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Synthesis, characterization and *in vitro* antioxidant activity of a sodium zinc metallo-chlorophyll obtained from the marine microalgae *Dunaliella tertiolecta*.

Síntesis, caracterización y actividad antioxidante *in vitro* de una metaloclorofila de sodio y zinc obtenida de la microalga marina *Dunaliella tertiolecta*

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ABSTRACT

Chlorophyll is unstable and prone to modifications during industrial processing, reducing its functionality and commercial value. Therefore, its conversion into water-soluble and thermally stable derivatives, called metallo-chlorophylls, allows for commercial exploitation. Here, a process is described for obtaining a sodium and zinc metallo-chlorophyll from the marine microalgae *Dunaliella tertiolecta*, a source with greater sustainability and profitability than traditionally used green vegetables. The process consisted of three stages: alkaline hydrolysis of chlorophyll, addition of the metal ion (Zn^{2+}), and conversion to chlorophyllin salts. The optimal concentrations for each stage were determined: ethanol-NaOH (15 %), $ZnSO_4$ (30 %), and ethanol-NaOH (5 %), respectively. The yield of sodium and zinc metallo-chlorophyll was 565 ± 2.3 mg/g of chlorophyll (57 %), exhibiting a water solubility of 94.66 ± 1.33 %. Through UV-VIS and FTIR spectroscopy, the metallo-chlorophyll obtained here showed absorption maxima and characteristic bands of metallo-chlorophylls. In addition, it exhibited high thermal stability (40-60 °C) to moderate (70-90 °C) over a wide range of time (5-60 min). Finally, its antioxidant capacity was determined by DPPH, FRAP and ABTS assays, showing the highest values through ABTS assay [44.402 ± 1.198 mmol TEAC/100 g (dw)], surpassing values reported in various foods considered important antioxidant sources. These results support the potential of sodium and zinc metallo-chlorophyll, generated from a sustainable source, such as marine microalgae, as a bioactive compound with potential application as a functional ingredient.

KEY WORDS: Chlorophyll; metallo-chlorophyll; bioactive compound; antioxidant; microalgae.

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R E S U M E N

Chlorophyll is unstable and prone to modifications during industrial processing, reducing itsLa clorofila es inestable y susceptible a modificaciones durante el procesamiento industrial, lo que reduce su funcionalidad y valor comercial. Por ello, su conversión a derivados hidrosolubles y termoestables, denominados metaloclorofilas, permite su aprovechamiento comercial. En este trabajo se describe un proceso para obtener una metaloclorofila de sodio y zinc a partir de la microalga marina *Dunaliella tertiolecta*, una fuente más sustentable y rentable que los vegetales verdes tradicionalmente utilizados. El proceso consistió en tres etapas: hidrólisis alcalina de la clorofila, adición del ion metálico (Zn^{2+}) y conversión a sales de clorofilina. Las concentraciones óptimas de cada etapa fueron: etanol-NaOH (15 %), $ZnSO_4$ (30 %) y etanol-NaOH (5 %), respectivamente. El rendimiento de conversión a metaloclorofilina de sodio y zinc fue de 565 ± 2.3 mg/g de clorofila (57 %), mostrando solubilidad en agua de 94.66 ± 1.33 %. Mediante espectroscopía UV-VIS y FTIR, la metaloclorofila obtenida presentó máximos de absorción y bandas características de este tipo de compuestos. Además, mostró alta estabilidad térmica entre 40-60 °C, y moderada entre 70-90 °C, esto durante un amplio intervalo de tiempo (5-60 min). Finalmente, se determinó su capacidad antioxidante mediante los ensayos de DPPH, FRAP y ABTS, mostrando ABTS los valores más altos [44.402 ± 1.198 mmol TEAC/100 g (dw)], superando los valores reportados en alimentos considerados fuentes importantes de antioxidantes. Estos resultados respaldan el potencial de una metaloclorofila de sodio y zinc generada a partir de una fuente sustentable, como una microalga marina, como un compuesto bioactivo con potencial aplicación como ingrediente funcional.

P A L A B L A S C L A V E : Clorofila; metaloclorofila; compuesto bioactivo; antioxidante; microalga.

Introduction

The high prevalence of chronic degenerative diseases, along with the persistence of infectious diseases and their well-established links to diet and lifestyle, has sparked growing global interest in food that not only provides basic nutrition but also promotes health. In recent decades, there has been a sustained interest in identifying and characterizing new food sources, supplements, and food ingredients containing bioactive compounds, that is, molecules that provide an added benefit for healthcare, including disease prevention and treatment (Barbulova *et al*., 2015; Kussman *et al*., 2023).

Revista Bio ciencias
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Marine organisms are among the main sources recognized as producers of bioactive compounds due to their wide distribution and availability since 70 % of the Earth's surface is covered with water, which provides a guaranteed supply (Ezquerra-Brauer & Chan-Higuera, 2021). Within this group, marine microalgae stand out, representing a highly sustainable source. In principle, during their production, they do not compete for soils for agri-food use, nor do they require freshwater, an important consideration given that only 2.5 % of the Earth's water is freshwater, and most of it is unavailable for consumption due to its presence in glaciers, snow or ice (CONAGUA, 2019). Furthermore, freshwater conditions represent an unfavorable environment for the maintenance of axenic cell cultures, an essential requirement for ensuring the quality of compounds. Another characteristic of interest in the cultivation of marine microalgae is their efficient nutrient assimilation, which enables high and sustained productivity throughout the year. In addition, certain species can be cultivated under extreme biotic and abiotic stress conditions, making them natural, promising, and virtually unlimited sources of bioactive molecules (Levasseur *et al*., 2020).

