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Recovery of β-Carotene from Microalga *Dunaliella* sp. by HPCCC

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Abstract: β-carotene, a high-value carotenoid widely used in the food, pharmaceutical, and cosmetics industries, is naturally synthesized by the microalga *Dunaliella* sp. However, the efficient extraction and purification of β-carotene from microalgae biomass remain a technical challenge. This study presents the development of a scalable and efficient isolation method employing high-performance countercurrent chromatography (HPCCC) to recover β-carotene from *Dunaliella* sp. The separation process was optimized by integrating two elution strategies (reverse phase and extrusion) using a biphasic solvent system of *n*-heptane and methanol (1:1, *v*/*v*). The upper phase served as the stationary phase, while the lower phase was used as the mobile phase. Two consecutive injections of 800 mg of microalgal extract each resulted in the isolation of 225.4 mg of β-carotene with a purity of 97% and a recovery of 98%. The developed HPCCC approach represents an efficient method for β-carotene purification and serves as a promising model for future scale-up in microalgae-based production platforms.

Keywords: β-carotene; *Dunaliella* sp.; high-performance countercurrent chromatography (HPCCC); countercurrent chromatography (CCC); centrifugal partition chromatography (CPC)

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1. Introduction

β-carotene is a valuable, naturally occurring carotenoid, with significant commercial importance in the global market due to its diverse applications in the food, pharmaceutical, nutraceutical, and cosmetics industries [1]. Its potent antioxidant properties and role as a precursor to vitamin A make it an essential component in combating various health problems, such as reducing oxidative stress, supporting immune function, and preventing vitamin A deficiency [2]. With increasing consumer demand for natural and sustainably produced compounds, there is growing interest in bio-based production methods that reduce reliance on synthetic chemicals and environmentally harmful processes. Among the natural sources of β-carotene, the microalga *Dunaliella* has emerged as one of the most promising candidates for sustainable, large-scale production. *Dunaliella* is a halotolerant unicellular algae capable of producing significant amounts of β-carotene under stressful conditions such as high salinity, intense light, or nutrient deficiency [3]. Its ability to thrive in hypersaline environments not only minimizes the risk of contamination from competing microorganisms, but also makes it suitable for cultivation on non-arable land, reducing pressure on agricultural resources. While the cultivation of *Dunaliella* for β-carotene production has been extensively researched, downstream processing, particularly

the extraction and purification of β-carotene, remains a bottleneck for cost-effective and scalable production.

β-carotene has been extracted from algal biomass using a variety of conventional techniques. These include pressurized liquid extraction with organic solvents such as ethanol and 2-methyltetrahydrofuran [4]; sequential extraction using methanol [5]; ultrasound-assisted extraction with a solvent mixture of hexane and ethanol [6]; extraction using tetrahydrofuran and acetone following cell disruption with glass beads [7]; ultrasound-assisted extraction combined with final purification via solid–liquid chromatography [8]; acetone extraction followed by high-performance thin-layer chromatography; and purification through flash chromatography [9]. In addition, β-carotene has been recovered using supercritical fluid extraction, including supercritical CO_2 [10–16]. While these methods have proven to be effective on a laboratory scale, several challenges remain when considering industrial implementation. Others are associated with high operational costs, complex instrumentation, or limited selectivity for hydrophobic compounds such as β-carotene.

High-performance countercurrent chromatography (HPCCC) is a liquid/liquid chromatography technique that offers a scalable and highly efficient solution for the separation and purification of bioactive compounds, including β-carotene. In contrast to conventional chromatographic methods, HPCCC does not use a solid stationary phase. This not only reduces operating costs, but also minimizes sample degradation and loss, making this method particularly suitable for compounds such as β-carotene. In addition, HPCCC offers high selectivity and scalability, making it an ideal technique for both research and industrial scale applications. In liquid/liquid chromatography, the separation process relies on the differential partitioning of target compounds between two immiscible liquid phases. The retention of the liquid stationary phase through centrifugal force can be achieved via two principal mechanisms: hydrodynamic and hydrostatic. In hydrodynamic systems, such as countercurrent chromatography (CCC), two rotational axes, a central axis and a planetary axis, create a dynamic, variable centrifugal field. In contrast, hydrostatic systems, such as centrifugal partition chromatography (CPC), operate with a single axis of rotation, producing a stable and constant centrifugal force field [17,18]. CCC has been used to recover β-carotene from carrots [19]; and CPC has been applied to obtain β-carotene from *Dunaliella salina* [20]. The present study aims to develop a sequential separation process for the recovery of β-carotene from *Dunaliella* sp. using high-performance countercurrent chromatography (HPCCC) as a production model, enabling the efficient recovery of this high-value carotenoid from microalgal biomass.

2. Materials and Methods

2.1. Biomass Production

Dunaliella sp. biomass was produced during the winter season in unilayer horizontal tubular photobioreactor (closed system) by A4F—Algae for future (Lisbon, Portugal), using A4F hypersaline industrial medium with limiting nutrient conditions, in an outdoor environment, with a maximum setpoint temperature of 30 °C. The biomass was harvested and concentrated by solid/liquid separation and then spray-dried to obtain a carotenoid-rich powder.

2.2. Preparation of Extract of Dunaliella sp. Biomass

Microalgal biomass extract was prepared by extracting 30 g of *Dunaliella* sp. biomass with a 1:1 (*v*/*v*) mixture of *n*-heptane (HiPerSolv Chromanorm, VWR Inc., Gliwice, Poland) and acetone (HiPerSolv Chromanorm, VWR Inc., Fontenay-sous-Bois, France). The extraction was carried out using an ultrasonic bath (K6 Kraintek, s.r.o., Podhájska, Slovakia) operating at 38 kHz frequency and an intensity of 47.77 W/cm^2, maintained at 25 °C, with a

total solvent volume of 3.6 L. The suspension was cleared of insoluble material by filtration. This extraction step was repeated three times on the same biomass using an equal volume of solvent each time. The resulting extracts were then combined, and the solvent was removed by rotary evaporation under reduced pressure at 38 °C. The concentration of β-carotene in the obtained extract was quantified using high-performance liquid chromatography coupled with diode array detection (HPLC-DAD), as described in Section 2.4. The obtained dried extract (6.6 g) was used as feedstock for the HPCCC separation of β-carotene.

