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Food-grade creatine-based deep eutectic solvent for sustainable r-phycoerythrin extraction from the red seaweed *Amansia* sp

Letícia S. Contieri [a,b], Giovanna Ribeiro Romanini [a,b], Renan Canute Kamikawachi [a,b], Fungyi Chow [a], Filipe H.B. Sosa [c], Leonardo M. de Souza Mesquita [a,b,*]

[a] Departamento de Botânica, Instituto de Biociências, Universidade de São Paulo, CEP 05422-970 São Paulo, Brazil
[b] Synthetic and Systems Biology Center, InovaUSP, Avenida Professor Lucio Martins Rodrigues, 370, São Paulo 05508-020, Brazil
[c] CICECO - Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal

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ABSTRACT

r-Phycoerythrin (r-PE) is a water-soluble fluorescent protein abundant in red macroalgae, with strong potential as a natural colorant aligned with clean-label trends and regulatory restrictions on synthetic dyes. Conventional extraction methods, however, face challenges in pigment stability and scalability, highlighting the need for innovative solutions. Deep Eutectic Solvents (DES) offer a versatile platform for selective recovery under mild conditions, though their food compatibility remains a limitation. This study investigates creatine-based DES for r-PE extraction from *Amansia* sp., a red macroalgae native to the Brazilian coast with recognized antioxidant properties. Response surface methodology was applied to optimize extraction, and the creatine: citric acid DES (1:6 M ratio) achieved superior efficiency compared to water and PBS. Under optimized extraction conditions: solid liquid ratio of 0.08 $g_{biomass}.mL_{solvent}^{-1}$; ultrasonic power of 450 W; 30% water in DES, and an extraction time of 13 min, the maximum yield reached 2.90 $mg_{r\text{-}phycoerythrin}.g_{biomass}^{-1}$. Antioxidant assays confirmed that DES not only enhanced recovery but also preserved pigment integrity, reinforcing its potential for food-grade applications.

1. Introduction

Rising global demand for sustainable energy, food, and materials is accelerating the transition toward renewable biomass-based economies [1,2]. In this context, marine resources represent an underexploited feedstock within the blue bioeconomy, offering opportunities to reduce dependence on terrestrial biomass while supporting Sustainable Development Goals 12 and 14 ([3]; [4]; [5]).

Marine macroalgae are particularly promising due to their rapid growth, lack of competition with arable land and freshwater resources, and rich biochemical composition [3]. They contain high-value compounds such as phycobiliproteins, carotenoids, chlorophylls, sulfated polysaccharides, and phenolic compounds, enabling their use in integrated biorefinery strategies [6]. Among these, r-phycoerythrin (r-PE), a water-soluble phycobiliprotein responsible for the red coloration (λ_{max} ≈ 565 nm) of Rhodophyta, has gained increasing interest as a natural colorant for food and biotechnology applications [7,8].

However, the industrial application of r-PE remains limited due to its low stability under heat, light, and oxidative conditions, as well as the lack of efficient, scalable, and environmentally friendly extraction methods [9]. Conventional aqueous and organic solvent-based systems often compromise pigment integrity or result in low selectivity, highlighting the need for alternative green extraction strategies.

In this context, Deep Eutectic Solvents (DES) represent a strategic platform for phycoerythrin recovery. DES are composed of hydrogen bond donors (HBDs) and hydrogen bond acceptors (HBAs) and have gained considerable attention as sustainable extraction media due to their biodegradability, low toxicity, negligible vapor pressure, ease of preparation, and customizable physicochemical properties [10,11] Their extraction efficiency can be further improved through integration with process-intensification technologies such as ultrasound-assisted extraction (UAE). The synergistic combination of DES and UAE enhances mass transfer and cell disruption, enabling higher extraction yields in shorter processing times while reducing energy consumption and solvent requirements [12]. DES systems may exhibit relatively high viscosity, which can limit mass transfer and extraction efficiency. This

* Corresponding author.
E-mail address: mesquitalms@gmail.com (L.M. de Souza Mesquita).

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limitation can be partially overcome through process intensification strategies such as UAE and systematic process optimization [13]. Also, most reported DES formulations are based on non-food-grade components, which limits their direct application in food, nutraceutical, and cosmetic products.

To address this limitation, food-compatible DES systems have been proposed in a UAE system. In particular, creatine-based DES represents a promising alternative due to the biocompatibility and functional properties of its components. Recently, Heaney et al. [14] reported the physicochemical characterization of a creatine and citric acid DES, demonstrating its stability, tunable polarity, and hydrogen-bonding capacity. However, its application as an extraction medium for bioactive compounds has not yet been explored.

Thus, the use of a creatine-based DES for r-PE recovery represents more than the first application of this solvent class for biopigment extraction. The proposed system integrates extraction and stabilization into a single step, while eliminating the need for solvent removal or exchange prior to application. Due to the biocompatible nature of citric acid and creatine, the DES remains as part of the final extract, acting not only as an extraction medium but also as a potential functional ingredient. Consequently, this approach reduces downstream processing requirements and aligns with the principles of process intensification, green chemistry, and circular bioeconomy.

