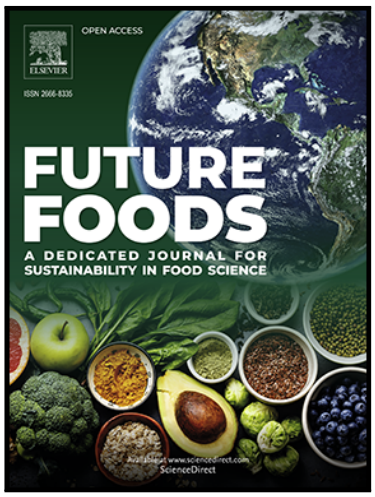
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Optimization of a Switchable Fatty Acid-Based NADES for Sustainable Recovery of Astaxanthin from Haematococcus pluvialis

Maja Živanović , Nadiia Khakimova , Nemanja Teslić , Aleksandra Mišan , Alena Stupar , Anamarija Mandić , Branimir Pavlić , Milica Pojić

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Highlights

- Fatty acid-based NADES were effective for astaxanthin extraction.
- Polarity switching enabled *in-situ* recovery of astaxanthin.
- Astaxanthin extraction was optimized using Box-Behnken design.
- Three astaxanthin esters were major components of the extract.
- Intensive stirring proved more efficient than ultrasound-induced cavitation.

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Optimization of a Switchable Fatty Acid-Based NADES for Sustainable Recovery of Astaxanthin from *Haematococcus pluvialis*

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Abstract: In this study, astaxanthin was extracted from the microalgae *Haematococcus pluvlias* using natural deep eutectic solvents (NADES). Seven NADES, based on fatty acids and menthol, were prepared and evaluated. NADES composed solely of fatty acids (caprylic:pelargonic acid, 1:1) was selected for further optimization. Optimization was performed using Box-Behnken design within response surface methodology, with total carotenoid content, astaxanthin content and energy consumption set as response variables. Optimal conditions were temperature of 31 °C, extraction time of 45 minutes and an algae/NADES ratio of 1.5:10 (w/w). The selected NADES demonstrated comparable performance to acetone (14.10 ± 3.23 compared to 14.23 ± 6.53 mg astaxanthin/g algae). Astaxanthin was successfully recovered via polarity switching, enabling *in situ* precipitation of over 90% astaxanthin within 1 hour. This recovery step relied on the switchable nature of the NADES used, whose hydrophobic character can be reversibly transformed into a hydrophilic form upon application of polyetheramine, thereby facilitating astaxanthin separation from the solvent phase. This method offers a simple, scalable, and sustainable alternative for astaxanthin extraction, with potential application in the food, nutraceutical, pharmaceutical, and cosmetic formulations.

Key words: astaxanthin; natural deep eutectic solvents (NADES); switchable NADES; carotenoids; extraction optimization

1. Introduction

Representing 24.31% of the global carotenoids market in 2024 and projected to expand at a compound annual growth rate (CAGR) of 7.11% through 2030, astaxanthin (3,30-dihidroxy-b-b-carotene-4-40-dione) is currently considered the most commercially valuable natural carotenoid (Mordor Intelligence, 2025). Highly desirable for its role in preventing oxidative stress-related diseases, astaxanthin belongs to the group of carotenoids known for their strong antioxidant capacity and ability to neutralize reactive oxygen species (Matić et al., 2025). Growing demand for astaxanthin is strongly supported by its superior antioxidant activity, which

is comparable, or in some cases may even surpass, the antioxidant potential of vitamin E (Kamezaki et al., 2016). Since the human body cannot synthesize this liposoluble carotenoid, it must be obtained through dietary sources or supplements. To meet the increasing market demand for this carotenoid with anticancer and neuroprotective effect (Chen et al., 2017; Masoudi et al., 2017), it is necessary to identify efficient and environmentally responsible production processes and raw materials.

Although astaxanthin is currently produced predominantly through chemical synthesis due to its lower production costs, natural astaxanthin remains of particular interest due to its stronger antioxidant capacity, enhanced bioactivity and a favorable safety profile (Shah et al., 2016; Rodríguez-Sifuentes et al., 2021). Natural astaxanthin can be extracted from biological sources, most commonly microalgae. However, these extraction methods predominantly use organic solvents such as acetone, hexane, ethanol, sulfuric acid and isopropyl alcohol (Molino et al., 2018; T. Zhao et al., 2019), which present limitations related to toxicity, residual solvents, and negative environmental impact. Moreover, the biotechnological production of natural astaxanthin involves complex downstream processing steps that can account for up to 70-80% of the total production cost (Rodríguez-Sifuentes et al., 2021). Another major challenge with natural astaxanthin production is its susceptibility to oxidation, as well as thermal and light-induced degradation due to its polyene structure (Shah et al., 2016). Therefore, considerable attention has been directed toward extraction approaches that aim to reduce the use of hazardous solvents and minimize processing time and costs, while preserving compound stability (Chemat et al., 2012; Ambati et al., 2014), among which natural deep eutectic solvents (NADES) have emerged as particularly promising green extraction media.

One such promising natural source of astaxanthin is the green freshwater microalgae *Haematococcus pluvialis*, considered the richest natural producer of astaxanthin (Borowitzka, 2010). Although typically green, when found in unfavorable environmental conditions, such as increased light exposure and elevated temperatures, it produces bright red carotenoid astaxanthin up to 4-5% of its dry biomass (Chekanov et al., 2014; Shah et al., 2016). It is particularly significant that microalgae can be cultivated on non-arable land, with minimal freshwater input (Khan et al., 2018). Moreover, wastewater streams such as domestic sewage and palm oil mill effluents (Selmani et al., 2013) can be used for the cultivation of microalgae, contributing to both biomass production and bioremediation (Tan et al., 2020).

Approximately 2.5 to 3.75 tons of palm oil mill effluent (POME) is generated during the production of crude palm oil (Chin et al., 2013). Due to its high organic content, POME represents a significant source of volatile fatty acids which hold promising potential for further conversion into medium-chain fatty acids (MCFAs) (Kurniawan et al., 2024). MCFAs, ranging from 6 to 12 carbon atoms, are known for their diverse biological activities and potential health-related applications (Chen et al., 2024; Shekhar & Thakur, 2025). Caprylic (octanoic) acid is naturally found in the coconut oil, palm kernel oil, and milk of various mammals (Chi et al., 2023; Roopashree et al., 2021), and has been associated with numerous health benefits, including neuroprotective activity, anti-inflammatory properties, and anticancer potential (Altinoz et al., 2020; Cansız et al., 2021; Kamata et al., 2007). Similarly, pelargonic (nonanoic) acid may also

be detected in mammalian milk (Chi et al., 2021; Zhang et al., 2024), but is more commonly utilized as a flavoring agent in food and animal feed (Bento et al., 2022). However, the relatively low concentrations of these MCFAs in milk, especially pelargonic acid, limit its feasibility as an industrial source.

These fatty acids are not only valuable bioactive compounds but can also serve as functional components in the design of NADES. NADES represent a promising alternative group of solvents, offering enhanced safety and environmental compatibility. High solubilization and selectivity can be achieved by rational selection of NADES components whose physicochemical properties closely match those of the target compound (Mišan et al., 2020; Stupar et al., 2021). In addition, certain NADES systems can provide protective effects for sensitive bioactive compounds during and after extraction (Fu et al., 2021), while many NADES systems have the ability to adjust their polarity, which further improves extraction efficiency and simplifies compound recovery (Dai et al., 2015; Stupar et al., 2021). Through their carboxylic functional groups, which participate in hydrogen-bond interactions responsible for eutectic structure stabilization, together with hydrophobic alkyl chains that modulate solvent polarity, MCFAs used as NADES components create an amphiphilic environment with enhanced extraction capacity for lipophilic compounds. Although representing an efficient and green extraction medium, the subsequent recovery of target molecules from NADES remains challenging. Anti-solvent addition (e.g. water) in hydrophilic NADES can induce analyte precipitation (Huang et al., 2017), however, it can disrupt intermolecular interactions within the solvent system, altering NADES properties and preventing their direct reuse. In contrast, polarity switching, particularly suitable for hydrophobic NADES, is based on reversible acid-base interactions and allows compound recovery without disrupting intermolecular interactions, making the NADES immediately reusable (Sed et al., 2018; Stupar et al., 2021).

This creates a dual opportunity in which POME can be valorized both as a nutrient-rich medium for cultivating *H. pluvialis* and as a source of MCFAs used in NADES formulations. Fatty acid–based NADES offer a biocompatible and sustainable solvent system, suitable for the extraction of lipophilic compounds such as astaxanthin from *H. pluvialis*. Integrated strategy of astaxanthin recovery by linking algal biomass production, genetic engineering, NADES extraction technology (Al Fuhaid et al., 2024), fatty acid recovery (Kurniawan et al., 2024), and switchable NADES technology (Cicci et al., 2018), warrants further investigation as a potential pathway towards a circular bioeconomy and waste-to-resource strategies.