2.3. High-Performance Countercurrent Chromatography (HPCCC) Separation

2.3.1. HPCCC Equipment

β-carotene was purified from the *Dunaliella* sp. extract using a high-performance countercurrent chromatography (HPCCC) system (Spectrum model, Dynamic Extractions Ltd., Slough, UK) equipped with a 134 mL coil constructed from PTFE tubing with an internal diameter of 3.2 mm. The rotational speed of the column was regulated via an integrated speed controller within the HPCCC unit. Column temperature was maintained using a H50/H150 Smart Water Chiller (LabTech Srl, Sorisole Bergamo, Italy). A Q-Grad pump (LabAlliance, State College, PA, USA) was utilized to deliver the mobile phase through the system. The real-time monitoring of the separation was achieved using a sapphire UV-VIS spectrophotometer (ECOM spol. s.r.o., Prague, Czech Republic) set at 440 nm. Data acquisition and process tracking were managed using the EZChrom SI software suite version number 3.3.2 SP2 (Agilent Technologies, Pleasanton, CA, USA).

2.3.2. Selection of the Suitable Biphasic Solvent System for HPCCC

Several biphasic solvent systems were prepared by mixing varying ratios of absolute ethanol (AnalaR Normapur, VWR Inc., Fontenay-sous-Bois, France), methanol (HiPerSolv Chromanorm, VWR Inc., Fontenay-sous-Bois, France), *n*-heptane (HiPerSolv Chromanorm, VWR Inc., Gliwice, Poland), and deionized water. Various biphasic solvent systems were assessed for their suitability in HPCCC. An optimal system should exhibit a favorable partition coefficient (*K*) for the target compound [21], a significant density difference between the two liquid phases, and a short settling time [21]. To measure the partition coefficient, 2 mg of the extract was dissolved separately in 1 mL of each phase (upper and lower) of the biphasic system. The mixtures were vigorously shaken and then allowed to settle until full phase separation was achieved. Each phase was then carefully sampled and analyzed by HPLC-DAD to quantify the β-carotene concentration. The partition coefficient (*K*) was calculated as the ratio of β-carotene peak areas between the upper and lower phases [22]. Settling times were determined according to established protocols [23], and phase densities were measured by weighing 1 mL of each phase with a precision microbalance [22].

2.3.3. HPCCC Separation Process

β-carotene isolation from the *Dunaliella* sp. biomass extract was carried out using HPCCC with an optimized biphasic solvent system of *n*-heptane and methanol. The solvent mixture was prepared by combining specific volumes of each solvent in a separatory funnel, followed by thorough mixing and phase separation to obtain two clear liquid phases. The upper liquid phase was used as the stationary phase, and the lower liquid phase served as the mobile phase. The HPCCC column was first filled with the stationary phase, then operated at a rotation speed of 1600 rpm and maintained at 30 °C. Once the column was filled, the mobile phase was pumped through the system until hydrodynamic equilibrium was established. Equilibrium was reached when the mobile phase eluted from the column without causing displacement of the stationary phase, indicating that the system was properly stabilized for injection. At this stage, the sample prepared by dissolving the

Dunaliella sp. extract in the mobile phase was loaded into the chromatographic system. Fractions were collected manually throughout the separation process and subsequently analyzed via HPLC-DAD.

The volume fraction of stationary phase retained in the HPCCC column (*Sf*) was determined according to the following formula:

$$Sf(\%) = \frac{Vs}{Vc} \times 100 \quad (1)$$

In this equation, *Vc* represents the total volume of the HPCCC column, while *Vs* denotes the volume of stationary phase retained in the column once hydrodynamic equilibrium is established [24].

2.4. HPLC-DAD Analysis of Extract and Fractions

The analysis of the *Dunaliella* sp. extract and the collected HPCCC fractions was carried out using a high-performance liquid chromatography system (Agilent 1100 Series, Santa Clara, CA, USA) equipped with a diode array detector (DAD). Chromatographic separation was achieved on a reversed-phase Luna® C8 column (100 × 4.6 mm, 3 µm particle size, 100 Å pore size) operating at a constant temperature of 30 °C. The chromatographic separation was carried out using a binary solvent system consisting of water (solvent A) and methanol (solvent B), with a constant flow rate of 0.8 mL/min. The gradient elution profile was programmed as follows: from 0 to 20 min, a linear decrease of solvent A from 20% to 0%; held at 0% A from 20 to 25 min; re-equilibrated from 0% to 20% A between 25 and 27 min; and maintained at 20% A until the end of the run at 30 min [25]. Detection was performed at a wavelength of 440 nm. The quantitative analysis and identification of β-carotene were conducted using a certified standard (Sigma Aldrich, Darmstadt, Germany). To determine the β-carotene concentration in the algal samples, a calibration curve was established using nine standard solutions ranging from 3.9 to 1000 µg/mL. Each sample was analyzed with a 20 µL injection volume. The linear regression of the standard curve was defined by the equation y = 6.6033x + 36.335 with a correlation coefficient of R^2 = 0.9999, where x represents the β-carotene concentration (µg/mL) and y corresponds to the peak area observed in the chromatogram. This same calibration model was used to assess the purity of the β-carotene isolated from the extract.

