Revista Brasileira de Farmacognosia
Brazilian Journal of Pharmacognosy
22(4): 854-860, Jul./Aug. 2012

Comparison of extraction and transesterification methods on the determination of the fatty acid contents of three Brazilian seaweed species

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Article

Received 28 Nov 2011
Accepted 12 Jan 2012
Available online 21 Jun 2012

Keywords:
seaweeds
fatty acids
extraction and transesterification methods

ISSN 0102-695X
http://dx.doi.org/10.1590/S0102-695X2012005000088

Abstract: Seaweeds are photosynthetic organisms important to their ecosystem and constitute a source of compounds with several different applications in the pharmaceutical, cosmetic and biotechnology industries, such as triacylglycerols, which can be converted to fatty acid methyl esters that make up biodiesel, an alternative source of fuel applied in economic important areas. This study evaluates the fatty acid profiles and concentrations of three Brazilian seaweed species, *Hypnea musciformis* (Wulfen) J.V. Lamouroux (Rhodophya), *Sargassum cymosum* C. Agardh (Heterokontophyta), and *Ulva lactuca* L. (Chlorophyta), comparing three extraction methods (Bligh & Dyer - B&D; AOAC Official Methods - AOM; and extraction with methanol and ultrasound - EMU) and two transesterification methods (7% BF_3 in methanol - BF_3; and 5% HCl in methanol - HCl). The fatty acid contents of the three species of seaweeds were significantly different when extracted and transesterified by the different methods. Moreover, the best method for one species was not the same for the other species. The best extraction and transesterification methods for *H. musciformis, S. cymosum* and *U. lactuca* were, respectively, AOM-HCl, B&D-BF_3 and B&D-BF_3/B&D-HCl. These results point to a matrix effect and the method used for the analysis of the fatty acid content of different organisms should be selected carefully.

Introduction

Seaweeds are photosynthetic organisms important to their ecosystem since they release O_2 into seawater and contribute to carbon fixation and nutrient cycling. In addition, they provide food and shelter for many animals. These organisms constitute a source of compounds with several different applications that can be used in the food, pharmaceutical and biotechnology industries (Gressler et a., 2009; 2011; Cardozo et al., 2007; 2008).

Among the compounds synthesized by macroalgae, fatty acids are highlighted because of their importance to human health (Cardozo et al., 2007). Thus, omega-3 (n-3) and omega-6 (n-6) are essential nutrient precursors of a group of eicosanoids that regulate developmental and regulatory physiology. Furthermore, triacylglycerols can be converted to the fatty acid methyl esters present in biodiesel (Chisti, 2007).

Biodiesel is an alternative source of fuel produced from plant and animal oils (Marchetti et al., 2007) and the use of algae for its production is of great interest due to their high photosynthetic rate compared to terrestrial plants and the possibility of cultivation in different conditions and in marine waters (Aresta et al., 2005), avoiding the use of arable land.

During the production of biodiesel, two steps are necessary: i. extraction of triacylglycerols from the raw material; and ii. transesterification to produces fatty acid methyl esters (FAME) (Chisti, 2007). Lipids can be extracted using solvent extraction or other techniques such as supercritical fluid extraction and microwave extraction. For the synthesis of FAME, acidic or basic catalysis, as well as other methods, can be used (Carrapiso & Garcia, 2000). Kumari et al. (2011) tested the extraction methods of Bligh & Dyer (1959), Folch (1957) and Cequier-Sánchez (2008) in three species of macroalgae and noted that the macroalgal matrix, the extraction method, and the buffer all influenced the content of fatty acid recovered, which shows that the method used should be selected with caution.

In Brazil, a country with a coastline of over 7000 km, the main raw material for biodiesel production

is soybean oil (80%), followed by bovine fat (10%). There is little information on the fatty acid composition of Brazilian species of marine benthic algae. This knowledge is very relevant, since the profile of fatty acids of raw materials influence biodiesel properties such as the cetane number, iodine value, cold filter plugging point, and oxidation stability (Ramos et al., 2009).