Only a limited number of studies have focused on the extraction of carotenoids using NADES (Ilić et al., 2025; Stupar et al., 2021). For astaxanthin extraction from *H. pluvialis*, Pitacco et al. (2022) explored NADES based on oleic acid combined with three different terpenes: thymol, menthol and geraniol, demonstrating their potential to improve extraction efficiency and compound stability. Al Fuhaid et al. (2024) investigated menthol-based NADES for astaxanthin extraction from wild-type *H. pluvialis*, without applying any pretreatment or sonication, while Pei et al. (2025) employed terpene- and fatty acid-based NADES following acidic pretreatment, aiming to optimize conditions for maximum astaxanthin yield from *H. pluvialis*.

Given these limitations, especially regarding astaxanthin extraction using NADES, this study aimed to identify the most suitable NADES formulation based on fatty acids for isolating astaxanthin from *H. pluvialis*, optimize the extraction process using NADES composed solely of fatty acids, and explore the possibility of applying polarity switching as an environmentally friendly strategy for selective compound separation and solvent recycling.

2. Materials and Methods

2.1. Algal biomass

Haematococcus pluvialis biomass used as a raw material in the present study was obtained from Algikey – Algae Based Solutions S.A. (Portugal), with an astaxanthin content of approximately 5%, according to the manufacturer's specification. To preserve bioactive compounds, the powdered biomass was stored in a freezer in its original packaging, protected from light, oxygen and moisture, as recommended by the manufacturer. No additional processing was performed prior to extraction.

2.2. NADES preparation

The selection and formulation of NADES were based on recent literature (Pitacco et al., 2022; Al Fuhaid et al., 2024), with particular focus on MCFAs due to their lipophilic nature and compatibility with non-polar astaxanthin. Special attention was given to fatty acids that could potentially be derived from specific industrial by-products (Kurniawan et al., 2024) and have some bioactivity (Altinoz et al., 2020; Cansız et al., 2021; Kamata et al., 2007). To address the limited research on NADES formulation for carotenoid extraction, combinations of highly lipophilic fatty acids and menthol were selected.

In total, seven different NADES were selected for the screening study. All NADES were prepared in erlenmeyer flask placed into a water bath at ~75 °C and stirred on a magnetic hot plate (C-MAG HS 7, IKA, Staufen, Germany), for ~10 minutes until homogeneous liquids were formed. The components were mixed in molar ratios given in Table 1, based on the literature data (Al Fuhaid et al., 2024; Stupar et al., 2021). The prepared NADES were slowly cooled and remained stable at room temperature (~20 °C) for more than seven days.

Table 1. Components and molar ratios of NADES used in the screening process for astaxanthin extraction.

Code	Components	Molar ratio
N1	Caprylic acid (C_8) – pelargonic acid (C_9)	1:1
N2	Caprylic acid (C_8) – capric acid (C_{10})	1:1
N3	Caprylic acid (C_8) – lauric acid (C_{12})	1:1
N4	Menthol – caprylic acid (C_8)	1:1
N5	Menthol – pelargonic acid (C_9)	1:1
N6	Menthol – capric acid (C_{10})	1:1
N7	Pelargonic acid (C_9) – capric acid (C_{10})	1:1

2.3. NADES characterization

In order to identify functional groups and the presence of hydrogen bonding in the prepared NADES, as well as to evaluate their structural and thermal properties, Fourier-transform (FTIR) spectroscopy and differential scanning calorimetry (DSC) were employed.

2.3.1. Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of the fatty acid-based NADES selected for the optimization of the extraction process (N1), and an additional representative terpene-fatty acid based NADES system (N4) were recorded using a Shimadzu IRTrace-100 spectrometer (Tokyo, Japan). All results were recorded at room temperature over the wavelength range of 4000-400 cm^{-1}, with a resolution of 4 cm^{-1}, 40 scans per sample, and background correction with ambient air.

2.3.2. Differential scanning calorimetry (DSC)

DSC was conducted based on a previously reported methodology (Nascimento et al., 2025), with minor modifications. Using a Netzsch DSC 204 F1 Phoenix calorimeter the samples were analyzed in 25 µL aluminum crucibles with pierced lids, with a heating rate of 5 °C min^{-1} from -20 °C to 50 °C.

2.4. Solid-liquid extraction (SLE) and ultrasound-assisted extraction (UAE)

Screening of the most suitable NADES formulation for astaxanthin extraction was performed using two extraction techniques: SLE and UAE.

UAE was performed using two selected NADES, N4 and N6, along with acetone as the control solvent. Extractions were carried out using a sonication water bath (Transsonic TI-H-10, 8.6 L, Elma Schmidbauer GmbH, Germany), operating at a frequency of 45 kHz. Each extraction was performed in triplicate at an algae/NADES ratio of 1:10 (w/w), at 40 ± 1 °C for 60 minutes in the dark.

SLE was performed to evaluate all seven prepared NADES. Acetone was used as the control solvent, while ethanol was also included as a commonly used solvent for carotenoid extraction. The extraction was carried out in a water bath at 40 ± 1 °C using a magnetic hot stirrer plate (C-MAG HS 7, IKA, Staufen, Germany) equipped with a thermocouple (EST-D5, IKA, Staufen, Germany). Glass vials containing the algal biomass, NADES (ratio 1:10 (w/w)) and a magnetic stir bar were tightly sealed with caps, wrapped in aluminum foil to prevent light-induced degradation of carotenoids, and immersed in the water bath for 60 minutes with constant stirring. After extraction, the samples were centrifuged (MiniSpin plus, Eppendorf, Hamburg, Germany) at 13,000 rpm for 5 minutes. The supernatants were separated from the solid residues and stored at -20 °C until further analysis.

2.5. Box-Behnken Design (BBD) optimization of extraction

To investigate the impact of key process parameters and optimize the efficiency of astaxanthin extraction, BBD was applied within the response surface methodology (RSM) framework. Three independent variables were selected: extraction temperature, extraction time and algae/NADES ratio. Each variable was evaluated at three levels (-1, 0, 1) to assess its effect

on the key responses: total carotenoid content (TCC), astaxanthin content and energy consumption. The selection of the three variables and their corresponding ranges was based on preliminary experiments, previous experience with NADES systems (Pavlić et al., 2022; Stupar et al., 2021; Teslić et al., 2022), and recent literature (Al Fuhaid et al., 2024). With three replicates at the central point, a total of 15 experimental runs were conducted. The actual values corresponding to the three factor levels, along with the 15 experimental runs, are presented in Table 2. All SLE were performed in triplicate, using the same extraction setup and post-processing procedures identical to those described in Section 2.3, with experimental conditions adjusted according to BBD.

The selection of optimal conditions was based on desirability function (*D*). Independent variables were coded according to the following equation:

$$X = \frac{X_i - X_0}{\Delta X} \quad (1)$$

where X represents the coded value of the process parameter (e.g., temperature), X_i is the corresponding actual value, X_0 represents actual value at the center point and ΔX represents increment of X_i corresponding to one coded unit. The experimental data were fitted to a second-order polynomial equation (Eq. (2)), which adequately describes the relationship between the responses and the independent variables:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_{ii}^2 + \sum_{i=1}^{2} \sum_{i<j}^{3} \beta_{ij} X_i X_j \quad (2)$$

where Y is predicted response, X_i and X_j are coded levels of independent variables, and β_i, β_{ii} and β_{ij} represent the regression coefficients for the intercept, linear, quadratic and interception terms, respectively (Bezerra et al., 2008). The relationship between the response and independent variables was demonstrated using a response surface plot.

Table 2. Experimental runs with coded and real values of independent variables according to the Box-Behnken design.

Run	A: Temperature [°C]		B: Time [min]		C: Algae/NADES ratio [g/g]	
1	0	45	0	60	0	1:10
2	0	45	1	100	1	1.5:10
3	0	45	0	60	0	1:10
4	-1	30	0	60	1	1.5:10
5	1	60	0	60	-1	0.5:10
6	1	60	0	60	1	1.5:10
7	0	45	-1	20	-1	0.5:10
8	-1	30	1	100	0	1:10
9	-1	30	-1	20	0	1:10
10	0	45	1	100	-1	0.5:10
11	0	45	-1	20	1	1.5:10
12	1	60	1	100	0	1:10
13	-1	30	0	60	-1	0.5:10
14	0	45	0	60	0	1:10
15	1	60	-1	20	0	1:10

2.6. Total carotenoid content (TCC)

Due to the presence of conjugated double bonds, carotenoids exhibit strong absorption in the visible spectral range (350-550 nm) (Finkel'shtein, 2016). Total carotenoid content (TCC) was quantified spectrophotometrically (UV 1800, Shimadzu, Japan) at a wavelength of 470 nm (Tolasa et al., 2005). Prior to analysis, the extracts were diluted in acetone. All extractions were performed in triplicate, and each measurement was conducted in duplicate. The results were expressed as milligrams of astaxanthin equivalents per gram of algal biomass (mg AE/g algae). A calibration curve was constructed using astaxanthin as the standard.

