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# Single-step extraction of carotenoids from brown macroalgae using non-ionic surfactants

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ABSTRACT

While there is an accrued interest in the production and application of bioactive compounds from macroalgae, several of these compounds with high industrial and commercial interest remain underexplored. Carotenoids (and specifically fucoxanthin) normally found in brown macroalgae are examples of these compounds. One of the major issues associated with the poor commercial exploitation of these biomolecules is the need for a highly performant and low cost extraction process to extract them selectively from the algal biomass. In this work, aqueous solutions of various non-ionic surfactants were screened for the carotenoids extraction. Moreover, and after the selection of the most performant surfactants, several processing conditions, namely the solid-liquid ratio, concentration of surfactant and time of extraction were evaluated. The optimal conditions were applied to the extraction of carotenoids for both Portuguese and Brazilian algae species. With the process of extraction developed in this work, that was optimized for the dry seaweed biomass but it is also applicable to the fresh one, extraction results of $5.28 \pm 2.01$ $mg_{carotenoids}/g_{biomass}$ for Tomadol 25-7 and $1.86 \pm 0.06$ $mg_{carotenoids}/g_{biomass}$ for Pluronic P-123 were obtained. Despite the higher extraction efficiency of the conventional (ethanol-based) method ($6.48 \pm 0.01$ $mg_{carotenoids}/g_{biomass}$), the methodology proposed in this work allows a much higher selectivity of the carotenoids extraction since, unlike with ethanol, less contaminants (in particular chlorophylls) are extracted along with the carotenoids.

This work proposes a simpler (with less extraction steps), more selective and organic solvent-free extractive process to recover carotenoids from brown macroalgae, directly applicable to fresh biomass.


## 1. Introduction

The United Nations of Food and Agriculture Organization estimated in 2011 the annual production of marine seaweeds and other aquatic plants to be over 22.1 million tons, valued at more than US$ 7 billion. The valorization of the Portuguese marine resources (Portugal has the 10th largest Exclusive Economic Zone in the world) is of crucial importance, due to their commercial potential. Macroalgae in particular, are one of the major natural and versatile marine resources, known by their applications in the food and feed industry [1]. In the context of a blue biorefinery, macroalgae are also valuable raw materials present all over the world, with high potential as sources of various added-value bioactive compounds. Due to the actual concerns of sustainability in processes and technologies in a cradle-to-grave approach, and taking into account the demands for a careful management and waste decrease, the blue biorefinery is of growing relevance, and with it, the demand for new technological platforms for the valorization of these marine biomasses. Brown seaweeds are abundant in Portugal, including the non-native *Sargassum muticum*, an algae widely spread in the Atlantic coast. This macroalgae is commonly found along the Portuguese coast, north of the Mondego river [2]. The north of Portugal is a biogeographic transition zone where many species of macroalgae have their distribution limits, which makes this region particularly interesting in terms of commercial exploitation of algal bio-derived products and materials. Thus, in Portugal and Brazil it is possible to find *Sargassum* species, abundant and currently un-explored [3,4]. This, associated to the valuable chemicals on its composition, namely carotenoids, makes this biomass commercially relevant. Fucoxanthin is a well-known carotenoid belonging to the group of xanthophylls [5], and found

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in several brown algae. This carotenoid in described as a non-toxic [6,7], anti-obesity [8], antitumoral [9], anti-diabetic [10], anti-inflammatory [11] and antioxidant compound.

However, one of the main problems inhibiting a wider commercial use of fucoxanthin is the low ratio among efficiency, selectivity, and cost associated with the extraction and purification processes used nowadays. Due to their selectivity and accurate resolution, chromatographic techniques are the most common approach described in literature, followed by the supercritical fluid extraction and pressurized liquid extraction [12] methods. However, the direct application of such techniques to the biomass or crude extracts may be technically challenging and limited in terms of economic viability and scalability [13]. Due to their hydrophobic nature, the extraction of carotenoids and fucoxanthin is commonly carried out from dry biomass with organic solvents such as acetone, methanol, ethanol, chloroform, and/or mixtures thereof [14–16]. In this context, a more effective and appropriate process to extract carotenoids, and to purify fucoxanthin from algae is needed.

Surfactants are amphiphilic molecules, meaning that they have a polar hydrophilic "head" and a non-polar hydrophobic "tail". When they are present above certain concentrations in water, known as the critical micelle concentration (cmc), the surfactant molecules form self-assembling aggregates [17,18]. When in aqueous solution, these aggregates/micelles formed have a non-polar interior formed by the hydrophobic "tails", while the polar "heads" are exposed to water molecules, forming the interface between the aqueous solution and the aggregates core [17–19]. The use of surfactants as solvents in the extraction of bioactive compounds is not new [20]. Moreover, and considering some of the process schemes, it is possible to identify some advantages considering the use of aqueous solutions of surfactants, namely the decreased number of solvents and concentrations used, which potentially minimizes the environmental impact and the cost associated with the process [21]. In this sense, this work aimed at to develop an extractive methodology in aqueous media using non-ionic surfactants for the recovery of carotenoids (among which fucoxanthin is included) from brown macroalgae. Aqueous solutions of non-ionic surfactants were screened for the carotenoids extraction, being the solid-liquid ratio, concentration of surfactant, and time of extraction the independent variables further optimized for the two surfactants with the best extractive performance. The extraction of carotenoids in the optimized conditions was applied to dry biomass samples for both Portuguese and Brazilian *Sargassum* species, followed by checking the feasibility of the method for fresh seaweed biomass.

## 2. Experimental

### 2.1. Materials

The company ALGAplus, Ltd provided the *Sargassum muticum* samples used in this work; *Sargassum cymosum* biomass was collected in the Santa Catarina Island (southern Brazil). The algal material (*Sargassum muticum* and *Sargassum cymosum*) was dried with continuous ventilation and controlled temperature at 25 °C until constant weight. Two different batches of *Sargassum muticum* were used: (i) batch S1.0315.D for the optimization process (first experiments and factorial planning), and (ii) batch S1.4915.D was used for the final tests comparing both dry and fresh biomass matrices. For more details about each batch please see Table A1 in Supporting information.

