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Seasonal biochemical fingerprints of *Kappaphycus alvarezii* grown in Southern Brazil: Efficient extraction of pigments and mycosporine-like amino acids using biosolvents

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ABSTRACT

This study investigates seasonal variations in pigment content of the red macroalga *Kappaphycus alvarezii* cultivated in southern Brazil and in the residual biomass resulting from the extraction of the aqueous fraction with claimed biostimulant properties. Bioactive compounds like chlorophylls, phycobiliproteins (PBPs), and mycosporine-like amino acids (MAAs) were extracted using biosolvents. Data were analyzed by the Scott-Knott test ($p < 0.05$) and multivariate approaches. Higher chlorophyll *a*nd phycobiliproteins concentrations were reached in fresh seaweed than in the residual biomass, highlighting an abundant amount of phycobiliproteins (PBP) and mainly phycoerythrin. Interestingly, MAA content was greater in the residue (135.64 $\mu g.mg^{-1}$) than in the fresh biomass (106.15 $\mu g.mg^{-1}$), suggesting potential accumulation during biostimulant production. PCA identified two distinct sample clusters: one associated with seaweed samples characterized by high chlorophyll *a*nd PBP levels, and another linked to residues with elevated MAA concentrations. In general, the fresh algal biomass exhibited higher levels of pigments compared to the residue. Regarding seasonality, the PCA revealed that winter samples formed a more homogeneous group, likely due to the significantly lower sea temperatures. These results underscore the dynamic nature of pigment biosynthesis in *K. alvarezii* and its biotechnological potential, particularly in sustainable extraction practices.

1. Introduction

Marine-derived pigments are generally categorized into three main groups: phycobiliproteins, chlorophylls, and carotenoids [1]. Phycobiliproteins, including phycoerythrin, phycocyanin, and allophycocyanin [2], are valued as natural bioactive compounds with applications in the food and biomedical industries due to their vibrant color [3] and in cosmetics for their anti-aging and antioxidant properties [4]. Chlorophylls and their derivatives are mainly utilized in biomedical contexts owing to their photophysical properties, which facilitate disease diagnosis and therapy [5]. Carotenoids are widely utilized in aquaculture, food, and cosmetic formulations due to their antioxidant and photoprotective properties [6].

Mycosporines-like amino acids (MAAs), although colorless, constitute an important family of photoprotective molecules predominantly found in red algae and widely employed in the cosmetic industry [7]. Their structural diversity is reflected in the absorption spectrum ranging from 268 to 362 nm [8] and in specific nitrogen or hydroxyl substituents that influence their mass-to-charge ratio (m/z) [9]. Seasonal variations can significantly affect both the content and structure of MAAs and pigments [10], emphasizing the need to analyze algal biomass collected during production peaks to ensure consistent quality for industrial use.

Among organisms producing natural pigments and MAAs, macroalgae stand out as one of the most important and economically viable sources, due to their sessile, multicellular, macroscopic, eukaryotic, and photoautotrophic nature [11]. They also produce various secondary

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metabolites, whose composition depends on species, environmental factors, and cultivation and harvesting conditions [12]. Consequently, large-scale macroalgal production for pigment and MAAs bio-prospecting is increasingly feasible and in demand across various industries.

Within this group, the red macroalga *Kappaphycus alvarezii* stands out. It exhibits three main color morphotypes, namely red, green, and brown/yellowish, reflecting the dominant pigment in each strain [13,14]. Cultivated in Asia since 1967, with Indonesia accounting for 65% of global supply [15], commercial farming in Brazil began in 2020, mainly in the southern region of the country, i.e., Santa Catarina state, reaching 751.09 tons, wet weight, by 2023–2024 harvest [16]. Environmental conditions such as pH, salinity, and temperature, particularly in southern Brazil, where winters are more pronounced, influence the biochemical diversity *of K. alvarezii* [17]. This raises the question of whether low temperatures might induce distinct biochemical phenotypes, thereby enhancing its commercial potential [18].

Management and harvesting practices also shape the metabolite and pigment profiles of marine algae, including chlorophylls, carotenoids, and phycobiliproteins, which concentrations varying according to water's pH, salinity, temperature [19], light intensity [20], depth, and seasonality [21]. For sustainable valorization, biorefinery approaches promoting eco-friendly extraction protocols have gained relevance [22]. This has fostered the adoption of biosolvents, such as organosolvents,

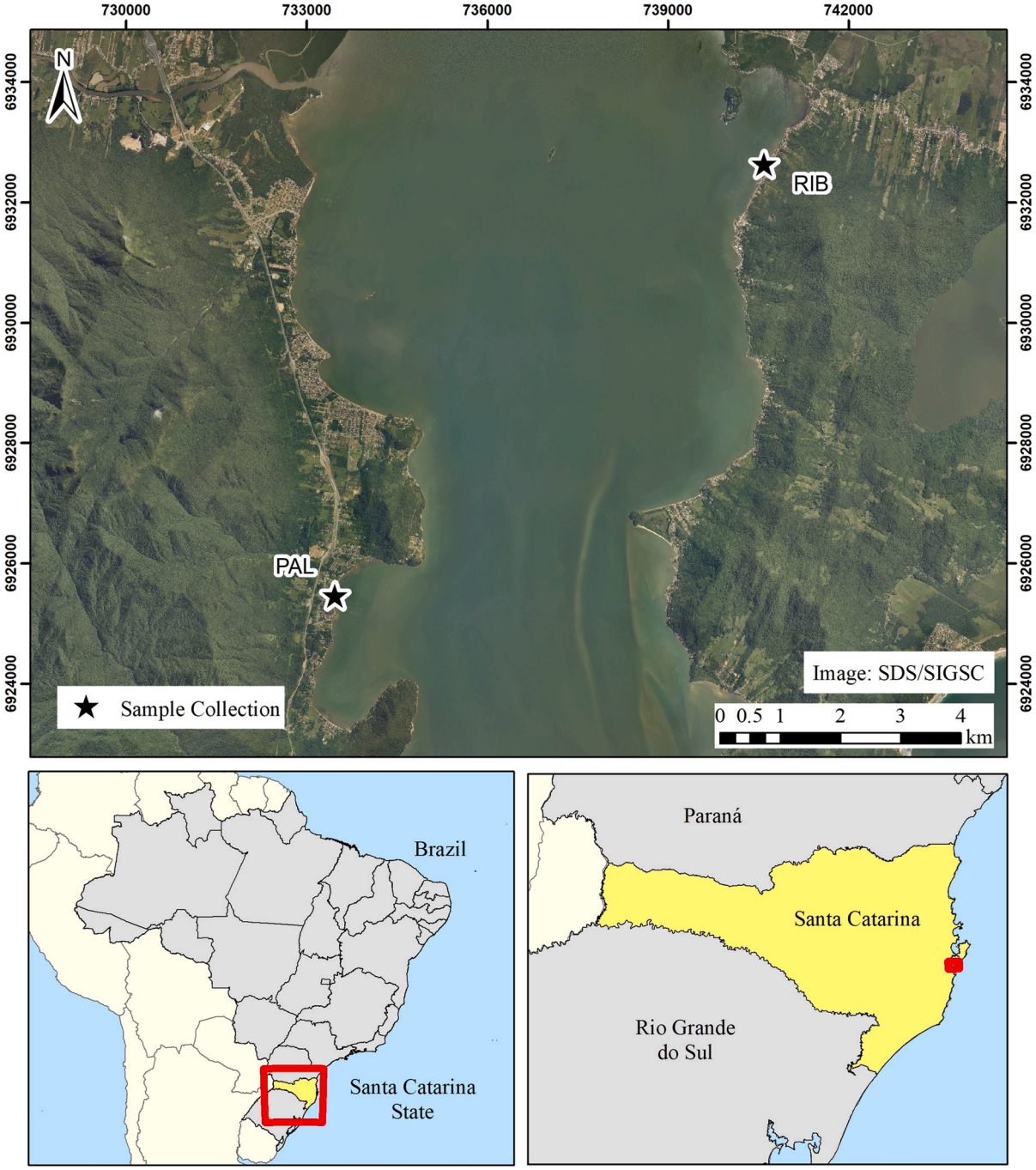

Fig. 1. Geographic location of the *K. alvarezii* sampling sites in Santa Catarina State, southern Brazil. Sampling sites are indicated in Florianópolis (Ribeirão da Ilha, RIB) and Palhoça (PAL). Symbols denote each sampling location, and municipal boundaries and coastline are shown to facilitate spatial reference. Source: Nunes et al. [33].

ionic liquids, and deep eutectic solvents, as alternatives to conventional solvents, minimizing environmental risks [23,24]. These green solvents are increasingly used in the natural products and pharmaceutical industries for the extraction and synthesis of high-value compounds, as well as in analytical methods aligned with green chemistry principles [25–27].

The literature largely reports the extraction of various types of metabolites using conventional solvents that exhibit polluting, flammable, and even toxic characteristics [28]. This also applies to pigment extraction, which commonly employs solvents such as acetone, chloroform [29], or methanol [30]. On the other hand, biosolvents may demonstrate selectivity toward the extraction of target metabolites [31] due to their unique physicochemical properties [32], which confer an additional advantage to their use.

In this context, this study aimed to evaluate the seasonal variation in the composition of pigments and MAAs in fresh and residual biomasses of *Kappaphycus alvarezii* cultivated in southern Brazil, to determine the yield and stability of these pigments and to compare the efficiency of biosolvents in the extraction of pigments and MAAs.

