Article

Improvement of the Extraction Process for High Commercial Value Pigments from *Desmodesmus* sp. Microalgae

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Considering the high interest and commercial value of pigments from microalgae, this study aimed to stablish the best lutein and β-carotene extraction method for *Desmodesmus* sp., a microalgae with high biomass production. The evaluation was done considering the best solvent or solvent mixture, number of extractions, pre-treatment of dry biomass with acetone, saponification in methanol and ethanol and stability evaluation of the obtained extract. Among achieved results, the best solvent extractor was a hexane:ethanol mixture in a 1:1 (v/v) proportion. In order to obtain maximum yield, the pattern stablished includes six extractions performed for 40 minutes. Stability tests presented that xanthophylls and carotenes pigments were more stable in methanol and that xanthophylls and carotenes pigments were less negatively affected than chlorophylls in the methanolic extract. Chromatographic identification and quantification of pigments from *Desmodesmus* sp. microalgae revealed that its major constituents are *trans*-zeaxanthin, chlorophyll b, β-carotene and *trans*-lutein.

Keywords: microalgae, pigments, extraction, saponification, HPLC-DAD

Introduction

Microalgae are single cell microorganisms capable of transforming sunlight into chemical energy by photosynthesis. Many bioactive compounds of commercial, medicinal and scientific interest are obtained from microalgae, such as pigments, antioxidants, fatty acids, enzymes, polymers, pharmaceuticals[1] and, most recently, fatty raw material for biodiesel production.[2,3]

Each microalgae species presents a specific class of pigments. Carotenoids and chlorophylls are the most common. Among chlorophylls, chlorophyll *a* is the major photosynthetic pigment in phytoplankton and it has been commonly used as an indicator of biomass production.

Carotenoids are classified as carotenes or hydrocarbon carotenoids made of carbon and hydrogen only,[1] such as lycopene and β-carotene, and xanthophylls or oxygenated carotenoids, which may contain epoxides, carbonylic, hydroxylic, methoxy and carboxylic acids groups. Examples of xantophylls are lutein, violaxanthin, canthaxanthin and zeaxanthin.[4]

Many carotenoids and xanthophylls have high commercial value, so there is a great interest in obtaining such substances directly from living organisms. Microalgae can provide pigments in an easy way, but is necessary finding the species that have the expected pigments and evaluate the best extraction method for these substances, in order to achieve high yields, using low-cost and toxicity solvents.

Extraction efficiency will depend on the solvent ability to solubilize pigments without modifying its structures.[5] Therefore, solvent selection is an important step in pigments extraction, presenting high efficiency and selectivity with the compounds being extracted.

Most microalgae carotenoids extraction studies involve a single solvent extraction. However, binary extraction system studies can be found in literature, as in Aburai *et al.*,[1] which used dichloromethane (DCM) and methanol to perform pigments extraction in four microalgae species. Solovchenko *et al.*[6] extracted microalgae *Desmodesmus* sp. pigments using chloroform and methanol. The use of

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ternary systems,[7] quaternary systems[8,9] and supercritical fluid extraction[10] are also found.

Other determinant factors for pigments extraction yield are: (*i*) time of contact between sample and solvent and (*ii*) the technique (mechanical or non-mechanical) applied in order to break cell wall.[11] Currently, some works show the use of ultrasound-assisted extraction (UEA)[12,13] and of microwave and vacuum microwave-assisted extraction (MAE and VMAE).[14,15]

Aiming to simplify pigments separation and to obtain free pigments, some methods suggest pigments saponification. Most of the methods use potassium hydroxide (KOH) alkaline saponification in ethanol or methanol.[1,9,16-18] However, this treatment leads to degradation and loss of information about the native compounds. In turn, the non-saponified extracts present more complex chromatograms and more pigment information, such as esterified xanthophyll.

Considering the various possibilities to accomplish microalgae pigments extraction and questioning which procedure would be more useful and suitable, the aim of this study was to evaluate the yield of β-carotene and lutein extraction from *Desmodesmus* sp. microalgae. For this, it were evaluated the following experimental parameters: (*i*) application of ultrasound technique using different proportions of the extractor solvents; (*ii*) evaluation of the number of extractions necessary to achieve the best extract yield; (*iii*) performing pre-treatment of dry biomass with acetone followed by solvent extraction; (*iv*) application of ethanolic and methanolic KOH saponification, both of them with or without pre-treatment of dry biomass with acetone and followed by solvent extraction; (*v*) evaluating extract stability in methanol, DCM, hexane and methanol:DCM (1:1, v/v); and (*vi*) evaluating of qualitative and quantitative pigments profile regarding to different extraction methods. Defining the best extraction system will be an important step, ascribing values to microalgae productive chain.

Experimental

Materials

Sodium sulfate, potassium hydroxide, hexane, ethanol and acetone were acquired at Neon Analytical Reagents (São Paulo, SP, Brazil). High performance liquid chromatography (HPLC) solvents (methanol, acetonitrile and dichloromethane) were obtained from J. T. Baker (Tokyo, Japan). Deionized water was obtained at Elix water purification system (Millipore, Billerica, MA, USA). The chlorophyll *a*, *trans*-lutein and *trans*-β-carotene standards were acquired from Sigma-Aldrich (St. Louis, MO, USA).

Extracts were filtered in a polytetrafluoroethylene (PTFE) sterile membrane (–0.22 μm × 25 mm). Analytical separation was performed in a YMC30 column (250 × 4.6 mm, 5 μm) by Waters (Milford, MA, USA).