The solvents used for the conventional extraction methodology were: ethanol (Analytical Reagent Grade Fisher Scientific), methanol (purity 100%, CHEM-LAB), ethyl acetate (purity 99%, VWR BDH-Prolabor), chloroform (purity 99%, Carlo Erba), n-hexane (HPLC grade, Carlo Erba e Acros), acetone (purity 100%, VWR Normapur), and acetonitrile of HPLC grade (Fisher Chemical). The chromatographic column was prepared with silica gel G-60 (Sigma-Aldrich). A commercial standard of fucoxanthin (Sigma-Aldrich) was purchased (see chemical structure in Fig. A1 in Supporting Information).

The non-ionic surfactants used were: Brij 30, Brij 93, Brij 98, Tomadol 25-7, Tomadol 91-6, Tomadol 1-9, Triton X-100, Triton X-114, Tween 80, Tergitol 15-S-7, Tergitol 15-NP-7, Tergitol NP-10, Tergitol 15-S-9, Pluronic F-127, Pluronic P-123, Pluronic L-35, and Pluronic F-108. All surfactants nominated as Tomadol were supplied by Prospector and the others were purchased from Sigma-Aldrich. The chemical structures of the surfactants and model molecules used in this work are provided in Table A2 presented in Supporting Information.

### 2.2. Organic solvent extraction (OSE)

A conventional methodology adapted from literature [22] was applied in this work for comparative purposes, by using pure ethanol. The dried biomass collected by ALGAplus was subjected to successive solvent extractions with ethanol-based solutions. Each extraction was carried during 120 min under constant agitation (250 rpm), at room temperature (25 °C) and in the dark. The successive solvent extractions were made until no peaks in an UV spectrophotometer Shimadzu-Pharma Spec 1700 were detected at 400–500 nm (including the carotenoids' characteristic peaks). The ethanol-based extracts obtained were analyzed in the maximum peak of absorbance at 417 nm in the crude extract (SHIMADZU, UV-1700 PharmaSpec). In this case, the total amount of carotenoids was quantified, being the experimental results expressed in $mg_{carotenoids}/g_{dried\ mass}$. Then, the ethanol-based extracts were applied in a liquid-liquid separation system, prepared by the addition of an aqueous solution of ethyl acetate 1:1 (v/v). The ethyl acetate extract now containing carotenoids (and fucoxanthin) was evaporated in a rotary evaporator (Heidophy - Laborota 4000) and the dried extract obtained was resuspended in methanol (1 mL). The yellowish fractions rich in carotenoids (and fucoxanthin) were separated from the ethyl acetate-soluble fraction by percolating the solution with a preparative silica gel in an open column chromatography (silica G-60) using a solution of chloroform-methanol-water 65:25:4 (v/v/v) as the mobile phase. After the elution of the mobile phase, the "orange" fraction (richer in fucoxanthin) was collected, evaporated to dryness and then, the residue was resuspended in 1 mL of acetone. The acetone extract was again percolated in a preparative silica gel in an open column chromatography (silica G-60) with a mobile phase consisting of n-hexane and acetone 6:4 (v/v). The final "orange" fraction pure in fucoxanthin was collected and analyzed by high-performance liquid chromatography (HPLC) to detect the standard peak of absorbance and to quantify the pure fucoxanthin content collected and isolated from the biomass. Aliquots of 20 μL of each fraction of pure fucoxanthin were analyzed by HPLC (Shimadzu LC - 10A) equipped with a reversed phase C18 column (Vydac 201TP54, 250 mm × 4.6 mm internal diameter), coupled to a pre-column (Vydac 218GK54, 5 μM), and UV–vis spectrophotometric detector operating at 450 nm. The elution was made with a methanol:acetonitrile solution (90:10 v/v) as mobile phase, being the flow rate at 1 mL/min. The identification of fucoxanthin was performed by comparison with the retention time of an analytical standard of fucoxanthin (Sigma-Aldrich, MO - USA) under the same experimental conditions. The quantification of the fucoxanthin was done through an external fucoxanthin analytical standard calibration curve. The values are shown as the average measure of three sequential sample injections. The average concentration of

fucoxanthin in the conventional methodology was expressed in $mg_{fucoxanthin}/g_{dried\ mass}$.

### 2.3. Screening of non-ionic surfactants for the carotenoids extraction

The screening was made with dried *Sargassum muticum* (batch S1.0315.D) collected in January 2015. The dry biomass was mixed with aqueous solutions of 18 non-ionic surfactants. The first experiments detailed the screening of the various tensioactive compounds in terms of their capacity to extract carotenoids from *Sargassum* sp., taking into account fixed conditions of surfactant concentration ($C_{surf}$) at 0.01 mol/L, time of extraction ($t$) of 90 min (i.*e*. time of contact between the biomass and the solvent), and solid-liquid ratio [$R_{(S/L)}$] of 0.02 (mass of biomass *per* volume of solvent). The conditions selected to start this work were adopted from the conventional method based in ethanol, here used as the control [22]. Briefly, the biomass was placed in contact with the various surfactant aqueous solutions under constant agitation (250 rpm) and 25 °C. The extractions performed in this work were done in the dark due to the high sensitivity of carotenoids to light. Then, these mixtures were centrifuged (5000 rpm, 4 °C, 40 min) in a Thermo Scientific, Megafuge 16R to efficiently separate the rests of biomass and the aqueous solutions of surfactants rich in carotenoids. After centrifugation, the supernatant was collected and further analyzed in a UV–vis spectrophotometer (SHIMADZU, UV-1700 PharmaSpec). The entire spectrum from 350 to 700 nm was determined in order to identify the carotenoids between 400 and 500 nm, and some other contaminants (namely chlorophylls) possibly present in the solutions obtained from the solid-liquid extractions performed. The carotenoid content on each supernatant was quantified at the maximum peak of absorbance characteristic of carotenoids, 417 nm, and in accordance with the one used in the ethanol-based conventional methodology. The determination of the carotenoids concentration was done in triplicate, being the results presented as the average of the three experiments (in $mg_{carotenoids}/g_{dried\ mass}$).