2. Material and methods

2.1. Sample collection

Fresh biomass of *K. alvarezii* and corresponding algal residue following the extraction of the biostimulant derivative were obtained from two sea farms located in Florianópolis, state of Santa Catarina, southern Brazil - Ribeirão da Ilha (RIB – 27° 42′ 32.724“ S, 48° 33’ 35.5” W) and Palhoça (PAL – 27° 46′ 29.928” S, 48° 37′ 50.7” W) (Fig. 1). Sampling was performed seasonally throughout the 2022/2023 cultivation cycle. A total of 32 samples were collected: four during autumn (early: RIB-1 and PAL-1; late: RIB-2 and PAL-2), four during winter (early: RIB-3 and PAL-3; late: RIB-4 and PAL-4), four during spring (early: RIB-5 and PAL-5; late: RIB-6 and PAL-6), and four during summer (early: RIB-7 and PAL-7; late: RIB-8 and PAL-8). Algal residues were derived from the biostimulant production process routinely employed by mariculture producers, wherein fresh seaweed biomass is chopped and mechanically pressed to obtain an aqueous extract with reported plant growth-promoting properties. The extraction was performed at room temperature using an industrial-scale blender followed by filtration of the aqueous extract through an industrial press. Detailed procedural information cannot be disclosed to trade secret restrictions associated with the company that supplied the samples. Samples representing both red and green *K. alvarezii* strains were collected, freeze-dried, ground with liquid N_2, and stored at −80 °C until further analysis.

The culture method used for *K. alvarezii* in the study was the tie-tie system, where cultivation ropes are anchored with stakes, keeping the algae in a movement system that oscillates between 30 cm and 1 m in depth. Additionally, *K. alvarezii* cultivation has been grown associated with other species, such as oysters, mussels, and scallops, in an integrated culture system. In southern Brazil's marine farms, a standard 50-m cultivation cable produces an average yield of 250 to 350 tons of *K. alvarezii* in summer. All data showing date, salinity, and sea surface temperature are provided in Table S1 – supplementary material, along with the harvest date of all samples (Table S2 – supplementary material).

2.2. Moisture content (M%)

To determine the moisture content in fresh seaweed and its residue, samples (20 g of each biomass) were freeze-dried for approximately 20 h, at −50 °C and 0.030 mbar (Labconco FreeZone 6 Liter Freeze Dryer). The moisture content (Table S3 – supplementary material) was calculated through Eq. 1 [34]:

$$M\% = 1 - \frac{Final\ weight}{Initial\ weight} x100 \quad (1)$$

Where, M% represents the moisture content in percentage, initial weight is 20 g of each sample and the final weight is the weight after freeze-drying each sample.

2.3. Extraction of bioactive compounds

Pigments were extracted in triplicate following a modified version of the protocol described by Osório et al. [35]. All extractions were performed using mixed algal samples comprising red, green, and brown algae. This approach reflects industrial practice, where production has not been strain-specific, and biomass availability depends on seasonal yield. The samples, kindly provided by local producers, were freeze-dried, macerated, and their pH measured using a Metrohm 913 pH meter. A single seasonal batch was used for extraction optimization.

For chlorophyll extraction, 25 mg of freeze-dried biomass (seaweed and residue) was placed into 2 mL microtubes, followed by the addition of 1 mL of solvent, in which biorenewable ethanol (EtOH), limonene (LIM), and cyrene (CYR) were included. Samples were vortexed and incubated at 80 rpm (Biosan RS24 multi-rotator) for varying times, under light-protected, and room temperature conditions. After extraction, samples were centrifuged (12,879 ×*g*, 15 min), and the absorbance of the supernatant (125 μL) was measured at 430 nm using a Spectramax® iD3 microplate reader. Quantification was performed using a chlorophyll *a* analytical standard (Sigma-Aldrich) and a calibration curve (1.6 $\mu g.mL^{-1}$–15.6 $\mu g.mL^{-1}$, $y = 64.622\times + 0.0168$, $r^2 = 0.998$). Additional solvents like cymene, m-THF, dimethyl isosorbide (DMISO), *gamma*-valerolactone (VAL), butyl lactate (98%, ButLac), and ethyl lactate (98%, EtLac) were also tested but excluded due to the absorbance values below the detection limit.

For the determination of phycobiliproteins, fresh or residual biomass samples (25 mg) were mixed with 1 mL of different bio-solvents at concentrations of 125, 250, and 500 mM. The samples were vortexed and gently centrifuged (80 rpm, Biosan RS24) for varying times under light-protected conditions at room temperature. The solvents (Merck – Portugal) used included EtOH, CYR, DMISO, VAL, ButLac, EtLac, ethyl acetate (99.5%, EtOAc), and phosphate-buffered saline (PBS) solution (50 mM, pH = 7.4). After centrifugation (12,000 xg, 15 min), the absorbance of the supernatant (125 μL) was measured at 498, 614, and 651 nm, and the total phycobiliproteins content was calculated according to Kursar et al. [36] (Eqs. 2 to 5). The results obtained were expressed in $\mu g.mL^{-1}$ for optimization of extraction variables.

$$\text{Allophycocyanin} : APC = 181.3\ A_{651} – 22.3\ A_{614} \quad (2)$$

$$\text{Phycocyanin} : PC = 151.1\ A_{614} – 99.1\ A_{651} \quad (3)$$

$$\text{Phycoerythrin} : PE = 155.8\ A_{498.5} – 40.0\ A_{614} – 10.5\ A_{651} \quad (4)$$

$$\text{Total} : APC + PC + PE \quad (5)$$

As *K. alvarezii* is a Rhodophyta macroalga, phycoerythrin content was seasonally determined for all samples using a calibration curve prepared with an analytical standard (Sigma-Aldrich, MO, USA) at 565 nm (10 $\mu g.mL^{-1}$–200 $\mu g.mL^{-1}$, $y = 5.0672\times$, $r^2 = 0.9998$).

The quantification of MAAs was achieved by measuring the absorbance of the phycobiliprotein extract in ethyl acetate (125 mM) at 330 nm. To perform a sequential extraction, allowing for the selective recovery of distinct pigments from the biomass, solvents that previously exhibited limited extraction efficiency for phycobiliproteins were tested, including VAL, ButLac, EtLac, and EtOAc. Quantification of MAAs was performed using a calibration curve at 330 nm (92 $\mu g.mL^{-1}$–774 $\mu g.mL^{-1}$, $y = 0.8537\times$, $r^2 = 0.9996$) based on a MAA analytical standard (Sigma-Aldrich, MO – USA).

2.4. Statistical analysis

For all statistical analyses, the Scott-Knott test (p-value <0.05) was employed using scripts written in R language (version 4.0.2). The pigment dataset, including chlorophylls, phycobiliproteins, and MAAs, was further analyzed through Principal Component Analysis (PCA) employing the Singular Value Decomposition (SVD) algorithm. This multivariate analysis was conducted using The Unscrambler X software (version 10.4), considering both the matrix type (seaweed or residue) and seasonal variation (autumn, winter, spring, and summer). Heatmap visualizations were generated in Microsoft Excel® software (v. 2206 build).

3. Results and discussion

3.1. Protocol optimization for chlorophyll extraction and seasonal analysis

To optimize chlorophyll extraction from *K. alvarezii* biomass samples, among the tested biosolvents (e.g., EtOH, LIM, CYR), EtOH yielded the highest extraction yields (0.3505 ± 0.0226 μg.mg^{-1}). This value was statistically higher than those obtained with LIM (0.2550 ± 0.0071 μg.mg^{-1}) and CYR (0.2773 ± 0.0076 μg.mg^{-1}), corresponding to a 37% and 26% increase, respectively (Fig. 2).

Ethanol is widely recognized for its efficiency in extracting chlorophyll derivatives from macroalgae [37], microalgae [38] and higher plants [39]. It consistently provides satisfactory yields compared to other common solvents such as acetone, methanol, and dimethyl sulfoxide. Its widespread use is further supported by favorable physicochemical properties, including a higher boiling point, lower volatility, lower cost, and reduced toxicity.

Regarding extraction time, ethanol, identified as the most effective biosolvent for chlorophyll recovery, showed no statistically significant differences among the evaluated durations for either the seaweed or the residue. Nevertheless, in all tested time intervals (from 0.5 h to 4 h), seaweed samples consistently exhibited chlorophyll concentrations over 200% higher than those of their respective residues (Fig. 3).

The chlorophyll *a* content significantly differed between the fresh and residual biomass samples of *K. alvarezii*, a finding that can be attributed to the industrial processing methods employed by the companies supplying the biosamples in study. During biostimulant production, the algal biomass undergoes cutting and mechanical pressing to obtain an aqueous extract, a procedure that effectively removes a substantial portion of intracellular components, which will probably help to explain the lower amounts of chlorophyll *a* detected in the residue. Considering these factors, the seasonal effect on the chlorophyll *a* content was subsequently evaluated using a 30 min-extraction with renewable ethanol and biomass concentration of 25 mg.mL^{-1}. For that, the seasonal analysis took into consideration, besides the harvest season, the production site of the algal biomass, i.e., Ribeirão da Ilha (RIB) and Palhoça (PAL) counties.

