



Evaluation of green microalgae isolated from central and north coast of Sao Paulo as source of oil

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INFO

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Palavras-chaves

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ABSTRACT

Evaluation of green microalgae isolated from central and north coast of Sao Paulo as source of oil.

Microalgae strains, newly isolated from freshwater in mangrove areas of Central and North Coasts of Sao Paulo State (Brazil), were evaluated regarding total protein and lipid content, and fatty acids profile. The biochemical composition was compared with that observed in strains obtained by UTEX Culture Collection (USA). Among seven identified green algae, *Monoraphidium contortum* (CCMA-UFSCar-701) presented the highest lipid content (43.60%), close to that observed in *Botryococcus braunii* (UTEX-2441; 48.85%). Protein content in isolated strains varied in the range of 13.90~23.60%. Finally, the most abundant fatty acids were palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2), and γ -linolenic acid (C18:3). *Chlorella* sp. (CCMA-UFSCar-697) may be highlighted for its high linoleic acid content (29%). On the other hand, *Elakatothrix* sp (CCMA-UFSCar-702) and *Scenedesmus obliquus* (UTEX-B2630) presented the highest content of oleic acid (41% and 43%, respectively), which is preferable for oils to be used as feedstock for biodiesel.

RESUMO

Avaliação de microalgas verdes isoladas nas costas central e norte de São Paulo como fonte de óleos.

Microalgas de água doce, isoladas em áreas de mangue nas costas central e norte do estado de São Paulo (Brasil), foram avaliadas considerando conteúdo de lipídios, perfil de ácidos graxos e conteúdo de proteínas. Essa composição bioquímica foi comparada com cepas obtidas da Coleção de Culturas da UTEX (EUA). Entre as sete algas verdes identificadas, *Monoraphidium contortum* (CCMA-UFSCar-701) apresentou o maior conteúdo lipídico (43,60%), valor próximo ao observado em *Botryococcus braunii* (UTEX-2441, 48,85%). O conteúdo de proteínas nos isolados variou entre 13,90 e 23,60%. Os ácidos graxos mais abundantes foram ácido palmítico (C16:0), ácido oleico (C18:1), ácido linoleico (C18:2) e ácido γ -linoléico (C18:3). *Chlorella* sp. (CCMA-UFSCar-697) destacou-se por seu alto conteúdo de ácido linoleico (29%), enquanto *Elakatothrix* sp (CCMA-UFSCar-702) e *Scenedesmus obliquus* (UTEX-B2630) apresentaram o maior conteúdo de ácido oleico (41% e 43%, respectivamente), sendo que este ácido se destaca como matéria prima para produção de biodiesel.

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INTRODUCTION

Microalgae may be found in marine environments, freshwater or even in soil (Norton et al., 1996) and they are believed to be responsible for at least 60% of primary productivity on Earth (Chisti, 2004). Tens of thousands of microalgal species have been classified, and this high diversity may represent a promising source of new bioproducts and applications (Norton et al., 1996; Pulz and Gross, 2004).

Several studies focus on a diversity of applications of microalgal biotechnology in food, cosmetics and pharmaceutical industries, including: food and feed supplements, anti-oxidative compounds, carotenoids, polyunsaturated fatty acids, vitamins, and immunologically active polysaccharide (Derner et al., 2006; Gouveia et al., 2011; Olaizola, 2003; Pulz and Gross, 2004). More recently, microalgae have been highlighted as valuable alternative and sustainable source of third generation biofuels (Chen et al., 2014; Chisti, 2007).

It is important to note that unicellular photosynthetic organisms are capable of using light energy more efficiently, in comparison with higher plants (Janssen et al., 2003), being considered very efficient for carbon dioxide bio-fixation (Brown and Zeiler, 1993). Moreover, they can be cultivated in controlled conditions, presenting desired and stable composition (Dunstan et al., 1993; Sassano et al., 2010) and favoring industrial/ commercial operations.

