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Monitoring the stability of the xanthophyll fucoxanthin in microalga and seaweed biomasses, and extracts stored at low temperatures

Monitoramento da estabilidade da xantofila fucoxantina em biomassas de microalga e algas marinhas e extratos armazenados a baixas temperaturas

Monitoreo de la estabilidad de la xantofila fucoxantina en biomasas de microalgas y algas y extractos almacenados a bajas temperaturas

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Abstract
The analysis of fucoxanthin (FCX) in algal biomasses presents some limitations, especially regarding the sample preservation after collection until extraction of the pigment. FCX stability was evaluated in biomass samples of one diatom and three brown seaweed species (Phaeophyceae) and their extracts stored at -20ºC and -80ºC, for 60 days. Fucoxanthin was quantified through UHPLC-DAD. The methanolic extracts of the micro/macroalga biomasses stored at -20ºC and -80ºC were more effective in preserving the xanthophyll, comparatively to the algal biomasses. Besides, the chromatographic analysis showed an increase in the FCX's peak retention time along the sample storage, possibly due to the *trans→cis* isomerization of the molecule. Such information is relevant for choosing the best protocol for storing and preserving at most FCX, because changes in the geometric conformations of the molecule over storage strongly affects its biological activities.
Keywords: Phaeophyceae; Fucoxanthin isomers; Carotenoids; UHPLC; Bacillariophyceae.

Resumo
A análise de fucoxantina (FCX) em biomassa de algas apresenta algumas limitações, principalmente quanto à preservação das amostras após a coleta até a extração do pigmento. A estabilidade da FCX foi avaliada em amostras de biomassas e extrato de uma diatomacea e três espécies de macroalgas marrons (Phaeophyceae) armazenadas à -20ºC e -80ºC, por 60 dias. Os conteúdos de FCX nas amostras foram determinados por UHPLC-DAD. Os extratos metanólicos das biomassas de micro/macroalgas armazenadas à -20ºC e -80ºC foram mais eficazes na preservação da xantofila, comparativamente às biomassas algais. A análise cromatográfica mostrou um aumento no tempo de retenção do pico de FCX nas amostras ao longo do armazenamento, possivelmente devido à isomerização *trans→cis* da molécula. Tal informação é relevante à escolha do melhor protocolo de armazenamento para preservar ao máximo os teores daquele oxicarotenoide, porque alterações nas conformações geométricas da molécula ao longo do armazenamento impactam fortemente nas atividades biológicas da FCX.
Palavras-chave: Phaeophyceae; Isômeros de fucoxantina; Carotenoides; UHPLC; Bacillariophyceae.

Resumen
El análisis de fucoxantina (FCX) en biomasas de algas presenta algunas limitaciones, especialmente en lo que se refiere a la conservación de la muestra después de su recolección hasta la extracción del pigmento. La estabilidad de FCX se evaluó en muestras de biomasa y extracto de una diatomea y tres especies de algas marrón (Phaeophyceae) almacenados a -20ºC y -80ºC, durante 60 días. La FCX se cuantificó mediante UHPLC-DAD. Los extractos metanólicos de las biomasas de micro/macroalgas almacenados a -20ºC y -80ºC fueron más efectivos en la conservación de FCX, en comparación a las biomasas de algas. Además, el análisis cromatográfico mostró un aumento del tiempo de retención del pico de FCX a lo largo del almacenamiento de la muestra, posiblemente debido a la isomerización *trans→cis* de la molécula. Esta información es relevante para elegir el mejor protocolo de almacenamiento y preservacion de FCX, porque los cambios de conformaciones geométricas de la molécula durante el almacenamiento afectam fuertemente sus actividades biológicas.
Palabras clave: Phaeophyceae; Isómeros de fucoxantina; Carotenoides; UHPLC; Bacillariophyceae.

1. Introduction

The carotenoid market worldwide has continuously increased, being concentrated essentially in the production of astaxanthin, β-carotene, and lutein (Yabuzaki, 2017; Novoveská et al., 2019). Among the various organisms that synthesize carotenoids, microalgae are recognized as the most effective source due to their high biosynthesis and accumulation (Butler et al., 2018). The technological application of carotenoids can be associated, for instance, to nutraceutical, feeding, and natural pigment's markets (Ambati et al., 2019; Honda et al., 2020).

