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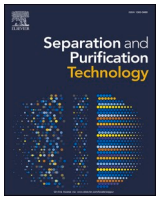

Extraction and separation of pigments from *Saccharina latissima* using eutectic solvents

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ABSTRACT

Harnessing the untapped potential of marine biomass has emerged as a pivotal strategy to address growing demands for natural bioactive compounds across many industries. Among these, *Saccharina latissima*, a prolific marine alga, is a rich source of fucoxanthin and chlorophylls — molecules of significant biotechnological interest. This work used *Saccharina latissima* as a source of pigments to develop an integrated platform to promote the extraction and separation of chlorophyll and fucoxanthin using eutectic solvents (ES). Hydrophobic ES were investigated in the extraction of these pigments, and operational conditions were optimised. Among four hydrophobic ES screened, the Ment:LevA system, enhanced with 20 % (v/v) of water, exhibited the greatest potential, yielding an optimised extract rich in fucoxanthin with 137.2 ± 2.6 $\mu g_{fucoxanthin} \cdot g^{-1}_{biomass}$. Additionally, a unique ES-ES biphasic system facilitated the selective partitioning of pigments: chlorophylls predominantly remained in the hydrophobic phase, while 95 % of fucoxanthin migrated to the hydrophilic. To further refine the quality of the extracted fucoxanthin, a subsequent purification step using water was implemented successfully, resulting in a concentrated pigment product. This work highlights ES as potential biocompatible solvents for the recovery of value-added compounds from marine biomass.

1. Introduction

The contemporary challenges faced by global enterprises highlight the intricate relationship between technological advancement and environmental sustainability [1]. As anthropogenic impacts exert immense pressure on our planet's ecosystems, there is a clear and urgent need to implement strategies that align economic objectives with ecological considerations. The 2030 Agenda for Sustainable Development [2] highlights the importance of creating sustainable products using natural raw materials and biomasses, providing alternatives for a more sustainable society [3]. Algae are excellent candidates as natural and renewable resources for the design of a biorefinery. Algae play an integral role in marine ecosystems, serving not only as primary producers but also as a valuable resource for human exploitation [4,5]. Their rapid growth rates, often exceeding those of terrestrial plants, result in high biomass productivity, making them an efficient feedstock for consistent bioprocessing. Moreover, their ability to thrive in non-arable land using brackish or saline water alleviates the concerns of freshwater usage and land competition with food crops. Also, algae possess the inherent capability to sequester carbon dioxide, making their cultivation a strategic move towards a sustainable and environmentally benign biorefinery process [6,7]. Most importantly, they are natural producers of several high-value bioactive compounds, including proteins, lipids, polysaccharides, and pigments, with interest in the food, pharmaceutical, biofuel and cosmetic sectors [4,8]. Among the diverse species of macroalgae, *Saccharina latissima*, a type of brown algae, is notable for its rich array of bioactive compounds, including polysaccharides and prominent pigments, namely, fucoxanthin and chlorophylls. Chlorophyll (Fig. 1**A**) is a ubiquitous pigment. It is found in most photosynthetic organisms, like plants and algae, and plays a crucial role in photosynthesis, allowing these organisms to transform light into energy. Moreover, it exhibits health-related important biological activities, from promoting wound healing to exhibiting anti-mutagenic properties [9]. The global chlorophyll extract market was substantial, valued at US $ 252.18 million in 2021, with an anticipated growth at a CAGR (compound annual growth rate) of 8.0 % [10]. Fucoxanthin (Fig. 1**B**), on

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the other hand, is a marine-specific xanthophyll predominantly found in brown algae. Its structural uniqueness lends itself to a range of health benefits. Previous studies have highlighted its antioxidant, anti-inflammatory, and anti-cancer properties [11]. Furthermore, research suggests its promising role in managing metabolic disorders, including obesity [12]. The market value of fucoxanthin is notable with estimates ranging from US$ 112.37 million (projected to reach USD 140.09 million by 2028) [13] to a staggering US$ 2.1 billion, projected to reach US$ 3.15 billion by 2030 at a CAGR of 5.2 % [14]. These variations reflect the diverse market analysis approaches highlighting the impact of pigment purity on market value. With their significant market value and biological activities both pigments find a wide range of potential applications in the food, feed, pharmaceutical, nutraceutical, and cosmetic fields [15–17].

The lack of cost-effective and environmentally sustainable processes with sufficient scalability has hindered the implementation of pigment recovery techniques [19]. Due to their perceived efficiency, organic solvents have long been favoured as the preferred extraction method for pigment extraction. However, these solvents frequently exhibit a variety of disadvantages, including their potential harm to the environment, and human safety concerns [20]. In the context of this work, an optimal solvent should be suitable for food and cosmetic applications, and present low toxicity. Moreover, it should have minimal environmental impact and be generated from renewable sources rather than petroleum. Also, the solvent should demonstrate a high capacity for dissolving and selectively targeting the desired molecules [21]. Eutectic solvents (ES) rise as promising alternatives for biomass valorisation. An ES is typically composed of two or more components, often a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA), that interact to form a eutectic mixture with a significantly reduced melting point compared to its components [22,23]. The features of the resulting solvent, such as cost, toxicity, and biodegradability, are contingent upon the appropriate selection of the HBA and HBD precursors [24,25]. Their potential also lies in their tunability, by the manipulation of its HBD and HBA selection and their mutual molar ratio, which enables the manipulation of certain physicochemical attributes, including polarity, surface tension, density, and viscosity, among others, leading to a more precise extraction process. Due to their high solubilisation power and stabilisation ability relative to volatile organic solvents [26–29], ES pave the way in a wide range of applications, including bioprocessing, chemical synthesis, and materials science [23]. Furthermore, it is worth noting that carotenoids and chlorophylls exhibit comparable polarity and are typically found in the same cellular structures, such as chromoplasts, chloroplasts, leucoplasts, and fat globules [30]. As a result, it is quite common to extract these compounds simultaneously, which can hinder the selectivity of the extraction process and ultimately compromise the purification of the compounds [31,32]. Consequently, further purification steps are necessary, which incur additional costs, energy consumption, and the specific types of equipment and materials [33]. Liquid-liquid extraction (LLE) methodologies are prominent in diverse purification protocols due to their distinct advantages over other traditional methods such as chromatography and saponification [34–36]. While numerous LLE systems have been explored, encompassing volatile organic solvents, aqueous biphasic systems, oily systems, IL-based systems and ES-based systems [32,37–39], a notable advancement is the integration of ES-ES biphasic systems. This innovative approach strategically pairs hydrophobic and hydrophilic ES phases to harness the benefits of both [40,41]. In this work, the feasibility of extracting fucoxanthin and chlorophylls from the algae *Saccharina latissima* using hydrophobic ES (HES) was explored. Mixtures of menthol (Ment) with four carboxylic acids, namely acetic acid (AcA), lactic acid (LacA), decanoic acid (DecA) and levulinic acid (LevA), were selected as HES candidates for the extraction and purification of the pigments. Following the initial extraction, the most effective HES mixture was identified. The extraction conditions were then optimised to achieve the maximum yield in terms of fucoxanthin extraction yield. The pigments were subsequently