Among the red macroalgae, the genus *Amansia* stands out, being widely distributed along the Brazilian coast. This macroalgae has attracted increasing interest due to its recognized antioxidant potential [15]. However, studies focusing on this genus remain limited, particularly those addressing the extraction and characterization of r-PE. The scarcity of research in this area highlights the need for further investigations exploring its bioactive properties and potential applications in different sectors.

2. Materials and methods

2.1. Materials

The macroalga *Amansia* sp. was collected from the northeast coast of Brazil at Stella Mares Beach, Salvador, Bahia State. Right after harvesting, epiphytic material along with impurities such as small invertebrates and algae, and sediments were thoroughly removed. The cleaning process was performed promptly to reduce the risk of degradation of the desired bioactive compounds. During this stage, the macroalgal material was continuously kept damp with seawater to maintain its structural integrity. After washing with purified water, the algal material was dried and immediately stored in opaque containers at −40 °C until further analysis. All experiments were performed using dried biomass; therefore, extraction yields were calculated and expressed on a dry-weight basis. To produce the eutectic solvents, the following starting materials were used, namely: creatine (N-(amino-iminomethyl)-*N*-methyl glycine) (Growth Supplements Produtos Alimentícios LTDA, Brazil), and citric acid (Synth, São Paulo, Brazil). Pure water was supplied by a Milli-Q Advantage water purifier system (Merck system). The pigment concentrations were determined based on the calculation using an external standard of r-phycoerythrin acquired from Sigma-Aldrich.

2.2. COSMO-RS model

The COSMO-RS model, a quantum chemistry-based thermodynamic approach, was employed to evaluate the optimal molar ratios and water content to be used in the screening of creatine: citric acid (C:CA) based eutectic solvents. For this purpose, the activity coefficients at infinite dilution ($\gamma\infty$) of r-phycoerythrin in different proportions of the solvents were calculated. The intermolecular interaction energies, specifically hydrogen bonding (HB), electrostatic misfit (misfit), and van der Waals (vdW) interactions, were also determined. Geometry optimization and charge density generation in the COSMO phase (COSMO-BP-TZVP) were performed using the Turbomole software (TmoleX25, Version 2025). -RS calculations were carried out using the COSMOtherm® software package (Version 25.0), employing the BP_TZVP_25 parameterization.

2.3. Creatine based solvent preparation

Creatine-based DES were prepared according to a procedure adapted from Heaney and co-workers (2022)[14]. Seven eutectic formulations were produced by combining creatine and citric acid at different molar ratios, followed by controlled heating and mixing until homogeneous liquids were obtained. This compositional screening enabled the investigation of the influence of molar ratio on DES formation and on the performance of extraction of r-phycoerythrin. The molar ratio between creatine and citric acid (C:CA, mol:mol) ranged from 1:4 to 1:6, while the water content was adjusted to 20–30% (*w*/w). 60 ± 2 °C and 500 rpm in an oil bath until a homogeneous and transparent liquid was formed. After preparation, the resulting DES were allowed to cool slowly to room temperature in order to reach equilibrium and were subsequently stored for 24 h prior to use.

2.4. r-Phycoerythrin extraction

The extraction performance of the different solvents was evaluated based on the r-PE yield, expressed as $mg_{\text{r-phycoerythrin}}.g^{-1}_{\text{biomass}}$ (dry biomass). Quantification was performed using a UV–Vis microplate reader (SpectraMax Mini, serial 3620) at 565 nm, with a corresponding calibration curve prepared with commercial r-phycoerythrin standard dissolved in water (Fig. S1, Supplementary Material). The absorbance of the DES was also evaluated at 565 nm, and no significant signal was observed at this wavelength. The purity of r-PE was estimated using the A_{565}/A_{280} ratio. All extractions were performed in technical triplicate, and the results are presented as mean ± standard deviation.

2.4.1. Solvent screening using ultrasonic Bath extraction

An initial screening was conducted to compare the extraction performance of the different DES formulations with pure water and phosphate-buffered saline (PBS, pH 7.4), used as control solvents. Extractions were performed using an ultrasonic bath (UAE_{bath}) (P60H, Elmasonic, Singen, Germany; 2.75 L, 37 kHz, 135 W) under fixed conditions: solid liquid ratio (SLR) of 0.33 $g_{biomass}$ $mL^{-1}_{solvent}$ (dry biomass) and extraction times of 30 and 120 min, according to previous studies [13,16]. After extraction, samples were centrifuged (Eppendorf 5416 R) at 4 °C and 12,000 rpm for 10 min. The supernatant was collected and analyzed by UV–Vis spectroscopy. The bulk extraction temperature was monitored using a thermocouple.