2.5. Confirmation of the Chemical Identity of the Purified Target Compound

The chemical identity of the purified compound was verified using a Dionex UltiMate 3000 high-performance liquid chromatography system (Thermo Scientific, Carlsbad, CA, USA) coupled with a high-resolution tandem mass spectrometer (Impact HD, Bruker, Billerica, MA, USA). The analysis was performed with an electrospray ionization (ESI) source operating in positive ionization mode (HPLC-ESI-HRMS). To enhance ionization performance, 0.1% formic acid was added to both solvent A and solvent B used during analysis. The mass spectrometry parameters were configured as follows: a capillary voltage of 2500 V, a drying gas temperature of 250 °C with a flow rate of 10 L/min, and a nebulizer pressure set to 58 psi. Full-scan mass spectra were acquired over an m/z range of 20–2200. For tandem MS analysis (MS/MS), β-carotene was fragmented using nitrogen as the collision gas at a collision energy of 30 eV. The identity of the isolated compound was verified by comparing the obtained mass spectra with reference data available in the literature. Detailed chromatographic conditions used for separation are provided in Section 2.4.

2.6. Evaluation of HPCCC Process Efficiency

The overall performance of the HPCCC system was evaluated based on a previously established methodology [26], using four key indicators: the process throughput (*Pt*), process efficiency (*Pe*), environmental risk factor (*Er*), and general evaluation factor (*Ge*).

The process throughput (*Pt*) quantifies the amount of algal extract processed per unit of time and was calculated using the following equation:

$$Pt = \frac{M_c}{t} \quad (2)$$

where *Mc* represents the total mass of algal extract processed in both cycles, while *t* denotes the cumulative time required to complete the two sequential separation runs.

The process efficiency (*Pe*) reflects the rate of isolated product formation over time, and was determined as follows:

$$Pe = \frac{M_t}{t} \quad (3)$$

with *Mt* representing the amount of purified β-carotene obtained and *t* representing the total processing time.

To assess the environmental sustainability of the separation, the environmental risk factor (*Er*) was used. This indicator estimates the volume of solvent waste generated per unit mass of product and was defined by the following:

$$Er = \frac{V}{M_t} \quad (4)$$

where *V* is the total solvent volume consumed and *Mt* is the mass of the isolated compound.

Finally, the general evaluation factor (*Ge*) integrates process efficiency and environmental impact to provide a relative measure of process performance. It is calculated as the ratio of *Pe* to *Er*:

$$Ge = \frac{Pe}{Er} \quad (5)$$

A higher *Ge* value indicates a more favorable balance between productivity and environmental impact.

Together, these indicators provide a comprehensive assessment of the HPCCC process. While *Pt* and *Pe* provide insights into productivity and separation efficiency, *Er* highlights environmental impact and *Ge* serves as an integrative metric for process optimization and sustainability.

3. Results and Discussion

3.1. Development and Optimization of HPCCC Separation

Multiple biphasic solvent systems, formulated by varying the volumetric proportions of selected solvents, were systematically evaluated to assess their suitability for the HPCCC-based isolation of β-carotene from *Dunaliella* sp. biomass extract (Table 1). The development of suitable solvent systems was guided initially by a known lipophilic mixture composed of *n*-heptane, ethanol, and water in a 6:5:1 ratio, previously reported in the literature [27]. Building upon this reference, several modified systems were formulated by increasing the proportion of ethanol to adjust polarity and enhance phase behavior. In parallel, an alternative biphasic solvent system, composed of *n*-heptane and methanol in a 1:1 ratio, was selected from the Arizona solvent system series [27]. This more apolar configuration was chosen to better match the hydrophobic characteristics of β-carotene, facilitating its preferential partitioning into the upper and lower phase. The selection of solvents for the biphasic systems also considered their compliance with European food safety regulations. While ethanol is widely accepted for use in the food industry, the application of *n*-heptane and methanol is subject to specific conditions. Heptane was chosen as a safer alternative to

hexane, with regulatory guidelines setting a maximum residue limit of 1 mg/kg in the final food product. For methanol, the maximum residue limit is 10 mg/kg [28]. These limits can be safely met by removing the solvents well during the final drying step. Following these rules is important to make sure the process is safe for food-related applications.

Table 1. Partitioning behavior of β-carotene in diverse biphasic solvent systems with measured density differences and settling times [25].

Solvent Systems	Composition	Relative Proportions of Solvents (*v*/*v*/*v*)	Phase Volume Ratio (UP/LP)	Settling Time (s)	Density Difference (LP − UP, g/mL)	Partition Coefficient (*K*) of β-Carotene
1	*n*-Hep–EtOH–H_2O	6/5/1	1.00	10	0.1284	1180.08
2	*n*-Hep–EtOH–H_2O	6/6/1	0.78	11	0.1125	1014.24
3	*n*-Hep–EtOH–H_2O	6/7/1	0.65	13	0.0953	220.46
4	*n*-Hep–EtOH–H_2O	6/8/1	0.50	14	0.0940	188.08
5	*n*-Hep–EtOH–H_2O	6/9/1	0.39	27	0.0910	98.16
6	*n*-Hep–EtOH–H_2O	6/10/1	0.27	20	0.0825	59.69
7	*n*-Hep–EtOH–H_2O	6/11/1	0.22	29	0.0797	54.40
8	*n*-Hep–EtOH–H_2O	6/12/1	0.13	29	0.0728	35.10
9	*n*-Hep–EtOH–H_2O	6/13/1	0.02	29	0.0642	24.03
10	*n*-Hep–EtOH–H_2O	6/14/1	No phases	-	-	-
11	*n*-Hep–MeOH	1/1	0.53	8	0.0700	9.00

LP: lower phase. UP: upper phase. *n*-Hep: *n*-heptane. EtOH: ethanol.

