ACS Sustainable Chemistry & Engineering

pubs.acs.org/journal/ascecg

Research Article

Open Access

This article is licensed under CC-BY 4.0

Unveiling the Use of Hydrophobic Eutectic Solutions as Task-Specific Solvents To Recover Bacterioruberin from *Haloferax mediterranei*

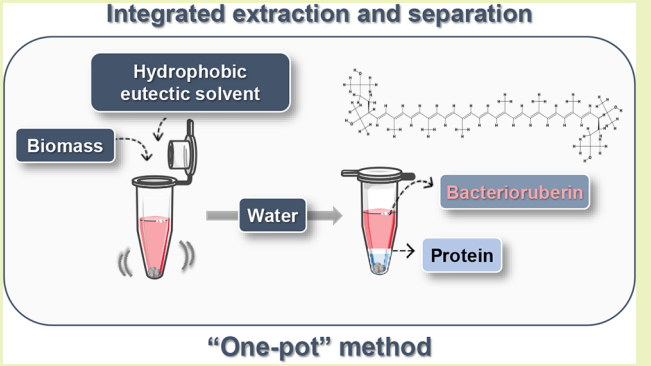
Mariam Kholany, Nicolas Schaeffer, Inês P. E. Macário, Telma Veloso, Tânia Caetano, Joana L. Pereira, Ana C. R. V. Dias, João A. P. Coutinho, and Sónia P. M. Ventura*

Cite This: *ACS Sustainable Chem. Eng.* 2023, 11, 13594−13605

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Haloarchaea make up a class of untapped marine microbial resources that constitute a promising source of valuable compounds with unique characteristics. Bacterioruberin is a C50 carotenoid produced by haloarchaea that possesses interesting antioxidant properties and bioactivities relevant to the food and cosmetic industries. As a substitute for the conventional organic solvents used in the extraction of nonpolar pigments, natural and biocompatible hydrophobic eutectic solvents (HES) based on the mixture of menthol with carboxylic acids were investigated for the extraction of bacterioruberin. Seven HES systems were screened both as neat solvents and in the presence of water. The menthol and levulinic acid mixture displayed a 4-fold improvement over both the ethanol control and the other HES, due to the dual action of its components. Additionally, the recovery of proteins could be achieved by the addition of water to the extract, resulting in a three-phase partition system and the formation of a protein-rich interfacial precipitate. The process intensification was assessed through the reuse of the eutectic phase over five successive extraction cycles, achieving a bacterioruberin-rich extract of 2.13 $mg_{bacterioruberin}$ mL_{HES}^{-1}. Finally, the carbon footprint of the process was determined. The results highlight the potential of HES as biocompatible solvents for the recovery of value-added compounds from marine biomass, while the use of three-phase partition allows the recovery of proteins producing a second product stream.



KEYWORDS: *Archaea, hydrophobic eutectic solvents, three-phase partition, bacterioruberin recovery*

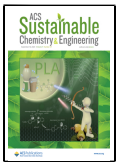
Downloaded via 213.13.252.251 on November 9, 2023 at 23:51:03 (UTC).
See https://pubs.acs.org/sharingguidelines for options on how to legitimately share published articles.

1. INTRODUCTION

Biotechnology, which harnesses the power of biological systems for diverse applications such as healthcare products and therapies, is becoming one of the most important high-tech industrial sectors with an estimated market size of US $1.02 trillion in 2021 and a projected annual growth rate of 13.9% from 2022 to 2030.[1] Fermentative production is expected to make up ∼10%−15% of the biotechnology sector. However, compared to the mature use of bacteria, fungal, or algae as cellular factories to produce value-added compounds, archaea have received little attention, despite their potential and advantages in a blue biotechnology context. The extremophile nature of many archaea microorganisms allows them to be readily cultivated under nonsterile and open-air conditions using inexpensive feedstocks often toxic to other microorganisms, thereby simplifying the cultivation process and its associated operating cost.[2]

Halobacteria, also known as haloarchaea, represent a class of euryarchaeota found worldwide in hypersaline environments (salinity of >15% (w/v) for optimal growth). These underexplored halophilic microorganisms are natural producers of several relevant high-demand products, including proteins, poly(3-hydroxybutyrate), polyhydroxyalkanoates (PHAs), and carotenoids.[3] Notably, most halophilic archaea produce bacterioruberin, a C50 carotenoid, in contrast to the C40 carotenoids identified from most natural sources such as bacteria, archaea, algae, fungi and plants.[4,5] Bacterioruberin contains 13 conjugated double bonds and 4 hydroxyl groups versus the 9 pairs of conjugated double bonds found in the C40 β-carotene, making bacterioruberin a better radical scavenger than β-carotene. It provides a higher protection against intensive light, gamma irradiation, and DNA damaging agents such as radiography, ultraviolet (UV) irradiation, and

Received: May 19, 2023
Revised: August 24, 2023
Published: September 8, 2023



© 2023 The Authors. Published by American Chemical Society

H_2O_2 exposure.[6,7] These characteristics award bacterioruberin interest in novel applications in the food, cosmetic, and pharmaceutical sectors.

The ambition of modern biorefineries is the creation of integrative separation platforms based on wastes with low environmental impact for the development of a range of products covering the full value chain to offset its operational costs.[8] This is, of course, easier said than done, considering the natural complexity of cellular factories and the variety of potential product streams including carbohydrates, lipids, fatty acids, proteins, vitamins, phenolics, flavonoids, chlorophylls, and carotenoids.[9] It is further worsened by the resource intensive nature of common separation processes, which account for an estimated 10%−15% of the world's energy consumption,[10] restricting the economic viability of smaller-scale biorefineries. An alternative solution to bypass the need for costly purification and solvent recovery steps is the direct inclusion of the biomass extract in formulations due to the enhancement in biological activity and bioavailability conferred by the solvent.[11,12] In this context, an ideal solvent must be compatible with food and cosmetic applications (low toxicity), present a limited environmental impact (biodegradable), obtained from renewable sources (nonpetroleum derived), and exhibit a high dissolving power and selectivity for the target compounds.[8] Unfortunately, carotenoids are conventionally extracted using organic solvents that do not meet the aforementioned characteristics, with hexane, acetone, and 4:3 ethanol:hexane mixtures being the most commonly used solvents for plant extracts.[13]

Eutectic solvents have emerged as promising alternative solvents for the valorization of biomass, due to their high solubilization power and stabilization ability, relative to volatile organic solvents.[11,12,14,15] Eutectic mixtures composed of a hydrogen bond donor (HBD) and acceptor (HBA) present interesting properties due to the enhanced liquefaction and solubility provided by the decrease of the melting point of the mixture relative to an ideal mixture and the pure constituents.[16,17] As for any mixture, the properties of the resulting solvent, including its cost, toxicity, and biodegradability, depend on the appropriate selection of the HBA and HBD precursors.[18,19] Furthermore, the nature of the eutectic components allows tuning the resulting physicochemical properties such as the polarity, surface tension, density, and viscosity (just to name a few), potentially providing a more targeted and selective extraction. The tunability of eutectic solvents, afforded by the simple manipulation of its HBD and HBA selection and their mutual molar ratio, has seen their widespread application for the recovery of numerous biomolecules from typically recalcitrant biomass.[20]

In this work, the recovery of bacterioruberin from the archaea *Haloferax mediterranei* was investigated using hydrophobic eutectic solvents (HES) in line with the lipophilicity of the C50 pigment. Guided by the ideal solvent requirements detailed above, mixtures of menthol (Ment) with various carboxylic acids were identified as promising HES for the one-pot extraction and purification of bacterioruberin. Ment-based HES were previously identified as suitable for direct dermatological use due to its negligible cytotoxicity and antibacterial action.[21] Mixtures of Ment with short-chained carboxylic acids could extract with comparable or better yields than volatile organic solvents a range of hydrophobic metabolites from *Arthrospira platensis*,[21] phytocannabinoids from cannabis plant material,[22] or phenolic compounds and flavonoids from *Ginko biloba* leaves.[23,24] Each HES component provides a specific contribution, with Ment shown to stabilize the more hydrophobic metabolite by dispersive interactions[23] while simple carboxylic acids are reported as permeation enhancers capable of destabilizing lignocellulosic membrane through hydrogen bonding.[25,26] Furthermore, Ment-based HES was already tested being combined with advanced separation techniques such as centrifugal partition chromatography for the separation of several hydrophobic analytes.[27]

Following an initial HES screening to identify the most promising HES combination, the extraction conditions were refined using a response surface methodology, considering both the bacterioruberin extraction yield and its stability. Finally, the extract was purified through the addition of water as a counter-solvent to yield a three-phase partition (TPP) system with a protein-rich interfacial precipitate. TPP is commonly applied for the precipitation of enzymes and proteins from aqueous solutions.[28,29] Usually, this is achieved by the precipitation of target proteins at the interface of an organic liquid phase, typically rich in aliphatic alcohols such as *tert*-butanol, and an aqueous solution of salt. The third phase formation has been explained by multiple effects including salting-out, alcohol-induced cosolvent precipitation, and osmolytic and kosmotropic precipitation of proteins.[30] In this case, Ment in the HES phase acts as the alcohol source, permitting the "one-pot" recovery of bacterioruberin and the protein fraction with greater yield than the ethanol control and providing a simple and transferable approach for the valorization of a blue biorefinery platform. In the end, the carbon footprint of the process was evaluated.