Considering the importance of a knowledge of the fatty acid composition of Brazilian seaweeds, as well as the choice of extraction and transesterification methods to be used, this study evaluates the fatty acid profile of three Brazilian seaweed species, comparing three methods of triacylglycerol extraction and two methods of transesterification to produces FAME.

Materials and Methods

Algal material

The study was conducted with *Hypnea musciformis* (Wulfen) J.V. Lamouroux (Rhodophya), *Sargassum cymosum* C. Agardh (Heterokontophyta) and *Ulva lactuca* L. (Chlorophyta), collected from the intertidal zone in Ubatuba, São Paulo State, Brazil. The specimens were cleaned by removing the epiphytic organisms and washing with seawater. Subsequently samples were frozen under liquid N_2, lyophilized, ground to a powder with liquid nitrogen and stored at -80 °C in the dark. All analyses were performed with three replicates. Voucher specimens were deposited in the SP herbarium under the numbers SP 427377 (*Hypnea musciformis*), SP 427378 (*Sargassum cymosum*), and SP 427379 (*Ulva lactuca*).

Total lipid extraction

Three different methods for lipid extraction were tested:

1. Bligh & Dyer (1959) (B&D): the macerated lyophilized algae (0.33g dry mass) was suspended in PBS and 125 µL of the C13:0 triacylglyceride standard (C13) solution (5 mg mL^{-1} in hexane) and 12.5 ml of chloroform:methanol:water (2:2:1) were added. The mixture was centrifuged and the chloroform phase was transferred to another flask and dried under N_2 (g).

2. AOAC Official Methods (2001) (AOM): to the macerated lyophilized algae (1 g dry mass) was added 0.5 mL of the C13:0 triacylglyceride standard solution (5 mg mL^{-1} in hexane), 50 mg pyrogallic acid and 1 mL of ethanol. This material was suspended in 5 mL of 8.3 M HCl, mixed and maintained in a shaker at 80 °C for 40 min. After cooling, the samples were extracted with ethyl ether (12 mL) and petroleum ether (12 mL). The sample was centrifuged and the ether phase was transferred to another flask and dried under N_2 (g).

3. Extraction with methanol and ultrasound (EMU): the macerated lyophilized algae (0.5 g dry mass) was suspended in 5 mL of methanol and then 125 µL of the triacylglyceride C13:0 standard solution (5 mg mL^{-1} in hexane) was added. The sample was submitted to ultrasound for 15 min and then dried under N_2 (g).

Fatty acid transesterification

Two different methods for fatty acid transesterification were tested:

1. BF_3 in methanol (BF_3): the dry lipid extracts obtained by the B&D and AOM methods were dissolved in 1 mL of BF_3 (7% in methanol) and 0.5 mL of toluene and heated to 100 °C for 45 min. After the reaction, 2.5 mL of water was added at room temperature and the FAME extracted with 1 mL of hexane.

2. HCl in methanol (HCl): to the dry lipid extracts obtained by the B&D, AOM and EMU methods were added 500 µL of 5% HCl in methanol and the mixture was incubated for 2 h at 100 °C. After the reaction, 1.25 mL of water was added at room temperature and the FAME extracted with 1.25 mL of hexane.

Chromatographic analysis

The FAME were analyzed by gas chromatography coupled with mass spectrometry (QP2010, Shimadzu, Kyoto, Japan) with a 30 m fused silica capillary column (HP-5MS with 0.25 µm film, Agilent). A sample (1 µL) was injected at temperature of 220 °C and with split of 1:10. Helium was used as the carrier gas at a flow rate of 1 mL min^{-1} with the following temperature ramp: initial temperature of 60 °C with an increase of 5 °C per min up to 260 °C, which was maintained for 10 min. The reference used for FAMEs was the standard Supelco 37 Component FAME Mix.

Quantification

Quantification of each fatty acid methyl ester (FAME) was based on the standard curve made with Supelco 37 Component FAME Mix. C13 recovery was calculated as $[(Cf \bullet 100) \bullet Ci^{-1}]$, where Cf is the final concentration obtained from the standard curve and Ci is the C13 concentration added to the sample.