After the first screening performed with the various aqueous solutions of different non-ionic surfactants, and the selection of the most promising surfactants regarding their higher capacity to extract carotenoids from the macroalgae, the optimization of the extraction conditions, namely the concentration of surfactant ($C_{surf}$), time of extraction ($t$) and solid-liquid ratio $R_{(S/L)}$, was carried out. To facilitate the simultaneous analysis of the various parameters [23], a $2^k$ factorial planning was carried, in which there are $k$ factors that can contribute to a different response regarding the final concentration of carotenoids in just one step of extraction. The experimental data was treated according to the second order polynomial equation (Eq. (1)) described as follows:

$$y = \beta_0 + \beta_i X_i + \beta_j X_j + \beta_{ii} X_i^2 + \beta_{jj} X_j^2 + \beta_{ij} X_i X_j \quad (1)$$

where $y$ is the dependent variable, namely the concentration of carotenoids extracted (in $mg_{carotenoids}/g_{dried\ mass}$), and $\beta 0$, $\beta i$, $\beta j$, $\beta ii$, $\beta jj$ and $\beta ij$ are the regression coefficients used respectively, to the intersection, linear, quadratic, and interaction of the terms. $X_i$ and $X_j$ represent the independent variables in the $2^k$ factorial planning. In this work, a $2^3$ factorial planning was used aiming at the optimization of the variables previously mentioned, namely solid-liquid ratio, surfactant' concentration, and time of extraction. The most significant extraction parameters and their interactions were evaluated. The central point (zero level), factorial points (1 and −1, level one), and axial points (level $\alpha$) were defined, being the $2^3$ factorial planning provided in Supporting Information. The axial points are encoded at a distance $\alpha$ (Eq. (2)) from the central point [23], being the specific values used in the experimental tests described in Table A3 in Supporting information.

$$\alpha = (2^k)\frac{1}{4} \quad (2)$$

The results obtained were statistically analyzed considering a confidence level of 95%. The adequacy of the model was determined [24]. In cases where the mathematical model was found to be inadequate, a new model was fitted excluding the non-significant variables. Three dimensional surface response plots were originated by changing two variables within the experimental range and maintaining the remaining factors at the central point. Each factorial planning developed used a central point experimentally obtained at least three times. Additional 12–20 and X–Y experiments were performed for each $2^3$ and $2^2$ factorial planning, being the various processing conditions repeated, when necessary, to guarantee the accuracy of the data. The Statsoft Statistica 8.0© software was applied in the statistical analysis, being the response surfaces and contour plots developed with the same software. The carotenoids theoretically predicted, the relative deviations considering the amount of carotenoids predicted and experimentally determined, the equations used to fit the results, and the regression coefficients and standard deviations of the $2^3$ factorial planning were determined.

Scanning electron microscopy (SEM) analysis was carried out using an Hitachi SU-70 microscope operating at 15 kV and freeze dried samples (1 mg) were deposited on carbon conducting tape and coated with carbon. The systems under study were cells oven dried and powered, cells after aqueous extraction, and cells after extraction with different surfactants.

### 2.4. Extraction of carotenoids from the fresh biomass of Sargassum muticum

The marine biomass used in this step belonged to the batch S1.4915.D. The biomass collected by ALGAplus was washed with fresh and distilled water at least 3 times to remove sand and other contaminants. The *Sargassum* samples were frozen in liquid nitrogen, grounded (in coffee grinder, Molinex brand), and homogenized in the different aqueous solutions of the non-ionic surfactants defined as the best solvents extracting carotenoids from the dried biomass. The dry weight of the algal biomasses was established by lyophilization. The extracts were then prepared considering the amount of water present in the macroalgae and the optimum conditions previously defined for the extraction process using the dry biomass, including the adequate concentration of each surfactant selected. Then, the same procedure previously described in Section 2.3 was followed.

## 3. Results and discussion

The main objective of this work is the development of an extraction process based on aqueous solutions of non-ionic surfactants to extract carotenoids from brown macroalgae. The conventional methodology described elsewhere [22] has several extraction steps with various organic solvents (in pure state) and/or mixtures of organic solvents. The alternative methodology proposed intends to overcome the drawbacks of conventional methods typically characterized by the use of organic solvents, long extraction times, complexity, and poor selectivity. Indeed, a simpler single-step and organic solvent-free method of extraction, more sustainable and environmentally friendly is presented, suitable to both dry and fresh seaweed biomasses.

### 3.1. Organic Solvent Extraction (OSE)

A conventional methodology recently described in literature [22] was used to determine the total amount of i) carotenoids

and ii) fucoxanthin of *Sargassum muticum* (Portugal) and *Sargassum cymosum* (Brazil) biomass samples.

The carotenoids were extracted (solid-liquid extraction) using pure ethanol and the ethanol-based extract obtained rich in carotenoids was further purified by a preparative open column silica gel chromatography. Using the OSE methodology, the total amount of carotenoids obtained for *Sargassum muticum* was 1.87 ± 0.02 $mg_{carotenoids}/g_{dried\ mass}$ and 0.91 ± 0.03 $mg_{carotenoids}/g_{dried\ mass}$ for *Sargassum cymosum*. The amount of pure fucoxanthin obtained was 0.100 ± 0.004 ($mg_{fucoxanthin}/g_{dried\ mass}$) for *Sargassum muticum* and 0.089 ± 0.003 ($mg_{fucoxanthin}/g_{dried\ mass}$) for *Sargassum cymosum*. These results suggest that the Portuguese species *Sargassum muticum* is richer in carotenoids than the Brazilian species, as the later showed lower fucoxanthin contents, i.e., 5.3% (*Sargassum cymosum*) and 9.8% for *Sargassum muticum*. These differences on the carotenoids and fucoxanthin contents might be attributed to the significant dependence of the macroalgae composition regarding their genotype traits, environmental stress [25], weather [26], season [27], and geographic location [28]. This is also verified when our values are compared with those found in literature, in which the differences on the fucoxanthin content increase significantly. Just to mention some examples: 1.01 ± 0.39 ($mg_{fucoxanthin}/g_{dried\ mass}$) for *Sargassum duplicatum*, 0.73 ± 0.39 ($mg_{fucoxanthin}/g_{dried\ mass}$) for *Sargassum bindei* [15], 0.080 ± 0.006 ($mg_{fucoxanthin}/g_{dried\ mass}$) for *Sargassum wightii*, 0.150 ± 0.012 ($mg_{fucoxanthin}/g_{dried\ mass}$) for *Sargassum ilicifolium* and 0.030 ± 0.006 ($mg_{fucoxanthin}/g_{dried\ mass}$) for *Sargassum longifolium* [29]. These results justify the demand for understanding the relation of the macroalgae composition and the adaptive characteristics of the *Sargassum* species regarding different seasons and geographic locations.