The commercial applications of carotenoids are directly related with their pharmacological properties in the prevention of human diseases, including cardiovascular and other chronic diseases (Christaki et al., 2013; Teixeira et al., 2014; Wood et al., 2005; Heo et al., 2010). In this sense, carotenoids have been widely used in the industry, both as ingredients, additives, food supplements, and cosmetics, mainly due to their photoprotective effects (Ambati et al., 2019; Rodriguez-Concepcion et al., 2018; Pangestuti, et al., 2018). In biotechnology, carotenoids from algae are recognized as potent natural antioxidants, performing the scavenging of singlet molecular oxygen and peroxyl radicals, due to their molecular structure composed of conjugated double bonds system (Foo et al., 2017).

Xanthophylls, one of the most important classes of carotenoids in algae, have shown positive effects on neurological, immunological, and ophthalmological systems, which translate them into new matrices for a pharmaceutical area (Aziz et al., 2020). Among the most studied xanthophylls, fucoxanthin (FCX) represents 10% of biogenic carotenoids, being found in greater quantities in brown algae and diatoms (Matsuno, 2001; Mikami & Hosokawa, 2013). FCX is a tetraterpenoid with a characteristic linear C40 molecular backbone containing up to 11 conjugated double bonds, including an allenic bond, an epoxide, and a conjugated carbonyl group in the polyene chain of the molecule (Rodriguez-Concepcion et al., 2018; Mikami & Hosokawa, 2013). The presence of conjugated double bonds in its structure contributes to the isomerization process of the molecule. In nature, FCX is found mostly in all trans forms, as cis conformers are regularly more thermodynamically unstable and prone to isomerization (Nakazawa et al., 2009). The molecular conformation of this compound is relatively unstable and sensitive to light, heating, oxygen, and storage conditions and time. Thus, FCX can be altered or even destroyed in biological samples depending on the conditions of its storage. The molecule can be altered by enzymatic or non-enzymatic oxidation and by isomerization, rearrangement, or other chemical reactions over the storage time (Schoefs, 2002; Piovan et al., 2013; Britton & Khachik, 2009).

Drying and freeze storage strategies influence the preservation and keep the predictable application of carotenoids in samples and marketed products, avoiding the oscillation in their contents and degradation. The choice method for preserving them shall consider the eventual effects on the chemical structure and stability of the molecules, and the involved costs and procedures. Usually, lyophilization has been pointed out as a more effective method for drying and preserving carotenoids in biological samples until the moment of their extraction. In laboratories, FCX molecules can also be extracted with organic

solvents and stored at low temperatures until the moment of usage (Ryckebosch et al., 2011).

In this study, FCX stability was investigated, over a 60-day experimental time, in three brown macroalgae samples (Phaeophyceae), e.g., *Colpomenia sinuosa* (Mertens ex Roth) Derbès and Solier, *Padina gymnospora* (Kützing) Sonder, and *Sargassum* sp. (Turner) C. Agardh, as well as in in vitro cultured cells of the diatom *Phaeodactylum tricornutum* Bohlin (Bacillariophyceae). For that, lyophilized biomass and methanolic extracts thereof were stored into freezers at -20ºC and -80ºC, following the chromatographic determination of their FCX contents to identify the best way to preserve that pigment for 60 days. Importantly, the algal species studied have FCX as their major carotenoid, reinforcing the importance of understanding the best strategy for preserving FCX in these marine organisms (Peng et al., 2011).