The results show that for both production sites the chlorophyll content was higher in the fresh seaweed samples than in the residual ones (Fig. 4; Table S4 from Supplementary Material). The chlorophyll *a* content ranged from 0.109 μg·mg^{-1} (RIB – spring) to 0.284 μg·mg^{-1} (RIB – summer) in fresh alga samples, representing a relative variation of approximately 160% between the lowest and highest values (Fig. 4). The highest chlorophyll *a* levels were found in summer-collected sample from RIB (0.284 ± 0.017 μg·mg^{-1}) and in spring for PAL (0.274 ± 0.030 μg·mg^{-1}). In their turn, residual biomass samples showed chlorophyll *a* concentration varying from 0.036 μg·mg^{-1} (PAL – spring) to 0.116 μg·mg^{-1} (RIB – winter), with a relative difference of 221%. The maximum content of that pigment was detected in the RIB-winter residue (0.117 ± 0.008 μg·mg^{-1}), statistically differing from the others. Finally, the comparative analysis of the chlorophyll *a* amount between fresh and residual alga biomass within the same season and production site (Fig. 4) consistently revealed superior values ($p < 0.05$) in the fresh samples, with increases ranging from 142% (RIB – spring) to 661% (PAL

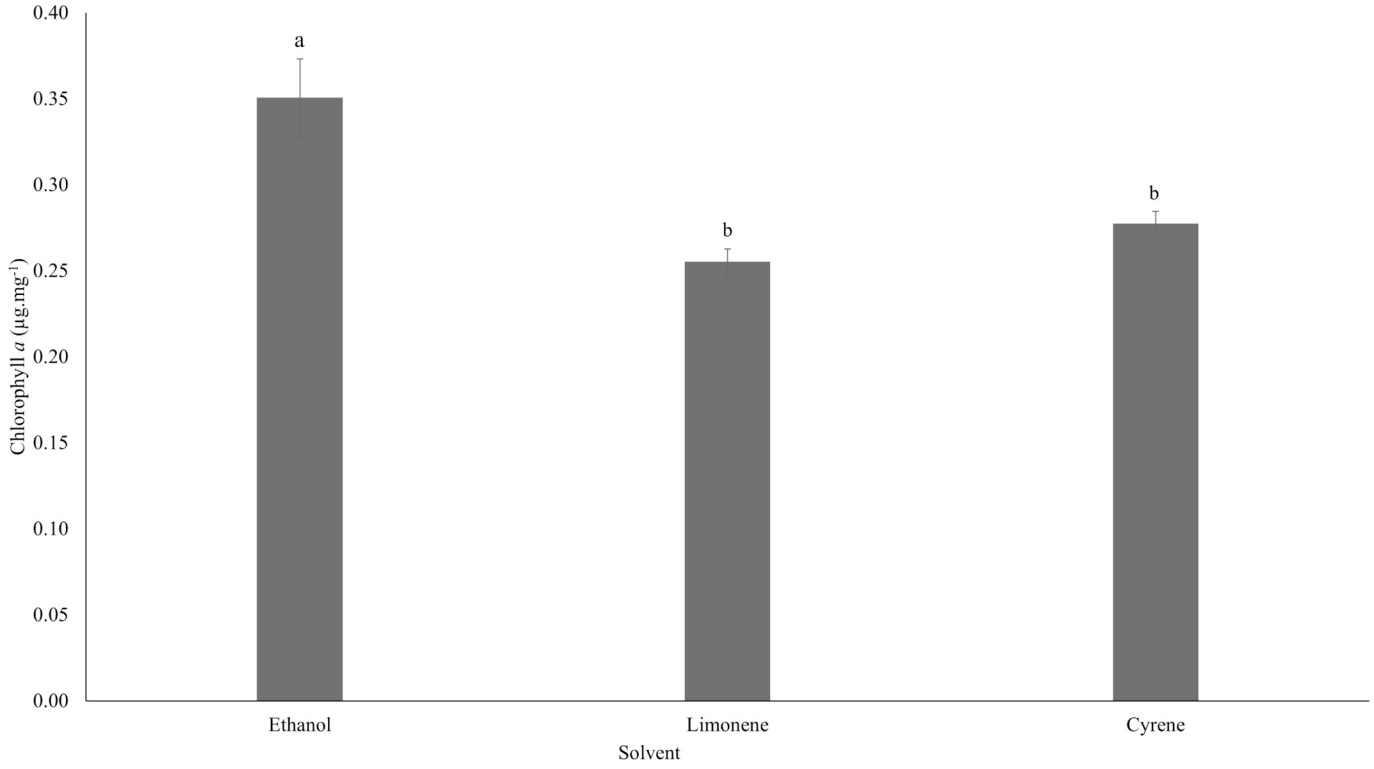

Fig. 2. Yield of chlorophyll (μg.mg^{-1}) extracted from *K. alvarezii* fresh biomass using ethanol, limonene and cyrene as biosolvents. Different letters denote statistically significant differences (Scott-Knott test, $p < 0.05$).

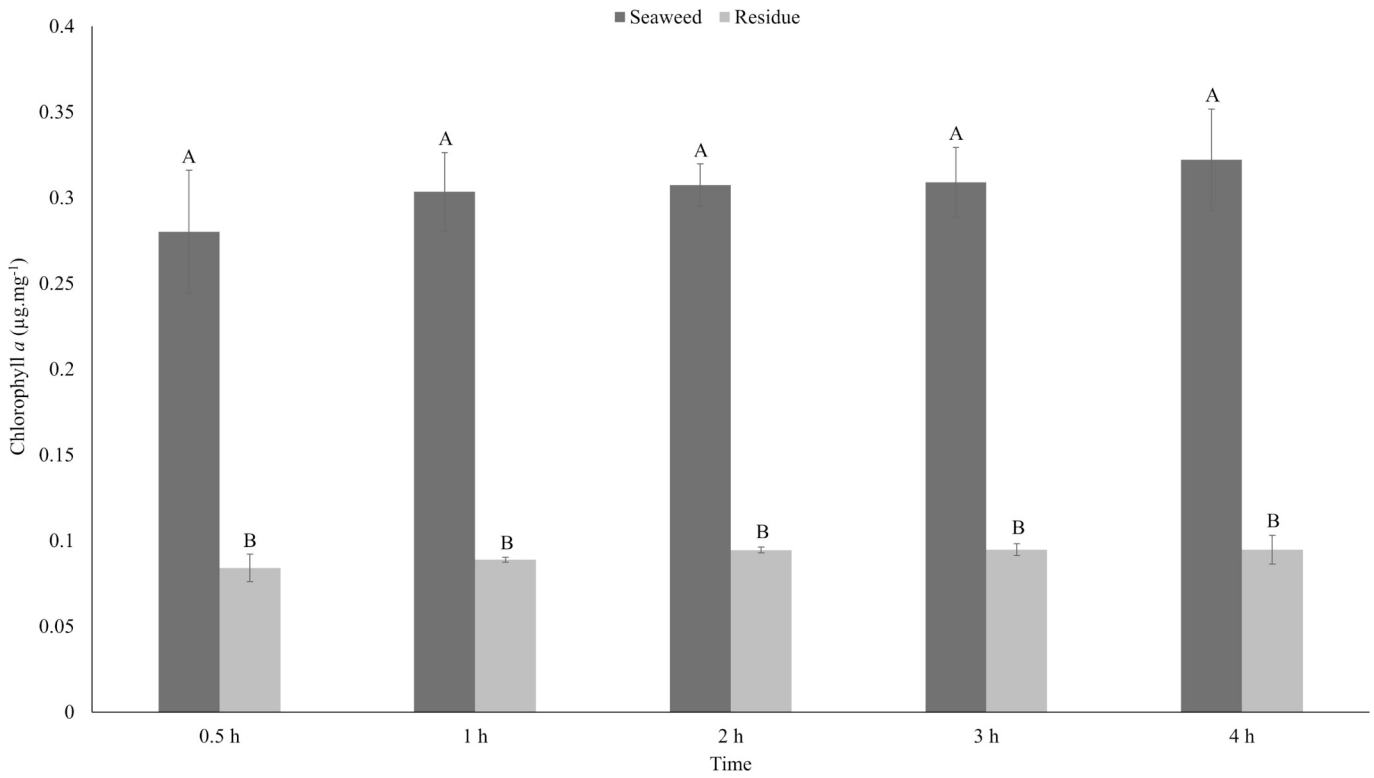

Fig. 3. Time optimization for chlorophyll *a* (µg.mg^{-1}) extraction using renewable ethanol as biosolvent.
Data are expressed as mean ± standard deviation. Scott-Knott test revealed no statistically significant differences within fresh seaweed or within residue groups ($p > 0.05$), as significant difference was observed between seaweed and residue ($p < 0.05$).