Dunaliella tertiolecta is a marine microalgae that has attracted great commercial interest due to its high production of bioactive compounds, such as chlorophylls, carotenoids, xanthophylls, and other pigments with documented antioxidant, antihypertensive, bronchodilator, hepatoprotective, and several bioactive properties (Medina-Félix *et al*., 2020; Bezerra-daSilva *et al*., 2021). Among these, chlorophyll is the most abundant, accounting for up to 10 % of its dry weight, making it one of the most commercially valuable metabolites in this species. In particular, the morphological and physiological characteristics of *D. tertiolecta* offer significant advantages for chlorophyll extraction when compared to traditional sources such as green vegetables such as spinach, bamboo leaves, lettuce, broccoli, watercress, wheat sprouts, and alfalfa sprouts; including freshwater microalgae such as Chlorella and Arthrospira. Unlike these sources, which require freshwater and possess complex cellular structures that complicate chlorophyll extraction and purification, *D. tertiolecta* can be cultivated in marine environments and exhibits a simple unicellular morphology lacking rigid cellulose walls, facilitating the more efficient recovery of high-purity chlorophyll through lower-energy and less chemically intensive processes (Santana-Moura *et al*., 2020; Martínez *et al*., 2022). These characteristics position *D. tertiolecta* as a more sustainable, cost-effective, and scalable alternative to traditional chlorophyll sources, while also offering added value through the potential use of microalgal biomass by-products in the food, pharmaceutical, and cosmeceutical industries (Li *et al*., 2016; Norzagaray-Valenzuela *et al*., 2018).

Chlorophyll has traditionally been used as a natural colorant and functional ingredient in several foods and beverages, representing a global market of 252.18 million dollars in 2021 (POLARIS, 2022). However, in its natural form, the chlorophyll molecule is highly photosensitive, water-insoluble, and sensitive to sudden changes in pH (Kang *et al*., 2018; Martins *et al*., 2023). To overcome these limitations, which reduce its commercial viability, numerous efforts have been made to convert chlorophyll into more stable, value-added derivatives with high potential for industrial application. Among these, metallo-chlorophylls such as sodium zinc chlorophyllin (SZC) stand out. SZC is structurally similar to natural chlorophyll but differs in that the phytol group has been eliminated and the central magnesium ion has been replaced with zinc, a more stable metal ion. These structural modifications confer SZC the ability to be water-soluble, tolerate

Revista Bio ciencias
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sudden pH changes, and remain stable at high temperatures without affecting its important biological properties. While metallo-chlorophylls are already used commercially, often labeled as “chlorophyll” despite being compounds like sodium copper chlorophyllin (SCC), scientific evidence specifically addressing the physicochemical and biological properties of these compounds remains limited. In fact, no previous studies have reported its synthesis from chlorophyll extracted from *Dunaliella tertiolecta* (Banu & Pavithra, 2017; Martins *et al*., 2023). This highlights the scientific interest in SZC and the importance of generating new insights into its potential applications as a functional ingredient.

In this context, the aim of the present study was to establish a methodology for obtaining SZC from chlorophyll extracted from the marine microalgae *D. tertiolecta* and subsequently confirm its chemical identity through UV–Vis and FTIR spectrometry. Additionally, its physicochemical properties and antioxidant capacity were evaluated to determine the potential application of SZC as a bioactive compound in the development of nutritional, nutraceutical, and cosmeceutical products or supplements. This approach not only enhances the value of marine microalgae as a source of functional ingredients, but also aligns with the growing consumer demand for innovative, sustainable and naturally derived alternatives with added value for healthcare.

Material y Methods

Microalgal strain and chemical reagents

The marine microalgae *Dunaliella tertiolecta* (strain DUT2) was obtained from Centro Biotecnológico de Microalgas de México SA de CV (MICROCELL®) in Culiacán, Sinaloa, Mexico. All solvents used for the extraction of chlorophyll were of analytical grade. Trolox, NaOH, $ZnSO_4$, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,4,6-tris-(2-pyridil)-s-triazine (TPTZ), and sodium copper chlorophyllin (SCC), were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA).

Microalgal culture and chlorophyll extraction conditions

The culture of *D. tertiolecta* was carried out in F/2 medium (Guillard & Ryther, 1962) at 25 °C under constant aeration (1 % CO_2) and continuous light (120–130 μmol photons m^{-2} s^{-1}). To establish the culture, the successive transfer methodology was employed, starting from the isolation of a single colony (CFU) present in solid medium, which was placed in 10 mL of liquid F/2 medium, and gradually scaled up until reaching a final volume of 16 L. Cell counts were obtained during each stage of scaling to monitor both the viability and density of the culture (Norzagaray-Valenzuela *et al*., 2018); this allowed the determination of the maximum day of the exponential phase of the culture to the final volume, at which time the biomass was harvested using chitosan as organic flocculant (Meza-Ayala, 2017). Chlorophyll was extracted from the recovered biomass. Due to the amphipathic nature of chlorophyll, the extraction method consisted of obtaining an oil-chlorophyll mixture, corresponding to the lipophilic fraction generated during oil extraction, following the methodology described by Germán-Báez *et al*. (2017). Subsequently, the recovered lipophilic

Revista Bio ciencias ISSN 2007-3380

fraction was subjected to rotary evaporation (65 cmHg/ 45 °C) until the solvents present were completely eliminated, thus obtaining chlorophyll in the solid state. The purity of the chlorophyll was then analyzed using thin-layer chromatography (data not shown), ensuring that the obtained chlorophyll consisted solely of different types of chlorophyll and their derivatives.

Obtaining sodium zinc chlorophyllin (SZC)