2. Material and Methods

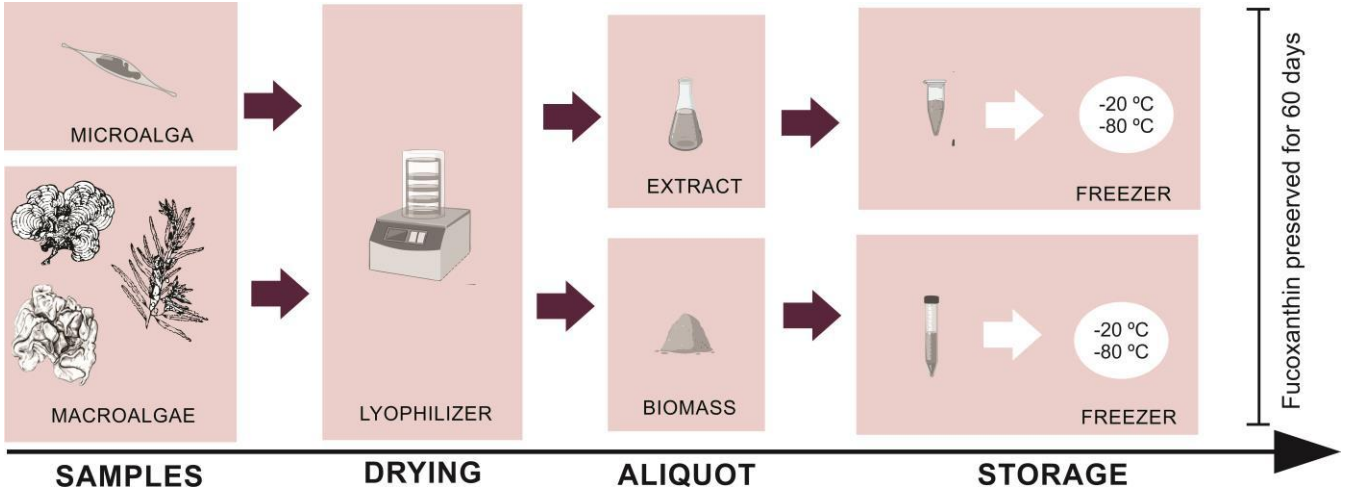
2.1 Biomass acquisition

The research is an experimental study with qualitative and quantitative data in order to evaluate the degradation of the FCX molecule. Samples of the *Padina gymnospora, Colpomenia sinuosa*, and *Sargassum* sp. were collected at Barra da Lagoa Beach, Florianópolis, and transported in thermo boxes to the Plant Morphogenesis and Biochemistry Laboratory (Federal University of Santa Catarina, Florianópolis, SC). Macroalgal epiphytes were eliminated by cleaning up the macroalgal thalli with a brush and washing them with filtered water. In its turn, the *Phaeodactylum tricornutum* cell cultures were grown in f/2 medium (500mL Erlenmeyer flask, 250mL working volume) for seven days (end of the log phase). After that, cell cultures were collected, centrifuged (4,000g x 9min), the supernatant discarded, and the pellet washed with with 0.5M ammonium formate solution for salt removing and centrifuged again (4,000g x 9min).

2.2 Sample drying and storage

For the seaweed biomasses, two thermal conditions of storage in freezer apparatus were tested (Figure 1). Initially, the fresh biomasses of macro- and microalgae were lyophilized for 55h and separated into two samples. The first one underwent an organosolvent extraction protocol, following storage at -80ºC or -20ºC. The second aliquot of the algal biomass was transferred to dark falcon tubes and reserved at -80ºC and -20ºC. Further, on the sampling days (1, 7, 21, 28, and 60), FCX was extracted and recovered for quantitation analysis to determine the most efficient sample type for storage, i.e., biomass or organosolvent extract.

Figure 1 - Workflow of the main steps of the study. Four different protocols for storage of the algal biomasses and methanolic extracts were investigated, regarding the FCX concentration and stability.



Source: Authors (2022).

2.3 Fucoxanthin extraction and quantification

The oxycarotenoid was extracted for 24h from the algal biomasses using methanol (p.a.) in a 1: 10 and 1: 50 dry biomass (mg) and solvent (mL) ratios for seaweeds and *P. tricornutum* cells, respectively. Following extraction, the FCX contents in the organosolvent extracts were chromatographically determined on days 1, 7, 21, 28, and 60 (Figure 1). An aliquot of each extracted sample (10 µL, n = 4) was injected into high-performance liquid chromatograph (Shimadzu LC-10 A), equipped with a C18 reverse-phase column (Vydac 218TP54, 250 mm x 4.6 mm, Ø 5 µm, 30°C), protected by a C18 reverse-phase guard column (Vydac 218GK54, Ø5 µm), and a UV-vis detector (λ = 450 ηm). Elution was performed with MeOH: CH_3CN (90: 10, v/v) at 1 mL min- flow rate. FCX identification was performed using the retention time and co-chromatography of analytical standard (all-trans fucoxanthin, CASS number 3351868 - Sigma-Aldrich, St. Louis, MO, EUA). Metabolite quantification was based on the standard curve of FCX (1- 25 µg mL-, $y = 0.0513x$, $r^2 = 0.999$). Readings were taken in quadruplicate and results were expressed as mean (mg FCX/g dry biomass) ± standard deviation.

