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Effect of drying biomass protocols on bioactive compounds, antioxidant and enzymatic activities of the red macroalga Kappaphycus alvarezii

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Abstract

Kappaphycus alvarezii is a red seaweed widely used throughout the world, with applications in various biotechnological processes. In this sense, the contents and stability of bioactive compounds of interest synthesized by this seaweed species must be guaranteed in post-harvest, by adopting suitable drying protocols of biomass (e.g.) that allow furnishing high-quality raw material for industrial usage. This study aimed to analyze the influence of commonly used drying methods, i.e., freeze-drying and oven-drying, on the total phenolic content (TPC), total flavonoid content (TFC), antioxidant activity (FRAP and DPPH assays), total carotenoid content (TC), and lipase (LA) and protease activities (PA) of *K. alvarezii* samples collected over the seasons in sea farms in southern Brazil. The freeze-drying technique showed to be more effective regarding superior contents of TPC and TC, besides antioxidant activity (DPPH assay). In its turn, oven-drying proved to be the best method regarding the TFC and PA, with better performance in the FRAP assay. In this way, it appears that the drying process of the algal biomass can be selected depending on the required traits of the biomass for the intended industrial application.

1 Introduction

Seaweeds have been used as foods and medicines for at least 14,000 years. However, large-scale cultivation is a relatively recent branch of aquaculture, dating back to the mid-20th century, when global industrial production of algae was close to zero tons (Ullmann and Grimm 2021). The increase of productivity of aquaculture production systems has occurred especially in the last 20 years, with a global production of algal biomass showing more than threefold growth, from 11.8 million tons to 35.8 million tons between 2000 and 2019 (FAO 2021). It was estimated that the global seaweed market could reach USD 24.9 billion in 2028, with a compound annual growth rate (CAGR) by 7.51% in the 2021–2028 period (Fortune Business Insights 2021).

Kappaphycus alvarezii, the fifth most cultivated seaweed in the world, has been widely studied for its use in food, pharmaceutical, and agricultural industries. Belonging to the *Florideophyceae* class, and *Rhodophyceae* division, such a species can be found with red, yellow, brown, and green colors according to its contents of phycoerythrin pigment. Due to its fast growth (4.5% per day), this species is the main industrial source of κ-carrageenan, a polysaccharide representing up to 37% of the seaweed's dry mass. In addition, it contains carbohydrates, fatty acids, proteins, lipids, sulfated polysaccharides, amino acids, and phenolic compounds (Rudke et al. 2020, Rudke et al. 2022). Due to its composition, the pharmacological potential of *K. alvarezii* has been recognized, with antioxidant and anti-inflammatory (Makkar and Chakraborty 2018), antitumor (Chang et al. 2017; Papitha et al. 2020), antiviral (Rudke et al. 2020; Álvarez-Viñas et al. 2021), and antidiabetic activities (Makkar and Chakraborty 2017), as well as for neural dysfunction therapy (Tirtawijaya et al. 2018). However, a series of genetic and environmental factors can interfere with the contents of bioactive compounds in the alga, which could directly impact its industrial applications, where composition standardization is required. Indeed, climatic factors (especially exposure to light, salinity, and heat) during cultivation, as well as biomass processing methods in post-harvest and inadequate storage can lead to composition modification and bioactive

compound degradation (Jimenez-Lopez et al. 2021). In this sense, the method adopted for drying biomass is of utmost importance to prevent the degradation of molecules with nutraceutical properties, for instance. Such an approach is particularly relevant for biomass drying of aquatic products, which can sum up to 85% water content, in order to minimize chemical and enzymatic reactions that lead to the reduction of nutrients, as well as microbial contamination. In this way, the maximization of biomolecule stability during biomass storage is possible through suitable drying techniques, ensuring the quality of raw material for industrial applications (Wang et al. 2019).

Among the commonly used drying techniques, lyophilization and oven drying can be found. These processes have different characteristics, especially in terms of cost, processing time, and efficacy in preserving the chemical composition of samples. Therefore, the choice between those techniques can be defined according to the final use of algal biomass (Oliveira et al. 2020). In this sense, this study aimed to investigate the effect of drying methods on the secondary metabolite profiles (e.g., phenolic, flavonoid, and carotenoid contents), antioxidant activity (DPPH and FRAP assays), and lipase and protease activities of *K. alvarezii* cultivated in two sites in southern Brazil, over the seasons of 2022. It has been hypothesized that by optimizing the biomass drying step, a raw material of superior quality will be offer to the market, optimizing the biotechnological potential and usage of *K. alvarezii.*

2 Material and Methods

2.1 Sample collection

Samples of *K. alvarezii* were obtained from two marine farms located in the municipalities of Florianópolis (RIB - Ribeirão da Ilha, 27° 42' 32.7" S, 48° 33' 35.5" W) and Palhoça (PAL – 27° 46' 29.9” S, 48° 37' 50.7” W), in the state of Santa Catarina, southern Brazil, in 2022. Santa Catarina state falls under the Cfa Köppen climate classification, with subtropical climate with hot summers, mild winters, and evenly distributed rainfall throughout the year. Samplings were carried out seasonally as follows: two samples were collected in the late autumn (RIB-2 and PAL-2), four in the winter (early: RIB-3, PAL-3; late: RIB-4, and PAL-4), four during the spring (early: RIB-5, PAL-5; late: RIB-6, and PAL-6), and two in the early summer (RIB-7 and PAL-7), resulting in a total of 12 samples (Fig. 1). Both, red and green thalli of *K. alvarezii* were sampled and mixed for the drying process.

2.2 Biomass drying

The collected alga biomass was firstly water-tap washed to remove salt, impurities, and encrusting organisms, followed by packaging in plastic bags and storage at -4°C for subsequent drying by freeze-drying and oven-drying. Biomass samples (15 g, fresh material) were utilized in triplicate for each site of collection and season, yielding an average dry weight of 2.5 g ± 0.7 g and 83.8% ± 1.7% moisture content for both drying methods investigated. Freeze-drying was carried out for 10h (0.040 mbar vacuum, condensation chamber at -50ºC), while the oven drying process was carried out for seven consecutive days at ~ 60ºC, until constant biomass dry weight. No significant differences were observed (*t*-student,

p = 0.678) between the drying protocols regarding the moisture content and dry weight of the *K. alvarezii* samples, regardless the seasons and site of collection.

2.3 Total phenolic content

The methodology proposed by Singleton and Rossi (1965) was used, with adaptations for the analysis of total phenolics. In short, 500 mg freeze-dried and oven-dried *K. alvarezii* samples were used, followed by adding 5 mL acidified methanol (80% methanol, 1% acetic acid, and 19% distilled water) as extracting solvent. The samples were submitted to ultrasonic bath (20 min), centrifuged (5,000 rpm, 10 min), and supernatants collected by filtration (filter paper 0.22 mm) under reduced pressure and stored in 10 mL amber glass flasks. The extraction process was repeated once again on precipitated material and supernatants were combined.