Instrumentation

HPLC analyses were conducted in Agilent equipment, 1220 Infinity LC (Agilent Technologies, Santa Clara, CA, USA), with column temperature control, binary bomb and diode array detection (DAD). It was used a rotatory evaporator model 344B2, from Quimis (Diadema, SP, Brazil) with vacuum pump, a Unique ultrasonic bath of 200 W model USC-5000 (Indaiatuba, SP, Brazil) and an incubator with heating and agitation system model 430, from Nova Ética (Vargem Grande do Sul, SP, Brazil).

Microalgae

The freshwater microalgae *Desmodesmus* sp. was isolated, identified, cultivated and supplied by Laboratory of Algae Cultivation (LCA) from Federal University of Santa Catarina (Florianópolis, SC, Brazil). The culture media for cultivating microalgae was WC medium (Wright's cryptophyte).[19] The cultures were grown in glass fiber cylinders containing 100 L of the respective culture media. Fixtures with fluorescent lamps were used and photosynthetically active radiation (PAR) was measured. Lighting was 150 μmol m^{-2} s^{-1} for a complete photoperiod. Pressurized atmospheric air continuously injected at a flow rate of 10 L min^{-1} into each cylinder was enriched with 1% CO_2 (100% purity). The temperature of the cultivation room was kept at 25 ± 2 °C using air conditioners. Biomass was separated by the addition of a polyacrylamide cationic flocculant Flopam FO 4400 SH in a 15 mg L^{-1} culture dosage, produced by SNF Floerger (Andrézieux-Bouthéon, France). After flocculation and sedimentation, biomass was filtered through a screen and dried in an oven at 50 °C for 24 h. The final residual water was 5%.

Pigments extraction using solvents in ultrasound

Pigments extraction was performed by ultrasound technique. Extractor solvent and extraction time were evaluated. Solvents were chosen based on their less toxicity and cost, on their higher ability to extract pigments, and their capacity to be recovered from distillation. Thus, the solvents tested were: (*i*) hexane; (*ii*) acetone; (*iii*) hexane:ethanol:acetone (10:6:7, v/v/v); (*iv*) hexane:ethanol in 1:1; 1:2, 1:3; 2:1, 3:1 and 4:1 (v/v) proportion; and (*v*) ethanol.

Extraction method was based on Inbaraj *et al.*[8] Approximately 500 mg of *Desmodesmus* sp. was weighted in an erlenmeyer flask and 15 mL of the extractor solvent to be tested were added. This mixture was left in ultrasonic bath for 40 min at room temperature (23 ± 2 °C). After, 7.5 mL of Na_2SO_4 10% aqueous solution was added, shaken by hand for 30 s and left to rest until phases were separated. The upper phase, rich in pigments, was collected and saved in a round-bottom flask.

The residue inside the erlenmeyer was again extracted in ultrasound with 10 mL of extractor solvent for 40 min and left to rest until phase separation. The upper phase was collected and added to the first extraction, which was in a round-bottom flask. This procedure was repeated for four times, totaling five extractions. The combined upper phases, which were rich in pigments, were evaporated in a rotatory evaporator, dissolved in 2.5 mL of a methanol:dichloromethane mixture (1:1, v/v) and filtered in a PTFE sterile membrane for HPLC analysis. Extractions were made in triplicate and HPLC-DAD analysis was made in duplicate. After selecting the best extractor solvent, extraction time was tested and varied from 120 to 320 min.

Pigments extraction with and without previous acetone treatment

The following procedures were evaluated: (*i*) biomass extraction using only hexane:ethanol (1:1, v/v), and (*ii*) biomass extraction using hexane:ethanol (1:1, v/v) after previous acetone treatment of dry biomass.

For experiments involving the previous acetone treatment, about 500 mg of dry *Desmodesmus* sp. sample was weighted in a 250 mL Erlenmeyer and 5 mL of acetone were added. The sample was left in ultrasonic bath at room temperature (23 ± 2 °C) for 30 min, 1 and 2 h. At the end of the pre-treatment process, acetone was recovered through simple distillation and the obtained extract was submitted to pigments extraction as described above, with hexane:ethanol (1:1, v/v) solution. This procedure was repeated six times, totaling 240 min.

Pigments saponification

Two experiments were evaluated for saponification process: the first one was alkaline saponification in (*i*) methanol and (*ii*) ethanol, followed by free pigments extraction with hexane:ethanol (1:1, v/v), repeated for six times of 40 min; and the second experiment tested the influence of previous pre-treatment of the biomass with acetone before alkaline saponification in (*i*) methanol and (*ii*) ethanol followed by free pigments extraction

with hexane:ethanol (1:1, v/v), repeated for six times of 40 min. Acetone treatment was performed as described in Pigments extraction with and without previous acetone treatment section.

For the saponification reaction, 8% (v/v) methanolic and ethanolic KOH solutions were tested. Five milliliter of alkaline alcoholic solution was added to both dry and acetone pre-treated biomass. The resulting mixture was stirred in an incubator at 200 rpm at 60 °C for one hour. After saponification, the extract inside the Erlenmeyer was submitted to pigment extraction as described above, using a hexane:ethanol (1:1, v/v) solution, for 40 min and repeated for six times, totaling 240 min.