2.4 Data analysis

Chromatographic data were collected, summarized, and submitted to a unifactorial analysis of variance (ANOVA), following by the Tukey *post-hoc* test ($p < 0.05$) when appropriate. Data were analyzed by mixed linear models and the correlation coefficient (r^2) was calculated to determine the goodness of fit between the experimental and predicted values. A mixed effects linear regression model was utilized to assess the influence of time and storage forms, as well as their interactions regarding the FCX contents. The metabolite concentration was used as the dependent variable, as time, temperature and their interactions as the fixed effect and the repeated measurement as random effect. For that, scripts written in R language (v. 4.1.0) were used for the regression models investigated.

3. Results and Discussion

Four protocols for storage of algal biomass and methanolic extracts thereof were investigated regarding their suitability for FCX conservation. The methanolic extract proved to be more effective in preserving the molecule in comparison to the dried biomasses, regardless the algal species (Table 1). After 60 days, the smallest reduction in FCX concentration noted was 0.1 mg/g for all species. Thus, this finding demonstrates that FCX storage in extraction solvent at low temperatures seems to be suitable for protecting the molecule from degradation processes.

Table 1 - Fucoxanthin contents (mg FCX/g dry weight biomass) determined by ultra-high performance liquid chromatography (UHPLC-DAD) in lyophilized algal biomasses and methanolic extracts thereof. Four different storage procedures were followed as described in Material and Methods section. In bold, values where significant responses were found ($p<0.05$) according to the ANOVA, as the letters represent the *post-hoc* Tukey test for days of storage.

Species	Storage	Days					
		1	7	21	28	60	Pr(>F)
Phaeodactylum tricornutum	Biomass (-20ºC)	8.1 ± 0.01 ab	8.5 ± 0.03 a	9.1 ± 0.3 a	6.9 ± 0.1 ab	6.5 ± 0.1 c	**7.9e-06**
	Biomass (-80ºC)	8.1 ± 0.01 ab	7.8 ± 0.03 ab	7.2 ± 0.4 b	8.6 ± 0.3 a	7.2 ± 0.1 b	**0.04**
	Extract (-20ºC)	8.1 ± 0.1 a	8.2 ± 0.1 a	8.2 ± 0.03 a	7.9 ± 0.02 a	8.0±0.1 a	0.12
	Extract (-80ºC)	8.1 ± 0.01 a	8.5 ± 0.05 a	8.6 ± 0.03 a	7.8 ± 0.07 a	8.1 ± 0.1 a	0.44
Padina gymnospora	Biomass (-20ºC)	1.0 ± 0.04 a	1.1 ± 2.7 a	1.2 ± 0.15 a	1.1 ± 0.03 a	1.0 ± 0.02 a	0.85
	Biomass (-80ºC)	1.0 ± 0.04 a	1.3 ± 0.04 a	1.1 ± 0.03 a	1.1 ± 0.01 a	1.0 ± 0.02 a	0.28
	Extract (-20ºC)	1.0 ± 0.04 a	0.9 ± 0.01 a	1.1 ± 0.02 a	0.9 ± 0.03 a	0.9 ± 0.01 a	0.06
	Extract (-80ºC)	1.0 ± 0.04 a	0.9 ± 0.01 bc	1.0 ± 0.01 ab	0.9 ± 0.003 bc	0.9 ± 0.01 c	**2.0e-04**
Colpomenia sinuosa	Biomass (-20ºC)	0.11 ± 0.01 bc	0.16 ± 0.0003 a	0.14 ± 0.01 ab	0.11 ± 0.005 bc	0.09 ± 0.003 c	**3.0e-04**
	Biomass (-80ºC)	0.11 ± 0.01 a	0.14 ± 0.004 a	0.08 ± 0.01 a	0.12 ± 0.09 a	0.12 ± 0.01 a	0.87
	Extract (-20ºC)	0.11 ± 0.01 a	0.11 ± 0.0004 a	0.14 ± 0.001 a	0.12 ± 0.001 a	0.11 ± 0.01 a	0.59
	Extract (-80ºC)	0.11 ± 0.01 a	0.11 ± 0.001 a	0.12 ± 0.001 a	0.11 ± 0.003 a	0.12 ± 0.002 a	0.01
Sargassum sp.	Biomass (-20ºC)	0.38 ± 0.01 a	0.33 ± 0.001 a	0.20 ± 0.01 b	0.16 ± 0.001 b	0.15 ± 0.002 b	**1.7e-09**
	Biomass (-80ºC)	0.38 ± 0.01 a	0.36 ± 0.004 a	0.19 ± 0.001 b	0.19 ± 0.0007 b	0.19 ± 0.001 b	**5.2e-07**
	Extract (-20ºC)	0.38 ± 0.01 a	0.37 ± 0.004 a	0.39 ± 0.01 a	0.38 ± 0.001 a	0.39 ± 0.008 a	0.71
	Extract (-80ºC)	0.38 ± 0.01 ab	0.37 ± 0.004 b	0.39 ± 0.001 a	0.39 ± 0.001 a	0.39 ± 0.01 a	**7.5e-03**

