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Lipids detection and quantification in oleaginous microorganisms: an overview of the current state of the art

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Abstract

Oleaginous microorganisms are among the most promising feedstocks for the production of lipids for biofuels and oleochemicals. Lipids are synthesized in intracellular compartments in the form of lipid droplets. Therefore, their qualitative and quantitative analysis requires an initial pretreatment step that allows their extraction. Lipid extraction techniques vary with the type of microorganism but, in general, the presence of an outer membrane or cell wall limits their recovery. This review discusses the various types of oleaginous microorganisms, their lipid accumulating capabilities, lipid extraction techniques, and the pretreatment of cellular biomass for enhanced lipid recovery. Conventional methods for lipid quantification include gravimetric and chromatographic approaches; whereas non-conventional methods are based on infrared, Raman, nuclear magnetic resonance, and fluorescence spectroscopic analysis. Recent advances in these methods, their limitations, and fields of application are discussed, with the aim of providing a guide for selecting the best method or combination of methods for lipid quantification.

Keywords: Oleaginous microorganisms, Microbial lipids, Lipid extraction, Lipid quantification, Conventional methods, High-throughput analysis

Introduction

In the search for alternatives to fossil-derived oils, oleaginous microorganisms have attracted attention as potential sources of oil feedstocks. Microalgae, yeasts, fungi, and bacteria, can accumulate high amounts of lipids (> 20% of cell dry weight) in their cellular compartments and are considered as oleaginous feedstocks for biofuel production [1]. A list of oleaginous microorganisms and their lipid accumulation ability is presented in Table 1.

Under nutrient-limiting conditions combined with an excess of carbon, oleaginous microorganisms accumulate significant quantities of lipids known as single cell oils [24, 25]. These lipids are composed predominantly of neutral triacylglycerols (TAGs), energy-rich fatty acids that can be used for biodiesel production. Depending on fatty acid composition, which is highly variable between species and strains, these lipids are suitable as raw materials for the food, pharmaceutical, nutraceutical, and cosmetic industries [26, 27].

Although oleaginous microorganisms are an attractive and renewable feedstock, the cost associated with microbial lipid production needs to be kept low. Consequently, the challenge for robust and cost-competitive microbial lipid production systems is to develop strains that are capable of converting low-cost substrates, grow quickly to high cell densities, and produce large amounts of neutral lipids [9]. This involves the identification of organisms with an inherent lipid accumulation capacity, as well as their genetic/metabolic engineering to direct carbon flow towards increased lipid biomass or to cope with adverse growth conditions on various carbon sources [28, 29]. Exploration of candidate microorganisms and establishment of favourable cultivation conditions often relies on laborious and time-consuming screening processes. Moreover, the analysis of a large number of samples generated from a screening assay represents a major bottleneck. Fast and accurate quantification of accumulated lipids is key to address this issue. Conventional methods for lipid quantification rely on

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Table 1 Examples of oleaginous microorganisms and their lipid accumulating capability on various feedstocks

Oleaginous microorganisms	Substrates	Lipid content (%, *w*/w)	References
Microalgae			
Chlorella sorokiniana	Beech wood *Fagus sylvatica* dilute-acid hydrolysate	4	[2]
Chlorella protothecoides	Glucose	49	[3]
Tetraselmis elliptica	Photoautotrophic (Flory medium)	14	[4]
Chlorella vulgaris NIES-227	Heterotrophic cultivation on glucose under nitrogen limitation	89	[5]
Auxenochlorella protothecoides	Organosolv pretreated wood biomass hydrolysates (birch and spruce)	66 and 63	[6]
Botryococcus braunii	Photoautotrophic (modified Chu 13 medium)	28	[7]
Chlamydomonas reinhardtii, CC1010	Photoheterotrophic ($TAPN^{-}$ + 0.1% glucose)	59	[8]
Yeast			
Rhodosporidium toruloides	Brewers' spent grain	56	[9]
Lipomyces starkeyi	Xylose and glucose	48	[10]
Rhodotorula glutinis	Monosodium glutamate with glucose	20	[11]
Cryptococcus curvatus	Waste cooking oil	70	[12]
	Glucose	53	
Lipomyces starkeyi CBS 1807	Sweet sorghum stalks juice	30	[13]
Fungi			
Fusarium oxysporum	Sweet sorghum stalks (12% w/w solid load)	22	[14]
	Glucose	42	
	Fructose	26	
	Sucrose	49	
	Glucose, fructose and sucrose mixture	53	
Fusarium equiseti UMN-1	Glucose	56	[15]
Sarocladium kiliense ADH17	Glucose and glycerol	33	[16]
Mortierella alpina LP M 301	Glucose with potassium nitrate	31	[17]
Microsphaeropsis sp.	Corncob waste liquor	22	[18]
Bacteria			
Rhodococcus opacus DSM 1069	Ethanol organosolv lignin	4	[19]
R. opacus PD630	Dairy wastewater	14	[20]
	Dextrose	70	
R. opacus DSM 43205	Biomass gasification wastewater	66	[21]
Gordonia sp. DG	Olive oil	13	[22]
	Sesame oil	50	
	Cotton oil	50	
	Peanut oil	40	
	Maize oil	40	
	Sunflower oil	52	
R. opacus PD630	Kraft hardwood pulp	46	[23]

solvent extraction and either gravimetric assays or chromatographic determination, which are time-consuming multi-step procedures unsuitable for high-throughput analysis and prompt response to changing culture conditions [30]. Moreover, adequate amounts of biomass and, in turn, large sample volumes are required, thus hindering screening and small-scale experiments. To select high-yielding strains, procedures based on lipid extraction are best avoided. At the same time, in situ screening of lipids is not possible with traditional solvent extraction methods, as cells are not in a viable state. There is a need to develop methods that allow for rapid screening of growth and oil accumulation by various strains and under varied conditions and with reduced sample

amounts [31, 32]. To this end, several alternative techniques enabling high-throughput and reliable lipid quantification have emerged recently [31, 33]. Fluorescence spectroscopy is considered the simplest and most cost-effective alternative to gravimetric methods for lipid quantification, and fluorescence detection methods have been developed for direct lipid measurements [34–36]. Fluorescent dyes such as Nile Red and boron-dipyrromethene (BODIPY) 505/515, which interact with intracellular lipids and emit fluorescence signals correlated to lipid content, are commonly used in such assays [31, 37]. These methods, based on a vital dye, offer a rapid and inexpensive approach for measuring lipid content and can be applied in various systems, in situ and in vitro [35]. Other non-destructive methods, such as infrared (IR), nuclear magnetic resonance (NMR), and Raman spectroscopy, have also been used to some extent for lipid determination in oleaginous microorganisms [38–42]. Techniques for lipid content determination in microalgae have been thoroughly described in several recent reviews [31, 33–35] and are also commonly applied to lipid quantification in other oleaginous microorganisms. Other important factors, such as the degree of unsaturation and saturation, the position and number of double bonds in the hydrocarbon chain, the amount of free fatty acids, TAGs, diacylglycerols (DAGs), monoacylglycerols (MAGs), and steryl esters, should also be taken into consideration when evaluating microbial lipids for biofuels [43]. Consequently, it is important to assess both TAG content and chemical composition of microbial lipids.

The aim of this review is to summarize some of the important aspects of conventional methods for total lipid analysis, including compositional analysis, and novel techniques that can be used for lipid detection and quantification (see Fig. 1).

Conventional lipid quantification

Conventional lipid quantification methods in oleaginous microorganisms include gravimetric assays, transesterification, and chromatography coupled with spectroscopy. Gravimetric methods for total lipid quantification encompass a series of events including harvesting of microbial biomass, drying, disruption of cellular integrity, and extraction of lipids. With this method, it is only possible to estimate the total amount of lipids, but not their composition. Hence, traditionally, the compositional analysis of lipids has been performed by chromatographic techniques such as gas chromatography (GC) coupled with an appropriate detector, following lipid derivatization into their esterified form [44, 45].

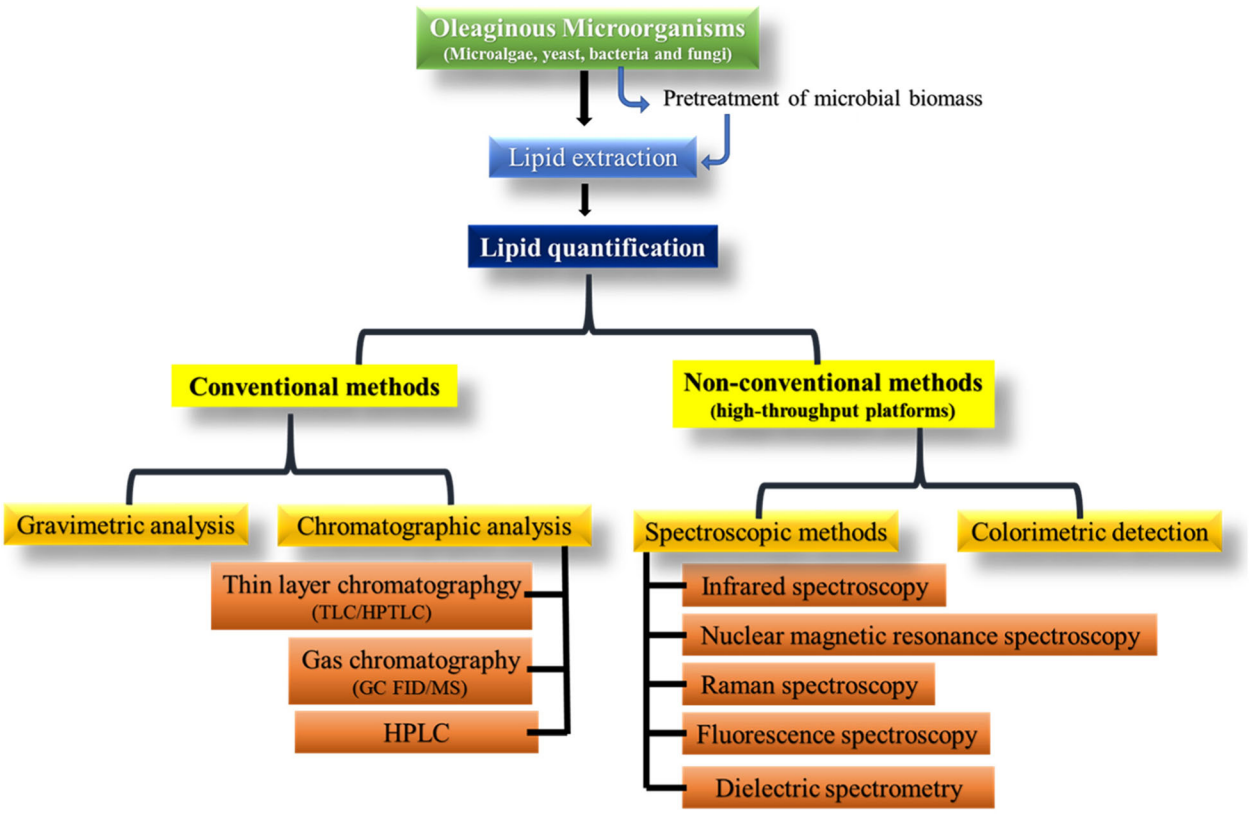

Fig. 1 Schematic diagram of lipid quantification by conventional and non-conventional methods

Gravimetric analysis

Gravimetric determination of total lipid content is the most widely used method for quantifying microbial lipids. In this method, lipids are extracted from microbial cells by one of the techniques listed in Table 2.

