower lipid content, it often underestimates lipid yield in dry biomass and can be limited due to emulsification issues [18].

The Folch Method (1957), which uses a higher solvent-to-sample ratio (chloroform-methanol 2:1, v/v), is better suited for lipid-rich samples and tends to extract more total lipids than Bligh and Dyer's method [10]. Despite this, its high volume of toxic solvents raises environmental and safety concerns.

Soxhlet Extraction is another classical method, which continuously washes dried biomass with organic solvents (e. g., hexane, petroleum ether) under reflux. Although this method provides relatively exhaustive extraction, it is time-consuming (typically 6–8 hours), energy-intensive, and its application is limited to dried biomass only [19].

These solvent-based methods, though widely used, present several challenges such as the requirement for toxic solvents, incomplete

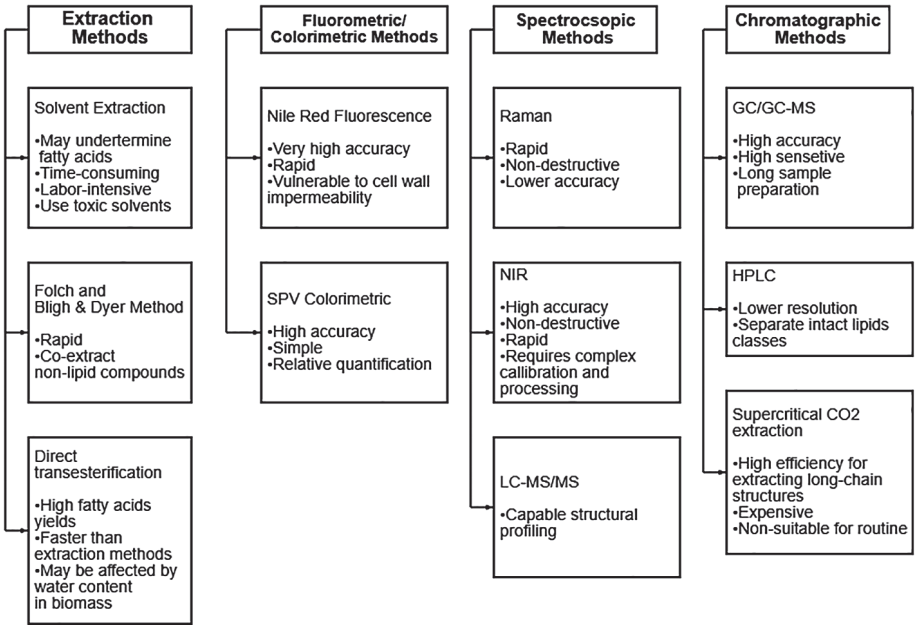

Figure 1. **Comparison of lipid quantification methods in microalgae**

Рисунок 1. **Сравнение методов количественной оценки объема липидов в микроводорослях**

extraction of bound lipids, and time-intensive procedures, which hinder high-throughput applications.

3.1.2. Direct transesterification

Direct transesterification bypasses the need for prior lipid extraction by directly converting fatty acids into fatty acid methyl esters (FAMEs) using an acid or base catalyst in the presence of methanol [20]. This technique has gained popularity due to its ability to more comprehensively convert both free and bound fatty acids in microalgae, especially for biodiesel-production oriented researches.

The primary advantages of this method include a shorter analysis time and higher total lipid yields compared to solvent extraction techniques. Nevertheless, the presence of water in the biomass can lead to saponification reactions and reduced FAME yields, requiring careful sample pretreatment and drying [21].

From the Table 2, it is evident that though the Soxhlet method features the highest extraction efficiency, it is impractical for large-scale or time-sensitive analyses. Bligh and Dyer's and Folch's methods offer a good balance between yield and time but are hindered by solvent toxicity and handling complexity. Direct transesterification, though sensitive to moisture, presents a more rapid and yield-efficient alternative, especially for biomass destined for transesterification-based downstream applications such as biodiesel production. Overall, method selection must balance extraction efficiency, safety, environmental impact, and suitability for the sample's type.

