CHROMATOGRAPHIC ANALYSIS OF DUNALIELLA SALINA DA23 CAROTENOIDS

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Abstract: *The increasing demand for sustainable and natural bioactive compounds in health and cosmetic industries has intensified interest in microalgae as renewable pigment sources. This study focused on the chromatographic characterization of carotenoids in the halotolerant green microalga Dunaliella salina DA23. Freeze dried biomass was extracted with methanol–acetone (1:1, v/v), and pigments were separated using thin layer chromatography (TLC), which revealed eight distinct fractions, including β-carotene, lutein, astaxanthin, chlorophyll a, chlorophyll b, pheophytin, violaxanthin, and neoxanthin. The major carotenoid fractions were further identified and quantified by high performance liquid chromatography (HPLC) on a C18 column using methanol:acetonitrile:dichloromethane (50:42:8) as the mobile phase. The chromatographic profiles confirmed β-carotene (68.35 ± 1.39%), lutein (12.60 ± 0.75%), and astaxanthin (9.05 ± 0.56%) as predominant pigments, corresponding to 2.73, 0.50, and 0.36 mg·g^{-1} DW, respectively. The total carotenoid content reached 4.00 ± 0.15 mg·g^{-1} DW, surpassing previously reported concentrations in common Dunaliella salina strains (typically 2–3 mg·g^{-1} DW). These results demonstrate Dunaliella salina DA23 exhibits enhanced carotenoid productivity and a diversified pigment profile. Given the established antioxidant, anti-inflammatory, and photoprotective properties of these carotenoids, this strain represents a promising and sustainable bioresource for the development of natural cosmeceutical and nutraceutical formulations.*

Keywords: *Carotenoids; Dunaliella salina; HPLC; TLC; β-carotene.*

1. Introduction

The cosmeceutical industry has increasingly shifted toward natural and sustainable bioactives, driven by consumer demand for safer, eco-friendly alternatives to synthetic chemicals (Harvey & Ben Amotz, 2020; Shahidi & Ambigaipalan, 2018; Yarkent et al., 2020) Among these bioactives, carotenoids are of significant interest due to their multifunctional properties. Carotenoids are natural pigments synthesized by plants, algae, and microorganisms, responsible for yellow, orange, and red coloration. In addition to their pigmentation roles, carotenoids act as potent antioxidants, protecting cells against reactive oxygen species (ROS) and oxidative stress (Lichtenthaler, 1987; Yarkent et al., 2020).

In skin care, carotenoids exhibit photoprotective effects, anti-inflammatory actions, and support skin elasticity and hydration. β-Carotene, the most widely studied carotenoid, serves as a provitamin A essential for epithelial health and has been linked to UV protection (Tominaga et al., 2017; Yarkent et al., 2020). Lutein has been associated with reducing oxidative damage in skin lipids and proteins, helping maintain elasticity and preventing wrinkle formation (Lichtenthaler, 1987; Yarkent et al., 2020). Astaxanthin is recognized as one of the most potent antioxidants, with radical scavenging capacity several fold higher than β-carotene and vitamin E (Higuera-Ciapara et al., 2006; Tominaga et al., 2017). Human clinical studies confirm that astaxanthin improves skin hydration, elasticity, and reduces UV-induced erythema, making it a valuable cosmeceutical active.

Microalgae are among the richest natural sources of carotenoids, with *Dunaliella salina* considered the premier β-carotene producer. This halotolerant alga accumulates large amounts of carotenoids under high salinity and light stress,

making it a reliable source of natural pigments (Borowitzka, 2013; Harvey & Ben Amotz, 2020). Importantly, microalgal cultivation does not compete with arable land, can use saline or wastewater, and contributes to carbon sequestration, aligning with the principles of sustainable biotechnology (Guedes et al., 2011; Harvey & Ben Amotz, 2020).

According to Grand View Research (2024), the global carotenoid market is valued at over USD 2 billion and is expanding steadily at an annual growth rate of approximately 5–6% (Grand View Research, 2024). This growth is primarily driven by the rising demand for natural colorants, antioxidants, and anti-aging compounds in the food, nutraceutical, and cosmetic industries. *Dunaliella salina* is among the most promising microalgal sources due to its exceptional β-carotene productivity; however, its full carotenoid spectrum, especially in local or novel isolates, remains underexplored. Their total carotenoid yields, detailed pigment profiles, and β-carotene–to–xanthophyll ratios have seldom been quantified. For the *Dunaliella salina* DA23 strain, no chromatographic data exist, despite the importance of these ratios for antioxidant and photoprotective performance in cosmetic applications. Since xanthophylls such as lutein and astaxanthin strongly influence anti-inflammatory and anti-photoaging activity, determining their levels relative to β-carotene is essential. Understanding this diversity is essential for identifying strains with superior pigment yields and functional properties aligned with sustainable, bio based product development.