Because lipids can be polar (phospholipids) or nonpolar (TAGs), their partitioning must be done according to differences in their polarity [64, 65]. In general, lipid extraction from oleaginous microorganisms can be performed through two different routes, the dry and wet route. The latter is advantageous over the former as it is more cost-effective and less energy-intensive because it eliminates the drying step prior to extraction [66, 67]. Bligh & Dyer and Folch are the oldest and most commonly used lab-scale methods for lipid extraction, whereby a 2:1 mixture of chloroform and methanol is used as solvent [46, 68, 69] and total lipids weight is determined gravimetrically after solvent evaporation. Various micro- and macro-gravimetric methods have been proposed. In macro-gravimetric methods, samples ranging from 0.5 to 5 g can be processed by the Bligh & Dyer or Folch techniques; whereas in micro-gravimetric methods, samples up to 100 mg can be processed according to Van Handel's protocol [70].

The extracted lipids are often contaminated with other cellular components, which affects their determination [71] and creates a discrepancy between gravimetric and fatty acid methyl ester (FAME) analysis. The most common contaminants are pigments such as chlorophyll; however, their interference can be eliminated by measuring the concentration of pigments by fluorometry [72]. Gravimetric methods for lipid quantification are applied on a routine scale as no special equipment or training are required and they are relatively fast. However, they require careful optimization and cannot provide information about lipid classes so, in the end, results have to be verified by other means such as GC [73].

Chromatographic techniques

The most common chromatographic methods for lipid analysis are thin-layer chromatography (TLC), GC, and high-performance liquid chromatography (HPLC), used alone or in conjugation with mass spectrometry (MS), tandem quadrupoles (MS/MS), flame ionization detector (FID), and time-of-flight (TOF).

Thin-layer chromatography

TLC is a valuable analytical tool for the qualitative and quantitative analysis of lipids that can rapidly resolve lipid components such as TAGs, DAGs, MAGs, and cholesterol esters in a single step without altering their chemical properties [74, 75]. The availability of commercial pre-coated chromotoplates has greatly improved the reproducibility of lipid separation over hand-made TLC plates. Moreover, lipid separation by TLC or its high-performance (HPTLC) variant, has benefitted substantially from the availability of good-quality absorbent materials, including high-performing silica bonded phases and impregnated layers [76]. Qualitative analysis can be performed on the basis of lipid components and their reactions with various types of reagents; e.g., acidic ferric chloride is used to detect cholesterol and cholesteryl esters, which appear as red to violet spots [77]. Some

Table 2 Overview of important extraction methods and their efficiency for lipid recovery

Method	Efficiency	Cost	Energy demands	Industrial perspective	References
Conventional solvents such as chloroform/methanol, hexane, or ether	Depends on the species of microorganism, pretreatment of biomass, moisture content, type of solvent, solvent: biomass ratio, treatment time, etc.	Mainly the cost of solvents and reactors, possible reuse of solvents, energy-intensive process	Drying of cellular biomass, heating of solvent, distillation of solvent	There are reports at a larger scale	[46]
Super critical CO_2	Varies with flow rate of CO_2, pressure, and exposure time	Mainly the cost of equipment and its maintenance	Distillation/heating of solvents if paired with co-solvent, maintaining high-pressure conditions	No report at an industrial scale	[47–51]
Liquid CO_2	Requires cell disruption to achieve better yield, usually lipid yield is low	Maintenance of high-pressure conditions (15 MPa), cell disruption	Distillation/heating of solvents if paired with co-solvent	No reports at a larger scale	[52, 53]
Microwave-assisted lipid extraction	Simple, rapid process, effective for robust species, easy to scale up; does not require dewatering of microorganisms; highly efficient at lab scale	Low operating costs but high maintenance costs of equipment	High energy consumption, recovery of thermolabile compounds may require cooling	No reports at a larger scale	[10, 46, 48, 54–56]
Ultrasonication-assisted lipid extraction	Short extraction time, simple to operate, highly reproducible results, energy-effective in small volume, cell wall hinders lipid recovery	Mainly cost of equipment	Large volume of sample, requires high energy	Not suitable for large-scale	[46, 56–63]

examples of lipid classes and their staining reagents are mentioned in Table 3 [78]. Quantitative lipid analysis by TLC can be done by either recovering the desired lipid component from the chromatoplate and analyzing it by established analytical tools, or through its direct analysis on the chromatoplate [75]. Quantitative analysis of lipid classes by TLC was performed after lipid extraction from oleaginous microalgae such as *Nannochloropsis oculata* and *Dunaliella salina* [58], as well as from oleaginous yeasts such as *Yarrowia lipolytica* and *Cryptococcus curvatus* [79, 80].

For the separation of TAGs, DAGs, MAGs, and cholesterol esters, a mobile phase of hexane:diethyl ether:acetic acid (85:15:1, *v*/v/v) is often used and the spots are typically visualized by staining with a methanolic $MnCl_2$ solution, followed by charring of the plate at 120 °C [74]. Different TAGs can be separated by TLC based on their molecular weight [81, 82]. Using a mixture of petroleum ether and diethyl ether, long-chain TAGs can be eluted first, followed by short-chain TAGs [82]. Normal-phase TLC containing silicic acid is often used to separate TAGs with varied degrees of saturated and unsaturated fatty acids [83]. TAGs molecular species can also be resolved by argentation TLC (Ag^+ TLC) or reverse-phase TLC [83].

Low costs for quick analysis of small samples, ease of handling, and reproducibility of results are the main advantages of TLC [78]. The main disadvantages are limitations of preparative applications for TLC/HPTLC and lipid oxidation (mainly unsaturated fatty acids) during exposure to atmospheric oxygen [78].

Gas and liquid chromatography coupled to mass spectrometry

Liquid chromatography (LC, HPLC, and ultra-high-performance liquid chromatography) and GC have been widely employed for lipid profiling and are usually coupled with MS for a more detailed analysis [84]. Other detectors coupled to GC for lipid profiling are the MS/MS, FID, and TOF MS; whereas HPLC can be coupled to an evaporative light scattering detector (ELSD). Among the different ionization techniques for MS, fast atom bombardment (FABMS) [85], electrospray ionization (ESI), matrix-assisted laser desorption and ionization (MALDI) [86], and paper spray ionization [87] have been applied for lipid analysis from oleaginous microorganisms.

Various conventional chromatographic/spectrometric methods for lipid quantification and profiling of oleaginous microorganisms are presented in Table 4. The most commonly applied steps before LC or GC are cell lysis and lipid extraction, as well as the conversion of saponifiable lipids directly into acyl esters of fatty acids via a transesterification reaction [33]. The reaction includes the addition of an acid or base as catalyst and an alcohol, most often methanol [99]. An alternative to the conventional process is direct or in situ transesterification, whereby lipid extraction and transesterification are performed in a single step. Factors critical for the efficiency of direct transesterification include alcohol type and alcohol:lipid ratio, cells' water content, reaction conditions (i.e., temperature and time), use of co-solvents, and role of the catalyst. For instance, catalyst-free supercritical processes using methanol have been applied for biodiesel production [100]. Therefore, the meticulous design of the transesterification step is critical for an adequate quantification of lipids by subsequent chromatographic methods.

GC/MS or LC/MS have excellent sensitivity, molecular specificity, and precision, and represent the current 'gold standard' for the quantification of microalgal cellular composition [101]. However, sample pretreatment makes these methods destructive, time-consuming, environmentally unfriendly, and does not allow for real-time lipid content monitoring. GC and/or MS have been employed successfully in oleaginous microorganisms for biodiesel production, such as the analysis of metabolic flux distribution in *Chlorella protothecoides* [102] and the automated biochemical analysis of aquatic microorganisms [103].