This study aimed to evaluate the potential of green microalgae as a source of oil-rich biomass. Seven green algae strains were isolated from freshwater in mangrove areas of Central and North Coast of Sao Paulo State. The lipid content, fatty acids profile, and protein content were compared with that of green microalgae obtained from Culture Collection.

MATERIAL AND METHODS

Sample collection and isolation

Using sterile glass bottles, freshwater samples (1 ~ 1.5 L) were collected in different mangrove areas within “Serra do Mar” State Park located in the cities of Cubatão and Ubatuba (Central and North Coast of São Paulo State – Brazil). Geographical coordinates are shown in table 1. Concomitantly with the collection of samples, temperature and pH (pH-indicator strips; Merck) were measured.

At the laboratory, microalgae containing water samples were used to inoculate enriched standard culture media: BOLD (UTEX, n.d.), CHU (UTEX, n.d.), Schlosser (Schlösser, 1982), and F/2 (Guillard and Ryther, 1962). When the culture

became visually greenish, the isolation took place by combining micropipette method and streaking cell across agar plates (Andersen and Kawachi, 2005), in order to isolate unique species of microalga from the water samples.

Isolates identification

Isolated strains were observed under Olympus system microscope model BX51 and images were captured with camera Olympus SC30. Microscopic identifications were based on the morphology of the individual cells and colonial characteristics.

Cultivation of isolate strains

Isolated microalgae strains were cultivated in the BOLD medium. Microalgae were grown in 6 L Erlenmeyer flasks containing 3 L of culture medium. Filtered air was continuously injected with the use of air stone diffuser. Room temperature was adjusted to 25 °C, and 30W fluorescent lamps were positioned above the flasks for providing light intensity of 60 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Initial pH was adjusted to 7.0.

Cell growth was indirectly measured by optical density at 550 nm (Becker, 1994) using a spectrophotometer (Femto 700 Plus), and the cultivation was finished when there was no further daily increase in optical density. Furthermore, four microalgae strains (Chlorophyceae) obtained from The Culture Collection of Algae at the University of Texas at Austin (UTEX) were cultivated in the same conditions: *Botryococcus braunii* (Utex 2441), *Chlorella vulgaris* (Utex 2714), *Neochloris oleoabundans* (Utex 1185), and *Scenedesmus obliquus* (Utex B2630).

Microalgal biomass evaluation

At the ended of each cultivation, biomass was recovered by centrifugation, washed twice with distilled water, and dried at 55 °C for 12h. Dry biomass was submitted to the determination of total lipids, employing organic solvents (chloroform: methanol 2:1, v/v) in Soxhlet extractor (Olguín et al., 2001; Piorreck et al., 1984). Lipid fraction, recovered with petroleum ether, was submitted to analysis of fatty acids content, after conversion to corresponding methyl esters (Hartman and Lago, 1973). The analysis of fatty acid methyl esters (FAME) was performed in a gas chromatograph (Agilent Model 7890 CX) in accordance with Rodrigues-Ract and Gioelli (Rodrigues-Ract and Gioelli, 2008) and Perez-Mora et al. (Pérez-Mora et al., 2016). FAME components were identified by comparing their retention time with the standard 37 FAME mix (Supelco).

Furthermore, total protein content in dry biomass was analyzed by the Kjeldahl method, and

factor 6.25 was adopted to convert from total nitrogen content (AOAC, 1984).

RESULTS AND DISCUSSION

In table 1, it is possible to see data from

Table 1 - Collection sites at Nucleo Itutinga-Pilões (Cb) in the city of Cubatão (S.P.) and Nucleo Picinguaba (P) in the city of Ubatuba (S.P.).