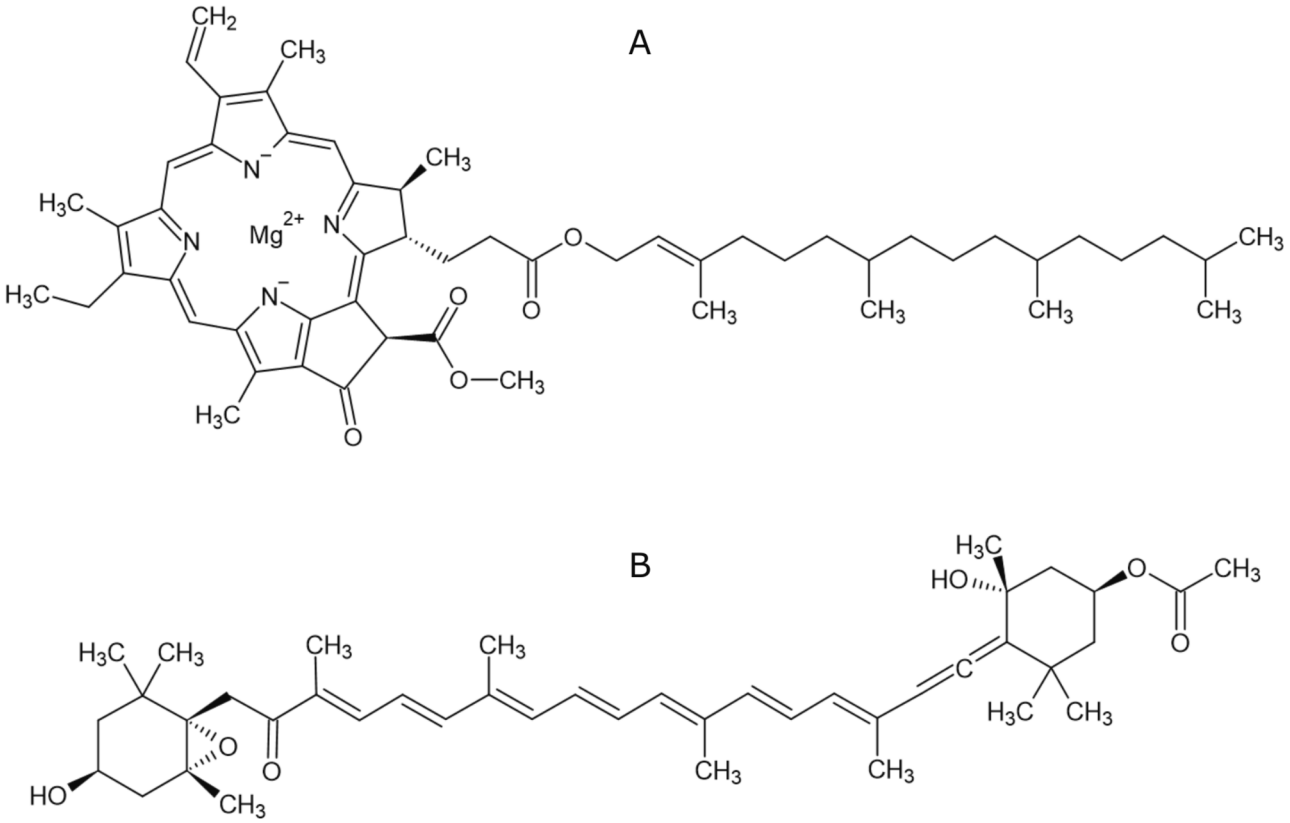

Fig. 1. Chemical structures of Chlorophyll *a* (A) and Fucoxanthin (B) [18].

isolated using an ES-ES biphasic system. The premise is straightforward yet innovative: by leveraging the two ES, it becomes possible to manipulate solute partitioning, thereby facilitating the partitioning of target molecules from a hydrophobic ES to another ES phase. The approach, using a Ment:LevA and ChCl:LevA combination as the ES-ES biphasic system, was shown to hold immense promise. Given the narrow scope of the solvents used, this study primarily served as a proof-of-concept. The aim was to demonstrate the potential utility of ES-ES biphasic systems in pigment extraction, even with a limited solvent selection. In the end, fucoxanthin and chlorophyll were separated. Further purification of fucoxanthin from the hydrophilic eutectic phase was pursued and achieved through the addition of water. The exploration of the ES-ES biphasic system in this context showcased a promising path towards blending efficiency with sustainability. Future work will investigate expanding the range of eutectic solvents used, building upon the foundation established by this preliminary study.

2. Experimental section

2.1. Chemicals

The biomass used in this work was provided by one of the industrial partners of project SeaSolv, Algaia SA (Saint- Lô, France). *Saccharina latissima* (Linnaeus) was collected in Brittany, France. The fresh biomass was harvested, washed, sun-dried, ground in a coffee grinder, and sifted to achieve a particle size of < 1 mm afterwards. The biomass was kept at room temperature under light protection until needed. The ES studied herein were prepared using Ment (purity, 95 %), cholinium chloride (ChCl) (purity, 98 %), and four carboxylic acids, namely: lactic acid (LacA) (purity, 90 %) levulinic acid (LevA) (purity, 98 %), acetic acid glacial (AcA) (purity, 100 %), and decanoic acid (DecA) (98 %) from Sigma-Aldrich. Methanol absolute (analytical reagent grade) used in the solid–liquid extraction (SLE) was acquired from Supelco™.