Thermal pyrolysis of biological macromolecules generates volatile compounds that can be detected by GC-MS/MS and characterized both quantitatively and qualitatively. Pyrolysis GC/MS was applied for the biochemical determination of proteins, carbohydrates, and lipids from 26 microalgae and cyanobacteria [91]. Its advantages include minimal sample preparation, reduced analysis time, and low amount of sample. Barupal et al. (2010) combined

Table 3 Qualitative estimation of lipid classes by specific reagents following separation by TLC [78]

Lipid class	Reagent	Results
Free fatty acids	2′,7′-Dichlorofluorescein/ AlCl3/FeCl3	Appears as rose color after few minutes
Glycolipids	α-Naphthol/sulfuric acid	Yellow spots
	Orcinol/sulfuric acid	Blue-purple spots
	5-Hydroxy-1-tetralone in 80% sulfuric acid	Glycolipids give yellow spots easily distinguishable from the light blue spots of phospholipids
Sphingolipids	Sodium hypochlorite/benzidine reagent	Blue spots on white background
Phospholipids	Molybdic oxide/molybdenum	Blue spots on white background
Cholesterol and cholesteryl esters	Acidic ferric chloride	Red to violet spots

Table 4 Examples of conventional chromatographic/spectrometric methods for lipid quantification and profiling of oleaginous microorganisms

Method	Microorganisms	Sample preparation	Compound detection and quantification	Correlation with method	Outcome	Reference
FABMS	*Chlorella vulgaris, Scenedesmus obliquus*	Algal cells, intact	Semi-quantitative analysis of fatty acids, TAGs	–	More flexible and versatile than GC	[85]
GC-MS	*Chlorella kessleri*, *C. vulgaris*, *Scenedesmus acuminatus*, *Spirulina platensis*	Solvent extraction of dried cells Transesterification	Saturated and unsaturated straight-chain methyl fatty acids, iso- and anteisoacids branched on other carbon atoms and cyclopropane fatty acids	–	Fatty acid profiling	[88]
GC-MS	*Chlamidomonas nivalis*	In situ transesterification	Fatty acid profiling	–	Fatty acid biomarkers correspond to response and adaptation to NaCl stress	[89]
Pyrolysis GC-MS	*Chlamydomonas reinhardtii*, *B. braunii*	Lyophilized algae culture, Immersion into isopropanol	C8 to C40 pyrolysis products	–	Automated manner without sample preparation 30 min run times, sequences up to 48 samples	[90]
Pyrolysis GC-MS	26 microalgae and cyanobacteria	Whole cells, freeze-dried	Biochemical composition (lipids, protein, carbohydrate)	Gravimetric	Fast analysis (1 h), minimal sample amount (< 0.1 mg), no sample preparation	[91]
GC-MS sub-microscale assay	*Mallomonas splendens*, *Nannochloropsis oculata*, *Chrysochromulina* sp., *Emiliania huxleyi*, *Rhodomonas sp.*, *Prorocentrum micans*	In situ transesterification	Saturated and unsaturated methyl fatty acids	BODIPY, flow cytometry	Minimal size requirement (250 μg dry biomass per sample), fast, less solvent than AOAC and AOCS	[44]
MALDI-TOF-MS, ESI-LTQ-Orbitrap MS	7 algal species	Solvent extraction	TAG profile	–	Direct analysis	[92]
DTD-GC x GC-TOF-MS	*Limnothrix* sp., *Scenedesmus acutus*, *Chironomous plumosus*, *Daphnia galatea*	Thermally assisted hydrolysis and methylation	FAMEs	–	Analysis of lipid markers for food-web, eco logical and clinical studies	[93]
MS/MS-CID employing paper spray ionization	*Kyo-Chlorella, Nannochloropsis*	Whole cells, lyophilized	Polar lipid profiling (glycolipids, phospholipids), double bond position determination	–	Structural determination of polar lipids, minimal sample preparation, low sample amount (0.5 μg)	[94]
UPLC-MS, ESI-MS/MS, ESI-MS	4 *Dunaliella* species	Solvent extraction	TAGs, free fatty acids	–	Low sample concentration, small-scale experiments	[95]
HPLC-ELSD	*C. reinhardtii*, *Chlorella* strains	Solvent extraction from dried pellets	TAGs, carotenoids, DAGs, free fatty acids, phospholipids, galactolipids	Comparison with TLC/GC, gravimetric, GC/MS	15 min run, detectable TAG levels > 0.5 μg/g dry weight	[96]
HPLC-ELSD, preparative	*Scripsiella* sp.	Solvent extraction by modified Bligh & Dyer, transesterification and FAME were purified with TLC	Polyunsaturated fatty acids	–	Polyunsaturated fatty acids purification, enrichment and purification of uncommon VLC polyunsaturated fatty acids	[97]
HPLC-ELSD/MS	*Chlorella*	Solvent extraction, Folch method	TAGs, hydrocarbons	–	No derivatization needed for determining lipid molecules in one peak	[98]

pyrolysis GC/MS with existing computational tools to perform a phenotypic analysis of the model organism *Chlamydomonas reinhardtii* and the lipid-rich *Botryococcus braunii* based on their hydrocarbon content. The method yielded a clear profile of pyrolysis products with a carbon-chain length of C8 to C40 [90].

A rapid, microscale, single-step in situ protocol for GC/MS lipid analysis, requiring only 250 μg dry mass per sample, was developed and applied in algae and aquatic organisms, combined with fluorescent techniques (BODIPY 505/515) and flow cytometry [44]. The assay included FAME preparation of lyophilized cells by exposing them to BF_3 in methanol, heating at 100 °C for 1 h, a two-step phase separation, and subsequent analysis. The in situ extraction required significantly less solvent and reagent (five to twenty times) than other conventional extraction methods, while a full profile of saturated and unsaturated fatty acids was obtained, thus monitoring the timing of fatty acid synthesis in different algal species.

MALDI-TOF MS is a fast and sensitive method applied in lipid research for apolar and polar lipids [86]. MALDI-TOF MS was used for the compositional analysis of intact, non-derivatized TAGs extracted from marine microalgae, along with ESI-LTQ-Orbitrap MS [92]. Further, direct thermal desorption GC/GC/TOF-MS has been applied for fatty acid detection in microalgae and aquatic meiofauna [93].

Ambient ionization MS analyzes samples by forming ions outside the mass spectrometer, thus avoiding the chemical separation of extracts, which in turn reduces analysis time and the need for solvents [104]. Paper spray ionization is a relatively new method that requires loading of the sample onto a triangular piece of chromatographic paper, the addition of solvent, and application of high voltage that creates ions for MS analysis [87]. MS/MS using paper spray ionization was employed for direct characterization of polar lipids in whole cells of two green microalgae species [94].

In spite of HPLC being coupled mostly with ultraviolet (UV)-visible or MS detectors, ELSD has been proposed to be more accurate for lipid quantification than UV-visible detectors, and less costly and easier to use than MS. Furthermore, ELSD allows the simultaneous quantification of not only TAGs, but also other neutral lipids that are of interest for biofuel or biodiesel production, which is not possible with MS. HPLC-ELSD has been used for the purification of methyl C16-C28 polyunsaturated fatty acids in *Scrippsiella* sp. [97]. Direct analysis of extracted lipids from *Chlorella* employing HPLC-ELSD-MS gave quantitative information on all lipid classes while reducing information and material losses associated with purification or derivatization steps required for GC analysis [98].

Non-conventional quantification of lipids, including high-throughput platforms

As previously stated, rapid and inexpensive methods for estimating lipid content in oleaginous microorganisms are required to screen numerous candidate strains and identify optimal cultivation conditions. To this end, various systems enabling the processing of large numbers of strains in a reasonable time are being developed and are discussed below.

Spectroscopic methods

Infrared spectroscopy

IR spectroscopy has gained ground as a fast and non-invasive lipid quantification method that can be applied on small amounts of whole cells for real-time monitoring and screening of cultures [40, 105]. Flow-cytometry and Fourier transform infrared spectroscopy (FTIR) supported by multivariate analysis, have been proposed as fast and reliable means to monitor and quantify lipids over time in the oleaginous yeasts *Cryptococcus curvatus*, *Rhodosporidium toruloides*, and *Lipomyces starkeyi* [38, 40]. Microtiter plate cultivation systems combined with FTIR have also been used as a high-throughput online platform for screening of oleaginous filamentous fungi [106] and to evaluate the efficiency of lipid extraction processes in oleaginous fungi [107]. FTIR analysis of various microorganisms and their sample preparation are presented in Table 5.

FTIR has been applied since 2000 to detect macromolecules in microalgae [112]. For example, Giordano et al. (2001) studied the carbon allocation pattern in *Chaetoceros muelleri* L. under nitrogen-limited conditions [112]. They suggested that, under such conditions, carbon was directed towards lipid rather than protein and chlorophyll synthesis, irrespective of the source of nitrogen [112]. A similar study by Herad et al. (2005) using synchrotron IR micro-spectroscopy examined the distribution of lipids and proteins in the microalgae *Micrasterias hardyi* when the supply of phosphorus and nitrogen was restored [113]. Sigee et al. (2007) studied carbon allocation during phosphorus limitation in *Scenedesmus subspicatus* using synchrotron-based FTIR micro-spectroscopy and suggested that lipid accumulation in this microalgae was more pronounced under cellular phosphorus limitation than extracellular phosphorus starvation [114]. FTIR micro-spectroscopy was used also by other authors to investigate the allocation of carbon towards lipids in various microalgal species [108, 115, 116]. Although these studies were based on a shifting ratio of macromolecules inside the cells, the exact quantification of lipids inside the cellular matrix by FTIR could not be achieved until a chemometric correlation was proposed by Laurens and Wolfrum [117]. These authors developed a multivariate calibration method that enabled the quantitative analysis of

Table 5 FTIR analysis of lipids from various microorganisms

Method	Microorganisms	Sample preparation	Compound detection and/or quantification	Correlation with method	Outcome	Reference
Micro- FTIR	*Microcystis aeruginosa, Croococcus minutus, Nostoc sp., Cyclotella meneghiniana, Phaeodactylum tricornutum*	Cell suspensions dried in the presence of ZnSe crystals for 30 min at 40 °C ± 0.5 °C; dried cells were used for spectral analysis	IR absorption and molecular vibrational modes for the identification and quantification of macromolecules	–	Detection of macromolecules during growth under different nutrient-limited condition	[108]
FTIR	*Chlamydomonas reinhardtii* and *Scenedesmus subspicatus*	Oven-dried cells at 40 °C for 30 min	Lipids carbohydrates and proteins	Effectiveness of FTIR was correlated with fluorescence spectrometer	Accumulation of lipids under nitrogen limitation can be detected at various growth phases of microorganisms	[109]
FTIR	*Chlamydomonas concordia* (PLY 491), *Chlamydomonas debaryana* (CCAP 11/70), *C. reinhardtii* (CCAP 1132C), *Chlorella luteoviridis* (CCAP 211/3), *Chlorella vulgaris* (CCAP 211/79), *Desmodesmus intermedius* (CCAP 258/38), *Dunaliella tertiolecta* (PLY 83), *Hindakia tetrachotoma* (CCAP 222/81) and *Parachlorella kessleri* (CCAP 211/11G) and two marine *C. concordia* and *D. teriolecta*,	Whole cells in 96-well silicon microplate	Lipids	FTIR data were exported in MATLAB and normalized using extended multiplicative scatter correction type two method (EMSC2)	Ability of FTIR spectroscopy to effectively differentiate closely related eukaryotic microalgae grown under different environmental conditions	[110]
FTIR	*Mucor circinelloides* and *Mortierella alpina*	Lipid extraction by Folch and Bligh & Lewis methods	Evaluation and monitoring of lipid extraction efficiency	Lipids were quantified by gravimetric analysis and FAME was quantified by GC FID	Identify compounds (e.g., polyphosphates) that have negative effect on the lipid extraction process	[107]
FTIR attenuated total reflectance	*Nannochloropsis* sp. (CCAP 211/78)	Freeze-dried biomass	Biomass (lipids, proteins, carbohydrates) composition	Conventional methods for the verification of FTIR data	Monitor biochemical composition during typical growth experiments	[111]
FTIR	*Rhodosporidium kratochvilovae HIMPA1*	Crude lipids extracted by Bligh & Dyer method	TAGs		Transmittance spectra similar to triolein	[74]

exogenous spiked neutral (trilaurin as a TAG) and polar lipids (phosphatidylcholine as phospholipid) in four different microalgae on the basis of near-infrared (NIR) and FTIR spectroscopy [117]. However, the correlation between the amount of phosphatidylcholine and FTIR signal was sometimes problematic, which might have been due to interference by TAGs whose spectra were similar to that of phosphatidylcholine [118]. Indeed, signal interference remains the major challenge of using FTIR to quantify lipids in microbial cells and the results should be cross-checked with other methods.