Collection site	Temperature	pH	Location
Cb1	26.0 °C	7.0	-23.8955 S -46.4621 W
Cb2	26.0 °C	7.0	-23.8988 S -46.4647 W
Cb3	30.0 °C	7.0	-23.9057 S -46.4285 W
P3	25.5 °C	6.5	-23.3556 S -44.8646 W

Cb1: Affluent of Pilões River; Cb2: Pilões River ; Cb3: Cotia-Pará Park; P3: Paciencia River.

In the same day at the laboratory, collected samples were used to inoculate different culture medium, generally used for photosynthetic microorganisms: BOLD (UTEX, n.d.), CHU (UTEX, n.d.), Schlosser (Schlösser, 1982), and F/2 (Guillard and Ryther, 1962). After 2 to 3 weeks, BOLD medium containing flasks showed the most intense green

collection sites. pH values were within 6.5 and 7.0, and temperature values were in the range 25.5 and 30.0. In fact, higher temperature (30.0) observed at Cotia - Pará Park may be attributed to the shallow water (approximately 5.0 cm).

color and these cultures were employed for isolation of 7 microalgae strains.

The identification of isolated strains was performed following the classification system by microscopic identifications based on morphological characteristics. As it is possible to see in table 2, all isolates belong to the Chlorophyceae class.

Table 2 - List of strains isolated from Nucleo Itutinga Pilões (Cb) in the city of Cubatão (S.P.) and Nucleo Picinguaba (P) in the city of Ubatuba (S.P.).

Our code	Organism	Accession number ^a
CB1a	<i>Chlorella vulgaris</i>	CCMA-UFSCar 698
CB2b	<i>Elakatothrix</i> sp.	CCMA-UFSCar 702
CB3c	<i>Monoraphidium contortum</i>	CCMA-UFSCar 696
CB2c	<i>Chlorella</i> sp.	CCMA-UFSCar 697
CB1Re	<i>Chlorella vulgaris</i>	CCMA-UFSCar 704
P3Fa	<i>Chlorella</i> sp.	CCMA-UFSCar 693
P3Fb	<i>Monoraphidium contortum</i>	CCMA-UFSCar 701

^a: Culture Collection of Freshwater Microalgae - Federal University of São Carlos.

Figure 1 and 2 shows photomicrographs of isolated microalgal strain from Nucleo Picinguaba (Ubatuba - S.P) and strain from Nucleo Itutinga-Pilões (Cubatão - S.P.), respectively. They were deposited in the Culture Collection of Freshwater Microalgae of The Federal University of São Carlos (CCMA-UFSCar). Some observed microscopic characteristics are described:

- *Chlorella* sp. (Beijerinck, 1890). (Figure 1A). Spherical, solitary cells with a single parietal chloroplast. It was also observed that reproduction takes place by autospores (disruption of the mother cell wall).
- *Monoraphidium contortum* (Komárková-Legnerová, 1969). (Figure 1B). Cells without mucilage and one parietal chloroplast, straight shape to lunate to sigmoid. Smooth cell wall.
 - with one parietal chloroplast.

- *Chlorella vulgaris* (Beijerinck, 1890). (Figure 2A). Single cells or colonies, spherical to subspherical, with one parietal chloroplast.
- *Elakatothrix*.sp. (Wille, 1898). (Figure 2B). Solitary or colonial cells. Random colony shape determined by cell arrangement. Hyaline and thin cell membrane. Fusiform cells with one parietal chloroplast.
- *Monoraphidium contortum* (Komárková-Legnerová, 1969) (Figure 2C). Cells with lunate form and elongate ends with single parietal chloroplast. Flat cell walls.
- *Chlorella* sp. (Beijerinck, 1890) (Figure 2D). Spherical to subspherical cells, single or in colonies. One parietal chloroplast.
- *Chlorella vulgaris* (Beijerinck, 1890) (Figure 2E). Single spherical cells