Table 2. **Comparison of extraction methods for lipid analysis in microalgae**

Таблица 2. **Сравнение методов экстракции для анализа липидов в микроводорослях**

Method	Sample Type	Accuracy (Yield%)	Solvent Use	Time Required	Key Limitations	Reference
Bligh and Dyer	Wet	80–90	Yes	~1–2 h	Underestimates in dry biomass, emulsification issues	[5]
Folch Method	Wet/ Dry	90–95	Yes	~2 h	Uses large solvent volumes, toxic solvents	[10]
Soxhlet Extraction	Dry	95–98	Yes	6–8 h	Long extraction time, not suitable for wet biomass	[19]
Direct Transeste-rification	Wet/ Dry	95–99	Minimal	~1–2 h	Affected by moisture, requires methylation step	[20]

3.2. Spectrophotometric and fluorometric methods

Spectrophotometric and fluorometric techniques represent the valuable tools in quantification of lipids extracted from microalgal biomass due to their relative simplicity, speed, and cost-effectiveness. These methods are typically applied when a rapid estimation of total lipid content is required, particularly in large-scale screening of strains or growth conditions. Although less detailed than chromatographic or spectroscopic analyses, they serve as instruments in initial assessments.

3.2.1. Sulfo-phospho-vanillin (SPV) colorimetric method

The SPV assay is based on the reaction between unsaturated lipid chains and a sulfuric acid-vanillin-phosphoric acid complex, producing a pink color that can be quantified at 520 nm using a spectrophotometer. This method is relatively sensitive and provides good linearity between lipid concentration and light absorbance ($R^2 \approx 0.99$) [22]. One major limitation, however, is its dependency on the degree of unsaturation in lipid samples, potentially leading to under- or overestimation based on lipid composition. Moreover, this method is destructive and involves the injection of corrosive reagents, such as concentrated sulfuric acid, which poses safety concerns and disposal challenges for environment [23].

3.2.2. Nile red fluorescence staining

Nile Red is a lipophilic dye that fluoresces strongly in non-polar environments such as lipid droplets, emitting at 575–600 nm when excited with blue light. This method is widely used for high-throughput lipid screening due to its rapidity and compatibility with flow cytometry and fluorescence microscopy. It provides high accuracy (R^2 up to 0.998) in species with permeable cell walls [12]. However, the method suffers from several drawbacks: cell wall impermeability in certain microalgal species can hinder dye penetration, leading to an underestimation of lipid content [24]; moreover, Nile Red fluorescence can be quenched or influenced by other cellular components, such as pigments, and it cannot distinguish between neutral and polar lipids [13].

3.2.3. BODIPY staining

BODIPY (boron-dipyrromethene) dyes are another class of fluorescent probes used for lipid detection in microalgae. Compared to Nile Red, BODIPY505/515 exhibits more consistent staining, reduced background fluorescence, and a greater specificity for neutral lipids [25]. Furthermore, it demonstrates improved performance in strains with thicker cell walls and is less susceptible to interference caused by autofluorescent pigments. However, the method is more expensive and requires advanced fluorescence imaging or plate-reader systems. The quantification accuracy is also slightly lower than Nile Red in some cases ($R^2 \approx 0.97–0.98$) [26].

Comparative analysis of the methods is presented in Table 2. These methods are optimal for comparative lipid estimation in microalgae and are often used in early-stage bio-prospecting studies. While colorimetric approaches such as SPV provide reliable quantification at low cost, fluorescence-based techniques, particularly Nile Red and BODIPY, offer superior speed and automation potential for large-scale screening. However, the variability in cell wall composition across microalgal taxa and the influence of interfering compounds demand cautious interpretation and, where possible, method of validation against gravimetric or chromatographic benchmarks.