Extract stability study

About 2 g of dry biomass was weighted and extracted with hexane:ethanol (1:1, v/v), as described above. Extract was dissolved in 10 mL of DCM and four aliquots (2.5 mL) were transferred to four previously weighted round-bottom flasks. These four solutions were concentrated in rotary evaporator and the extract obtained in each flask was dissolved in 2.5 mL of the test solvent (DCM, methanol, hexane or DCM:methanol (1:1, v/v). This procedure led to four new extracts with final concentrations of 22.8 mg mL^{-1} for extracts dissolved in methanol and DCM, 26 mg mL^{-1} for DCM:methanol solution and 26.4 mg mL^{-1} for hexane solution. Each solution was filtered in a sterile PTFE membrane and stored in an amber vial for HPLC-DAD chromatographic analysis. Each sample was analyzed every four hour totalizing eight points in 32 h. The autosampler in HPLC-DAD system was maintained at temperature of 23 ± 2.0 °C and in absence of light.

Chromatographic conditions

Chromatographic analysis method was based on Inbaraj *et al.*[8] HPCL-DAD mobile phase was (A) methanol:acetonitrile:water (84:14:2, v/v/v) and (B) dichloromethane, using the gradient conditions: 100% A and 0% B for 14 min; 95% A for 25 min; 75% A for 30 min; 74% A for 35 min; 45% A for 50 min; and returning to 100% A at 55 min, in 60 min of analysis. Column oven was kept at 23 ± 2 °C. Extract injection volume was 20 µL. Mobile phase was pumped in 1 ml min^{-1} flow. DAD was operated from 250 to 700 nm and detection wavelength at 450 nm. Comparison between retention times and peaks absorption spectrum were used for pigments identification. *cis* and *trans* isomers of pigments were identified based on previous studies.[9,20-22]

Pigments quantification

Pigments were quantified by HPLC using calibration curve from chlorophyll *a*, *trans*-lutein and *trans*-β-carotene standards, with six different concentrations (1, 5, 10, 15, 30 and 60 μg mL^{-1}). The curve was prepared plotting standard concentration *vs.* area. Linear regression was calculated at Microsoft Excel (Microsoft Corp., Redmond, WA, USA), leading to a correlation coefficient (r) larger than 0.9995.

Analysis of variance (ANOVA) was carried out to determine if at least one pigment extraction conditions were different from the others. Then, the *t*-test was employed to identify differences among mean values. The ANOVA were performed using Microsoft Excel (Microsoft Corp., Redmond, WA, USA). Hypotheses were tested with a level of significance of $p < 0.05$.

Determining limits of quantification (LOQ) and detection (LOD)

Three *trans*-β-carotene solutions standards were prepared (1, 5 and 10 μg mL^{-1}) and analyzed by HPLC in triplicate, resulting in three calibration curves plotting concentration *vs.* area. Limit of quantification (LOQ) was calculated based on the equation 1:

$$LOQ = 10\left(\frac{\sigma}{s}\right) \qquad (1)$$

and limit of detection (LOD) was calculated based on equation 2:

$$LOD = 3.3\left(\frac{\sigma}{s}\right) \qquad (2)$$

where in both equations s is the interception standard deviation and σ is the straight line inclination average, from which values of 0.066 and 0.022 μg mL^{-1} were obtained, respectively.

Results and Discussion

Effect of solvent type

Figure 1 shows lutein and β-carotene extraction yields from *Desmodesmus* sp. biomass in different extractor solvent mixtures. The analysis of variance revealed that lutein and β-carotene extractions methodologies were significantly different. The best condition for these pigments extraction was the hexane:ethanol (1:1,v/v) mixture, which led to 324.65 ± 11.77 μg g^{-1} to lutein and 158.44 ± 8.51 μg g^{-1} to β-carotene content. It is important to highlight that the second best condition used

for lutein extraction was the hexane:ethanol (4:1, v/v) mixture and for β-carotene was hexane:ethanol:acetone (10:6:7, v/v/v), which presented 305.83 ± 4.92 μg g^{-1} and 125.47 ± 6.7 μg g^{-1} yield, respectively. For lutein extraction, the *t*-test showed no significant difference between the highest extraction yields, however for β-carotene it was observed distinction among the mean values.
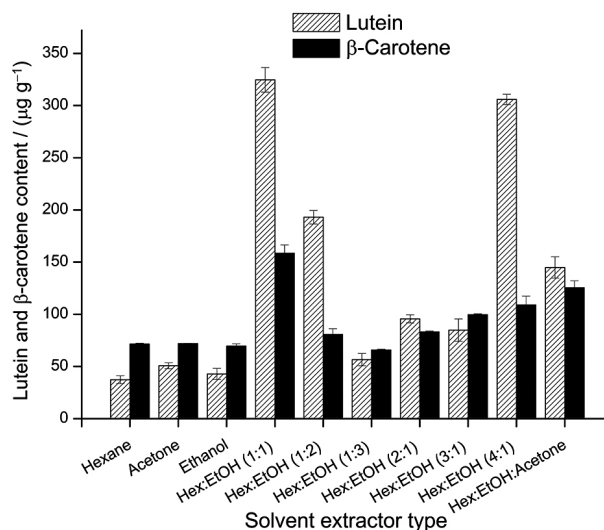
Figure 1. Lutein and β-carotene content in relation of extractor solvent type. Hex: Hexane; EtOH: ethanol.

By increasing ethanol content in hexane:ethanol mixture, a decrement in β-carotene and lutein extract yield for *Desmodesmus* sp. microalgae was observed. This decrement may be explained due to the greater mixture polarity in the presence of ethanol. Furthermore, ethanol is miscible with the sodium sulfate solution water, thus part of the extract may have been retained in aqueous phase. Hexane:ethanol (1:4, v/v) mixture was tested, but when sodium sulfate solution was added, it was not generated a two-phase system, leading to an undefined phase separation.