For a biphasic solvent system to be suitable for HPCCC, it must meet specific criteria. Firstly, the system should yield an appropriate partition coefficient (*K*) within the optimal range of 0.5 to 3.5 [21], ensuring the effective distribution of the target compound between the two immiscible liquid phases of the chosen biphasic solvent system. Secondly, it must enable the adequate retention of the stationary phase in the HPCCC column, which requires a sufficient density difference between the phases (greater than 0.08 g/mL) [22,24], along with a rapid phase separation characterized by a settling time of less than 30 s [21]. Among the biphasic solvent systems evaluated (Table 1), none exhibited a partition coefficient (*K*) within the ideal operational range. Nevertheless, system 11 emerged as the most promising candidate, as it demonstrated a *K* value closest to the desired range, coupled with the shortest settling time and a sufficiently acceptable density difference between the upper and lower phases. Although elevated *K* values are typically associated with prolonged elution times, this limitation can be mitigated by employing the highest permissible flow rate of the mobile phase. As such, system 11 was chosen as the most suitable option and subsequently implemented in the HPCCC system to optimize the separation of β-carotene.

It is widely recognized that maintaining high stationary phase retention is essential for achieving an effective resolution in countercurrent chromatography [17]. Since both the flow rate of the mobile phase and the quantity of sample introduced in HPCCC can significantly influence the volume of stationary phase retained within the column [24], these operational parameters were systematically optimized in this study to ensure maximum separation efficiency. The column was operated at a rotation speed of 1600 rpm, the maximum achievable on the HPCCC instrument. This high-speed setting promoted the optimal retention of the stationary phase, thereby enhancing chromatographic resolution [29]. The loop volume was at 0.5 mL, and the separation was operated at 30 °C. The separation was carried out in reverse elution mode, wherein the mobile phase consisted of the lower liquid phase of the biphasic system, and the upper phase was retained as the stationary phase.

Based on the evaluation of how different mobile phase flow rates influenced stationary phase retention (Figure 1), a flow rate of 10 mL/min was identified as the most suitable for achieving effective separation of β-carotene. This setting enabled a more time-efficient operation, while preserving a satisfactory retention of the stationary phase within the

column (*Sf*: 50%, as calculated by Equation (1)), and ensured that system pressure remained safely below the maximum threshold of 250 PSI.

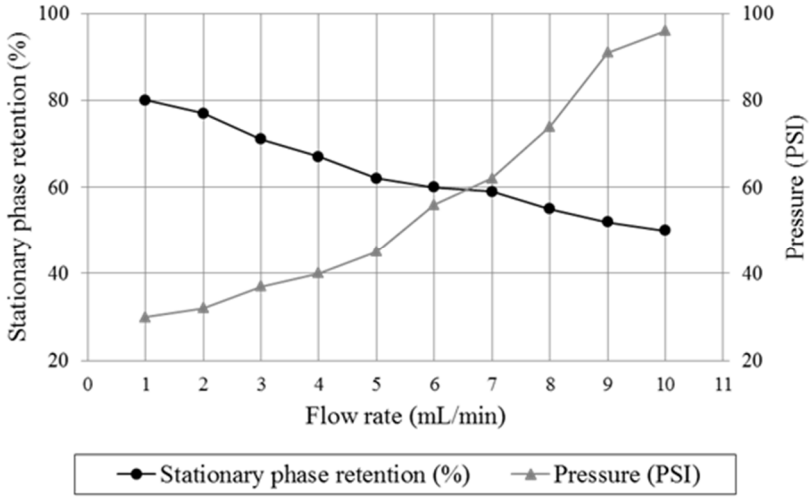


Figure 1. Effect of mobile phase flow rate on stationary phase retention in high-performance countercurrent chromatography (HPCCC). The mobile phase corresponds to the lower phase of solvent system 11, composed of *n*-heptane and methanol in a 1:1 volume ratio. The stationary phase is the upper phase of the same solvent system, also composed of *n*-heptane and methanol in a 1:1 (*v*/*v*) ratio.

The impact of sample loading on stationary phase retention was also assessed (Figure 2, Table 2), revealing that effective separation of the target compound could be maintained across a loading range of 100 to 800 mg of algal extract. All experiments were performed at a constant flow rate of 10 mL/min and a column temperature of 30 °C, with an initial retention of the stationary phase (*Sf*) of 50% at the hydrodynamic equilibrium.

Table 2. Optimization of β-carotene recovery using HPCCC at different sample loadings [25].

Optimization Experiments	Flow Rate (mL/min)	*Sf* at the Hydrodynamic Equilibrium in HPCCC (%)	Loading per Injection (mg)	*Sf* at the End of the HPCCC Separation Run (%)	β-Carotene HPLC Purity (%)	β-Carotene Yield (mg)
a	10	50	100	47	98	13.5
b	10	50	200	36	98	28.1
c	10	50	400	29	97	55.6
d	10	50	800	21	97	112.7

Experiments were performed using a 134 mL semi-preparative coil and biphasic solvent system 11 (*n*-heptane/methanol, 1:1 *v*/*v*). The reversed phase elution was performed with the lower phase as mobile phase. The column was kept at 30 °C, detection was performed at 440 nm, and the volume of the injection loop was 3 mL. Stationary phase retention: *Sf*.

With an increasing injection load, a progressive decrease in stationary phase retention was observed at the end of each HPCCC run, dropping from 47% at 100 mg to 21% at 800 mg. Despite this decrease, the system maintained high separation efficiency throughout, as evidenced by the purity of β-carotene of 97% to 98% in all cases. Importantly, the yield of β-carotene increased proportionally with the sample load, reaching a maximum of 112.7 mg at the 800 mg injection, the highest amount tested. Ultimately, 800 mg was selected as the optimal loading amount, as it maximizes processing efficiency and enhances overall throughput.