Source: Authors (2022).

By comparing the results of FCX amounts in the investigated species, one can see higher values for the microalga species *P. tricornutum* sample, i.e., 8.1 mg/g dry weight biomass. Among the macroalgae species, *P. gymnospora* showed the highest amount with 1 mg FCX/g dry weight biomass. On their turn, *Sargassum* sp. and *C. sinuosa* presented contents of 0.38 and 0.11 mg FCX/g, respectively. These species were selected for the study because they present FCX as their main carotenoid, being widely recognized in the group of brown algae as sources of that pigment (Foo et al., 2017; Prabhasankar et al., 2009). However, it is notorious the discrepancies between the FCX contents detected in the macroalgae samples in respect to the microalga one. Indeed, among the algal species studied, *P. tricornutum* proved to be much more effective in the synthesis and accumulation of FCX, which reinforces its importance in biotechnological applications.

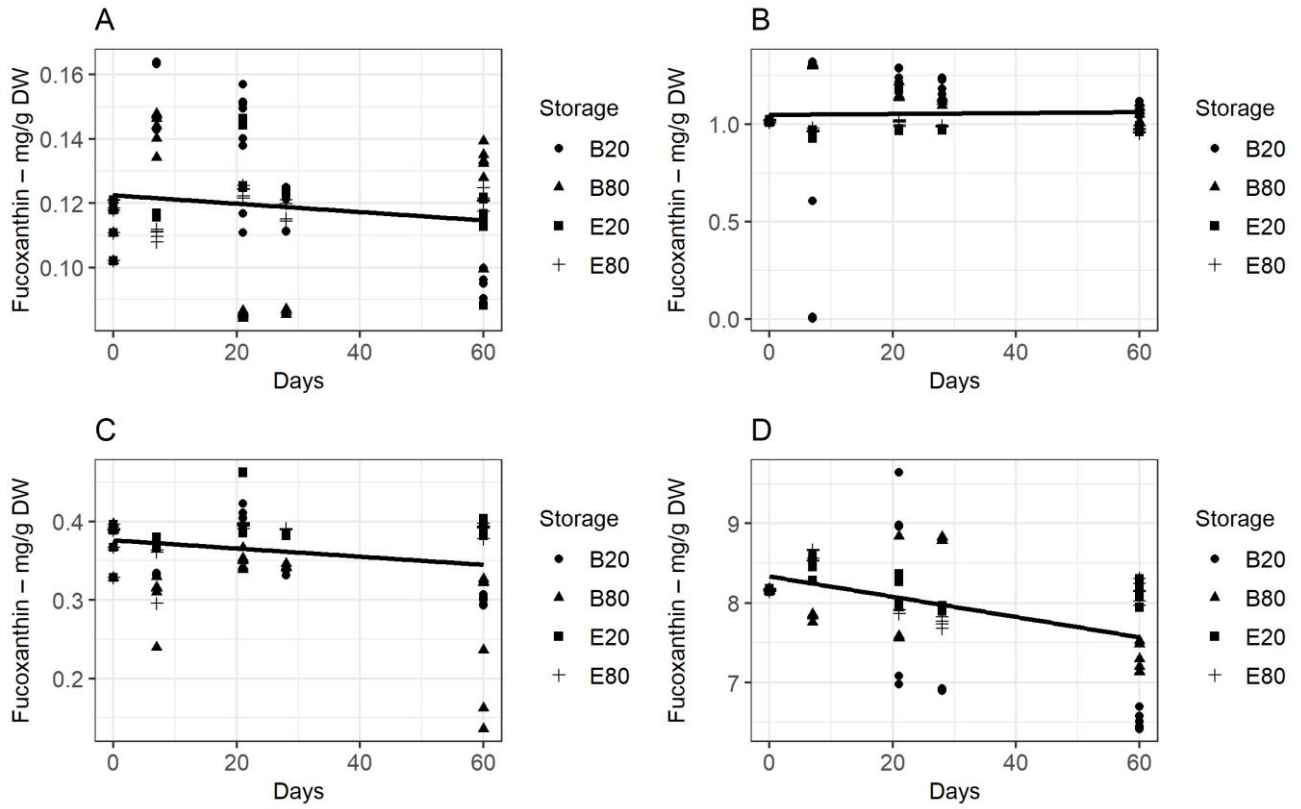
The results demonstrate that by storing lyophilized algal biomasses, even in a low-temperature freezer (i.e. -20ºC), the reduction in the loss of FCX might be achieved in a certain extension. This finding also showed to be dependent on the algal species. Indeed, FCX contents in *C. sinuosa* and *P. gymnospora* lyophilized biomasses and methanolic extracts seemed to