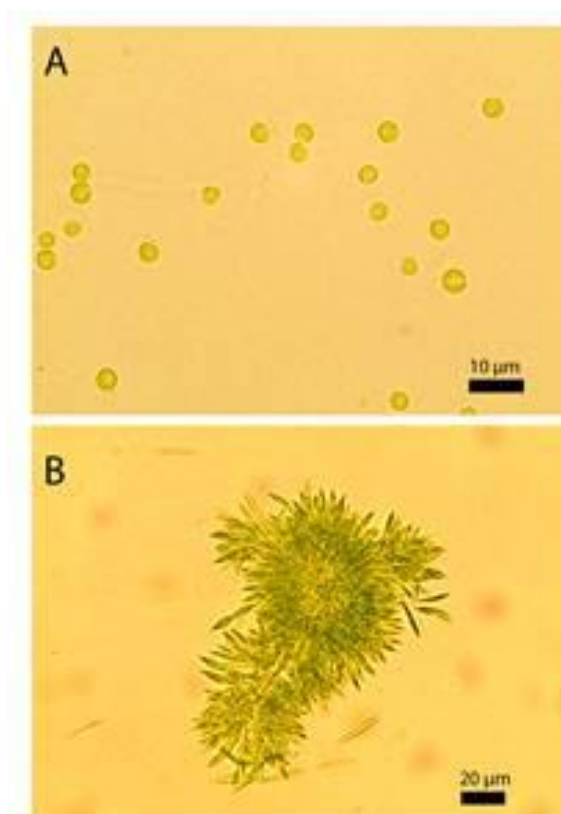


Figure 1 - Photomicrographs of isolated microalgal strain from Nucleo Picinguaba (Ubatuba - S.P.). A: *Chlorella* sp. (CCMA-UFSCar 693; our code: P3F-a). B: *Monoraphidium contortum*. (CCMA-UFSCar 701; our code: P3F-b).