Data analysis

Data were subjected to analysis of variance (ANOVA) of one factor, followed by the comparison test of Student-Newman-Keuls, considering a confidence level of 95%.

Results

Comparison of extraction and transesterification methods

There was significant difference in the C13 recovery between the different methods tested only for *Sargassum cymosum*, and it was higher in the B&D-BF_3 method. There was no significant difference in the recovery of C13 among species (Figure 1).

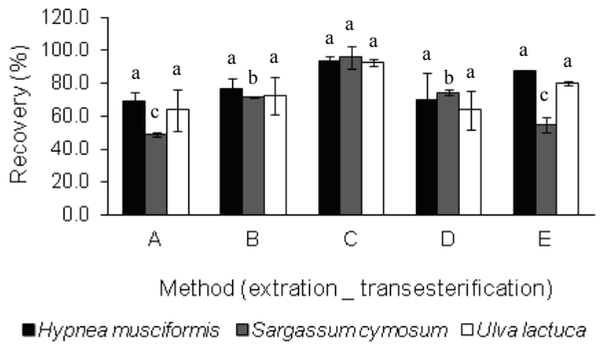

Figure 1. Recovery of the C13:0 triacylglyceride internal standard in extraction and transesterification by the different methods with *Hypnea musciformis, Sargassum cymosum* and *Ulva lactuca* collected from Ubatuba, São Paulo, Brazil. The extraction_transesterification methods are: A. EMU-HCl; B. B&D-HCl; C. B&D-BF_3; D. AOM-HCl; E. AOM-BF_3. Each data point is the mean of three replicates and the bars are the standard deviation For each species, distinct letters indicate significant differences between the methods tested according to one-way ANOVA and to the comparison test of Student-Newman-Keuls (p<0.05).

H. musciformis showed a higher saturated fatty acid content in the AOM-BF_3 and AOM-HCl methods (Table 1 A), which is a consequence of the highest concentrations of palmitic and myristic acid methyl esters when extracted and transesterified by these methods (Figure 2 A and B). The contents of monounsaturated fatty acid and of palmitoleic acid methyl ester also were higher in the AOM-HCl method (Table 1 A and Figure 2 B), while the content of oleic acid methyl ester was higher in the B&D-BF_3, AOM-HCl and AOM- BF_3 methods (Figure 2 B). The contents of polyunsaturated fatty acid, represented by arachidonic acid methyl ester, and of total fatty acid were higher in the AOM-HCl, and AOM- BF_3 methods (Table 1 A and Figure 2 B).

S. cymosum showed higher contents of saturated fatty acid and of myristic and palmitic acid methyl esters in the B&D-BF_3 method (Table 1 B and Figure 3 A and B). The contents of monounsaturated fatty acid and of oleic acid methyl ester were also higher in the B&D-BF_3 method (Table 1 B and Figure 3 B), while the content of palmitoleic acid methyl ester was higher in the B&D-BF_3 and B&D-HCl methods (Figure 3 B). The contents of polyunsaturated and total fatty acid and of aracdonic and linoleic acid methyl esters were higher in the B&D-BF_3 method (Table 1 B and Figure 3 B).