2.4.2. Process optimization using ultrasonic probe extraction

After identifying the most promising DES molar ratio during the screening step, process optimization was performed using an ultrasonic probe (UAE_{probe}) (Ultronique, 550 W, 20 kHz). The probe system was selected to increase extraction efficiency and reduce processing time compared with the ultrasonic bath. The initial extraction time was fixed at 3 min, following the methodology proposed by Contieri et al.[13]. A Central Composite Design (CCD) was employed to optimize the extraction conditions. The experimental design included three central-point replicates, resulting in a total of 17 experiments. Three independent variables were evaluated: ultrasonic power (170.5–540 W), DES water content (30–90%, *w*/w) and SLR (0.08–0.92 $g_{biomass}.mL^{-1}_{solvent}$, dry biomass). The optimal conditions predicted by the model were experimentally validated through three independent extractions. Model adequacy was assessed using ANOVA ($p < 0.05$; $R^2 > 0.70$), and predictive accuracy was evaluated by comparing predicted and experimental values using the coefficient of variation and percent deviation. A control experiment was conducted using an aqueous citric acid solution prepared at the same effective concentration as present in the DES system

(3.9 mol.L^{-1}), corresponding to the citric acid content derived from the 1:6 creatine:citric acid molar ratio with 30% water. The extraction was performed under identical optimized conditions to those applied for the DES.

To evaluate the applicability of the optimized extraction method, the extraction protocol established for *Amansia* sp. was applied to four additional red macroalgae species: *Hypnea* sp., *Halymenia* sp., *Gracilaria* sp., *and Kappaphycus* sp. All biomass samples were previously freeze-dried, ground to obtain homogeneous particles, and stored under dry conditions until use. Extractions were performed under the optimized conditions determined by the experimental design using the creatine: citric acid deep eutectic solvent (1,6 M ratio, 30 wt% water) and ultrasound-assisted extraction. The solidliquid ratio, ultrasonic power, and extraction time were maintained identical to those optimized for *Amansia* sp. to enable a direct comparison among species. The r-phycoerythrin content was quantified spectrophotometrically according to the method previously described.

2.5. FTIR and ^{1}H NMR of Creatine based eutectic solvent

Fourier-transform infrared spectroscopy (FTIR) was employed to characterize the functional groups and intermolecular interactions of the individual components (citric acid and creatine) and the deep eutectic solvent (DES) formulation. Spectra were recorded using a PerkinElmer Spectrum BX spectrometer equipped with a horizontal Golden Gate attenuated total reflection (ATR) cell and a diamond crystal. All samples were analyzed under identical instrumental conditions, using a spectra range of 4000–400 cm^{-1}, a resolution of 4 cm^{-1} and 32 scans per sample to ensure an adequate signal-to-noise ratio. Before each measurement, a background spectrum of ambient air was collected. The same acquisition and processing procedure was applied to the DES and to the individual components, allowing direct comparison of the spectra. Shifts in band positions and changes in intensity or broadening were interpreted as evidence of hydrogen bonding and eutectic formation. Additionally, proton nuclear magnetic resonance (^{1}H NMR) was employed to characterize the molecular environments of citric acid, creatine, and the DES formulation. Spectra were recorded using a Bruker AVANCE 300 MHz^{1}H NMR spectrometer (Bruker, Billerica, MA) at room temperature. Samples of pure citric acid, creatine, and the DES mixture were dissolved in deuterated water (D_2O) as the solvent and tetramethylsilane (TMS) as an internal reference.

2.6. Kinetic study (temperature x time (min))

The extraction time was subsequently optimized under the selected operational conditions by evaluating six-time intervals (1–21 min). The selected time intervals were adapted from the methodology proposed based on previously methodologies [13,17]. Kinetic extraction curves were obtained by plotting pigment yield ($mg_{r\text{-}phycoerythrin}.\ g^{-1}_{biomass}$) versus time (min), enabling assessment of mass-transfer dynamics throughout the process. Temperature was continuously monitored using a thermocouple positioned in direct contact with the extraction medium and fixed adjacent to the ultrasound probe tip. Therefore, the recorded temperatures represent the region surrounding the cavitation zone, where the highest ultrasonic energy densities are expected.

2.7. Extract evaluation

2.7.1. Thermal stability

Extracts were submitted at three temperatures (55, 75, and 95 °C) using either the DES or pure water as solvents to enable direct comparison. Pigment stability was assessed by monitoring r-PE concentration ($mg_{r\text{-}phycoerytrhin}.\ g^{-1}_{biomass}$) over time at each temperature. Aliquots were collected at predetermined intervals and immediately cooled in an ice bath to rapidly quench thermal effects and prevent further heat-induced degradation [16]. The collected vials were analyzed by UV–vis spectrometry at a wavelength of 565 nm to check r-PE losses caused by heating over time.

2.7.2. Antioxidant activity (DPPH, ABTS and FRAP assays)

The antioxidant activity of the extracts was evaluated using three complementary assays: DPPH, ABTS, and FRAP. All analyses were performed in 96-well microplates using a microplate reader. The DPPH radical scavenging assay was conducted according to the method described by Brand- Williams et al.[18]. while the ABTS radical cation decolorization assay followed the procedure reported by Pellegrini et al. [19]. The ferric reducing antioxidant power (FRAP) assay was performed according to Urrea-Victoria et al.[20].For all assays, the antioxidant capacity was calculated based on the percentage reduction in absorbance relative to the control. Calibration curves were constructed using Trolox as standard, and regression equations were obtained for each assay (Fig. S2- Supplementary Material). The results were expressed as Trolox equivalents ($mg_{Trolox\ equivalent}.mL^{-1}_{extract}$). All measurements were performed in triplicate.