The total amount of carotenoids experimentally obtained by the ethanol-based method will be further compared with the results obtained for the use of the different aqueous solutions of non-ionic surfactants. Since *Sargassum muticum* has the highest content on carotenoids, this species was used in further studies regarding the optimization of the extraction conditions.

### 3.2. Carotenoids' extraction with aqueous solutions of non-ionic surfactants

For the development of the alternative extraction process, the biomass of *Sargassum muticum* was used, due to their abundance and accessibility, as well as its higher content on carotenoids. The screening of the various surfactants was performed at room temperature and by maintaining the main conditions of surfactant concentration, $C_{surf}$ at 0.01 mol/L, solid-liquid ratio $R_{(S/L)}$ of 0.02, and the time of extraction (t) at 90 min. The concentration of carotenoids extracted by each aqueous solution of surfactant was experimentally determined and the data are depicted in Fig. 1.

From these results, it is possible to conclude that the non-ionic surfactants have a different effect on the cell disruption and/or in the carotenoids solubilization, being the worst extractive performances obtained for Brij 30 [carotenoids = 0.04 ($mg_{carotenoids}/g_{dried\ mass}$)], and the best for Tomadol 25-7 [carotenoids = 1.37 ± 0.05 ($mg_{carotenoids}/g_{dried\ mass}$)] and Pluronic P-123 [carotenoids = 1.310 ± 0.001 ($mg_{carotenoids}/g_{dried\ mass}$)]. By comparing the results obtained by the conventional (1.87 ± 0.02 $mg_{carotenoids}/g_{dried\ mass}$ for *Sargassum muticum*) and alternative methodologies, it is observed that the latest is extracting lower amounts of carotenoids. The lower capacity of the non-ionic tensioactives to extract carotenoids from this brown macroalgae can be attributed either to their poor ability to disrupt the macroalgae cells, to act efficiently in the solubilization of the carotenoids, or simply by the use of inadequate conditions in the extraction.

To understand the impact of surfactants on the cell disruption, SEM images of the cells were taken in various scenarios. Through the analysis of Fig. 2A it is possible to infer about the effects of different conditions, like the drying process and the biomass grinding to powder as the image suggests, the physical processing of the macroalgae biomass promoted the disruption of the cells, exposing the intracellular content. It was possible to observe that the cell wall remains almost intact. Fig. 2B shows the cells after their contact with water and in the same conditions used in the surfactants screening. From this image, it seems that only the presence of water has a great impact on the cells disruption. In addition to the effect of mechanical disruption processes and the use of water, SEM images of the macroalgae cells after contact with aqueous solutions of different surfactants were taken. In this context, the worst and best surfactants extracting carotenoids were focused, respectively the Brij 30 represented in Fig. 2C, and Tomadol 25-7 and Pluronic P-123 in Fig. 2D and E. An analysis of the SEM images suggests that the cellular disruption does not results from the use of surfactants, contrarily to what was recently described in literature [20] for microalgae, but from the mechanical grinding process. The results suggest that the role of the surfactants is the increase of the carotenoids solubility in water.

### 3.3. Optimization of the operational conditions: response surface methodology

After the proper selection of the surfactants with higher capacity to extract carotenoids from the algal biomass, the optimization of different process conditions was conducted, through a $2^3$ factorial planning. This analytical methodology allows the simultaneous study of various parameters of the solid-liquid ratio of the extraction process and their relationship with the extraction of carotenoids. These models were developed using the most efficient surfactants extracting carotenoids, namely Tomadol 25-7 and Pluronic P-123. The uncoded and coded coefficients representing the central, factorial, and axial points for the $2^3$ factorial planning experiments are presented in Tables A3 and A4, respectively. The variables $R_{(S/L)}$, t and $C_{surf}$ were the conditions optimized. The model equations used to fit the experimental results obtained for the dependent variable (concentration of carotenoids), the carotenoids concentration experimentally obtained, and those predicted by the respective model (using the correlation coefficients obtained in the statistical treatment), as well as all the statistical analyses are presented in the Supporting Information (Eqs. A1 and A2 and Tables A5–A14). The response surface and the contour plots for Pluronic P-123 and Tomadol 25-7, respectively are represented in Figs. 3 and 4.

Based on the results obtained for the $2^3$ factorial planning carried for Pluronic P-123, and according to the experimental design (Tables A5 in Supporting Information), the statistical analysis (Tables A6 and A7 in Supporting Information) was developed. The experimental data obtained in the $2^3$ factorial planning is depicted in Fig. 3. The main results presented in the response surface and contour plots suggest that the most significant condition studied was the $R_{(S/L)}$. The experimental data obtained indicates that when the $R_{(S/L)}$ increases, the carotenoids content extracted from the macroalgae increases. In this sense, the response curves indicate that *t* and the $C_{surf}$ are conditions without statistical significance, which is also confirmed by the Pareto's Diagram (Fig. A2 in Supporting Information). No significant differences (p-value 0.05) were observed between the predicted and experimental values obtained, as observed in Fig. A3 and Table A5 in Supporting information), showing the high accuracy and the precision of the model equations defined in this factorial planning. The accuracy and the precision of the model equations were validated by comparing the carotenoids contents experimentally extracted with those theoretically predicted by equations defined (Eqs. A1 and A2 in