2.2. Methods

2.2.1. ES preparation

The ES formed by two-component mixtures (HBA and HBD) were accurately prepared at the desired molar ratio. All systems were prepared gravimetrically using an analytic balance Sartorius Cubis ® scale (with an uncertainty of $\pm 10^{-4}$ g). These mixtures were placed in sealed vials with constant stirring while heated at 60 °C for 1 h or until a homogeneous transparent liquid was obtained. Following heating, the mixture was allowed to return to room temperature and stored in sealed vials until used to prevent any water absorption from the atmosphere until use.

2.2.2. Cell disruption and solid–liquid extraction

Cell disruption and SLE were performed simultaneously. The extractions were performed at a fixed temperature of 25 °C under constant agitation (1800 rpm), protected from light exposure using an Eppendorf Thermomixer C – ThermoTop®. A fixed time of 120 min and solid–liquid ratio (SLR) of 0.05 [mass of dry cells (in g) *per* volume of solvent (in mL)] was established for all extractions. The screening was performed using four Ment-based HES composed of the terpene and the bio-derived carboxylic acids, LacA, LevA, AceA and DecA. The HES were tested at an initial molar ratio of 1:1 to evaluate their capacity to release fucoxanthin from the biomass both as a pure solvent and as an emulsion with the addition of a controlled volume of water. Finally, a pure methanol control extraction was also carried out. After the extraction, the samples were centrifuged (11000*g*, 10 min) in an Eppendorf Centrifuge 5424 at 25 °C. The supernatant fraction was recovered and analysed, and the biomass debris were discarded. In the case of biphasic HES + H_2O systems, only the HES phase was analysed due to the complete partitioning of fucoxanthin to the latter and the results corrected for the change in phase volume.

2.2.3. Pigment quantification

The quantification of fucoxanthin was later determined using Ultra-high-performance liquid chromatography with diode-array detection (UHPLC-DAD), on a Shimadzu Nexera X2 (Kyoto, Japan), equipped with a quaternary pump and an analytical Kinetex® C18 column (5 µm 100 Å, 150 x 4.6 mm) from Phenomenex ®, using similar conditions to those described in the method previously reported [42]. Briefly, 20 µL were injected into the LC at a 1 mL·min^{-1} flow rate. The elution solvents were (A) 0.5 M ammonium acetate in methanol:Milli-Q water (85:15 v/v), (B) 90 % of an aqueous acetonitrile solution and (C) 100 % of ethyl acetate. Each run took 53 min, with an elution program as described in Table 1.

For fucoxanthin, the detection wavelength was set at 448 nm in methanol and 431 nm in ES, possibly due to solvent interference. This will be further investigated in future studies. Chlorophyll was consistently measured at 431 nm. Fucoxanthin and Chlorophylls showed up at 5.67 and 23.34 min, respectively, within the chromatogram. The contents of fucoxanthin and chlorophyll were determined by Equation (1) using calibration curves for both methanol- and ES-based solutions. Fucoxanthin (Sigma-Aldrich™) and chlorophyll (Supelco) standards were used to determine the calibration curves.

$$\text{Yield of extraction}\left(\mu g_{\text{Pigment}} \cdot g_{\text{biomass}}{}^{-1}\right) = \frac{[\text{Pigment}] \times \text{Volume}}{\text{Weight}} \quad (1)$$

"[Pigment]" corresponds to the concentration of either fucoxanthin or chlorophyll in the extract (µg·mL^{-1}), "Volume" is the volume of solvent (mL) and "weight" is the weight of the biomass (g).

2.2.4. Optimisation of the cell disruption/solid–liquid extraction steps

The most promising solvent was selected. Several parameters were investigated to appraise the most appropriate conditions to improve the extraction yield: the molar fraction of the eutectic components (0.3 to 0.7, x_{Ment}), the SLR (0.05 to 0.3, mass of dry cells (in g) *per* volume of solvent (in mL)), the time of extraction (20 to 120, min), the varying amounts of water added to the system (water content, 10 to 40, %), and the temperature (25 to 45, °C). The agitation was kept constant at 1800 rpm.

2.2.5. Pigment Fractionation: ES-ES biphasic system

After optimising the solid–liquid extraction with the selected solvent to extract the pigments, a second step comprising a liquid – liquid extraction system was applied and obtained by adding and mixing the hydrophobic ES-based extract with a hydrophilic ES. The system was arbitrarily proposed at a ratio of 1:1 (in volume) of LevA:Ment-based extract to LevA:ChCl. The two phases were formed in an Eppendorf Centrifuge 5424 at 11000 × *g* for 10 min at 25 °C, and both phases were analysed.

2.2.6. Polishing of fucoxanthin-rich extract

The fucoxanthin-rich extract recovered from the biphasic system was further purified to fully separate the pigment from the ES. To this end, cold water was added to the phase at a ratio of 1:1 (in volume) of fucoxanthin-rich phase to water. A small "oil-type" phase was formed at the top with an evident yellowish colour, while a colourless aqueous phase was formed at the bottom.

Table 1
UHPLC-DAD mobile phase gradient elution condition.

Time (min)	Proportion of mobile phases
5	A: 60 %, B: 40 %, C: 0 %
10	A: 0 %, B: 100 %, C: 0 %
40	A: 0 %, B: 30 %, C: 70 %
46	A: 0 %, B: 0 %, C: 100 %
47	A: 0 %, B: 100 %, C: 0 %
48	A: 60 %, B: 40 %, C: 0 %
53	Stop

2.2.7. COSMO-RS

The compositions of two immiscible liquid phases, denoted as α and β, at thermodynamic equilibrium are governed by the following relationship ([43]):

$$x_i^{\alpha}\gamma_i^{\alpha} = x_i^{\beta}\gamma_i^{\beta} \tag{2}$$

where x_i^{α} and x_i^{β} represent the mole fractions of component i in the liquid phases α and β, respectively, while γ_i^{α} and γ_i^{β} are the corresponding activity coefficients. From Equation (2), the so-called thermodynamic partition coefficient (K_i) can be defined as ([43]):

$$K_i = \frac{x_i^{\alpha}}{x_i^{\beta}} = \frac{\gamma_i^{\beta}}{\gamma_i^{\alpha}} \tag{3}$$

The Conductor like Screening Model for Real Solvents (COSMO-RS) is a statistical thermodynamics model designed to predict the activity coefficients of components in complex liquid mixtures ([44–46]). To do so, COSMO-RS relies on sigma surfaces and profiles, which are obtained from quantum chemistry calculations and quantify the screened electrostatic potential of molecular surfaces. As such, the fully predictive COSMO-RS model can be integrated with Equations (2) and (3) to predict the liquid–liquid equilibrium (LLE) behaviour of the eutectic solvents studied, as well as partition coefficients for the solutes examined.