For determination of the total phenolic content, sample (0.5 mL) was added of 0.5 mL distilled water, 0.5 mL Folin-Ciocalteau reagent, and 2.5 mL sodium carbonate (20%, w/v). The test tubes were vortexed and the reaction was allowed to proceed for one hour. The absorbance reading was performed in glass cuvettes using an UV-vis spectrophotometer (BEL photonics®, SP 2000 UV/vis) at 725 nm. For the calibration curve ($y = 0.0282x$, $r^2 = 0.9965$), gallic acid (GAE, Sigma-Aldrich, MO, USA) was used as analytical standard, at 0, 5, 15, 25, and 35 µg/mL concentrations.

2.4 Total flavonoid content

Total flavonoid content (TFC) was determined according to the methodology described by Awad et al. (2000), with adaptations. Thus, 500 mg freeze-dried and oven-dried seaweed samples were transferred to 10 mL amber glass flasks, followed by the addition of 4 mL acidified methanol. After homogenization and ultrasonic bath treatment (30 min), 1 mL aluminum chloride (5%) was added, and a new homogenization was carried out. The obtained sample was kept in a dark environment for 30 min. Afterward, the sample was centrifuged (6,000 rpm, 25 min) at 5ºC and an aliquot (4 mL) was collected, followed by the addition of 1 mL aluminum chloride. The supernatant was removed and the absorbance read (UV-vis spectrophotometer - BEL photonics®, SP 2000 UV/vis) at 425 nm. The calibration curve ($y = 0.013x$, $r^2 = 0.9788$) was performed using quercetin as analytical standard (QE, Sigma-Aldrich, MO, USA) at 20, 40, 60, 80, and 100 µg/mL concentration.

2.5 Antioxidant activity

The antioxidant activity of the methanolic extract of *K. alvarezii* was determined using the ferric reducing antioxidant power assay (FRAP, Benzie et al. 1996) and the 2,2-diphenyl-1-picryl-hydrazyl-hydrate one (DPPH, Rossetto et al. 2009). For both analyzes, 500 mg freeze-dried and oven-dried seaweed samples were used. For the FRAP assay, 10 mL acidified methanol were added to 500 mg algal sample. After being submitted to an ultrasonic bath (10 min), the material was centrifuged (6,000 rpm, 10 min at 5ºC). In test tubes, 900 µL FRAP reagent, 30 µL sample, and 90 µL distilled water were added. The blank was prepared in the same way, replacing the seaweed extract with 30 µL extracting solvent (acidified methanol). The absorbance reading was performed at 594 nm (UV-vis spectrophotometer, BEL

photonics®, SP 2000 UV/vis). The calibration curve was built with iron sulfate ($FeSO_4$ – y = 0.0007x, r^2 = 0.9973) at 166.7, 333.3, 500.0, 666.7, 833.3, and 1000 mmol (m/v) concentration. The activity was expressed in mmol $FeSO_4$- reduced per g fresh mass.

For the DPPH assay, biomass samples were weighed (500 mg) and transferred to falcon tubes (15 mL). Then, 10 mL methanol were added, followed by stirring (vortex) until homogenization and an ultrasonic bath treatment (15 min), The methanolic extract was recovered by centrifuging (3,000 rpm, 10 min, 5ºC). In a test tube, 500 µL supernatant, 3 mL methanol, and 300 µL DPPH were added. The blank was prepared in the same way, by replacing the sample extract with 500 µL extracting solvent (acidified methanol). The reading of absorbance was performed at 517 nm (UV-vis spectrophotometer, BEL photonics®, SP 2000 UV/vis). For the DPPH scavenging activity calculation, the following equation was used:

$$DPPH\% = \frac{Blankabsorbance - Sampleabsorbance}{Blankabsorbance} x100$$

After obtaining the % reduced DPPH, a standard curve with Trolox at concentrations of 0.65, 1.31, 1.97, 2.63, 3.28, and 3.90 µg/mL (y = 23.606x, r^2 = 0.9961) was built.

2.6 Total carotenoids

For the analysis of total carotenoids (TC), the methodology proposed by Sims and Gamon (2002) was used. Oven-dried and freeze-dried *K. alvarezii* samples (100 mg) were centrifuged (2,000 rpm, 5 min) after the addition of 5 mL acetone, buffered with Tris hydrochloride (Tris-HCl) and vortex homogenization (5 min). After removing the supernatant, filtration was performed and the absorbance of the organosolvent extract was measured in a glass cuvette (UV-vis spectrophotometer, BEL photonics®, SP 2000 UV/vis) at 663 nm (chlorophyll a), 647 nm (chlorophyll b), 537 nm (anthocyanin), and 470 nm (carotenoids). The calculation was performed according to the following equation:

$$TC = \frac{A_{470} - (17.1x\,(Chlorophylla + chlorophyllb) - 9.479xAnthocyanin)}{119.26}$$

2.7 Lipase and protease activities

The lipolytic activity of *K. alvarezii* samples was determined according to the method described by Okino-Delgado and Fleuri (2014). Briefy, samples (0.25 g) were mixed with 5 mL extra virgin olive oil and 7% Arabic gum solution (1: 4, v/v) and 3 mL phosphate buffer solution (0.1 M, pH 7.0). Controls consisted of the same emulsion volume and phosphate buffer. The reaction mixture was incubated for 30 min at 40ºC in a thermostatic bath with agitation at 130 opm (oscillations per minute). The reaction was stopped by the addition of an acetone: ethanol (1: 1, v/v) solution. Fatty acids released by the hydrolysis of olive oil lipids were titrated with 0.05 M NaOH solution using phenolphthalein as indicator. For the evaluation of the enzymatic activity, a lipolytic activity unit was defined as the amount of lipase able to release 1 µmol fatty acid per minute (U/g), under the described test conditions.

For the proteolytic activity, casein was used as substrate as described by Obata et al. (1977), Rowley and Bull (1977), and Ferracini-Santos and Sato (2009), with modifications. The reaction mixture containing 1.5 mL casein solution (2% w/v), 1 mL sodium phosphate buffer (0.1 M, pH 7.0), and 50 mg sample was incubated in a water bath (SL 150/10 – Solab) at 30°C for 30 min. The reaction was stopped by adding 2 mL trichloroacetic acid (10% w/v), followed by centrifuging at 6000 rpm, for 15 min, at 4°C. The absorbance was determined at 280 nm (UV-vis spectrophotometer, BEL photonics®, SP 2000 UV/vis), and an activity unit was defined as the amount of protease able to increase the absorbance by one unit, under the test conditions, according to the following equation.

$$U/g = \frac{(Sampleabsorbance - Blankabsorbance)/0.1)}{0.05}$$

The results were expressed in U/g.

2.8 Statistical analysis

All freeze-dried and oven-dried *K. alvarezii* samples were analyzed in triplicate (n = 3). Data were individually analyzed considering the two drying methods by ANOVA and Bonferroni correction ($p \leq 0.05$) for the total contents of phenolic compounds, flavonoids, and carotenoids, besides antioxidant activity (i.e., DPPH and FRAP assays). For the lipase and protease activities, ANOVA was performed followed by Sidak's multiple comparison test ($p \leq 0.05$).

The Scott & Knott test (Scott & Knott, 1974) with a 5% error probability was applied for comparing means in order to analyze the collection sites, without considering the drying process, using scripts written in R language (Version 4.0.2). Subsequently, all variables were subjected to the principal component analysis (PCA) in order to verify the influence of drying methods between collection sites. PCA was calculated using the singular value decomposition (SVD) algorithm for matrix factorization. For the generation of PCAs, Unscrambler® X (Version 10.4) statistical software was used. Besides, linear discriminant analysis (LDA) was performed with support of the Past software (Version 4.03) as a classification model.