3.3. Chromatographic methods

Chromatographic techniques have been widely adopted as key analytical tools for the qualitative and quantitative assessment of lipid content and composition in microalgae. Among the most prevalent are gas chromatography (GC), high-performance liquid chromatography (HPLC), and thin-layer chromatography (TLC). Each of these methods offers unique advantages depending on the type of lipids being analyzed, the relevant resolution, and the sample complexity.

Gas Chromatography (GC) is considered as the gold standard for fatty acid methyl ester (FAME) analysis, providing high sensitivity, reproducibility, and resolution for volatile and thermally stable compounds. The process typically involves derivatization of lipids into FAMEs, followed by separation on capillary columns with flame ionization detection (FID) or mass spectrometry (MS) detection [27]. GC-FID is commonly used due to its simplicity and cost-efficiency, whereas GC–MS enables more precise structural representation of results [28]. However, the derivatization step can introduce variability and is not suitable for intact lipid class analysis.

High-Performance Liquid Chromatography (HPLC) allows for the direct analysis of intact lipid classes without the need for derivatization. HPLC coupled with evaporative light scattering detection (ELSD), diode-array detection (DAD), or tandem mass spectrometry (MS/MS) is particularly effective for profiling neutral lipids, glycolipids, and phospholipids [29]. Reversed-phase HPLC is typically applied for the separation of lipid classes based on chain length and saturation, while normal-phase HPLC is more suited for class-based lipid separation [30]. The method, however, is less efficient in resolving isomeric species compared to GC–MS, and often requires longer analysis times.

Thin-Layer Chromatography (TLC) is a low-cost, rapid screening method used for semi-quantitative lipid profiling. It is particularly useful for preliminary assessments of lipid class distribution. TLC can separate major lipid categories such as triacylglycerols (TAGs), free fatty acids (FFAs), and phospholipids by using specific solvent systems on silica gel plates. Detection can be performed using iodine vapor, charring, or

Table 3. **Comparison of spectrophotometric and fluorometric methods for lipid analysis in microalgae**

Таблица 3. **Сравнение спектрофотометрических и флуоресцентных методов для анализа липидов в микроводорослях**

Method	Principle	Accuracy (R^2)	Advantages	Limitations	References
SPV Colorimetric	Reaction with vanillin and sulfuric acid	~0.99	Simple, inexpensive, relatively accurate	Not lipid-class specific; hazardous reagents; destructive	[22,23]
Nile Red Fluorescence	Fluorescence in hydrophobic environment	0.995–0.998	Rapid, high-throughput, widely used	Cell wall permeability issues; signal interference	[12,13,24]
BODIPY505/515	Neutral lipid-specific fluorescence	0.97–0.98	Improved signal-to-noise; less pigment interference	Expensive; requires advanced instruments	[25,26]

densitometry after plate development [31]. Although TLC offers simplicity, it suffers from limited resolution, poor reproducibility, and lacks sensitivity compared to GC and HPLC.

A comparison of these chromatographic methods is summarized in the Table 4.

Among chromatographic techniques, GC offers the highest precision for fatty acid profiling but is limited with its requirement for derivatization and its inability to assess intact lipid structures. HPLC stands out as a powerful method for intact lipid profiling and identification of bioactive lipids, especially when coupled with mass spectrometry. Its limitations lie in the cost and analytical complexity. TLC, on the other hand, provides a cost-effective and fast solution for general lipid class assessment but lacks the precision and reproducibility required for detailed quantitative analysis. Therefore, the choice of method is highly context-dependent: GC is preferred for detailed fatty acid profiling, HPLC for comprehensive class-based lipidomics, and TLC for preliminary process or high-throughput screening purposes.

3.4. Spectroscopic methods

Spectroscopic techniques have emerged as powerful tools in the field of lipid quantification and characterization due to their non-destructive nature, high specificity, and potential for rapid analysis. These methods are based on the interaction of electromagnetic radiation with lipid molecules, which allows for the elucidation of molecular structures, functional groups, and even quantitative assessment of lipid content in microalgae. Compared to traditional extraction-based techniques, spectroscopy offers the advantage of minimal sample preparation and is increasingly being integrated into real-time and high-throughput lipid analysis pipelines [32].