be stable for longer periods in respect to the other algal species investigated. However, *Sargassum* sp. and *P. tricornutum* presented a meaningful lowering in their FCX contents in the biomasses stored both at -20ºC and -80ºC. *Sargassum* sp. reduced by ~60% (from 0.38 → 0.15 mg/g) in the biomass samples when stored at -20ºC and by 50% (0.38 → 0.19 mg/g), following a 60 day-storage at -80ºC. *P. tricornutum* lyophilized biomass presented lower FCX losses, ranging from 8.1 mg/g to 6.5 mg/g (~20%) and 7.2 mg/g (~11%) after 60 days stored at -20ºC and -80ºC, respectively.

The difficulty of drying biomass through lyophilization, especially in remote places, makes the search for alternative methods for biomass and FCX conservation a continuous effort. Thus, two lower cost methods eventually more accessible were investigated, e.g., oven (45ºC) and silica gel (room temperature), for water removal from algal biomass. Interestingly, oven proved to be the drying method with greater degradation of FCX, practically extinguishing the compound just after 25h exposure at 45ªC (data not shown). For instance, losses of about 90% and 84% FCX were detected in *P. gymnospora* and *Sargassum* sp. samples. Moreover, only at day 21 silica gel-dried samples presented similar FCX amounts in respect to the oven (data not shown). The negative effects on FCX stability of drying biomass in the oven and exposure to air were already expected. The degradation of these molecules as a function of positive temperatures as an isolated factor follows the first-order kinetics of Arrhenius law (Bechoff et al., 2010). When samples are oven- or silica gel-dried, one can have the synergistic effect of temperature and oxygen on the degradation kinetics. These findings make clear that when looking for the use of carotenoids such as FCX, drying biomass in an oven or on silica gel pads is strongly discouraged.

To understand how time (measured in days) and the storage method (biomass and methanolic extract) influence the FCX content individually, a linear mixed-effect model by restricted maximum likelihood was performed. The mixed linear regression models indicate the continued degradation of FCX in both biomass samples and methanolic extracts over the 60-day experimental time (Figure 2). These mathematical models allowed us to describe a time trend considering the correlation between factors time and storage. Interestingly, among the four species herein investigated, only *Sargassum* sp. (r^2=0.5006, p=1.82e^{-11}) and *P. tricornutum* (r^2=0.4405, p=6.708e^{-11}) afforded mathematical models suitable for the study by mixed linear models (Table 2). The regression models support the results found through ANOVA of the storage methods, where *C. sinuosa* and *P. gymnospora* demonstrated stability of the FCX content, not differing in their contents of that secondary metabolite (Table 1). The content of that oxycarotenoid was negatively influenced by the factor time, being the microalga *P. tricornutum* the most impacted (angular coefficients= -0.0338, p = 3.00^{e-7}) in comparison to *Sargassum* sp. (angular coefficient= -0.0010735, p = 0.0010).