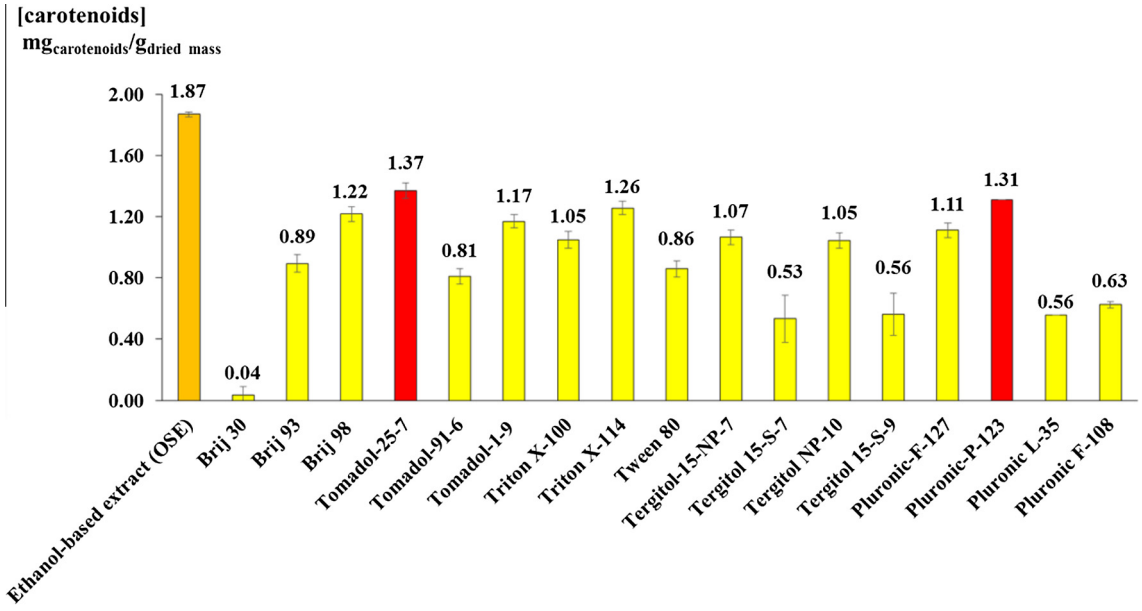


**Fig. 1.** Screening of the extractive capacity of non-ionic surfactants applied in the carotenoids extraction from the *Sargassum muticum* dried biomass.

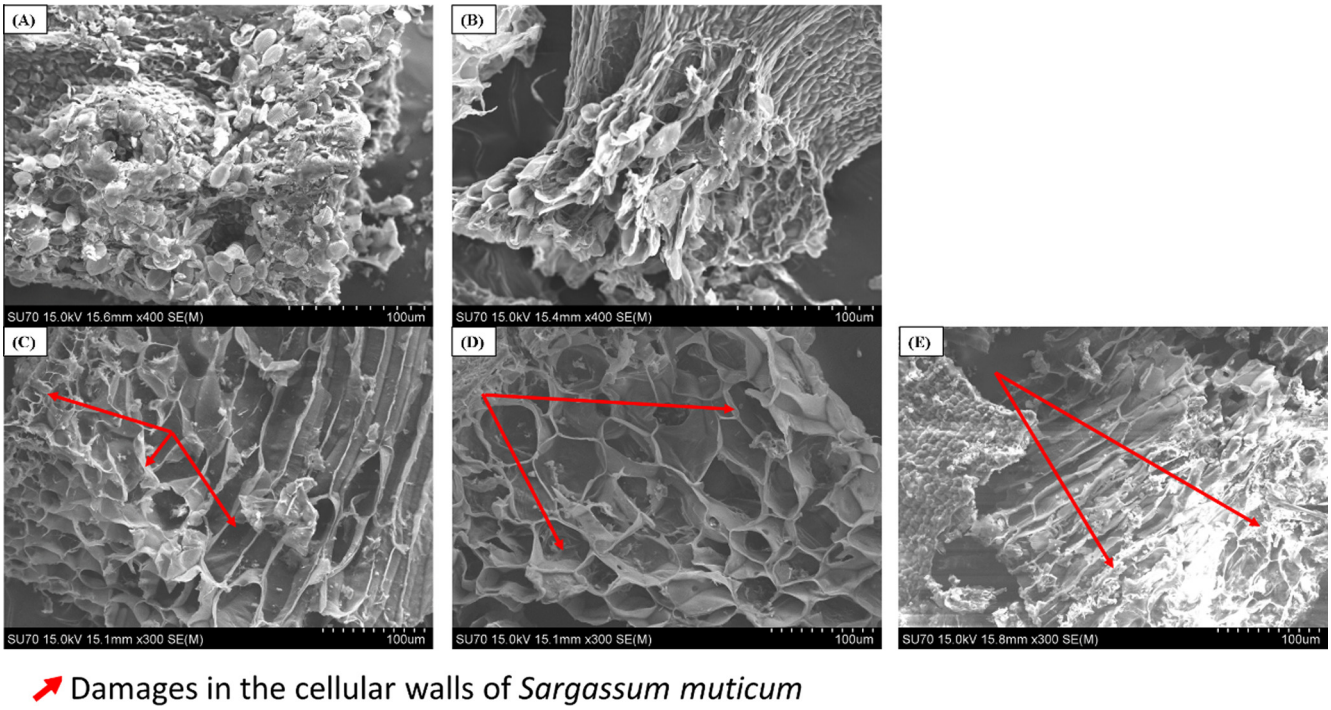


**Fig. 2.** Scanning Electron Microscope (SEM) of the *Sargassum muticum* biomass cells, being (A) cells oven dried and powered; (B) cells after aqueous extraction; (C) cells after extraction with Brij 30 aqueous solution; (D) cells after extraction with Tomadol 25-7 aqueous solution; and (E) cells after extraction with Pluronic P-123 aqueous solution. Images (A) and (B) were expanded 400X while (C)–(E) were expanded 300×. Scale for SEM images: 100 μm.

Supporting information) for the results obtained for Pluronic P-123 (Tables A6 and A7 in Supporting Information).

By the analysis of the recoveries of carotenoids (Table A8 in supporting information), it is possible to identify some conditions represented by 100% of recovery, namely the experiments 5–6 and 14. In fact, these points represent different values of $C_{surf}$, respectively, 0.005 mol/L, 0.015 mol/L, and 0.010 mol/L. Again, these results proved the significance and best performance of the higher conditions of $R_{(S/L)}$, because for points 5–6 and 14, independently of the concentration of surfactant, 100% of carotenoids recovery is achieved. The same is found for the time of exposure.