Nuclear magnetic resonance spectroscopy

NMR spectroscopy can be used to determine the composition of complex biological samples. It requires minimal sample preparation and is of low complexity as it does not require chromatographic separation or derivatization of the sample, plus it can be applied to liquid or solid samples (liquid- or solid-state NMR). NMR-active nuclei in lipid molecules include carbon (^{13}C), hydrogen (^{1}H), oxygen (^{17}O), and phosphorus (^{31}P), with ^{1}H NMR being the most common for lipid profiling of algal samples [119]. ^{1}H NMR allows the quantification of different lipid classes such as steroids and some pigments (carotenoids and chlorophylls), which contain protons with non-overlapping peaks [120]. ^{13}C NMR can be used for the quantification of different lipid components in an extract by way of particular pulse sequences such as inverse gated decoupling and an appropriate standard [120]. An advantage of NMR is that it provides detailed structure-specific information, while the risk of chemical alterations such as oxidation is kept minimal [120]. Other advantages include the high speed of analysis compared to traditional techniques (i.e., GC, MS, UV or IR spectroscopy) and that it is non-destructive, enabling recovery of the sample for further analysis.

^{1}H and ^{13}C NMR have been applied for profiling the main components of whole algal cells [39, 119–124]. Besides a complete lipid quantification profile, Sharpal et al. (2015) performed a cost and practicability analysis of the method [121]. While NMR required five-fold less time than GC/MS and a similar operating cost, it was noted that its initial investment was also five times higher. Davey et al. (2012) used a liquid-state ^{1}H NMR with a quantitative method previously developed by Henderson et al. (2012), in which the reference compound was kept in coaxial inserts to avoid interaction with the sample solution. This strategy was applied to quantify TAGs in living microalgae in their growth medium [125]. NMR is ideal for high-throughput calibration of flow cytometry methods aimed at the quantification of cellular TAGs in various algal species [126]. Further, semi-solid NMR techniques, such as high-resolution magic-angle spinning (HR-MAS) and intermolecular multiple-quantum coherence (iMQC), can be used to detect non-TAG lipids [126].

There are several NMR methods and each of them has its advantages and disadvantages. For example, HR-MAS can be used on intact biological samples and results in well-resolved spectra with narrow peaks when the sample is spun at low frequencies at a 54.7° angle relative to the magnetic field [127]. ^{1}H HR-MAS and ^{13}C NMR have been applied to detect and identify whole algal cells and extracted lipids [125, 128, 129]. A method employing low-field ^{1}H NMR was developed for in vivo online monitoring and real-time tracking of lipid dynamics in *C. protothecoides* [130].

Time domain (TD) NMR is based on the different relaxation times of hydrogen nuclei in various phases of the analyzed sample [131]. Solids, such as carbohydrates and proteins, show short relaxation times in the order of microseconds, whereas bound water, free water, and lipids have relaxation times in the order of, respectively, a few hundred microseconds, seconds, and a few hundred milliseconds [132]. In TD-NMR, the hydrogen nuclei signal from lipids is separated from that derived from other compounds by applying a spin-echo NMR pulse sequence, and lipid content can be quantified using an appropriate calibration [132]. TD-NMR has already been applied for lipid quantification in heterotrophically grown algae [132]. A drawback of this method is the high amount of sample required (up to 1 gram of lyophilized biomass), and its incapacity to differentiate between neutral lipids (i.e., TAGs) and polar or membrane lipids [125]. Nevertheless, TD-NMR has shown generally good agreement with both lipid extraction and Nile Red staining.

Nuzzo et al. (2013) reported an efficient, fast, and reproducible method for the identification and quantification of different classes of lipids (free fatty acids, TAGs, glycolipids, phospholipids) in microalgae [133]. Their approach used a modified Folch method to extract lipids from cells, followed by direct analysis of the extract with ^{1}H NMR and a reference electronic signal as external standard (ERETIC method) [133]. Accordingly, it was possible to quantify total lipid content, degree of saturation, and class distribution during both high-throughput screening of algal cultures and metabolic analysis during genetic studies. A further simplification of the method was introduced by evaluating sample extracts without partition against water of the chloroform-ethanol phase. The obtained spectra retained the capacity to provide information on TAGs, free fatty acids, unsaturated, and saturated fatty acids, making this simplified method suitable for analysis of lipid classes with potential applications in crude oil production or for monitoring biodiesel synthesis. NMR spectroscopy methods applied to various microorganisms for the detection of lipids are presented in Table 6.

Raman spectroscopy

Raman spectroscopy is based on the inelastic scattering of monochromatic light produced by a laser in the visible, NIR or UV range [135]. Raman spectroscopy is a valuable tool for in vivo lipid biomonitoring; however, it can also generate broad spectra for the identification of lipids, proteins, carbohydrates, nucleic acids, and pigments [136]. Different techniques have been developed to boost the Raman signal of target molecules, such as coherent anti-Stokes Raman scattering (CARS), resonant Raman spectroscopy, confocal Raman spectroscopy (CRS), and laser-trapping Raman spectroscopy (LTRS), of which each has its benefits and limitations [119].

Raman spectroscopy is a rapid and non-destructive method for quantifying the degree of lipid unsaturation. The vast majority of chemical composition studies of algal species employs the ratio between 1656 cm^{-1} (C=C) and 1445 cm^{-1} (C-H deformation), often denoted as I_{1656}/I_{1445}, to determine fatty acid composition. This is done after constructing calibration models based on C=C and CH_2 coupled with differential calorimetry and melting point data [136]. A general limitation of Raman spectroscopy for lipid detection and quantification is the strong Raman signal of fluorescent pigments and carotenoids, which overlaps with the lipid peaks and interferes with the analysis. This is a major drawback particularly in oleaginous microorganisms with a high photosynthetic signature. To this end, routine Raman signal post-processing includes cosmic noise removal and fluorescence background subtraction, prior to any further spectral analysis.

Traditional Raman spectroscopy is a widely used and simple technique, with strong potential for industrial implementation. However, its application in living systems is limited by the use of significantly higher excitation laser power to produce distinguishable signals, which could lead to photo-damage of specific samples [137], as well as long integration times (100 ms – 1 s per pixel) [138]. Stokes Raman spectroscopy was employed to identify carotenoids and TAGs produced by *Chlorella sorokiniana* and *N. oleoabundans* [139]. However, unexpected variations in fluorescence background levels were observed during prolonged laser exposure, possibly due to photo-bleaching of pigments in chloroplasts and unexplained sudden spikes of high-intensity fluorescence [139]. The lipid content and degree of unsaturation have been determined by NIR-Raman spectroscopy in *Chlorella vulgaris* [140] and by Raman spectroscopy in *B. braunii* [138].

CRS is another valuable tool for determining the composition of a single individual cell in vivo, including in algae, as it can provide molecular information by simple, fast, non-invasive, and multiplex measurements [141]. In CRS, the laser light is focused onto a sample through a microscope objective while the backscattered signal is refocused onto a spatial pinhole aperture before returning to the spectrometer, where it is dispersed on a charge-coupled device array to produce a spectrum [135]. CRS generates highly spatially resolved 3D images with little background disturbance [135]. CRS was combined with ordinary least squares analysis to determine the relative composition of different fatty acids in a TAGs mixture from a single cell of *Thalassiosira pseudonana*. Such approach overcame the limitation of a conventional method based on the I_{1656}/I_{1445} ratio, which is not reliable for samples containing three or more different fatty acids [142]. Indeed, CRS identified four major lipid moieties, including myristic, palmitic, palmitoleic, and eicosapentaenoic acid, with high correlation (> 0.9) to GC. Although some spectra revealed strong pre-resonant Raman bands of carotenoids, ordinary least squares analysis provided reliable quantitative information. Sharma et al. (2015) benchmarked an integrative approach for lipid biomonitoring via CRS [143]. The method quantified saturated and unsaturated fatty acids (I_{1650}/I_{1440}) and was cross-validated by LC-MS experiments, demonstrating high linear correlation. Recently, a non-destructive and label-free single-cell Raman micro-spectroscopy method was applied for high spatial resolution monitoring of in vivo spatiotemporal dynamics of lipids and astaxanthin in *Haematococcus pluvialis* cells induced with 15% CO_2 and high light intensity [141]. Raman micro-spectroscopy has been applied also to quantify the degree of unsaturation by means of the ratiometric method in six species of *Mortierella* [144]; in situ analysis of lipids accumulated in *Botryococcus sudeticus*, *Chlamydomonas* sp., and *Trachydiscus minutus* [145]; in vivo lipid and carbohydrate quantification of single *Chlamydomonas* sp. [101]; TAG accumulation in single *Nannochloropsis oceanica* cells [146]; and quantitative analysis of lipid unsaturation in immobilized *Fistulifera solaris* [147]. Urban et al. (2011) performed a proof-of-concept study for an integrated and straightforward multidimensional analysis of single *Euglena gracilis* and *C. reinhardtii* cells by combining fluorescence and Raman micro-spectroscopy with laser desorption/ionization MS [148].