In this work, all quantum chemistry calculations were carried out using the software package TURBOMOLE ([47]). Specifically, sigma surfaces were obtained by performing geometry optimizations and single-point calculations employing density functional theory (DFT) with the def-TZVP basis set, the BP-86 functional, and the COSMO solvation environment (infinite permittivity). These sigma surfaces are depicted in Fig. 2. Following standard practices in the field, the geometry of choline chloride was optimized as an ionic pair, adopting a specific conformation known to yield accurate results in the prediction of phase equilibria behaviour for deep eutectic solvents ([48]). Finally, all COSMO-RS calculations were performed using the software package COSMOtherm ([49]) with the BP_TZVP_21 parametrization that is consistent with the DFT level of theory used.

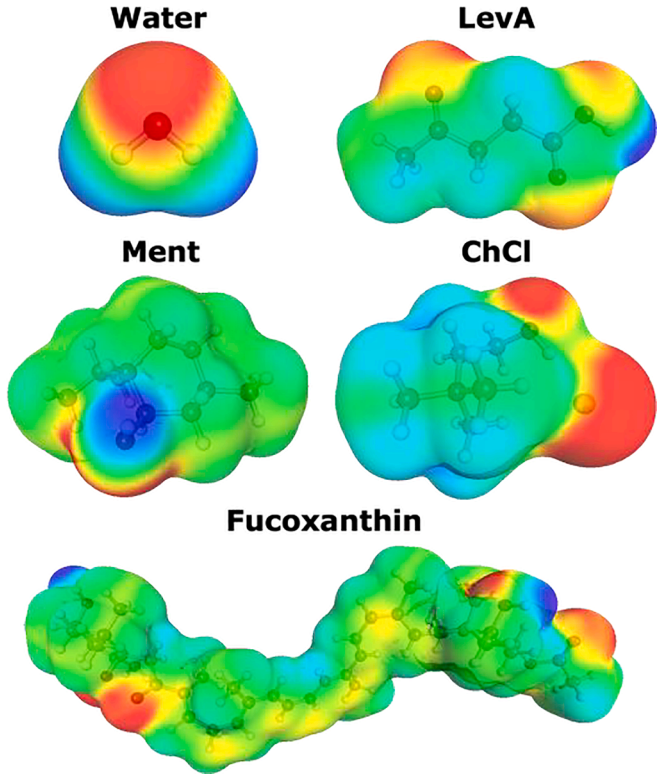

Fig. 2. Sigma surfaces of water, levulinic acid (LevA), menthol (Ment), choline chloride (ChCl), and fucoxanthin.

3. Results and discussion

3.1. Screening of eutectic solutions

The screening was performed using four Ment-based HES composed of the terpene and the bio-derived carboxylic acids AcA, LacA, DecA and LevA. The HES evaluated in this study were pre-selected based on our prior research [50], in which these solvents demonstrated the ability to extract xanthophylls. These molecules are not only biocompatible but also approved to be used in food and cosmetic formulations [51–54]. This not only simplifies the purification of the resulting extracts but may even eliminate the necessity for solvent removal. In this study, the term "hydrophobic" specifically refers to mixtures that manifest a biphasic separation in the presence of water. While a reduction in water solubility was noted for compounds when incorporated into HES [55], the smaller carboxylic acids are expected to have a significant partitioning to the aqueous phase. Thus, any specific Ment to carboxylic acid ratio mentioned in this study pertains to the HES composition before water addition.

An initial molar ratio of 1:1 of HBA:HBD was arbitrarily selected for an extraction screening as these mixtures were all previously reported to form liquid solutions at room temperature and this composition [55,56]. The different HES were applied to the extraction of pigments from dry *Saccharina latissima* both in the presence and absence of water and compared with methanol as the control. Fig. 3 depicts the yields of extraction of fucoxanthin ($\mu g_{fucoxanthin} \cdot g_{biomass}^{-1}$) and chlorophylls ($\mu g_{chlorophyll} \cdot g_{biomass}^{-1}$) in four HES systems at 0 % and 20 % of water added.

Examining the extraction yields of the various systems, none of the pure ES showed extraction ability for either pigment. The addition of water was crucial to initiate the extraction process. This may be due to different factors, such as: (i) the dispersion of the biomass in the solvent may be improved with water due to a decrease in viscosity; (ii) water may rehydrate dried algal cells, making their membranes more susceptible to rupture and facilitate the release of intracellular content; (iii) solubilisation of chlorophyll and fucoxanthin may be enhanced in a solvent system with dual characteristics – polar (from water) and non-polar (from HES), given the slight polarity of these pigments. Nevertheless, in the presence of water all systems displayed some extracting capability. Our emphasis was mainly placed on fucoxanthin due to its higher commercial value compared to chlorophylls. According to Fig. 3, Ment:DecA, rich in decanoic acid with its longer alkyl chain, performed slightly better than Ment:AcA. This behaviour is somewhat expected due to the tensioactive properties of DecA, aiding in the disruption of cell membranes and subsequently improving pigment extraction efficiency [37,57]. Interestingly, a similar extraction yield was obtained in the Ment:DecA and Ment:LacA. These three HES tested, invariably resulted in inferior yields compared to the control. However, the Ment:LevA system presented a remarkable yield of 80 $\mu g_{fucoxanthin} \cdot g_{biomass}^{-1}$ for fucoxanthin, not only matching but surpassing the methanol benchmark. This trend has been seen in previous works [50], suggesting a unique attribute or synergy inherent to levulinic acid that amplifies extraction efficiency. This highlights the potential of this system as an effective extractant. Regarding chlorophyll extraction, methanol outperformed all HES systems. Interestingly, a parallel trend to that observed with fucoxanthin extraction became evident, when moving from AcA to DecA, further stressing the influence of the carbon chain length in the process of cell lysis. Within the HES-based, the highest extraction yields obtained were with Ment:DecA and Ment:LevA at approximately 34 $\mu g_{chlorophyll} \cdot g_{biomass}^{-1}$. Due to its very efficient capacity at extracting pigments from the macroalga, the following work employed Ment:LevA (1:1) + 20 % (v/v) of water as the best solvent.