2.8. Sustainability assessment by the Path2Green metric

The sustainability evaluation of the developed method for the r-PE recovery was carried out using the *Path2Green* metric proposed by De Souza Mesquita et al; (2024). This metric was designed in accordance with the 12 principles of green biomass extraction. Within this framework, parameters were defined to assess attributes across both pre- and post-extraction stages, namely: (1) Biomass; (2) Transport; (3) Pretreatment; (4) Solvents; (5) Scaling; (6) Purification; (7) Yield; (8) Post-treatment; (9) Energy; (10) Application; (11) Repurposing; and (12) Waste management. Each principle received a score ranging from −1.00 to 1.00, with values closer to 1.00 reflecting stronger compliance with green extraction principles. The assessment was performed through the *Path2Green* mobile application. The resulting pictogram displays the overall score and the performance of the extraction process, highlighting potential "red-flag" areas where improvements in sustainability are required. Finally, the overall score is converted into $g_{CO_2}.g^{-1}_{biomass}$.

2.9. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (*one-way* ANOVA) followed by Bonferroni's post hoc test to compare extraction yields across different extraction unit operations and to identify the optimal extraction time. A separate one-way ANOVA was also conducted to evaluate the effect of different extraction solvents on yield. Statistical significance was defined at the 95% confidence level ($p < 0.05$), with all experiments performed in triplicate *($n = 3$)*. All analyses were carried out using JAMOVI software (version 2.3.21).

3. Results and discussion

3.1. Creatine based eutectic solvent design

The choice of a creatine-based DES was motivated by its innovative character, food-grade composition, and the limited exploration of its potential as an extraction medium for high-value biomolecules. Creatine is widely used as an ergogenic ingredient in the supplement industry, with a market valued at $350–550 million in the U.S. [14]. It exhibits zwitterionic character, strong hydrogen-bonding capability, and high biocompatibility, making it a suitable and functional candidate for DES design [21].

The use of a creatine-based DES presents distinctive features compared to the most commonly reported DES systems, particularly choline chloride (ChCl)-based and sugar-based formulations. In comparison, ChCl-based DESs are widely explored due to their versatility and extraction efficiency, but often rely on components not directly intended for ingestion without further processing [22]. Sugar-based

DESs offer excellent biocompatibility but may suffer from high viscosity, which can negatively affect mass transfer and extraction performance [23]. Similarly, the creatine-based DES investigated in this work may also present relatively high viscosity, which can limit solvent penetration and diffusion of target compounds. To overcome this limitation, the solvent system was experimentally optimized in combination with UAE, allowing improved mass transfer and enhanced extraction efficiency under reduced processing times.

The creatine-based eutectic solvents were formulated according to Heaney and collaborators (2022)[14], who demonstrated that mixtures containing 10–60 mol% creatine (HBA) with citric acid form stable deep eutectic systems, with eutectic behavior retained upon addition of up to 70 mol% water. Citric acid was selected as the HBD primarily for its molecular architecture: three carboxylic acid groups and one hydroxyl group provide multiple sites for strong hydrogen bonding with creatine's guanidino and amine functionalities, facilitating significant melting point depression. Its pKa (3.1, 4.8, 6.4) enables partial proton transfer to creatine without excessive acidity that could degrade the base component. Furthermore, citric acid (E330), authorized under Regulation (EC) No 1333/2008 and specified in Commission Regulation (EU) No 231/2012, is biodegradable, exhibits low toxicity (GRAS status), and aligns with green chemistry principles, attributes critical for developing sustainable extraction media compatible with food and pharmaceutical applications [24]. Citric acid is commonly employed as a preservative, acidulant, flavor enhancer, buffering agent, and stabilizer [25].

Building on the favorable physicochemical and safety profiles of creatine (HBA) and citric acid (HBD), COSMO-RS simulations were employed as a rational screening tool to identify eutectic compositions with enhanced affinity toward r-PE. Previous work demonstrated that creatine–citric acid mixtures form stable deep eutectic systems with elevated melting temperatures (363 K for certain compositions), supporting their structural robustness and potential applicability[14].

The screening strategy was conducted in two steps. First, the effect of water content was evaluated for the 1:1 M ratio by calculating the activity coefficient at infinite dilution ($\gamma\infty$) of r-PE at 30, 50, 70, and 90% water (Table S1- Supplementary Material). The results show that $\ln(\gamma\infty)$ becomes progressively less negative as the water content increases, indicating a reduction in solute–solvent interaction strength due to dilution effects. The formulation containing 30% water presented the most negative $\ln(\gamma\infty)$ value (−4.89), corresponding to the highest predicted thermodynamic affinity. The pure DES was not considered due to its very high viscosity, which limits mass transfer and practical handling, as previously reported [14].Therefore, 30% of water was selected as a compromise between molecular affinity and process feasibility.