In CARS, two high-powered laser beams are focused together on a sample generating stronger spectra due to the coherence of the beam. CARS is a non-linear method that provides high 3D sectioning capability for video-rate imaging. Moreover, CARS signals can be easily distinguished from the fluorescent background due to a blue shift of the response signal. New developments in CARS microscopy have removed the primary non-resonant background problem, by employing complex experimental procedures or post-image data processing [149]. The ability to visualize lipids in microalgae by CARS microscopy was demonstrated as proof-of-principle on *Coccomyxa subellipsoidea* [150]. However, signal overlap between the excited fluorescence of the chlorophyll two-photon and CARS limited the analysis. In subsequent experiments on

Table 6 Nuclear magnetic resonance spectroscopy methods

Method	Microorganisms	Sample preparation	Compound detection and/or quantification	Correlation with method	Outcome	Reference
TD-NMR	*Chlorella protothecoides*	Whole cells	Quantitative	Nile Red, lipid extraction/ gravimetric	Less restriction than Nile Red, which cannot determine lipid content < 38%	[132]
^{1}H NMR, ^{13}C NMR, 2D ^{1}H/^{13}C NMR, liquid state	*Ulva rigida*, *Gracilaria longa*, *Fucus virsoides*, *Codium tomentosum*	Crude extracted lipids, Solvent extraction by Folch method	Mono- and polyunsaturated fatty acids, carotenes and carotenoids, steroids, chlorophylls; Qualitative	–	–	[120]
^{13}C NMR Solid state	*Chlamydomonas reinhardtii*, *Pavlova lutheri*, *Nannochloropsis oculata*	Whole cells enriched with $NaH^{13}CO_3$	Lipids, saccharides; Quantitative	–	Could not distinguish cell wall components with mobility	[123]
^{1}H LF-NMR	*C. protothecoides*	Whole cells, crude extracted lipids	Quantitative glyceryl trioleate internal standard	GC/MS	Extremely sensitive, limits of quantification 1.18 g/L, < 2% RSD, limit of detection 0.32 g/L (algal broth)	[130]
^{1}H NMR, 1H HR-MAS, liquid state	*Chlorella*, *C. reinhardtii*	Whole cells	TAGs; Quantitative, oleic TAG standard internal standard	GC	Biomonitoring	[125]
1D, 2D ^{1}H/^{13}C solution state NMR	*C. reinhardtii*, *Chlorella vulgaris*, *Synechocystis*	^{13}C labeled whole cells	Lipids, TAGs, among metabolites; Qualitative	–	Metabolic profiles	[124]
Liquid state ^{13}C NMR, ^{31}P NMR	*Neochloris oleoabundans*	Whole cells	TAGs; Qualitative, glyceryl trioleate standard	–	Interference from MAGs and DAGs, no contribution of phospholipids	[122]
ERETIC ^{1}H NMR	*Thalassiosira weissflogii*, *Cyclotella cryptica*, *Nannochloropsis salina*	Solvent-extracted lipids by Folch method	Free fatty acids, TAGs, glycolipids, phospholipids; Quantitative	–	Saturation degree, total lipid content, class distribution, profiling	[133]
^{1}H, ^{13}C NMR, 1D, 2D	*C. vulgaris*, *Scenedesmus ecornis*	Extracted lipids	Unsaturated and saturated fatty acid esters, TAG, free fatty acids, polar lipids, standard: TGO, soybean, *Jatropha* oil and Mixoil14; Quantitative	GC/MS	Lipid profiling and quantification of extracts	[121]
^{1}H, ^{13}C NMR	*C. vulgaris*, *S. ecornis*	Solvent-extracted lipids by chloroform:methanol:water	Neutral lipids (TAGs, free fatty acids), polar lipids (glyceroglycolipids, phospholipids) and unsaturation degree; Quantitative	GC/MS	Monitoring cultivations for biodiesel potential	[39]
HR MAS ^{1}H NMR	*Dunaliella* sp., *Amphidinium carterae*, *Phaedodactylum tricorutum*, *Thalassiosira pseudonana*	Whole cells	Lipids, saturated, unsaturated; Qualitative	–	Profiling of whole cells	[128]
^{1}H NMR, ^{1}H HR MAS	*Thalassiosira pseudonana*	Extracts and whole cells	Important metabolites such as polyunsaturated fatty acids, eicosapentaenoic acid and docosahexaenoic Acid; Qualitative	–	Lipid profiling	[134]
^{1}H NMR, ^{1}H HR MAS, ^{13}C-NMR	*Chaetoceros muelleri*	Extracted lipids and whole cells	Important unsaturated fatty acids such as eicosapentaenoic and docosahexaenoic acids, phospholipids, and glycerols; Qualitative	–	Lipid profiling	[129]

Phaenodactylum tricornutum, the CARS signal and chlorophyll fluorescence were separated by time-gated detection [151]. Jaeger et al. (2016) reported for the first time the direct analysis of lipids in *Monoraphidium neglectum* by successful separation of the lipid-specific CARS signal and the interfering two-photon excited fluorescence of chlorophyll [152]. Raman spectroscopic methods applied for the quantification of lipids from different microorganisms are presented in Table 7.

Stimulated Raman scattering microscopy has emerged as an alternative to CARS. Unlike CARS, it offers straightforward image interpretation and quantification with improved signal-to-noise ratio. Moreover, it exhibits linear concentration dependence and thus high potential for the quantification of individual molecules in a multicomponent system; its main limitation remains the capacity to probe only a single Raman band at a time. Fu et al. (2012) reported a multiplexing approach that allowed the simultaneous measurement of multiple Raman bands, offering better chemical specificity and quantitative multicomponent analysis [149]. Importantly, they achieved a three-fold higher implementation of multispecies chemical mapping of multispecies than with CRS after in situ or external calibration.

LTRS, a combination of Raman micro-spectroscopy and NIR optical trapping, was introduced for the chemical

Table 7 Raman spectroscopic methods

Method	Microorganisms	Sample preparation	Compound detection and quantification	Correlation with method	Excitation wavelegth (nm), resolution (cm^{-1})	Reference
Raman micro-spectroscopy	*Haematococcus pluvialis* mutant	Culture cells were immobilized in agarose	Total lipids, astaxanthin; Qualitative	–	532, n/a	[141]
CRS	*Thalassiosira pseudonana*	Culture cells	TAGs, saturated and unsaturated fatty acids; Quantitative	GC	785, n/a	[142]
NIR Raman spectroscopy	*Chlorella vulgaris*	Culture cells, fresh and lyophilized	Total lipids; Quantitative	–	1064, n/a	[140]
Raman micro-spectroscopy integrated with MS	*Euglena gracilis, Chlamydomonas reinhardtii*	Culture cells	Total lipids; Quantitative	–	532, n/a	[148]
Single-cell Raman miscro-spectroscopy	*Fistulifera solaris*	Immobilized cells on agarose	Unsaturation degree; Quantitative	GC/MS	532, n/a	[147]
Single-cell LTRS	*Botryococcus braunii, Neochloris oleoabundans, C. reinhardtii*	Culture cells	Unsaturation degree; Quantitative	–	785, n/a	[153]
CARS	*Phaenodactylum tricornutum*	Culture cells	Lipids and unsaturation degree; Quantitative	GC/MS	532, n/a	[151]
Raman micro-spectroscopy	6 *Mortierella* species	Culture cells	Unsaturation degree; Quantitative	GC	532, 8	[144]
Multiplex stimulated Raman scattering microscopy (SRS)	*B. braunii*	Culture cells	Total lipids, pigments, proteins; Quantitative	–	n/a, 33	[149]
Confocal Raman spectroscopy and microscopy	*Chlorella sorokiniana, N. oleoabundans*	Culture cells	TAGs, carotenoids, chlorophyll; Qualitative	–	532, 2.4	[139]
Single-cell Raman spectroscopy	*Chlamydomonas* sp.	Culture cells	Lipid/starch content; Quantitative	GC/MS	532, 6	[101]
Single-cell Raman micro-spectroscopy	*Nannochloropsis oceanica*	Culture cells	TAGs; Quantitative	LC/MS	532, 2	[146]
Confocal Raman microscopy	*C. reinhardtii*	Culture cells	Saturated and unsaturated fatty acids; Quantitative	LC/MS	532, 3	[143]
CARS scattering microscopy	*Monoraphidium neglectum*	Culture cells	Saturated and unsaturated fatty acid composition; Quantitative	GC/MS, gravimetric	n/a	[152]
Raman micro-spectroscopy	*Botryococcus sudeticus, Chlamydomonas* sp., *Trachydiscus minutus*	Culture cells	Unsaturation degree; Qualitative	GC/MS	785, n/a	[145]
Raman spectroscopy	*B. braunii*	Culture cells	Lipids; Qualitative	–		[138]
CARS and spontaneous Raman spectroscopy	*Coccomyxa subellipsoidea*	Culture cells	Lipids; Qualitative	–	514, n/a	[150]

analysis of single living cells in suspension [154]. Laser tweezers allow the immobilization of an individual cell in suspension away from any surfaces, and simultaneous acquisition of a Raman spectrum. Wu et al. (2011) applied LTRS for the analysis of lipid composition in single microalgae cells of *B. braunii*, *N. oleoabundans*, and *C. reinhardtii*, along with quantification of the degree of lipid unsaturation using the I_{1656}/I_{1445} ratio [146]. LTRS is limited by the weak intensity of the Raman signal compared to fluorescence and interference from the laser-tweezer [146].

Fluorescence spectroscopy

Many of the high-throughput platforms for measuring neutral lipids produced by oleaginous microorganisms are based on fluorescence detection. These tools commonly employ small-molecule dyes, such as BODIPY 505/515 and Nile Red, for in situ screening. The dyes have their respective advantages and disadvantages in terms of photo/chemical stability, optical properties, and solubility. Moreover, their applicability is highly species- and strain-specific, so the method has to be tailored accordingly to ensure that fluorescence values can be properly correlated to lipid content [34, 155]. Intracellular lipid content determination requires the dye to be dissolved in a solvent carrier, adding the solution to the harvested cells, staining in a dark room at optimal temperature, centrifugation and washing of the cells, resuspension, and spectrometric analysis. For quantitative measurements, calibration curves correlating lipid concentration with emission intensity can be easily prepared.

Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one) is a relatively photostable lipophilic fluorescent dye that was first used by Cooksey et al. (1987) in microalgae [37]. Nile Red has some limitations, such as spectral interference with the autofluorescence of chlorophyll, non-specific fluorescence, and inconsistent cell uptake. The latter is exacerbated in algal species with thick cell walls. This complicates the development of consistent fluorescence protocols, while dye uptake is dependent on cell viability [126]. Further, Nile Red is intensely fluorescent in organic solvents, but has a low quantum yield in water [156]. Due to susceptibility of its chromophore to changes in solvent polarity and dielectric constant, this dye fluoresces at defined wavelengths depending on solvent polarity [157]. Emission wavelengths vary with the hydrophobicity degree of lipids and a blue shift of emission maxima is observed with decreased solvent polarity. Hence, by choosing proper excitation and emission wavelengths, it is possible to use more hydrophobic conditions to obtain correct neutral lipid measurements and distinguish them from polar lipids [156]. Interaction of Nile Red with neutral lipids results in shorter emission wavelengths (< 590 nm) than with polar lipids (> 590 nm), enabling differentiation between them [140].

The main factors affecting Nile Red fluorescence, uptake, and intensity have been established. The permeability of cell walls can be improved by using stain carriers, such as dimethyl sulfoxide (DMSO; 4–25% *v*/v), glycerol (0.1–0.125 mg/mL) or ethylenediaminetetraacetic acid (EDTA; 3.0–3.8 mg/mL). Temperatures ranging from 30 to 40 °C have also been shown to facilitate Nile Red diffusion across the cell wall. The interaction between Nile Red and lipids can be further improved by using a low dye:cell ratio (0.25–2.0 μg dye/mL) and cell concentrations > 5×10^4 cells/mL. Finally, to determine the maximum Nile Red fluorescence intensity, it is recommended to scan excitation and emission for up to 40 min [35].

Several Nile Red-based methods have been developed recently for rapid screening of lipid production in algal, yeast and fungal strains [156, 158–160]. Chen et al. (2011a) proposed a high-throughput multi-well microplate-based method with low sample requirements that allows for rapid analysis of multiple samples simultaneously. Unfortunately, lack of reproducibility of fluorescence measurements together with a non-linear response between intensity and oil content has limited the use of this method [159]. In an attempt to address measurement variability and to avoid using a calibration standard, Bertozzini et al. (2011) suggested the use of known amounts of internal standard (triolein) in combination with Nile Red staining [161]. However, this method is not applicable to algal species with thick cell walls that restrict the uptake of Nile Red. Furthermore, differences in relative Nile Red fluorescence by various lipid classes and the different distribution between dye and triolein globules, may result in under- or overestimation of cell lipid content. Sitepu et al. (2012) developed an improved high-throughput assay using DMSO but eliminated the washing step, reduced the Nile Red concentration, and proposed kinetic readings rather than a single time point [162]. Several cell treatment approaches have been tested aiming to increase the permeability of Nile Red; these include microwaves [159], ultrasonic treatment [163], EDTA [164], and mild cell pre-bleaching [165].

An alternative to Nile Red is the dye BODIPY 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene), a highly lipophilic green fluorophore. BODIPY 505/515 can stain a variety of algae irrespective of cell wall properties [166]. Compared to Nile Red, it has higher fluorescent quantum yield and a narrower emission spectrum, higher sensitivity, and better reproducibility [167]. BODIPY 505/515 has been the fluorescent marker of choice for the evaluation of intracellular lipids in algae [166, 168]. TAG content in BODIPY 505/515-stained *C. vulgaris* by single-cell fluorescence strongly correlated with the result obtained by ^{1}H NMR [126]. BODIPY 505/515 dissolved in DMSO at a final concentration of 1–10 μM was shown to selectively stain

lipid bodies of several taxa of live algae. Moreover, the concentration of DMSO itself was only in the range of 0.02–0.2%, much less than 20–30% for Nile Red [166, 169]. Another advantage of BODIPY 505/515 is that it selectively binds to lipid bodies, whereas Nile Red binds also to other cellular compartments [166]. As BODIPY 505/515 has a higher oil-to-water partition coefficient than Nile Red, it can bind more specifically to neutral lipids [168]. Further, with an excitation wavelength of 488 nm and an emission maximum at 515 nm, its emission spectrum is distinct from that of chloroplasts [166, 169]. Disadvantages of BODIPY 505/515 include medium background fluorescence, overlap with chlorophyll, and dye-dye interactions that lead to variability during measurements [166]. The ideal incubation temperature for BODIPY 505/515 staining is 25 °C and the optimal biomass concentration is 10^6 cells/mL for 1.12 μg/mL of dye [169]. BODIPY 505/515 has also been shown to stain oil-containing organelles within minutes and maintain fluorescence efficacy over at least 20–30 min, being more photostable than Nile Red [166, 168, 169].

Both dyes have been compared for their accuracy; however, their applicability depends on the experimental conditions and microorganism species. For example, both were evaluated in situ in a microbioreactor system to monitor real-time production of intracellular lipids in the yeast *Y. lipolytica*. While online detection of BODIPY 505/515 revealed a linear correlation between fluorescence and intracellular lipid concentration, no fluorescence was observed when Nile Red was used. This could be explained by the reduced photostability of Nile Red [170]. In contrast, BODIPY 505/515 was found unsuitable in a Nile Red staining protocol for various microalgae species that used a microplate fluorescence reader [171].

Recently, the blue fluorescent neutral lipid-specific dye AC-202 was evaluated as an alternative for labelling lipid droplets in the model green alga *C. reinhartii* and the model diatom *P. tricornutum* [172]. AC-202 belongs to a class of dyes that are analogs of thalidomide (2,6-diisopropyl phenyl-4/5-amino-substituted-4/5,6,7-trifluorophthalimides) and show very low toxicity to plant cells. It can reveal subtle intracellular modifications in neutral lipid content, either alone or in combination with Nile Red or BODIPY 505/515. Moreover, owing to its elevated lipid specificity, high sensitivity, and low background signal, it could identify metabolic and signalling pathways implicated in lipid formation, greatly surpassing the results obtained by Nile Red and BODIPY 505/515 [172].

Droplet-based microfluidic methods have been recently developed for high-throughput analysis and screening of lipid content in algal cultures. Such systems are based on the formation of aqueous droplets in an immiscible fluid (carrier oil) that are capable of encapsulating one or more cells, so they can be processed and analyzed in parallel within a short period. Moreover, in combination with fluorescence staining, they have been proven useful to analyze oil accumulation in a high-throughput manner in different species and in single cells [173]. A digital microfluidic system for high-throughput screening of growth and lipid production in *Cyclotella cryptica*, evaluating hundreds of culture conditions has been demonstrated [171]. In another study, alginate hydrogel microcapsules containing BODIPY 505/515-stained single cells were used to analyze the in situ lipid content of individual *C. vulgaris*, *Chlamydomonas sp.*, and *B. braunii* algae [174].

Flow cytometry allows to simultaneously measure fluorescence and sort individual stained cells, resulting in the selection and isolation of target populations [171]. Staining with either Nile Red or BODIPY 505/515 has been applied for in vivo flow cytometry quantification of lipids in individual microalgae [36, 166, 167]. Fluorescence-activated cell sorting (FACS) has been applied for the high-throughput sorting of algal cells with high lipid accumulation such as hyperaccumulating mutants of *C. reinhardtii* [175]. Terashima et al. (2015) proposed the *Chlamydomonas* high-lipid sorting (CHiLiS) strategy for isolating viable high-lipid cells from a pool of 60,000 mutants stained by Nile Red [176]. An advantage of FACS is that the cell sorter takes into account the fluorescence of each cell individually rather than bulk fluorescence measurements. Moreover, by vitally staining with Nile Red or BODIPY 505/515, processed cells can be seeded to produce new algal lineages with desired characteristics such as lipid hyperaccumulation [166]. For example, *Nannochloropsis* strains have been vitally stained with Nile Red for high-throughput screening and flow cytometric cell sorting, to obtain pure high-lipid producing cultures [160]. In this case, glycerol was used instead of DMSO as a carrier solvent because it did not inhibit cell growth.

Furthermore, high-content image analysis methods have been developed for the screening of oleaginous yeasts [177]. Specifically, lipid droplet accumulation was analyzed by the acquisition of high-quality confocal images of Nile Red-stained cells. The ensuing morphological information enabled a detailed study of lipid droplet dynamics on a single-cell scale. Davis et al. (2014) applied hyperspectral confocal fluorescence spectroscopy for the in vivo localization of lipid bodies in four microalgae and to quantify lipid yield at a single-cell level [178]. Various lipophilic fluorescence dyes used in fluorescence spectroscopic analysis of lipids from different microorganisms are presented in Table 8.