The development and refinement of spectroscopic methods have enabled researchers to overcome some of the major drawbacks associated with classical lipid extraction and quantification techniques, such as solvent usage, long processing times, and poor reproducibility. In particular, spectroscopic techniques such as Nuclear Magnetic Resonance (NMR), Fourier Transform Infrared (FTIR) spectroscopy, Raman spectroscopy, and Near-Infrared (NIR) spectroscopy have been extensively explored due to their potential to provide qualitative and quantitative insights into the lipidome array of various microalgal species [33,34].

Each of these methods offers unique advantages: for example, FTIR and NIR are highly amenable to high-throughput screening due to their speed and minimal sample processing requirements, while Raman spectroscopy provides molecular-specific fingerprints without the need for dyes or labels. NMR, despite its high cost, is unmatched in its ability to elucidate detailed molecular structures and quantify lipid classes directly in crude extracts or even intact cells [35]. However, no single spectroscopic method offers a universal solution, and its selection depends on analytical objectives, sample's complexity, and instrumentation availability.

This section focuses on a detailed review of spectroscopic methods applied in the analysis of lipids in microalgae.

3.4.1. Nuclear magnetic resonance (NMR) spectroscopy

NMR spectroscopy is a highly reliable analytical technique that provides both qualitative and quantitative information about lipids in biological matrices. It operates based on the magnetic properties of atomic nuclei, typically ^1H and ^13C, and their response to external magnetic fields. When applied to lipid analysis, NMR enables the identification of different lipid classes, degrees of unsaturation, and positional isomerism with high accuracy and reproducibility [36].

One of the most significant advantages of NMR over chromatographic and colorimetric methods is its capacity for direct analysis of lipid extracts without the need for derivatization. This makes NMR perfect for structural elucidation and comprehensive lipid profiling. Moreover, NMR can be used quantitatively due to its linear response to concentration changes, providing reliable data on total lipid content and composition [37]. For instance, the integration of specific chemical shifts corresponding to methylene, methyl, or olefinic protons can be used to estimate saturation levels and fatty acid profiles in lipid extracts.

In the context of microalgae, several studies have demonstrated the efficacy of NMR in characterizing complex lipid mixtures. For example, Fan et al. used ^1H NMR to quantify neutral and polar lipids in *Chlorella vulgaris* and *Nannochloropsis oceanica*, showing that NMR can yield comparable or superior results to conventional gas chromatography (GC) methods, especially in terms of reproducibility and speed [38]. Another study by Yunus et al. applied ^13C NMR to analyze lipid accumulation under nitrogen starvation in *Scenedesmus* species, revealing significant shifts in carbon skeleton composition that correlated with triacylglycerol production [39].

High-resolution NMR techniques such as two-dimensional (2D) correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and total correlation spectroscopy (TOCSY) further expand the capabilities of NMR, allowing for detailed assignment of overlapping signals and identification of minor lipid species. These advanced techniques are particularly useful for resolving complex lipidomes in microalgae, where the diversity of lipid classes and structural isomers poses analytical challenges [40].

Despite its analytical power, NMR spectroscopy features limitations that restrict its widespread application in routine lipid analysis. The primary challenges include the high cost of instrumentation and maintenance, the requirement for skilled personnel, and lower sensitivity compared to mass spectrometry-based methods. Additionally, the interpretation of complex spectra can be time-consuming, particularly when dealing with heterogeneous samples or unknown compounds [41].

Nevertheless, NMR remains an indispensable tool for comprehensive lipid analysis in research settings, especially when detailed structural information is required. Recent advancements in cryogenically cooled probes, automation, and pulse sequence optimization are gradually improving sensitivity and throughput, making NMR more accessible for lipidomics applications [41].