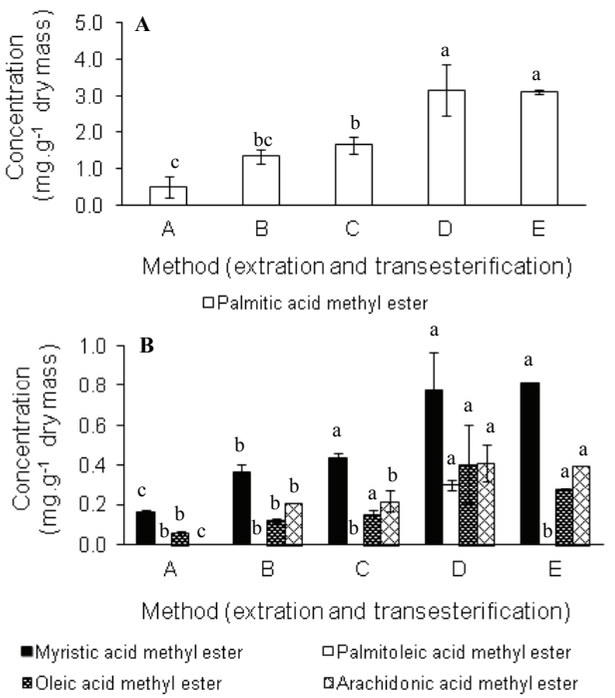

Figure 2. Fatty acid methyl ester concentrations (mg g^{-1} dry weight) of *Hypnea musciformis* collected from Ubatuba, São Paulo, Brazil. The extraction- transesterification methods are: A. EMU-HCl; B. B&D-HCl; C. B&D-BF_3; D. AOM-HCl; E. AOM-BF_3. Each data point is the mean of three replicates and the bars are the standard deviation. For each fatty acid, distinct letters indicate significant differences between the methods tested according to one-way ANOVA and to the comparison test of Student-Newman-Keuls (p<0.05).

There was no significant difference in saturated fatty acid and palmitic acid methyl ester contents among the different methods tested for *U. lactuca* (Table 1 C and Figure 4 A). The same was observed for the monounsaturated fatty acid and oleic acid methyl ester (Table 1 C and Figure 4 B). However, the content of palmitoleic acid methyl ester was higher when extracted with the B&D-BF_3 and B&D-HCl methods (Figure 4 B). The content of polyunsaturated acid was higher in the B&D-BF_3, B&D-HCl and AOM-HCl methods (Table 1 C). The content of linoleic acid methyl ester was higher when extracted with the B&D-BF_3 and B&D-HCl methods and the content of linolenic acid methyl ester was lower when extracted with the AOM- BF_3 method (Figure 4 B).

Table 1. Fatty acid concentrations (mg g^{-1} dry weight) obtained by different extraction and transesterification methods for *Hypnea musciformis* (A), *Sargassum cymosum* (B) and *Ulva lactuca* (C) collected from Ubatuba, São Paulo, Brazil.

	Fatty acid	EMU	B&D-HCl	B&D-BF_3	AOM-HCl	AOM-BF_3
A	Saturated	0.69±0.36^{b}	1.71±0.16^{b}	1.63±0.44^{b}	3.93±0.73^{a}	3.94±0.05^{a}
	Monounsaturated	0.06±0.01^{b}	0.12±0.01^{b}	0.15±0.02^{b}	0.80±0.09^{a}	0.28±0.01^{b}
	Polyunsaturated	n.d.^{c}	0.21±0.01^{b}	0.22±0.04^{b}	0.41±0.08^{a}	0.40±0.01^{a}
	Total	0.72±0.38^{b}	2.04±0.37 ^{b}	2.08±0.27^{b}	4.95±1.02^{a}	4.62±0.06^{a}
B	Saturated	0.82±0.27^{c}	2.32±1.00^{bc}	3.85±0.24^{a}	2.30±0.17^{b}	1.50±0.37^{bc}
	Monounsaturated	0.20±0.10^{c}	0.87±0.18^{b}	1.19±0.06^{a}	0.61±0.05^{b}	0.33±0.15^{c}
	Polyunsaturated	n.d.^{d}	0.61±0.10^{b}	0.83±0.03^{a}	0.42±0.02^{c}	0.11±0.08^{d}
	Total	1.01±0.37^{d}	3.80±1.26^{b}	5.87±0.32^{a}	3.33±0.24^{bc}	1.94±0.59^{cd}
C	Saturated	1.85±0.42^{a}	2.13±0.45^{a}	2.49±0.19^{a}	2.01±0.31^{a}	2.28±0.51^{a}
	Monounsaturated	0.52±0.11^{a}	0.72±0.15^{a}	0.79±0.01^{a}	0.52±0.08^{a}	0.46±0.07^{a}
	Polyunsaturated	0.68±0.15^{b}	0.91±0.12^{ab}	1.09±0.01^{a}	0.83±0.09^{ab}	0.27±0.03^{c}
	Total	3.05±0.68^{a}	3.76±0.71^{a}	4.37±0.19^{a}	3.36±0.47^{a}	3.02±0.61^{a}