2.7. Astaxanthin quantification and identification by HPLC analysis

Identification and quantification of astaxanthin and its esters in the extracts obtained were performed following the protocol described by Matić et al. (2024). HPLC analysis was carried out using a liquid chromatograph (Agilent 1200 series, Agilent, Santa Clara, CA, USA), equipped with a diode array detector (DAD) and a Zorbax Eclipse C18 column (4.6 mm × 250 mm, 5 μm particle size; Agilent Technologies, Santa Clara, CA, USA). The separation of carotenoids was performed at ambient temperature (24 ± 1 °C), with an injection volume of 5 μL. Chromatographic separation was achieved using a solvent gradient by varying the proportion of solvent A (25% (v/v) water and 75% (v/v) acetone) and solvent B (25% (v/v) methanol and 75% (v/v) acetone) at a flow rate of 1.5 mL/min, with the following gradient profile: from 0 to 25% B in 10 min, from 25 to 100% B in 35 min, and 100% B for 10 min. Prior to injection, samples were diluted with a 50:50 (v/v) acetone-isopropanol mixture and filtered through a 0.22 μm PTFE membrane filter. Detection was performed at 480 ± 4 nm (Jurčacková et al., 2023). Quantification was performed using a calibration curve constructed with astaxanthin as standard (R^2 = 0.9999), prepared at five concentration levels ranging from 0.76 to 45.5 μg/mL. The results were expressed as astaxanthin equivalents per gram of algae.

2.8. Energy consumption

Energy consumption during the extraction process was measured using a digital power consumption monitoring device (Trotec BX09 EU, Trotec GmbH, Germany). The measurement was conducted throughout the extraction period, excluding the energy required to reach the target temperature, and the results were expressed in kilowatt-hours (kWh).

2.9. Switching NADES polarity

Mechanism of astaxanthin recovery from the NADES extract was related to the fatty acids ability to switch polarity (hydrophobic to hydrophilic system) in presence of basic compound. A 5% (v/v) aqueous solution of Jeffamine D-230 (poly(propylene glycol) bis(2-aminopropyl ether)) was added to the hydrophobic NADES extract at a volume ratio of 13:1 (Jeffamine D-230:NADES extract), according to a procedure described by Sed et al. (2018). The mixture was vortexed and subsequently placed in a hot water bath for 5 minutes to facilitate acid-base interactions between the fatty acids and Jeffamine D-230. The system was then stirred on an orbital shaker (PSU-10i Biosan, Riga, Latvia) for 2 hours and left to stand in the dark for 24 hours.

To monitor the crystallization and sedimentation kinetics of astaxanthin and its esters from the extract matrix, time-resolved analyses were performed. Aliquots were collected immediately after the addition of Jeffamine D-230 and after 1, 2, 4, 12 and 24 hours of incubation. Prior to each analysis, samples were centrifuged at 9000 rpm for 10 minutes (Eppendorf 5804 R, Hamburg, Germany) to separate the precipitate from the supernatant. Astaxanthin recovery was determined by spectrophotometric and HPLC analysis of the supernatant, while the precipitated astaxanthin and its esters were isolated and further characterized by HPLC.

Finally, an attempt was made to reverse the polarity of the now hydrophilic NADES and regenerate its original hydrophobic state. This was achieved by adding 0.1 g of citric acid to 1 mL of the supernatant. The reappearance of a hydrophobic phase was visually observed, indicating successful polarity switching.

2.10. Statistical analysis

All experiments were performed in triplicate, and the results were expressed as the mean ± standard deviation (SD). For the screening of different NADES formulations, one-way analysis of variance (ANOVA) followed by *post-hoc* Tukey's HSD test was performed in order to evaluate statistically significant differences between samples. Differences were considered significant if $p < 0.05$. Analyses were conducted using Statistica 12 software (StatSoft Inc., Tulsa, OK, USA).

BBD within the RSM framework was employed using Design-Expert 12 (Stat-Ease, Minneapolis, MN, USA) to investigate the impact of extraction parameters and optimization of extraction conditions. Model fitness was assessed by ANOVA, while model adequacy was evaluated using the coefficient of determination (R^2), coefficient of variance (CV), and *p*-values for the model and lack of fit. A significance level of $\alpha = 0.01$ was used for the BBD and RSM.

3. Results and Discussion

3.1. NADES Screening

The selection and formulation of NADES were based on recent literature (Pitacco et al., 2022; Al Fuhaid et al., 2024), with particular focus on MCFAs due to their lipophilic nature and compatibility with non-polar astaxanthin. Special attention was given to fatty acids that could potentially be derived from specific industrial by-products (Kurniawan et al., 2024) and have some bioactivity (Altinoz et al., 2020; Cansız et al., 2021; Kamata et al., 2007). To address the limited research on NADES formulation for carotenoid extraction, combinations of highly lipophilic fatty acids and menthol were selected.

Based on the premise that ultrasound waves could effectively disrupt algal cell walls and facilitate astaxanthin release, UAE was initially selected as the primary extraction technique (Zou et al., 2013). This approach was chosen in order to intensify the extraction process, as UAE is widely recognized for its ability to enhance mass transfer, reduce processing time, increase yields and improve the quality of the extract (Mišan et al., 2020). Two NADES, N4 and N6, were first tested using a sonication bath, yielding 47.84 ± 5.66 and 80.05 ± 1.60 mg AE/g algae

(spectrophotometrically determined), respectively. However, significantly higher astaxanthin yields were observed: 96.44 ± 3.64 mg AE/g algae for N4 and 90.68 ± 10.51 mg AE/g algae for N6 when these results were compared to those obtained using SLE under identical conditions (40 ± 1 °C, 60 min) in a water bath with intensive continuous stirring. Due to the lower efficiency of UAE, further screening of NADES formulations was carried out using SLE. These findings suggest that, under the applied conditions, intensive continuous mixing during extraction played a more critical role in enhancing extraction efficiency than sonication in an ultrasonic bath alone. In ultrasonic bath systems, the effective exposure of biomass particles to cavitation can be limited without sufficient mixing, especially at solid-to-solvent ratios of 1:5 or 1:10, where additional mechanical agitation may be required (Fu et al., 2020). The rigid cell wall of *H. pluvialis* represents a major barrier to solvent penetration and astaxanthin release, while the intracellular localization of lipophilic astaxanthin esters makes their extraction highly dependent on efficient solvent-biomass contact (Shah et al., 2016). Therefore, since the ultrasonic bath used in this study did not provide mechanical stirring, the higher yields obtained with SLE may be attributed to improved dispersion and continuous interaction between the algal biomass and NADES and more efficient mass transfer during extraction.

All NADES, along with ethanol and acetone, were tested again using only SLE in a water bath with intense continuous stirring. TCC, expressed as astaxanthin equivalents, are shown in Figure 1a. Among the tested NADES, the lowest yield was obtained using N2, with 79.35 ± 13.74 mg AE/g algae, while the highest yield was obtained using N4, with 96.44 ± 3.64 mg AE/g algae. The fact that the same NADES formulation was the most effective for astaxanthin extraction in a previous HPLC-based study by Al Fuhaid et al., (2024) lends additional support to extraction potential of N4. The highest extraction capacity for astaxanthin remained obtained with the conventional solvent acetone, reaching 104.11 ± 10.53 mg AE/g algae. While the one conventional solvent showed the best extraction capacity, safe, non-toxic, and environmentally friendly ethanol yielded the lowest amount of astaxanthin (58.25 ± 6.77 mg AE/g algae). Its polarity limits its efficiency in extracting the highly nonpolar astaxanthin. Therefore, the use of alternative, less polar solvents is more appropriate for achieving higher extraction yields.

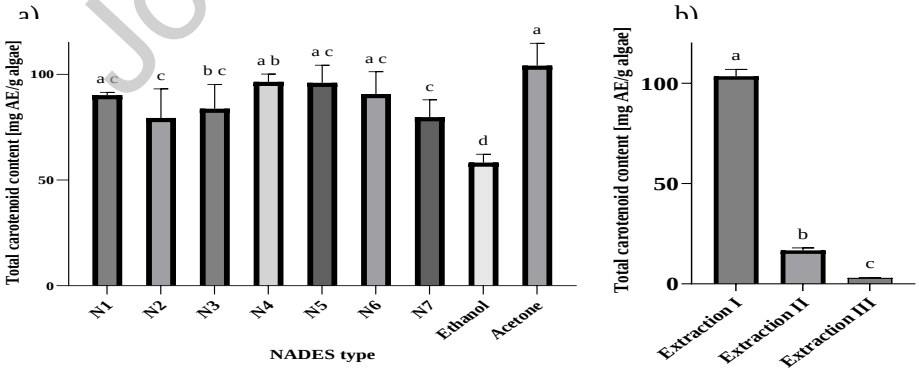


Figure 1. Total carotenoid content expressed as astaxanthin equivalents (mg AE/g algae): a) comparison of different NADES with ethanol and acetone (T = 40 ± 1 °C; t = 60 min; algae/NADES ratio = 1:10 w/w); b) evaluation of the efficiency of three successive extraction steps using N1. Results are presented

as mean ± standard deviation (SD). Bars labeled with different letters represent statistically significant differences ($p < 0.05$). AE: astaxanthin equivalents.