3.4.2. Fourier-transform infrared (FTIR) spectroscopy

Fourier-transform infrared (FTIR) spectroscopy has emerged as a reliable, non-destructive analytical technique in the field of lipid quantification due to its capacity to provide detailed molecular fingerprints of complex biological samples. FTIR measures the absorption of infrared radiation by molecular bonds, which results in characteristic vibrational spectra which correspond to specific functional groups. In lipid analysis, the most informative regions are those related to CH2 and CH3 stretching vibrations, typically found between 2800 and 3000 cm^{-1}, and ester carbonyl that stretches around 1740 cm^{-1} [42].

One of the primary advantages of FTIR is its rapid sample processing, with minimal preparation. Samples, whether in solid or liquid form, can be directly analyzed or immobilized on attenuated total reflectance (ATR) crystals, enhancing throughput and reproducibility. FTIR has been applied for monitoring lipid accumulation in microalgae such as *Chlorella vulgaris* and *Nannochloropsis oculata*, with strong correlations to traditional gravimetric and chromatographic techniques [43].

However, the limitations of FTIR should not be overlooked. The technique is semi-quantitative unless calibrated against reference methods like gas chromatography (GC). Moreover, overlapping spectral bands in complex biological matrices can complicate spectral interpretation. Despite these limitations, recent studies have shown that multivariate calibration techniques such as partial least squares regression (PLSR) can significantly improve the accuracy of lipid quantification via FTIR [44].

3.4.3. Raman spectroscopy

Raman spectroscopy, based on inelastic scattering of monochromatic light (typically from a laser), has also gained significant attention in microalgal lipid analysis. The technique provides complementary information to FTIR, especially valuable in aqueous environments where water exhibits strong IR absorption but minimal Raman scattering [45]. Characteristic Raman bands for lipids include those at 1440 cm^{-1} (CH2 scissoring), 1655 cm^{-1} (C=C stretching), and 1300 cm^{-1} (CH2 twisting), thus enabling the assessment of lipid saturation and unsaturation [46].

Table 4. **Comparison of chromatographic methods for lipid analysis in microalgae**

Таблица 4. **Сравнение хроматографических методов анализа липидов в микроводорослях**

Method	Sample Preparation	Detection Type	Lipid Type	Advantages	Limitations	Sensitivity (LOD)	R^2	Reference
Gas Chromatography (GC-FID/MS)	FAME derivatization	FID/MS	Fatty acids	High resolution, excellent quantification	Requires derivatization, not for intact lipids	~1 ng	0.995–0.999	[27,28]
HPLC (RP or NP, ELSD/MS)	Minimal, no derivatization	ELSD/DAD/MS	Intact lipid classes	No derivatization, suitable for class profiling	Expensive, lower resolution for isomers	~10–100 ng	0.990–0.998	[29,30]
Thin-Layer Chromatography (TLC)	Minimal	Visual/Densitometry	Lipid classes	Inexpensive, rapid screening	Semi-quantitative, low resolution	~1 µg	~0.95	[31]

Raman spectroscopy offers exceptional spatial resolution, making it suitable for single-cell lipid profiling. This is particularly advantageous in studies of microalgal heterogeneity, where the lipid content can vary significantly between cells even under the same cultivation conditions. Confocal Raman microscopy allows for subcellular imaging of lipid bodies, thereby providing both qualitative and semi-quantitative insights into lipid accumulation [47].

Nevertheless, the Raman technique also has its drawbacks. The weak Raman signal often necessitates long acquisition times or signal enhancement strategies, such as surface-enhanced Raman spectroscopy (SERS), which can increase complexity and cost. Additionally, fluorescence interference caused from pigments such as chlorophyll can mask Raman signals, particularly in photosynthetic organisms like microalgae [48].

3.4.4. Near-infrared (NIR) spectroscopy

Near-infrared (NIR) spectroscopy is another vibrational spectroscopic technique increasingly used in lipid quantification. NIR operates within the 780–2500 nm range and detects overtones and combinations of molecular vibrations primarily involving CH, OH, and NH bonds. In microalgae, NIR spectroscopy has been applied for rapid extimation of total lipid content, with minimal sample preparation [49].