3 Results and Discussion

Drying methods commonly used for terrestrial plants and marine organisms can differently avoid degradation processes of bioactive compounds in harvested biomass, depending on the specific organism. In regards to seaweeds, such organisms are well-known for their high-water content (60 to 94%) as their commercialized products are usually proposed in a dried form, which implies the drying of the alga biomass, both for sale *in natura* or even for inclusion as additive in other products (Gaidhani et al. 2015). Water content is also one of the main causes of the progression of unwanted chemical and enzymatic reactions, which can compromise the contents and stability of bioactive compounds. This occurs because a high-water activity is a prerequisite for all enzymatic reactions in cells and eventual

contaminant microorganisms. Moreover, it causes an increase of O_2 transfer rate, which accelerates the degradation of bioactive molecules through oxidative reactions, for instance (Cao et al. 2021).

Due to the growing market demand of seaweeds, the drying process needs to be optimized for industrial applications. Ideally, for large scale adoption, a biomass drying protocol shall be fast, cost-effective, environmentally-friendly, and also causing minimum loss of targeted chemical constituents in the raw material. Thus, the degradation of molecules with biological effects in seaweed biomass is an obvious goal and must be minimized for their further applications in food, cosmetic, pharmaceutical, agriculture, and feed sectors (e.g.). Commonly, in several parts of the world, industry has adopted drying protocols by exposure of biomass to sun/open air or using forced air tunnels. However, even though these methodologies are sustainable and cost-effective, they are conditioned by several factors which can interfere with the quality and the composition of the final product (Silva et al. 2019). The adoption of more refined techniques for drying, even somewhat more expensive, such as freeze-drying, can be essential for the development of high added-value products, as these methods can guarantee higher amounts and stability of bioactive molecules (Gaidhani et al. 2015).

The site of seaweed production, as well as the season of harvesting biomass are relevant factors able to modify the profiles and amounts of compounds of interest, since these environmental factors can affect the availability of organic and inorganic carbon, the light intensity, temperature, salinity, and pH (Kumar et al. 2021). Additionally, it is worth highlighting that the first large scale cultivations of *K. alvarezii* in southern Brazil were authorized by the Brazilian Institute of the Environment and Renewable Natural Resources only very recently, i.e., 2020 (IBAMA – Brazil 2020), making it a brand-new economic activity. Importantly, despite the continuous growth of the seaweed biomass produced over the past three years, from 102.30 tons (2021/2022) to 300.35 tons (2022/2023) (Santos 2023), the effect of drying processes on the chemical profile of antioxidant compounds (e.g., phenolics, flavonoids, and carotenoids) and enzymatic activities (protease and lipase) of *K. alvarezii* raw material produced in southern Brazil remains unknown so far. Therefore, considering this scenario, the sites of cultivation and the collection seasons should be scrutinized for guaranteeing high quality *K. alvarezii*-based products. For these reasons, in the present study two representative production sites were selected for harvesting macroalga biomass over the seasons, following determining the total contents of phenolic compounds, flavonoids, and carotenoids, besides measuring the antioxidant and the protease and lipase activities of lyophilized- and oven-dried-*K. alvarezii* samples.

Freeze-dried *K. alvarezii* samples exhibited higher total phenolic contents than oven-dried ones, regardless the collection site (Table 1). TPC of for freeze-dried samples showed superior and a wider range of values (39.23 to 127.74 mg/100 g) in respect to the oven-dried ones (13.40 to 39.11 mg/100 g - Table 1). In fact, despite the difference between minimum and maximum TPC values of samples to be around three-fold regardless the site and season of collection, it appears that those secondary metabolites are better preserved by the freeze-drying algal biomass than oven-drying it. A comparative analysis applied to the contents of the secondary metabolites of interest for the freeze-dried samples from the two collection sites revealed higher TPC in the PAL-5 sample (127.74 mg/100 g), followed by

PAL-4 (105.57 mg/100 g), and RIB-5 (98.51 mg/100 g), which statistically differed from other samples. Importantly, PAL-5 and RIB-5 samples came from samples collected during early spring and PAL-4 during late winter.

Regarding total flavonoid content (TFC), all samples, except RIB-3 and PAL-4, differed statistically according to the drying method. Freeze-dried samples presented a wider range (4.10 to 15.62 mg/100 g) of TFC in respect to the oven-dried ones (4.99 to 12.29 mg/100 g). After oven-drying, RIB-2, RIB-3, RIB-7, PAL-2, PAL-3, PAL-4, and PAL-6 samples showed higher TFC values, namely 58% of all samples (Table 1). On the other hand, freeze-dried biomass revealed superior TFC amounts for the winter- (RIB-3, RIB-4), and early spring- (RIB-5) collected samples. Thus, in respect to the seasonality, it appears that *K. alvarezii* cultured under mild temperatures (i.e., winter and early spring – southern Brazil) synthesizes and accumulates higher amounts of TFC values, as noted for the freeze-dried samples. Interestingly, similar results were found for the TPC of early-spring collected samples. In turn, oven-dried samples showed higher variations in the concentrations of those secondary metabolites and a clear pattern resulting from the seasonality effect was not detected. Finally, regarding the collection sites, RIB-4 and RIB-2 samples showed the highest TFC values for both freeze-drying (15.62 mg/100 g) and oven-drying (12.29 mg/100 g) processes, respectively.

Table 1
Total phenolic content (TPC, mg GAE/100 g) and total flavonoid content (TFC, mg QE/100 g) of freeze-dried and oven-dried *Kappaphycus alvarezii* biomass.

Sample	Phenolics		Flavonoids	
	Freeze-dried	Oven-dried	Freeze-dried	Oven-dried
RIB-2	59.54 ± 0.39 e	18.44 ± 0.11 g	9.05 ± 0.16 e	12.29 ± 0.17 a
RIB-3	67.42 ± 0.19 d	17.56 ± 0.46 h	10.92 ± 0.21 c	11.02 ± 1.14 b ns*
RIB-4	49.05 ± 0.96 f	22.52 ± 0.41 e	15.62 ± 0.12 a	4.99 ± 0.29 e
RIB-5	98.51 ± 0.37 c	21.20 ± 0.28 f	13.23 ± 0.06 b	5.86 ± 0.30 d
RIB-6	44.30 ± 0.16 h	13.40 ± 0.44 j	6.75 ± 0.10 h	5.05 ± 0.04 e
RIB-7	44.45 ± 0.14 h	14.51 ± 0.50 i	9.31 ± 0.05 d	10.51 ± 0.08 b
PAL-2	39.23 ± 1.27 j	23.04 ± 0.39 d	6.37 ± 0.04 i	7.81 ± 0.07 c
PAL-3	41.82 ± 0.29 i	23.52 ± 0.04 d	5.58 ± 0.06 j	6.45 ± 0.41 d
PAL-4	105.57 ± 0.31 b	29.68 ± 0.17 c	7.77 ± 0.06 f	8.37 ± 0.08 c ns*
PAL-5	127.74 ± 0.41 a	39.11 ± 0.23 a	7.05 ± 0.12 g	5.45 ± 0.38 e
PAL-6	43.99 ± 0.73 h	14.90 ± 0.19 i	4.10 ± 0.08 k	5.31 ± 0.09 e
PAL-7	45.90 ± 0.23 g	31.97 ± 0.93 b	6.79 ± 0.07 h	5.90 ± 0.15 d

Means followed by the same letter in the column do not differ statistically by the Scott-Knott test ($p \leq 0.05$). ns* = not significant for analysis between types of drying method within the same variable (TPC and TP – line) by Bonferroni correction ($p \leq 0.05$).