An increase in hexane content in the hexane:ethanol mixture was tested and the proportion 4:1 (v/v) provided the best result, even though it was not enough to extract all pigments. Single solvent extractions (hexane, acetone or ethanol) offer similar efficiency in the extract yield for both lutein and β-carotene. This can explain low yields obtained in the industrial process, as described by Dey and Rathod.[18] In addition to that, Wang and Liu[23] described that the acetone:petroleum ether mixture (1:1, v/v) presented better results in pigment extraction of colza biomass when compared to single solvent extractions.

The chromatogram of pigments extracted from *Desmodesmus* sp. microalgae biomass using hexane:ethanol (1:1, v/v) is shown in Figure 2a. Mobile phase gradient developed by Hibaraj *et al.*[8] and used in this study for

Desmodesmus sp. microalgae allowed the separation of 23 carotenoids in 50 min. The pigments were identified considering the ultraviolet-visible spectra characteristics, chromatographic behavior, co-elution with standards and comparison of data from the literature. *trans*-zeaxanthin, chlorophyll *b*, carotenes and *trans*-lutein are the major pigments, representing 23.9, 21.7, 20 and 11.4% of total pigments, respectively. Violaxanthin, antheraxanthin, neoxanthin were identified as demonstrated Solovchenko *et al.*[6] and it was observed some other minor peaks (Table 1). Hexane:ethanol (1:1, v/v) extraction led to 2842.7 µg g^{-1} yield of total pigments from dry microalgae biomass.

Chlorophyll and its derivatives present two major absorption bands in the visible region due to π electrons

delocalization of the porphyrin, which are a band in the red region (Q band) and another in the blue region (Soret or B band). The absorption bands values in the red and blue region for chlorophyll *a* are 664 and 432 nm, respectively. For chlorophyll *b*, these values are 652 and 468 nm, respectively. Chlorophyll *b* has two types of maximum absorption: from 642 to 652 nm at red region and a second absorption of 465 to 470 nm at blue region.[20,24]

Pheophytin is a chlorophyll *a* or *b* derivative without the central magnesium atom. Pheophytin *a* presents maximum absorption at 409 and 665.9 nm, whereas in pheophytin *b*, the values are 434 and 653 nm. *Desmodesmus* sp. extract obtained by using hexane:ethanol (1:1, v/v) presented maximum absorption in 666 and 412 nm for pheophytin *a* (Table 1). The absorbance intensity rate for Soret and Qy
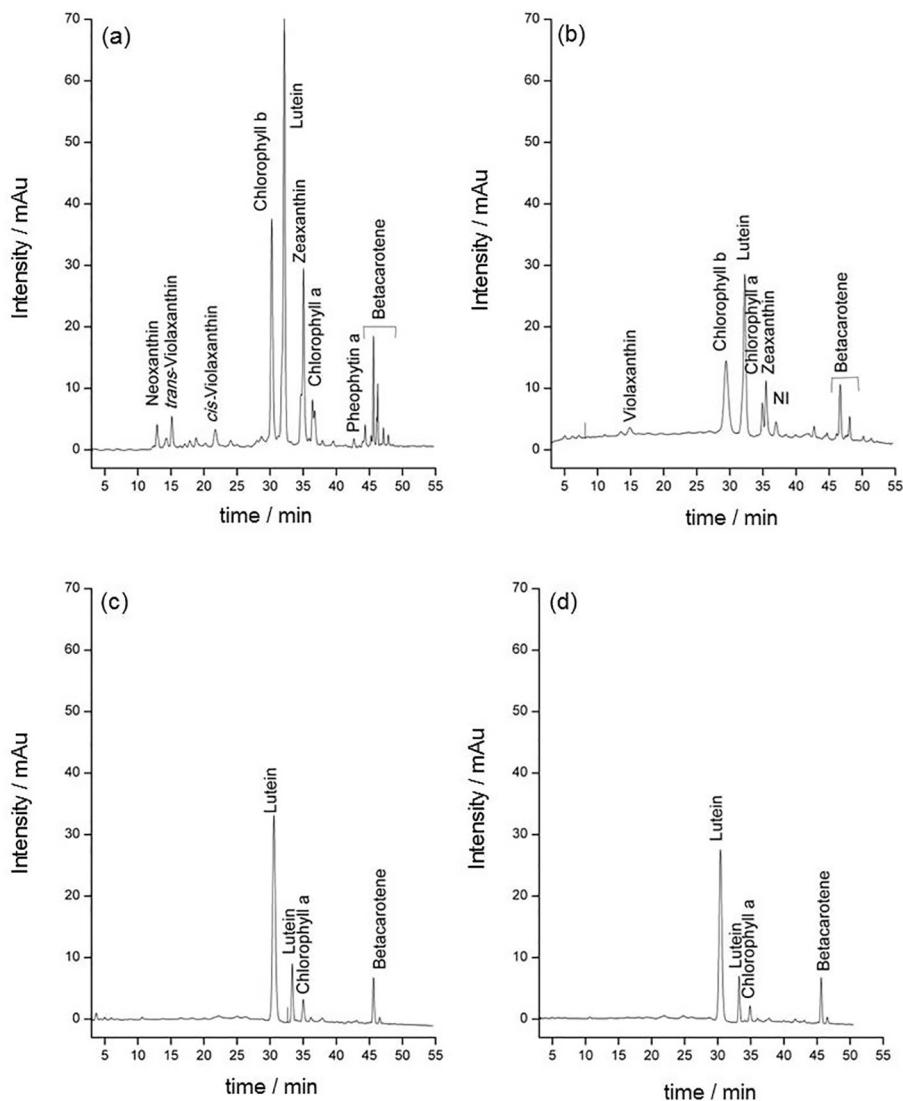
Figure 2. Chromatogram obtained by high performance liquid chromatography with diode array detection (HPLC-DAD) of dry biomass from *Desmodesmus* sp. microalgae (a) extracted with hexane:ethanol (1:1, v/v); (b) treated with acetone for 1 h followed by hexane:ethanol (1:1, v/v) extraction; (c) treated with acetone for 1 h followed by methanolic saponification and subsequent hexane:ethanol (1:1, v/v) extraction; (d) treated with acetone for 1 h followed by ethanolic saponification and subsequent hexane:ethanol (1:1, v/v) extraction.