The objective of this study was to identify and quantify the major carotenoids of *Dunaliella salina* DA23 and to assess its potential for applications in sustainable health and cosmetic development.

2. Research overview

Microalgae have increasingly gained attention as renewable and sustainable bioresources rich in high value metabolites with applications in health and cosmetic industries. Among these metabolites, carotenoids stand out for their multifunctional biological activities, particularly their antioxidant, anti-inflammatory, and photoprotective effects, which are highly relevant to skin protection and rejuvenation (Guedes et al., 2011). These compounds, naturally synthesized under stress conditions, can provide safer and more eco-friendly alternatives to synthetic antioxidants in cosmeceutical formulations.

Harvey et al. (2020) identified *β*-carotene, lutein, and astaxanthin as the most prominent carotenoids with cosmeceutical potential, highlighting their synergistic effects in neutralizing reactive oxygen species and reducing photoaging (Harvey & Ben Amotz, 2020). Similarly, Guedes et al. (2011) emphasized the pharmacological and biotechnological importance of microalgal carotenoids and lipids, noting their role in anti-aging, UV protection, and cellular defense mechanisms. These studies collectively demonstrate that the biochemical versatility of microalgae represents a promising foundation for the development of bioactive skincare ingredients (Guedes et al., 2011).

From a commercialization standpoint, Borowitzka (2013) provided an overview of the economic feasibility of high value products derived from microalgae, recognizing *Dunaliella salina* as one of the most efficient natural producers of *β*-carotene (Borowitzka, 2013). Subsequent work by Harvey et al. (2020) and the industrial product documentation from BASF further confirmed the success of large scale production and marketing of *Dunaliella salina* derived carotenoids, particularly Betatene® - a natural *β*-carotene product widely used in dietary supplements, functional foods, and cosmetics . These developments exemplify how research on *Dunaliella salina* has effectively transitioned from laboratory studies to sustainable industrial applications (Harvey & Ben Amotz, 2020; Yarkent et al., 2020).

Despite these advances, most previous studies have concentrated on standard or industrial *Dunaliella salina* strains, such as CCAP 19/18, UTEX 1644, and DCCBC 15, which are optimized for β-carotene production under saline stress (Borowitzka, 2013; Harvey & Ben Amotz, 2020; Liang et al., 2023). Comparative analyses reveal that these strains differ in pigment composition while CCAP 19/18 predominantly accumulates β-

carotene, other isolates exhibit higher lutein or zeaxanthin levels depending on salinity and light intensity (Borowitzka, 2013; Harvey & Ben Amotz, 2020; Yarkent et al., 2020). Recent findings indicate that total carotenoid contents in these industrial strains range from 1,8 to 2,4 $mg \cdot g^{-1}$ DW under optimized conditions, with β-carotene accounting for up to 40-80% of the total pigments. However, indigenous or newly isolated strains remain underexplored, though they may harbor unique carotenoid profiles shaped by environmental adaptations.

Recent omics and bioprocess studies have identified gene clusters regulating carotenoid biosynthesis and stress induced accumulation pathways in *D. salina*, revealing strain specific metabolic signatures (Liang et al., 2023). Nevertheless, systematic chromatographic profiling of such novel strains remains scarce, especially regarding their potential for cosmeceutical and nutraceutical applications.

Therefore, the present study focuses on *Dunaliella salina* DA23 a newly isolated halotolerant strain to fill this gap. Using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC), the research aims to characterize its carotenoid diversity, identify key pigments, and assess their potential for sustainable health and cosmetic utilization. This work contributes novel insights into carotenoid variability among *Dunaliella salina* strains and provides a scientific foundation for developing eco-friendly, bioactive products from renewable algal resources.

3. Research methods

3.1. Microalgae strain and cultivation

Dunaliella salina DA23, which was isolated from salt ponds in Bach Long commune, Giao Thuy district, Nam Dinh province during mid summer (dry season), was cultured in F/2 medium under controlled laboratory conditions. Cultures were maintained at a salinity of 1.5 M NaCl, room temperature, with continuous aeration, a 12:12 h light–dark cycle, and an illumination intensity of 10,000 Lux for 16 days to promote carotenoid biosynthesis.