Dielectric spectrometry

Dielectric methods are based on the different permittivity and conductivity of cell components and measure the dielectric properties of a medium as a function of

Table 8 Fluorescence spectroscopy methods

Method	Microorganism	Range	Sample preparation	Correlation with method	Internal Standard	Excitation/ emission (nm)	Dye concentration	Reference
Nile Red	*Amphora coffeaformis, Navicula* sp., *Tropidoneis* sp.	n/a	Whole cells	Gravimetric, GC	–	Exc. 488–525 Em. 570–600	1 μg /mL/acetone	[37]
Nile Red (high-throughput microplate assay with mild bleach solution addition)	3 *Chlorella* species	0.75–40 μg	Lipid extracts suspended in chloroform; whole cells; extracted lipids (25 μL diluted in isopropanol); cells (200 μL OD_{600} 0.35–1.0)	GC/MS, gravimetric, TAG determination with TLC	Corn oil	Exc. 530 Em. 575	4 μg/mL/ DMSO	[165]
Nile Red	*Lipomyces starkeyi, Rhodosporidium toluroides, Cryptococcus curvatus*,3 *Mortierella* species	2–5000 μg lipid/mL broth	Whole cells, 100 μL culture broth suspended in 2 mL buffer	GC	–	Exc. 400 Em. 565–585	0.24–0.47 μg/mL/ acetone	[179]
Nile Red (High-throughput microplate assay)	9 microalgal species	2.0–20.0 μg/mL	Whole cells, 3×10^6 cells/mL, $OD_{750} = 0.06$	Gravimetric	Triolein	Exc. 530 Em. 575	0.5 μg/mL /DMSO	[180]
Nile Red	*Nannochloropsis* sp.	n/a	Whole cells, 3 mL of culture	–	–	Exc. 486 Em. 570	0.26 μM/acetone	[156]
Nile Red	*Skeletonema marinoi, Chaetoceros socialis, Alexandrium minutum*	0.12–12 μg/mL	Whole cells, 2 mL resuspended in isopropanols and internal standard	–	Triolein	Exc .547 Em.580	0.25 μg/mL	[161]
Nile Red (High-throughput microplate)	*Saccharomyces cerevisiae, Rhodosporidium diobovatum, Pichia manshurica, Rodosporidium paledigenum, Cryptococcus victoriae*	n/a	Whole cells, 250 μL at $OD_{600} = 1$ in 300 μL total volume	Gravimetric	–	Exc.530 Em. 590	5 μg/mL /DMSO	[162]
Nile Red (High-throuput microplate assay, two-step, microwave assisted)	*Pseudochlorococcum* sp., *Scenedesmus dimorphus, Chlorella zofigiensis*	n/a	Whole cells, 5–10 μL algal cells	–	–	Exc. 490 Em. 580	1 μg/mL /DMSO and acetone	[159]
Nile Red (High-throughput assay)	*Auxenochlorella protothecoides, Chlorella vulgaris, Scenedesmus dimorphus, Scenedesmus obliquus*	n/a	Whole cells, 5 g/L dry weight, 10 μL algal suspension in 120 μL total volume	–	–	Exc. 530 Em. 604	10 μg/mL /ethanol	[171]
Nile Red	*A. protothecoides*	Up to 70% dry weight	Whole cells, 1.6 mL	SPV	–	Exc. 495 Em. 540–750	6 μg /mL/ DMSO	[181]
Nile Red (cell treatment with EDTA)	*Tetraselmis suecica*, 6 microalgal stains	–	Whole cells, 250 μL	–	–	Exc. 530 Em. 590	1.3 μg/mL / acetone	[164]
Nile Red	*Nannochloropsis* sp.	–	Whole cells, 2 mL			Exc. 480 Em. 575	0.3 and 0.7 μg/mL for glycerol and DMSO, respectively	[160]
Nile Red (combining ultrasonic treatment and 3D excitation emission matrix fluorescence spectroscopy)	*Scenedesmus* sp.	n/a	Whole cells, 5 mL	Gravimetric	–	Exc. 530 Em. 568	1.5 mg/mL /acetone	[163]
Nile Red	*Nannochloropsis oculata, Tisochrysis lutea*	n/a	Whole cells, $OD_{750} = 0.2$	Gravimetric	–	Exc. 570 Em. 670	1.67 or 3.33 μg/mL/ glycerol	[158]

Table 8 Fluorescence spectroscopy methods *(Continued)*

Method	Microorganism	Range	Sample preparation	Correlation with method	Internal Standard	Excitation/ emission (nm)	Dye concentration	Reference
BODIPY 505/515	*C. vulgaris*, *Dunaliella primolecta*, *Chaetoceros calcitrans*	n/a	Whole cells, 10^6 cells/mL	Nile Red	–	Exc. 480 Em. 590	0.067 μg/mL/DMSO	[168]
AC-202	*Chlamydomonas reinhardtii*, *Phaeodactylum tricornutum*	n/a	Whole cells, 1 mL	Nile Red, BODIPY	–	Exc. 395 Em. BP445/50	10 μM/DMSO	[172]

n/a Not available

frequency. They provide a rapid, non-invasive, and label-free method for determining the composition of lipids in a cell, as the critical frequency is sensitive to cellular lipid content but is not affected by changes in cell concentration [182]. Dielectric spectra have been shown to correlate with the oil content in *Mortierella alpina* cells [183], whereas different *C. protothecoides* electrorotation spectra have been correlated with changes in lipid content [184]. Accordingly, dielectrophoresis was applied for the binary separation of *Chlorella* cells with different lipid contents, suggesting that lipid accumulation influenced the dielectric properties of algal cells [185]. Cells from the model alga *C. reinhardtii* have been dielectrically characterized and correlated with variations in lipid content induced by nitrogen starvation [186]. The application of dielectric spectrometry requires simple instrumentation such as a vector network analyzer and minimal sample preparation as cells are centrifuged and resuspended in fresh medium. Automated single-cell dielectric characterization methods for online lipid measurements could be used for the biomonitoring of industrial biodiesel production.

Other methods

A simple and sensitive spectrophotometric method was developed for the quantification of fatty acids derived from microalgal cultures of *N. oculata* and *D. salina* [187]. The procedure includes cell lysis, followed by lipid saponification, fatty acid extraction, and formation of copper-fatty acid complexes after reaction with triethanolamine-copper salts. The organic phase was detected by measuring absorbance at 260 nm. The developed method was reproducible and provided quantification of nanomoles of lipids using only 1 mL of culture. However, it is a multi-step method, which requires the use of solvents for cell lysis, saponification, and extraction of fatty acids.

Colorimetric detection

Colorimetric methods for lipid quantification constitute an attractive choice due to their fast response and simplified sample handling; however, they also usually require other preliminary steps such as cell disruption and lipid extraction. The sulfo-phospho-vanillin (SPV) method that was initially developed by Charbol and Charonnat (1937) for the quantification of serum total lipids is the most commonly used colorimetric technique for lipid quantification in oleaginous microorganisms [188]. The SPV reaction is performed in three steps: (1) phosphoric acid reacts with vanillin to form phosphate esters with higher reactivity of the carbonyl group, known as the SPV reagent; (2) unsaturated lipids react with concentrated sulfuric acid (≥95%) at high temperatures (90–100 °C) to form a carbonium ion; (3) the ensuing lipid products react with the SPV reagent, whereby the carbonium ion reacts with the carbonyl group of a phosphovanillin ester to form a pink chromophore compound that is stabilized by resonance [189]. Quantification is then performed by measuring absorbance at 530 nm.

The SPV method has been recommended for routine analysis; however, the presence of either double bonds or free hydroxyl groups is required for a positive SPV reaction [190]. To this end, its accuracy depends primarily on the reference standard being used. Color intensity varies between different lipids due to structural differences in fatty acids. A higher unsaturated fatty acids content leads to higher intensities; however, the presence of polyunsaturated fatty acids does not translate in a more intense color because of steric hindrance [191]. For example, triolein resulted in higher color intensity compared to the polyunsaturated eicosapentaenoic and docosahexaenoic acids, whereas unsaturated fatty acids did not react with the SPV reagent, as expected. [191]. This observation led the authors to conclude that the reaction was affected by the samples' concentration and chemical structure. Various colorimetric methods applied for lipid quantification of oleaginous microorganisms are presented in Table 9.

The SPV method has been modified for high-throughput analysis of total lipids in a 96-well microplate using solvent-extracted and purified lipid samples [193]. The microplate procedure included the addition of < 100 μL of sample and had a detection range of 5–120 μg lipids. The microplate assay showed a strong correlation with the gravimetric method for the analysis of four green microalgae strains. Anschau et al. (2017) adapted the SPV method to a 96-well microplate assay, eliminating the first step of organic solvent addition (chloroform), and verified the impact of unsaturated and saturated fatty acids as a reference on the validation parameters of the method [190]. No significant difference in lipid determination was found between this and the gravimetric method, suggesting that the SPV approach could be a useful analytical tool to quantify lipids in lyophilized yeast and microalgae cells.

The SPV assay has advantages over traditional extraction and gravimetric analysis, as well as high-throughput fluorescence spectroscopy. Compared to Folch extraction and gravimetric quantification, both of which may lead to overestimating lipid content due to inclusion of other lipophilic compounds (e.g., lipophilic proteins and pigments), the SPV assay eliminates these issues as these products are degraded by acid-thermal treatment. Compared to Nile Red, which can undergo rapid photobleaching, the color formed in the SPV reaction remains stable for several hours even under abundant light [194]. One drawback of the SPV method is that it requires lipid extraction through addition of chloroform and subsequent sample purification.

Table 9 Colorimetric methods for lipid quantification of oleaginous microorganisms

Method	Assay	Microorganism	Range	Outcome	Standard	High correlation with	Reference
Copper extraction in chloroform	Centrifuge cells and freeze pellet, thaw and saponify pellet (with 25% methanol in 1 N NaOH), add 59 mg glass beads for cell disruption, heat at 100 °C for 30 min, add 200 µL neutralization reagent and 200 µL copper reagent, add 250 µL chloroform and centrifuge, add 50 µL of organic phase and 50 µL sodium diethyldithiocarbamate in 2-butanol, measure absorbance at 440 nm	*Phaeodactylum tricornutum*, *Chlorella vulgaris*	0.02–0.8 µmol fatty acid	Small amout of culture (1–2 mL culture); adaptation to microcentrifuge format allows up to 30 samples in < 2 h; requires cell disruption	Laurate, decanoate	–	[192]
SPV, modified for microplate high-throughput screening	Evaporate solvent, add 100 µL sulfuric acid, and incubate at 90 °C for 20 min, cool microplate for 2 min on ice, add 50 µL vanillin-phosphoric acid, wait 10 min, measure absorbance at 540 nm	4 *Chlorella* species	5–120 µg	Small amount of sample (< 100 µL), less time (< 1 h), color development is more consistent due to modification of reagent concentrations; requires extraction and purification	Corn oil	Gravimetric	[193]
SPV	Prepare sample (extraction), evaporate solvent, add 100 µL sample, add 2 mL sulfuric acid, heat at 100 °C for 10 min, cool for 5 min in ice bath, add 5 mL vanillin-phosphoric acid, incubate at 37 °C for 15 min at 200 rpm, measure absorbance at 530 nm	*Chlorella* sp., *Monoraphidium* sp., *Ettlia* sp., *Nannocloropsis* sp.	100–1000 mg	Applicable to wide range of microalgae	Canola oil	GC/MS	[194]
Automated quantification on spinning disc with TAG colorimetric detection based on a commercial kit	Automated process	*Chlamydomonas reinhardtii*	5–30 µg	Includes automated cell sedimentation lysis and extraction; reduced time (< 13 min); only TAGs; small amount of sample (≤500 µL)	Triglyceride lipid standard	GC/MS, gravimetric	[195]
SPV	Add 0.2–1 mg dried biomass in distilled water, incubate at 100 °C for 10 min, cool for 5 min in ice bath, add 5 mL vanillin-phosphoric acid, incubate at 200 rpm and 37 °C for 15 min, measure absorbance at 530 nm	*Schizochytrium* sp., *Thraustochytrium* sp.,	0.2–1 mg	No solvent addition, applied to whole cells; reduced time (30 min)	Commercial *Schizochytrium* oil	Gravimetric	[191]
SPV, modified for microplate high-throughput screening	Prepare sample, dissolve 20 µL sample in 180 µL concentrated H_2SO_4, heat at 100 °C for 10 min and cool to room temperature, add 0.5 mL vanillin-phosphoric acid, heat at 37 °C for 15 min and cool to room temperature, store for 45 min in dark box, measure absorbance at 530 nm	*C. vulgaris*, *Lipomyces starkeyi*, fresh and lyophilized	up to 20 mg/mL	Small amount of sample (20 µL); no solvent addition as preliminary step; reduced time (< 1 h)	Oleic acid, palmitic acid	Gravimetric	[196]