In the second step, the influence of the C:CA molar ratio was evaluated at fixed 30% water. All investigated ratios (1:1 to 1:6) exhibited low $\gamma\infty$ values, indicating strong predicted interactions between r-PE and the eutectic medium (Table S1- Supplementary Material). Seven mixtures of creatine, citric acid, and water were formulated to identify compositions yielding a stable, homogeneous eutectic phase suitable for r-PE extraction from *Amansia* sp., as summarized in Fig. 1. The initial formulation adopted a 1:6C:CA molar ratio (14 mol% creatine) with 20% of water, conditions selected to ensure phase stability while remaining within the eutectic window reported by Heaney et al. [14]. Besides, this relatively low hydration level was chosen deliberately to preserve strong hydrogen-bonding interactions within the solvent network, which are critical for maintaining extraction efficiency, while still allowing systematic evaluation of water content effects on both phase behavior and pigment recovery [14].

However, the initial 1:6C:CA mixture containing 20% water did not form a stable homogeneous phase. Instead, crystallization was observed immediately after preparation (Fig. 1A), indicating the presence of a solid crystalline. This behavior is consistent with the phase diagram reported in the literature, where compositions outside the stable eutectic region exhibit coexistence of crystalline domains [14]. To improve phase stability, the water content was increased to 30% water and maintained constant while varying the molar ratio. This water content was also supported by COSMO-RS predictions, which indicated favorable intermolecular interactions between the hydrated DES system and r-PE, suggesting enhanced solvation capability at this composition.

The 1:1, 1:2, and 1:3 (C:CA) systems still showed phase separation accompanied by crystalline phase formation (Figs. 1B–D). Although the 1:3 mixture initially appeared transparent, crystallization occurred within 24 h, suggesting metastability. In contrast, ratios of 1:4, 1:5, and 1:6 yielded optically clear, thermodynamically stable single phases that remained homogeneous over time (Figs. 1E–G). These three stable

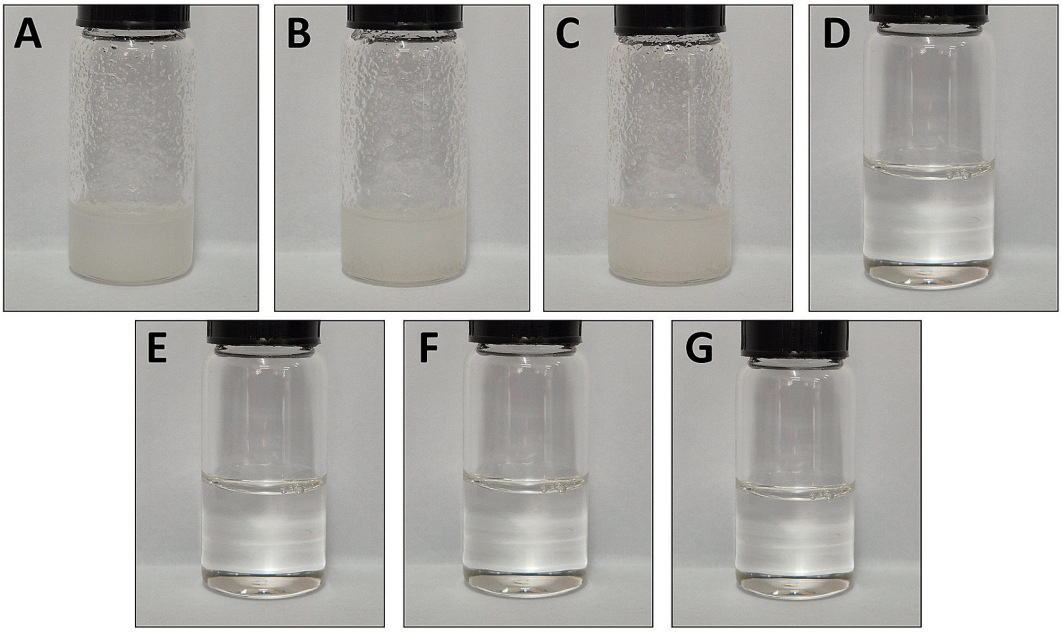

Fig. 1. Different mixtures using creatine, citric acid and water. (A) 1:6 creatine: citric acid (mol/mol) and 20% of water. (B) 1:1 creatine: citric acid (mol/mol) and 30% of water. (C) 1:2 creatine: citric acid (mol/mol) and 30% of water. (D) 1:3 creatine: Citric acid (mol/mol) and 30% of water. (E) 1:4: creatine: citric acid (mol/mol) and 30% of water. (F) 1:5 creatine: citric acid (mol/mol) and 30% of water. (G) 1:6 creatine: citric acid (mol/mol) and 30% of water.

formulations were selected for subsequent evaluation of their capacity to extract r-PE from *Amansia* sp.