3.2. Carotenoid extraction

Freeze dried microalgal biomass (approximately 50 mg) was extracted using 10 mL solvent mixture of methanol and acetone (1:1, v/v). The samples were vortexed for 2 min to ensure complete pigment release and centrifuged at 5000 rpm for 10 min. The supernatant was carefully collected and used directly for thin layer chromatography analysis. The extraction was repeated until the residue became colorless, indicating complete pigment recovery (Davies, 1965; Ludwig et al., 2021).

3.3. Thin layer chromatography (TLC) identification

Carotenoid extracts (10 µL) and standards (β-carotene, lutein, astaxanthin) were spotted on silica gel 60 F254 plates. Plates were developed in acetone:hexane (3:7, v/v) and pigment bands were visualized under visible and UV light. Identification was based on coloration, and comparison with standards following Davies (1965) and Lichtenthaler (1987). TLC analyses were repeated three times to confirm the consistency of pigment separation, and representative bands were scraped for HPLC identification (Davies, 1965; Lichtenthaler, 1987).

3.4. High performance liquid chromatography (HPLC) quantification

Scraped TLC bands were transferred into amber vials and eluted with 1mL methanol:acetone. The mixture was vortexed for 1 min and centrifuged at 5000 rpm for 10 min to remove silica residues. The clear supernatant was carefully collected and used directly for HPLC analysis. Before injection, samples were filtered through a 0.22 µm PTFE membrane, and 20 µL was injected. The injection concentration reflected pigments extracted from 50 mg dry biomass into 10 mL solvent, corresponding to 20 µg/mL total carotenoids.

Carotenoids were analyzed using a C18 reverse phase column. The mobile phase consisted of methanol: acetonitrile: dichloromethane (50:42:8, v/v/v) at a flow rate of 1.0 mL/min. Detection was performed at 450 nm using a photodiode array detector (Fazeli et al., 2009). Retention times and absorption spectra were compared with authentic standards of β-carotene, lutein, and astaxanthin. Quantification was carried out based on peak area.

The carotenoid content in the *Dunaliella salina*

DA23 sample was calculated according to the following equation:

$$\text{Content (mg/g DW)} = \frac{(Asample - b) \times Ve}{a \times 1000 \times DW}$$

where:

A_{sample} = peak area of the sample,

a = slope of the calibration curve,

b = intercept of the calibration curve,

V_e = extraction volume (mL),

DW = dry weight of microalgal biomass (g).

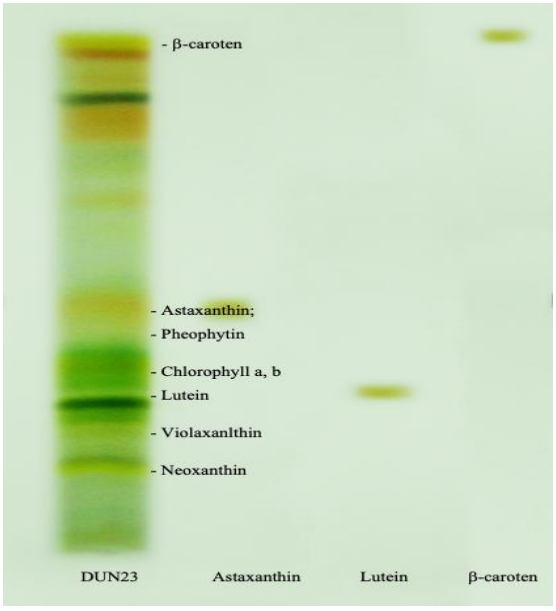
3.5. Data analysis

Each result was expressed as the mean value of three independent replicates. Data analyses were performed using Microsoft Excel. The significance of differences among samples was determined at a confidence level of 95% or 99%.

4. Research results

4.1. Thin layer chromatography (TLC) profile

Figure 1. Representative TLC profile of carotenoid extracts from D. salina DA23



The thin layer chromatographic (TLC) profile of *Dunaliella salina* DA23 pigment extract revealed eight distinct pigment fractions, reflecting the alga's high carotenoid diversity. Comparison with authentic standards identified three major pigments: β-carotene, lutein, and astaxanthin. The upper bright orange band corresponded to β-carotene, which migrated near the solvent front due to its strong hydrophobicity. A reddish orange band at a lower Rf value matched astaxanthin, while the mid green band coincided with the lutein standard (Figure 1). Additional greenish yellow fractions observed below were attributed to chlorophyll *a*, chlorophyll *b*, pheophytin, violaxanthin, and neoxanthin compounds typically found in photosynthetic microalgae.