The process for obtaining SZC from the extracted chlorophyll was divided into three main stages: (1) *alkaline hydrolysis of chlorophyll*, which was performed using 1 g of microalgal chlorophyll with 20 mL of ethanol-NaOH at four different concentrations (5.0, 7.5, 10.0 and 15.0 %) under constant agitation (350 rpm) at 25 °C overnight. The reaction was stopped by adding pure acetone (1:1; v/v), followed by centrifugation (Thermo Forma 1L GP Centrifuge) at 3600 x *g*, 30 min. The resulting pellet was recovered and resuspended in 65 % acetone, then centrifuged again (3600 x *g*, 30 min) to collect the supernatant containing water-soluble chlorophyllins. In this stage, the response variable was the water solubility of each treatment. (2) *Incorporation of the metal ion*. In this stage, the previously obtained water-soluble chlorophyllins were acidified with 10 N HCl to a pH of 2-3. $ZnSO_4$ was then added at three different concentrations (10, 20 and 30 %) and stirred in a Varimix for 20 min. The sample was subsequently placed in a water bath at 60 °C for 1 h, resulting in the formation of a water-insoluble zinc-chlorophyllin (Zn-Chl) precipitate, which was recovered by centrifugation (3600 x *g*, 30 min). The response variable for this stage was the amount of Zn incorporated into the molecule, which was quantified by flame atomic absorption spectroscopy (FAAS) with an air–acetylene flame (Shimadzu AA-6800 spectrophotometer equipped with an ASC-6100 autosampler). Measurements were carried out at 213.9, 285.2, and 324.8 nm using hollow cathode lamps specific for Zn, Mg, and Cu, respectively; the integration time was 5 s and the gas flow rate was 1.8 L min^{-1}. These operating conditions are consistent with methodologies previously reported for the analysis of metallo-chlorophylls and related bioactive compounds (AOAC, 1999; Suzuki *et al*., 2016). Calibration was performed using an external multipoint curve (0.1–5.0 mg L^{-1}) prepared from certified standard solutions, and quality control included blanks, duplicate samples, and spike recovery tests. (3) *Conversion to chlorophyllin salts*. During this stage, the Zn-Chl precipitate was dissolved in ethanol-NaOH at three different concentrations (2.5, 5.0 and 10.0 %); the pH was adjusted to 11 using 1 N NaOH, and the mixture was placed in an incubator at 45 °C to remove the solvents, thereby obtaining dry SZC. The response variables for this stage were color and water solubility in each treatment. The concentration ranges used in each of the three stages were selected based on preliminary trials, which were determinate by previous studies on the alkaline hydrolysis of esters (Theodorou *et al*., 2007) and their application in the transformation of certain microalgal pigments (Gómez-Luna, 2007), as well as in protocols for obtaining other types of metallo-chlorophylls (Li *et al*., 2016). To determine the optimal process for obtaining SZC, the best treatment for each of the aforementioned stages was selected, allowing the establishment of a proposal to SZC production, and the resulting combination was used to generate SZC from the chlorophyll of *D. tertiolecta*. From this optimized process, a batch of SZC was prepared for all subsequent analyses. To evaluate the efficiency of the process, the SZC yield (mg/g of chlorophyll) was calculated taking into account the initial weight of the microalgal chlorophyll on a dry weight (dw).

Revista Bio ciencias
ISSN 2007-3380

Characterization by UV–Vis and FTIR spectrometry

UV–Vis and FTIR spectra of SZC were obtained. For the UV–Vis analysis, an aqueous solution of SZC (125 µg/mL) was used, and absorbance readings in the range of 200-800 nm, with a step resolution of 2 nm, were obtained using a Thermo Scientific Biomate 3S spectrophotometer. For FTIR analysis (400-3900 cm^{-1} range, 2 cm^{-1} resolution), transmittance spectra were obtained from 1 mg of SZC fully dried, ground, and sieved, using a Cary 660 FTIR/ATR spectrometer equipped with an attenuated total reflectance (ATR) accessory, with the powdered samples placed directly onto the ATR crystal (Agilent Technologies, Santa Clara, CA, USA). The samples analyzed by both methods included SZC, natural chlorophyll extracted from *D. tertiolecta* (the starting material for SZC production), and sodium copper chlorophyllin (SCC) from Sigma-Aldrich Co., used as commercial standard; these latter two were included as reference controls due to their well-documented spectral profiles, which supported the qualitative analysis of SZC by allowing comparison of its main characteristic bands and absorption patterns with previously reported data. Notably, all spectral measurements and analyses of SZC were performed using samples obtained under the conditions described in this study, without the use of external standards for calibration. All samples were analyzed in triplicate and scanned on a single day to avoid instrument drift effects.

Physicochemical properties

Within the sensory and physicochemical properties of the obtained SZC, the color, texture, solubility in different solvents, and thermal stability were evaluated. The color and texture of the SZC in the solid state were determined according to the methodology reported by Singh *et al*. (2020), as well as through absorbance units of SZC (125 µg/mL) in solution at the maximum absorption wavelength (405 nm), obtained by analyzing its UV–Vis spectra using the NMX-AA-017-SCFI-2021 standard as a reference. To determine the solubility of the SZC, the methodology reported by Anchundia *et al*. (2016) was used; for this purpose, 5 mg of SZC was dissolved in 5 mL of the solvent to be evaluated (acetone, chloroform, ethanol, and water), and the samples were homogenized in a Varimix for 30 min, and centrifuged at 25 °C/3500 x *g*/15 min. Subsequently, the supernatant was recovered, and the resulting pellets were placed in an oven at 60 °C for 24 h to dry. For the analysis, the weight of the solids obtained after drying the pellet for each treatment was evaluated, and the percentage of solubility was calculated. The analysis of the thermal stability of SZC at high temperatures was carried out using the methodology reported by Ferruzzi & Schwartz (2005), using a solution of SZC in water at a concentration of 125 µg/mL, which was subjected to temperatures of 40, 50, 60, 70, 80, 90, and 100 °C at different times (5, 15, 30, 45, and 60 min). Finally, the absorbance at 635 nm was measured for each of the treatments to calculate the percentage of degradation using the equation reported by Singh *et al*. (2020).

Evaluation of antioxidant capacity

The *in vitro* antioxidant capacity of SZC was determined using the DPPH, ABTS and FRAP assays following the methodology proposed by Martínez-Montaño *et al*. (2022). In each assay, a Trolox standard curve was obtained, the results were calculated by linear regression and expressed as mmol Trolox equivalents per 100 g of SZC on a dry weight [mmol TE/100 g (dw)].

Statistical analysis

The data for each evaluated parameter were analyzed by analysis of variance (ANOVA), and the means were compared using Fisher's test (LSD, $\alpha = 0.05$) performed with STATGRAPHICS Centurion XVI (Statpoint, Inc., Warrenton, Virginia). Prior to conducting the ANOVA, the assumptions of normality and homogeneity of variances were verified through visual inspection of residual plots and variance distribution graphs provided by the software. These plots indicated that the residuals followed a normal distribution and that the variance was homogeneous across groups. All treatments and evaluations were carried out in triplicate.