According to the experimental design (Table A9 in Supporting Information), defined for Tomadol 25-7, the statistical analysis (Tables A10 and A11 in Supporting Information) properly developed. Fig. 4 shows the contour and surface plots obtained for Tomadol 25-7. The results represented in Fig. 4 show that both the $R_{(S/L)}$ and $C_{surf}$ are statistically relevant (as shown by the Pareto's chart in Fig. A4 in Supporting Information). Actually, it is observed that

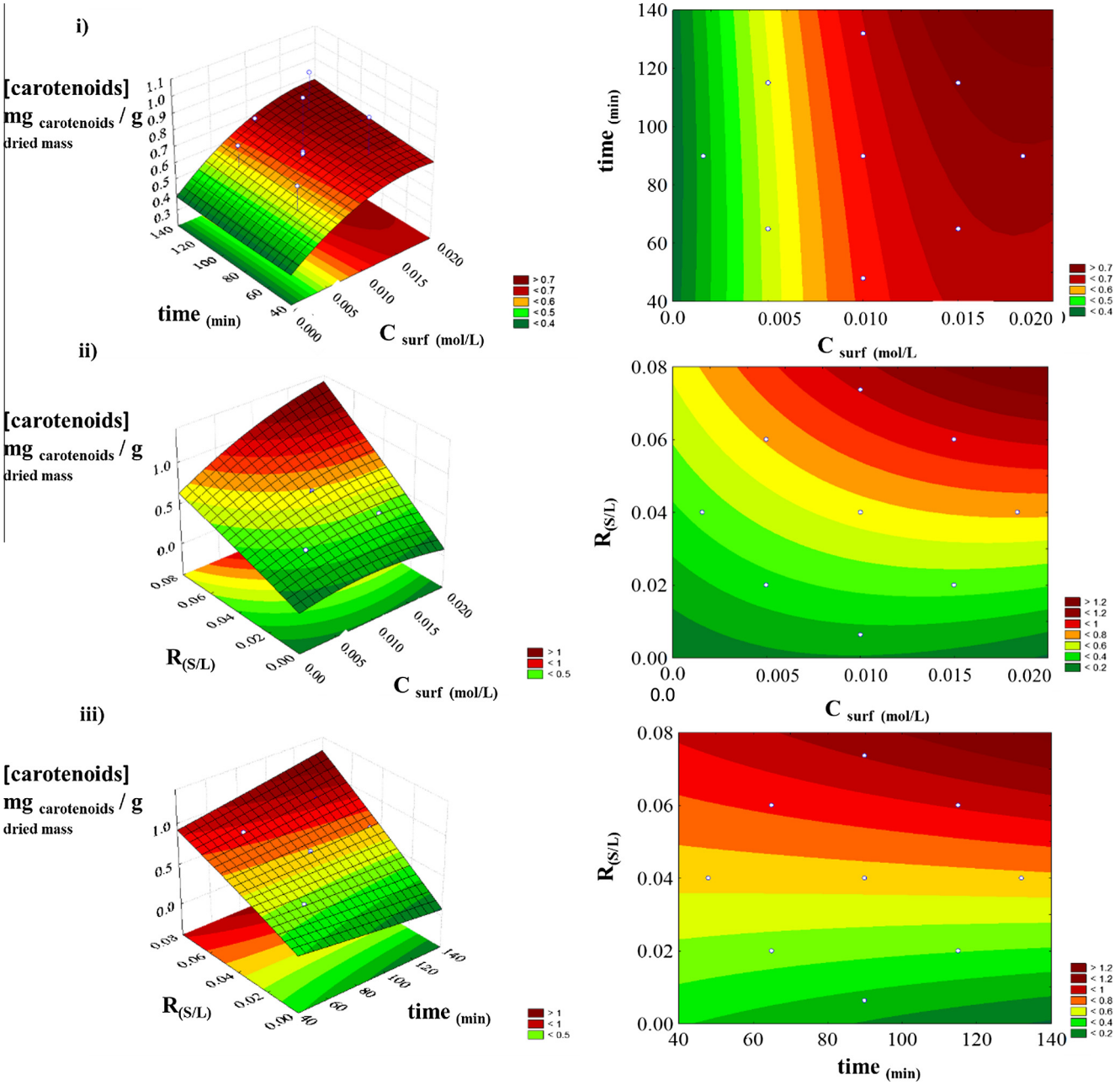


**Fig. 3.** Response surface plots (left) and contour plots (right) on the quantity of carotenoids ($mg_{carotenoids}/g_{dried\ mass}$) with the combined effects of (i) $C_{surf}$ (mol/L) and *t* in minutes, (ii) $C_{surf}$ (mol/L) and $R_{(S/L)}$ and (iii) $R_{(S/L)}$ and *t* in minutes, using aqueous solutions of Pluronic P-123.

the carotenoids extraction is directly correlated with these variables, i.e., when both the $R_{(S/L)}$ and $C_{surf}$ increase, the extraction is favored. Contrarily, the time of extraction (t), at least for the interval of time selected in this work (less than 48 min), was defined as non-statistically significant. No significant differences ($p < 0.05$) were observed between the predicted and experimental values obtained (as concluded from Figs. A4 and A5 and Table A9 in Supporting information), showing the high accuracy and the precision of the model equations defined in this factorial planning. The model described 96% of the variability in the experimental data. For this factorial planning the maximum extraction of carotenoids was achieved for $R_{(S/L)} > 0.06$ and $C_{surf} > 0.01$ mol/L. Meanwhile, and because the time of extraction (*t*) was not statistically significant for Tomadol 25-7, a new statistical analysis was carried using the $R_{(S/L)}$ and the $C_{surf}$. This new model answered by 95% of the variability in the experimental data, being described by a new fit regarding Eq. A3 (in Supporting information). Taking into account these new results found for Tomadol 25-7 (statistical analysis provided in Tables A12 and A13, and Figs. A5 and A6 in Supporting Information), again the statistical significance of both parameters was proved. Furthermore, the percent recovery was determined as the ratio between the experimental and theoretical amount of carotenoids recovered (Table A14 in Supporting information). Analyzing the recoveries of carotenoids around 100%, namely for the assays 7–8, 10, 14–17), it is possible to identify again the $R_{(S/L)}$ (also depicted in the Pareto chart, Fig. A6) as the statistically significant condition. In fact, the assays 7–8, 10, 14–17 are representing different values of $R_{(S/L)}$, namely $R_{(S/L)} = 0.04$ for the assays 10, 15–17; $R_{(S/L)} = 0.06$ for assays 7–8 and $R_{(S/L)} = 0.07$ for assay 14. In other words, this means that lower amounts of Tomadol 25-7 can be used if higher $R_{(S/L)}$ are used.