The TLC pattern confirmed that *Dunaliella salina* DA23 synthesizes both carotenes and oxygenated xanthophylls, supporting a multifunctional photoprotective system (Lichtenthaler, 1987) . β-carotene acts as the major singlet oxygen quencher and lipid membrane stabilizer, while lutein and astaxanthin contribute to blue-light absorption, antioxidant defense, and prevention of UV induced skin damage (Borowitzka, 2013). This pigment composition agrees with previous findings in *Dunaliella* species known for β-carotene dominance (Harvey & Ben Amotz, 2020).

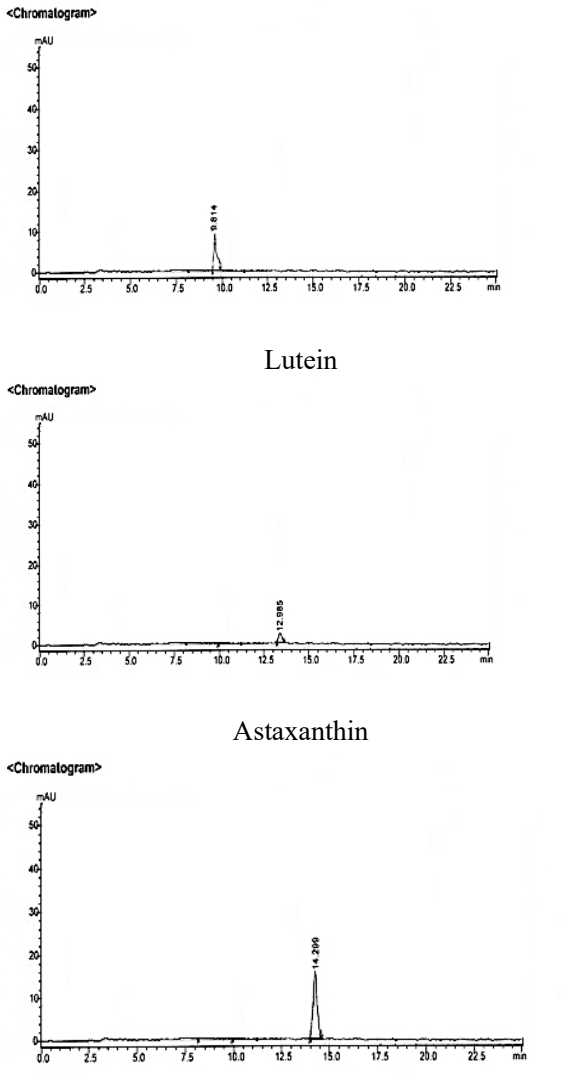
The coexistence of primary carotenoids and accessory chlorophyll derivatives implies potential for integrated utilization of the biomass, minimizing waste and maximizing bioactive recovery (Lichtenthaler, 1987). Collectively, the TLC results demonstrate that *Dunaliella salina* DA23 represents a rich and sustainable natural source of carotenoids with strong potential for eco-friendly health and cosmetic applications.

4.2. HPLC chromatographic analysis

High-performance liquid chromatography (HPLC) provided clear chromatographic separation and reproducible quantification of the major carotenoids present in *Dunaliella salina* DA23 extracts. The results of the standard analysis of Lutein, Astaxanthin β-carotene and *Dunaliella salina* DA23 extracts are presented Table 1, Figure 2 and Figure 3.

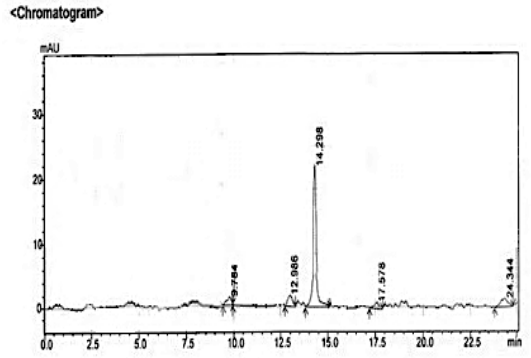
Extracts used for HPLC were obtained by elution of silica scraped from thin layer chromatography bands; all eluates were clarified by centrifugation prior to injection. HPLC was run on a C18 reverse phase column with methanol :acetonitrile :dichloromethane (50:42:8, v/v/v) at 1.0 $mL.min^{-1}$ and detection at 450 nm. Chromatographic identities were confirmed by matching retention time and UV-visible absorption spectra with authentic standards under identical chromatographic conditions.