Phenolic compounds of seaweeds can be considered highly attractive for applications for health-promoting products and their preservation is essential during initial phases of industrial processing (Silva et al. 2019). Several phenolic compounds have been identified in *K. alvarezii*, such as apigenin, cinnamic acid, chlorogenic acid, hispidulin, isoorientin, isoquercitrin, kaempferol, methoxyphenylacetic acid, naringenin, salicylic acid, scopoletin, rutin, and xanthohumol (Rudke et al. 2022). However, few studies performed a systematic and rigorous analysis of these compounds and most of the investigations reported in literature addressed the total quantification of phenolics evidencing variations based on collection sites. For example, Araújo et al. (2020) found contents between 40.80-58.49 mg GAE 100 g^{-1} in cultivated *K. alvarezii* biomass from southeastern Brazil, while Nurshahida et al. (2020) reported TPC at 1917 mg GAE 100 g^{-1} in samples produced in Malaysia. As herein shown, the comparison of contents of *K. alvarezii*'s phenolic compounds in samples harvested in two collection sites (PAL and RIB) situated in the same bay and 10 km far from each other (Fig. 1), revealed a high variability within the sampling sites, without a clear difference between the production areas. These findings reinforce the effect of the production and harvest seasons on the metabolic profile of *K. alvarezii* cultured in southern Brazil. Indeed, seasonality seems to be a relevant factor in the definition of the content of phenolics in the algal biomass investigated, mostly under mild temperatures over the winter and early spring, especially in the freeze-dried samples (Table 1). These data agree as to the seasonality effect with previous findings of Araújo et al. (2022), where higher phenolics contents in green and red phenotypes of *K. alvarezii* grown in n southeastern Brazil were recorded in summer and autumn. Thus, the season of seaweed harvest can impact on the content of those bioactive compounds, eventually affecting the intended quality of the raw material for a given industrial application.

Regarding total flavonoid content, samples also presented a high variability over the seasons and for the sites of collection. Additionally, a clear pattern of contents for those bioactive compounds in the algal biomass did not come out from the drying processes investigated. However, previous studies suggested that TFC of *K. alvarezii* was higher in oven-dried samples. Ling et al. (2015) reported that samples of that macroalga oven-dried at 40ºC and 80ºC presented higher contents of flavonoids, reaching 25.67 and 23.67 mg/100 g, respectively, in comparison to the freeze-dried biomass (i.e., 12.33 mg/100 g). These results were also corroborated by Charles et al. (2020), who found superior flavonoid content in oven-dried *K. alvarezii* biomass (0.13 mg/g), comparatively to the freeze-dried alga (0.9 mg/g).

Conversely, the drying method can drastically interfere with phenolics content in algae as reported in previous studies. For example, TPC showed a drastic reduction by drying *K. alvarezii* biomass in an oven, as compared to the freeze-dried samples, an effect attributed to the high processing temperature, which can result in the fast oxidation of those secondary metabolites (Santhoshkumar et al. 2023). Thus, oven drying, commonly used to reduce operating processing costs, generally leads to phenolic content reduction (Cao et al. 2021). In turn, freeze-drying, based on the phenomenon of water sublimation under

reduced pressure of a previously freeze sample, though more expensive, can preserve product quality attributes in terms of nutrients, color, and flavors. In fact, lyophilization guarantees minimal thermal damage, good preservation of flavors, nutrients and bioactivities, besides low final moisture content to the product (Cao et al. 2021). Low temperatures (e.g., < 4ºC) might promote greater stability of phenolics for long periods, which may be related to the inhibition of phenol oxidase activity, thus reducing oxidation and, consequently, condensation and degradation. These results were confirmed by studies carried out with other seaweed species such as *Padina pavonica* (Čagalj et al. 2021), *Cladophora glomerata* (Yarnpakdee et al. 2022), *Sargassum fusiforme* (Zhao et al. 2022), and *Cytosphora* sp. (Subbiah et al. 2023).

Among the methodologies used to analyze antioxidant activity, FRAP method allows measuring the reduction of a ferric ion complex (Fe^{+3}-2, 4, 6-tripyridyl-*S*-triazine) to the intense blue ferrous complex (Fe^{+2}-2, 4, 6-tripyridyl-*S*-triazine) by antioxidants in an acidic environment. Alternatively, DPPH method involves the measurement of the reduction capacity of the 2, 2-diphenylpicrylhydrazyl stable radical by the donation of electrons from antioxidant molecules (Munteanu and Apetrei 2021). These methods can be considered complementary techniques by measuring antioxidant properties of compounds through mechanistically distinct ways.

In the FRAP analysis, all samples, but RIB-6, PAL-6 (late spring) and RIB-7, PAL-7 (early summer), differed ($p < 0.05$) in their antioxidant activity between the two employed methods (Table 2). The most of samples (i.e., 58%) showed a higher FRAP antioxidant activity when freeze-dried (RIB-4, RIB-5, RIB-7, PAL-2, PAL-3, PAL-4, and PAL-7–0.37 to 0.62 mmol/100 g) in comparison to the oven-dried ones (0.28 to 0.70 mmol/100 g). Among the samples, freeze-dried PAL-4 (late winter) and oven-dried PAL-5 (early spring) presented the highest antioxidant activity (0.62 and 0.70 mmol/100 g, respectively), statically differing from others (Table 2).

All the samples analyzed differed in their antioxidant activity determined by the DPPH assay according to the drying methods, with superior values for the freeze-dried ones (6.12 to 8.91 mg/100 g) in respect to the oven-dried samples (0.40 to 3.63 mg/100 g – Table 2). Interestingly, as noted in the FRAP assay, the early spring-collected sample PAL-5 presented the highest DPPH activity for both freeze-drying (8.91 mg/100 g) and oven-drying (3.63 mg/100 g) methods, statically differing from other samples treated with the same drying method.

Table 2
Antioxidant activity by FRAP (expressed in mmol/100 g) and DPPH reducing capacity (expressed in mg/100 g and %) of *Kappaphycus alvarezii* macroalga biomass after freeze-drying and oven-drying.