2. EXPERIMENTAL SECTION

2.1. Material. *2.1.1. Chemicals.* In this work, we studied eutectic solutions composed of menthol and different carboxylic acids to extract bacterioruberin. The HES were prepared by mixing Ment (purity, 99.5%) with different carboxylic acids, namely, acetic acid (purity, >99.9%) from Honeywell, decanoic acid (purity, 99%), and lactic acid (purity, 85%) from Acros Organics, and butyric acid (purity, PA) and levulinic acid (LevA) (purity, 98%) from Sigma−Aldrich. Ethanol absolute (analytical reagent grade) used in solid−liquid extraction was acquired from Fisher Scientific. For *H. mediterranei* culturing, yeast extract was acquired from Organotechnie; peptone from meat from Alfa Aeser; casamino acids from U.S. Biological, Life Sciences; and NaCl from J. T. Baker; $MgSO_4{\cdot}7H_2O$, $CaCl_2$, and KOH were obtained from VWR Chemicals, $MgCl_2{\cdot}6H_2O$ and Tris-HCl were from Biochem Chemopharma, and KCl was from J.M.G.S. For the SDS-PAGE analysis, glycerol (purity, 99.9%) was acquired from Fisher Chemical, bromophenol blue sodium salt from Merck, dithiothreitol (DTT, purity, 98%) from NZYtech, and tris(hydroxymethyl)-aminomethane (purity, 99%) from Alfa Aesar. RunBlue Teo 20x Teo Tricine SDS and RunBlue SDS gel 4−12% 10 cm × 10 cm were supplied by Expedeon. BlueSafe used to stain the proteins and NZYColour Protein Marker I were acquired from NZYtech.

2.1.2. Archaea Cultivation. Cell cultivation was accomplished following a protocol previously developed by us.[31] Briefly, *Haloferax mediterranei* ATCC 33500 was cultivated under controlled conditions aiming at maximum bacterioruberin yield, in YPC-Hv (yeast, peptone, and casamino acids media) culture medium: peptone of meat, 0.1% (w/v); casamino acids, 0.1% (w/v); yeast extract, 0.5% (w/v); NaCl, 14.4% (w/v); $MgSO_4{\cdot}7H_2O$, 2.1; $MgCl_2{\cdot}6H_2O$, 1.8% (w/v); KCl, 0.42% (w/v); Tris-HCl, 12 mM (pH 7.5); KOH, 1 M; and 0.5 M $CaCl_2$.[32] The medium was sterilized at 121 °C prior to archaea culturing. A single colony was selected from the agar plate (YPC-Hv broth with 1.5% agar) and inoculated into 25 mL of YPC-Hv broth in a 100 mL Erlenmeyer flask and cultured at 37 °C, 180 rpm, under

https://doi.org/10.1021/acssuschemeng.3c02997
ACS Sustainable Chem. Eng. 2023, 11, 13594−13605

continuous light (4000 lux) for 72 h. To increase the cell mass concentration, 20 mL of this preinoculum were resuspended in 400 mL of fresh broth in a 1 L Erlenmeyer flask and incubated under the same conditions (i.e., 37 °C, 180 rpm, 4000 lux) for 96 h. Archaea growth was monitored by the optical density determined at 600 nm. At the end of the cultivation period, the biomass was centrifuged in a Thermo Scientific Heraeus Megafuge 16R centrifuge at 18894*g* for 15 min at room temperature (20−25 °C). The supernatant was discarded, and the pellet was stored in darkness at −20 °C until use.

2.2. Methods. *2.2.1. HES Preparation.* The HES was prepared by mixing Ment with different carboxylic acids at the desired molar ratio. All chemicals were dried under vacuum for 24 h prior to the preparation of the eutectics. All systems were prepared gravimetrically using an analytic balance Mettler Toledo XP205 scale (with an uncertainty of $\pm 10^{-4}$ g). These mixtures were placed in sealed glass vials with constant stirring and heated at 5 °C above the melting point of Ment (42.1 °C) for 1 h until a homogeneous transparent liquid was obtained. Following heating, the mixture was allowed to return to room temperature and stored in sealed vials until use to prevent any water absorption from the atmosphere until use. In addition, the operation liquidus range at 25 °C of the Ment:LevA system was evaluated by preparing mixtures encompassing the full composition range, x_{Ment} from 0.1 to 0.9 in 0.1 intervals.

2.2.2. Cell Disruption and Solid−Liquid Extraction. The cell disruption and solid−liquid extraction steps were performed at the same time. The extractions were performed at a fixed temperature of 25 °C under constant agitation (1800 rpm), protected from light exposure using Eppendorf Thermomixer Comfort equipment. A fixed time of 60 min and solid−liquid ratio (SLR) of 0.1 (mass of wet cells (in g) per volume of solvent (in mL) were established for all extractions. The screening was performed using five hydrophobic Ment-based HES composed of the terpene and bioderived carboxylic acids, namely, acetic acid (AcA), butyric acid (ButA), decanoic acid (DecA), lactic acid (LA), and levulinic acid (LevA). The different HES were screened at an initial molar ratio of 1:1 Ment to carboxylic acid to evaluate their capacity to release bacterioruberin from the biomass both as a pure solvent and as an emulsion with the addition of controlled volumes of water. Additionally, bacterioruberin extraction was also studied using aqueous solutions with carboxylic acids used as HES hydrogen bond donors for comparison. Finally, a pure ethanol control extraction was also carried out. After the extraction, the samples were centrifuged (16200*g*, 10 min) in a Micro Star 17 centrifuge (VWR), at room temperature (20−25 °C). The supernatant fraction was recovered and analyzed, and the biomass debris was discarded. In the case of biphasic HES+H_2O systems, only the HES phase was analyzed due to the complete partition of bacterioruberin to the latter and the results corrected for the change in phase volume. All of the assays were performed in duplicate.

The quantification of bacterioruberin was determined using a UV−vis microplate reader (Synergy HT microplate reader-BioTek). The absorption spectra of the analyzed extracts were measured between 350 and 700 nm, and the bacterioruberin content was determined by eq 1 using calibration curves at the maximum peak of absorbance observed, 494 nm, for both ethanol- and eutectic-based solutions. The bacterioruberin standard, used to determine the calibration curves, was obtained by preparative thin-layer chromatography (TLC), as previously reported by us.[31]

$$\text{yield of extraction}\left(\text{mg}_{\text{bacterioruberin}}\,\text{g}_{\text{wet biomass}}^{-1}\right) = \frac{[\text{bacterioruberin}] \times \text{volume}}{\text{weight}} \quad (1)$$

Here "[Bacterioruberin]" corresponds to the concentration of bacterioruberin in the extract (mg mL^{-1}), "volume" is the volume of solvent (mL), and "weight" is the weight of the wet cells tested (g).

The residual water content in the biomass was determined by freeze-drying. A fixed amount of biomass was weighed, and the sample was freeze-dried and weighed again. The amount of water present was calculated by a mass balance.

2.2.3. Optimization of the Cell Disruption/Solid−Liquid Extraction Steps. The most promising solvent, meaning the solvent with the highest extraction yield for bacterioruberin, was selected. The following conditions were investigated to appraise the most appropriate range of each parameter to improve the extraction yield: eutectic composition (0.2−0.7 x_{Ment}), SLR (from 0.1 to 0.25), and extraction time (from 20 min to 60 min). At this point, the stability of the extract was followed over 8 h to rule out any conditions liable to induce rapid chemical alterations of bacterioruberin.

A response surface methodology (RSM) was then applied to simultaneously analyze different variables and to identify the most significant parameters and their interaction, aiming at finding the optimum conditions to maximize the yield of bacterioruberin extraction. The optimization of the process was achieved by applying a central composite rotatable design (CCRD-2^3), totalling 20 extractions with 4 replicates at the central point. The independent variables considered were the time of extraction (t, min), the molar fraction of the eutectic components (x_{Ment}), and the SLR, with the dependent variable being the yield of extraction of bacterioruberin (yield of extraction, $\text{mg}_{\text{bacterioruberin}}\,\text{g}_{\text{wet biomass}}^{-1}$). The temperature and agitation were kept constant as described for the screening of solvents, i.e., 25 °C and 1800 rpm, respectively. The results were statistically analyzed with a 95% confidence level. The Statistica 7 software was used for all statistical analyses and to represent the response surfaces and contour plots. The real values are presented in Table S1 in the Electronic Supporting Information (ESI). Finally, the optimum conditions determined were validated in triplicate using the means of relative deviation (%).

2.2.4. Protein-Induced Precipitation. Following extraction, protein precipitation was achieved by adding varying amounts of water as a counter-solvent to the initial extract (obtained from the solid−liquid extraction step). The samples were centrifuged using a Thermo Scientific Heraeus Megafuge 16R centrifuge at 4700*g* for 10 min, resulting in three different fractions: a solid interphase and two liquid phases (eutectic-rich and water-rich). After removal of the HES phase, the solid fraction from the optimized precipitation was manually removed with a spatula and rinsed with ethanol to remove the excess HES. This interfacial precipitate was then resuspended in 1 mL of PBS (1×, pH 7.4) for further analysis. The quantification of the protein was evaluated by UV−vis using the Pierce BCA Protein Assay Kit. The concentrations were calculated by using a calibration curve previously determined. The protein yield of extraction and the protein recovery, respectively, were determined according to eqs 2 and 3:

$$\text{yield of extraction}\left(\text{mg}_{\text{protein}}\,\text{g}_{\text{wet biomass}}^{-1}\right) = \frac{[\text{protein}]_{\text{phase}} \times \text{volume}}{\text{weight}} \quad (2)$$

$$\text{recovery}\,(\%) = \frac{[\text{protein}]_{\text{solid interface}}}{[\text{protein}]_{\text{solid interface}} + [\text{protein}]_{\text{aqueous phase}}} \times 100 \quad (3)$$

where "$[\text{Protein}]_{\text{phase}}$" corresponds to the concentration of protein in the solid phase or the aqueous phase (mg mL^{-1}), "volume" is the volume of solvent (mL), and "weight" is the weight of the wet cells tested (g).

This fraction was further evaluated by SDS-PAGE following the procedure of Laemmli.[33] The sample was diluted (1:1) in loading buffer (4% (w/v) of SDS, 20% (w/v) of glycerol, 0.5 mM of Tris-HCl pH 6.8, 0.02% (w/v) Bromophenol Blue, 3.1% (w/v) of DTT), and then incubated for 5 min at 95 °C for protein denaturation. The samples were then loaded onto the polyacrylamide gel with the molecular weight marker (NZYColour Protein Marker I) and left to run for 2 h at 110 V. Finally, to stain the proteins, an incubation with BlueSafe was performed under mild agitation for 3 h.