Figure 2. Representative standard HPLC chromatogram showing the retention times of Lutein (9.814), Astaxanthin (12.985), and β-carotene (14.299); X axis: Retention time (min); Y axis: Absorbance (mAU)



Three major carotenoid peaks were consistently observed in the sample chromatograms and were assigned to lutein, astaxanthin and β-carotene based on standard retention times of 9.784, 12.986 and 14.298 minutes, respectively (Figure 2). Integration of peak areas (triplicate injections per biological replicate, three independent biological replicates) yielded relative peak area distributions of 12.60 ± 0.75% for lutein, 9.05 ± 0.56% for astaxanthin, and 68.35 ± 1.39% for β-carotene; the remaining detected pigment fraction (10.00 ± 0.93%) corresponded to minor or coeluting carotenoids not fully resolved by the current protocol.

Figure 3. Representative HPLC chromatogram of Dunaliella salina DA23 (Retention times: Lutein 9.784; Astaxanthin 12.986; β-carotene 14.298); X axis: Retention time (min); Y axis: Absorbance (mAU)



Absolute contents were calculated by converting the measured relative proportions to mass using the experimentally determined total carotenoid concentration in the extract (20 μg·mL^{-1} in the working extract) and the known extraction parameters (50 mg dry weight biomass, extraction volume 10 mL). Using these values, the content of individual carotenoids in dry biomass was determined as follows β-carotene 2.73 ± 0.06 mg·g^{-1} DW, lutein 0.50 ± 0.03 mg·g^{-1} DW, astaxanthin 0.36 ± 0.02 mg·g^{-1} DW, and other carotenoids combined 0.40 ± 0.04 mg·g^{-1} DW. The sum of identified and minor carotenoids yielded a total carotenoid content of 4.00 ± 0.15 mg·g^{-1} DW for the strain and culture conditions used in this study.

Analytical reproducibility was high, relative standard deviations for triplicate injections of each biological replicate were low (<3% for main peaks), and interreplicate variability across independent biological replicates produced standard deviations as reported above. Representative chromatograms and the TLC plate images are presented in Figure 1 and Figure 3, respectively; the TLC plates showed banding patterns that matched the HPLC identifications and guided fraction scraping. No significant coelution was observed for the three target compounds under the chromatographic conditions used, as verified by the photodiode array spectra associated with each peak.

The combined TLC - HPLC workflow allowed reproducible identification and quantification of the three major carotenoids in *Dunaliella salina* DA23 biomass, with β-carotene dominating the pigment profile and total carotenoid yield of approximately 4 mg·g^{-1} DW under the tested conditions.

Table 1. Major carotenoids Dunaliella salina DA23

Carotenoid	Retention time (min)	Relative content (%)	Content (mg/g DW)
β-Carotene	14.298	68.35 ± 1.39	2.73 ± 0.06
Lutein	9.784	12.6 ± 0.75	0.50 ± 0.03
Astaxanthin	12.986	9.05 ± 0.56	0.36 ± 0.02
Others	-	10.0 ± 0.93	0.40 ± 0.04

5. Discussion

The chromatographic profile obtained from *Dunaliella salina* DA23 revealed a diverse composition of carotenoids, with β-carotene, lutein, and astaxanthin being the dominant pigments. The high proportion of β-carotene (68.35%) is consistent with the well known capability of *Dunaliella* species to accumulate large amounts of this pigment under environmental stress. However, the relatively high lutein and astaxanthin levels observed in this study suggest that *Dunaliella salina* DA23 possesses a broader carotenoid biosynthetic potential than typical strains.

A noteworthy aspect is that the *D. salina* DA23 strain was isolated from natural salt ponds characterized by extreme salinity. Such hypersaline environments expose cells to osmotic stress, high light intensity, and oxidative pressure, all of which are known triggers for carotenoid accumulation. These environmental factors likely stimulated the expression of key enzymes in the carotenoid biosynthetic pathway such as phytoene synthase (PSY), lycopene β-cyclase (LCYB), and β-carotene hydroxylase (BCH) leading to enhanced synthesis of β-carotene, lutein, and astaxanthin. This adaptive mechanism enables *Dunaliella* to protect its photosynthetic apparatus against photooxidative damage by dissipating excess light energy and scavenging reactive oxygen species.