Compared to mid-IR methods like FTIR, NIR offers deeper penetration and faster result acquisition times, making it suitable for high-throughput screening. Furthermore, NIR instruments can be integrated into process monitoring systems, facilitating real-time observation of lipid production during microalgal cultivation [50].

Despite these advantages, NIR is inherently less specific than FTIR or Raman spectroscopy due to the broad and overlapping nature of overtone bands. This necessitates the application of advanced chemometric tools for data interpretation. The calibration of NIR models also requires a large and diverse dataset covering a broad range of lipid concentrations and species-specific variability [4].

The comparative analysis presented in the Table 5 of the spectroscopic techniques highlights the diversity of the available tools for lipid quantification in microalgae, each suited for specific contexts. FTIR offers a rapid, non-destructive option with reasonable accuracy, but it suffers from signal overlap with other macromolecules, necessitating thorough calibration. Raman spectroscopy, particularly in its confocal variant, stands out for its high specificity and spatial resolution, enabling lipid visualization at the single-cell level. However, this comes at the cost of more sophisticated equipment and lower throughput.

Near-infrared (NIR) spectroscopy is notable for its portability and throughput, making it an excellent candidate for real-time and in-line industrial monitoring, although its specificity is more limited than mid-infrared or Raman techniques.

In terms of quantitative reliability, all methods demonstrate relatively high correlation coefficients ($R^2 > 0.85$), while confocal Raman and fluorescence methods typically achieve the highest values. Ultimately, the choice of method depends on the analytical goal — be it process monitoring, cellular imaging, or rapid screening. Combining complementary techniques may often yield the most reliable analytical outcomes.

3.5. Comparative analysis of lipid quantification methods

Quantification of lipids in microalgae is a cornerstone in evaluating their potential for biofuel production and bioproduct applications. Multiple analytical techniques — gravimetric, colorimetric, chromatographic, spectroscopic, and fluorimetric — have been developed and applied in this context, each featuring certain advantages and limitations. A holistic comparison is essential for selecting an appropriate methodology tailored to the specific research or industrial goals, especially considering the parameters such as accuracy, specificity, required instrumentation, sample throughput, and environmental sustainability.

Gravimetric methods, particularly those based on solvent extraction (e. g., Bligh and Dyer or Folch methods), are traditionally employed due to their simplicity and directness in quantifying total lipid content [5]. These methods are cost-effective and relatively easy to perform but lack specificity and can overestimate lipid content due to co-extraction of non-lipid materials such as pigments and carbohydrates [18].

Colorimetric methods, including the sulfo-phospho-vanillin (SPV) and Nile Red assays, offer rapid assessment of lipid content and are suitable for high-throughput screening [11,51]. SPV is advantageous for estimating total lipids with relatively high sensitivity, while Nile Red is used for intracellular lipid localization and semi-quantitative analysis [13]. However, colorimetric techniques can be influenced by interfering substances, and the reproducibility may vary based on species and cellular conditions.

Chromatographic methods, especially gas chromatography (GC) and high-performance liquid chromatography (HPLC), provide high-resolution lipid profiling, enabling both qualitative and quantitative analysis of fatty acid methyl esters (FAMEs) and complex lipid species [27,52]. GC coupled with flame ionization detection (GC-FID) or mass spectrometry (GC–MS) is widely regarded as the gold standard for fatty acid quantification. These methods, however, require derivatization steps and advanced instrumentation, thus limiting their accessibility in resource-limited laboratories [14].

Spectroscopic methods, such as nuclear magnetic resonance (NMR) and Fourier-transform infrared (FTIR) spectroscopy, enable non-destructive analysis of lipid components and structural information without chemical derivatization [53]. NMR provides detailed insights into lipid classes and chain composition with high reproducibility, albeit with significant instrument costs and lower sensitivity compared to chromatographic approaches [54]. FTIR and Raman spectroscopy are advantageous for rapid screening and potential in-line monitoring applications but generally provide semi-quantitative data and are susceptible to spectral overlap [55].