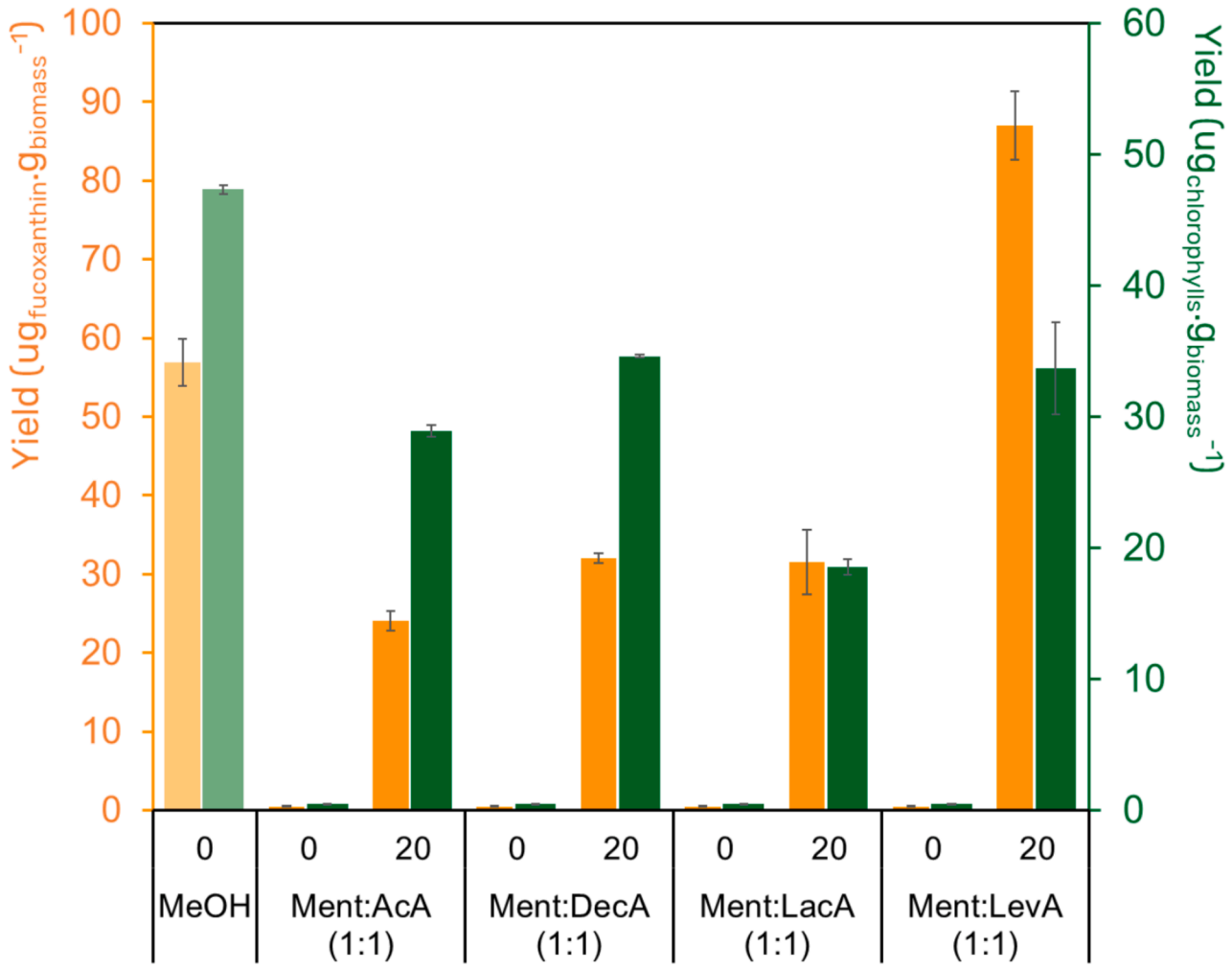

Fig. 3. Yields of extraction of fucoxanthin using HES as well as methanol, used as a control solvent. X-axis represents the percentage (0 and 20 %) of water added to each ES. The tests were done at 25 °C, under constant agitation at 1800 rpm, for 2 h, protected from light exposure, and at a fixed SLR of 0.05 mass of dry cells (in g) per volume of solvent (in mL).

3.2. Optimisation/evaluation of the solid–liquid extraction parameters

Given fucoxanthin's considerable market value (attributed to its unique biological activities) and its primary sourcing from brown seaweeds, emphasis should be placed on this pigment during the valorisation of such biomass [11,13,14,58]. As such, the optimisation of the extraction process was primarily focused on the fucoxanthin yield. A set of preliminary assays was conducted to understand the effects of specific parameters on the extraction of fucoxanthin, namely the Ment molar fraction (x_{Ment}), the SLR, the extraction time, the % (v/v) of water added, and the temperature of extraction. Unless otherwise specified, standard conditions of x_{Ment} = 0.5, SLR of 0.05, t = 120 min, water added = 20 % (v/v), and T = 25 °C were applied. Although the phase diagram of the eutectic mixture Ment:LevA was not explicitly measured, all the binary mixtures fell in the liquidus range at room temperature [50]. Fig. 4 depicts the obtained results, with the extracts quantified in terms of yield of extraction of fucoxanthin ($\mu g_{fucoxanthin} \cdot g^{-1}_{biomass}$). The x_{Ment} displayed a clear linear relationship, peaking at 0.3, suggesting that an increase in LevA promotes a more efficient extraction, which is consistent with experimental results previously obtained by some of us [50]. To understand better this behaviour, the molecular interactions of the system were analysed through COSMO-RS calculations where it was proved the positive effect of the presence of LevA on improving the extraction of fucoxanthin (more details in ESI).