Table 1. Identification of pigments obtained by high performance liquid chromatography with diode array detection (HPLC-DAD) of dry biomass from *Desmodesmus* sp. microalgae by hexane:ethanol (1:1, v/v) extraction

Pigment	λ / nm[a,9]	λ / nm[b]	Concentration[c] / (μg g^{-1})
trans-neoxanthin	416, 444, 472	414, 442, 470[9]	77.9 ± 1.6
Neochrome	400, 422, 442	398, 422, 440[8]	9.6 ± 0.1
trans-violaxanthin	416, 440, 468	416, 440, 468[9]	101.7 ± 1.1
9- or 9'-*cis*-neoxanthin	416, 438, 466	412, 436, 464[9]	18.2 ± 1.5
cis-neoxanthin	406, 428, 456	404, 428, 458[8]	22.2 ± 1.3
cis-violaxanhtin	420, 444, 468	416, 440, 468[9]	98.7 ± 3.6
Anteraxanthin	420, 444, 474	418, 442, 472[9]	19.3 ± 0.6
Chlorophyll b or b'	468, 652	465, 652[22]	615.8 ± 6.2
13- or 13'-*cis*-lutein	418, 468	415, 440, 464[8]	2.1 ± 1.4
cis-lutein	424, 446, 472	422, 446, 470[8]	44.8 ± 1.1
trans-lutein	424, 446, 474	423, 446, 470[8]	324.7 ± 11.7
cis-zeaxanthin	422, 448, 472	422, 446, 474[26]	142.5 ± 4.1
trans-zeaxanthin	426, 452, 478	427, 452, 476[8]	679.4 ± 3.2
Chlorophyll a or a'	432, 664	430, 662[22]	109.1 ± 0.3
Pheophytin a or a'	412, 666	409, 665[22]	7.6 ± 1.1
9- or 9'-*cis*-α-carotene	422, 454, 478	422, 452, 476[8]	70.0 ± 0.5
trans-α-carotene	428, 450, 478	426, 449, 476[8]	114.9 ± 3.0
13- or 13'-*cis*-β-carotene	428, 448, 476	422, 448, 474[26]	80.9 ± 2.1
trans-β-carotene	428, 454, 480	430, 458, 482[8]	158.4 ± 8.5
9- or 9'-*cis*-β-carotene	426, 452, 478	428, 452, 476[8]	74.4 ± 1.8
9- or 9'-*cis*-β-carotene	426, 452, 478	428, 452, 476[8]	70.5 ± 1.5
Total / (μg g^{-1})	–	–	2842.7

[a]Inline; [b]reported; [c]avarage ± SD. [8]Mobile phase system of methanol:acetonitrile:water (84:14:2, v/v/v) and methylene chloride (100%); [9]mobile phase system of methanol:acetonitrile:water (79:14:7, v/v/v) and methylene chloride (100%); [22]mobile phase system of acetonitrile:methanol:ethyl acetate (60:20:20 v/v/v, isocratic); [26]mobile phase system of methanol:acetonitrile:water (84:14:5, v/v/v) and methylene chloride (100%).

(A_{Soret}/A_{Qy}) bands regarding chlorophyll *a*, chlorophyll *b* and pheophytin *a* is 1.25, 2.8 and 2.0, respectively, as already reported.[22]

Violaxanthin, neoxanthin, zeaxanthin, β-carotene and *trans* and *cis*-lutein were identified by comparison with literature data, based on absorption spectrum[8,9,21,25-28] and retention times.

Extraction time effect

Hexane:ethanol (1:1, v/v) solvent mixture presented the best extract yield for lutein and β-carotene in *Desmodesmus* sp. microalgae. Therefore, the effect of extraction time on extract yield was tested using this solvent mixture. The presented data in Figure 3 show lutein and β-carotene extraction yield in *Desmodesmus* sp. for different extraction time. The ANOVA showed significance ($p < 0.05$) for both pigments in these extraction times tested.

Lutein extraction yield increases until 240 min (equivalent to 6 repetitions of 40 min). For β-carotene, the yield increased till 280 min (equivalent to 7 repetitions of 40 min) and it was kept constant up to 320 min. But *t*-test showed no difference between these times and also for 240 min. Increased extraction times leads to higher
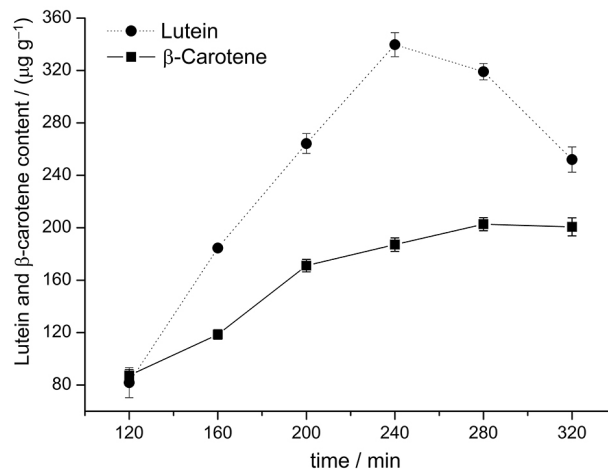
Figure 3. Lutein and β-carotene content in relation of extraction time.

extract content because a contact between pigments and solvent eases extraction process up to the point where concentration gradient between microalgae cell and solvent reaches balance.[11] Lutein yield decrement after reaching the curve peak may be related to extract degradation, after a long contact time with the extractor solvent. For further experiments the procedure will consist of 6 extractions of 40 min, totaling 240 min.