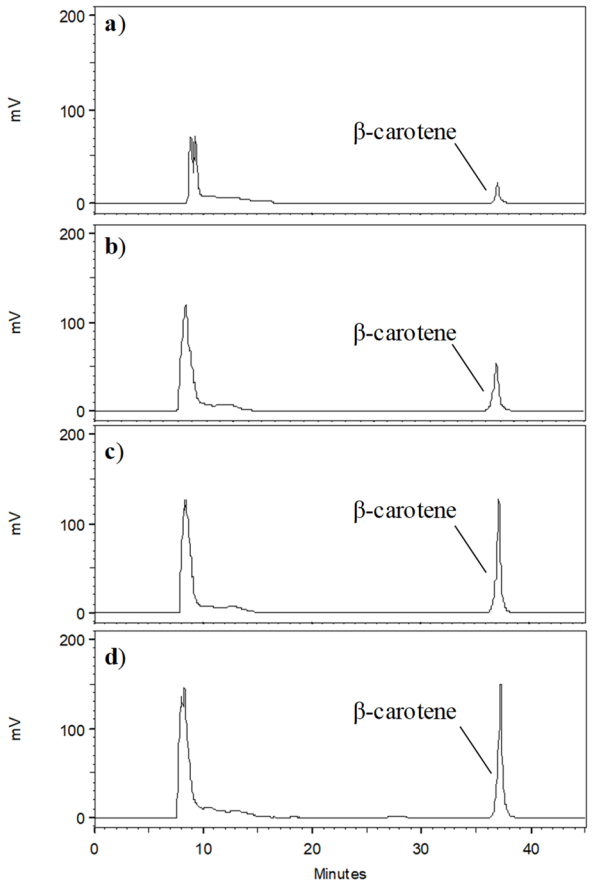


Figure 2. Evaluation of sample load impact on β-carotene isolation from *Dunaliella* sp. extract using HPCCC. Injection volumes tested: (**a**) 100 mg, (**b**) 200 mg, (**c**) 400 mg, and (**d**) 800 mg. Experiments were performed at 1600 rpm rotor speed, employing biphasic solvent system 11 (*n*-heptane:methanol, 1:1 *v*/*v*). Reverse-phase elution was conducted using the lower phase as mobile phase at 10 mL/min flow rate. Column temperature was maintained at 30 °C, with detection at 440 nm. Samples were prepared by dissolving in 3 mL of stationary phase and injected through a 3 mL loop.

To further increase the efficiency and output of the optimized process, a dual-injection strategy was implemented. This approach involved re-establishing the hydrodynamic equilibrium between runs, enabling consecutive separations of the target compound. After the first elution, the system was reset by applying extrusion mode. This was performed by switching from mobile phase pumping to stationary phase pumping while keeping the column in continuous rotation [25,30]. This technique effectively refills the column with fresh stationary phase without interrupting its motion, allowing the formation of a new equilibrium. By doing so, a second separation cycle can be seamlessly initiated (Figure 3). The use of stationary phase extrusion not only removes any residual compounds from the column coil but also ensures that each injection is made into a clean, regenerated stationary phase. This contributes to high reproducibility, which is crucial for processes intended for repeated or continuous operation.

Using the previously optimized conditions, a sequential two-cycle HPCCC protocol was successfully applied for the isolation of β-carotene from *Dunaliella* sp. biomass extract (Figure 3). In each cycle, 800 mg of algal extract was introduced, processing a total of 1.6 g over two runs. The complete HPCCC procedure lasted a total of 137.5 min. This duration encompassed 13.5 min for initially filling the first column with the upper phase at a flow rate of 10 mL/min, followed by a 10 min equilibration step in which the lower phase was introduced at 10 mL/min to establish liquid phase balance within the column. The remaining 114 min was dedicated to conducting two consecutive separation cycles.

Each of the two HPCCC cycles consisted of three phases: (1) the separation of β-carotene using reverse-phase elution with the lower phase pumped at 10 mL/min for 42 min; (2) the extrusion of the stationary phase by switching to upper phase flow for 20 min; and (3) the achievement of the hydrodynamic equilibrium of two immiscible liquid phases inside the column for another 10 min. The overall chromatographic profile of the entire process is presented in Figure 3. It is relevant to note that for future scale-up into production, the duration of the steps could likely be optimized in further studies and, thus, reduced. Throughout the two-cycle process, 1.375 L of solvents were consumed, yielding 225.4 mg of β-carotene at a purity of 97%. Given that the β-carotene concentration in the dried *Dunaliella* sp. extract was measured at 14.396 mg per 100 mg extract, the recovery efficiency of the developed HPCCC method reached 98%. Figure 4a displays the HPLC-DAD chromatogram of the *Dunaliella* sp. biomass extract, while Figure 4b presents the chromatogram of the corresponding isolated fraction. The comparison highlights the effectiveness of the HPCCC technique in achieving the selective separation of β-carotene.

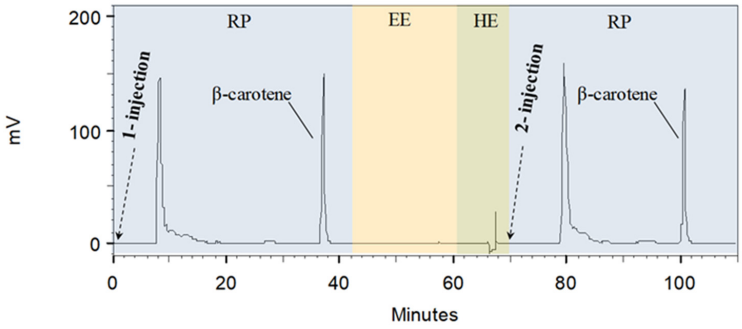


Figure 3. Sequential two-cycle HPCCC process for β-carotene isolation from *Dunaliella* sp. extract using biphasic solvent system 11 (*n*-heptane:methanol, 1:1 *v*/*v*). The separation involved reverse-phase (RP) elution followed by elution-extrusion (EE), with hydrodynamic equilibrium (HE) established prior to each run. Each cycle included injection of 800 mg extract dissolved in 3 mL stationary phase via a 3 mL injection loop. Operating conditions: mobile phase flow rate 10 mL/min, column rotation speed 1600 rpm, temperature 30 °C, and detection at 440 nm.