2.2.5. Solvent Characterization. To infer about the integrity of the Ment:LevA components in the HES in the presence of a secondary aqueous phase and over repeated extraction cycles, ^{1}H nuclear magnetic resonance (NMR) spectroscopy was carried out. The

https://doi.org/10.1021/acssuschemeng.3c02997
ACS Sustainable Chem. Eng. 2023, 11, 13594−13605

spectra were recorded using a Bruker Avance 300 spectrometer (Bruker Corporation, Billerica, MA, USA) operating at 300.13 MHz for ^{1}H NMR, and at 75.47 MHz for ^{13}C NMR. The HES phases were dissolved in DMSO. The HES was further analyzed by Fourier transform infrared−attenuated total reflectance (FTIR-ATR) spectroscopy. The spectra of Ment, LevA and the Ment:LevA (1:1) were obtained on a PerkinElmer spectrometer (PerkinElmer Inc., Waltham, MA, USA) equipped with a single horizontal Golden Gate ATR cell and a diamond crystal. The data were recorded by the accumulation of 32 scans performed at room temperature in the range of 4000−400 cm^{-1}, with a resolution of 4 cm^{-1} and an interval of 1 cm^{-1}. All spectra were subtracted against background air spectrum and recorded in transmittance mode.

2.2.6. Carbon Footprint. The carbon footprint of the process proposed for the recovery of bacterioruberin using the most promising HES (Ment:LevA) was calculated based on ISO 14067.[34] The carbon footprint is the sum of greenhouse gas emissions, expressed as carbon dioxide equivalents (CO_2 equiv), associated with the production of the energy, water, and reagents consumed in the steps of HES preparation, cell disruption and solid−liquid extraction, and protein-induced precipitation (Table 1). The consumption of electricity was estimated based on the power of the equipment used, the time of operation, and the ratio between the capacity occupied by the samples and the total capacity of the equipment. Data on the carbon footprint of the production of electricity (Portuguese mix), water, and Ment were taken from the Ecoinvent 3.7.1 database.[35] In the absence of data specific to Ment, a dataset to produce organic chemicals was used instead. Data on the carbon footprint of levulinic acid was retrieved from ref 36. The conversion of the emissions of individual greenhouse gases into CO_2 equiv was performed using the global warming potentials for a time horizon of 100 years from ref 37.

Table 1. Consumption of Energy, Water, and Reagents To Recover Bacterioruberin from 1 g of Biomass

step	units	amount
HES Preparation		
menthol	g	5.35
levulinic acid	g	3.98
electricity	W h	22.92
Cell Disruption and Solid−liquid Extraction		
biomass	g	1
Ment:LevA[a]	g	9.33
electricity	W h	9.31
Protein-Induced Precipitation		
water	g	8.40
electricity	W h	12.46

[a]Produced in the step of HES preparation.

RESULTS AND DISCUSSION

Screening of Eutectic Solutions. The screening was performed using five hydrophobic Ment-based HES composed of the terpene and bioderived carboxylic acids, specifically AcA, ButA, DecA, LA, and LevA. A summary of the explored systems and their extraction capacities is presented in Table 2. These compounds were selected due to their biocompatibility and existing food and cosmetic applications, with the final aim of producing an extract suitable for direct application without the need for solvent removal. Furthermore, the carboxylic acids were selected to assess the influence of structural parameters on the extraction including the alkyl chain length going from AcA to DecA and the presence of additional hydroxyl and ketone functional groups in LA and LevA, respectively. It is important to note that the term "hydrophobic" is here employed to denote that these mixtures form a biphasic system in the presence of water. This is a practical simplification reflecting the system's macroscopic behavior given the differing aqueous solubilities of the HES components. Although a synergistic decrease in water solubility was observed for compounds upon their inclusion in HES,[38] a significant aqueous phase partition of the smaller carboxylic acid can be expected.[39] As such, any specific Ment-to-HBD ratio discussed in this work refers to the HES composition prior to the addition of water.

Table 2. Summary of Screened HES and Their Capacity To Extract Bacterioruberin from *H. mediterranei* in the Absence (χ) and Presence ($\surd$) of Added Water (10, 25, and 50 vol %)

HES(1:1)	without water	with water
Ment:AcA	χ	$\surd$
Ment:ButA	χ	$\surd$
Ment:DecA	χ	$\surd$
Ment:LA	χ	$\surd$
Ment:LevA	$\surd$	$\surd$
Ment:Thymol	χ	χ
water	χ	

An initial Ment-to-carboxylic acid molar ratio of 1:1 was arbitrarily selected for an extraction screening as these mixtures were all previously reported to form liquid solutions at room temperature and this composition.[38,39] The different HES were applied to the extraction of bacterioruberin from *H. mediterranei* and compared with ethanol as the control in terms of the bacterioruberin extraction yield. Due to the halophilic nature of *H. mediterranei*, the addition of deionized water (from 0% to 50% of the final volume) was considered in the first step of extraction, to promote the cell rupture by osmotic pressure.[2] Importantly, control extraction assays using deionized water resulted in complete cell lysis and the formation of a viscous gel-like phase, which prevented any bacterioruberin recovery and quantification. Figure 1 shows the yields of bacterioruberin extraction ($mg_{bacterioruberin}\ g_{wet\ biomass}^{-1}$) in four HES systems as a function of the final added water percentage. The Ment:DecA system did not display any measurable extracting capability in the presence or

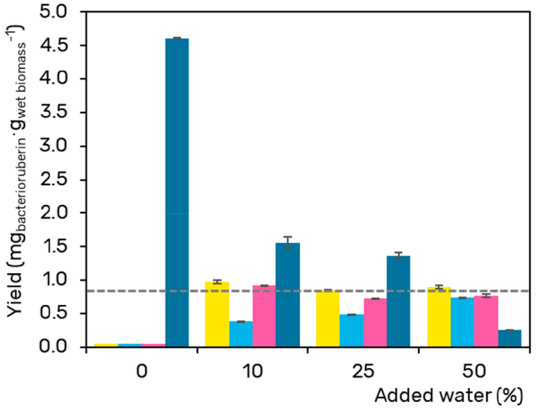


Figure 1. Bacterioruberin yield of extraction using HES as well as ethanol, used as a control solvent at 25 °C under a constant agitation at 1800 rpm, for 1 h, protected from light exposure, and at a fixed SLR of 0.1: menthol:acetic acid (yellow), menthol:butyric acid (teal), menthol:lactic acid (mauve), menthol:levulinic acid (blue-gray), and ethanol (dotted line (---))).

https://doi.org/10.1021/acssuschemeng.3c02997
ACS Sustainable Chem. Eng. 2023, 11, 13594−13605

absence of added water, and, as such, it is not presented in Figure 1. By analyzing the trend of AcA, ButA, and DecA, it is possible to infer that the elongation of the carbon chain lowered the extraction success. This is somewhat unexpected given the reported extraction of bacterioruberin from *H. mediterranei* using aqueous solutions of anionic or neutral surfactants, albeit at a lower concentration of 250 mM.[31]

For most systems, the addition of water was necessary to induce the extraction process, which can be ascribed to the difference in osmotic pressure between the hypersaline cellular environment of *H. mediterranei* and the extraction media. Interestingly, a similar extraction yield compared to that obtained using the ethanol control was obtained in the Ment:AcA and Ment:LA systems after addition of a small (10% v/v) volume of water. It appears that a certain degree of solvent polarity is required, most likely to effectively solvate the lysis products, including its ionic salts. However, the opposite trend was observed in the Ment:LevA system in which the addition of water and the visually observed emulsification of the system hampered the extraction yield. Promising results were obtained for neat Ment:LevA with a 4-fold increased extraction yield, compared to the conventional ethanol extraction method, indicating significant potential as an extractant. As a result, the following work was performed using neat Ment:LevA as the best solvent.

The improved pigment extraction in the Ment:LevA system is surprising, given the reported poor enrichment factor of carotenoids in the same solvent from *Spirulina*, when compared to other hydrophilic and hydrophobic eutectic solvents.[21] To better understand the unexpected increase in bacterioruberin extraction yield upon substitution of AcA by LevA, *H. mediterranei* extractions were performed using 50 vol % of carboxylic acid solutions (AcA, LA, and LevA) in water (see Figure S1 in the Supporting Information). The potential chemical degradation of bacterioruberin at higher temperatures prevented the extraction using liquid Ment (T_m = 42.1 °C). As an alternative, the extraction was assessed in the liquid HES composed of 1:1 Ment:Thymol mixture with 0 to 50% added water (not shown). The extraction results in these systems do not indicate any measurable recovery of bacterioruberin, clearly indicating an important synergistic increase in the extraction yield for the Ment:LevA system relative to that of its individual components. Furthermore, comparison of Kamlet–Taft solvent descriptors of the various Ment-based HES in Table 3 does not indicate any appreciable variation that could justify the significantly improved recovery yield in the Ment:LevA system. Although no obvious explanation is found to justify Ment:LevA as the best performing HES, the recovery yield trends in Figure 1 hint at the compromise in solvent properties required for bacterioruberin extraction and its subsequent stabilization. Given the lipophilic nature of a C50 pigment and the well-known rule of thumb "like dissolves like", it would be expectable for bacterioruberin to present the highest partition in the more hydrophobic solvents (Ment:DecA and Ment:Thy). However, the haloarchaea biomass characteristics must be considered. This includes its halophilic nature and the presence of bacterioruberin in the cell membrane.[3] Similarly to ethanol, small carboxylic acids were reported to act as "adjuvants" for weakening biomass cell walls and enhancing a subsequent pigment extraction.[40] Furthermore, the important water content of the wet biomass (68 wt % as determined by lyophilization in this work) requires a solvent capable of solubilizing this excess water and salt content, both to create an osmotic pressure gradient and to ensure a homogeneous dispersion of the substrate during extraction. Excessive clumping of the biomass was observed in the most hydrophobic HES, namely, Ment:DecA and Ment:Thy.