Several of the tested NADES, particularly those based on menthol, showed high extraction efficiency, yielding between 90 and 96 mg AE/g algae. Despite the best extraction efficiency demonstrated by the menthol-based NADES, N1 composed solely of fatty acids, with the yield of 90.15 ± 1.31 mg AE/g algae was selected for further optimization. While DL-menthol-based NADES have been previously recognized as effective solvents for carotenoid extraction, our results, aligned with those of Stupar et al. (2021), indicate that fatty acid–based NADES offer superior practical advantages. Stupar and colleagues similarly evaluated menthol-based NADES but ultimately favored fatty acid–based systems for β-carotene extraction from pumpkin due to their enhanced biocompatibility and solvent recyclability. Since no statistically significant differences were observed among the best-performing solvents, N1 was selected based on the following considerations collectively contributing: 1) polarity tunability – unlike systems based on menthol, NADES based on fatty acids contain carboxylic groups that allow potential polarity switching, making the downstream processes and recovery of astaxanthin easier (Cicci et al., 2018); 2) biocompatibility and metabolic relevance – MCFAs, such as caprylic acid, are naturally present in various food products and are rapidly metabolized by the human body (Schönfeld & Wojtczak, 2016); 3) cost-effectiveness – available market-pricing data suggest that fatty acid components may offer an economic advantage over menthol-based systems. For instance, caprylic acid is generally reported at lower market prices than menthol, although prices may vary depending on purity, supplier, region, and market conditions (Argus Media, 2026; IMARC Group, 2026); and 4) sensory properties – menthol is solid at room temperature, and exhibits a strong, persistent odor, which can be unpleasant, while caprylic and pelargonic acid are liquid at room temperature, have a milder odor and are more convenient for practical use.

After one cycle of extractions, the algal biomass remained visibly colored, indicating the presence of residual pigments. Therefore, prior to the optimization of astaxanthin extraction conditions, two additional successive extraction cycles were performed using fresh portions of the selected NADES (N1) under the same conditions previously described for solid-liquid extraction. The results of the successive extraction are presented in Figure 1b. Nevertheless, it was observed that the second and third extraction steps contributed minimally to the overall yield, with the majority of astaxanthin being recovered during the first extraction. These findings suggest that a single-step extraction may be sufficient for efficient recovery, either due to the high solubility of astaxanthin in the selected NADES, or possible partial degradation caused by repeated exposure to air during the successive extraction steps, despite the effort to minimize oxygen contact.

3.2. Characterization of selected NADES

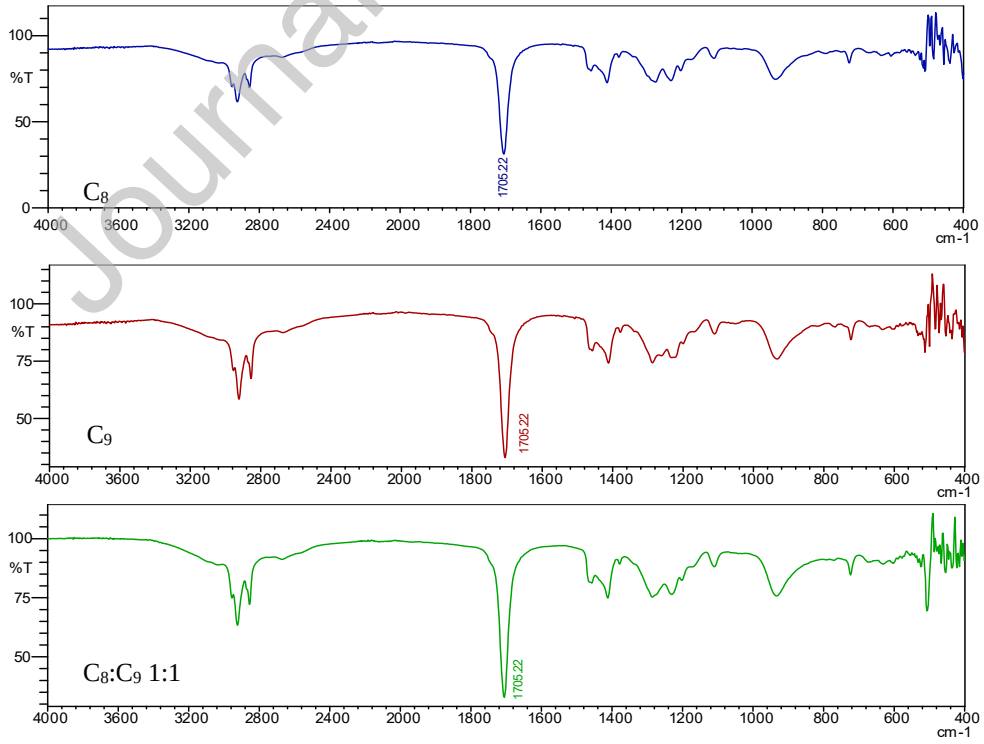
Following the NADES screening step, N1 and N4 were selected for further characterization. N1 was selected as the optimal NADES for extraction optimization, while N4

was chosen as a representative terpene-fatty acid based NADES to enable comparison with the fatty acid – fatty acid NADES system.

Comparison of the FTIR spectra of the N1 and its individual components (Figure 2a) revealed identical characteristic absorption bands, indicating that no chemical reactions occurred between the C_8 and C_9 fatty acid components. The characteristic carbonyl stretching band (C=O) of both fatty acids remained unchanged at 1705.22 cm^{-1} in the N1. Additionally, other typical peaks of fatty acids were preserved, including the alkyl C – H stretching vibrational bands (at 2955.09, 2924.09 and 2856.72 cm^{-1}), as well as C – H bending vibrational bands (at 1458.33 and 1412.04 cm^{-1}) (Arcon & Franco, 2020). To the best of our knowledge, the C_8:C_9 based NADES has not been previously reported, however, its spectral features are consistent with those described for other hydrophobic fatty acid-based NADES, particularly systems combining C_{12} with C_8, C_9 or C_{10} fatty acids, in which no significant FTIR band shifts were observed (Arcon & Franco, 2020).

In contrast, the FTIR spectrum for N4, composed of menthol and caprylic acid, exhibited noticeable shifts in characteristic absorption bands (Figure 2b), suggesting the establishment of intermolecular interactions between the terpene and fatty acid component (Fraterrigo Garofalo et all., 2025). The hydroxyl group of menthol showed an absorption band of 3335.07 cm^{-1}, however in the NADES, this band was no longer clearly resolved, indicating the involvement of the menthol hydroxyl group in hydrogen bonding. Furthermore, the carbonyl stretching band C = O of fatty acid shifted from 1705.22 cm^{-1} to 1711.01 cm^{-1}, upon NADES formation, reflecting changes in the hydrogen-bonding environment. The alkyl C-H stretching bands between 2850-3000 cm^{-1} present in both precursors were also reflected in NADES. Overall, these spectral changes support the formation of a terpene-fatty acid NADES.

a)



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b)

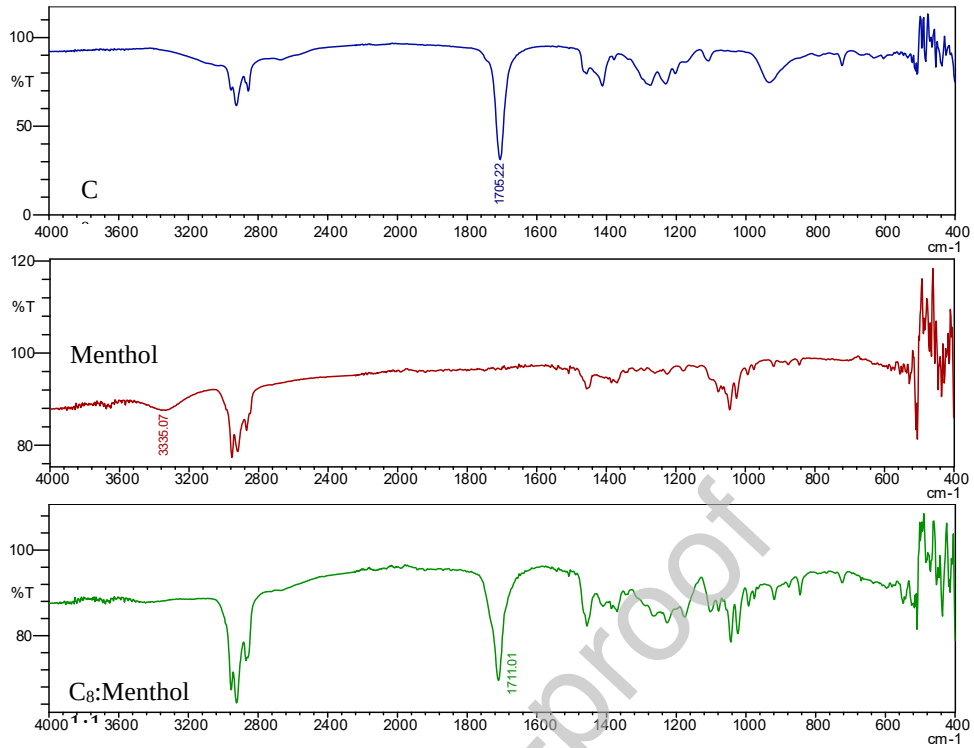


Figure 2. FTIR spectra of N1 (a) and N4 (b) and their individual components.