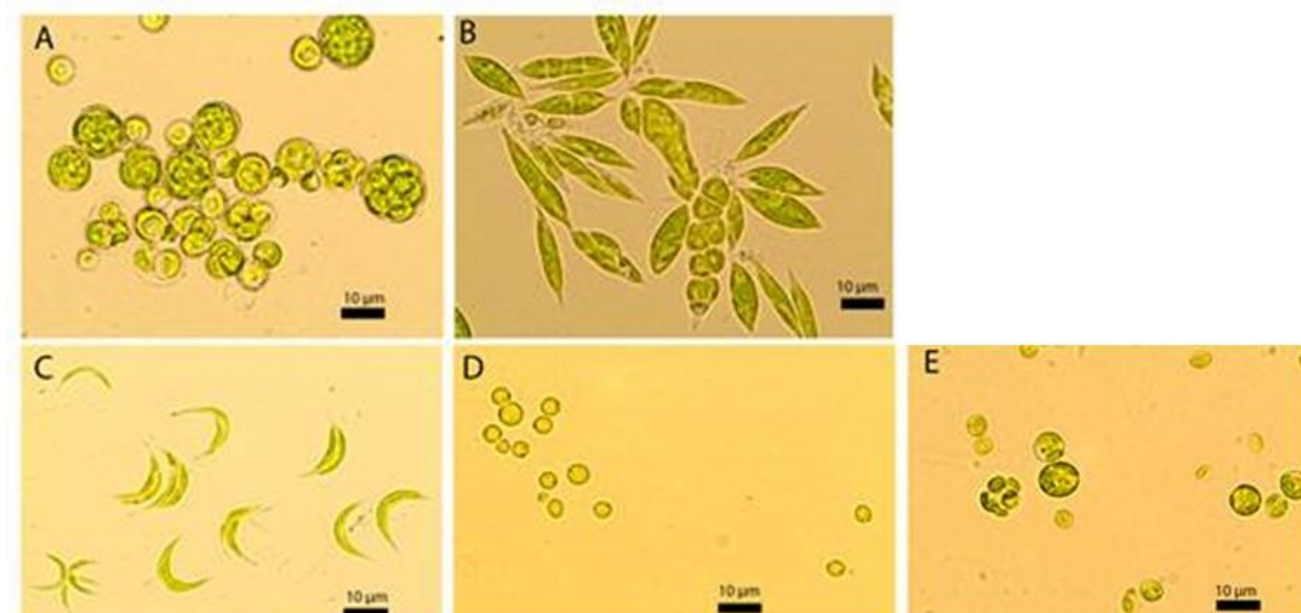


Figure 2 - Photomicrographs of isolated microalgal strain from Nucleo Itutinga-Pilões (Cubatão - S.P.). A: *Chlorella vulgaris* (CCMA-UFSCar 698; our code: CB1a); B: *Elakatothrix* sp. (CCMA-UFSCar 702; our code: CB2b); C: *Monoraphidium contortum*. (CCMA-UFSCar 696; our code: CB3c); D: *Chlorella* sp. (CCMA-UFSCar 697; our code: CB2c); E: *Chlorella vulgaris*. (CCMA-UFSCar 704; our code: CB1-Re).

All these isolates and the four microalgae strain obtained from Culture Collection (UTEX) were cultivated in 6L Erlenmeyer flasks containing 3L of BOLD Basal Medium for obtaining sufficient biomass for biochemical analysis: total protein content, total lipid content, and fatty acids profile.

Table 3 shows results from the analysis of total lipid and total protein content. Total lipid content varied from 18.07 to 43.60% in isolated strains and

from 21.40 to 48.85% in microalgae strains obtained from Culture Collection (UTEX)

The fatty acid profile of each strain was obtained by analysis of fatty acid methyl esters (FAME) by gas chromatography. The results in table A1 (Annex 1) presented palmitic acid (16:0), oleic acid (18:1n9), linoleic acid (C18:2n6), and γ -linolenic acid (C18:3n6) as the most abundant fatty acids for these strains.

Table 3 - Total lipid and total protein contents in the biomass of isolated strains and microalgae from Culture Collection (UTEX).

Strain	Total Lipid (%)	Total Protein (%)
CB1-a: <i>Chlorella vulgaris</i>	25.29 \pm 1.16	23.60 \pm 0.41
CB2b: <i>Elakatothrix</i> sp.	30.10 \pm 1.86	13.90 \pm 0.27
CB2c: <i>Chlorella</i> sp.	18.07 \pm 0.95	19.31 \pm 0.14
CB1-Re: <i>Chlorella vulgaris</i>	27.09 \pm 0.51	16.44 \pm 0.19
CB3c: <i>Monoraphidium contortum</i>	34.53 \pm 0.86	20.34 \pm 2.32
P3Fa: <i>Chlorella</i> sp.	26.02 \pm 2.88	20.88 \pm 0.37
P3Fb: <i>Monoraphidium contortum</i>	43.60 \pm 0.69	14.80 \pm 0.14
<i>Botryococcus braunii</i> (UTEX 2441)	48.85 \pm 0.72	14.05 \pm 0.78
<i>Chlorella vulgaris</i> (UTEX 2714)	21.59 \pm 2.13	26.23 \pm 2.72
<i>Neochloris oleoabundans</i> (UTEX 1185)	21.40 \pm 0.34	29.28 \pm 0.73
<i>Scenedesmus obliquus</i> (UTEX B2630)	29.09 \pm 2.31	15.16 \pm 0.72

The isolated *Monoraphidium contortum* (CCMA-UFSCar 701) and the purchased *Botryococcus braunii* (UTEX 2441) presented the highest values of lipid content (43.60 % and 48.85 %, respectively). The lack of nutrients at the end of the cultivation may have contributed to the increase in lipid content, as a result of the well-known phenomenon of triacylglycerol synthesis in response to stress condition (Liu and Benning, 2013). However, further studies have to be performed for a specific strain, since lipid metabolism seems not to be uniform in the algal realm (Liu and Benning, 2013).