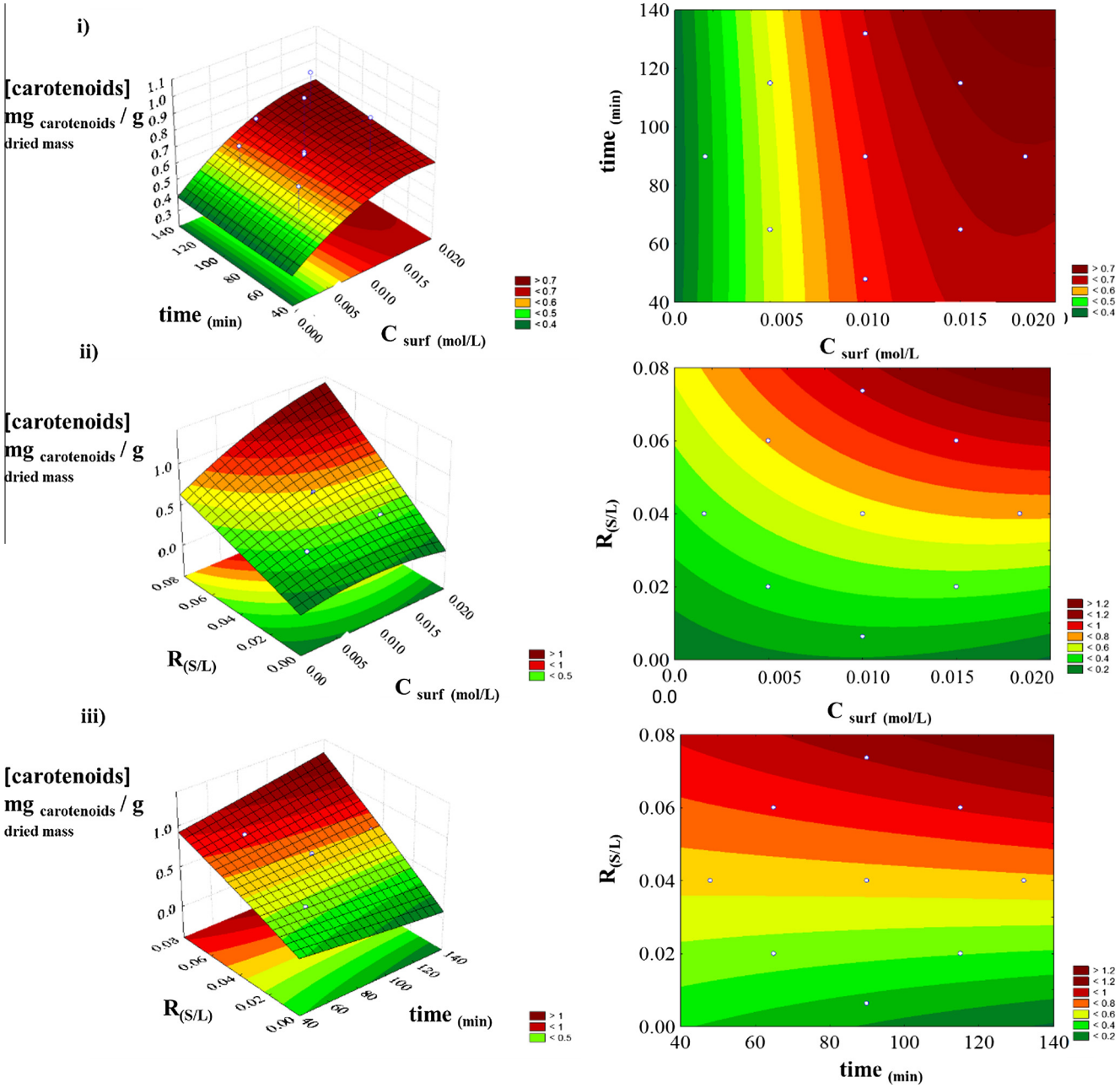


**Fig. 4.** Response surface plots (left) and contour plots (right) on the content of carotenoids ($mg_{carotenoids}/g_{dried\ mass}$) with the combined effects of (i) $C_{surf}$ (mol/L) and *t* in minutes, (ii) $C_{surf}$ (mol/L) and $R_{(S/L)}$ and (iii) $R_{(S/L)}$ and *t* in minutes, using aqueous solutions of Tomadol 25-7.

### *3.4. Extraction of carotenoids from both Sargassum species under the optimized conditions*

Once the optimum conditions identified (Table 1), the Portuguese and Brazilian species were processed and the amount of carotenoids determined for the dried matrices (Fig. 5A). Fig. 5A shows that there is a difference in the amount of carotenoids present in *Sargassum muticum* and *Sargassum cymosum*. The results demonstrate that the increase in the concentration of Tomadol 25-7 from 0.01 to 0.02 mol/L does not significantly change the amount of carotenoids extracted obtained after (Fig. 5A) and before (Fig. 1) the optimization of the experimental conditions. It seems that lower $R_{(S/L)}$ are good for Pluronic P-123 to extract similar amounts of carotenoids when compared with the non-optimized conditions (Fig. 1). By comparing the extraction of carotenoids obtained by the conventional (ethanol-based extract) and alternative methodology (using Tomadol 25-7 or Pluronic P-123 at the optimal conditions), it is concluded that the *Sargassum cymosum* has equivalent contents on carotenoids (0.91 ± 0.01, 1.00 ± 0.01, and 0.80 ± 0.03 $mg_{carotenoids}/g_{dried\ mass}$ respectively, for the ethanol-based extract, Tomadol 25-7 and Pluronic P-123).

**Table 1**
Optimal conditions determined for a factorial planning $2^3$ regarding Tomadol 25-7 and Pluronic P-123.

| Surfactants | $C_{surf}$ (mol/L) | t (min) | $R_{(S/L)}$ |
|---|---|---|---|
| Tomadol 25-7 | 0.02 | 90 | 0.1 |
| Pluronic P-123 | 0.01 | 90 | 0.02 |