Fluorimetric methods, such as those using BODIPY and Nile Red fluorescent dyes, allow real-time, *in vivo* imaging of lipid accumulation with high spatial resolution, and are widely used in strain screening and metabolic studies [25]. These techniques are fast and relatively simple but can be affected by dye penetration issues and autofluorescence emitted from algal pigments, which complicates data interpretation [12].

From the comparative assessment, it is evident that no single technique is universally optimal for all research or industrial scenarios. Gravimetric and colorimetric methods remain suitable for preliminary estimations and low-resource settings due to their cost-effectiveness and simplicity. Chromatographic techniques provide unequalled specificity and accuracy in lipid profiling but require specialized infrastructure and expertise. Spectroscopic and fluorimetric methods offer promising non-destructive and high-throughput capabilities, making them perfect for rapid screening, particularly in biotechnology and bioengineering contexts. Ultimately, the selection of a quantification method should consider the balance between analytical rigor, resource availability, and the specific research objectives.

Table 5. **Comparison of spectroscopic methods for lipid analysis in microalgae**

Таблица 5. **Сравнение спектроскопических методов анализа липидов в микроводорослях**

Method	Principle	Sample Preparation	Specificity to Lipids	Sensitivity	Throughput	Quantitative Accuracy (R^2)	Advantages	Limitations	References
FTIR (Fourier-Transform Infrared)	Measures absorbance of lipid-specific bonds (e.g., C=O stretch)	Minimal (dry biomass)	Moderate	Moderate	High	~0.90–0.96	Non-destructive, fast, requires small sample volume	Interference from proteins/carbohydrates; requires calibration	[43,44]
Raman Spectroscopy	Scatters monochromatic light to detect vibrational modes of lipid molecules	Minimal (live or fixed cells)	High	High	Medium	~0.95–0.98	Label-free, can analyze living cells, high spatial resolution	Fluorescence background; limited depth penetration	[45–47]
Confocal Raman Microscopy	Raman with spatial mapping capability	Moderate	Very High	High	Medium	~0.97	Single-cell resolution, enables 3D lipid mapping	Expensive, complex instrumentation	[48]
NIR (Near-Infrared Spectroscopy)	Measures overtone vibrations, especially CH bonds	Low (intact biomass or slurry)	Moderate	Moderate	Very High	~0.85–0.92	Rapid, portable instruments available, suitable for real-time monitoring	Lower molecular specificity; needs robust chemometric calibration	[49,50]

3.5.1. Selection of the method for specific applications

The appropriate selection of a lipid quantification method in microalgae depends on several interrelated factors, including the biological material, desired data resolution, available instrumentation, cost, and required throughput. For example, when absolute quantification is required for biofuel yield estimation, gravimetric methods or GC-based fatty acid methyl ester (FAME) analysis are typically used due to their robustness and reproducibility [56]. In contrast, rapid screening of high-lipid-producing strains under variable environmental conditions is better served by high-throughput techniques such as Nile Red fluorescence or NIR spectroscopy [11,13].

Industrial-scale applications benefit from methods that offer real-time monitoring, such as FTIR or NIR, which can be integrated into process analytical technology (PAT) frameworks [55]. These methods, while often less precise than chromatographic analyses, enable fast decision-making for harvesting and processing steps.

Research requiring structural elucidation — e. g., lipid biosynthesis pathway elucidation or representation of membrane lipid composition under stress — typically uses NMR, HPLC–MS, or GC–MS techniques due to their high specificity and structural insight [52,53]. Fluorescence microscopy with dyes such as BODIPY is also commonly used in metabolic engineering studies, where spatial localization of lipid droplets provides critical information [25].

3.5.2. Combined and sequential approaches

No single method comprehensively meets all analytical needs. Therefore, sequential or complementary approaches are often applied to balance speed, resolution, and quantification accuracy. For instance, SPV or Nile Red staining may be used for preliminary screening of multiple strains or treatments, followed by gravimetric or chromatographic quantification for the selected samples [27,27]. This two-tiered strategy is particularly efficient in bioprospecting and mutagenesis-based lipid productivity enhancement programs.