Figure 2 - Data distribution of FCX contents (mg/g) during storage of algal biomasses and their methanolic extracts at -20º and -80ºC (n = 5 per species/time). The mixed linear models built (in black) indicate a wide range of values detected for the variable in study and suggest a specie-specific dependence thereof over the storage time. The mathematical models represent the distribution of the response variable (FCX content) by species and storage form (caption) in relation to the continuous variable time (day), where a) *Colpomenia sinuosa* model, b) *Padina gymnospora* model; c) *Sargassum* spp. model, and d) *Phaeodactylum tricornutum* model.



Source: Authors (2022).

The regression models rejected the null hypothesis with p-values significant ($p<0.05$) for the fixed factor time (Table 2). By associating the fixed factor (time) with the forms of storage, a tendency it was noted a greater stability of FCX in the methanolic extract, with lower p-values compared to those associated with the biomass method. The temperatures of storage (-20ºC and -80ºC) did not prove to be a critical point for maintaining stable FCX in the methanolic extract over 60 days. However, when FCX was stored into the algal biomasses, the data show that 21 days was the cut-off point for being kept at -20ºC (Table 1). Thus, when storing FCX in biomass for longer periods (over 21 days), it is advisable to use lower temperatures, as in the case -80ºC. In summary, the methanolic extract seems to be the best way to preserve the FCX molecule at -80ºC. On the other hand, by storing FCX in the algal biomass, lower temperatures are required to preserve it longer, since biomass storage at -20ºC showed to be the most harmful way of retaining the integrity of the pigment's molecular structure according to the models investigated.

Table 2 - Fitting mixed linear models considering the range of fucoxanthin contents in biomass samples and methanol extracts (n = 5 per species/time) stored in freezer at -20ºC or -80ºC. Two variables were used for building the mathematical model, days, and methods of storage. In bold, values where significant responses were found ($p < 0.05$).

Species	Residual	r^2	F value	p-value	Variable	Estimate	SD	t value	Pr(>|t|)
C. sinuosa	0.0173	0.181	3.458	**0.002**	Intercept	0.1388	0.0049	28.12	**2.00E-16**
					day	-0.0006	0.0001	-3.890	**2.08E-03**
					storageB80	-0.0262	0.0068	-3.838	**0.0002**
					storageE20	-0.0147	0.0068	-2.168	0.0323
					storageE80	-0.0235	0.0068	-3.465	**0.0007**
					day: StorageB80	0.0005	0.0002	2.757	**0.0068**
					day: StorageE20	0.0005	0.0002	2.489	**0.0143**
					day: StorageE80	0.0007	0.0002	3.366	**0.0010**
P. gymnospora	0.0304	0.126	2.307	**0.0310**	Intercept	0.987	0.0468	21.111	**2.00E-16**
					day	0.003	0.0015	1.968	**0.0516**
					storageB80	0.177	0.0662	2.675	**0.0086**
					storageE20	0.046	0.0662	0.690	0.4917
					storageE80	0.017	0.0662	0.250	0.8030
					day: StorageB80	-0.004	0.0021	-1.953	0.0533
					day: StorageE20	-0.003	0.0021	-1.568	0.1196
					day: StorageE80	-0.003	0.0021	-1.617	0.1086
Sargassum sp.	0.0016	0.500	16.04	**1.820E^{-11}**	Intercept	0.375	0.0098	38.382	**2.00E-16**
					day	-0.001	0.0003	-3.392	**0.0010**
					storageB80	-0.011	0.0138	-0.791	0.4306
					storageE20	0.018	0.0138	1.270	0.2068
					storageE80	-0.002	0.0138	-0.157	0.8753
					day: StorageB80	-0.001	0.0005	-1.312	0.1922
					day: StorageE20	0.001	0.0005	2.948	**0.0039**
					day: StorageE80	0.001	0.0005	3.315	**0.0012**
P. tricornutum	0.4829	0.440	11.7	**6.708E^{-11}**	Intercept	8.650	0.1304	66.335	**2.00E-16**
					day	-0.033	0.0042	-8.007	**1.78E-12**
					storageB80	-0.463	0.1861	-2.487	**0.0144**
					storageE20	-0.404	0.1844	-2.196	**0.0303**
					storageE80	-0.385	0.1905	-2.022	**0.0457**
					day: StorageB80	0.022	0.0059	3.799	**0.0002**
					day: StorageE20	0.031	0.0059	5.324	**5.89E-07**
					day: StorageE80	0.029	0.0059	4.991	**2.42E-06**

Source: Authors (2022).