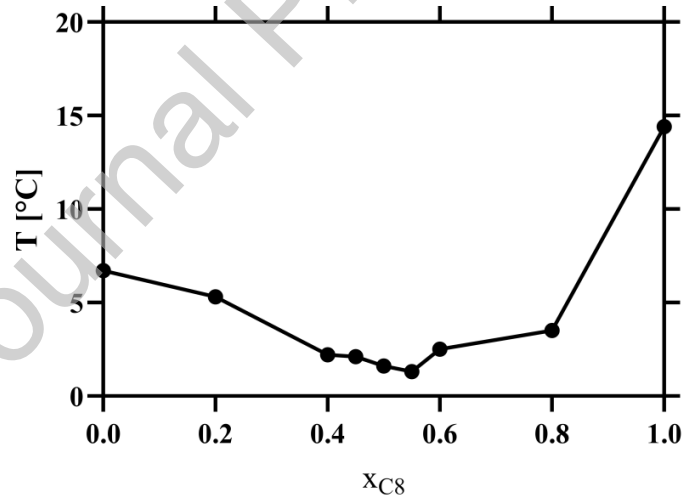


Figure 3. Solid-liquid phase diagram of the binary system C_8 + C_9 fatty acids. x_{C8} – mass fraction of C_8 fatty acid.

Any mixture that exhibits a certain degree of miscibility in the liquid phase, while remaining immiscible in the solid phase, regardless of its chemical composition, shows eutcetic phase behaviour (Van den Bruinhorst and Gomes, 2022). Despite the absence of new bond formation as shown by FTIR analysis, the DSC analysis was performed on the C_8:C_9 mixture to

further evaluate its thermal properties. The results demonstrated that the mixture of these fatty acids nevertheless exhibits eutectic behavior. This observation is consistent with the solid-liquid phase diagram reported by Wang et al. (2017), which confirms that caprylic and pelargonic acid are fully miscible in the liquid phase, whereas in the solid phase they crystallize separately. The formation of eutectic system was further confirmed by the appearance of a distinct melting point at a temperature lower than those of the individual components, as shown in Figure 3.

3.3. Box-Behnken Design (BBD) and Response Surface Methodology (RSM) Optimization

The extraction of bioactive compounds is strongly affected by various process parameters, particularly temperature, extraction time and the solid-to-liquid (S/L) ratio (Milošević et al., 2024; Pojić et al., 2024; Stupar et al., 2021). Determination of the most influential parameters was performed using a BBD within RSM framework. A total of 15 experimental runs were conducted, including three replicates at the central point.

3.3.1. Model Adequacy

The adequacy of the applied models was evaluated using analysis of variance (ANOVA) and descriptive statistic, including the coefficient of determination (R^2) and the coefficient of variation (CV), as summarized in Table S1. A quadratic polynomial model was used to describe the relationship between the extraction parameters and the observed responses. With high R^2 values, ranging from 0.9109 to 0.9956, the models successfully predicted carotenoid extraction outcomes. CV values below 10% are generally considered acceptable. Hence, the low CV values (2.60-7.29%) suggested minimal variation between the predicted and experimental data.

Model validity was statistically confirmed using F-tests, which showed highly significant *p*-values (< 0.0001) for TCC and energy consumption. Although the model for astaxanthin yield displayed a slightly higher *p*-value (0.0351), it remained statistically significant ($p < 0.05$). Additionally, lack-of-fit tests for all responses were greater than 0.05, confirming that the residual variability originated from pure experimental error rather than model inadequacy.

3.3.2. Effect of Independent Variables on Investigated Responses

According to ANOVA results, statistical significance of the independent variables (significant if $p < 0.05$) differed for each targeted response: TCC, astaxanthin content and energy consumption. The experimentally obtained values for these responses are presented in Table 3.

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Table 3. Experimental response obtained with BBD, along with the corresponding coded levels of the independent variables.

Run	A: Temperature [°C]	B: Time [min]	C: Algae/NADES ratio [g/g]	Total carotenoid content [mg AE/g algae]	Astaxanthin content [mg /g algae]	Energy consumption [kWh]
1	0	0	0	100.67	13.37	0.03
2	0	1	1	104.52	14.52	0.05
3	0	0	0	100.75	13.09	0.03
4	-1	0	1	104.91	14.37	0.01
5	1	0	-1	101.13	13.60	0.04
6	1	0	1	99.57	13.00	0.04
7	0	-1	-1	101.60	13.90	0.01
8	-1	1	0	94.45	13.59	0.04
9	-1	-1	0	92.59	13.19	0.01
10	0	1	-1	92.24	12.04	0.05
11	0	-1	1	95.58	12.97	0.01
12	1	1	0	92.08	12.52	0.08
13	-1	0	-1	94.56	14.18	0.01
14	0	0	0	101.45	13.45	0.03
15	1	-1	0	93.77	12.65	0.02

Among all linear effects, only algae/NADES ratio had the significant influence on TCC (Figure 4a; Table 4). The highest TCC (104.91 mg AE/g algae) was obtained in experimental run 4, at the highest tested algae/NADES ratio of 1.5:10, temperature of 30 °C, and extraction time of 60 min. Increasing the amount of biomass, while keeping the solvent volume constant, positively affected carotenoid extraction, as higher concentration of solid material present in the system resulted in a greater quantity of carotenoids available for extraction. Although it is known that increasing the S/L ratio generally improves extraction efficiency by enhancing concentration gradient (Milošević et al., 2024; Pojić et al., 2024; Teslić et al., 2022), in this case higher amounts of solvent would not benefit overall TCC yield. A similar dilution effect was observed in carotenoid extractions from other matrices as well (Stupar et al., 2021). Furthermore, this approach reduced both solvent consumption and waste generation aligning with green extraction principles. On the other hand, it should be noted that excessively high biomass content may lead to solvent saturation with the target compound, limiting further mass transfer (Teslić et al., 2022). Hence, ensuring sufficient biomass in the extraction system is crucial for maximizing yield. In contrast, according to ANOVA results (Table 4), neither temperature nor extraction time showed statistically significant linear effects on TCC (p = 0.9792 and p = 0.8852, respectively). However, all quadratic terms: temperature, extraction time and algae/NADES ratio, as well as all interaction terms between investigated parameters (Figure 4b, 4c, 4a, 4d, 4e, 4f; Table 5), demonstrated statistically significant effects. This indicates that temperature and time affect TCC primarily through non-linear and synergistic mechanisms, rather than direct linear trends. This observation aligns with the lowest TCC (92.08 mg AE/g algae), which was

observed in experimental run 12 (algae/NADES ratio 1:10, temperature 60 °C, time 100 minutes). Although the algae/NADES ratio in this case was not the lowest tested, the combination of elevated temperature and extended extraction time likely caused degradation of thermolabile carotenoids, thereby negating the potential positive effect of biomass concentration. The relative contributions of linear, interaction and quadratic terms were evaluated based on their corresponding sum of squares. These findings emphasize the importance of considering the interactive and non-linear behavior of process variables when optimizing extraction condition. It should also be noted that TCC was determined using a spectrophotometric method, which generally exhibits lower precision and specificity. Therefore, optimal parameter combinations are essential to maximize yield, while avoiding losses due to degradation or saturation effects.

Table 4. Significance of linear, combined and quadratic terms on TCC, astaxanthin content and energy consumption.