Expectedly, the SLR was optimised at the lowest point of 0.05. Such a result underlines the idea that the extraction yield decreases linearly with the increase in SLR due to the potential solvent saturation and more inefficient mixing at higher loadings. As for the extraction time, there was a straightforward relationship: the longer the extraction process, the better the yield, up to the 120 min tested. One of the most intriguing findings was related to the volume of water added. As indicated before, the complete absence of extraction at 0 % (v/v) highlights the indispensable role of water in the process. While fucoxanthin may exhibit sensitivity to solvent purity, the linear decrease in extraction efficiency from 10 % (v/v) to 40 % (v/v) suggests an optimal balance between the solvent's polarity and its capacity to interact with the biomass and simultaneously solubilise and extract fucoxanthin, where minimal amounts are found to be the best. The temperature parameter offered little to no significant effect on extraction yields between 25 to 45 °C. The extraction process for fucoxanthin from *Saccharina latissima* is somewhat temperature agnostic, within this range. Such a characteristic is beneficial for broader applications, as strict temperature controls might not be a pressing concern. Conclusively, after finding the optimal operational conditions (0.3 x_{Ment}, SLR 0.05, 120 min, 10 % (v/v) of water added, and 25 °C), these were combined in a single extraction attempt, resulting in a robust yield of 137.2 ± 2.6 $\mu g_{fucoxanthin} \cdot g^{-1}_{biomass}$.

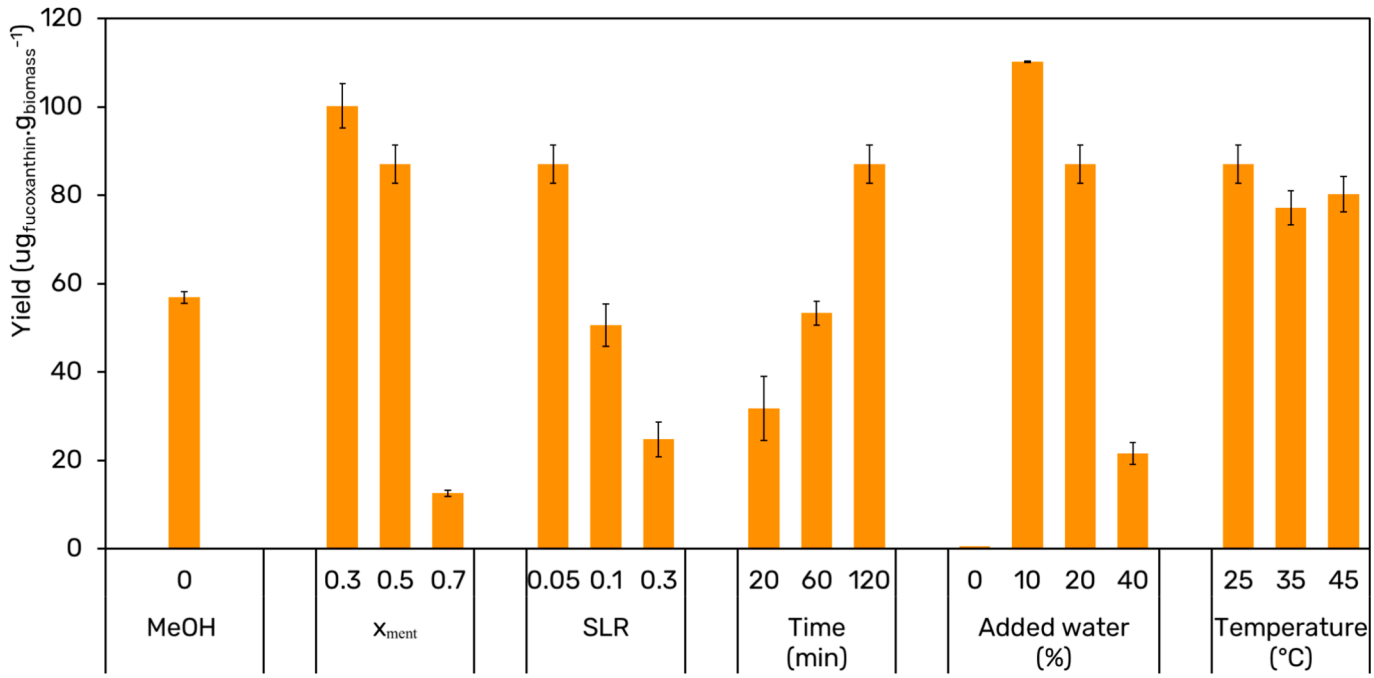

Fig. 4. Yields of extraction of fucoxanthin under varying conditions, namely: molar fraction of the eutectic components (x_{Ment}), SLR ($g_{biomass}{\cdot}mL^{-1}_{solvent}$), time of extraction (min), water content (% (v/v)) and temperature (°C).

3.3. Separation of fucoxanthin from chlorophylls by applying an ES-ES biphasic system

As previously mentioned, both fucoxanthin and chlorophyll were co-extracted from *Saccharina latissima* using the hydrophobic ES. This co-extraction is significant given the distinct properties and functionalities of each pigment. The hydrophobic nature of the selected eutectic solvents facilitated the simultaneous extraction of these two pigments, showcasing their versatility. While fucoxanthin is the primary target of this work, the concurrent extraction of chlorophyll emphasises the broad-spectrum efficacy of the solvent system. To achieve a selective and efficient separation of fucoxanthin and chlorophyll, an ES-based biphasic system was introduced. The initial hydrophobic extract, rich in Ment:LevA, was combined with a hydrophilic ES, namely ChCl:LevA (1:2). Upon mixing, these two eutectic solvents manifested a clear biphasic separation, and a yellowish bottom phase and a greenish top phase were formed. From the results already reported in literature, it seems possible that LevA migrates between the phases, with some loss to the bottom phase, creating new equilibrium conditions [50]. To understand exactly the equilibrium conditions of the ES-based biphasic system, COSMO-RS was applied. From this analysis, it was proved some migration of ChCl towards the hydrophobic phase and Ment to the hydrophilic phase (for more details about this evaluation lease check ESI).