Sample	FRAP		DPPH	
	Freeze-dried	Oven-dried	Freeze-dried	Oven-dried
RIB-2	0.37 ± 0.01 h	0.43 ± 0.01 d	6.12 ± 0.35 g (38.69)	0.40 ± 0.05 f (7.29)
RIB-3	0.40 ± 0.01 g	0.43 ± 0.01 d	6.75 ± 0.04 f (42.42)	0.80 ± 0.03 e (11.98)
RIB-4	0.48 ± 0.01 e	0.45 ± 0.02 c	7.99 ± 0.25 c (49.77)	1.06 ± 0.06 d (15.04)
RIB-5	0.45 ± 0.01 f	0.33 ± 0.02 g	6.24 ± 0.07 g (39.46)	0.71 ± 0.01 e (11.00)
RIB-6	0.41 ± 0.00 g	0.42 ± 0.01 d ns*	7.58 ± 0.07 d (47.37)	0.41 ± 0.07 f (7.42)
RIB-7	0.38 ± 0.00 h	0.36 ± 0.01 f ns*	7.64 ± 0.14 d (47.73)	0.87 ± 0.02 e (12.83)
PAL-2	0.54 ± 0.02 c	0.28 ± 0.01 h	6.56 ± 0.08 f (41.33)	1.07 ± 0.26 d (15.17)
PAL-3	0.52 ± 0.00 d	0.41 ± 0.01 e	6.83 ± 0.02 e (42.92)	0.81 ± 0.15 e (12.17)
PAL-4	0.62 ± 0.01 a	0.49 ± 0.00 b	8.25 ± 0.06 b (51.32)	1.57 ± 0.09 c (21.16)
PAL-5	0.60 ± 0.01 b	0.70 ± 0.01 a	8.91 ± 0.10 a (55.18)	3.63 ± 0.12 a (45.38)
PAL-6	0.39 ± 0.01 g	0.40 ± 0.00 e ns*	6.90 ± 0.08 e (43.32)	1.01 ± 0.08 d (14.52)
PAL-7	0.45 ± 0.01 f	0.43 ± 0.01 d ns*	6.99 ± 0.06 e (43.87)	2.30 ± 0.15 b (29.69)

Means followed by the same letter in the column do not differ statistically by the Scott-Knott test ($p \leq 0.05$). ns* = not significant for analysis between types of drying method within the same variable (TPC and TP – line) by Bonferroni correction ($p \leq 0.05$).

The present study demonstrates higher DPPH antioxidant activity in freeze-dried samples compared to the FRAP method. However, no relationship was found between this variable and the locations of algal biomass collection, indicating wide variation between locations.

Previous reports indicate that *K. alvarezii* exhibits an excellent radical scavenging effect both by FRAP and DPPH assays (Kumar et al. 2008; Ling et al. 2015; Mohamed and Abdullah 2016). In fact, the antioxidant activity determined by the FRAP assay reported in the present study was higher (0.28–0.70 mmol/100 g) than that recorded in *K. alvarezii* cultivated in two sites in Malaysia, e.g., 0.010 mmol/100 g (Langkawi, Kedah) and 0.017 mmol/100 g (Semporna, Sabah) (Mohamed and Abdullah 2016). On the other hand, higher values have also been found for the same species cultivated in Indonesia, where FRAP activity varying from 45 to 97 mmol/100 g was detected among sun-dried, oven-dried, dried-vacuum, and freeze-dried samples (Charles et al. 2020). Regarding the DPPH test, Araújo et al. (2020) observed that *K. alvarezii* cultivated in southeastern Brazil exhibited values ranging from 7.15–31.21%,

which are similar to the observations herein found. In the research by Vaghela et al. (2022), a DPPH reducing capacity of ~ 0.35 mg/100 g was recorded in the methanolic extract of *K. alvarezii* cultivated in Simar, India. Taking together, these results indicate that large variations of the antioxidant activity of *K. alvarezii* have been detected in samples originated from climatically distinct producing regions worldwide.

In a second approach, correlation analysis was applied to the TPC, TFC, FRAP, and DPPH dataset, considering the separation of samples according to their collection site (RIB and PAL) and drying methods (freeze-drying and oven-drying). The results are presented in Table 3. Significant correlations were found only between TPC and DPPH variables for the freeze-dried PAL (r^2 = 0.9886) and oven-dried (r^2 = 0.7857) samples, and when grouping RIB and PAL oven-dried samples (r^2 = 0.7998).

Table 3
Correlation analysis of the antioxidant capacity and total phenolic and flavonoid contents of freeze-dried and oven-dried *Kappaphycus alvarezii* samples cultivated in Florianópolis (RIB) and Palhoça (PAL), southern Brazil.

Sample	Drying method	Phenolic content	Correlation coefficients	
			FRAP	DPPH
RIB	Freeze-dried	TPC	y = 0.0006x + 0.3782	y = -0.0283x + 8.7698
			r^2 = 0.0867	r^2 = 0.5494
		TFC	y = 0.0111x + 0.2951	y = 0.0226x + 6.8088
			r^2 = 0.677	r^2 = 0.0083
	Oven-dried	TPC	y = 0.0004x + 0.3956	y = 0.0346x + 0.0871
			r^2 = 0.0008	r^2 = 0.2274
		TFC	y = 0.0004x + 0.3987	y = -0.0149x + 0.8307
			r^2 = 0.001	r^2 = 0.0367
PAL	Freeze-dried	TPC	y = 0.0017x + 0.4079	y = 0.0241 x + 5.7845
			r^2 = 0.5512	**r^2 = 0.9886**
		TFC	y = 0.0551x + 0.1755	y = 0.4371x + 4.665
			r^2 = 0.651	r^2 = 0.3578
	Oven-dried	TPC	y = 0.0128x + 0.1052	y = 0.113x + 1.3253
			r^2 = 0.5708	**r^2 = 0.7857**
		TFC	y = -0.0432x + 0.7332	y = -0.3212x + 3.8339
			r^2 = 0.1497	r^2 = 0.1458
RIB + PAL	Freeze-dried	TPC	y = 0.0016x + 0.363	y = 0.0131x + 6.3952
			r^2 = 0.3259	r^2 = 0.2112
		TFC	y = -0.0035x + 0.4979	y = -0.0004x + 7.2343
			r^2 = 0.0178	r^2 = 2E-06
	Oven-dried	TPC	y = 0.0088x + 0.229	y = 0.1052x + 1.1477
			r^2 = 0.4331	**r^2 = 0.7998**
		TFC	y = -0.0079x + 0.4852	y = -0.1205x + 2.1126

	$r^2 = 0.0385$	$r^2 = 0.1146$

TPC: Total phenolics content; TFC: Total flavonoids content.

The low correlation found between polyphenol content and antioxidant activity for most of the studied samples suggests that other compounds than phenolics are responsible for the antioxidant activity of the *K. alvarezii* samples investigated, such as the carrageenan polysaccharide, pigments (chlorophylls, carotenoids, and phycobiliproteins), proteins, and peptides (Rajaram et al. 2021).

Regarding the total carotenoid contents, firstly it was noted a wide range of values in samples between the collection sites (i.e., RIB and PAL) and over the seasons studied, irrespective of the drying method adopted (Table 4). For this class of secondary metabolites, undoubtedly the freeze-dried protocol afforded better results, with superior amounts found in all samples, except for RIB-2 and PAL-6, which did not differ statistically ($p < 0.05$). In fact, oven-drying revealed to be ineffective for preserving carotenoids in *K. alvarezii* biomass, leading to quite important losses of those bioactive compounds in comparison to the lyophilization protocol. Interestingly, freeze-dried samples harvested over the winter and early-spring presented the highest amounts of those pigments, regardless the collection sites.