Table 3. Reported Kamlet–Taft Solvatochromic Parameters of the Investigated HES and of Common Solvents[a]

solvent	α	β	π^*	ref
water	1.17	0.14	1.09	41
ethanol	0.83	0.75	0.51	41
menthol[b]	0.53	0.66	0.42	42
Ment:AcA	1.64	0.60	0.53	43
Ment:LevA	1.56	0.58	0.66	43
Ment:OctA	1.77	0.50	0.41	43
Ment:Thy[b]	0.84	0.28	0.77	42

[a]Legend: α, hydrogen bond acidity; β, hydrogen bond basicity; π^*, polarizability; OctA, octanoic acid. [b]Measured at 50 °C.

Variable Screening. A set of preliminary assays were conducted to understand the effects of specific parameters, namely, the extraction time, the Ment molar fraction (x_{Ment}), and the SLR. Unless otherwise specified, standard conditions of 60 min, x_{Ment} = 0.5, and SLR of 0.1 were applied. Although the phase diagram of the Ment:LevA HES was not explicitly measured, the liquidus range at room temperature was determined by preparing the binary mixture along the full composition range at x_{Ment} intervals of 0.1. The results presented in Figure S2 in the Supporting Information indicate a liquid composition range at 25 °C of x_{Ment} from 0.1 to 0.7. Comparison of the experimental liquidus range with the ideal phase diagram in Figure S2 suggests the quasiideal nature of the Ment:LevA system. Based on these preliminary assays (Figure 2), it was possible to identify the most suitable ranges for these parameters to optimize the reaction conditions further and improve the bacterioruberin extraction yield by an RSM. The extraction kinetics are fast, with no increase in the recovery yield going from 20 min to 60 min. As is often the

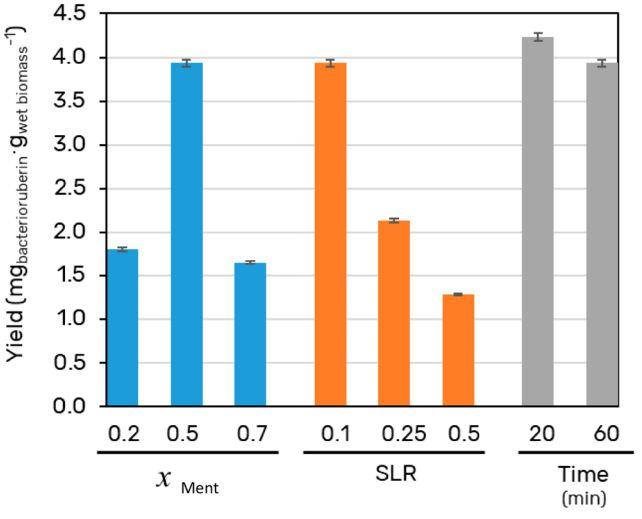


Figure 2. Initial screening of extraction conditions upon the bacterioruberin yield in the Ment:LevA HES, namely, the ratio of Ment to LevA (x_{Ment}, blue), the SLR (orange), and the time of extraction (gray).

https://doi.org/10.1021/acssuschemeng.3c02997
ACS Sustainable Chem. Eng. 2023, 11, 13594–13605

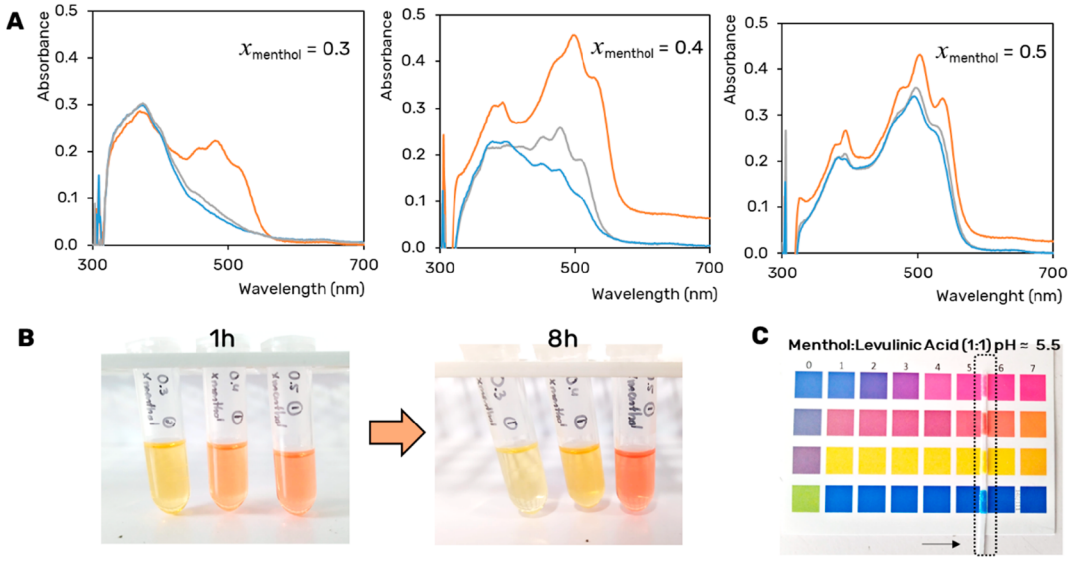


Figure 3. Bacterioruberin stability after extraction in the Ment:LevA HES as a function of time and x_{Ment} by (A) by UV−vis spectroscopy (1 h, blue line; 4 h, gray line; 8 h, orange line) and (B) visual inspection (from left to right, x_{Ment} = 0.3 to 0.5). (C) Estimation of the HES phase pH by for x_{Ment} = 0.5 using a universal pH indicator (pH strip in dotted black box).

case, the extraction yield decreases linearly with the increase in SLR due to the potential solvent saturation and more inefficient mixing at higher loadings.

A distinct nonlinear behavior in the extraction yield was observed as a function of x_{Ment} with a clear maximum of bacterioruberin recovery at x_{Ment} = 0.5. These initial assays also showcased the importance of the different operating conditions on the stability of the pigment with the molar ratio of the eutectic components being crucial in bacterioruberin structure preservation. The pigment stability was compromised in the presence of an excess of levulinic acid, with the three characteristic UV−vis peaks of bacterioruberin at 472, 494, and 530 nm disappearing after 1 h for x_{Ment} = 0.3 (Figure 3A). This was accompanied by a reduction in the redness of the extract and the appearance of a yellowness (Figure 3B). A similar behavior with pH was reported for astaxanthin at pH values below 5.0.[44] The approximate pH of the HES phase at x_{Ment} = 0.5, representing the composition at which bacterioruberin was stable for over 8 h, is 5.5 and suggests a similar negative influence of pH as described for astaxanthin. The HES pH is higher than the LevA aqueous pK_a value of 4.64. As such, the eutectic composition was restricted to those enabling a minimum pigment stability duration of 8 h. This period of 8 h was defined as the maximum time needed for the processing and possible isolation of the pigment if necessary. Based on these results, the parameters time, molar ratio of the components and SLR were then further optimized by RSM within the identified set ranges.

Optimization of the Solid−Liquid Extraction. An optimization of the operational conditions was performed based on a central composite rotatable design (CCRD-2^3) consisting of three independent variables, namely, molar fraction of eutectic components (x_{Ment}, X1), SLR (X2), and extraction time (t (in min), X3). This methodology allows the optimization of the response (bacterioruberin extraction yield) as a function of independent variables. Twenty assays with four central (level 0) and axial points (−1.68 and +1.68 levels) were investigated in terms of bacterioruberin yield of extraction (in $mg_{bacterioruberin}\ g_{wet\ biomass}^{-1}$) (Table S1 in the ESI). The fitted model described in eq 2, obtained using the analysis of variance (ANOVA) to estimate the statistical significance of the variables and their interactions, shows good predictability at a confidence level of 95% with R^2 = 0.9392 and F-calculated > F-tabulated. The impact of these three variables on the bacterioruberin yield is illustrated in Figure 4, in the Pareto chart in Figure S3 in the ESI and the predicted vs observed values in Figure S4 in the ESI.

$$\text{yield of extraction } (mg_{bacterioruberin}\ g_{wet\ biomass}^{-1}) = 15.5889 - 20.7895(X1) - 36.4542(X2) + 0.0104 + 50.3125(X1) \times (X2) \quad (4)$$

The response surfaces plotted in Figure 4 show a small impact of the extraction time on the yield, as expected from the screening results. Nevertheless, more extended periods yielded better results. As suggested by the preliminary screening assays, the yield of extraction is positively influenced to a certain extent by a lower SLR, with the maximum yield located below an SLR of 0.1, as seen in Figure 4a. The molar ratio of the eutectic components greatly influences the extraction process, where an optimum value was reached at 0.5 × Ment, the lowest ratio tested. Although these results suggest that a higher extraction yield could be achieved at Ment ratios of <0.5, such conditions would not be suitable due to the stability issues noticed (Figure 3). As such, a critical and practical analysis of the best conditions was required. After finding the optimal operational conditions (110.4 min, 0.54 × Ment, and SLR 0.07), we validated the model (Table S2 in the ESI). A bacterioruberin yield of extraction of 4.74 ± 0.01 $mg_{bacterioruberin}\ g_{wet\ biomass}^{-1}$ was obtained experimentally, encompassing a mean relative deviation of 2.61%. Finally, an optimal point was chosen at the following operational conditions (110.4 min, 0.5 × Ment, and SLR 0.1), achieving a maximum yield of 5.16 ± 0.01 $mg_{bacterioruberin}\ g_{wet\ biomass}^{-1}$ (see Table S2) This represents a significant increase relative to the obtained extraction yields using pure ethanol (0.76 $mg_{bacterioruberin}\ g_{wet\ biomass}^{-1}$) and aqueous solutions of nonionic surfactants

https://doi.org/10.1021/acssuschemeng.3c02997
ACS Sustainable Chem. Eng. 2023, 11, 13594−13605