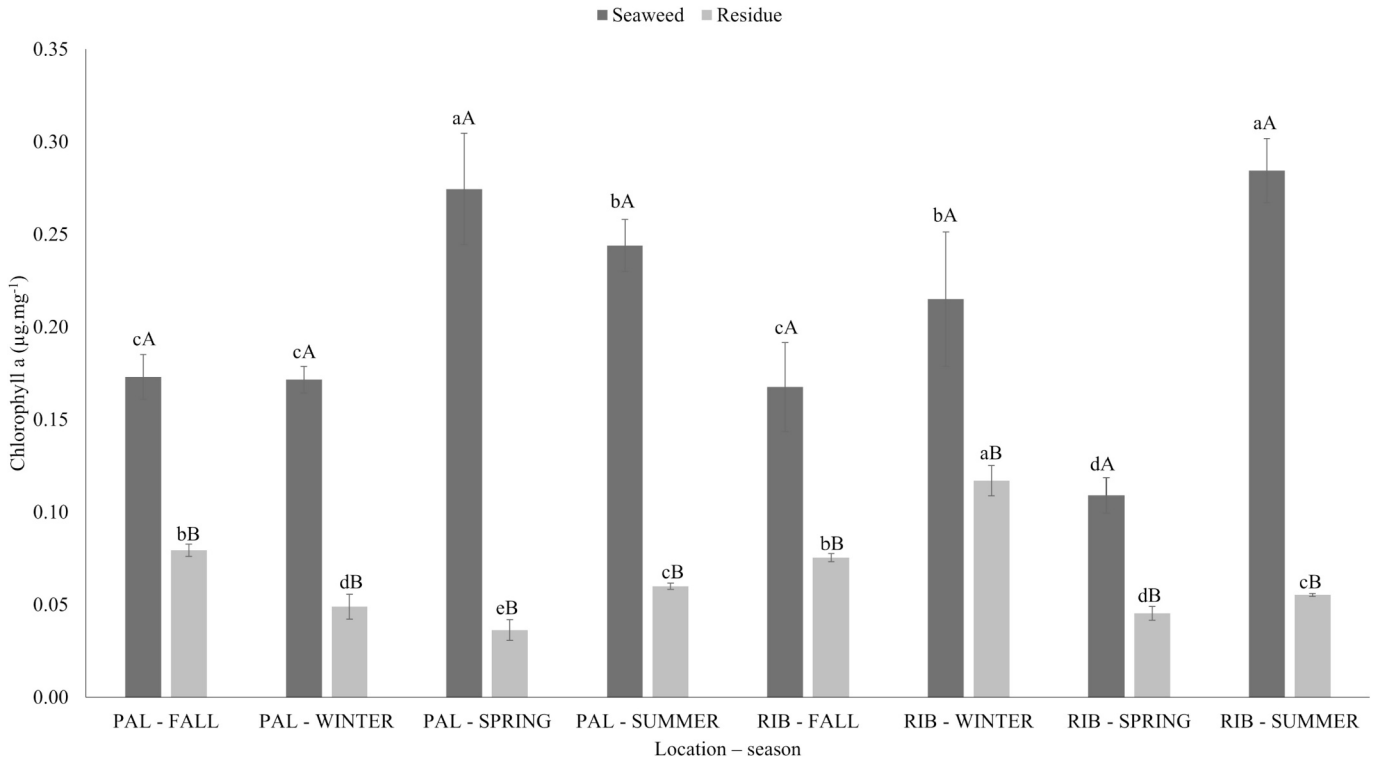

Fig. 4. Chlorophyll content (µg.mg^{-1}) of seaweed and residual biomasses of *K. alvarezii* grown in Palhoça (PAL) and Ribeirão da Ilha (RIB), across harvest seasons (2022−2023) in southern Brazil.
Data are expressed as mean ± standard deviation. Means were grouped using the Scott-Knott test ($p < 0.05$). Lowercase letters indicate statistical differences between seaweed or residue samples within each season, while uppercase letters denote differences between algae and residues for each respective season.

– spring).

The chlorophyll content in macroalgae is influenced by multiple exogenous factors, including cultivation depth [40,41], sea surface temperature [42], nutrient availability, wind direction, and even the presence of marine debris, which can reduce the irradiance the algal photosynthetic apparatus [43]. Consequently, variations in chlorophyll concentrations among macroalgae are common across different seasons and geographical regions. When compared with other studies, Rajaram et al. [44] reported higher chlorophyll levels in *K. alvarezii* cultivated in Palk Bay, on the southeast coast of India. The lowest pigment concentration recorded was 0.68 μg.mg^{-1} during the pre-monsoon period, increasing to 0.87 μg.mg^{-1} during the monsoon season. These values reflect India's tropical climate, characterized by two main seasons, largely differing from those found in southern Brazil, where four distinct seasons occur, including winters with temperatures below zero. Globally, *K. alvarezii* cultivation sites display an even wider range of chlorophyll concentrations due to regional and seasonal variability. For example, Paransa et al. [45] reported chlorophyll contents ranging from 0.00614 to 0.01832 μg.mg^{-1} during weekly cultivation cycles in Indonesia. Similarly, Periyasamy et al. [46] found concentrations between 0.79 and 1.10 μg.mg^{-1}, with an annual average of 0.93 μg.mg^{-1} along the Indian coastline. Iskandar et al. [41] observed chlorophyll levels from 0.006 to 0.013 μg.mg^{-1} in algae grown at varying depths, while Aslin et al. [47], using 80% acetone as extraction solvent, reported values between 1.86 and 6.21 μg.mg^{-1} in samples cultivated in Indonesia. This broad variation of contents underscores the importance of monitoring chlorophyll levels, as they serve as indicators of physiological stress within the organism. In fact, periods of environmental stress can lead to significant pigment fluctuations, potentially compromising algal growth and productivity [48].

3.2. Protocol optimization for phycobiliproteins extraction and seasonal analysis

The biomass-to-biosolvent ratio was standardized at 25 mg.mL^{-1} after it was observed that carrageenan, naturally present in *K. alvarezii*, interfered with the extraction process. This hydrocolloid significantly increased the viscosity of the extraction medium, hindering sample handling and reducing pigment recovery efficiency. Absorbance measurements of the extracts were taken after 30 min of extraction and again after 24 h, with samples kept refrigerated until the second reading. When evaluating the phycobiliproteins content (μg.mL^{-1}) in macroalgae and residue samples at both extraction times (i.e., 0.5 h and 24 h), calculated according to Kursar et al. [36], higher amounts were consistently obtained with 24 h extractions. In seaweed samples, the phycobiliproteins concentration ranged from 0.56 to 34.04 μg.mL^{-1} after 0.5 h, thus representing an increase by 5971% across treatments. For the longer extraction time, i.e., 24 h, values ranged from 9.34 to 58.33 μg.mL^{-1}, showing a 524% increase for the lowest concentration. Besides, the maximum PBP yield at 24 h (58.33 μg.mL^{-1}) was 71% higher than that achieved at 0.5 h (34.04 μg.mL^{-1}) (Table 1).

Regarding solvent performance, for seaweed samples extracted over 0.5 h, only water and EtOAc at 250 mM yielded significantly higher phycobiliproteins contents compared to the other solvents. After 24 h, higher recoveries were obtained using water and EtOH at all concentrations, CYR at 125 and 250 mM, EtOAc at all concentrations, and DMISO at 125 and 250 mM (Table 1).

For the residual biomass samples extracted over 0.5 h, higher phycobiliproteins yields were observed with EtOH at 500 mM, ButLac at 125 mM, EtOAc at 125 and 250 mM, and DMISO at 250 and 500 mM. After 24 h, only water and EtOAc exhibited superior extraction performance. Samples extracted with ButLac (500 mM) and VLC at 250 and 500 mM were excluded from the analysis due to turbidity in the aqueous phase, which interfered with spectrophotometric readings.

For the optimization of the extraction time, the same biomass-to-biosolvent ratio (25 mg·mL^{-1}) was maintained. Absorbance readings were taken every 60 min up to 4 h. Only the solvents that previously demonstrated the highest yields in phycobiliproteins extraction were selected for this test, as follows: water, PBS, EtOH, CYR (125 and 250 mM), EtOAc (125 and 250 mM), and DMISO (125, 250, and 500 mM) (Table 2). Overall, for both seaweed and residue samples, EtOAc at 125 mM consistently produced the highest phycobiliproteins concentrations, except for the 1-h extraction of the residue. The maximum amount of those pigments in the seaweed was 44.00 μg mL^{-1} after 3 h of extraction,

Table 1
Phycobiliprotein content (μg.mL^{-1}) of fresh and residual biomass samples of *K. alvarezii* according to the biosolvent systems and times of extraction.

Solvent	Concentration (mM)	Seaweed PBP (μg.mL^{-1})		Residue PBP (μg.mL^{-1})	
		0.5 h	24 h	0.5 h	24 h
Water	–	32.33 ± 2.42 a	50.90 ± 3.99 a	20.05 ± 1.43 b	29.35 ± 1.19 c
PBS	–	26.48 ± 0.97 b	40.26 ± 4.00 b	21.00 ± 1.00 b	30.20 ± 1.19 c
EtOH	125	26.37 ± 0.98 b	45.49 ± 7.558 a	16.72 ± 0.34 b	28.91 ± 1.25 c
	250	25.88 ± 3.20 b	44.20 ± 6.65 a	21.12 ± 1.63 b	36.93 ± 6.05 c
	500	25.72 ± 1.46 b	50.91 ± 4.40 a	26.05 ± 1.73 a	35.22 ± 1.21 c
CYR	125	27.69 ± 3.30 b	51.38 ± 16.45 a	19.19 ± 8.78 b	33.84 ± 0.89 c
	250	22.52 ± 3.27 c	48.04 ± 12.39 a	13.35 ± 0.94 c	37.03 ± 1.89 c
	500	16.11 ± 0.59 d	38.06 ± 2.51 b	12.18 ± 0.40 c	30.98 ± 1.45 c
ButLac	125	15.53 ± 1.79 d	38.70 ± 10.62 b	25.13 ± 7.40 a	15.53 ± 1.79 d
	250	1.18 ± 0.83 f	23.09 ± 7.07 b	2.18 ± 2.04 d	1.18 ± 0.83 e
EtOAc	125	26.56 ± 3.21 b	58.33 ± 2.74 a	28.05 ± 0.54 a	47.41 ± 3.85 a
	250	34.04 ± 13.27 a	49.98 ± 10.28 a	27.74 ± 1.32 a	40.36 ± 1.04 b
	500	15.02 ± 0.67 d	50.33 ± 9.41 a	19.05 ± 1.17 b	30.12 ± 0.52 c
DMISO	125	20.83 ± 1.50 c	44.69 ± 2.35 a	18.75 ± 1.07 b	40.15 ± 2.55 b
	250	20.08 ± 1.55 c	48.14 ± 14.93 a	23.47 ± 2.01 a	50.78 ± 18.56 a
	500	17.77 ± 1.72 d	28.22 ± 8.54 b	23.47 ± 0.27 a	41.35 ± 0.51 b
EtLac	125	11.29 ± 0.30 e	31.68 ± 3.83 b	14.76 ± 0.71 b	11.73 ± 0.28 d
	250	0.56 ± 0.98 f	9.34 ± 2.36 c	0.811 ± 0.31 d	1.04 ± 0.15 e
	500	1.41 ± 0.64 f	12.58 ± 5.26 c	0.043 ± 0.20 d	0.80 ± 0.53 e
VLC	125	12.69 ± 1.45 e	30.58 ± 3.37 b	11.96 ± 0.61 c	13.01 ± 0.50 d
	250	3.63 ± 0.67 f	-*	5.83 ± 3.31 d	-*
	500	3.15 ± 0.45 f	-*	4.37 ± 1.32 d	-*

PBS = phosphate buffer 50 mM; EtOH = ethanol; CYR = cyrene; ButLac = Butyl lactate; EtOAc = ethyl acetate; DMISO = dimethyl isosorbide; EtLac = Ethyl lactate; VLC = γ-valerolactone. Data are expressed as mean ± standard deviation. Means followed by distinct letters in the columns indicate statistically significant differences ($p < 0.05$) according to the Scott-Knott test.