Term	TCC [mg AE/g algae]	Astaxanthin content [mg/g algae]	Energy consumption [kWh]
A-Temperature [°C]	0.9792	0.0151*	< 0.0001*
B-Time [min]	0.8852	0.9724	< 0.0001*
C-Ratio [g algae/g NADES]	0.0004*	0.3006	1.0000
AB	0.0358*	0.4862	0.0011*
AC	0.0002*	0.3063	1.0000
BC	< 0.0001*	0.0045*	1.0000
A^2	0.0002*	0.7583	0.3318
B^2	< 0.0001*	0.0947**	0.0030*
C^2	0.0011*	0.0645**	0.0030*

**$0.05 < p < 0.1$ – moderately significant; *$p < 0.05$ – significant

Astaxanthin content determined by HPLC was considered as the most relevant among all measured responses, due to the method's high selectivity and accuracy in quantifying the target compound. For astaxanthin content, the linear term of temperature was statistically significant ($p = 0.0151$), indicating a consistent influence on yield across the experimental range. Regardless of the applied extraction technique and solvent system, temperature is generally recognized as a critical parameter influencing extraction efficiency (Pavlić et al., 2022; Pojić et al., 2024; Tomšik et al., 2017). Carotenoids are thermolabile compounds, yet they can remain stable under moderately elevated temperatures, depending on the exposure duration and matrix conditions (Stupar et al., 2021). Increasing the temperature had a negative effect on astaxanthin content in the extracts due to thermal degradation (Figure 5a; Table 4). Similar decrease in carotenoid yields at higher temperatures has been reported in previous studies (Gu et al., 2008; Norshazila et al., 2017). However, the quadratic term of temperature was not statistically significant. In contrast, quadratic terms for extraction time and algae/NADES ratio showed moderate significance ($p = 0.0947$ and $p = 0.0645$, respectively) (Table 4; Figure 5b, 5c), while the interaction between the extraction time and algae/NADES ratio (Table 4; Figure 5d) was statistically significant ($p = 0.0045$). This helps explaining why both, the highest and lowest, astaxanthin yields were obtained at the same temperature of 45 °C, under identical extraction times (60 minutes), but with different algae/NADES ratios. In experimental run 2, the highest

yield (14.52 mg AE/g algae) was achieved at the highest tested algae/NADES ratio (1.5:10), whereas the lowest content (12.04 mg AE/g algae) was observed in experimental run 10, involving the lowest tested algae/NADES ratio (0.5:10). Although the algae/NADES ratio did not exhibit a statistically significant linear effect (p = 0.3006), and its quadratic effect was only moderately significant (p = 0.0645), these results suggest that algae/NADES ratio may still influence astaxanthin recovery when considered in interaction with other process parameters. The significant interaction between extraction time and algae/NADES ratio explains that the impact of time depends on the amount of solvent relative to biomass. At higher algae/NADES ratios, extended extraction time negatively affects astaxanthin recovery, causing saturation or compound degradation, while at lower algae/NADES ratios, increased solvent availability improves mass transfer and prevents saturation. Overall, these results confirm that while temperature contributes to astaxanthin extraction, the final yield depends on complex interaction between process parameters. As for TCC, optimal recovery can only be achieved when temperature, time and algae/ NADES ratio are balanced to avoid degradation and ensure sufficient solvent availability.

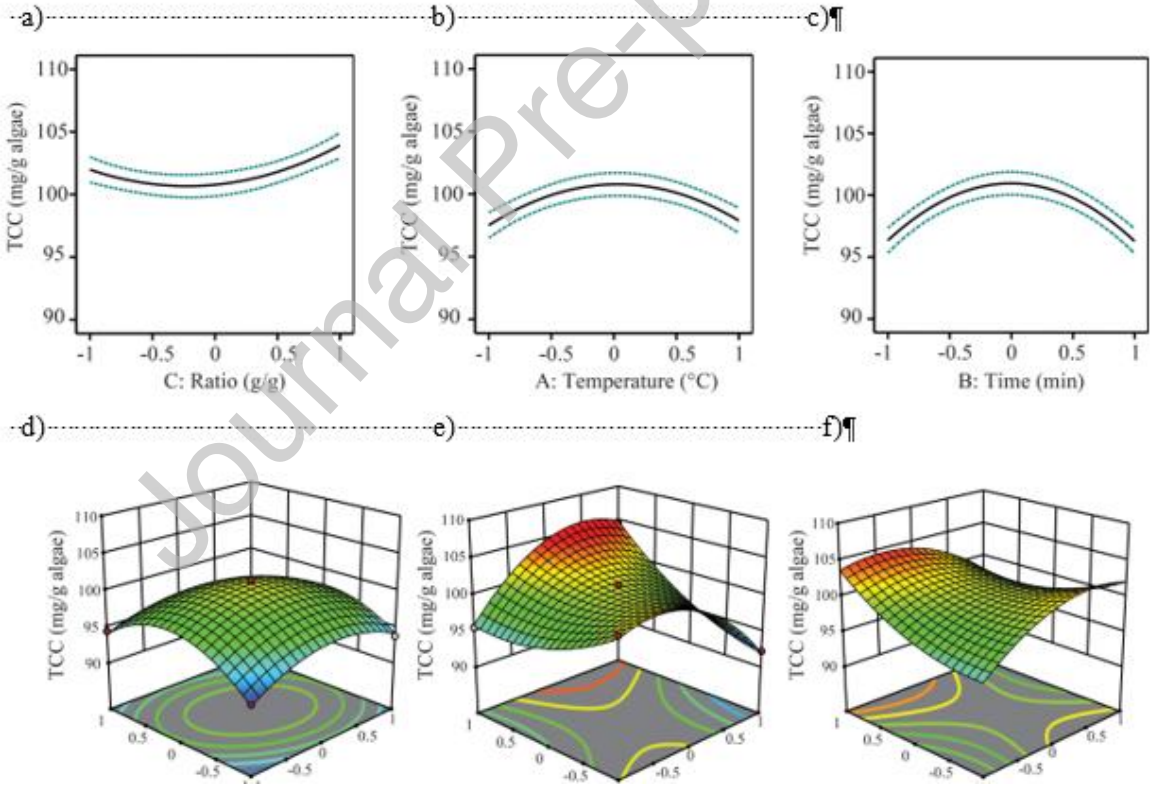


Figure 4. Effect of significant NADES extraction parameters on total carotenoid content TCC: a) Influence of the algae/NADES ratio as an individual factor; b) Influence of the temperature as an individual factor; c) Influence of the extraction time as an individual factor; d) Statistically significant interaction between extraction time and temperature; e) Statistically significant interaction between

algae/NADES ratio and extraction time; f) Statistically significant interaction between algae/NADES ratio and temperature

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Often neglected in research focused on extraction optimization, energy consumption is a critical factor in industrial processes, directly affecting both the scalability and economic feasibility of the process. Given its strong influence on operational costs, minimizing energy demand even at the expense of reduced yields can represent a more sustainable and economically favorable option in large-scale production. As predicted, higher temperatures and prolonged extraction time led to increased energy consumption. Longer extraction times result in a cumulative increase in energy usage (Bouhzam et al., 2024), while elevated temperatures require more input energy to maintain the desired thermal conditions. As expected, the algae/NADES ratio did not exhibit any significant linear effect on energy consumption, as confirmed by its *p*-value of 1.00 (Table 4; Figure 5c), while the linear terms of temperature and extraction time, as well as their interaction, significantly influenced energy consumption ($p < 0.0001$ and $p = 0.0011$, respectively) (Table 4; Figure 5a, 5b, 5d). At the central point of the experimental design (45 °C, 60 minutes, algae/NADES ratio 1:10; runs 1, 3 and 14), the measured energy consumption for SLE was 0.03 kWh, whereas under the same conditions, UAE required 0.20 kWh. It is important to note that energy consumption was measured only during the extraction period, excluding the energy needed to reach the target temperature. In the case of SLE, in addition to stirring, heating remained active throughout the extraction time to maintain the set temperature. On the other hand, for UAE, the water was preheated at 45 °C prior to extraction, and the heating system was then turned off, as ultrasound maintained the target temperature. Yet, energy consumption for UAE remained considerably higher compared to SLE. Interestingly, the

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quadratic term of temperature was not statistically significant (p = 0.3318). Although the algae/NADES ratio is not expected to have a mechanistic influence on energy consumption, the statistical significance of its quadratic term may reflect model-fitting effects rather than a true physical relationship.

Figure 5. Effect of significant NADES extraction parameters on astaxanthin content: a) Influence of the temperature as an individual factor; b) Influence of the extraction time as an individual factor; c) Influence of the algae/NADES ratio as an individual factor; d) Statistically significant interaction between algae/NADES ratio and extraction time.