Table 4
Total carotenoid concentration (µg/g) of *Kappaphycus alvarezii* biomass after freeze-drying and oven-drying.

Sample	Carotenoids	
	Freeze-dried	Oven-dried
RIB-2	13.01 ± 1.80 g	14.37 ± 0.40 c ns*
RIB-3	72.76 ± 0.17 a	13.15 ± 0.70 d
RIB-4	47.02 ± 0.34 c	27.19 ± 0.64 a
RIB-5	75.33 ± 10.22 a	2.19 ± 0.14 h
RIB-6	15.76 ± 2.22 f	6.05 ± 0.12 g
RIB-7	39.35 ± 2.12 d	11.42 ± 0.80 e
PAL-2	19.31 ± 2.68 f	9.89 ± 1.30 f
PAL-3	37.96 ± 1.27 d	11.18 ± 0.03 e
PAL-4	34.73 ± 0.87 d	14.79 ± 0.58 c
PAL-5	54.37 ± 0.55 b	21.47 ± 1.42 b
PAL-6	10.27 ± 1.99 g	9.69 ± 0.63 f ns*
PAL-7	28.93 ± 1.27 e	4.92 ± 0.58 g

Means followed by the same letter in the column do not differ statistically by the Scott-Knott test ($p \leq 0.05$). ns* = not significant for analysis between types of drying method within the same variable (TPC and TP – line) by Bonferroni correction ($p \leq 0.05$).

The freeze-drying method proved to be more efficient for the preservation of carotenoids in the algal biomass, showing a lower degradation of these pigments, a factor which can be explained by the high susceptibility of these compounds to heat and oxidation related decay (Boon et al. 2010), as also occurring for polyphenols. Studies have reported higher contents of total carotenoids (0.26 to 0.52 mg g^{-1} – Periyasamy et al. 2019, and 0.38 to 0.53 mg g^{-1} – Rajaram et al. 2021) than those herein found. Those discrepant values with respect to the present work can be ascribed to the fact that the above-mentioned authors carried out measurements on fresh algal biomass, differently from those performed in this study.

In addition to determining the contents of secondary metabolites, a second set of variables was measured regarding the analysis of lipase and protease activities in the *K. alvarezii* biomass investigated. Specifically, lipases catalyze the hydrolysis of triglycerides, oils and fats, and can be involved in the synthesis of fat acids, for instance, depending on the water content of the reaction system. Differently, proteases hydrolyze the peptide bond (Camargo et al., 2022). Notably, lipases and proteases are the most used enzymes by food, beverages, detergents, animal feed, personal care, cosmetics, nutraceuticals, and textiles industries (Adetunji and Olaniran 2021; Gimenes et al. 2021; Mehta et al. 2021).

Marine organisms have attracted increasing attention as sources of enzymes, which are considered more stable and active than their counterparts derived from plants and animals (Zhang and Kim 2010). Therefore, the analysis of the lipolytic and proteolytic activities in *K. alvarezii* biomass was carried out aiming to shed some light on the biotechnological potential of that seaweed as source of those enzymes for industrial purposes.

Surprisingly, lipase activity was not found in any studied samples, regardless the method used for drying biomass. This result may be related to the low concentration of lipids (0.36%-1.95%) of *K. alvarezii*, as previously reported (Xiren and Aminah 2017; Ndawala et al. 2022). In turn, protease activity was found in all examined samples, with values up to 886 times higher in oven-dried samples (RIB-2, Table 5). In fact, most samples (75%) presented higher catalytic activity ($p < 0.05$) following oven-drying in respect to lyophilization, except RIB-7. Additionally, the highest catalytic activity detected in the oven-dried samples, i.e., RIB-2–1485.09 ± 26.84 U/g, was notoriously superior to that found in the freeze-dried one with the best performance, i.e., PAL-5, 579.25 U/g. Finally, as noted in the analysis of the secondary metabolites, protease activity seems not to be related to the collection site of *K. alvarezii* and also to the seasonality, since a clear pattern of enzymatic activity was not detected in respect to those factors.

Table 5
Protease activity (U/g) of *Kappaphycus alvarezii* biomass after freeze-drying and oven-drying.

Sample	Protease activity	
	Freeze-dried	Oven-dried
RIB-2	150.47 ± 1.43 g	1485.09 ± 26.84 a
RIB-3	204.01 ± 8.47 e	744.46 ± 53.19 b
RIB-4	308.18 ± 10.53 c	431.70 ± 13.53 e
RIB-5	174.40 ± 1.08 f	298.50 ± 6.67 g
RIB-6	75.98 ± 2.31 i	221.71 ± 28.27 h
RIB-7	92.50 ± 0.62 h	119.50 ± 9.50 i ns*
PAL-2	155.34 ± 11.01 g	393.82 ± 2.60 f
PAL-3	245.65 ± 7.23 d	654.07 ± 13.92 c
PAL-4	466.86 ± 8.44 b	366.77 ± 1.23 f
PAL-5	579.25 ± 0.45 a	503.97 ± 22.82 d
PAL-6	90.00 ± 8.41 h	131.59 ± 6.59 i
PAL-7	474.62 ± 16.36 b	315.29 ± 1.80 g

Means followed by the same letter in the column do not differ statistically by the Scott-Knott test ($p \leq 0.05$). ns* = not significant for analysis between types of drying method within the same variable (TPC and TP – line) by Bonferroni correction ($p \leq 0.05$).

Proteases have been used especially in the detergent and leather industry and for pharmaceutical applications, such as digestive and anti-inflammatory drugs (Zhang and Kim 2010). *K. alvarezii*, especially oven-dried biomass, showed relevant values of proteolytic activity, which may be of great interest for industry. Possibly, oven-drying protocols may increase the availability of proteases and their activity due to the efficient biomass demolition at high temperatures, 50–60ºC (Magalhães et al. 2019; Naveed et al. 2021). In comparison to other macroalga species, *K. alvarezii* presents superior protease activity than *Ulva rigida*, *Codium decorticatum*, *Stypocaulon scoparium*, *Dictyota dichtoma*, *Pterocladiella capillacea*, and *Gracilaria* sp. (Pérez-Lloréns et al. 2003).

In a secondary approach to data analysis, multivariate statistical techniques were used, namely principal component analysis (PCA) and linear discriminant analysis (LDA) to verify the presence of clusters between the methodologies used. Protease activity was deemed irrelevant to the descriptive models built and thus excluded from the analysis. Further, PCA applied to the total phenolics, total flavonoids, FRAP, DPPH, and total carotenoids data set revealed a total variance of 80%, with PC1 accounting for

56% and PC2 for 24% (see Fig. 2). Notably, the oven-dried samples (91.66% - n = 11) were distinctly separated along PC2 and were not associated with metabolites. This indicates that, in general, they exhibited lower concentrations of these compounds. Conversely, freeze-dried samples (clustered in PC1) were correlated with the computed variables. DPPH and phenolics showed a close association with carotenoids, while the flavonoid content exhibited an opposite relationship with FRAP. Regarding the harvest site and seasonality effects, no sample grouping was detected by PCA. Nevertheless, it is crucial to underscore the significance of assessing the bioactive compounds, antioxidant capacity, and enzymatic activity of this biomass across different seasons, particularly considering its recent cultivation in Brazil, in order to better understand and address the potential of usage of that raw material by industry.