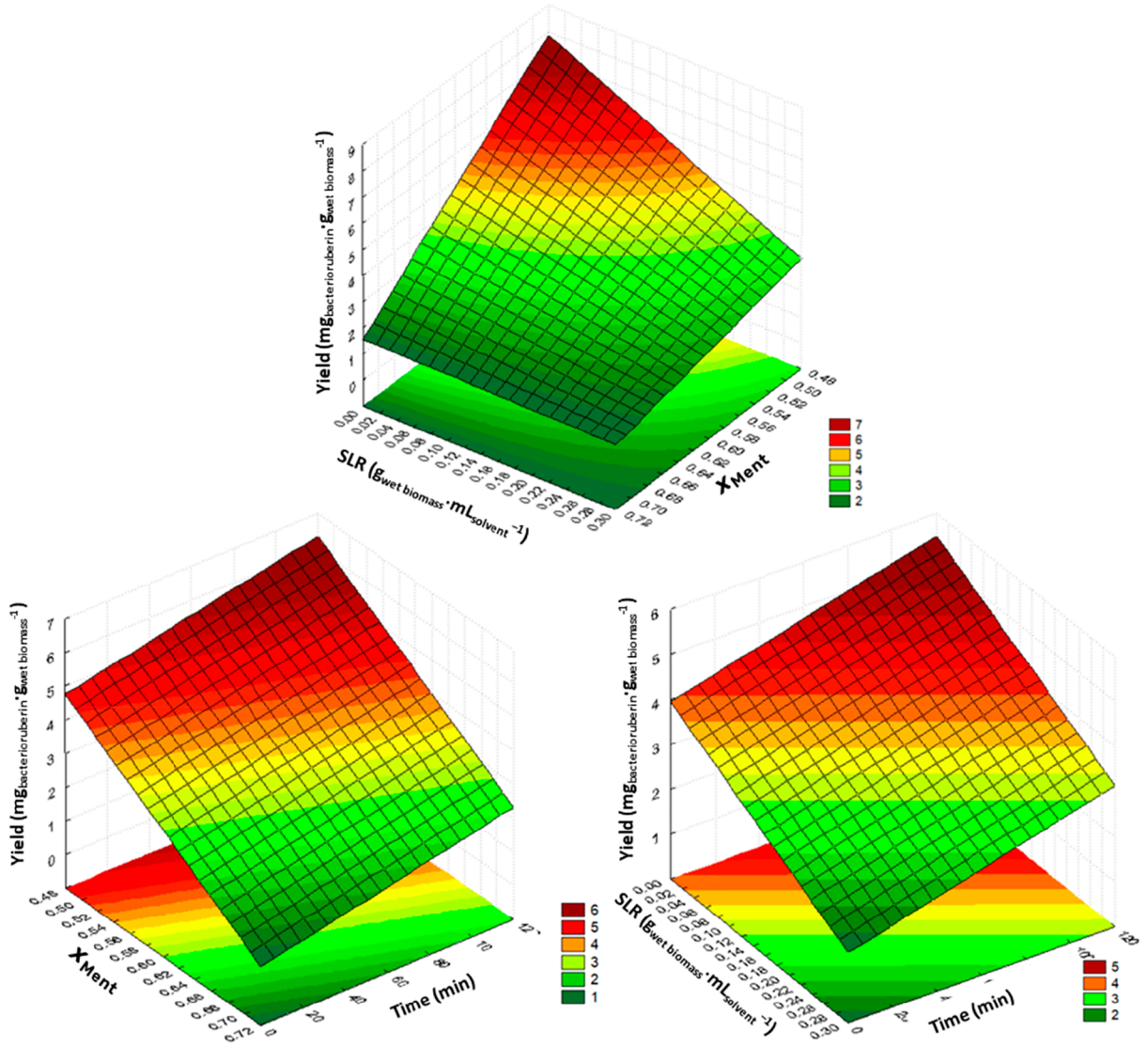


Figure 4. Response surface plots of the bacterioruberin yield of extraction ($mg_{bacterioruberin}\ g_{wet\ biomass}^{-1}$) with the combined effects of SLR ($g_{wet\ biomass}\ mL_{solvent}^{-1}$), molar fraction of the eutectic components (x_{Ment}), and time of extraction (t, min).

(0.37 $mg_{bacterioruberin}\ g_{wet\ biomass}^{-1}$).[31] Moreover, the Ment:LevA eutectic was extensively characterized by Wils et al.[21] and was shown to be biocompatible with human epidermal keratinocytes below concentrations of 200 $\mu g\ mL^{-1}$. Furthermore, Ment:LevA acts as a potential regulating agent for the skin microbiota targeting mainly *Corynebacterium xerosis*, due to the well-known antimicrobial properties of Ment against human and plant microbes.[21] To summarize, Ment:LevA as an extraction solvent presents a 4-fold increase in bacterioruberin recovery compared to ethanol and presents suitable properties for a direct cosmetic/dermatological use.[21,45,46]

Protein Extraction. During the initial screening for the extraction of bacterioruberin using Ment:LevA, a white interfacial precipitate was detected in all systems after centrifugation including that with 0 vol % added water (see photo presented as Figure S5 in the ESI). The appearance of amide bands at 1626 cm^{-1} (amide I) and 1525 cm^{-1} (amide II) on the FTIR spectra indicates the presence of protein in this third phase (Figure S6 in the ESI). This was further confirmed by performing an SDS-PAGE electrophoresis of the precipitated fraction after dissolution in PBS (1×, pH 7.4) (Figure S7 in the ESI). Both results suggest the possibility to valorize protein as a coextracted product in lower value applications such as animal feed, allowing the projection of a multiproduct exploitation scenario. The precipitation of proteins to the interface can be explained by the nature of the two liquid phases that form the system. Notably, important similarities exist between the studied HES system and three-phase partition processes (TPP) commonly applied for the precipitation of enzymes and proteins from aqueous solutions.[28,29] Similar to classical TPP systems, the HES extract is rich in the cyclic alcohol Ment while the inherent water (68 wt %) and salt content (>18% (w/v) salt, cf. section 2.2.1) present in the wet biomass from the residual culture media provide a salt-rich polar phase. The extracted proteins are insoluble in the HES-rich top phase. This characteristically apolar solvent reduces the hydration of the protein by removing the water molecules, causing protein aggregation and consequent precipitation. Although proteins generally present high solubility in the aqueous phase the salt can lead to

https://doi.org/10.1021/acssuschemeng.3c02997
ACS Sustainable Chem. Eng. 2023, 11, 13594−13605

protein aggregation. Due to its halophilic profile, biomass is produced under very high salinity conditions (>18% salt, *cf.* section 2.2.1), negatively affecting the protein solubility. The hostile environment surrounding the proteins causes most of them to promptly precipitate as an isolated coproduct. Note that a similar white precipitate was observed for all the Ment-based solvents tested and was also reported in a previous work during the purification of violacein by Ment:Thy, although it was not further investigated at that time.[47] Considering this, a more systematic study was performed looking at the effect of the addition of small volumes of water as a counter solvent on the yield and efficiency of protein precipitation and further recovery. The protein content was measured in the aqueous and precipitate phases after resuspension, and the HES phase concentration was calculated through a mass balance. Different amounts of water were evaluated to maximize the amount of protein precipitated to the solid interface. The results suggest a maximum recovery plateauing at 78% of the total proteins precipitating (Figure 5).

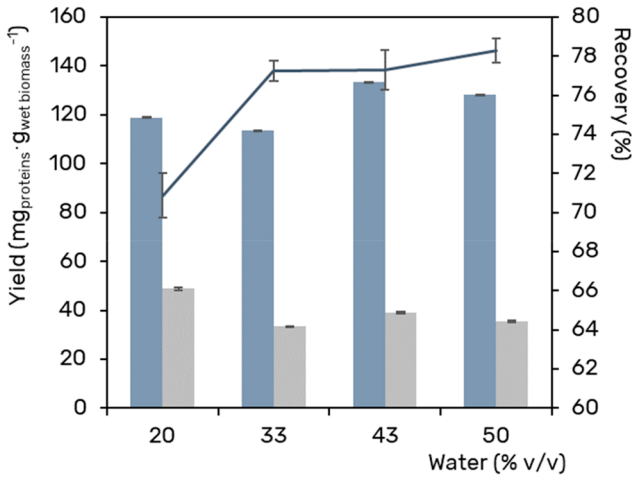


Figure 5. Yield of protein recovered ($mg_{protein}$ $g_{wet\ biomass}^{-1}$) to the solid interface (blue-gray) and aqueous phase (gray); and precipitation recovery (%) (solid line (—)).

After the addition of water to the system, a TPP system was formed, with most of the proteins being precipitated as a solid fraction in the interface between the two liquid phases. This solid fraction can be used (as obtained) in lower value applications such as animal feed, However, this was not the focus of the present work.

To the best of our knowledge, this represents the first application of a HES-based TPP (HES-TPP) to promote the separation of two cellular fractions from the biomass (bacterioruberin and proteins). Moreso, a simple integration of the initial solid−liquid extraction step with a subsequent TPP process was demonstrated. However, considering that the components of Ment:LevA HES have different water solubilities and that Ment and proteins may coprecipitate, the application of TPP in HES likely triggers a change in its final composition. To highlight this, the relative partition of the HES components after equilibration at two organic-to-aqueous (O/A) phase ratios (O/A) of 9:1 and 1:1 was estimated by ^{1}H NMR (Figure 6). The results clearly indicate the loss of LevA to the aqueous phase even for the larger O/A ratios, resulting in a non-negligible change in the HES composition, relative to its initial one, potentially impacting its reusability. This can be partially mitigated by the high salt content of typical TPP systems.