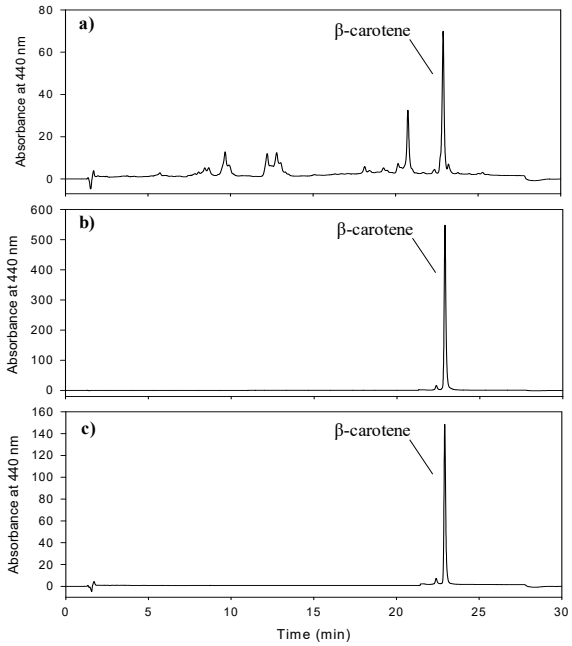


Figure 4. (**a**) HPLC-DAD chromatogram of the crude *Dunaliella* sp. extract, (**b**) chromatogram of the β-carotene-enriched fraction obtained after HPCCC purification, and (**c**) chromatogram of the *β*-carotene reference standard (Sigma Aldrich). All chromatograms were recorded at 440 nm.

3.2. Confirmation of β-Carotene Identity Isolated by HPCCC

The compound isolated via HPCCC was conclusively identified as β-carotene through high-resolution electrospray ionization mass spectrometry (ESI-HRMS) and UV-Visible spectroscopy. The obtained spectral profiles matched those of previously reported profiles in the literature [31,32], as well as a commercial all-*trans*-β-carotene standard, confirming the compound's identity. The ESI-HRMS spectrum of the isolated compound showed a distinct molecular ion peak at m/z 536.4484 $[M]^{+\bullet}$, characteristic of a radical cation. The fragmentation analysis revealed two major diagnostic ions at m/z 444 and m/z 429 (Figure 5a). The formation and stability of β-carotene molecular ions, whether as protonated species $[M+H]^+$ or as radical cations $[M]^{+\bullet}$, are strongly influenced by its extensive polyene conjugation, the parameters of the ESI ion source, and the composition of the HPLC mobile phase. When using a mobile phase of MeOH:H_2O (20:80, v/v), we observed a predominance of the radical molecular ion $[M]^{+\bullet}$, while the signal for the protonated molecular ion $[M+H]^+$ was negligible. As with many carotenoids, β-carotene undergoes in-chain eliminations that follow Woodward–Hoffmann rules. The loss of toluene (−92 Da) from the radical molecular ion occurs via a fully radical mechanism, yielding a fragment at m/z 444 $[M-92]^{+\bullet}$. In contrast, the loss of xylene (−106 Da) may proceed through a partially protonated mechanism, resulting in a fragment at m/z 429 $[M-(106+H)]^{+\bullet}$.