The colorimetric quantification of algal fatty acids dissolved in chloroform has been described by Wawrik and Harriman (2010) based on a method initially developed by Duncombe (1963) [192]. The assay includes the hydrolysis of algal lipids to their corresponding fatty acids, followed by extraction of their copper salts with chloroform. Finally, the amount of copper is determined colorimetrically at 440 nm following addition of diethyldithiocarbamate and generation of a yellow product. Adaptation of the protocol to a microcentrifuge tube with 1 mL of log-phase algal culture allowed quantification of 24–30 samples in less than 1 h. The assay produced a linear response for all long-chain fatty acids. Whereas the extinction coefficient was significantly higher in C12:0 than C10:0 fatty acids; it remained largely indistinguishable in C12:0, C14:0, and C16:0 fatty acids. Moreover, unsaturation had a small but significant effect, as did functional groups, suggesting that chemical modification could lead to an underestimation of the total lipid content with this method.

Kim et al. (2015) developed a fully integrated centrifugal microfluidic device for rapid on-site automated quantification of lipids from microalgal samples, involving small sample volumes (≤500 μL) and short times (≤13 min) [195]. The serial process included optimized steps of cell sedimentation and lysis, liquid-liquid extraction using *n*-hexane:ethanol, and colorimetric detection by a commercial TAG assay kit. The colorimetric assay involved the following reactions: enzymatic hydrolysis of lipids by a lipase generating glycerol and free fatty acids, the phosphorylation of glycerol to glycerol-3-phosphate by a kinase, oxidation of the latter to dihydroxyacetone phosphate by a glycerol phosphate oxidase, and finally the conversion of the oxidized product to quinoneimine dye by a peroxidase. The dye product was quantified by measuring absorbance at 540 nm. The highly linear correlation ($R^2 > 0.99$) between the quantity of lipids determined by the fully automated lab-on-a-disc and either manual extraction or conventional GC/MS suggested high accuracy of the developed device. This, however, was countered by a relatively high error in the measured amounts of microalgal lipids, particularly at low sample concentrations. The error was attributed mainly to sample preparation and the contribution of other lipids during GC/MS detection.

Comparison of conventional and high-throughput lipid measurements

As discussed previously, the selection of an appropriate method depends on its suitability for the chosen microbial strain and the specific experimental conditions. Conventional methods of lipid quantification rely on lipid extraction by solvents, which is often time consuming, insensitive to small differences in lipid content, and requires a substantial amount of cell material and toxic solvents. Furthermore, as the traditional gravimetric method involves several steps, neutral lipids can be lost along the way [180]. Another drawback of gravimetric analysis is that it estimates all lipids, not just neutral ones. Nevertheless, the gravimetric method remains the most common way to quantify lipids and it can be considered a standard when developing new screening strategies. Compared to the traditional gravimetric method, fluorescence measurements can offer a simpler, faster, and less biomass-requiring way of determining lipid content. Also, sensitivity is higher, allowing for small differences in lipid content to be distinguished [137]. On the downside, fluorescence-based detection is highly species- and strain-specific, and these methods require optimization before they can become reliable quantification tools. The Nile Red method might either over- or underestimate neutral lipid content as it strongly depends on the dye diffusing into cells, and the presence and concentrations of pigments or other lipophilic compounds affecting the fluorescence background [180]. Other methods such as TD-NMR and SPV are species-specific and their accuracy varies among species. Accordingly, their protocol requires some modifications before unknown samples can be accurately analyzed [33]. Raman spectrometric methods can be applied to single cells, but they do not show good accuracy with samples containing fluorescent pigments and carotenoids [145]. Moreover, lipid quantification is based on the degree of unsaturation of fatty acids and further calibration with other methods such as the gravimetric one is needed [197]. High costs associated with sample preparation for TLC/HPTLC hinder their application on a large scale. A comparison of all conventional and non-conventional methods is presented in Table 10 and is aimed to provide a guide for identifying the most suitable method.

Conclusions

Methods for microbial lipid analysis should be selected based on their suitability to the species being examined. In oleaginous microorganisms, the choice depends on the desired level of identification. If crude lipid content or total neutral lipids and/or lipid classes are the targets, gravimetric, fluorescence, and FTIR methods, respectively, should be attempted. Conversely, the types of fatty acids, their length, and level of saturation/unsaturation can be quantified most reliably by NMR, Raman spectrometry, and GC FID/MS, respectively. The estimation may vary with the size and stage (wet or dry) of samples required for a particular method: 1 g of dry biomass for NMR, 5 to 500 mg wet biomass for chromatography or MS, 1 ml of culture for colorimetric methods, 5 μL of culture for fluorescence estimation, and single cells for Raman spectrometry. Lipid quantification can be relative or absolute depending on the method used. Absolute quantification can be done by gravimetric analysis, GC,

Table 10 Comparison between conventional and non-conventional methods for lipids determination

Methods	Advantages	Disadvantages
Conventional		
Gravimetric	The easiest method to quantify total lipids in a short time, no need for special equipment or training	Requires cell disruption, extraction, not suitable for small amounts of sample
Chromatographic coupled with detector	Well-established procedures for lipid profiling	Requires cell disruption, extraction, and (in situ) transesterification, multi-step process; Depends on microalgae strains and cellular extraction method
Non-conventional		
FTIR	Identification of lipid classes on the basis of standards, cellular content determination without disruption of cells, small amount of samples	FTIR band shifts are species-specific, cannot differentiate different species when a mixture of cultures is used
Raman spectroscopy	Label-free, no sample preparation, in vivo analysis, real-time, non-destructive	Fluorescent pigments and carotenoids interfere with analysis; quantification is focused mainly on determining the degree of unsaturation, there is need for calibration based on other methods such as gravimetric analysis; difficult to translate to an industrial setting
NMR	Can be non-destructive, application to whole cells, extracted and transesterified lipids	Need for internal standards for quantification; difficult to translate to an industrial setting
Dielectric spectroscopy	Rapid, non-invasive, and label-free method; potential for automated process for biomonotring	Total lipid detection, no profiling
Fluorescence spectrometry	High-throughput potential	Uptake and intensity depends on dye and cell wall; only detects neutral lipids
Nile Red	Mostly used in literature	Photo-bleaching of agent; poor penetration of cells; interference with chlorophyll autofluorescence and green fluorophores; limited specificity to lipids
BODIPY 505/515	More lipid-specific, narrower emission spectrum, higher sensitivity, and better reproducibility, requires lower concentration of solvent carrier	Interference with green fluorophores
AC-202	More sensitive than BODIPY; low-background signal	Not used for quantification yet
Colorimetry	High-throughput potential Low cost	Total lipid detection only
SPV	Color formation is stable for hours Fast, low detection limit Solvent extraction can be omitted depending on the assay	Requires presence of double bond or free hydroxyl groups (unsaturated lipids) Is dependent on standard
TAG kit	Fast, automated process	Detects only TAGs; requires cell disruption and extraction
Copper extraction	Fast, high detection limit, can be adapted to microcentrifuge format	Requires solvent addition and cell disruption

MS, and by direct transesterification methods; whereas relative quantification (unless using a standard) can be done with FTIR, fluorescence probes, colorimetric, NMR, and Raman spectrometry. With some methods, cultures remain viable at the end of the quantification procedure, as in the case of NMR, Raman spectrometry, and sometimes Nile Red staining. If a fast screening of lipid content is required, a high-throughput Nile Red flurospectrophotometric method can be used, however it is often not comparable between different species. Finally, the selection of a lipid quantification method is dictated by the accessibility of equipment and resources in the laboratory; preferably, it should favor the most significant and universally accepted ones.

Abbreviations

BODIPY: Boron-dipyrromethene; CARS: Coherent anti-Stokes Raman scattering; CRS: Confocal Raman spectroscopy; DAGs: Diacylglycerols; DMSO: Dimethyl sulfoxide; DTD: Direct thermal desorption; EDTA: Ethylenediaminetetraacetic acid; ELSD: Evaporative light scattering detector; ESI: Electrospray ionization; FABMS: Fast atom bombardment mass spectrometry; FACS: Fluorescence-activated cell sorting; FAME: Fatty acid methyl esters; FID: Flame ionization detector; FTIR: Fourier transform infrared spectroscopy; GC: Gas chromatography; HPLC: High-performance liquid chromatography; HPTLC: High-performance thin-layer chromatography; HR-MAS: High-resolution magic angle spinning; iMQC: Intermolecular multiple-quantum coherence; IR: Infrared; LTRS: Laser-trapping Raman spectroscopy; MAGs: Monoacylglycerols; MALDI: Matrix-assisted laser desorption and ionization; MS: Mass spectrometry; NIR: Near-infrared; NMR: Nuclear magnetic resonance; SPV: Sulfo-phospho-vanillin; TAGs: Triacylglycerols; TD: Time domain; TLC: Thin-layer chromatography; TOF: Time-of-flight; UV: Ultraviolet

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Authors' contributions
LM, UR and PC conceived the content of the article and revised the text. AP, IA, and JE analyzed the literature and wrote the paper. All authors read and approved the final manuscript.

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