Each value is the mean±SD of three replicates. For each fatty acid (saturated, monounsaturated, polyunsaturated and total) distinct letters indicate significant differences between the different methods tested according to the one-way ANOVA and to the comparison test of Student-Newman-Keuls ($p< 0.05$).

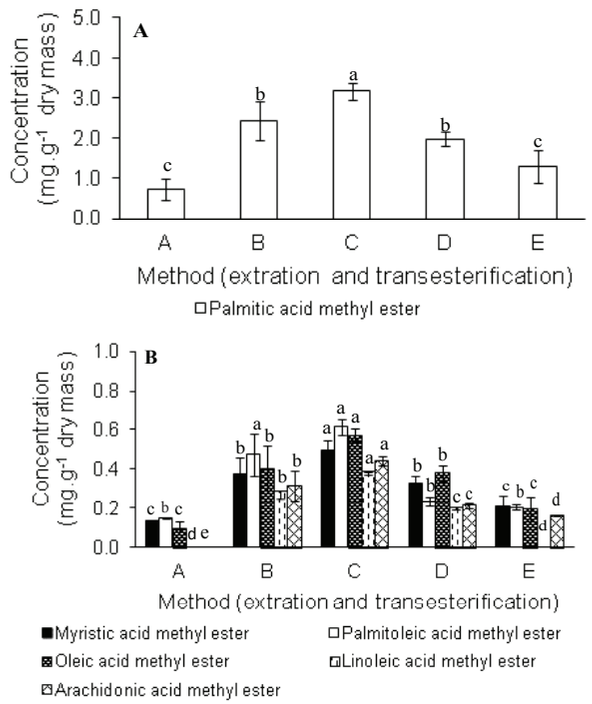

Figure 3. Fatty acid methyl ester concentrations (mg g^{-1} dry weight) of *Sargassum cymosum* collected from Ubatuba, São Paulo, Brazil. The extraction- transesterification methods are: A. EMU-HCl; B. B&D-HCl; C. B&D-BF_3; D. AOM-HCl; E. AOM-BF_3. Each data point is the mean of three replicates and the bars are the standard deviation. For each fatty acid, distinct letters indicate significant differences between the methods tested according to one-way ANOVA and to the comparison test of Student-Newman-Keuls ($p<0.05$).