Extractor solvents, when first extractions were observed, presented a darker color than the subsequent extracts, indicating a higher pigment concentration in initial extracts. During the experiment, the increase volume in aqueous phase throughout the extraction process is due to the interaction of ethanol with sodium sulfate solution.

Evaluation on the influence of acetone pre-treatment of dry biomass

Extraction efficiency of pigments and other compounds from microalgae are related to microalgae cell wall destruction, releasing these substances and allowing their interaction with extractor solvent.[29] Therefore, a biomass pre-treatment with acetone in ultrasonic bath was considered a possible influence in β-carotene and lutein extraction yield when compared to extraction using only hexane:ethanol (1:1, v/v).

Analyzing the chromatogram from Figure 2b, pre-treatment with acetone followed by hexane:ethanol (1:1, v/v) extraction led to a reduction in peaks number and intensity when compared to extraction using only hexane:ethanol (1:1, v/v; Figure 2a). According the identified pigments (Table 2), the predominant presence of β-carotene, α-carotene, chlorophyll and lutein can be observed, as well as smaller quantities of violaxanthin and zeaxanthin. The total pigments concentration extracted from biomass varied from 798.6 to 1282.6 µg g⁻¹, corresponding to a reduction of 66.4% when compared to the extraction using only hexane:ethanol (1:1, v/v).

The statistical analysis performed for acetone treatment showed that the effects for both pigments were significant, with $p = 0.000074$ to lutein and $p = 0.00043$ to β-carotene. By analyzing Figure 4, hexane:ethanol (1:1, v/v) extraction for lutein was the highest yield method when compared to procedures involving acetone pre-treatment. Pre-treatment with acetone lead to a reduction of 73.5% in lutein content when conduced for 2 h. This reduction is related to pigment degradation in face of greater contact with acetone and, then, with the extractor solvent.

For β-carotene extraction, the biomass pre-treatment of 1 h followed by hexane:ethanol (1:1, v/v) extraction led to greater yield than the other tested methodologies. Thus, pre-treatment with acetone decreases lutein content but it improves β-carotene yield. Therefore, considering that the t- test for the highest yields from β-carotene was slightly different ($p = 0.047$) and also one more step for pigments extraction, the procedure with hexane:ethanol (1:1, v/v)

Table 2. Identification of pigments obtained by high performance liquid chromatography with diode array detection (HPLC-DAD) of dry biomass from *Desmodesmus* sp. microalgae treated with acetone followed by hexane:ethanol (1:1, v/v) extraction; methanolic and ethanolic saponification with or without acetone treatment followed by hexane:ethanol (1:1, v/v) extraction

| Pigment | Concentration / (µg g⁻¹) | | | | | | | | | | |
| | Acetone treatment | | | Methanolic saponification | | | | Ethanolic saponification | | | |
	30 min	1 h	2 h	No AT	30 min AT	1 h AT	2 h AT	No AT	30 min AT	1 h AT	2 h AT
cis-violaxanthin	29.4	57.8	32.6	–	–	–	–	–	–	–	–
Chlorophyll b	280.3	257.9	153.8	–	–	–	–	–	–	–	–
9-*cis*-lutein	18.9	17.9	12.1	27.2	31.5	31.0	29.9	24.9	31.2	25.7	31.8
trans-lutein	130.5	155.8	90.9	180.9	194.2	203.0	192.7	179.8	208.9	225.8	181.8
Chlorophyll a or a'	31.1	4.8	10.4	5.15	7.0	9.2	7.1	6.4	3.9	3.4	3.3
trans-zeaxanthin	40.9	70.5	49.1	–	–	–	–	–	–	–	–
Not identified	5.5	7.7	5.0	–	–	–	–	–	–	–	–
9-*cis*-α-carotene	66.1	135.7	69.5	–	–	–	–	–	–	–	–
trans-β-carotene	146.1	268.6	157.9	124.1	121.3	163.2	136.2	145.8	167.2	156.2	140.1
9-*cis*-β-carotene	125.9	123.6	122.6	63.2	63.5	69.8	65.5	65.2	68.8	65.2	65.7
cis-β-carotene	90.9	180.0	91.9	–	–	–	–	–	–	–	–
cis-β-carotene	2.9	2.2	2.8	–	–	–	–	–	–	–	–
Total / (µg g⁻¹)	968.5	1282.6	798.6	400.6	417.5	476.2	431.4	422.1	480.0	476.3	422.7

AT: Acetone treatment.

presents as a better method. On financial aspects, there are no advantages in this kind of treatment, since lutein presents higher price than β-carotene.
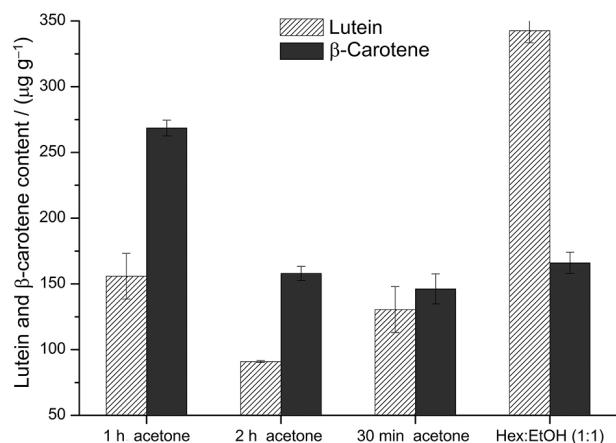
Figure 4. Influence of acetone treatment of dry *Desmodesmus* sp. microalgae on lutein and β-carotene content. Hex: Hexane; EtOH: ethanol.