* Cloudy sample.

Table 2
Heatmap of phycobiliproteins concentration (μg.mL^{-1}) of *K. alvarezii* fresh and residual biomass samples according to the biosolvent systems and times of extraction.

Solvents	Seaweed				Residue			
	1 h	2 h	3 h	4 h	1 h	2 h	3 h	4 h
Water	27.14 ± 0.80 ab	30.53 ± 2.27 b	32.00 ± 2.02 b	34.09 ± 3.48 a	21.83 ± 0.42 c	25.11 ± 2.02 d	21.13 ± 1.06 e	24.70 ± 1.75 e
PBS	17.98 ± 2.61 c	24.49 ± 4.59 c	28.16 ± 2.15 b	26.07 ± 3.28 b	32.55 ± 2.32 a	31.15 ± 0.82 a	28.32 ± 0.78 c	28.37 ± 0.69 c
125 mM EtOH	23.03 ± 2.93 bc	32.24 ± 0.57 b	37.59 ± 2.79 a	37.87 ± 4.83 a	19.37 ± 1.12 d	22.68 ± 0.61 d	24.23 ± 2.22 d	24.11 ± 1.48 e
250 mM EtOH	26.05 ± 0.89 ab	31.54 ± 2.00 b	31.11 ± 1.01 b	34.66 ± 4.97 a	19.01 ± 0.10 d	23.52 ± 0.63 d	26.18 ± 1.45 d	26.79 ± 1.30 d
500 mM EtOH	28.08 ± 1.47 ab	34.23 ± 3.25 b	38.55 ± 6.35 a	34.30 ± 1.51 a	26.03 ± 0.18 b	29.63 ± 0.82 b	33.81 ± 3.60 b	31.55 ± 1.57 b
125 mM CYR	25.11 ± 3.97 abc	32.11 ± 1.91 b	31.93 ± 1.10 b	36.17 ± 0.98 a	15.68 ± 0.36 e	20.12 ± 0.38 e	22.76 ± 0.19 e	23.24 ± 0.38 e
250 mM CYR	24.05 ± 2.41 bc	32.13 ± 3.70 b	30.30 ± 4.00 b	36.63 ± 4.18 a	13.98 ± 0.17 e	17.63 ± 0.44 f	21.42 ± 1.78 e	22.42 ± 1.69 e
125 mM EtOAc	32.31 ± 3.65 a	43.02 ± 3.17 a	44.00 ± 7.35 a	39.10 ± 1.68 a	27.01 ± 0.21 b	31.85 ± 0.51 a	38.43 ± 5.49 a	35.79 ± 0.44 a
250 mM EtOAc	27.98 ± 0.99 ab	32.24 ± 4.38 b	39.80 ± 4.24 a	35.75 ± 3.08 a	25.16 ± 1.07 b	29.71 ± 1.20 b	31.50 ± 1.46 c	30.87 ± 1.16 b
125 mM DMISO	25.41 ± 0.78 ab	29.65 ± 1.91 b	33.74 ± 0.78 b	32.54 ± 2.94 a	20.93 ± 0.58 c	23.86 ± 1.20 d	26.43 ± 1.15 d	26.14 ± 1.91 d
250 mM DMISO	23.41 ± 3.39 bc	24.68 ± 0.32 c	28.35 ± 1.17 b	28.11 ± 2.06 b	25.26 ± 0.60 b	26.85 ± 1.46 c	29.40 ± 1.03 c	30.36 ± 1.65 b
500 mM DMISO	23.11 ± 1.96 bc	26.73 ± 2.46 c	25.43 ± 2.06 b	26.15 ± 2.16 b	24.48 ± 0.63 b	27.21 ± 0.99 c	29.13 ± 0.31 c	28.83 ± 1.34 c

Means followed by the same letter, in the column, do not differ statistically by the Scott-Knott test ($p \leq 0.05$). PBS = phosphate buffer 50 mM; EtOH = ethanol biorenewable; CYR = Cyrene; EtOAc = ethyl acetate; DMISO = dimethyl isosorbide.

while the residue reached a maximum of 38.43 μg mL^{-1}. Notably, for the residue, phycobiliproteins also yielded relatively high values at 1 and 2 h, reaching a maximum content of 32.55 μg.mL^{-1}; a value ca. 15.85% lower than the maximum obtained at 3 h (Table 2).

Optimal conditions for phycobiliproteins extraction were established using 125 mM EtOAc for 3 h, allowing for the achievement of the maximum yield within the shortest extraction time. The efficiency of EtOAc in disrupting macroalgal cell structures, and consequently facilitating the release of intracellular compounds, has been well documented for various biomolecules, including lipids [49], phlorotannins [50], and other polyphenols [51]. In contrast, PBS remains a widely used aqueous medium for phycobiliproteins extraction from seaweed, particularly when combined with physical disruption techniques such as ultrasonication [52] or repeated freeze-thaw cycles [53], which are responsible for the increase in the protein release.

After selecting EtOAc (125 mM) as the optimal biosolvent and establishing an extraction time of 3 h, the total concentration of phycobiliproteins was determined in samples according to the season in which the *K. alvarezii* biomass was harvested (Fig. 5). In seaweed samples, phycobiliproteins content varied from 11.04 μg.mg^{-1} in spring (PAL) to 92.80 μg.mg^{-1} in summer (PAL), representing an increase of around 840% between the minimum and maximum values. The highest concentration was found during summer at the PAL site (92.80 ± 1.28 μg.mg^{-1}), showing a statistically significant difference from all other seaweed samples (Fig. 5). For the residual biomass, phycobiliproteins content ranged from 6.93 μg.mg^{-1} in spring (RIB) to 28.03 μg.mg^{-1} in fall (RIB), corresponding to an increase of approximately 405% between the lowest and highest values. The highest concentrations were recorded during winter for both locations, RIB (28.03 ± 1.42 μg.mg^{-1}) and PAL (26.55 ± 0.28 μg.mg^{-1}), with no statistical difference between them,