Results and Discussion

Obtaining sodium zinc chlorophyllin (SZC)

From 56 g (dw) of whole *D. tertiolecta* biomass, 10.64 g (dw) of chlorophyll was extracted, representing a yield of 19 %, which is slightly lower than the 25 % yield reported by Li *et al*. (2016) from chlorophyll extracted from the whole biomass of *Scenedesmus sp*; however, in addition to inherent differences due to the species characteristics and cultivation conditions, it is important to highlight that the authors performed the extraction using column chromatography, a method that, although it may be more efficient than extraction with organic solvents like that used in the present investigation, is costly, laborious and has a longer execution time. Furthermore, *Scenedesmus sp*. is a freshwater microalga; therefore, its cultivation competes with water resources intended for human consumption. Therefore, the yield obtained in the present study suggests that *D. tertiolecta* is a chlorophyll source with high scalability potential under a sustainable production model based on the use of seawater.

In order to perform a comparative analysis between the chlorophyll extraction yield from the microalgae model used here (19%), and the spinach cultivation, considered the traditional vegetable source of chlorophyll with the highest commercial impact and the one most commonly used to meet market demand; we took into account the findings reported by Heras-Cervantes (2006), who starting from 52 g (dw) of spinach leaves, obtained 10 g (dw) of chlorophyll, equivalent to a yield of 19.2 %, revealing that both models exhibit very similar yields. However, although the yields are similar, it is important to take into account that the cost of producing 52 g of dry spinach leaves is significantly higher than that required to produce the same amount of *D. tertiolecta* biomass. According to the CCB (2015), the number of plants needed to obtain that amount of spinach biomass under protected agricultural conditions requires approximately 1 m^2 of cultivation soil and 180 L of freshwater for irrigation, in addition to other inputs such as fertilizers, herbicides, and pest management. Indeed, spinach cultivation may well be considered a challenge because, like other crops, its growth is affected by many factors that are difficult to control, such as inclement weather and exposure to pests and diseases, as well as long production times. In addition, as

Revista Bio Ciencias
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indicated in the same report, spinach requires approximately 60 days to reach harvest maturity, and it is a seasonal crop available only 6 months of the year, with high demands regarding the climate and type of soil. In contrast, *D. tertiolecta* has emerged as an efficient and sustainable source of chlorophyll and its derivatives, in this case, metallo-chlorophylls such as SZC, due to its cultivation characteristics and cellular morphology, which facilitate the extraction of bioactive compounds through simple and low-cost techniques.

Once the native chlorophyll of *D. tertiolecta* was obtained, tests were carried out for the conversion of its water-soluble derivative SZC. This conversion process can be divided into three stages. First, alkaline hydrolysis of the native chlorophyll was carried out to eliminate the phytol group corresponding to the hydrophobic portion of the molecule, as well as the alkyl radicals that are replaced by hydrophilic sodium ions, thus obtaining the derivative water-soluble sodium magnesium chlorophyllin. In this regard, during the development of this stage, a statistically significant positive correlation ($p < 0.05$) was observed between ethanol–NaOH concentration and both solubility and absorbance (Table 1). Solubility increased progressively with increasing ethanol–NaOH concentration, and this behavior was reflected by the absorbance values. The highest absorbance, at the peak wavelength (405 nm) was recorded in the ethanol–NaOH 15 % treatment (2.845 AU) corresponding to the maximum concentration tested.

In the *sodium magnesium chlorophyllin* obtained in the first stage, the Mg atom causes instability in the molecule; thus, the second stage consisted of replacing Mg with a more stable metal ion, such as Zn, and converting *sodium magnesium chlorophyllin* into *zinc chlorophyllin*, which became water-insoluble again due to the elimination of sodium ions from the molecule. Under the conditions used herein, the displacement of Mg by Zn within the molecule was achieved, resulting in greater integration in the treatment with the highest concentration of $ZnSO_4$ evaluated (30 %), with a total Zn content of 13,600 ppm detected in the compound (Table 2). Likewise, when comparing the other elements evaluated, Mg and Cu, in both the different treatments and the controls (Table 1), the displacement percentage could be determined, where SZC showed that 96.25 % of Mg was displaced, a percentage higher than that of *sodium copper chlorophyllin* (SCC), with 93.18 %.

The third and last stage of the process consisted of the reincorporation of sodium ions to convert the *zinc chlorophyllin* obtained into chlorophyllin salts, in order to obtain the final product: SZC, by the addition of NaOH. During this stage, a greater solubility (94.66 ± 1.33 %) of the sample was observed at an ethanol-NaOH concentration of 5 %, which coincided with the best result in terms of color (1.004 ± 0.016 AU). Table 1 shows the solubility percentages and color values for each treatment. The increase in the concentration of ethanol-NaOH was not proportional to the increase in solubility and color, which could be due to the excessive use of NaOH complicated the pH adjustment required for this type of reaction, leading to unfavorable results.

Based on the best results obtained in each of the three stages, it was possible to establish a process to obtain SZC from the native chlorophyll extracted from *D. tertiolecta,* which consists of: stage 1, alkaline hydrolysis of chlorophyll using 15 % ethanol-NaOH; stage 2, addition of the metal ion Zn to replace Mg using 30 % $ZnSO_4$; and stage 3, conversion to a chlorophyllin salt, that is, the final product SZC, using 5 % ethanol-NaOH. The yield obtained under this process was approximately 0.565 g of SZC per 1 g of chlorophyll, corresponding to an efficiency of 57%, calculated based on the total SZC mass obtained which is much greater than that obtained by Li *et al*. (2016), who obtained only 39.6 % SCC as a chlorophyll derivative from the freshwater microalga *Scenedesmus sp.*

Table 1. Main stages of the process for obtaining SZC from *D. tertiolecta*.