Saponification and pigments extraction

There is a great interest from the industry in free pigments, in other words, non-esterified, which is achieved by saponification of microalgae biomass. Therefore, alkaline saponification was tested in both solvents, methanol and ethanol. Even though, β-carotene is a non-esterified carotenoid and it is important to evaluate the influence of saponification process in non-esterified pigments content.

By using saponification, β-carotene and lutein extraction has been favored when acetone pre-treatment is applied in both methanolic and ethanolic solution for saponification, but the increased was slightly significant. The highest lutein yield was obtained when dry biomass was pre-treated with acetone for 1 h, followed by saponification in ethanol and subsequent hexane:ethanol (1:1, v/v) extraction (Figure 5 and Table 2).

For β-carotene, a better yield was reached within 30 min of acetone pre-treatment when ethanol was used as the saponification reaction solvent (Figure 5 and Table 2). When it was used methanol as solvent on alkaline solution, β-carotene yield achieved its maximum in 1 h of pre-treatment with acetone.

The *t*-test for methanol and ethanol with or without acetone pre-treatment showed no significance difference ($p > 5$). Based on these results, for β-carotene extraction the content is similar in 1 h of pre-treatment with acetone in either methanol or ethanol. However, for lutein extraction the yields under same conditions is slightly different and it is better in ethanol solution. So, considering the methanol toxicity, it is suggested to obtain free pigments from *Desmodesmus* sp. biomass the use of ethanol saponification reaction.

According to Table 2, which identifies chromatograms peaks from Figures 2c and 2d, saponification process releases free pigments (non-esterified), simplifying pigments extraction. In addition, saponification also provides esterified pigments identification, as shown for the identification of xanthophyll esters.[16,17]

Methanol (Figure 2c) and ethanol (Figure 2d) alkaline saponification also led to the disappearance of some peaks that were usually present in ordinary solvent extractions. Saponification reaction, either from dry biomass or acetone
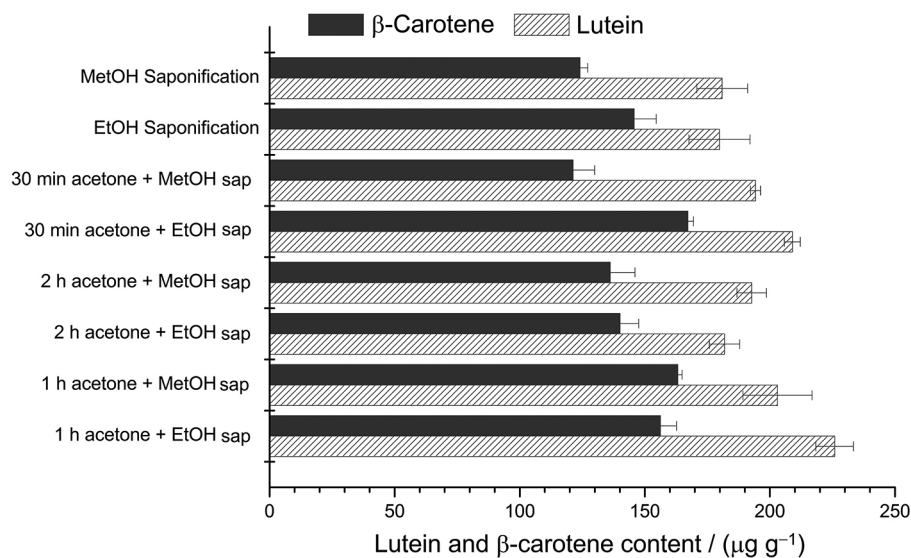
Figure 5: Influence of the methanolic and ethanolic saponification with or without acetone treatment of dry *Desmodesmus* sp. microalgae on lutein and β-carotene content. MetOH: Methanol; sap: saponification; EtOH: ethanol.

treatment biomass, led to a considerable decrease of total pigments yield, around 84.7% regarding hexane:ethanol (1:1, v/v) without any treatment.

Pigment saponification allowed simplified non-esterified pigment chromatographic identification, emerging as an important strategy to be adopted for microalgae pigment analysis; however, it led to lower pigment yields.