As a complementary model, LDA was applied, revealing that axes 1 and 2 accounted for 99.99% of the variance, with four distinct groupings identified, where RIB and PAL oven-dried samples (left quadrants) and RIB and freeze-dried PAL (right quadrants) appeared, as also seen via PCA (Fig. 3). Total carotenoids and total flavonoids were the most relevant variables for the Group 2 samples (RIB) quadrant, while FRAP, DPPH, and total phenolics were determinant for those samples in Group 4 (PAL) quadrant. In general, it is clear that the oven-dried samples, as seen in the PCA, were separated in the model, demonstrating that they have lower concentrations of compounds.

In general, it can be concluded that the freeze-drying process more effectively preserves important bioactive compounds of *K. alvarezii* biomass in respect to the oven-drying technique. However, depending on the specific application, oven drying may be preferable for certain industrial applications of the algal biomass, making it suitable for various uses. According to Stratta et al. (2020), a comprehensive economic analysis of the freeze-drying process suggests that the costs involved may not be as prohibitive as the initial investment required for setting up operations. However, in adopting industrial drying processes based on lyophilization, it is essential to consider whether significant advantages in preserving compounds are relevant or if a risk exists of compromising quality when opting for alternative and low-cost drying methods, such as oven-drying. Additionally, the selection of industrial dryers must consider other factors like energy consumption and environmental impact, particularly when prioritizing sustainability and the utilization of natural products (Murugan et al. 2021).

This consideration is particularly important due to the cost-effectiveness of industrial applications involving the seaweed species herein investigated. In previous study conducted by our research group (Nunes et al. 2024), diverse applications of *K. alvarezii* biomass have been described, such as cosmetics, animal nutrition, human food, health/medicine, agriculture, and general industry, in connection with the quality of the seaweed raw material used. This underscores the significance of investigating the impact of the drying process on the quality of the *K. alvarezii* produced, particularly in regions where its cultivation is still in nascent stages, as is the case in Brazil.

4 Conclusions

Comparison between freeze-drying and oven-drying methods for processing Kappaphycus alvarezii biomass, as reported here, indicates that freeze-dried samples generally exhibited higher compound levels. These results underscore the significance of the drying process in preserving bioactive compounds in biomass, enabling the selection of the most appropriate method for specific industrial applications. Free-drying is recommended for industries such as pharmaceuticals and cosmetics, where the aim is to obtain biomass with higher compound richness or greater stability.

Further investigations are warranted to assess the impact of drying methods on other bioactive compounds present in *K. alvarezii* biomass. Additionally, exploring the influence of geographic origin and seasonality might help optimize cultivation practices across different regions and seasons of the year.

Declarations

Competing interests

We declare that there are no competing interests.

Author Contribution

Aline Nunes: Conceptualization, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing; Felipe de Souza Dutra, Sinara de Nazaré Santa Brito, Milene Stefani Pereira, Gadiel Zilto Azevedo, Alex Ricardo Schneider, Eva Regina de Oliveira Rodrigues e Alex Alves dos Santos: Formal analysis, Investigation, Writing - review & editing; Fábio Vianello: Supervision, Writing – review & editing; Marcelo Maraschin and Giuseppina Pace Pereira Lima: Conceptualization, Funding acquisition, Resources, Project administration, Supervision, Writing - review & editing.

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Data Availability Statement

The results of the systematic review analyses conducted for this manuscript are available and can be provided to researchers upon request. Interested parties are invited to contact the authors of the manuscript for access to the data.

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Figures

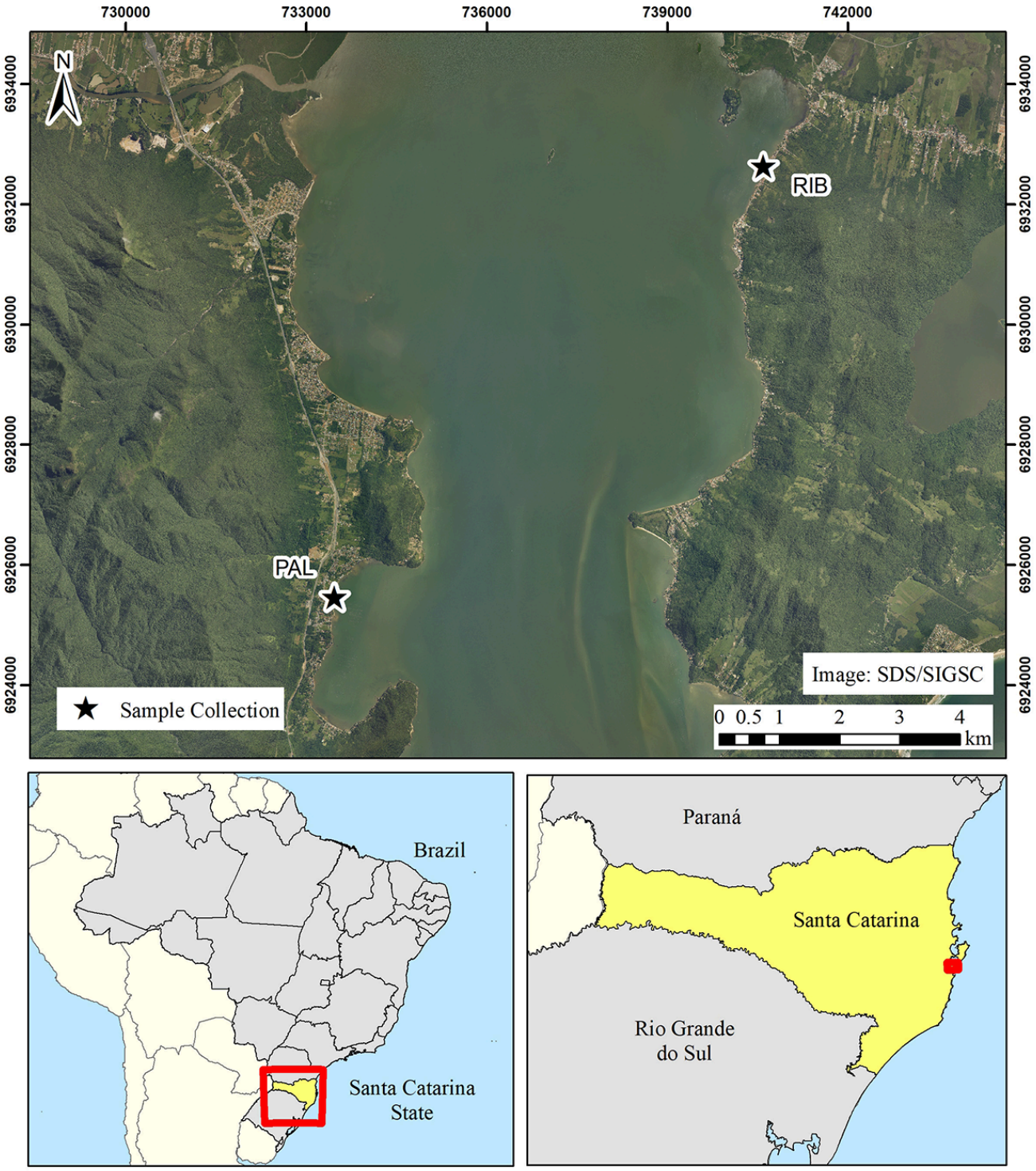


Figure 1

Details of the sites of collections of the macroalga *Kappaphycus alvarezii* in Florianópolis (RIB - Ribeirão da Ilha) and Palhoça (PAL), state of Santa Catarina, southern Brazil.