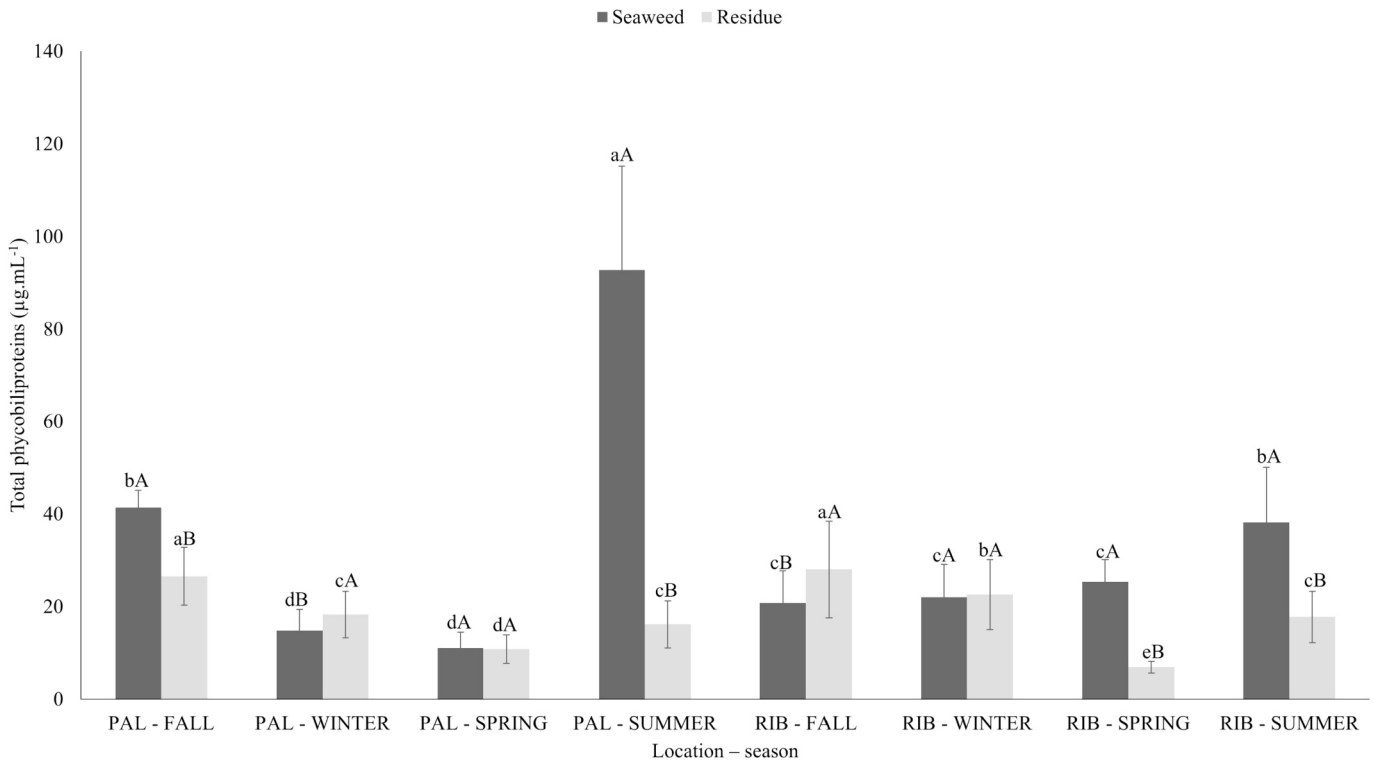

Fig. 5. Total phycobiliproteins content (μg.mL^{-1}) in seaweed and residual biomasses of *K. alvarezii* grown in Palhoça (PAL) and Ribeirão da Ilha (RIB), across harvest seasons (2022–2023) in southern Brazil.
Data are expressed as mean ± standard deviation. Means were grouped using the Scott-Knott test ($p < 0.05$). Lowercase letters indicate statistical differences among seaweed or residue samples within season, while uppercase letters denote differences between seaweed and residue for each respective season.

though both samples differed significantly from the other along the seasons (Fig. 5). When comparing seaweed and residue samples within the same location and season, significant variations were observed. Seaweed samples from PAL (fall and summer) and RIB (spring and summer) exhibited higher phycobiliprotein contents than their respective residues. Conversely, residue samples from PAL (winter) and RIB (fall) showed higher concentrations than the corresponding seaweed samples. No statistical differences were detected between seaweed and residue for PAL in spring or RIB in winter (Fig. 5).

As observed for chlorophylls, total phycobiliprotein contents are strongly influenced by environmental factors. Variables such as nutrient availability and temperature play key roles in regulating the biosynthesis and accumulation of these pigments [54,55]. On *K. alvarezii*, the occurrence of distinct color morphotypes must also be considered when interpreting pigment content, as red algae typically contain phycocyanin, phycoerythrin, and allophycocyanin [56,57]. The considerable variation observed among our samples reflects the color heterogeneity of the harvested biomass across different seasons.

Among the phycobiliproteins, phycoerythrin was the predominant pigment in *K. alvarezii*, and its concentration in the samples was quantified using a calibration curve at 565 nm (Fig. 6; Supplementary Material – Table S4). In the fresh seaweed samples, phycoerythrin content ranged from 0.569 $\mu g.mg^{-1}$ (RIB – spring) to 4.03 $\mu g.mg^{-1}$ (RIB – summer), corresponding to a 707% increase between the lowest and highest values. The highest concentrations occurred during summer at both sites, differing statistically from the other seasons. The maximum content was recorded at PAL (4.03 ± 0.04 $\mu g.mg^{-1}$), followed by RIB (1.78 ± 0.15 $\mu g.mg^{-1}$). For the alga residues, phycoerythrin levels varied from 0.400 $\mu g.mg^{-1}$ (PAL – spring) to 1.392 $\mu g.mg^{-1}$ (PAL – fall), representing a 348% relative increase. The highest concentrations were found in fall-harvested samples for both sites, with RIB showing the greater value (1.39 ± 0.07 $\mu g.mg^{-1}$) compared to PAL (1.19 ± 0.02 $\mu g.mg^{-1}$). These discrepancies were statistically significant relative to each other and to the remaining samples (Fig. 6).

Martino et al. [58] reported phycoerythrin concentrations ranging from 1.51 ± 0.49 to 6.37 ± 1.45 $\mu g.g^{-1}$ for a green strain of *K. alvarezii* cultivated indoors during autumn. The extraction was performed using a 50 mM PBS solution, and pigment quantification was conducted spectrophotometrically following the equations proposed by Kursar et al. [36]. Similarly, Lumbessy et al. [59] observed a maximum phycoerythrin content, i.e., 0.405 $\mu g.mg^{-1}$ in *K. alvarezii* cultivated in vitro from Indonesian strains using a fertilizer specifically designed for photosynthetic studies. These findings highlight the considerable variability in phycoerythrin content arising from differences in cultivation conditions and extraction methodologies, underscoring the importance of methodological standardization in future research. Despite its biological and industrial relevance, phycoerythrin content in *K. alvarezii* remains underreported in the literature. This gap likely results from the predominant research focus on other pigments, the pigment's relatively low abundance, and the technical challenges associated with its extraction and quantification. Beyond its role in photosynthesis, phycoerythrin exhibits promising biotechnological applications, including its use as a natural colorant in the food industry [60], as well as for its antioxidant and antimicrobial properties [61]. Consequently, further studies specifically targeting this pigment in *K. alvarezii* are essential to better elucidate its potential across diverse industrial and biomedical fields.

3.3. Protocol optimization for MAA, extraction and seasonal analysis

MAA extraction was optimized concurrently with phycobiliproteins, as these metabolites are co-extracted in aqueous media. MAA quantification was performed using the full absorption spectra of the phycobiliproteins, specifically monitoring absorbance at 330 nm. Based on these analyses, the optimal extraction conditions were determined to be 125 mM EtOAc for 3 h. MAAs are characteristic metabolites of red macroalgae, widely recognized for their strong UV-absorbing capacity, which

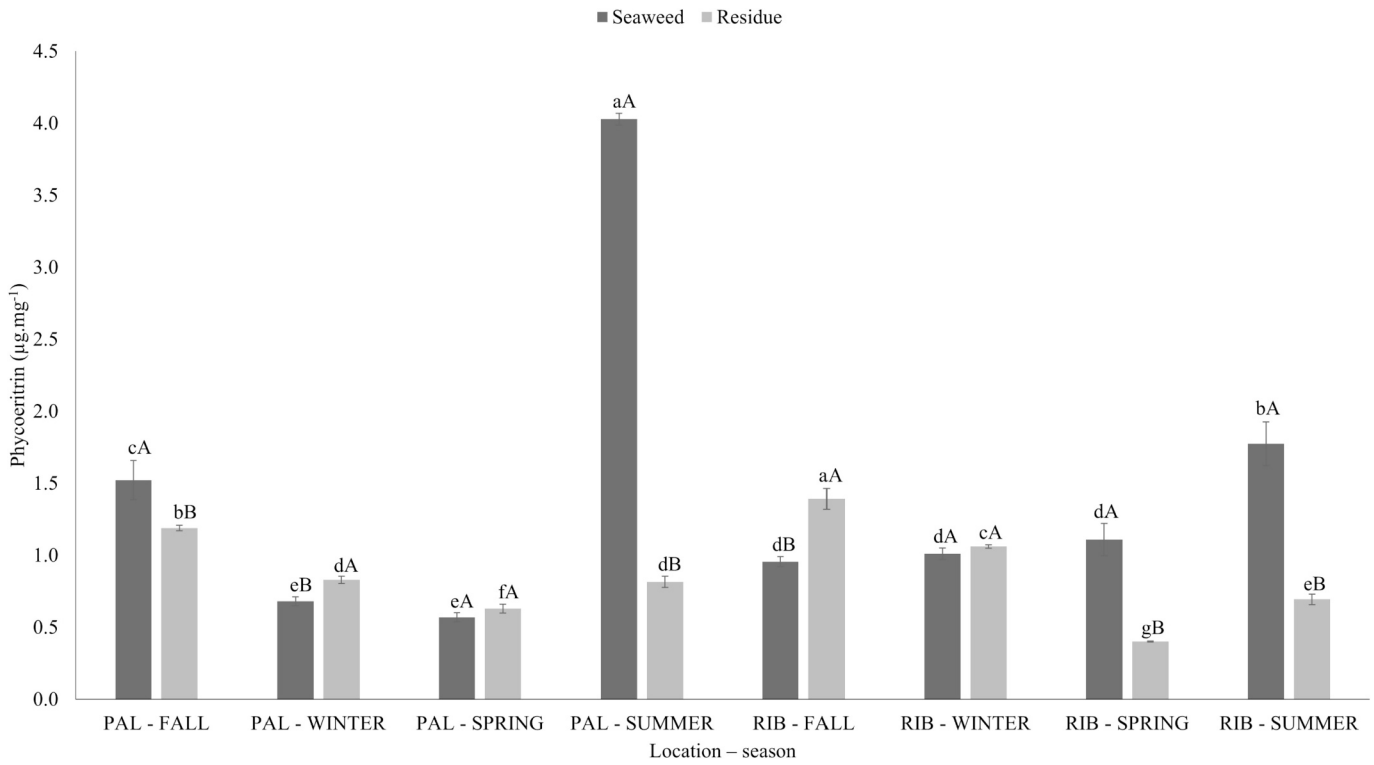

Fig. 6. Phycoerithrin content ($\mu g.mg^{-1}$) in seaweed and residual biomasses of *K. alvarezii* grown in Palhoça (PAL) and Ribeirão da Ilha (RIB), across harvest seasons (2022–2023) in southern Brazil.
Data are expressed as mean ± standard deviation. Means were grouped using the Scott-Knott test ($p < 0.05$). Lowercase letters indicate statistical differences among seaweed or residue samples within each season, while uppercase letters denote differences between seaweed and residue for each respective season.

imparts photoprotective properties and potential applications in preventing UV-induced skin damage [62,63]. Their maximum absorbance in the UV–Vis spectrum typically occurs around 330 nm, depending on the molecular structure of the specific MAA, following the absorbance region associated with phenolic compounds and proteins at approximately 280 nm [18]. Flavonoids, on the other hand, also exhibit strong absorption within the 240–385 nm spectral range, varying according to their chemical structure [64,65]. The presence of flavonoids in *K. alvarezii* has been previously reported [66,67], raising the possibility that the absorbance values measured in this study may not exclusively represent MAAs but could also include contributions from flavonoid compounds. To address this, Fig. S1 – Supplementary material presents the UV–Vis absorption spectra of both seaweed and residual biomasses of *K. alvarezii* within the spectroscopic region of interest, allowing for a more accurate interpretation of the overlapping signals.