	Ethanol-NaOH (%)	% Solubility		AU[1] (405 nm)
STAGE 1 Alkaline hydrolysis of chlorophyll	**5.0**	86.66 ± 0.58[b]		0.612 ± 0.022[b]
	7.5	92.67 ± 1.53[c]		1.630 ± 0.013[c]
	10.0	92.00 ± 1.00[c]		2.016 ± 0.051[d]
	15.0	96.70 ± 0.20[d]		2.245 ± 0.033[d]
	C+	96.66 ± 0.58[d]		2.998 ± 0.025[e]
	C-	1.27 ± 0.17[a]		0.085 ± 0.012[a]
	$ZnSO_4$ (%)	**Element content (ppm)[2]**		
		Mg	**Zn**	**Cu**
STAGE 2 Incorporation of the metal ion	**10.0**	130.0	6,000.0	73.0
	20.0	150.0	6,700.0	36.0
	30.0	82.4	13,600.0	19.0
	C+	150.0	16.0	35,500.0
	C-	2,200.0	23.4	22.4
	Ethanol-NaOH (%)	**% Solubility**		**AU[1] (405 nm)**
STAGE 3 Conversion to chlorophyllin salts	**2.5**	76.00 ± 2.00[b]		0.675 ± 0.022[b]
	5.0	94.66 ± 1.33[d]		1.004 ± 0.016[d]
	10.0	86.00 ± 2.00[c]		0.930 ± 0.019[c]
	C+	95.33 ± 1.15[d]		2.722 ± 0.014[e]
	C-	2.00 ± 0.00[a]		0.100 ± 1.670[a]

C+, Sodium copper chlorophyllin (positive control); C-, unprocessed chlorophyll from *D. tertiolecta* (negative control); [1]Absorption units; [2]Values obtained by atomic absorption spectroscopy. The values in stages 1 and 3 represent the mean of three replicates ± standard deviation. Different letters in the same column for each stage represent statistically significant differences by Fisher's test ($p < 0.05$).

Source: Own elaboration based on experimental results.

Characterization by UV–Vis and FTIR spectrometry

As mentioned above, SCC is one of the most studied metallo-chlorophylls and is used as a reference standard for the analysis of other metallo-chlorophylls due to its similarity in its molecular structure, differing only in the central metal ion (García-Rodríguez & Altamirano-Lozano, 2001; Banu & Pavithra, 2017). For this reason, in the present investigation, SCC purchased from Sigma–Aldrich was used as a reference standard in both the UV–Vis and FTIR assays. Figure 1 shows the UV–Vis spectra (200 to 800 nm) of the obtained SZC and the SCC control, revealing, in both cases, wavelengths of maximum absorption (λmax) within the typical ranges reported for metallo-chlorophylls (250, 400-430 and 620-660 nm), with λmax at 250, 405 and 635 nm, respectively.

These values are similar to those reported by Ferruzzi & Schwartz (2005), who obtained λmax values at 250, 406 and 635 nm for Sigma–Aldrich SCC. These results are also similar to those reported by Li *et al*. (2016) for SCC obtained from the microalga *Scenedesmus sp*., and for a SCC standard (GB26406-2011), which reported λmax values at 418 and 642 nm. Farag (2006) carried out optical absorption studies of thin films of SCC prepared by spray pyrolysis, observing λmax values at 250, 430 and 650 nm. Thus, the comparison of the λmax values determined for SZC and the SCC control confirms that the SZC obtained herein exhibits the typical absorption pattern of metallo-chlorophylls and their constituent compounds. In this sense, Farag (2006) reported that the λmax at 250 nm corresponds to the presence of the central atom in association with another SCC band, which could explain why it differs from the absorption maxima reported for chlorophylls (400-450 nm and 600-690 nm), specifically those observed for the native chlorophyll of *D. tertiolecta*, as shown in Figure 1.

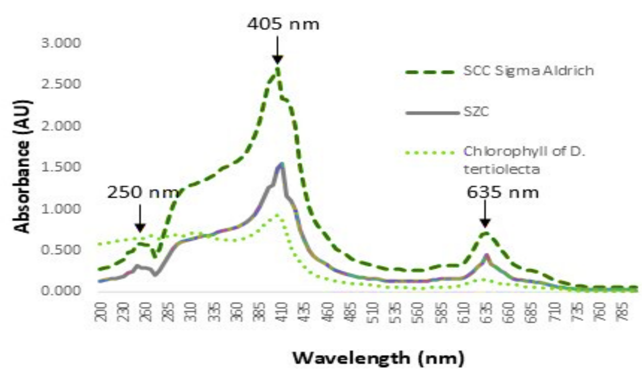

Figure 1. UV–Vis spectra (200-800 nm) of SZC; SCC Sigma-Aldrich (reference standard); and unprocessed chlorophyll from *D. tertiolecta* (process control).

Arrows indicate the wavelengths of maximum absorbance peaks in the spectra.

Source: Own elaboration based on experimental results.

Regarding the λmax of SZC (0.328 AU at 250 nm, 1.503 AU at 405 nm and 0.452 AU at 635 nm) and the λmax of the control SCC (0.592 AU at 250 nm, 2.705 AU at 405 nm and 0.723 AU at 630 nm), the highest absorption of both metallo-chlorophylls was observed at 405 nm, which coincides with the values reported by Zhan *et al*. (2014), who observed the highest absorption of three different metallo-chlorophylls generated from pine needles, SZC, SCC and sodium iron chlorophyllin (SIC), at wavelengths of 415, 402 and 405 nm, respectively.

Revista Bio ciencias
ISSN 2007-3380

The IR spectra of the SZC obtained under the conditions described herein showed bands typical of metallo-chlorophylls and was used to identify the presence of the main functional groups in the molecule, which were similar to those reported by several authors for metallo-chlorophylls obtained from different sources (Table 2). The bands of greatest intensity in the spectra of both the SZC and the SCC control (Figure 2 a and b, respectively) were observed between 1390 and 2919 cm^{-1}, which may correspond to aromatic rings (C=C), secondary amines (C=N), aliphatic groups, and aromatic interactions (CH). In addition, moderate widening between 1112 and 1440 cm^{-1} was observed, which could be related to the presence of primary amines (CN) and simple bonds between the pyrrole rings (CC) (Suresh *et al*., 2022).

Table 2. Absorption bands in the IR spectra of SZC and their correspondence with previous reports of different types of metallo-chlorophylls.

Bands SZC (cm^{-1})	Functional group	Related compounds	Reference bands	References
3285	O-H	stretching vibration of water	3362	Holt & Jacobs (1955)[d]
			3366	Farag (2006)[a], Kumar et al. (2015)[d]
			3239	Suresh et al. (2022)[a,b]
2919	C-H	Aromatic groups	2920	Holt & Jacobs (1995)[d]
			2973	Kumar et al. (2015)[d]
2851	C-H	Stretching of aldehyde group and aliphatic groups	2852	Farag (2006)[a]
			2856	Kumar et al. (2015)[d]
1564	C=C C=N	Stretching vibrations of the aromatic system,	1610	Holt & Jacobs (1995)[d]
			1592	Farag (2006)[a]
		aromatic rings	1602	Petrovic et al. (2006)[b,c]
		and conjugates amine	1562	Suresh et al. (2022)[a,b]
1440	$-CH_3$	Methyl group	1454	Farag (2006)[a]
			1457	Petrovic et al. (2006)[b,c]

Revista Bio ciencias
ISSN 2007-3380

Continuation

Table 2. Absorption bands in the IR spectra of SZC and their correspondence with previous reports of different types of metallo-chlorophylls.