Process Optimization. Following the individual optimization of an efficient extraction step for the recovery of bacterioruberin and a simple protein recovery by TPP, the integration of both steps was studied over multiple extraction cycles. As mentioned, the HES components were selected to produce a biocompatible extract, thereby eliminating the requirement for solvent removal and polishing. Rather, continuous extractions were performed until solvent saturation to produce a concentrated, pigment-rich product. In short, after the first extraction cycle, the extract solution was mixed with fresh biomass, maintaining the same SLR and time during the following extraction cycles. Figure 7 shows the increase in pigment concentration until solvent saturation. After four extraction cycles, the pigment concentration increased by 3-fold. A maximum concentration of 2.13 $mg_{bacterioruberin}$ mL_{HES}^{-1} was obtained after five extraction cycles. It is possible that this value does not represent the solvent saturation limit but it is rather a consequence of the change in HES composition (increase in x_{Ment}) with each successive cycle. The linear decrease in the incremental bacterioruberin concentration at each extraction (Figure 7), could be due to the detrimental reduction in the LevA molar fraction for each cycle, as previously shown in Figure 6. For comparative purposes, the average extraction yield for five cycles was 3.49 ± 0.01, compared to 6.91 ± 0.01 $mg_{bacterioruberin}$ $g_{wet\ biomass}^{-1}$ after the first cycle (Figure S8 in the ESI). However, even after the fourth cycle, the incremental bacterioruberin concentration in HES still outperforms the control represented by ethanol over a single extraction step. More importantly, a single-step extraction at a SLR five times higher (SLR = 0.5 $g_{wet\ biomass}$ $mL_{solvent}^{-1}$), preserving the remaining extraction conditions, did not provide similar or higher concentrations of bacterioruberin, when compared to five successive cycles of extraction. Indeed, the concentration of 1.01 $mg_{bacterioruberin}$ mL_{HES}^{-1} obtained at a SLR of 0.5, is less than half of the concentration obtained with successive extractions (of 2.13 $mg_{bacterioruberin}$ mL_{HES}^{-1}). Thus, for an equal amount of biomass used, successive extractions are shown to exceed a single extraction step by a factor of 2.

Aiming to propose an integrated process that allows one to simultaneously recover and purify bacterioruberin and proteins from *H. mediterranei*, the use of HES in a "one-pot" approach using water as a counter-solvent was investigated. The aim is to integrate the HES as part of the product due to the biocompatibility of the solvent and its existing food and cosmetic applications. A final diagram of the process developed in this work is proposed (Figure 8), in which all of the steps are considered. Here, bacterioruberin is efficiently extracted to the HES-rich phase. The concentration of this product can be enhanced by 3-fold after four consecutive extraction cycles. In the end, the pigment extracted can be directly incorporated into various product formulations. Simultaneously, a protein fraction is precipitated and isolated to the interface as a second product. As a last step, the reuse of the aqueous phase was envisioned. Due to the salt-rich nature of the aqueous phase, it could potentially be recycled back to induce the formation of the TPP. The presence of levulinic acid in this phase would also minimize the loss of levulinic acid from the HES-rich phase.

Carbon Footprint. The total carbon footprint of the process proposed for the recovery of bacterioruberin using

https://doi.org/10.1021/acssuschemeng.3c02997
ACS Sustainable Chem. Eng. 2023, 11, 13594−13605

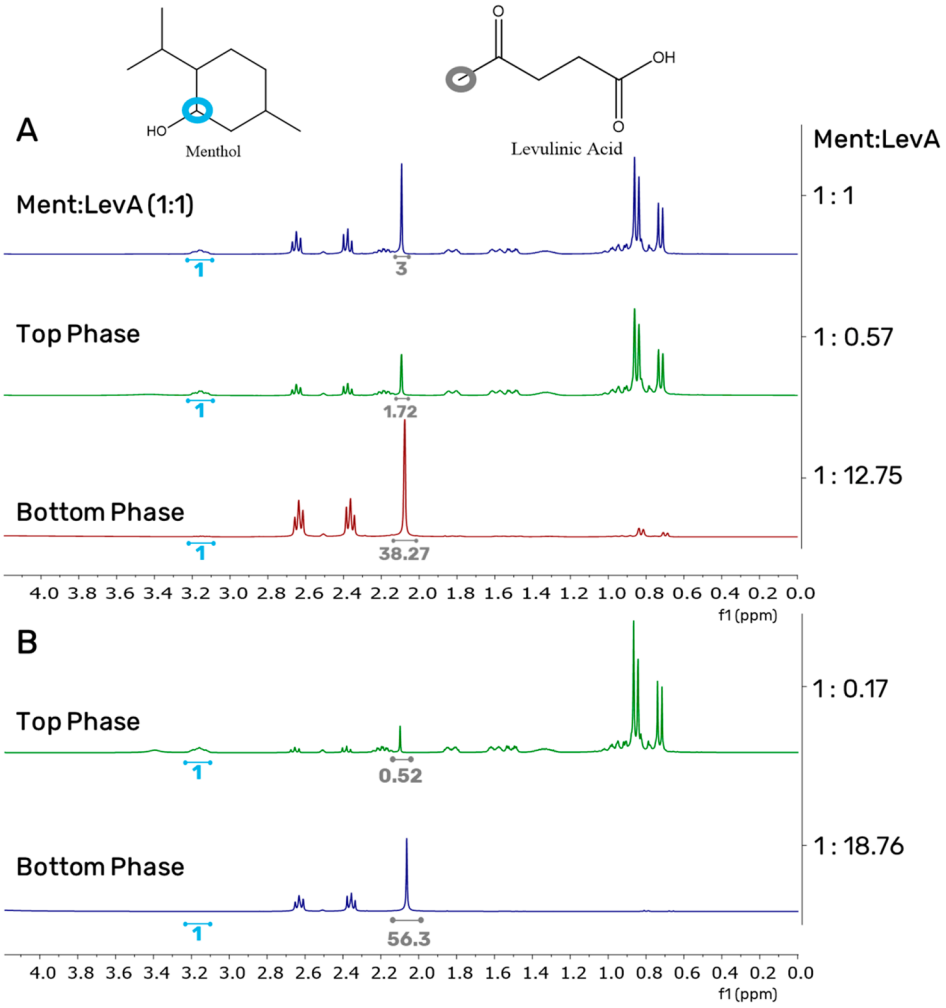


Figure 6. ^{1}H NMR spectra of Ment:LevA (1:1) and the Ment:LevA HES and water systems at (A) 10% water and (B) 50% water. The hydrogen atoms used to compare the menthol and levulinic acid molar ratio are identified in blue and gray, respectively.

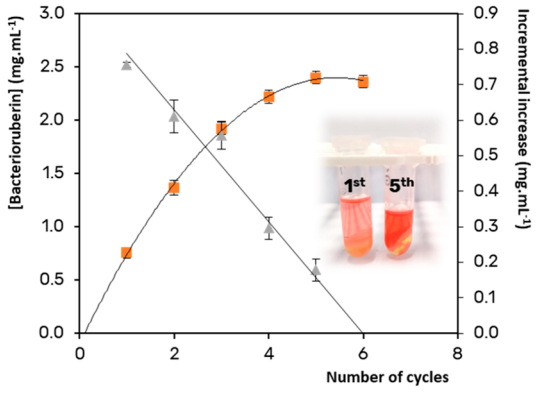


Figure 7. Concentration of bacterioruberin obtained from consecutive extractions using Ment:LevA (orange squares, ■) and consequential yield of extraction (gray triangles, ▲).

Ment:LevA amounted to 44 g of CO_2 equiv per g of biomass. About 75% of this carbon footprint is derived from the HES preparation step, while 14% and 11% result from the protein-induced precipitation, and cell disruption and solid–liquid extraction steps, respectively. Within the HES preparation step, 37% of the carbon footprint is due to levulinic acid production, 32% to electricity production, and 31% to Ment production. In the other steps, the main contribution to the carbon footprint is from electricity production. The lack of published data on the carbon footprint of similar or alternative systems for bacterioruberin recovery prevents a comparison with the results of the carbon footprint obtained in this study.

CONCLUSIONS

This work describes the recovery of bacterioruberin, an unusual C50 pigment with interesting properties relevant to the food and cosmetic industries using HES. The HES mixtures were selected with the intention of designing a "one-pot" extraction and separation process using water as a counter solvent, where ultimately the HES could be integrated as part of the product due to the biocompatibility of the solvent. From the seven HES screened, the Ment:LevA mixture displayed a 4-fold improvement over both the ethanol control and the other HES. Interestingly, a synergistic increase in the extraction yield of bacterioruberin was found in the Ment:LevA system, relative to its individual components. The extraction parameters were optimized to achieve a yield of 5.16 ± 0.01 $mg_{bacterioruberin}\ g_{wet\ biomass}^{-1}$, with the x_{Ment} parameter being particularly important. The recovery of proteins was further demonstrated by the addition of water to the extract, resulting in the

https://doi.org/10.1021/acssuschemeng.3c02997
ACS Sustainable Chem. Eng. 2023, 11, 13594−13605