The content of MAAs varied markedly across seasons and sampling sites in both the seaweed and residual biomasses of *K. alvarezii*. In seaweed samples, MAA levels ranged from 42.82 μg.mg^{-1} (PAL – summer) to 106.15 μg.mg^{-1} (RIB – spring), corresponding to a relative increase of 248%. The highest concentration, recorded in spring for RIB (106.15 ± 1.36 μg·mg^{-1}), differed statistically from all other samples (Fig. 7). For the residual biomass samples, MAAs concentrations ranged from 35.54 μg.mg^{-1} (RIB – spring) to 135.64 μg.mg^{-1} (PAL – fall), representing a substantial 381.6% variation. The maximum concentration observed in fall for PAL (135.64 ± 1.12 μg.mg^{-1}) was also statistically distinct from all other samples (Fig. 7). The comparison of seaweed and residue samples by season and location revealed significant differences across all cases. A heterogeneous pattern was observed: higher MAA levels occurred in seaweed during winter and spring at PAL and during spring at RIB, whereas the residues showed higher concentrations in fall and summer at PAL and in fall, winter, and summer at RIB. Overall, the greater MAA concentrations detected in the residues suggest incomplete extraction or potential retention and preservation of these compounds even after biostimulant production.

Notably, MAAs have been identified in several red marine algae (Rhodophyta), such as *Porphyra umbilicalis* and *Grateloupia turuturu*, both of which are widely consumed as nutritious food sources, particularly in Asian countries [68,69]. Numerous studies have reported variable extraction yields of MAAs from red seaweeds, influenced by genotypes, solvent composition, and extraction parameters. Sun et al. [70], for example, evaluated four species (*Bangia fusco-purpurea*, *Gelidium amansii*, *Gracilaria confervoides*, and *Gracilaria* sp.) and reported extraction yields ranging from 9% to 32%, with the highest yield obtained from *Gracilaria* sp. using 25% ethanol under optimized conditions (40 °C, 2 h, biomass-to-solvent ratio of 1:20 g.mL^{-1}). Similarly, Zwerger et al. [71] isolated 15.7 mg of shinorine and 36.2 mg of porphyra-334 from 4 g of crude *Porphyra* sp. extract using fast centrifugal partition chromatography, corresponding to a total MAA recovery of approximately 1.3%. These findings underscore the considerable variation in MAA yields among red algae species and highlight the importance of optimizing extraction processes to enhance recovery efficiency, particularly for applications involving high-value bioactive compounds within a biorefinery framework. A more targeted analytical approach could be applied in future studies to confirm the presence and precise identification of MAAs in *K. alvarezii*. Nonetheless, as observed for the other pigments analyzed in this study, MAAs content also exhibited seasonal variation across seaweed and residue samples.

3.4. Principal component analysis

Principal Component Analysis (PCA) was performed on the dataset comprising chlorophylls, phycobiliproteins, and MAAs contents. The descriptive model revealed that the first two principal components, PC1 (48%) and PC2 (31%), together explained 79% of the total data variance (Fig. 8). Two distinct clusters were observed primarily along the PC1 axis: one comprising the algal residue samples (PC1–, PC2–/PC2+) and

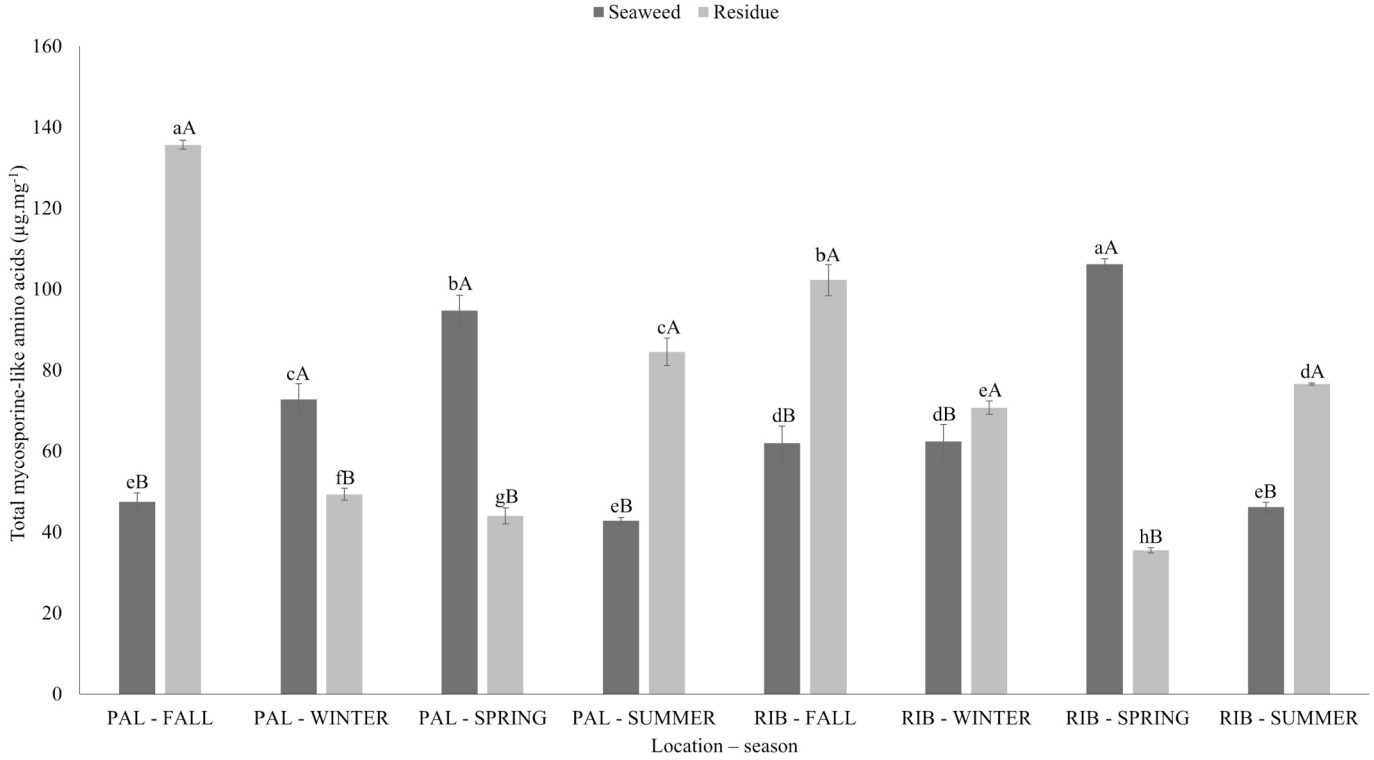

Fig. 7. MAAs content (μg.mg^{-1}) of seaweed and residual biomasses of *K. alvarezii* grown in Palhoça (PAL) and Ribeirão da Ilha (RIB), across harvest season (2022–2023) in southern Brazil.
Data are expressed as mean ± standard deviation. Means were grouped using the Scott-Knott test ($p < 0.05$). Lowercase letters indicate statistical differences among seaweed or residue samples within each season, while uppercase letters denote differences between seaweed and residue for each respective season.