The hydrophobic and hydrophilic phases in this biphasic system display differential affinities for the two pigments under focus. Different proportions of HES-based extract to hydrophilic ES were proposed for the systems (from 1:1 to 1:6 of each ES) to evaluate the partitioning of each pigment between the phases. The efficiency of this separation was

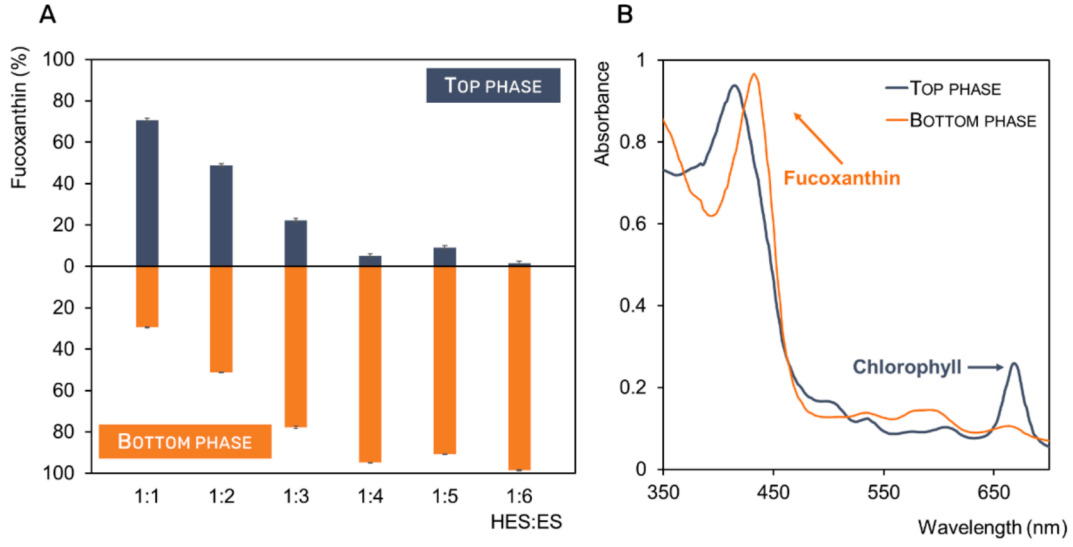

Fig. 5. Liquid – liquid extraction systems composed of the extract obtained using the Ment:LevA-rich extract and the ChCl:LevA (1:2): **(A)** partitioning of fucoxanthin within the system at different ratios of hydrophobic ES (Ment:LevA) to hydrophilic ES (ChCl:LevA); **(B)** UV – vis spectra of top and bottom phases of the system.

quantitatively analysed by monitoring the concentrations of fucoxanthin and chlorophylls in each phase. Notably, chlorophylls fully remained in the more hydrophobic phase (originated from Ment:LevA) in all systems. This can be attributed to the higher hydrophobicity of chlorophyll (LogP = 11.95) [59] compared with fucoxanthin (LogP = 6.83) [59]. On the other hand, fucoxanthin exhibited a strong propensity to migrate towards the more hydrophilic phase (ChCl:LevA-rich), which can be seen in Fig. 5. Given the slight polarity of fucoxanthin, the hydrophilic ES likely offers a more favourable environment, possibly through polar interactions or hydrogen bonding, facilitating the transfer. An aqueous solution of ChCl at the same proportion of the hydrophilic ES was tested to further understand this partitioning behaviour. It was observed that, unlike the ES-ES system, the system composed of HES-rich extract and ChCl aqueous solution showed no significant partition of fucoxanthin to the hydrophilic phase. Fig. 6 provides a direct visual comparison between the partitioning behaviour of fucoxanthin in both the water-based and ES-based systems. This suggests that the presence of levulinic acid in the ES might play a crucial role in adjusting the phase polarity to a degree that is more favourable for fucoxanthin partitioning. Moreover, the efficiency of this partitioning was closely tied to the ratio of Ment:LevA to ChCl:LevA. Initial trials employing a 1:1 ratio of each ES revealed that most fucoxanthin (around 70 %) remained within the Ment:LevA-rich phase, with only around 30 % migrating to the hydrophilic bottom phase. However, with the ES ratio transitioning towards 1:6 in favour of ChCl:LevA, a significant increase in fucoxanthin migration was observed. This trend showcased a clear linearity, with the concentration increment of ChCl:LevA positively influencing the fucoxanthin's partitioning towards the hydrophilic phase. This trend plateaued at a ratio of 1:4, where 95 % of fucoxanthin migrated to the bottom phase (Fig. 5**A**). Furthermore, the selectivity of the ChCl:LevA-rich phase for fucoxanthin over chlorophylls emphasises its significant potential as a separating agent in this biphasic system. The findings revealed that most fucoxanthin migrated to the hydrophilic phase, whereas chlorophylls were non-detectable (Fig. 5**B**).

This ES-ES biphasic system not only signifies an advance in the selective separation of pigments but also underscores the potential ES have in green and sustainable extraction and purification processes. The chlorophyll-rich extract present in the Ment:LevA-rich phase, presents a distinct advantage. Ment-based HES have been previously identified as suitable for direct dermatological use owing to their negligible cytotoxicity and inherent antibacterial properties [53]. This allows the extract to be directly applied in cosmetic or food applications without solvent removal. Consequently, the recovered chlorophyll can be seamlessly integrated into various product formulations, providing immediate applicability and value. On the other hand, though the fucoxanthin was successfully purified within the ChCl-based ES phase, its use in some applications, namely cosmetics, might be constrained by recent legislative changes pertaining to ChCl [60]. Considering this, further separation of the pigment from the solvent was applied to ensure compliance and broader applicability. The desired end-use will influence the extent of this additional step, reinforcing the versatility of our extraction approach.

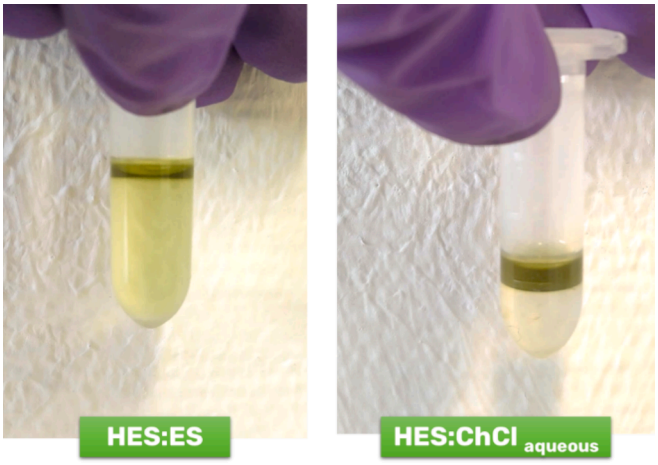

Fig. 6. Pigment partition in the ES-ES system at a ratio of 1:1 (v/v) of each solvent (left), and ES-ChCl aqueous solution at a ratio of 1:1 (v/v) of each solvent (right).