3.2. r-Phycoerythrin extraction in ultrasonic bath (UAE_{bath})

To assess the extraction performance of the stable eutectic formulations, r-PE recovery from *Amansia* sp. was evaluated and benchmarked against water and PBS, which are conventional polar media widely used for phycobiliprotein extraction due to their compatibility with the pigment's hydrophilic and ionic characteristics [26]. Fig. 2 presents the extraction yields ($mg_{r\text{-}phycoerythrin}.g_{biomass}^{-1}$) obtained with the different solvents. For this stage, ultrasound-assisted extraction was performed in a bath system (UAE_{bath}) using a SLR of 0.50 $g_{biomass}.mL_{solvent}^{-1}$, following prior protocols [13,17]. Extractions were performed at 30 min and 120 min to evaluate the effect of sonication time on pigment recovery kinetics and to determine whether equilibrium extraction yields were attained within this timeframe.

The screening of these different extraction times for recovery r-PE from *Amansia* sp. revealed a distinct time-dependent behavior between eutectic solvents and conventional media. While water and PBS achieved higher yields after 30 min of UAE_{bath}, likely due to their low viscosity and rapid diffusion into algal cells, the C:CA eutectics required extended sonication to reach peak performance. After 120 min, the 1:6 (C:CA) formulation surpassed PBS and matched water, delivering the highest yield (~1.25 $mg_{r\text{-}phycoerytrhin}.g_{biomass}^{-1}$). This delayed but ultimately superior extraction efficiency reflects the eutectic's molecular design, meaning that a denser hydrogen-bonding network, facilitated by excess citric acid, gradually disrupts pigment–biomass interactions more effectively than simple polar solvents. The results demonstrate that eutectic solvents are not immediate substitutes for water but function as tunable, interaction-driven media whose extraction potential is unlocked through optimized process time, highlighting the importance of aligning solvent chemistry with operational parameters in biorefinery applications. Beyond the time-dependent extraction kinetics, a critical observation emerged once PBS exhibited a 10% decrease in r-PE yield after 120 min compared to 30 min (from 1.3 to 0.9 $mg_{r\text{-}phycoerythrin}.g_{biomass}^{-1}$), while water showed a similar behavior. This reduction strongly suggests that heat generated during UAE induced thermal degradation of the pigment in conventional aqueous media, as r-PE is known to be thermally labile above 40 °C [27]. Crucially, all eutectic formulations (1:4, 1:5 and 1:6C:CA) defied this trend, with yields increasing by 100–300% over the same period, most notably the 1:6 mixture (0.45 to 1.25 $mg_{r\text{-}phycoerythrin}.g_{biomass}^{-1}$). The temperature measurements were performed using a thermocouple immersed in the bulk extraction medium. After 120 min of sonication, the water system exhibited a temperature increase of approximately 40 °C, whereas the citric acid–creatine DES showed a lower temperature variation of approximately 33 °C, suggesting a greater thermal buffering capacity of the DES. This result demonstrates that DES is able to maintain a more stable temperature throughout the extraction process, highlighting a pivotal advantage of the eutectic system. Its dense hydrogen-bonding network likely acts as a thermal buffer, dissipating localized heat generated by cavitation and stabilizing the pigment's structure [28]. The contrast underscores that eutectics are not merely alternative solvents but functionally superior media that mitigate process-induced degradation, a critical consideration for heat-sensitive biomolecules like phycobiliproteins. This thermal resilience, coupled with their tunable chemistry, positions creatine-citric acid eutectics as robust, sustainable platforms for high-value pigment recovery where conventional solvents fail under prolonged processing.

3.3. FTIR e ^1H NMR of Creatine: Citric acid formulation

The spectroscopic analyses performed by FTIR (Fig. S3-Supplmentary Material) and ^1H NMR (Fig. 3) provide complementary evidence for the formation of the creatine: citric acid DES. Spectroscopic analyses of the crude extracts were not performed because the complexity of the extract matrix would likely generate highly overlapped signals, limiting the ability to obtain meaningful structural information regarding the extracted compounds. FTIR and ^1H NMR analyses provide clear evidence for the formation of the C:CA (1:6 mol/mol, 30% water) DES. The FTIR spectrum of the DES exhibits broader absorption bands than those of the individual components, particularly in the O–H/N–H stretching region (3000–3500 cm^{-1}) and in the 1400–1700 cm^{-1} region associated with C═O, COO^- and N—H vibrations. These spectral changes indicate the establishment of an extensive hydrogen-bonding network between citric acid and creatine. Furthermore, no new absorption bands were observed after DES formation, suggesting that the eutectic solvent is formed through strong intermolecular hydrogen-bonding interactions rather than the formation of new covalent bonds. Similar FTIR features have been widely reported for deep eutectic solvent systems and are considered characteristic of DES formation [29,30]. The ^1H NMR spectrum of the DES preserves all characteristic signals of both components, with no new resonances or disappearance of peaks, but with slight chemical shift variations and peak broadening, particularly for protons adjacent to hydrogen-bonding sites. These results confirm that DES formation occurs through strong non-covalent interactions rather than chemical reactions, leading to molecular reorganization while maintaining the structural integrity of creatine and citric acid.