Another example involves combining FTIR or NIR with GC-FID. FTIR/NIR models can be calibrated using a representative sample set analyzed via GC, allowing subsequent FTIR/NIR predictions to provide rapid, non-destructive lipid estimates [54]. Such hybrid workflows are also applicable to machine learning-based prediction models, which increasingly use spectral data to predict biochemical parameters with high accuracy [53].

3.5.3. Emerging trends and future trends directions

The field of lipid analysis is witnessing a shift toward automation, miniaturization, and integrative omics. Microfluidic platforms combined with Raman or fluorescence microscopy now enable real-time, single-cell lipid analysis, thus making them powerful tools for evolutionary screening or synthetic biology applications [13]. Likewise, biosensor-based technologies that couple selective lipid binding proteins with optical or electrochemical readouts are under active development [12].

Moreover, advanced data analytics, including chemometrics and machine learning, are enhancing the interpretability of complex spectral datasets, especially those obtained from FTIR and NIR platforms [54]. This is paving the way for deployment of intelligent lipid monitoring systems in algal biorefineries.

Finally, sustainability concerns are encouraging the development of greener methods, reducing solvent use and hazardous chemicals. Techniques such as supercritical CO_2 extraction, green solvents, and direct-*in situ* lipid estimation methods are gaining prominence [14].

4. Conclusion

A critical assessment of the methodologies used for lipid quantification in microalgae highlights the analytical complexity and multidimensional character of this task. As microalgae increasingly emerge as viable platforms for sustainable production of biofuels, nutraceuticals, and lipid-derived compounds, the need for accurate, reproducible, and context-adapted quantification strategies becomes essential — both in basic research and in the process scale-up.

The examined techniques — ranging from classical gravimetric approaches to advanced spectroscopic, chromatographic, and fluorometric systems — each offer distinctive benefits and pose particular limitations, often dictated by their specificity, sensitivity, required infrastructure, and throughput potential. While solvent-based extraction methods remain widely used for estimating total lipid content, they are limited in selectivity and may overestimate due to co-extraction of non-lipid substances. Chromatographic techniques, although highly informative in terms of lipid class composition and fatty acid distribution, are labor-intensive and often require complex instrumentation and derivatization steps. Spectroscopic approaches provide a non-destructive and relatively fast alternative, but they frequently require calibration models and may not deliver adequate resolution when used alone. Fluorescent staining techniques, particularly those compatible with live-cell analysis, have become indispensable for high-throughput screening applications but are often affected by variability in cell permeability and pigment background.

In practice, no single method meets all analytical needs. A combined approach -wherein rapid screening is followed by targeted, high-resolution analysis — often represents the most efficient and reliable strategy. The integration of multiple techniques allows for both qualitative and quantitative lipid insights, enhancing data reliability and enabling more informed decision-making process in experimental workflows.

Ultimately, method selection must be guided by the specific goals of the research — whether focused on rapid strain screening, absolute lipid quantification, or detailed lipidomic profiling. Considerations such as scalability, reproducibility, time efficiency, and operational cost should further inform this choice. At the same time, the lack of standardized protocols keep being a barrier to cross-study comparability and broader industrial implementation.

Looking forward it is obvious, that methodological innovation will likely involve increased automation, the use of integrated lab-on-chip platforms, and the application of AI-driven analytics to handle complex datasets and support real-time decision-making. In parallel, collaborative efforts aimed toward harmonizing the protocols and validating techniques across laboratories will be the key to ensuring data consistency and accelerating development across the algal biotechnology sector.

In conclusion, the reliable and standardized lipid quantification is a cornerstone of successful microalgal bioprocess development. The future of this field will rely not only on refining individual techniques but also on strategically integrating them into cohesive analytical pipelines that match the scale and complexity of emerging biotechnological challenges.

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