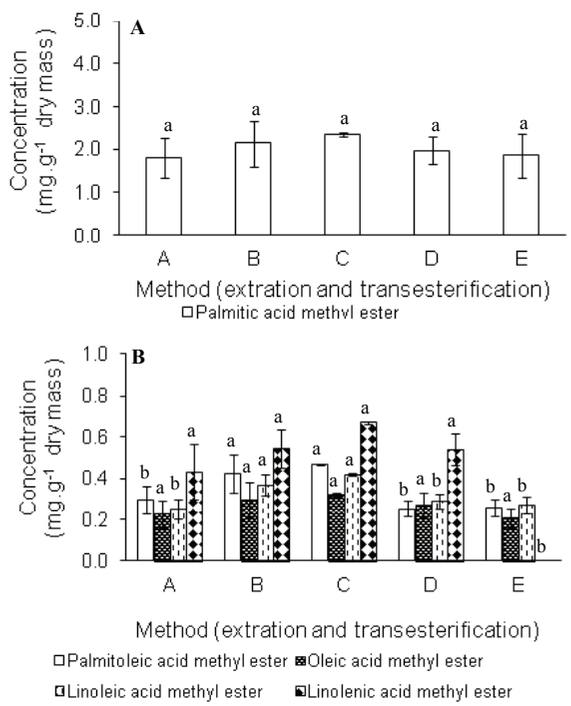

Figure 4. Fatty acid methyl ester concentrations (mg g^{-1} dry weight) of *Ulva lactuca* collected from Ubatuba, São Paulo, Brazil. The extraction- transesterification methods are: A. EMU-HCl; B. B&D-HCl; C. B&D-BF_3; D. AOM-HCl; E. AOM-BF_3. Each data point is the mean of three replicates and the bars are the standard deviation. For each fatty acid, distinct letters indicate significant differences between the methods tested according to one-way ANOVA and to the comparison test of Student-Newman-Keuls ($p<0.05$).

Comparison between the species studied

The comparison between the species studied was made by choosing two extraction methods. For *Hypnea musciformis*, the best extraction method, which yielded the highest value of saturated, unsaturated and polyunsaturated fatty acids, was the AOM-HCl method. For *Sargassum cymosum* it was B&D-BF_3 and for *Ulva lactuca* the B&D-BF_3 and B&D-HCl methods. Thus, comparison of the fatty acid profile of these species was made considering the AOM-HCl and B&D-BF_3 methods.

In both methods tested, the three species showed higher concentrations of saturated fatty acids and palmitic acid was the major fatty acid (Figure 5 A and B). However, when comparing species, and when the extraction and transesterification were made using the B&D-BF_3 method, *S. cymosum* and *H. musciformis* showed the highest and lowest contents of this fatty acid, respectively. *S. cymosum* also showed higher concentrations of palmitoleic, oleic and arachidonic acid methyl esters when the B&D-BF_3 method was used (Figure 5 A).

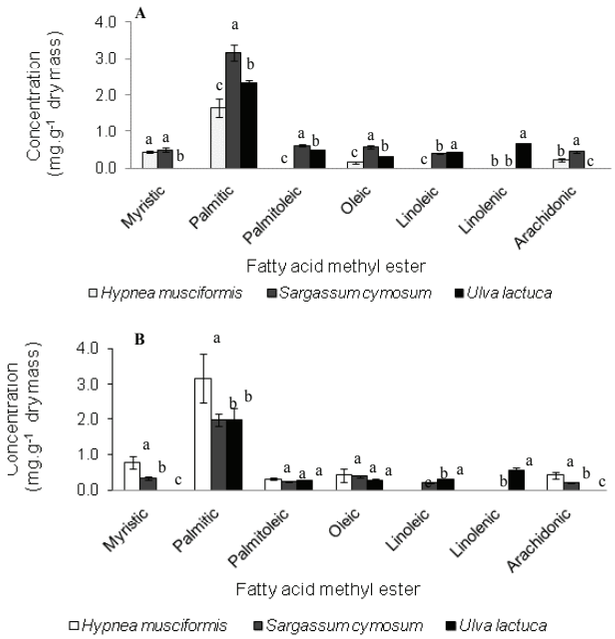

Figure 5. Fatty acid methyl ester concentrations (mg g^{-1} dry weight) of *Hypnea musciformis, Sargassum cymosum* and *Ulva lactuca* collected from Ubatuba, São Paulo, Brazil. **A**. B&D-BF_3 method; **B**. AOM-HCl method. Each data point is the mean of three replicates and the bars are the standard deviation. For each fatty acid, distinct letters indicate significant differences between species according to one-way ANOVA and to the comparison test of Student-Newman-Keuls (p<0.05).

However, when the AOM-HCl method was used, *H. musciformis* showed higher concentrations of palmitic, myristic and arachidonic acid methyl esters than the other species and significant differences between the species for palmitoleic and oleic acid methyl esters were not observed (Figure 5 B). Moreover, this species did not yield palmitoleic acid methyl ester when B&D-BF_3 method was used (Figure 5 A) and linoleic and linolenic acid methyl esters were not detected, independent of the method used.

Ulva lactuca was the only species in which linolenic acid methyl ester was detected.