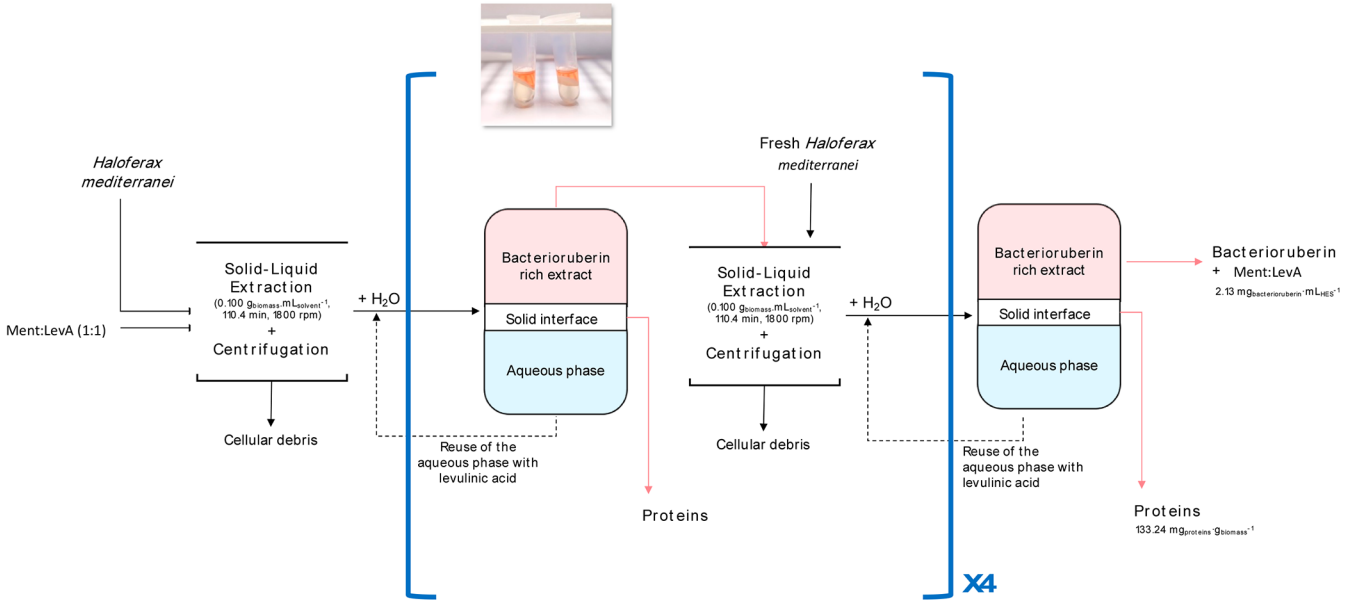


Figure 8. Schematic of the integrated process for the extraction and purification of the different fractions from *H. mediterranei*. The dashed lines indicate that the step was not performed experimentally. The same eutectic phase was reused over five extraction cycles with fresh biomass used at each step.

formation of protein-rich interfacial precipitate (a TPP system). Finally, the process intensification was assessed through the reuse of the eutectic phase over five successive extraction cycles, achieving a bacterioruberin rich extract of 2.13 $mg_{bacterioruberin}$ mL_{HES}^{-1} and an average yield of extraction of 3.49 ± 0.01 $mg_{bacterioruberin}$ $g_{wet\ biomass}^{-1}$ with suitable properties for direct cosmetic/dermatological use. The results herein highlight the potential of HES as biocompatible solvents for the recovery of value-added compounds from biomass while the use of TPP with HES as the organic phase allows the recovery of the proteins to produce a second product stream.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.3c02997.

Detailed analysis of the central composite rotatable design; photographs and UV−vis spectra of the extracts obtained with carboxylic acid solutions; liquidus range of Ment + LevA HES at room temperature and its ideal phase diagram; photograph of the initial Ment + LevA HES systems; solid fraction characterization; yields of extraction obtained from the consecutive extractions (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Sónia P. M. Ventura − *CICECO−Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal;* orcid.org/0000-0001-9049-4267; Email: spventura@ua.pt

Authors

Mariam Kholany − *CICECO−Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal*

Nicolas Schaeffer − *CICECO−Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal;* orcid.org/0000-0002-0747-2532

Inês P. E. Macário − *CICECO−Aveiro Institute of Materials, Department of Chemistry and CESAM−Centre for Environmental and Marine Studies, Department of Biology, University of Aveiro, 3810-193 Aveiro, Portugal*

Telma Veloso − *CICECO−Aveiro Institute of Materials, Department of Chemistry and CESAM−Centre for Environmental and Marine Studies, Department of Biology, University of Aveiro, 3810-193 Aveiro, Portugal*

Tânia Caetano − *CESAM−Centre for Environmental and Marine Studies, Department of Biology, University of Aveiro, 3810-193 Aveiro, Portugal;* orcid.org/0000-0001-9157-8761

Joana L. Pereira − *CESAM−Centre for Environmental and Marine Studies, Department of Biology, University of Aveiro, 3810-193 Aveiro, Portugal;* orcid.org/0000-0001-7573-6184

Ana C. R. V. Dias − *CESAM−Centre for Environmental and Marine Studies, Department of Environment and Planning, University of Aveiro, 3810-193 Aveiro, Portugal*

João A. P. Coutinho − *CICECO−Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal;* orcid.org/0000-0002-3841-743X

Complete contact information is available at: https://pubs.acs.org/10.1021/acssuschemeng.3c02997

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was developed within the scope of the project CICECO-Aveiro Institute of Materials, UIDB/50011/2020, UIDP/50011/2020, and LA/P/0006/2020, financed by national funds through the FCT/MEC (PIDDAC). The authors

acknowledge FTC/MCTES for the financial support to CESAM, UIDB/50017/2020 + UIDP/50017/2020 + LA/P/0094/2020, financed by national funds. The authors are also grateful to the FCT for the doctoral grants to M.K. (SFRH/BD/138413/2018), I.P.E.M. (SFRH/BD/123850/2016), and T.V. (SFRH/BD/147346/2019). T.C. was funded by national funds (OE), through FCT, in the scope of the framework contract foreseen in the numbers 4, 5, and 6 of Article 23, of the Decree-Law 57/2016, of August 29, changed by Law 57/2017, of July (CEECIND/01463/2017). The National NMR Network, funded within the frame- work of the National Program for Scientific Re-equipment, contract REDE/1517/RMN/2005 with funds from POCI 2010 (FEDER) and FCT, is also acknowledged.

REFERENCES

(1) *Biotechnology Market Size, Share & Trends Analysis Report By Technology (Nanobiotechnology, DNA Sequencing, Cell-Based Assays), By Application (Health, Bioinformatics), By Region, and Segment Forecasts, 2022−2030*. Grand View Research Report, May 2023

(2) Pfeifer, K.; Ergal, İ.; Koller, M.; Basen, M.; Schuster, B.; Rittmann, S. K. M. R. Archaea Biotechnology. *Biotechnol. Adv.* **2021**, *47*, No. 107668.

(3) Giani, M.; Garbayo, I.; Vílchez, C.; Martínez-Espinosa, R. M. Haloarchaeal Carotenoids: Healthy Novel Compounds from Extreme Environments. *Mar. Drugs* **2019**, *17* (9), 524.

(4) Rodrigo-Baños, M.; Garbayo, I.; Vílchez, C.; Bonete, M. J.; Martínez-Espinosa, R. M. Carotenoids from Haloarchaea and Their Potential in Biotechnology. *Mar. Drugs* **2015**, *13* (9), 5508.

(5) Serrano, S.; Mendo, S.; Caetano, T. Haloarchaea Have a High Genomic Diversity for the Biosynthesis of Carotenoids of Biotechnological Interest. *Res. Microbiol.* **2022**, *173* (3), No. 103919.

(6) Kottemann, M.; Kish, A.; Iloanusi, C.; Bjork, S.; DiRuggiero, J. Physiological Responses of the Halophilic Archaeon Halobacterium Sp. Strain NRC1 to Desiccation and Gamma Irradiation. *Extremophiles* **2005**, *9* (3), 219−227.

(7) Shahmohammadi, H. R.; Asgarani, E.; Terato, H.; Saito, T.; Ohyama, Y.; Gekko, K.; Yamamoto, O.; Ide, H. Protective Roles of Bacterioruberin and Intracellular KCl in the Resistance of Halobacterium Salinarium against DNA-Damaging Agents. *J. Radiat. Res.* **1998**, *39* (4), 251−262.

(8) Clarke, C. J.; Tu, W. C.; Levers, O.; Bröhl, A.; Hallett, J. P. Green and Sustainable Solvents in Chemical Processes. *Chem. Rev.* **2018**, *118* (2), 747−800.

(9) de Souza Mesquita, L. M.; Martins, M.; Pisani, L. P.; Ventura, S. P. M.; de Rosso, V. V. Insights on the Use of Alternative Solvents and Technologies to Recover Bio-Based Food Pigments. *Compr. Rev. Food Sci. Food Saf.* **2021**, *20* (1), 787−818.

(10) Sholl, D. S.; Lively, R. P. Seven Chemical Separations to Change the World. *Nature* **2016**, *532*, 435−437.

(11) Murador, D. C.; de Souza Mesquita, L. M.; Vannuchi, N.; Braga, A. R. C.; de Rosso, V. V. Bioavailability and Biological Effects of Bioactive Compounds Extracted with Natural Deep Eutectic Solvents and Ionic Liquids: Advantages over Conventional Organic Solvents. *Curr. Opin. Food Sci.* **2019**, *26*, 25−34.

(12) Radošević, K.; Ćurko, N.; Gaurina Srček, V.; Cvjetko Bubalo, M.; Tomašević, M.; Kovačević Ganić, K.; Radojčić Redovniković, I. Natural Deep Eutectic Solvents as Beneficial Extractants for Enhancement of Plant Extracts Bioactivity. *LWT* **2016**, *73*, 45−51.

(13) Amorim-Carrilho, K. T.; Cepeda, A.; Fente, C.; Regal, P. Review of Methods for Analysis of Carotenoids. *TrAC Trends Anal. Chem.* **2014**, *56*, 49−73.

(14) Dai, Y.; Verpoorte, R.; Choi, Y. H. Natural Deep Eutectic Solvents Providing Enhanced Stability of Natural Colorants from Safflower (Carthamus tinctorius). *Food Chem.* **2014**, *159*, 116−121.

(15) de los Angeles Fernández, M.; Boiteux, J.; Espino, M.; Gomez, F. J. V.; Silva, M. F. Natural Deep Eutectic Solvents-Mediated Extractions: The Way Forward for Sustainable Analytical Developments. *Anal. Chim. Acta* **2018**, *1038*, 1−10.