3.4. r-Phycoerythrin extraction optimization using an ultrasonic probe (UAE_{probe})

Based on its confirmed eutectic behavior, and its superior performance in r-PE recovery, the 1:6C:CA (mol/mol) formulation was selected for process optimization. This solvent not only achieved the highest extraction yield (~1.25 $mg_{r\text{-}phycoerytrhin}.g_{biomass}^{-1}$), after 120 min of ultrasound treatment (UAE_{bath}) but also demonstrated thermal

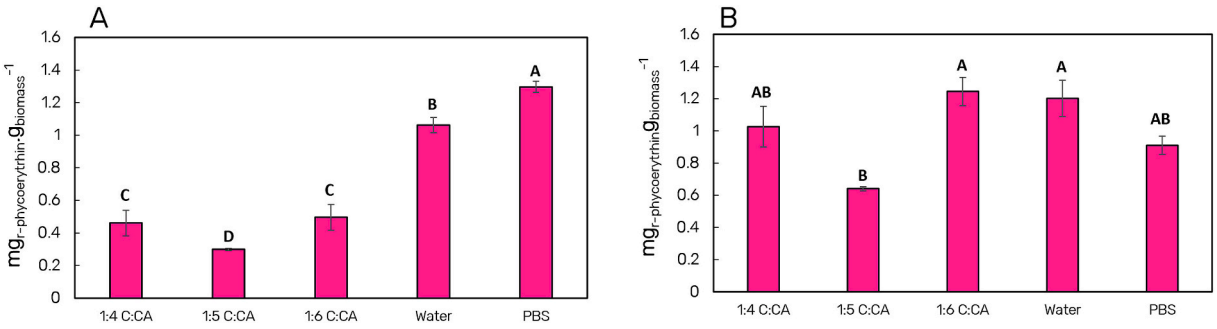

Fig. 2. r-Phycoerythrin extraction performed at UAE bath (600 W; SLR of 0.33 $g.mL^{-1}$). (A) Extraction made by 30 min. (B) Extraction made by 120 min. The bars show the extraction yield in $mg_{r\text{-}phycoerithrin}.g_{biomass}^{-1}$ with the standard deviation of the extraction made in triplicate ($n = 3$). Different letters above bars indicate statistically significant differences ($p < 0.05$). Bars sharing the same letter are not significantly different.

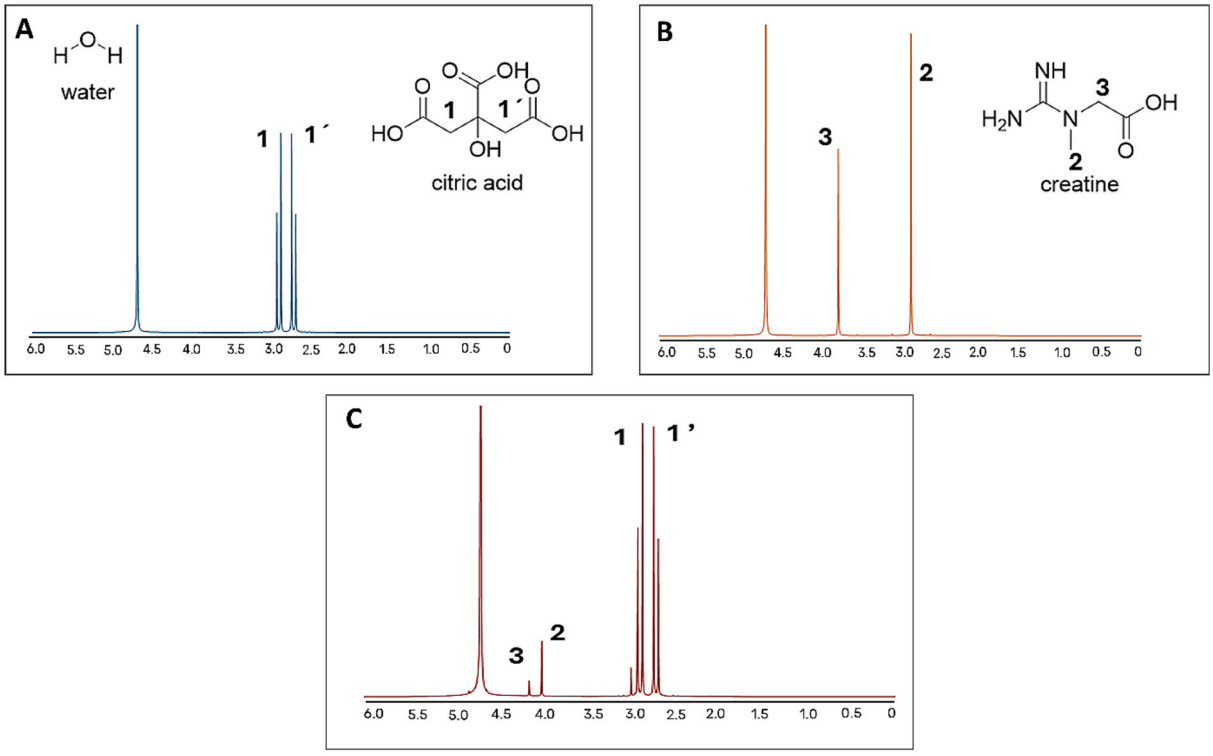

Fig. 3. ^{1}H NMR spectra of (A) citric Acid, (B) creatine, and (C) DES formulation (creatine:citric acid, 1:6 mol/mol with 30% water). The combined spectra confirm the presence of characteristic proton signals from both components, with no evidence of new peaks, indicating physical mixing and preservation of molecular identity within the eutectic system.