It has been reported that temperature influences on the oxidative degradation of all-*trans* molecules and allow their isomerization into *cis* conformation (Zhao et al., 2014). This occurs because the effect of temperature on FCX causes more intense geometric isomerization, producing more unstable Z isomers, while the total content of the oxycarotenoid is not changed significantly, but more prone to generate apocarotenoids (Aparicio-Ruiz; et al., 2011).

Regarding the amounts of FCX found in this study, one can note that by storing that pigment in organosolvent, i.e., methanol, it seems to be the best way to preserve it in comparison to the algal biomass. However, by analyzing UHPLC profiles of the methanolic extracts, it was possible to note the increase in the retention time of that compound for all the samples investigated, eventually demonstrating a change in the FCX's molecular conformation (Table 3). The increase in retention times of compounds in liquid chromatography methods is typically found as result of the conversion of *trans* isomers into *cis* ones (Nakazawa et al., 2009; Britton & Khachik, 2009). For instance, FCX was extracted from *Fucus* sp. and over 80% of the pigment content occurred as E (*trans*) isomers. When kept at low temperature (5°C) for two months, the FCX molecule underwent geometric isomerization for the Z (*cis*) conformation, increasing its retention time in the chromatographic profile (Nakazawa et al., 2009), corroborating the findings of this study. Those authors, using HPLC and NMR spectroscopy indicated that the main *cis* isomers found were 13-*cis*, 13′-*cis*, and 9′-*cis* FCX. Thus, by storing FCX at low temperatures influences the molecule's geometric conformation, eventually without expressive reduction in its content in samples. Importantly, the isomerization of the molecule can subsequently affect its stability in the extract and its biological activities.

Table 3 - Retention times (min) for monitoring the all-*trans* fucoxanthin conversion to its *cis* conformer in methanol extracts stored during 1, 7, 21, 28, and 60 days by UHPLC-DAD. Fucoxanthin was extracted from *P. tricornutum*, *P.gymnospora*, *C. sinuosa*, and *Sargassum* sp. biomasses, following the protocols described in Material and Methods section and summarized in Figure 1.

Species	Storage (days)	Retention time (min)				
		1	7	21	28	60
P. tricornutum	Biomass (-20ºC)	2.053	2.057	2.090	2.107	2.083
	Biomass (-80ºC)	2.053	2.063	2.093	2.113	2.083
	Extract (-20ºC)	2.053	2.067	2.093	2.103	2.083
	Extract (-80ºC)	2.053	2.073	2.097	2.107	2.083
P. gymnospora	Biomass (-20ºC)	2.053	2.057	2.087	2.103	2.083
	Biomass (-80ºC)	2.053	2.063	2.093	2.113	2.083
	Extract (-20ºC)	2.053	2.070	2.093	2.103	2.083
	Extract (-80ºC)	2.053	2.073	2.097	2.103	2.083
C. sinuosa	Biomass (-20ºC)	2.053	2.057	2.083	2.097	2.083
	Biomass (-80ºC)	2.053	2.063	2.093	2.113	2.083
	Extract (-20ºC)	2.053	2.067	2.093	2.103	2.083
	Extract (-80ºC)	2.053	2.070	2.097	2.103	2.083
Sargassum sp	Biomass (-20ºC)	2.060	2.063	2.093	2.107	2.083
	Biomass (-80ºC)	2.060	2.067	2.093	2.103	2.08
	Extract (-20ºC)	2.060	2.073	2.097	2.103	2.083
	Extract (-80ºC)	2.060	2.073	2.097	2.107	2.083

Source: Authors (2022).

4. Conclusion

The methanolic extracts kept at -20ºC and -80ºC preserved the contents of FCX longer than the biomasses of the algal species herein studied. Besides, the type of the FCX's geometric conformation resulting from each form of storage, i.e., biomass or extract, also must be considered, knowing that the conformation of molecule strongly determines its bioactivity. This information is relevant in connection with quality control protocols of stored samples containing that pigment, especially because the bioactivity of the different geometric conformations of the molecule is well known and important as to their applications in biotechnological products and processes. The present research verified the isomerization of the FCX molecule in relation to the retention time in the chromatographic spectrum. For new studies of stability and isomerization of the molecule, it is necessary to study the patterns of the different FCX isomers in order to qualify these molecules in terms of their molecular conformation.

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