Pigments extract stability

After extraction, extract stability in different solvents was evaluated by successive injections until 32 h after first of each vial. Figure 6 shows the concentration of pigments (mg mL^{-1}) expressed in percentage regarding to first injection, with a 100% extraction yield for chlorophyll *b* and lutein. β-Carotene curve stability was not plotted because the peak area from hexane extract presented low concentration values, thus chlorophyll *b* area was chosen.
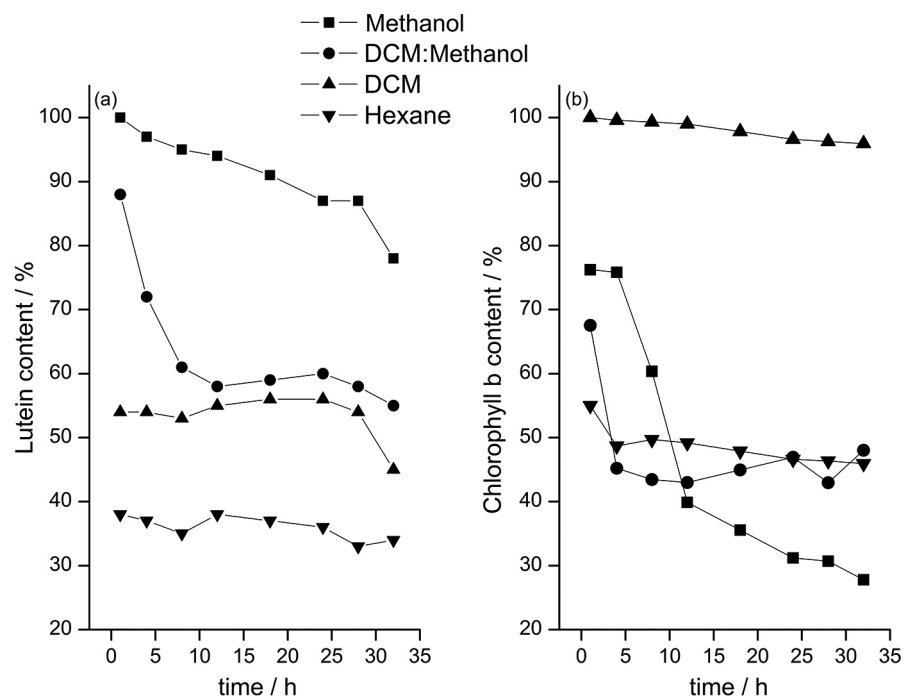
Lutein data for first injection in DCM:methanol, DCM and hexane are related to the methanol dissolved extract concentration in the first injection, once this extract provided higher lutein yield. In contrast, for chlorophyll *b* concentration data are related to DCM dissolved extract. A long term decay in both extracts was observed and it can be related to isomerization and degradation pigments.

For lutein pigment, hexane and DCM extract solution data show good stability, however with low concentration values when compared to methanol and DCM:methanol

data. These extracts present decay until 7% in DCM and between 3 and 8% in hexane at the first 24 h. The extract in methanol presented higher yield when compared to other studied solvents, with a linear decay of 22% in its concentration during 32 h. DCM:methanol solution extract exhibited lower stability in the first 8 h, with 31% reduction in lutein extract when related to first injection, presenting stability since then. Therefore, it was not possible using this solution to maintain the obtained extract from hexane:ethanol (1:1, v/v).

Considering chlorophyll b, the extract dissolved in hexane and DCM showed stability with linear decay between first and last injection, corresponding to a 4% loss in DCM and 16% loss in hexane in 32 h after the first injection. For DCM:methanol dissolved extract, there is a large decay (33%) between first and second injection, however the stability value in the following injections was maintained. The extract dissolved in methanol showed instability, mainly in first 12 h with 47% reduction in concentration content. This result for methanol confirms instability obtained by van Leeuwe *et al.*[30] for five microalgae species.

The ANOVA indicated that all the extracts tested had a significant effect on peak area of lutein and chlorophyll *b* concentration. In this experiment, it was also observed differences in the chromatographic profile for different pigments extracts. For the DCM and DCM:methanol dissolved extracts, no large difference was observed;

Figure 6. (a) Lutein and (b) chlorophyll b content over 24 h in extracts from *Desmodesmus* sp. Pigments content are presented as a percentage of the concentration of the first injection. DCM: Dichloromethane.

however, DCM:methanol showed higher resolution peaks. Hexane dissolved extract did not present good resolution and separation of peaks in first 35 min of analysis. The extract dissolved in methanol presented better resolution and separation of chromatographic pigments peaks.

These inequalities in chromatographic profiles among different dissolved extracts affected these pigments concentrations and stability. However, in order to maintain the compound integrity, the use of dissolved extract in methanol until 18 h after ending extraction was determined, because xanthophyll and carotenes were less affected than chlorophyll. Thus, by determining extract stability time, the appearance of degradation products and compounds structure reorganization are avoided and pigments primary characteristics are maintained.

Conclusions

For *Desmodesmus* sp. dry biomass, hexane:ethanol (1:1, v/v) biphasic extraction system showed better yields for lutein and β-carotene when compared to other tested solvents. About extraction times, lutein maximum yield was obtained with 6 extractions of 40 min using hexane:ethanol (1:1, v/v). For β-carotene, the best condition occurred with 7 extractions of 40 min. Even though it provided an increase of β-carotene extraction, pre-treatment of dry biomass with acetone caused a decrement in lutein extraction, making this pre-treatment step commercially unviable.

Saponification before hexane:ethanol (1:1, v/v) extraction led to chromatographic profile simplification by converting esterified pigments in free pigments. Pre-treatment with acetone for 1 h followed by saponification in ethanolic solution and extraction with hexane:ethanol (1:1, v/v) was the best procedure to obtain better yields when extracting pigments from *Desmodesmus* sp. dry biomass. Intend an extract with attractive pigments, the saponification method can be adopted because it will facilitate the next steps (separation and purification) to obtain the final compound with high purity.

For the stability tests, the use of methanol dissolved extract up to 18 h after the end of extraction was determined, because xanthophyll and carotenes pigments were more stable than chlorophyll and affording a better chromatographic profile. Chromatographic analysis allowed identifying that the main pigments available in *Desmodesmus* sp. are *trans*-zeanxanthin, chlorophyll *b*, carotenes and *trans*-lutein. Considering that most industrial processes use only one type of solvent for pigments extraction, the methods tested (hexane:ethanol, saponification and treatment with acetone) improved β-carotene and lutein yields for *Desmodesmus* sp.

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