Discussion

The present study showed significant differences in the observed fatty acid contents of three species of seaweeds, *Hypnea musciformis, Sargassum cymosum* and *Ulva lactuca*, when extracted and transesterified by different methods. Moreover, the best method for one species was not necessarily the best for the other species. The best extraction and transesterification methods for *H. musciformis, S. cymosum* and *U. lactuca* were, respectively, AOM-HCl, B&D-BF_3 and B&D-BF_3/B&D-HCl. Similar results were observed by Kumari et al. (2011), where the content of total lipids and fatty acids of *Ulva fasciata* Delile, *Gracilaria corticata* (J. Agardh) J. Agardh and *Sargassum tenerrimum* J. Agardh varied with the extraction method tested and the best method differed for each species. These results may be due to the matrix effect, since these species differ in their content of carbohydrates and proteins, which can interact with triacylglycerides.

When comparing the fatty acid contents of the three species of seaweed studied using the AOM-HCl method, *H. musciformis* showed higher concentrations of palmitic, myristic and arachidonic acid methyl esters than the other species. Significant differences between the species for palmitoleic and oleic acid methyl esters were not observed. The same was not the case for the B&D-BF_3 method. These results highlight the matrix effect and show that different results are generated as a consequence of the method used, which can produce false results. If only the results generated by the AOM-HCl method were considered, *H. musciformis* would be the species that produces more palmitic acid methyl ester. However, if only the B&D-BF_3 method were considered, *S. cymosum* would be the species that produces more of this fatty acid. Thus, care must be taken in studies that compare the content of fatty acids from different algal species since differences in the results can be due to the matrix effect rather than to differences in intrinsic concentrations in the sample.

The three species studied showed higher concentrations of saturated fatty acids, and palmitic acid was the major fatty acid, which is in agreement

with the findings for other red algae (Guaratini et al., 2007; Gressler et al., 2010; Gressler et al., 2011), green algae (Ortiz et al., 2006; Yaich et al., 2011) and brown algae (Khotimchenko, 1991; Noviendri et al., 2011).

S. cymosum had a higher content of monounsaturated than polyunsaturated fatty acids. Similar results were found for *Ochtodes secundiramea* (Montagne) M.A. Howe (Gressler et al., 2011) and *U. lactuca* (Yaich et al., 2011). However, the opposite was observed for *U. lactuca* (present study) and for species of red algae such as *Plocamium brasiliense* (Greville) M.A. Howe & W.R. Taylor (Gressler et al., 2011), *Gracilaria domingensis* (Kützing) Sonder ex Dickie, G. birdiae Plastino & E.C. Oliveira, *Laurencia dendroidea* J. Agardh (as L. filiformis (C.Agardh) Montagne), and *Laurencia* sp. (as *L. intricata* J.V. Lamouroux) (Gressler et al., 2010), brown algae such as *Sargassum aquifolium* (Turner) C. Agardh (as S. binderi Sonder ex J. Agardh) and *S. ilicifolium* (Turner) C.Agardh (as *S. duplicatum* (J. Agardh) J. Agardh) (Noviendri et al., 2011) and green algae such as *Ulva pertusa* Kjellman (Floreto et al., 1993). It is important to note that the fatty acid profile of many organisms can vary according to environmental changes. For example, Floreto et al. (1993) observed an increase in the composition of saturated fatty acids and a decrease in unsaturated fatty acids of *U. pertusa* collected from mid-spring to early summer (March-June).

The results of the fatty acid profiles suggest that *S. cymosum* is the most suitable species to be used for biodiesel production, since biodiesel with a higher monounsaturated fatty acid content has a greater oxidative stability and does not precipitate when subjected to lower temperatures (Serdari et al. 1999). However, further studies of the growth of this species, as well as the yield, are needed to evaluate the economic viability of production of the compounds of interest.

Acknowledgements

This work was supported by FAPESP, CAPES, CNPq, Ministério da Saúde, Ministério de Ciência e Tecnologia, NAP-USP de Biodiversidade Marinha and CNPq-INCT-Redoxoma.

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