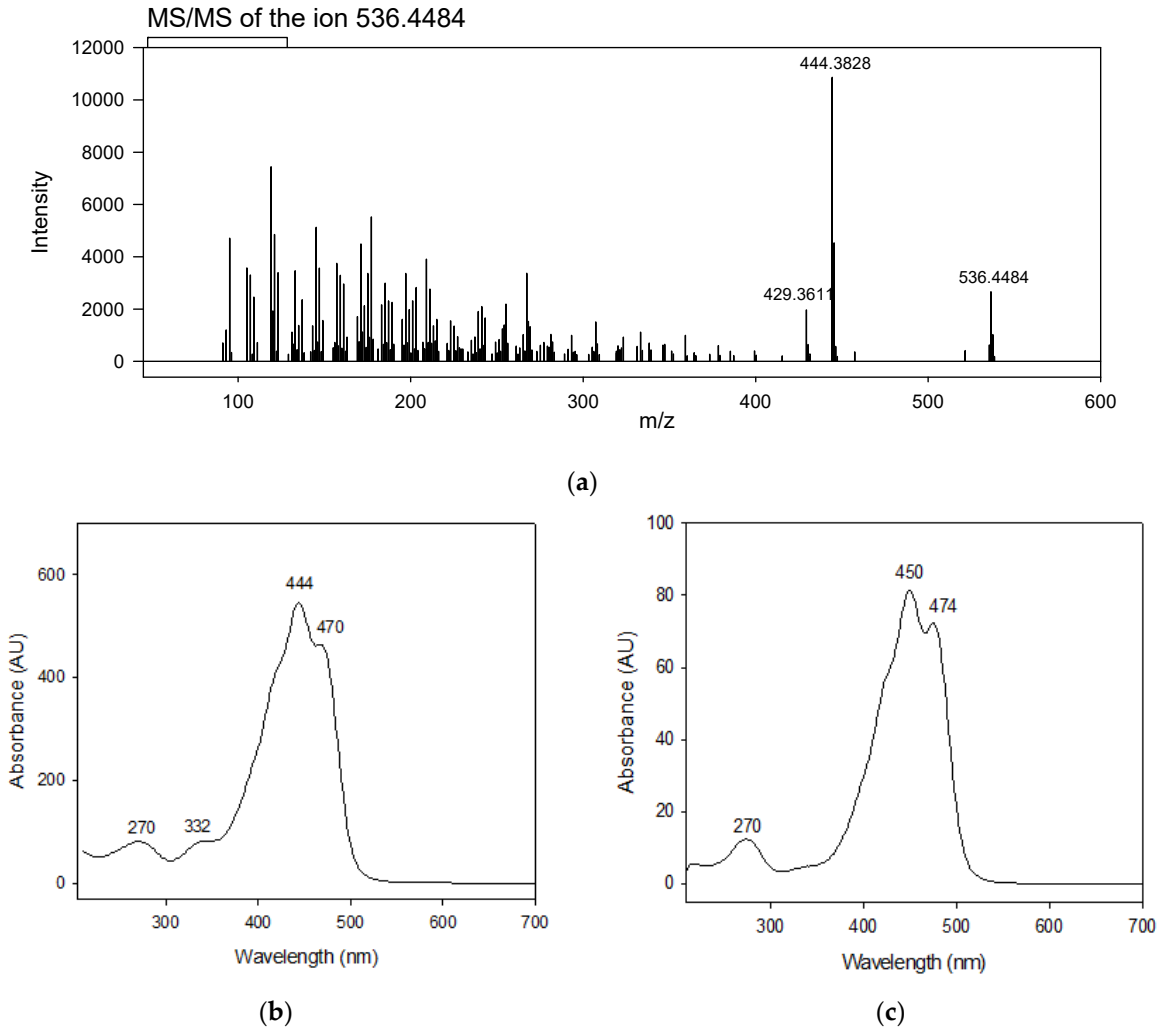
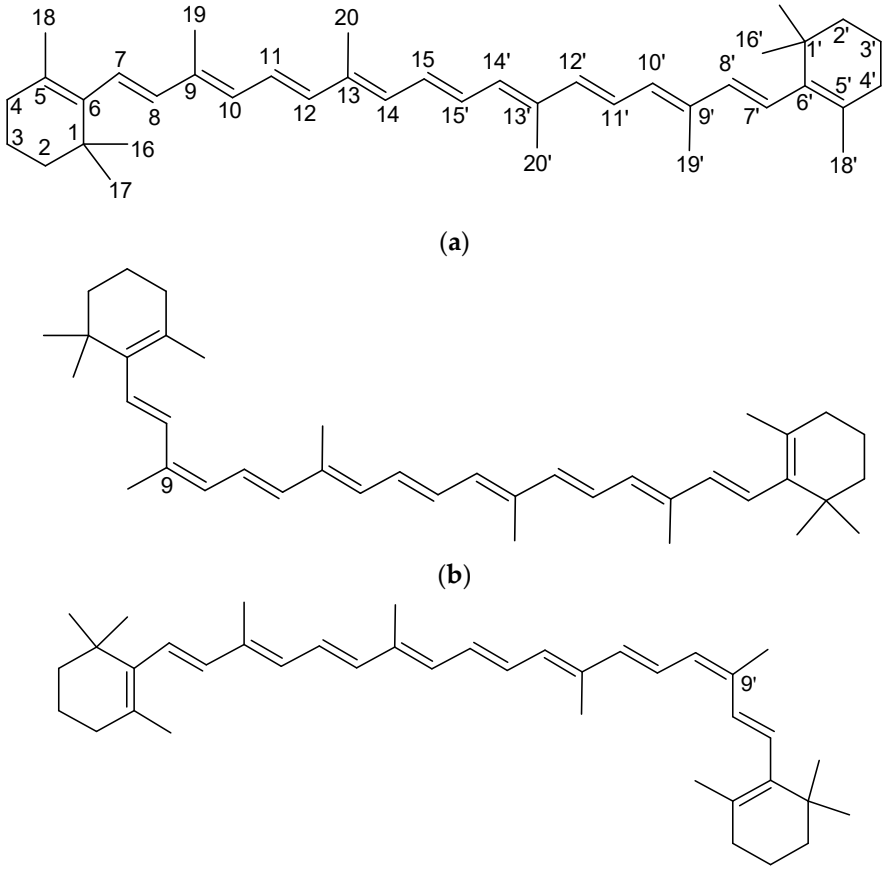


Figure 5. (**a**) High-resolution electrospray ionization tandem mass spectrometry (ESI-HRMS/MS) analysis of β-carotene isolated by HPCCC. (**b**) UV-Vis absorption spectrum of β-carotene obtained via HPCCC. (**c**) UV-Vis spectrum of the all-trans-β-carotene reference standard.

Although *Dunaliella* is recognized for producing β-carotene predominantly in the 9-*cis* configuration [33], the identification in this study was referenced against an all-*trans* commercial standard. Therefore, while mass spectrometry confirmed the molecular identity as β-carotene, it did not allow for a discrimination between *cis* and *trans* isomers. To gain further insight into the isomeric nature of the isolated compound, a UV-Vis spectral analysis was performed. The purified β-carotene exhibited characteristic absorbance maxima at 444 and 470 nm (Figure 5b), while the all-*trans* β-carotene standard displayed its typical peaks at 450 and 474 nm (Figure 5c). This noticeable hypsochromic shift (i.e., shift to shorter wavelengths) in the absorption maxima of the isolated compound suggests a structural variation compared to the all-*trans* form. In addition to the spectral shift, the UV-Vis spectrum of the isolated compound revealed a distinct *cis*-peak, indicative of a *cis* isomer. The intensity ratio between the *cis*-peak (D_B) and the main absorption peak (D_{II}) was calculated at 10.61%, further supporting the presence of a *cis* configuration. These spectral features are consistent with previously reported data for the 9-*cis* or 9′-*cis* isomers of β-carotene [32]. Therefore, based on the combined spectral evidence, it is likely that the isolated pigment corresponds to one of these naturally occurring *cis* isomers, which are known to be prevalent in *Dunaliella*. The chemical structure of β-carotene isomeric forms are shown in Figure 6.



(**a**)

(**b**)

(**c**)

Figure 6. Chemical structure of all-*trans*-β-carotene (**a**), 9-cis β-carotene (**b**) and 9′-cis β-carotene (**c**).