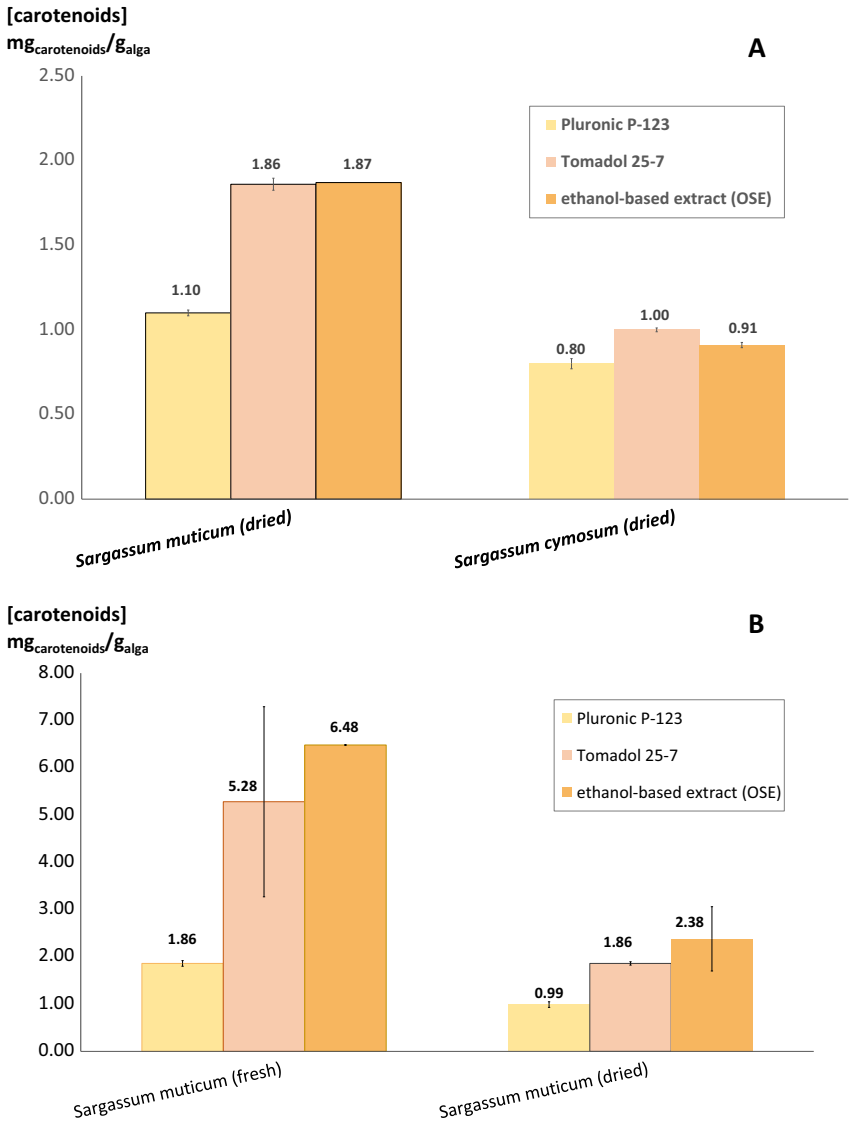


**Fig. 5.** Concentration of carotenoids obtained in different extraction scenarios: (A) by the conventional methodology OSE (ethanol-based extract) and by the use of the best surfactants (Tomadol 25-7 and Pluronic P-123) selected in the optimum extractive conditions for both and *Sargassum cymosum* (Brazilian species) and *Sargassum muticum* (Portuguese species) and (B) representing the results of extraction of carotenoids obtained from the dried and fresh (wet) algal biomass of *Sargassum muticum*, by applying the conditions optimized for the dry biomass.

Moreover, the same optimal conditions selected for the dried biomass of *Sargassum muticum* were tested on the extraction of carotenoids from its fresh biomass, being the results depicted in Fig. 5B. From these results it is concluded that, despite the idea generally conveyed that any extraction process is much more complicated starting from the fresh biomass, due to the mechanisms of cell disruption, salt effects, availability/solubility of key components that would be very different, in this work, this rule is contradicted. In other words, it is demonstrated that the conditions previously optimized for the ethanol-based extraction and the alternative process, using aqueous solutions of Pluronic P-123 or Tomadol 25-7, are not only adequate to extract carotenoids from the wet biomass but also allow better results of extraction from the fresh biomass, without any further optimization (for the ethanol-based extract $6.48 \pm 0.01$ $mg_{carotenoids}/g_{alga}$ and for the surfactants $5.28 \pm 2.01$ $mg_{carotenoids}/g_{alga}$ for Tomadol 25-7 and $1.86 \pm 0.06$ $mg_{carotenoids}/g_{alga}$ for Pluronic P-123). It should be also highlighted that the use of liquid nitrogen is helpful in the algal cellular disruption. However, the use of the surfactants aqueous solutions allows the recovery of carotenoids in a more selective way, which could be observed by the analysis of the photos taken from the cells after each extraction process (Fig. A7 in Supporting information). In this case, if the amount of carotenoids extracted by ethanol is the highest ($6.48 \pm 0.01$ $mg_{carotenoids}/g_{alga}$), the selectivity of this extractive process is low, which is proved by the white color of the biomass. Essentially, the white color of the biomass is indicative of the complete depigmentation (at least indicating the removal of chlorophylls), phenomenon that did not occur in the extractions performed with the selected surfactants. On the other hand, and despite the fact that the amount of carotenoids extracted by both surfactants is lower than that obtained with ethanol, the chlorophylls are not significantly removed, which is a great advantage from the technological, industrial and economic points of view. Using the alternative approach herein developed, less steps of purification, and less solvents are required. The pure ethanol is replaced by water, creating a more sustainable process. The water-based alternative process to extract carotenoids is as effective in extracting carotenoids, but it is shown to be far more selective, avoiding the complete depigmentation of the biomass. In the proposed process the chlorophylls are not extracted

simultaneously with carotenoids providing a more pure extract that will require a simpler purification.

## 4. Conclusions

Macroalgae are natural raw materials, rich in a variety of bioactive compounds of economic and industrial interest. In this work, a more effective and sustainable process to extract carotenoids and fucoxanthin from two brown macroalgae species was developed. Since one of the major limitations of the algae processing is their large water content that often compromises the economic viability of the extraction processes, in this work the use of aqueous solutions of various non-ionic surfactants was adopted. The main experimental data suggest that the best performance of extraction of carotenoids from brown seaweeds was achieved for Tomadol 25-7 and Pluronic P-123. The best extraction conditions were also selected for Tomadol 25-7 and Pluronic P-123. For both surfactants, the $R_{(S/L)}$ was statistically significant, meanwhile, the $C_{surf}$ was only significant for Pluronic P-123. It was also shown that, while the process defined in this work was developed on a dry biomass base, the use of aqueous solutions of surfactants for the extraction of carotenoids from macroalgae can be straightforwardly extended to wet algal samples using for its advantage the water content of the biomass, in a more sustainable way. Summing up, in this work an efficient and more selective water-based method of extraction of carotenoids was developed.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.seppur.2016.07.052.

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