Previous studies have reported similar patterns in *Dunaliella salina* strains isolated from hypersaline habitats in Israel and China, where β-carotene content reached 2–3 mg·g^{-1} DW (Fazeli et al., 2009; Harvey & Ben Amotz, 2020; Liang et al., 2023; Lichtenthaler, 1987; Yarkent et al., 2020). The *Dunaliella salina* DA23 strain in the present study, however, exhibited even higher total carotenoid content (4.00 mg·g^{-1} DW), indicating superior pigment productivity. This may be attributed to both genetic adaptation and selective pressure from its saline environment, resulting in metabolic optimization toward carotenoid accumulation.

The balanced ratio between β-carotene and xanthophylls (such as lutein and astaxanthin) observed in *Dunaliella salina* DA23 is particularly advantageous for biotechnological applications, since xanthophylls play important roles in photoprotection and antioxidant activity. In addition, the coexistence of chlorophyll derivatives (chlorophyll *a*, chlorophyll *b*, and pheophytin) reflects the dynamic regulation between photosynthetic pigments and protective carotenoids, suggesting that *Dunaliella salina* DA23 can finely modulate pigment biosynthesis in response to stress.

Overall, the results demonstrate that *Dunaliella salina* DA23, due to its natural adaptation to high salt environments, has developed a robust carotenoid-producing system. Its elevated pigment diversity and concentration not only highlight its ecological resilience but also underscore its potential as a sustainable source of natural carotenoids for nutraceutical, cosmetic, and pharmaceutical industries.

6. Conclusions

This study successfully characterized the carotenoid composition of *Dunaliella salina* DA23 using an integrated TLC–HPLC approach. Three major pigments β-carotene, lutein, and astaxanthin were identified, with β-carotene accounting for the majority (68.35 ± 1.39%) of total carotenoids. The total carotenoid content reached 4.00 ± 0.15 mg·g^{-1} DW, indicating that

this strain is a promising natural source of bioactive pigments.

These findings contribute to understanding pigment diversity in halotolerant microalgae and highlight the potential of *Dunaliella salina* DA23 for developing sustainable, health promoting, and cosmeceutical products based on renewable algal resources. However, its biosynthetic origin warrants further biochemical or molecular confirmation, such as LC-MS/MS identification or gene expression analysis of ketocarotenoid biosynthetic enzymes, and future work could also explore pilot scale cultivation to optimize pigment production.

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PHÂN TÍCH THÀNH PHẦN CAROTENOID CỦA VI TẢO DUNALIELLA SALINA DA23 BẰNG KỸ THUẬT SẮC KÝ

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Tóm tắt: *Nhu cầu ngày càng tăng về các hợp chất tự nhiên và bền vững trong lĩnh vực chăm sóc sức khỏe và mỹ phẩm đã thúc đẩy sự quan tâm mạnh mẽ đến vi tảo như một nguồn sinh học tái tạo đầy tiềm năng. Nghiên cứu này tiến hành phân tích thành phần carotenoid của loài vi tảo Dunaliella salina DA23 bằng kỹ thuật sắc ký. Sinh khối đông khô được chiết bằng hỗn hợp methanol:acetone (1:1, v/v). Phân tích sắc ký bản mỏng (TLC) cho thấy tám phân đoạn sắc tố riêng biệt, bao gồm β-carotene, lutein, astaxanthin, chlorophyll a, chlorophyll b, pheophytin, violaxanthin và neoxanthin. Các phân đoạn carotenoid chính được tiếp tục xác định và định lượng bằng sắc ký lỏng hiệu năng cao áp (HPLC) trên cột C18 với pha động methanol:acetonitrile:dichloromethane (50:42:8). Kết quả phân tích HPLC xuất hiện β-carotene (68,35 ± 1,39%), lutein (12,60 ± 0,75%) và astaxanthin (9,05 ± 0,56%) là ba sắc tố chính, tương ứng với hàm lượng 2,73; 0,50 và 0,36 mg·g^{-1} DW. Tổng hàm lượng carotenoid đạt 4,00 ± 0,15 mg·g^{-1} DW, cao hơn so với các chủng Dunaliella salina thông thường (2–3 mg.g^{-1} DW). Kết quả cho thấy Dunaliella salina DA23 có khả năng tích lũy carotenoid cao và thành phần sắc tố đa dạng, là nguồn nguyên liệu tiềm năng, bền vững cho phát triển các sản phẩm dược mỹ phẩm và thực phẩm chức năng.*

Từ khóa: *Carotenoids; Dunaliella salina; HPLC; TLC; β-carotene.*