Bands SZC (cm^{-1})	Functional group	Related compounds	Reference bands	References
1390	C-H	Stretching of aliphatic groups (bending)	1354	Farag (2006)[a]
			1380	Petrovic et al. (2006)[b,c]
			1391	Suresh et al. (2022)[a,b]
1112	C-N C-O	Stretching of amines, oxygen bonds	1122	Petrovic et al. (2006)[b,c]
			1086	Kumar et al. (2015)[d]
			1094	Suresh et al. (2022)[a,b]
830	Zn+	Metal-ligand	888	Ahmad & Collins (1991)[a]
			879	Farag (2006)[a]
			809	Suresh et al. (2022)[a,b]

[a]Sodium copper chlorophyllin; [b]Copper chlorophyllin; [c]Zinc chlorophyllin; [d]Chlorophyll derivatives without metal ions. Source: Adapted from the authors cited in the table.

Finally, a wide band was observed at 3285 cm^{-1}, which suggests the presence of OH groups bonded to other hydrogen atoms, amplifying the signal or the extension vibration and, therefore, creating a band broadening. However, the presence of bands with these characteristics in the FTIR spectra of hygroscopic pigments is often due to their interaction with atmospheric water molecules, which prevents a correct appreciation of the functional groups present in the range of 3200-3500 cm^{-1} (Farag, 2006; Petrovic *et al.*, 2006).

Regarding the results herein, the FTIR spectra of SZC showed bands similar to those reported between 1000 and 2000 cm^{-1} for complexes generated from chlorophyll extracted from spinach leaves, specifically copper chlorophyllin and zinc chlorophyllin. Both exhibited maxima at 1118 and 1122 cm^{-1}, respectively, which are characteristic of oxygen bonds (CO). Likewise, bands at 1380, 1457 and 1602 cm^{-1} in both compounds are typical of aliphatic groups (CH), methyl groups ($-CH_3$), aromatic rings (C=C) and conjugated amines (C=N), respectively. However, they differ in their FTIR spectra, in the existence of two additional bands corresponding to esters groups (1288 and 1271 cm^{-1}), which are also observed in the FTIR spectra of chlorophyll at 1285 cm^{-1}. These bands are associated with the formation of metallo-chlorophylls and have been attributed to the oversaturation of metal ions during the production process. At high concentrations, the metal can bind to the cyclopentanone ring, which detaches during the hydrolysis of chlorophyll,

Revista Bio ciencias
ISSN 2007-3380

generating a cyclic chelate complex (Petrovic *et al*., 2006). These two bands described above were not observed in the FTIR spectra of SZC; however, they were present in the spectra of native chlorophyll from *D. tertiolecta* (Figure 2c), as well as in the FTIR spectra of spinach leaves and in the FTIR spectra of the SCC control used here, which also showed a band at 1285 cm^{-1}, attributed to ester groups (Kang *et al*., 2018). Therefore, the concentration of Zn used during the production process described here does not appear to induce the formation of secondary compounds such as cyclopentanone chelate. Furthermore, the FTIR spectra of SZC coincides with those reported by Suresh *et al*. (2022), who analyzed copper chlorophyllin and SCC obtained from *Aloe vera* leaves, both spectra were similar to each other, and all the bands reported by the authors (809, 1094, 1391, 1562 and 3239 cm^{-1}) were also observed in the spectra of SZC obtained in this study.

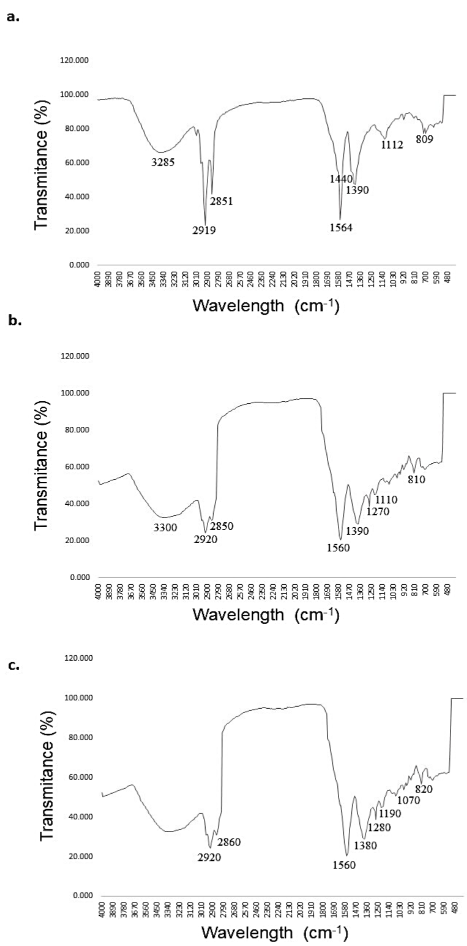

Figure 2. FTIR spectra (400-3900 cm^{-1}).

a. Sodium zinc chlorophyllin (SZC); b. Sodium copper chlorophyllin (SCC) of Sigma-Aldrich (reference standard); c. Unprocessed chlorophyll from ***D. tertiolecta*** (process control).

Source: Own elaboration based on experimental results.