Mahmoud et al. (Mahmoud et al., 2015) evaluated microalgae strains isolated in different locations in Egypt. Among fifteen isolates, they highlighted *Chlorella vulgaris*, *Scenedesmus quadricauda* and *Trachelomonas oblonga* as the most suitable candidates for oil production, with 37%, 34% and 29% of lipid (w/w), respectively.

Several studies have been done, not only submitting microalgae to stress conditions but also with genetic approaches. For example, increases in lipid accumulation were observed when the starch synthesis pathway was blocked in mutants of *Chlamydomonas* (Li et al., 2010) and *Chlorella* (Ramazanov and Ramazanov, 2006).

After the extraction of oil, defatted biomass may contain hydrophilic compounds with commercial interests, such as sugars and proteins (Bellou et al., 2014). Microalgal proteins may be used in pharmaceutical products, cosmetics or mainly for food and

feed supplements (Derner et al., 2006; Pulz and Gross, 2004). In the present study, total protein content varied in the range 13.90~23.60% in biomass of isolated microalgae strains and 14.05~29.28% in biomass of microalgae obtained from Culture Collection (UTEX).

The values of protein content obtained in this study were much lower than the values observed among the data presented by Barka & Blecker (Barka and Blecker, 2016), where some species of Chlorophyceae strains present protein content higher than 40% or even higher than 50%. However, to produce Single Cell Protein, for example, it would worth it to evaluate the physico-chemical condition for increasing the biosynthesis of these biomolecules, mainly light intensity and nitrogen content. In this context, there are several studies showing the possibility of increasing protein content in *Arthrospira platensis* in different types of photobioreactor by fed-batch process (Cruz-Martínez et al., 2015); repeated fed-batch (Matsudo et al., 2009) or continuous process (Avila-Leon et al., 2012; Matsudo et al., 2012)

Concerning fatty acids profile, linoleic and γ -linolenic acid, commonly known as ω -6 fatty acids, are precursors of arachidonic acid, which is important in the synthesis of eicosanoids in human body (Verlengia and Lima, 2002). These eicosanoids may play a role as mediators in processes associated to inflammation, immune system modulation, and platelet aggregation (Kus et al., 2011).

Isolated *Chlorella* sp. (CCMA-UFSCar 697) showed the highest content of linoleic acid (29%) and, among the strains from UTEX culture collection, *Neochloris abundans* (UTEX 1185) presented the highest content of this fatty acid (36%) (Table A1 – Annex 1).

In the case of γ -linolenic acid, *Chlorella* sp. (CCMA-UFSCar 697), *Chlorella vulgaris* (CCMA-UFSCar 698), and *Neochloris oleoabundans* (UTEX 1185) presented the highest values (23, 22, and 19%, respectively) (Table A1 - Annex 1). It is important to note that, the consumption of this fatty acid may be useful in the case of deficiency in delta-6-desaturase, responsible for its ordinary synthesis in the human body (Horrobin, 1992). In this sense, it is worth using one of these strains for further studies aiming to increase unsaturated fatty acids. For example, Ronda & Lele (Ronda and Lele, 2008) observed that increasing light intensity and decreasing temperature have a positive effect on the content of γ -linolenic acid.

Palmitic acid was above 20% in all the evaluated strains, but the highest contents were observed in that from UTEX culture collection, *Chlorella vulgaris* (46%) and *Scenedesmus obliquus* (34%) (Table A1 - Annex 1). The abundance of this fatty acid was also found by Molino et al. (Molino et al., 2018) in the green algae *Chlorella vulgaris* and *Dunaliella salina*.

The most abundant FAME, presented in the strains evaluated in the present study, are within the most common fatty esters in biodiesel, in accordance with Knothe (Knothe, 2008). Moreover, *Elakatothrix* sp (CCMA-UFSCar 702) and *Scenedesmus obliquus* (UTEX B2630) presented the highest content of oleic acid (41% and 43%, respectively), which is preferable for oils to be used as feedstock for biodiesel (Knothe, 2008; Mahmoud et al., 2015).