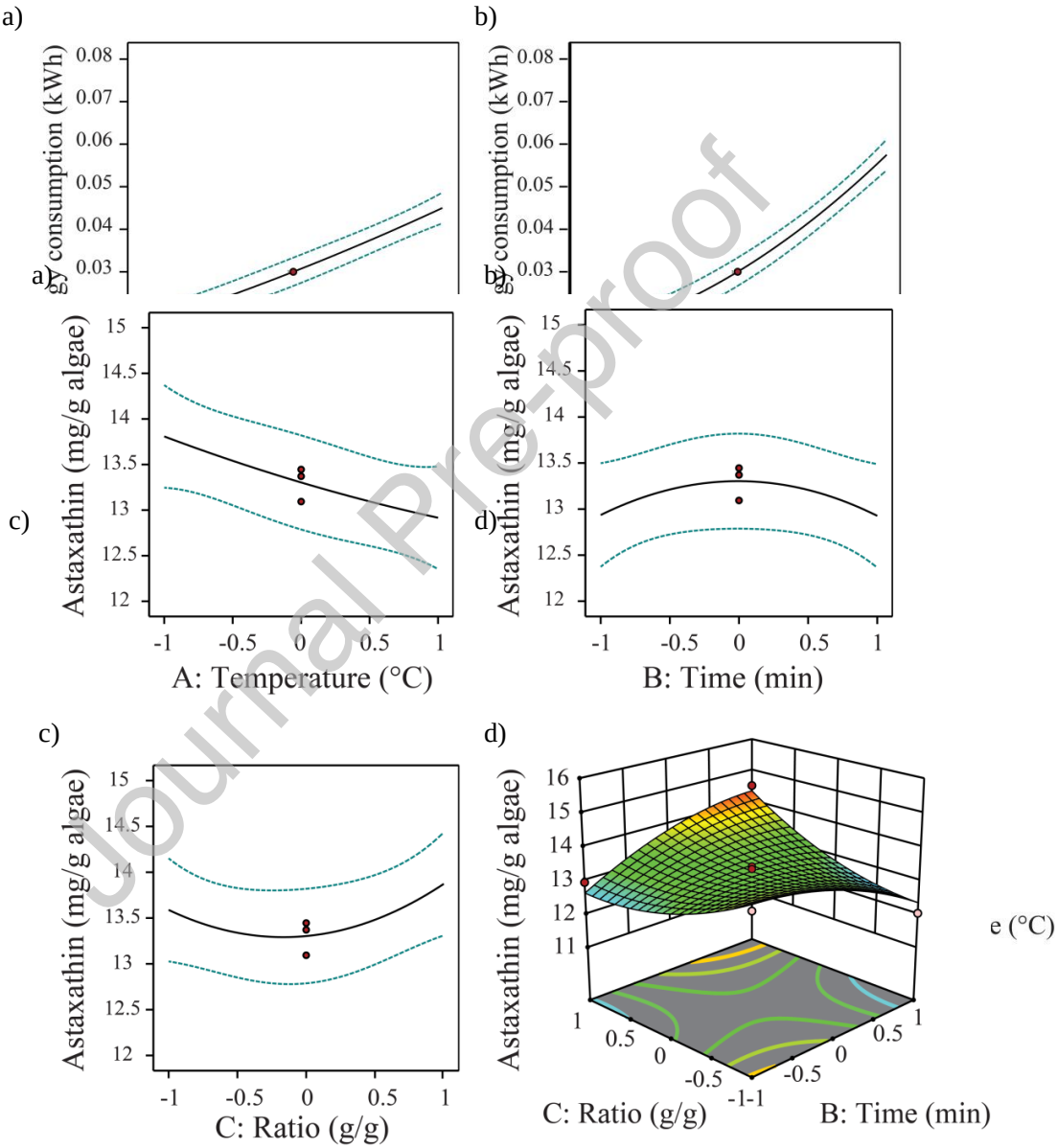


Figure 6. Effect of significant NADES extraction parameters on energy consumption: a) Influence of the temperature as an individual factor; b) Influence of the extraction time as an individual factor; c) Influence of the algae/NADES ratio as an individual factor; d) Statistically significant interaction between temperature and extraction time.

3.3.3. Multi-Response Optimization

After the identification of key parameters affecting the extraction, RSM was employed to simultaneously maximize the TCC and astaxanthin content, while minimizing energy consumption. A set of optimal process parameters: temperature of 31 °C, extraction time of 45 minutes and algae/NADES ratio of 1.5:10 (w/w), was selected based on the desirability function (*D*) approach, with value of 0.990. The high desirability value reflects the model's ability to simultaneously satisfy the targeted objectives. Predicted and experimentally obtained responses are presented in Table 5.

According to the given model and based on a significance level of $\alpha = 0.01$, the agreement between experimental and predicted values was very good, with a minor disagreement with the predicted value for the TCC. The experimentally obtained TCC value of 99.64 mg AE/g algae did not correspond with the predicted value (105.30 mg AE/g algae) for TCC response. The observed deviation may be attributed to the inherent variability of the system, particularly considering the complexity and sensitivity of the spectrophotometric method used for quantification. On the other hand, the experimentally obtained values for astaxanthin content (14.10 mg/g algae) and energy consumption (0.02 kWh) indicated good agreement with predicted values. Therefore, despite the slight disagreement observed for TCC, the overall outcome can be considered successful, as the combination of all responses under the selected conditions remains within acceptable experimental limits.

Table 5. Experimental validation of optimal conditions obtained with response surface methodology.

Input and output parameters	Goal	Lower limit	Upper limit	Predicted values	Experimental values
				Optimal conditions	
Temperature [°C]	in range	30	60	31	
Time [min]	in range	20	100	45	
Ratio [g algae/g NADES]	in range	0.5:10	1.5:10	1.5:10	
TCC [mg AE/g algae]	maximize	92.08	104.91	105.30 ± 0.62	99.64 ± 1.28
Astaxanthin [mg/g algae]	maximize	12.04	14.51	14.52 ± 0.35	14.10 ± 3.23
Energy consumption [kWh]	minimize	0.01	0.08	0.01	0.02

Extraction under optimal conditions was performed using both the selected NADES and acetone. The NADES achieved a higher yield (99.64 ± 1.28 mg AE/g algae) in terms of TCC, outperforming acetone (91.65 ± 14.51 mg AE/g algae). HPLC analysis further confirmed that NADES was comparable to acetone in astaxanthin recovery, yielding 14.10 ± 3.23 mg/g algae, compared to 14.23 ± 6.53 mg/g algae obtained with acetone. Based on a declared astaxanthin content in the biomass (5% w/w), this corresponds to an astaxanthin recovery of 28.2%. A similar astaxanthin content in *H. pluvialis* was reported by Al Fuhaid et al. (2024), obtained with menthol-based NADES. In their study, extracted yields ranged from 10.4 to 13.4 mg astaxanthin/g dry biomass, corresponding to recovery of 13.2-20.8%. The highest yield was achieved with menthol:caprylic acid 1:1 (mol/mol) NADES, which also showed promising results during the screening phase of our study. However, following the optimization of

extraction parameters, our NADES made of fatty acids outperformed this menthol-based NADES, further justifying its selection. Pitacco at al. (2022) achieved similar astaxanthin recoveries, ~30% from *H. pluvialis* culture (2.7% w/w astaxanthin) and ~60% from *H. pluvialis* freeze-dried biomass (1.6% w/w astaxanthin), using three oleic acid-terpenes based NADES. Although their yields were higher, their extraction time was significantly longer, with maximum recovery reached only after 6 hours. However, this study shows the relevance of fatty acids used for astaxanthin extraction. Another recent study by Pei et al. (2025) investigated terpen-fatty acid NADES in order to extract astaxanthin from *H. pluvialis.* The optimized conditions included a temperature of 70 °C, extraction time of 2 hours and algae/NADES ratio of 1:55, resulting in a notably high yield of 43.1 mg astaxanthin/g. Prior to extraction, an acid and thermal pretreatment using 4M HCl at 70 °C, was performed in this study in order to disrupt algal cell wall. Although effective, this method involves aggressive and energy-consuming approach compared to our pretreatment-free approach.

To enhance extraction yields, green pretreatment methods such as pulsed electric field (PEF) (Martínez et al., 2019) or enzymatic treatment (X. Zhao et al., 2019) can be employed. PEF induces reversible or irreversible membrane electroporation of the cell membrane by applying high-voltage pulses. This treatment increases cell wall permeability and enables the selective release of intracellular compounds while preserving the general morphological integrity of the biomass (Ahirwar et al., 2023). On the other hand, enzymatic pretreatment targets specific polysaccharide components in the cell wall matrix improving extraction efficiency in less time (X. Zhao et al., 2019). Such mechanical or biochemical disruptions of the robust cell wall of *H. pluvialis* significantly enhance the accessibility of intracellular astaxanthin to the extraction solvent, thereby improving overall recovery of astaxanthin. As a potential direction for future research, application of these techniques can be considered.

Astaxanthin in *H. pluvialis* predominantly occurs in esterified forms (approximately 95%), mostly as monoesters (~70%) and to a lesser extent as diesters (~25%), with only about 5% present as the free compound (Lorenz & Cysewski, 2000). Three dominant peaks eluting at approximately 25, 27 and 29 minutes (Figure S1a) were detected in the optimal extract. These peaks were identified as astaxanthin esters based on the obtained DAD spectra and by comparison with literature data (Al Fuhaid et al., 2024; Jurčacková et al., 2023). Their quantification was expressed as astaxanthin equivalents. Following the approach described by Al Fuhaid et al. (2024), based on peak integration, the areas of the three major ester peaks were summed, resulting in 14.10 ± 3.23 mg astaxanthin equivalent/g algae. Al Fuhaid et al. (2024) also reported a similar chromatogram, featuring three dominant peaks in *H. pluvialis*. Based on literature data (Al Fuhaid et al., 2024; Jurčacková et al., 2023), it is assumed that the three detected peaks in optimal extract correspond to astaxanthin monoesters. However, due to the differences in chromatographic conditions and the lack of mass spectrometry data, definitive structural confirmation was not possible. Additionally, no peak at approximately 7 minutes, corresponding to free astaxanthin was observed in optimal extract, which is consistent with its low abundance in *H. pluvialis* biomass.