Revista Bio ciencias
ISSN 2007-3380

Physicochemical properties of SZC

The SZC obtained under the process developed herein was a fine powder with a dark Jasper-green color, as described by Li *et al*. (2016) and Singh *et al*. (2020) for SCC obtained from the microalgae *Scenedesmus sp*. and *Chlorella minutissima*, respectively. Regarding its solubility in different types of solvents (Table 3), an increase was observed as the polarity of the solvent increased, with the highest solubility of SZC observed in water (91.33 ± 1.15 %) and the lowest solubility in chloroform (2.667 ± 1.15 %), confirming the polar nature of metallo-chlorophylls, and its contrast with native chlorophyll, which is completely water-insoluble. These findings align with those reported for SCC obtained from *C. minutissima*, which was completely water-soluble, partially soluble in ethanol and insoluble in chloroform (Singh *et al*., 2020). The aforementioned structural resemblance between SCC and SZC could explain the similarities in their sensory and physicochemical characteristics. This comparability supports the use of the National Food Safety Standard for Food Additives (GB 2760-2014), as described in the GAIN REPORT (2011), established by the Ministry of Health of China, which regulate the production and safe use of SCC (E141), as technical references for the assessment of SZC. SCC is currently approved as a food colorant or additive under European Directive 94/36/EC, the food sanitary regulation of Chile (Decree 977/96), and by the Codex Alimentarius Commission (2024) through CODEX STAN 192-1995. In this context, and based on comparison with technical requirements established by the aforementioned regulatory bodies for sodium copper chlorophyllin (SCC, E141), the SZC produced in this study meets several of the key physicochemical criteria specified for this type of food additive. These include complete water solubility, insolubility in chloroform, an alkaline pH range (9.5-11 in 1 % aqueous solution), intense jasper-green color, and clear aqueous solution without sedimentation. These characteristics, commonly associated with water-soluble metallo-chlorophylls, support the technical comparability of SZC with SCC and its potential use in food applications as natural colorant or bioactive compound, while acknowledging that specific regulatory definitions for SZC are not yet established.

Table 3. Solubility of SZC in different types of solvents

Types of solvents	% Solubility SZC	% Solubility C+
Chloroform	2.66 ± 1.1^{5}a	1.26 ± 0.68[a]
Acetone	24.00 ± 2.00[b]	19.00 ± 1.85[b]
Ethanol	27.33 ± 1.15[c]	25.31 ± 1.55[c]
Water	91.33 ± 1.1^{5}d	93.33 ± 1.07[d]
Chlorophyll in water[1]	0.00 ± 0.00[a]	0.00 ± 0.00[a]

C+, Sodium copper chlorophyllin (positive control); [1]Unprocessed chlorophyll from *D. tertiolecta* in water (negative control); values represent the mean of three replicates ± standard deviation. Different letters in the same column represent statistically significant differences by Fisher's test ($p < 0.05$).

Source: Own elaboration based on experimental results.

Revista Bio ciencias
ISSN 2007-3380

Thermal stability at high temperatures

The evaluation of the behavior of SZC under high temperature fluctuations is of great importance when considering its potential use in the formulation of industrialized products. The thermal stability exhibited will serve as a guideline for determining the types of products in which it can be used (Ferruzzi & Schwartz, 2005; Mohd-Amin *et al*., 2023). In this context, the SZC obtained in the present work was subjected to high temperatures, and degradation was found to increase with longer exposure times at a given temperature. However, the degradation of SZC at different temperatures was not always significantly different when evaluated at similar time points (Table 4). This behavior was also observed in the SCC control used in this study and aligns with the results reported by Ferruzzi & Schwartz (2005), for the same compound, SCC, in aqueous solution at the same concentration used in our tests.

Table 4. Degradation percentage of SZC under different temperature and time conditions.

T (°C)	Degradation of SZC (%)				
	5 min	**15 min**	**30 min**	**45 min**	**60 min**
40	12.97 ± 0.74^{a}	15.07 ± 0.88 ^{b}	20.59 ± 0.94^{efgh}	20.82 ± 0.74^{fghi}	22.84 ± 0.61^{jk}
50	19.11 ± 0.23^{c}	19.65 ± 0.13^{cd}	19.96 ± 0.35^{de}	21.13 ± 0.35^{ghi}	23.69 ± 0.48^{lm}
60	20.04 ± 0.84^{def}	20.43 ± 0.13^{defg}	21.28 ± 0.97^{hi}	21.52 ± 0.13^{i}	23.85 ± 0.35^{mn}
70	20.59 ± 0.58^{hi}	22.54 ± 0.56^{j}	23.54 ± 0.46^{klm}	24.63 ± 0.81^{no}	26.88 ± 0.26^{q}
80	22.68 ± 0.13^{j}	22.99 ± 0.53^{jkl}	24.24 ± 0.23^{mno}	25.79 ± 0.13^{p}	26.96 ± 0.71^{q}
90	29.21 ± 0.48^{r}	29.370 ± 0.003^{r}	29.91 ± 0. 0.35^{r}	31.70 ± 0.40^{s}	34.49 ± 0.40^{t}
100	33.72 ± 0.26^{t}	38.228 ± 0.004^{u}	42.73 ± 0.13^{v}	48.79 ± 0.35^{w}	52.13 ± 0.35^{x}

Values represent the mean of three replicates ± standard deviation. Different letters represent statistically significant differences between treatments by Fisher's test ($p < 0.05$).

Source: Own elaboration based on experimental results.

SZC was stable at temperatures of 40, 50, and 60 °C, showing degradation values ranging from 12 to 21 % during the first 30 min. This is quite favorable because thermal pasteurization stands out among food conservation and safety processes that comply with sanitary regulations worldwide. In addition to being efficient, thermal pasteurization has been demonstrated to maintain the properties of foods intact, unlike other non-thermal techniques, such as high pressure processing (HPP), which uses high levels of cold pressure (Bogahawatha *et al*., 2018). In this sense, thermal pasteurization methods mostly used for food products like juices, beer, milk, and other liquids involve subjecting the product to temperatures between 60 and 70 °C for short periods, typically ranging from 5 to 10 min. Some techniques even require only seconds of exposure, such as high-temperature short-time (HTST) pasteurization, which uses temperatures of 70 ± 5 °C (Donalisio *et al*., 2018). In the present study, after 5 min of exposure, SZC degradation was 20.59 ± 0.58 % at 70 °C and 22.68 ± 0.13 %, at 80 °C, remaining constant up to 15 min of exposure, indicating stability close to 80 % under industrial processing conditions and suggesting its potential use in the formulation of beverages, food supplements, and other products. The maximum degradation of SZC (52.13 ± 0.35 %) was observed at 100 °C after 60 min of exposure, which is lower than the degradation reported by Ferruzzi & Schwartz (2005), who reached a similar percentage at 90 °C after only 20 min of exposure, using commercial-grade SCC.