3.3. Process Performance

To evaluate the performance of the developed HPCCC process, Table 3 presents a comparative analysis of key productivity and sustainability indicators (as calculated by Equations (2)–(5)) against a previous study [19], which employed a dual-mode countercurrent chromatography (CCC) method using a ternary solvent system comprising *n*-hexane,

benzotrifluoride, and acetonitrile. The values derived from the previous study [19] were estimated in the present study based on the data reported in that publication. In contrast, the HPCCC method developed in the present study utilizes a binary solvent system composed of *n*-heptane and methanol, eliminating the need for fluorinated solvents and reducing overall chemical complexity. All values related to solvent consumption, sample loading, and carotenoid yield from the comparative CCC process were obtained directly from the original publication. In terms of productivity, the HPCCC system achieved a process throughput (*Pt*) of 0.698 g/h and a process efficiency (*Pe*) of 0.09836 g/h, representing more than a 25-fold and 7-fold improvement, respectively, over the corresponding values calculated from the previous study (Table 3) These improvements are primarily attributed to optimized sample loading (800 mg per run) and the enhanced stationary phase retention achieved under high-performance centrifugal conditions. The environmental impact of the HPCCC process was also markedly lower. The environmental risk factor (*Er*), which quantifies solvent use per gram of product, was calculated at 6.1 L/g, representing a reduction of more than 50% compared to the 14.1 L/g estimated from the previous study (Table 3). Furthermore, the overall evaluation factor (*Ge*), which integrates both productivity and environmental efficiency, reached 0.0161 $g^2 \cdot h^{-1} \cdot L^{-1}$ in this study, an almost 17-fold improvement over the 0.000956 $g^2 \cdot h^{-1} \cdot L^{-1}$ calculated from the previous CCC study (Table 3). Another study [20] also reported the recovery of β-carotene from *Dunaliella salina*, using centrifugal partition chromatography (CPC) with an ethyl oleate-based solvent system containing 5% dichloromethane; however, their approach was specifically designed for continuous extraction while minimizing the impact on microalgal viability. In contrast, the current HPCCC method prioritizes high-yield recovery and enhanced environmental and operational performance, highlighting its suitability for integration into scalable microalgae-based biorefinery systems.

Table 3. Performance evaluation of countercurrent chromatography methods for the isolation of *β*-carotene.

HPCCC Process	Purity (%)	*Pt* (g/h)	*Pe* (g/h)	*Er* (L/g)	*Ge* (g^2 h^{-1} L^{-1})
Method in this paper	97	0.6980	0.09836	6.1003	0.0161
[19]	95	0.0270	0.01348	14.1000	0.0009

Pt: process throughput. *Pe*: process efficiency. *Er*: process environmental risk factor. *Ge*: general process evaluation factor. Estimation of *Pe* and *Er* uses the mass of isolated β-carotene.

Overall, the results demonstrate that under optimized conditions, HPCCC offers a more productive, scalable, and environmentally sustainable platform for the purification of β-carotene from microalgae. These findings underscore its strong potential for industrial implementation within integrated biorefinery workflows targeting the multiproduct valorization of algal biomass.

3.4. Theoretical Throughput Projections for β-Carotene Separation from Dunaliella sp. via HPCCC at Multiple Scales

Table 4 provides a theoretical projection of the β-carotene separation throughput from *Dunaliella* sp. using HPCCC across different operational scales, ranging from laboratory (semi-preparative) to pilot-scale configurations. These estimations are derived from volumetric scaling principles and are expressed in terms of the mass of algal extract processed per unit time. The calculations take into account a realistic weekly operating schedule tailored to each scale of operation. Using the Spectrum system (134 mL coil volume) as used in this study, the estimated throughput is 0.698 g/h, which corresponds to approximately 28 g of processed extract per week under standard laboratory conditions (40 h/week). When scaling up to commercially available systems, including a preparative

HPCCC unit such as the 980 mL column, productivity increases more than sevenfold, reaching 5.105 g/h or approximately 204.2 g/week. Further scale-up to pilot-scale systems, specifically the 4.6 L and 8.82 L column HPCCC units, substantially enhances the weekly processing capacity to over 500 g and more than 1 kg, respectively. The highest throughput is achieved with the 18L system, which processes 93.765 g/h and over 2 kg of extract per week, assuming 24 h continuous operation. These projections underline the scalability of the HPCCC process. The consistent linear increase in throughput with the column volume indicates that the optimized parameters established at the semi-preparative level can be adapted for larger systems with minimal modification. This demonstrates that HPCCC can be used not only for research-scale purification, but also in preparative and industrial settings where continuous and cost-effective processing is essential.

Table 4. Estimated throughput of HPCCC-based β-carotene recovery from *Dunaliella* at semi-preparative, preparative, and pilot scales [25].

Column Volume (mL)	Throughput (g/h)	Throughput (g/week)
134	0.698	27.92 [a]
980	5.105	204.19 [a]
4.6	23.962	575.09 [b]
8.820	45.945	1102.67 [b]
18	93.765	2250.37 [b]

The throughput values indicate the amount of algae extract processed per hour. [a] Estimates for lab-scale systems are based on 40 h of operation per week. [b] Pilot scale estimates are based on 24 h per week of continuous operation when equipment is available.

4. Conclusions

This study successfully established a sequential two-cycle high-performance counter-current chromatography (HPCCC) process for the efficient recovery of β-carotene from autotrophically cultivated *Dunaliella* sp. The optimized HPCCC method produced a high-purity β-carotene fraction without the use of solid-phase materials, demonstrating enhanced separation efficiency and selectivity compared to conventional chromatographic techniques. The process achieved reproducible recovery across both cycles, highlighting the robustness and scalability of HPCCC for continuous operation. These results validate HPCCC as a cost-effective and sustainable alternative for carotenoid purification, delivering a solvent-free product suitable for high-value applications in the food, nutraceutical, and cosmetic sectors. Furthermore, the successful implementation of HPCCC in this context illustrates its broader potential as a core technology in algae-based biorefineries, enabling the selective and efficient extraction of bioactive compounds from microalgal biomass.

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