3.4. Switching NADES polarity

One of the key challenges in the extraction of astaxanthin is obtaining solvent-free compounds, which often requires additional purification steps that increase both process complexity and production costs (Rodríguez-Sifuentes et al., 2021). For this reason, NADES represent promising extraction solvents not only because of their nontoxic nature but also due to their switchable physiochemical properties. Ability to undergo polarity switching allows these solvents to selectively recover targeted compounds without the need for conventional solvent removal methods (Jessop et al., 2010). Specifically, when extracting hydrophilic compounds, the polarity of NADES can be shifted toward a more hydrophobic state to induce phase separation and facilitate extract recovery (Cicci et al., 2018). On the other hand, for lipophilic compounds, increasing the hydrophilicity of the solvent can promote the release of the lipophilic extract (Sed et al., 2018; Stupar et al., 2021).

The addition of Jeffamine-D230 to the extract induced a pH change that consequently led to a transformation in the system's polarity, affecting the hydrophobic character of the NADES extract, as well as its solubility and phase behavior. Mixing of the phases, resulting in a monophasic system stable over time, confirmed successful polarity switching. This behavior can be attributed to the formation of ion pairs between the amine groups $–NH_2$ of Jeffamine-D230 and the carboxylic –COOH groups of fatty acids present in NADES extract. Due to the electrostatic interactions between deprotonated carboxylic group (COO^-) and protonated amine groups ($–NH_2$) hydrophilic complex is formed (Q. Chen et al., 2017; Niu et al., 2024). The kinetics of precipitation was monitored over a 24-hour period, with the majority of carotenoid precipitation within the first hour following polarity switching. Immediately after the addition of Jeffamine-D230, the astaxanthin recovery, indirectly assessed by monitoring the decrease in astaxanthin concentration in the supernatant, was 21.30% according to spectrophotometric measurements, and 21.85% based on HPLC analysis. A progressive decrease in astaxanthin concentration was observed in the analyzed supernatant aliquots collected over time, indicating that an increasing amount of astaxanthin underwent crystallization and remained localized at the surface as the process advanced. After just one hour, the recovery increased to 93.41% for the spectrophotometric measurement and 97.11% for the HPLC analysis (Figure 7a, 7b). Interestingly, instead of settling at the bottom of the tube as typically expected (Stupar et al., 2021), the precipitated carotenoids were primarily observed along the walls of the tube and on the surface of the liquid. This behavior can be attributed to their lower density and low solubility in the changed polarity medium, causing them to remain at the interface. In systems where carotenoids easily sediment, the crystals obtained this way can be separated from the medium using simple techniques such as filtration, simplifying downstream process by reducing the need for energy-demanding, time-consuming and costly downstream operations. However, the formed astaxanthin crystals were lightweight and adhesive, which hindered their efficient collection, highlighting the need for further optimization of process.

After completing the monitoring of carotenoid crystallization kinetics, the original hydrophobic character of the system was preliminarily restored by the addition of citric acid through protonation of the fatty acid-amine complex, enabling phase re-separation (Niu et al., 2024). This step was performed as a proof of concept, while optimization of the acid amount

required for efficient phase re-separation and assessment of solvent reuse remain subjects for future studies.

3.5. HPLC analysis of carotenoids in supernatant and precipitate after extract polarity switching

HPLC analysis was performed on both the supernatant and the obtained crystals to monitor the distribution of astaxanthin following polarity switching and crystallization. The three astaxanthin esters that were dominant in the *H. pluvialis* extract remained the major components in the supernatant collected immediately after polarity switching. However, a minor peak corresponding to free astaxanthin appeared at a retention time of approximately 9 minutes. The intensity of this peak increased over time, suggesting that, in addition to the change in solvent polarity, the accompanying pH shift likely contributed to partial alkali hydrolysis of a smaller fraction of astaxanthin esters. As crystallization progressed, the intensity of ester peaks in the supernatant gradually decreased, as a result of two concurrent processes: crystallization of astaxanthin and partial hydrolysis of a minor ester fraction to free astaxanthin. After 24 hours mostly free astaxanthin was detected in the supernatant, indicating that the majority of astaxanthin esters had crystallized (Figure S1b). Despite the observed increase in free astaxanthin in the supernatant over time, this did not indicate a significant loss of the target compound, as its overall concentration in the supernatant remained low (~0.05 mg/g algae). The astaxanthin crystals were collected after 24 hours, re-dissolved in acetone, and analyzed by HPLC, confirming that the three astaxanthin esters remained the dominant components in the crystal fraction (Figure S1c). The later elution of both free astaxanthin in the supernatant and esters in crystals is most likely due to the pH change induced by polarity switching.

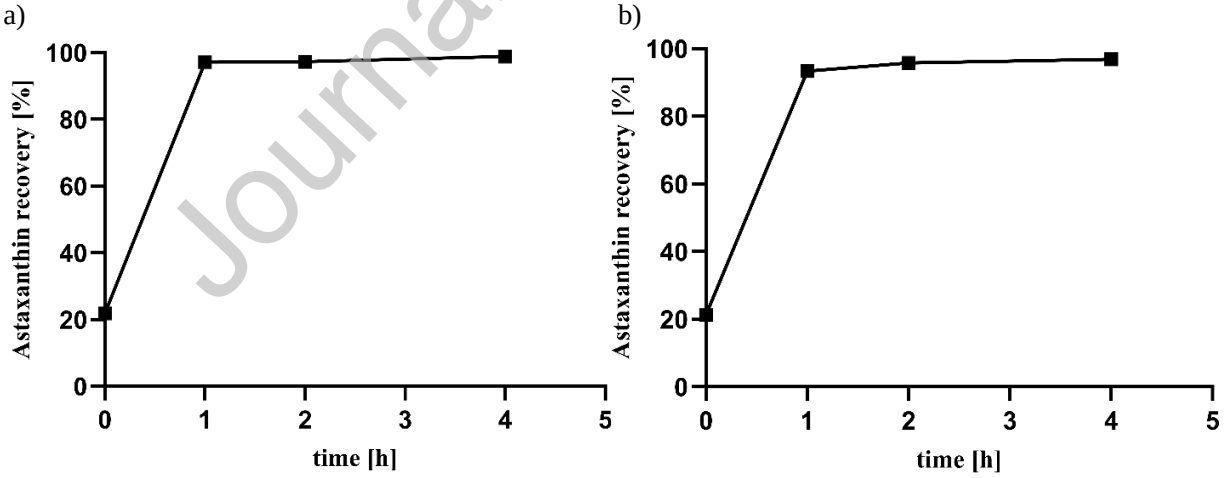


Figure 7. a) Kinetics of astaxanthin recovery monitored by HPLC after the polarity switching of the NADES system; b) Kinetics of astaxanthin recovery monitored by spectrophotometry under the same conditions.

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4. Conclusions

Astaxanthin was successfully extracted from *Haematococcus pluvialis* using novel NADES based on fatty acids. Although several NADES containing menthol demonstrated great potential for astaxanthin extraction, the one composed solely of mid-chain fatty acids was selected for further optimization due to its switchable polarity and potential health benefits. Using a BBD and RSM, the optimal extraction conditions were set at temperature of 31 °C, an extraction time of 45 minutes, and an algae/NADES ratio of 1.5:10 (w/w). The optimized fatty acid-based NADES yielded a comparable amount of astaxanthin to acetone, confirming its potential as a sustainable and less toxic alternative to conventional organic solvents. Extracts obtained this way can be directly incorporated into final products due to the non-toxic nature of NADES. Their compatibility with various delivery systems makes them especially attractive for such applications, where they serve as both carriers and functional ingredients, enhancing the stability of the final product. Alternatively, if a solvent-free formulation is required, the solvent can be easily removed from the carotenoid-rich extract through polarity switching, offering flexibility in downstream processing.

Accordingly, this study demonstrates an environmentally sustainable approach for carotenoid extraction, fully aligned with green chemistry principles by utilizing an efficient solvent in reduced quantities and operating under mild energy conditions. Given the limited number of studies exploring NADES for astaxanthin extraction, especially with the fact that most of them, including ours, report around 30% recovery of total astaxanthin, this work represented an ideal opportunity to address the current knowledge gap. While the potential of NADES as competitive alternatives to conventional solvents for astaxanthin extraction has been demonstrated, further research is needed to improve extraction yields and evaluate their applicability at larger scale. To further enhance extraction efficiency, the application of green pretreatment technologies, such as PEF and enzymatic treatment, should be considered, as these methods disrupt the cell wall and facilitate astaxanthin recovery. Additionally, the polarity switching process can be further optimized by selecting the most suitable basic component, its appropriate volume, and the optimal type and amount of acid required to successfully restore solvent's original polarity.

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Ethical statement – studies in humans and animals

This study did not involve research on humans or animals. Therefore, ethical approval was not required.

Declaration of Interest Statement

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

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