Antioxidant capacity (AOXC) of SZC

To evaluate AOXC it is necessary to consider the different mechanisms of antioxidant activity and the respective methodologies used to measure each one. Therefore, determining AOXC by a single method is not sufficient. The AOXC of SZC produced here, was evaluated using three different methods and results were expressed as Trolox Equivalent Antioxidant Capacity (TEAC). Values of 17.717 ± 0.32 mmol TEAC/100 g (dw) by DPPH, 38.008 ± 1.835 mmol TEAC/100 g (dw) by FRAP and 44.402 ± 1.198 mmol TEAC/100 g (dw) by ABTS were determined. The highest result was obtained using the ABTS assay, suggesting that this method has the highest affinity for this molecule. It also indicated that SZC exhibits a strong capacity to oxidize the cationic chromophore radical 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS•+), which has solubility in polar and nonpolar media and is not affected by ionic strength, allowing it to evaluate both hydrophilic and lipophilic antioxidants. For this reason, the value obtained through the ABTS method [44.402 ± 1.198 mmol TEAC/100 g (dw)] can be considered the total AOXC of SZC (Pérez-Gálvez *et al*., 2020). Consequently, it can conclude that SZC exhibits a high antioxidant capacity, given that this value is comparable to those reported for vegetables such as artichokes, beans (red and pinto), and soybeans, as well as fruits like blackberries and blueberries. All of these are considered good references in terms of their AOXC and located in the first position [>9 mmol TEAC/100 g (dw)] of the classification proposed by Pennington and Fisher (2009), who suggested a classification of 6 levels for fruits and vegetables according to their total AOXC.

According to the AOXC value obtained using the FRAP assay, Carlsen *et al*. (2010) proposed a classification comprising 24 levels divided into 3 categories: plant, animal or mixed origin. Based on the FRAP value reported here for SZC [38.008 mmol TEAC/100 g (dw)], it falls within the first category, which includes foods of plant origin. This category contains the group with the highest contribution of antioxidants; but due to its nature, the SZC is better located more specifically at level 3, corresponding to spices and herbs, which includes foods with an average AOXC of 29.02 mmol TEAC/100 g (dw). This value is higher than those reported for several varieties of laurel, cinnamon, nutmeg, ginger, dill, basil and saffron, with average AOXC values of 27.8, 26.5, 26.4, 20.3, 20.2, 19.9 and 17.5 mmol TEAC/100 g (dw), respectively. It is worth noting that Carlsen *et al*. (2010) analyzed a total 3139 foods to propose this classification, in which AOXC of the SZC obtained in the present study was higher than that of approximately 2524 of them, representing nearly 80 % of all evaluated foods. It even exceeds the values reported in many foods and beverages considered important sources of antioxidants, such as black tea [1.0 mmol TEAC/100 g (dw)], black coffee [2.5 mmol TE/100 g (dw)], sunflower seeds [6.4 mmol

TEAC/100 g (dw)], pomegranate juice [2.1 mmol TEAC/100 g (dw)], and *Moringa stenopetala* leaves [11.9 mmol TEAC/100 g (dw)].

In a study by Ferruzzi *et al*. (2002), chlorophyll derivatives lacking metallic ions as well as metallo-chlorophylls containing different metal ions were obtained and their AOXC were evaluated through DPPH and ABTS assays. The AOXC values for the chlorophyll derivatives without metal ions were lower than those for the native chlorophyll from which they were generated; while the different metallo-chlorophylls evaluated showed the highest AOXC values of all the molecules tested including native chlorophyll. This strongly suggests that the presence and type of metal ion in the molecules, whether the native Mg in chlorophyll or metals added during the formation of metallo-chlorophylls, play an important role in their AOXC. Supporting this, several reports indicate that AOXC varies among different types of metallo-chlorophylls (Zhan *et al*., 2014; Mohd-Amin *et al*., 2023). Furthermore, emerging technologies for the industrial processing of fruits, vegetables and foods rich in chlorophylls, whose processes include stages involving the addition of specific metal ions for preservation of color and biological properties (Kang *et al*., 2018; Mohd-Amin *et al*., 2023).

Taken together, the high antioxidant capacity observed for SZC, along with its favorable physicochemical characteristics and thermal stability, supports its potential as a food additive aligned with current demand for natural compounds with value-added in healthcare. Nonetheless, to fully substantiate this potential, further research will be necessary, particularly studies addressing its bioaccessibility, safety profile, and performance when applied in real food systems. Such investigations would provide a more robust foundation for considering SZC a viable functional ingredient, enhancing its potential acceptance in both industrial and nutritional applications.

Conclusions

In the present study, an efficient process for obtaining SZC from chlorophyll extracted from the marine microalga *Dunaliella tertiolecta* was developed, with yields of up to 52 %. Based on UV–Vis and FTIR spectrometry analyses, the correct chemical conformation corresponding to SZC was confirmed. Likewise, the physicochemical properties coincided with those reported for metallo-chlorophylls from other sources and complied with the requirements established by the National Food Safety Standard for Food Additives, specifically those applicable to SCC. Furthermore, the good stability of SZC at temperatures traditionally required for the processing of many foods was also demonstrated. Finally, the AOXC of the SZC produced here exceeded that of many fruits and vegetables traditionally regarded as excellent sources of antioxidants, which suggests its potential use as a functional food ingredient. Based on the results described here, the SZC obtained from chlorophyll extracted from the marine microalgae *D. tertiolecta*, is a metallo-chlorophyll that could effectively contribute to meeting the global demand for functional products, offering a more sustainable and profitable alternative to those currently derived from green vegetables.

Author's contribution

Work conceptualization: VOA, GBLJ; Methodological performance: MAKA, MGS, GTJA, GBLJ; Software management: MAKA, GTJA, GBLJ; Experimental validation: MAKA, GBLJ; Results analysis: MAKA, VOA, VOR, GBLJ; Data management: MAKA, GBLJ; Manuscript writing and preparation: MAKA, VOA, GBLJ; Reading, revision, and editing: MGS, VOR, GTJA; Project manager: VOA, GBLJ; Funding acquisition: VOA, GBLJ. All the authors read and accepted the public version of this manuscript: MAKA, VOA, MGS, VOR, GTJA, and GBLJ.

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Declaration of interests

The authors declare no potential conflict of interests.

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