Source: Elaborated by the author (2023).

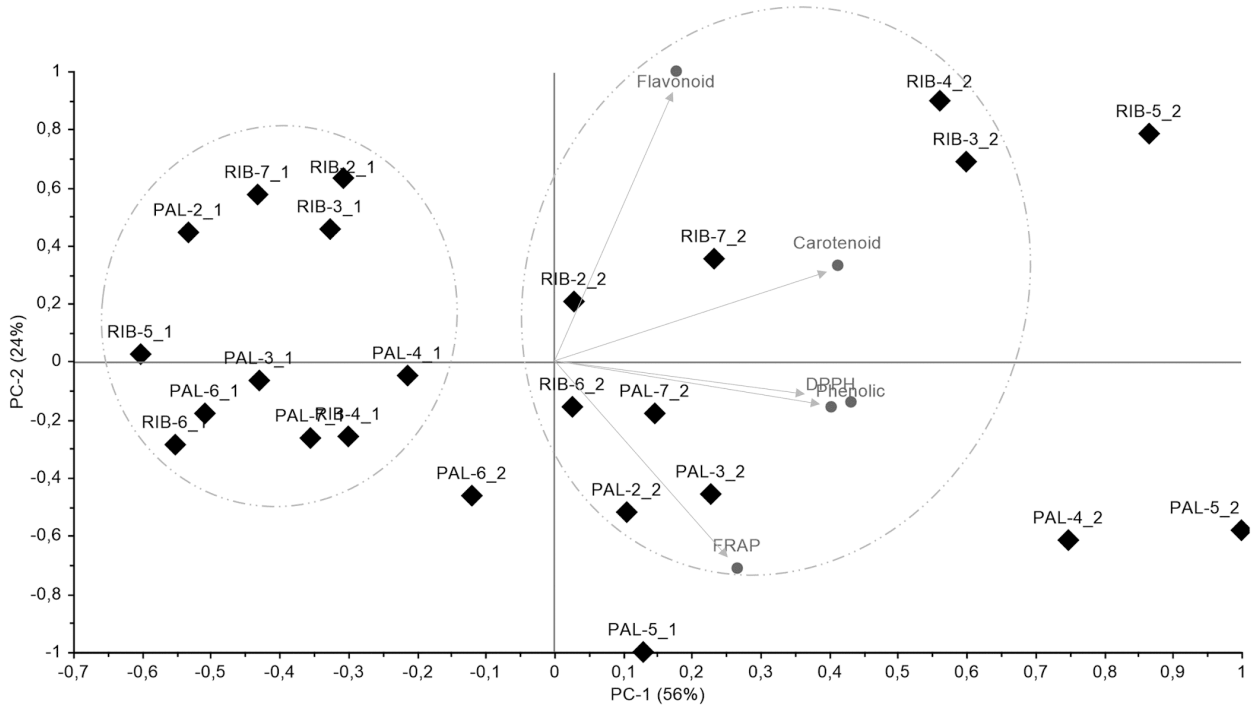


Figure 2

Scores scatter plot and factor loadings of principal component analysis (PCA) of oven-dried and freeze-dried samples of *Kappaphycus alvarezii* cultivated in Ribeirão da Ilha (RIB, Florianópolis county) and Palhoça (PAL), southern Brazil.

PAL-x_1 and RIB-x_1 – Oven-dried; PAL-x_2 and RIB-x_2 – Freeze-dried.

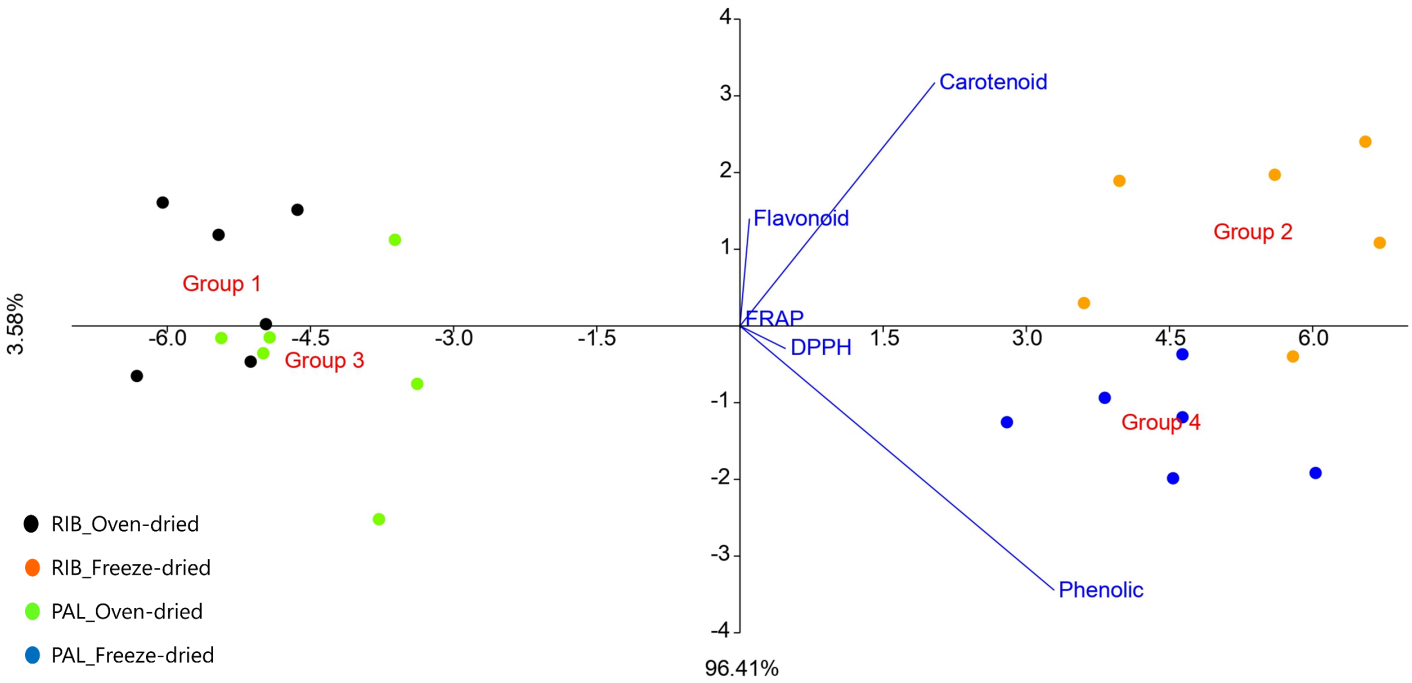


Figure 3

Linear discriminant analysis (LDA) of oven-dried and freeze-dried samples of *Kappaphycus alvarezii* cultivated in Ribeirão da Ilha in Florianópolis (RIB) and in Palhoça (PAL).