3.4. Separation of fucoxanthin from the solvent

The successful extraction and preferential partitioning of fucoxanthin to the most hydrophilic ChCl:LevA-rich phase sets the way for a subsequent critical step, its separation from the solvent. Given the high commercial value of fucoxanthin, particularly in cosmetics [61,62], and the stringent regulatory landscape exemplified by the European Union's Regulation (EC) No 1223/2009 [60], which bans the use of ChCl in cosmetic formulations, it is critical to isolate fucoxanthin from the ES. To this end, water was intended to be used as anti-solvent [63]. Upon mixing the fucoxanthin-rich ChCl:LevA extract with water at a 1:1 ratio (v/v), the hydrophilic ES was fully mixed with water, expelling fucoxanthin. The absence of fucoxanthin in the water phase aligns with its known insolubility in water. Contrary to the expected precipitation of fucoxanthin, a distinctly pigmented and concentrated top phase emerged. This observation is consistent with findings from another study [64], where antisolvent dilution led to the formation of a pigmented top phase rather than straight carotenoid precipitation, indicating the existence of a concentrated carotenoid top layer. The "oily" layer in the sample displayed a significant lipidic content, suggesting that the carotenoids were suspended in a lipid solution, preventing their precipitation [64]. Similarly, in our case, the emergence of this pigmented top phase could potentially be attributed to the co-extraction of lipidic compounds along with fucoxanthin during the extraction process. Given the amphiphilic nature of many lipids, they could entrap or solubilise the fucoxanthin, thus leading to the formation of a concentrated top "oily" phase. The exact mechanisms and interplays will be a subject for future evaluation and consideration. Nevertheless, the outcomes from the water-based polishing step illustrate the nuanced and multifaceted challenges associated with pigment purification using eutectic solvents.

3.5. Process design

The process described in this work outlines an environmentally friendly approach for extracting and purifying fucoxanthin and chlorophylls from the brown macroalgae *Saccharina latissima*. A final diagram of the process developed in this work is proposed (Fig. 7), in which all steps are considered. A mixture of a Ment-based hydrophobic ES and water was used to efficiently co-extract both pigments to the ES-rich phase. The subsequent introduction of a hydrophilic ChCl-based ES to this extract induced the formation of a distinct ES-ES biphasic system. Within this system, chlorophyll remains in the Ment-rich hydrophobic phase, thus being ready for further use in cosmetic applications, for example. Fucoxanthin migrates to the ChCl-based hydrophilic phase being submitted to further refinement due to possible legal constraints related with ChCl. By introducing water to this hydrophilic extract, a distinct phase partitioning occurs. Water seamlessly dissolves the ES, leading to the appearance of a distinct top layer, abundant in fucoxanthin and free from the eutectic solvent, which is then ready for further applications.

4. Conclusions

This work offers an innovative approach to the extraction and purification of fucoxanthin and chlorophylls from brown seaweed using ES. Of the four hydrophobic ES screened in the presence and absence of

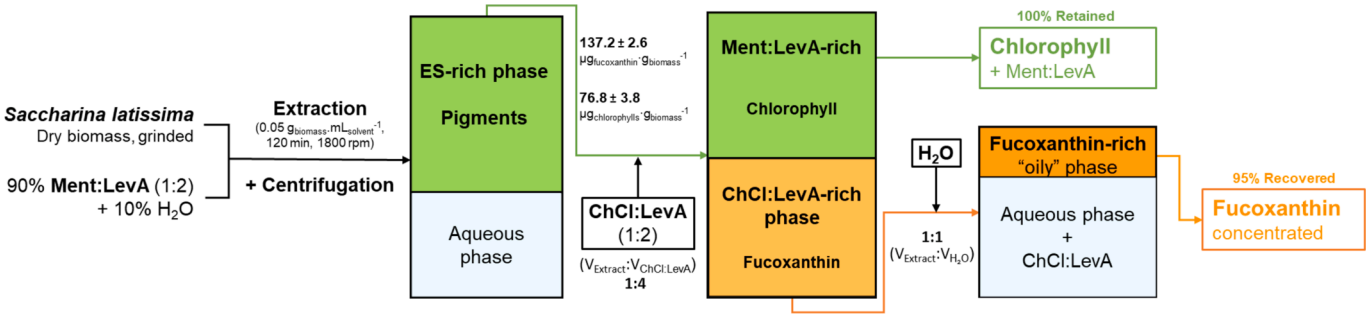

Fig. 7. Schematic representation of the integrated process for the recovery of fucoxanthin and chlorophylls from *Saccharina latissima*.

water, Ment:LevA system, enriched with 20 % (v/v) of water, yielded the best extraction yield result for fucoxanthin. The extraction parameters were then evaluated, and an optimised extract was obtained leading to yields of extraction of fucoxanthin and chlorophyll of 137.2 ± 2.6 $\mu g_{fucoxanthin} \cdot g_{biomass}^{-1}$ and 76.8 ± 3.8 $\mu g_{chlorophyll} \cdot g_{biomass}^{-1}$, respectively. The highly selective separation of the pigments was proposed through an ES-ES biphasic system. The achieved separation allowed chlorophylls to remain in the hydrophobic Ment-based phase, directly suited for various applications, while fucoxanthin underwent further refinement from its ChCl-rich phase. The biphasic separation strategy was both effective and efficient, with 95 % of the fucoxanthin in the system migrating to the hydrophilic phase and chlorophyll retention in the hydrophobic phase being noteworthy at 100 %. For the final polishing of fucoxanthin, water was applied as anti-solvent, however, instead of precipitating the fucoxanthin, it resulted in the appearance of a highly concentrated pigment "oily" phase. In the end, this work provides an optimised, efficient and more sustainable process to recover and separate the hydrophobic pigments from *Saccharina latissima*.

Declaration of competing interest

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

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.seppur.2024.130053.

Data availability

Data will be made available on request.

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