(16) Martins, M. A. R.; Pinho, S. P.; Coutinho, J. A. P. Insights into the Nature of Eutectic and Deep Eutectic Mixtures. *J. Solution Chem.* **2019**, *48* (7), 962−982.

(17) Abbott, A. P.; Capper, G.; Davies, D. L.; Rasheed, R. K.; Tambyrajah, V. Novel Solvent Properties of Choline Chloride/Urea Mixtures. *Chem. Commun.* **2003**, No. 1, 70−71.

(18) Halder, A. K.; Cordeiro, M. N. D. S. Probing the Environmental Toxicity of Deep Eutectic Solvents and Their Components: An in Silico Modeling Approach. *ACS Sustain. Chem. Eng.* **2019**, *7* (12), 10649−10660.

(19) Radošević, K.; Cvjetko Bubalo, M.; Gaurina Srček, V.; Grgas, D.; Landeka Dragičević, T.; Redovniković, R. I. Evaluation of Toxicity and Biodegradability of Choline Chloride Based Deep Eutectic Solvents. *Ecotoxicol. Environ. Saf.* **2015**, *112*, 46−53.

(20) Chen, Y.; Mu, T. Application of Deep Eutectic Solvents in Biomass Pretreatment and Conversion. *Green Energy Environ.* **2019**, *4* (2), 95−115.

(21) Wils, L.; Leman-Loubière, C.; Bellin, N.; Clément-Larosière, B.; Pinault, M.; Chevalier, S.; Enguehard-Gueiffier, C.; Bodet, C.; Boudesocque-Delaye, L. Natural Deep Eutectic Solvent Formulations for Spirulina: Preparation, Intensification, and Skin Impact. *Algal Res.* **2021**, *56*, No. 102317.

(22) Křížek, T.; Bursová, M.; Horsley, R.; Kuchař, M.; Tůma, P.; Čabala, R.; Hložek, T. Menthol-Based Hydrophobic Deep Eutectic Solvents: Towards Greener and Efficient Extraction of Phytocannabinoids. *J. Clean. Prod.* **2018**, *193*, 391−396.

(23) Wang, L. T.; Yang, Q.; Cui, Q.; Fan, X. H.; Dong, M. Z.; Gao, M. Z.; Lv, M. J.; An, J. Y.; Meng, D.; Zhao, X. H.; Fu, Y. J. Recyclable Menthol-Based Deep Eutectic Solvent Micellar System for Extracting Phytochemicals from Ginkgo Biloba Leaves. *J. Clean. Prod.* **2020**, *244*, No. 118648.

(24) Jin, Y.; Jung, D.; Li, K.; Park, K.; Lee, J. Mixing of Menthol-Based Hydrophobic Deep Eutectic Solvents as a Novel Method to Tune Their Properties. *J. Mol. Liq.* **2020**, *301*, No. 112416.

(25) Liu, Y.; Deak, N.; Wang, Z.; Yu, H.; Hameleers, L.; Jurak, E.; Deuss, P. J.; Barta, K. Tunable and Functional Deep Eutectic Solvents for Lignocellulose Valorization. *Nat. Commun. 2021 121* **2021**, *12* (1), 1−15.

(26) Mussagy, C. U.; Santos-Ebinuma, V. C.; Gonzalez-Miquel, M.; Coutinho, J. A. P.; Pereira, J. F. B. Protic Ionic Liquids as Cell-Disrupting Agents for the Recovery of Intracellular Carotenoids from Yeast Rhodotorula Glutinis CCT-2186. *ACS Sustain. Chem. Eng.* **2019**, *7* (19), 16765−16776.

(27) Bezold, F.; Minceva, M. A Water-Free Solvent System Containing an L-Menthol-Based Deep Eutectic Solvent for Centrifugal Partition Chromatography Applications. *J. Chromatogr. A* **2019**, *1587*, 166−171.

(28) Dennison, C.; Lovrien, R. Three Phase Partitioning: Concentration and Purification of Proteins. *Protein Expr. Purif.* **1997**, *11* (2), 149−161.

(29) Li, Z.; Jiang, F.; Li, Y.; Zhang, X.; Tan, T. Simultaneously Concentrating and Pretreating of Microalgae Chlorella Spp. by Three-Phase Partitioning. *Bioresour. Technol.* **2013**, *149*, 286−291.

(30) Saxena, L.; Iyer, B. K.; Ananthanarayan, L. Three Phase Partitioning as a Novel Method for Purification of Ragi (Eleusine Coracana) Bifunctional Amylase/Protease Inhibitor. *Process Biochem.* **2007**, *42* (3), 491−495.

(31) Vaz, B. M. C.; Kholany, M.; Pinto, D. C. G. A.; Macário, I. P. E.; Veloso, T.; Caetano, T.; Pereira, J. L.; Coutinho, J. A. P.; Ventura, S. P. M. Recovery of Bacterioruberin and Proteins Using Aqueous Solutions of Surface-Active Compounds. *RSC Adv.* **2022**, *12* (47), 30278−30286.

(32) Allers, T.; Ngo, H. P.; Mevarech, M.; Lloyd, R. G. Development of Additional Selectable Markers for the Halophilic Archaeon Haloferax Volcanii Based on the LeuB and TrpA Genes. *Appl. Environ. Microbiol.* **2004**, *70* (2), 943−953.

https://doi.org/10.1021/acssuschemeng.3c02997
ACS Sustainable Chem. Eng. 2023, 11, 13594−13605

(33) Laemmli, U. K. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* **1970**, *227*, 680−685.
(34) International Organization for Standardization. *ISO 14067:2018—Greenhouse Gases—Carbon Footprint of Products—Requirements and Guidelines for Quantification*; ISO Report, Geneva, Switzerland, 2018.
(35) Wernet, G.; Bauer, C.; Steubing, B.; Reinhard, J.; Moreno-Ruiz, E.; Weidema, B. The Ecoinvent Database Version 3 (Part I): Overview and Methodology. *Int. J. Life Cycle Assess.* **2016**, *21* (9), 1218−1230.
(36) Cañon, C.; Sanchez, N.; Cobo, M. Sustainable Production of Ethyl Levulinate by Levulinic Acid Esterification Obtained from Colombian Rice Straw. *J. Clean. Prod.* **2022**, *377*, No. 134276.
(37) Myhre, Gs.; Bréon, F.; Collins, W.; Fuglestvedt, J.; Huang, J.; Koch, D.; Lamarque, J.; Lee, D.; Mendoza, B.; Nakajima, T.; Robock, A.; Stephens, G.; Takemura, T.; Zhang, H. Anthropogenic and Natural Radiative Forcing. In *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*; Cambridge University Press, 2013; pp 659−740.
(38) Martins, M. A. R.; Crespo, E. A.; Pontes, P. V. A.; Silva, L. P.; Bülow, M.; Maximo, G. J.; Batista, E. A. C.; Held, C.; Pinho, S. P.; Coutinho, J. A. P. Tunable Hydrophobic Eutectic Solvents Based on Terpenes and Monocarboxylic Acids. *ACS Sustain. Chem. Eng.* **2018**, *6* (7), 8836−8846.
(39) Florindo, C.; Branco, L. C.; Marrucho, I. M. Development of Hydrophobic Deep Eutectic Solvents for Extraction of Pesticides from Aqueous Environments. *Fluid Phase Equilib.* **2017**, *448*, 135−142.
(40) Pitacco, W.; Samorì, C.; Pezzolesi, L.; Gori, V.; Grillo, A.; Tiecco, M.; Vagnoni, M.; Galletti, P. Extraction of Astaxanthin from Haematococcus Pluvialis with Hydrophobic Deep Eutectic Solvents Based on Oleic Acid. *Food Chem.* **2022**, *379*, No. 132156.
(41) Jessop, P. G.; Jessop, D. A.; Fu, D.; Phan, L. Solvatochromic Parameters for Solvents of Interest in Green Chemistry. *Green Chem.* **2012**, *14* (5), 1245−1259.
(42) Martins, M. A. R.; Silva, L. P.; Schaeffer, N.; Abranches, D. O.; Maximo, G. J.; Pinho, S. P.; Coutinho, J. A. P. Greener Terpene-Terpene Eutectic Mixtures as Hydrophobic Solvents. *ACS Sustain. Chem. Eng.* **2019**, *7* (20), 17414−17423.
(43) Florindo, C.; McIntosh, A. J. S.; Welton, T.; Branco, L. C.; Marrucho, I. M. A Closer Look into Deep Eutectic Solvents: Exploring Intermolecular Interactions Using Solvatochromic Probes. *Phys. Chem. Chem. Phys.* **2018**, *20* (1), 206−213.
(44) Liu, X.; McClements, D. J.; Cao, Y.; Xiao, H. Chemical and Physical Stability of Astaxanthin-Enriched Emulsion-Based Delivery Systems. *Food Biophys.* **2016**, *11* (3), 302−310.
(45) Patel, T.; Ishiuji, Y.; Yosipovitch, G. Menthol: A Refreshing Look at This Ancient Compound. *J. Am. Acad. Dermatol.* **2007**, *57* (5), 873−878.
(46) Trombetta, D.; Castelli, F.; Sarpietro, M. G.; Venuti, V.; Cristani, M.; Daniele, C.; Saija, A.; Mazzanti, G.; Bisignano, G. Mechanisms of Antibacterial Action of Three Monoterpenes. *Antimicrob. Agents Chemother.* **2005**, *49* (6), 2474.
(47) Schaeffer, N.; Kholany, M.; Veloso, T. L. M.; Pereira, J. L.; Ventura, S. P. M.; Nicaud, J. M.; Coutinho, J. A. P. Temperature-Responsive Extraction of Violacein Using a Tuneable Anionic Surfactant-Based System. *Chem. Commun.* **2019**, *55* (59), 8643−8646.

https://doi.org/10.1021/acssuschemeng.3c02997
ACS Sustainable Chem. Eng. 2023, 11, 13594−13605