CONCLUSION

In this study, 7 green microalgae strains, isolated from freshwater in mangrove areas of Central and North Coasts of Sao Paulo State (Brazil), were evaluated regarding total lipid content, fatty acids profile, and total protein content. 4 strains obtained by UTEX Culture Collection (USA) were also evaluated for comparisons. *Monoraphidium contortum* (CCMA-UFSCar 701) presented the highest lipid content (43.60%), close to the lipid content observed in *Botryococcus braunii* (UTEX 2441; 48.85%). Protein content in isolated strains varied in the range of 13.90 ~ 23.60%. The most abundant fatty acids were palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2), and γ -linolenic acid (C18:3). *Chlorella* sp. (CCMA-

UFSCar 697) may be highlighted for its high linoleic acid content (29%). On the other hand, *Elakatothrix* sp (CCMA-UFSCar 702) and *Scenedesmus obliquus* (UTEX B2630) presented the highest content of oleic acid (41% and 43%, respectively), which is preferable for oils to be used as feedstock for biodiesel.

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Annex 1

Table A1 - Fatty acids profiles found in the isolated species and microalgae from Culture Collection (UTEX)

Fatty acid (%) ^a	<i>Chlorella vulgaris</i> CB1a	<i>Elakatothrix</i> sp. CB2b	<i>Chlorella</i> sp. CB2c	<i>Chlorella vulgaris</i> CB1-Re	<i>Monoraphidium contortum</i> CB3-c	<i>Chlorella</i> sp. P3Fa	<i>Monoraphidium contortum</i> P3Fb	<i>Botryococcus braunii</i> UTEX 2441	<i>Chlorella vulgaris</i> UTEX 2714	<i>Neochloris oleoabundans</i> UTEX 1185	<i>Scenedesmus obliquus</i> UTEX B2630
C12:0	-	-	-	-	-	-	3.43	-	-	-	-
C16:0	22.57	21.61	21.19	23.06	25.57	27.48	22.43	29.72	46.92	21.72	34.92
N.I. ^b	-	-	9.71	-	5.23	-	-	-	-	-	-
C16:1	-	-	-	2.69	-	4.09	-	-	4.01	2.60	3.20
N.I. ^b	8.42	-	-	-	-	4.57	5.40	-	-	-	-
C17:0	-	3.79	-	-	3.88	-	5.88	-	-	-	-
C17:1	-	-	7.37	3.12	-	6.66	-	-	4.08	10.31	1.44
N.I. ^b	-	-	-	6.60	-	-	-	-	-	-	-
C18:0	-	-	-	2.95	-	-	-	18.29	5.32	-	3.28
C18:1n9	36.69	41.67	5.73	35.40	33.28	23.48	31.39	33.37	14.87	9.22	43.68
C18:2n6	9.82	14.10	29.40	4.95	21.75	21.12	11.07	12.74	17.52	36.28	9.55
C18:3n6	22.51	14.59	23.78	21.21	10.19	12.61	13.09	5.85	7.31	19.89	3.96
N.I. ^b	-	4.24	-	-	-	-	4.32	-	-	-	-
C20:3n6	-	-	2.85	2.32	-	-	3.00	-	-	-	-

^a Percentage of fatty acids relative to the total content (weight/weight).

^b Unidentified compound. Absent in 37 MIX patterns.

C12:0 lauric acid; C16:0 palmitic acid; C16:1 palmitoleic acid; C17:0 heptadecanoic acid; C17:1 cis-10-heptadecenoic acid; C18:0 stearic acid; C18:1n9 oleic acid; C18:2n6 linoleic acid; C18:3n6 γ -linolenic acid; C20:3n6 eicosatrienoic acid.

- : Not detected