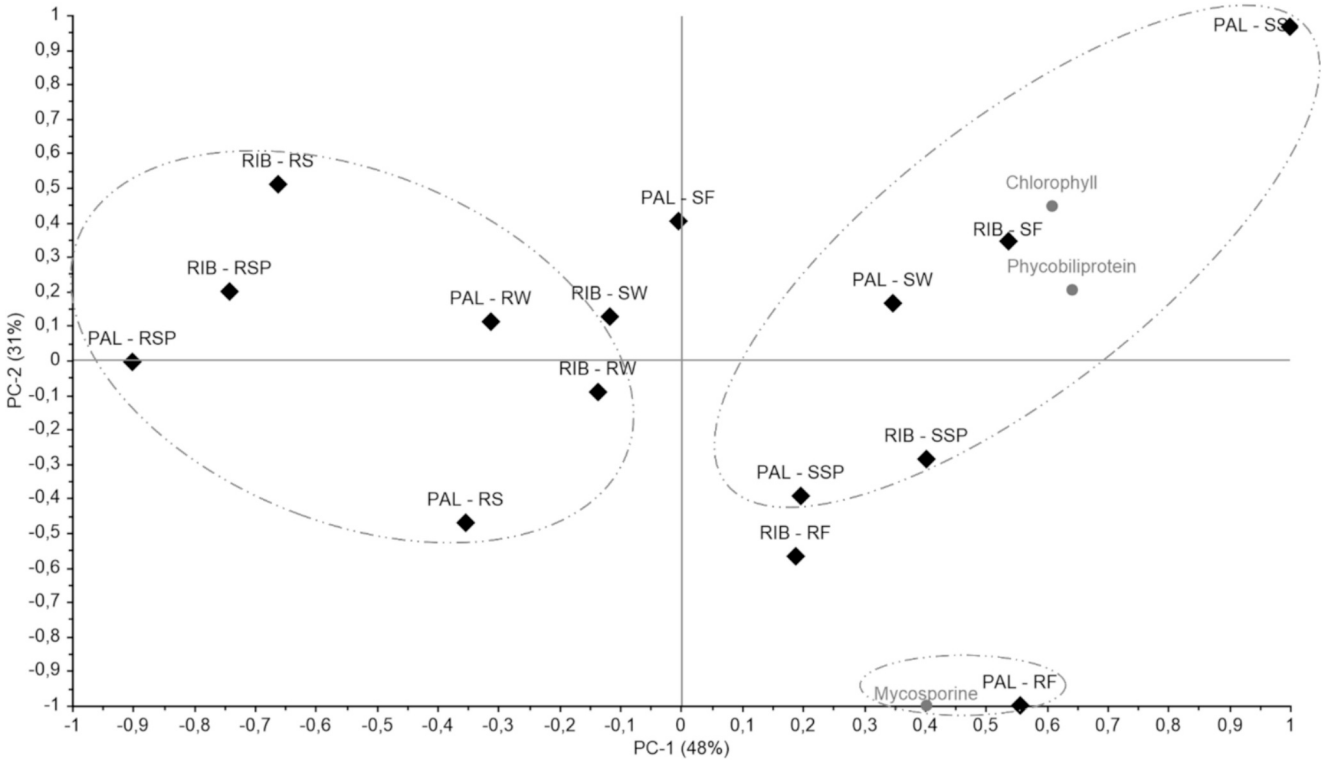

Fig. 8. Scatter plot of principal component scores (PC1 and PC2) derived from the dataset of total contents of chlorophylls, phycobiliproteins, and MAAs in seaweed and residual biomasses of *K. alvarezii* grown in Palhoça (PAL) and Ribeirão da Ilha (RIB), across harvest seasons (2022–2023) in southern Brazil.
SF – Seaweed Fall; SW – Seaweed Winter; SSP – Seaweed Spring; SS – Seaweed Summer; RF – Residue Fall; RW – Residue Winter; RSP – Residue Spring; RS – Residue Summer.

another grouping the seaweed samples (PC1+, PC2+/PC2–), mainly driven by differences in pigment composition. The loading plot indicated that chlorophyll and phycobiliproteins' concentrations were key variables in differentiating between seaweed and residual biomass. Notably, the MAA-rich fall sample from PAL showed a distinct position (PC1+/PC2–), separating it from all other samples. These findings suggest that chlorophylls and phycobiliproteins are predominantly associated with seaweed biomass and vary seasonally, whereas the association of MAAs with the residue samples may reflect their relative concentration following the bio-stimulant production process.

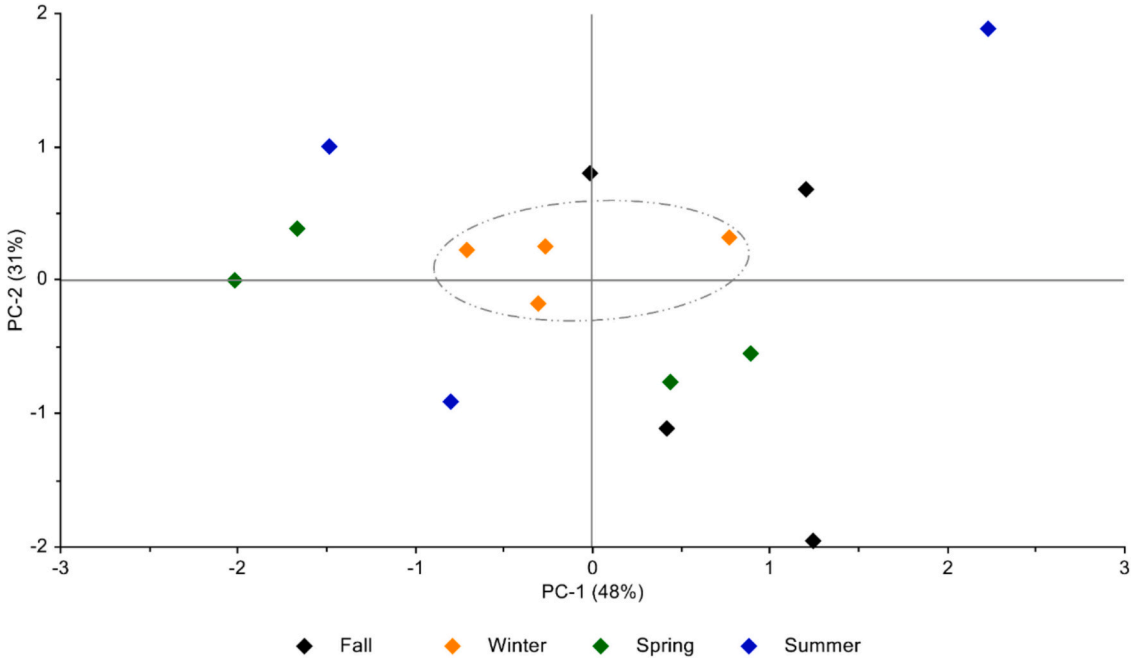

Fig. 9. Scatter plot of principal component scores (PC1 and PC2) derived from the samples of *K. alvarezii* grown during autumn, winter, spring, and summer in Palhoça (PAL) and Ribeirão da Ilha (RIB) in Southern Brazil.

Importantly, no statistically significant differences were found in the contents of Chl, PE, and MAA when comparing seaweed and residue samples across the different locations (Table S5 – supplementary material).

A second PCA was conducted using the pigment dataset of the macroalga, considering the seasonal origin of the samples. This model explained a total variance of 79%, with PC1 alone accounting for 48% of the variation. Among the four seasons, only the winter samples displayed a distinct clustering pattern, positioned near the center of the PCA plot (between −1 and 1 on both PC1 and PC2 axes). This tighter grouping suggests that the pigment profiles of winter samples were more homogeneous compared to those from other seasons, which exhibited greater dispersion. Such uniformity may be attributed to the marked temperature decrease typical of southern Brazil's subtropical climate during the winter months.

Analysis of the results in Fig. 9 reveals a clear seasonal dependence on *K. alvarezii* growth, closely related to temperature. The species reaches its growth peak between 28 and 36 °C [72], with a daily biomass increase of 0.94% at 30 °C [73]. However, the production of antioxidant metabolites and pigments is highly influenced by exogenous factors such as depth, temperature, light intensity, and cultivation practices [74]. Consequently, the lower temperatures typically found of southern Brazil may induce stress in the macroalga, promoting the accumulation of metabolites essential for survival. Other environmental variables, including salinity, precipitation, and hydrodynamics, warrant further investigation to better understand their contribution to seasonal variations in pigment and metabolite profiles.

3.5. Limitations and perspectives

Although the present study demonstrates good reproducibility, some limitations should be acknowledged. First, the absence of key environmental measurements intrinsically related to pigment and MAA biosynthesis, such as light intensity and water pH, represents a limitation. These parameters could not be monitored because they were not routinely measured by the farms and companies responsible for biomass production. Second, the experimental design was primarily oriented toward an industrial context, in which macroalgal biomass is typically not segregated by color strain, given its broad applicability in biotechnological processes. Consequently, potential pigment variability associated with different color morphotypes was not addressed in this study.

Another important limitation is that the seasonal analysis was based on a single year of sampling, which restricts the ability to capture interannual variability in pigment production. Future studies should therefore extend the temporal monitoring period and include additional cultivation regions, as the present work focused exclusively on *K. alvarezii* cultivated in southern Brazil.

From an academic perspective, future investigations could also benefit from color-strain-based biomass segregation, which may provide deeper insights into the behavior and distribution of different pigment classes and MAAs under varying environmental conditions. As reported by Araújo et al. [75], in addition to seasonality, pigment extraction with segregation between *K. alvarezii* lineages (green and red) results in quantitative differences in the extracted compounds. This allows for seasonal selectivity to more efficiently exploit the different compounds produced by the macroalga. Such an approach would enable seasonal and strain-specific selectivity, contributing to more efficient exploitation of the macroalga's biochemical potential and supporting the development of optimized and sustainable biotechnological applications.

4. Conclusions

This study optimized the extraction of pigments from fresh *K. alvarezii* biomass and its post-processing residue, highlighting the potential of sustainable, bio-based solvents. Biorenewable ethanol yielded the highest chlorophyll recovery, while ethyl acetate presented the highest extraction efficiency for PBP and MAAs. The integration of green extraction strategies, seasonal assessments, and residue valorization significantly contributes to circular economy and biorefinery concepts.

Quantitative analyses revealed distinct pigment distributions between the fresh biomass and the residual fraction. Chlorophylls and phycobiliproteins were more abundant in the fresh biomass (40% and 30% respectively), whereas the residue exhibited elevated MAA contents (78%), indicating that these compounds remain stable even after biostimulant production.

Seasonal evaluation demonstrated that chlorophyll and phycobiliproteins reached higher concentrations during warmer months, particularly in spring and summer. In contrast, MAAs show an opposite pattern, with higher concentrations at lower temperatures, suggesting a possible physiological stress response by *K. alvarezii*. Overall, these findings support advanced sustainable extraction methods and highlight the added value of seaweed-derived by-products, contributing to waste minimization and promoting the full valorization of seaweed biomass by the recovery of high-value compounds.

CRediT authorship contribution statement

Alex Schneider: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Conceptualization. **Aline Nunes:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Alex Alves dos Santos:** Writing – review & editing. **Sónia P.M. Ventura:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Marcelo Maraschin:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Funding declaration

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2026.104713.

Data availability

Data will be made available on request.

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