resilience. These combined attributes, establish the 1:6C:CA (mol:mol) as the most promising candidate for developing a robust, green extraction protocol for thermolabile phycobiliproteins. However, to enhance extraction performance and markedly shorten the processing time (from 120 min to only 5 min), the ultrasonic bath (UAE_{bath}) was substituted with an ultrasonic probe (UAE_{probe}) (Ultronique, 550 W, 20 kHz) [13]. In comparison with the bath system, the probe concentrates acoustic energy, generating stronger cavitation and promoting faster extraction kinetics [31]. Nevertheless, the higher intensity also increases the likelihood of pigment degradation, highlighting the need for careful optimization of both sonication power and exposure time.

A Central Composite Design (CCD) with three independent variables, namely, ultrasonic power (170.5–540 W), water content in the DES (30–90%), and solid liquid ratio (SLR) (0.08–0.92 $g_{biomass}{\cdot}mL_{solvent}^{-1}$) was employed to optimize r-PE extraction using the 1:6C:CA eutectic solvent. The design comprised 14 axial/factorial points plus three center-point replicates, totaling 17 experimental runs. The response variable was extraction yield ($mg_{r\text{-}phycoerytrhin}{\cdot}g_{biomass}^{-1}$). Regression analysis yielded a statistically significant predictive model (Eq. (1); $p < 0.05$ for all terms) with high explanatory coefficient ($R^2 = 0.91$) (Table S2 – Supplementary Material). Pareto analysis (Fig. S4- Supplementary Material) identified water content and SLR as the dominant factors influencing yield, while response surface contours (Fig. 4) visualized their interactive effects. Model-predicted optimum conditions were experimentally validated through four replicate extractions. Full experimental matrix and results are provided in Table S3- Supplementary Material).

$$Y = 0.31 + 0.06\,(Pw) - 0.37\,(\%w) + 0.08\,(\%w)^2 - 0.21\,(SLR) + 0.13\,(SLR2) \quad (1)$$

The response surface analysis (Fig. 4) elucidates the intricate interplay between ultrasonic power and water percentage in the C:CA (1:6 mol/mol), and SLR on r-PE extraction. Fig. 4A reveals that maximal pigment recovery occurs at moderate-to-high power (400–550 W) coupled with low water percentage (20–30%), where the DES maintains a robust hydrogen-bonding network essential for solubilizing r-PE; yields decline sharply beyond 30% water due to dilution of critical H-bond interactions, while excessive power (>550 W) could induce thermal degradation despite the eutectic's inherent thermal buffering capacity. Fig. 4B demonstrates that low SLR (0.1–0.2 $g_{biomass}{\cdot}mL_{solvent}^{-1}$) combined with high power optimizes extraction by ensuring sufficient solvent volume for complete biomass wetting and minimizing pigment re-adsorption, whereas SLR >0.3 causes rapid yield reduction due to biomass saturation. Crucially, Fig. 4C identifies a narrow synergistic optimum at water percentage of 20–30% and SLR of 0.1–0.2 $g_{biomass}{\cdot}mL_{solvent}^{-1}$, confirming that extraction efficiency depends on maintaining solvent excess to prevent competitive binding. This relationship, validated by the high predictive accuracy of the model ($R^2 = 0.91$), highlights the DES's unique advantage over conventional solvents meaning that its tunable physicochemical properties enable precise control over pigment-solvent interactions, allowing optimization of both solvent composition and process parameters to achieve >1.3 $mg{\cdot}g^{-1}$ yield while mitigating thermal degradation.

Thus, the optimal conditions for maximizing r-phycoerythrin extraction from *Amansia* sp. were identified as 30% water content in the DES, in 450 W, and a SLR of 0.08 $g_{biomass}{\cdot}mL_{solvent}^{-1}$. Under these conditions, the experimental yield reached 1.65 ± 0.03 $mg_{r\text{-}phycoerythrin}{\cdot}g_{biomass}^{-1}$ ($n = 3$), closely matching the predicted value of 1.72 $mg{\cdot}g^{-1}$ obtained from Eq. (1). This corresponds to a low relative error of 4.6%, with a difference of only 0.1 $mg_{r\text{-}phycoerythrin}{\cdot}g_{biomass}^{-1}$ between observed and predicted, indicating excellent model accuracy and reliability. This validation is presented in Table S4 - Supplementary material, and the predictive vs. experimental results are shown in Fig. S4- Supplementary material. Following optimization of key operational parameters (0.08 $g_{biomass}{\cdot}mL_{solvent}^{-1}$, 450 W and 30% water), the extraction duration was systematically evaluated to maximize r-PE recovery. Six time points (1,

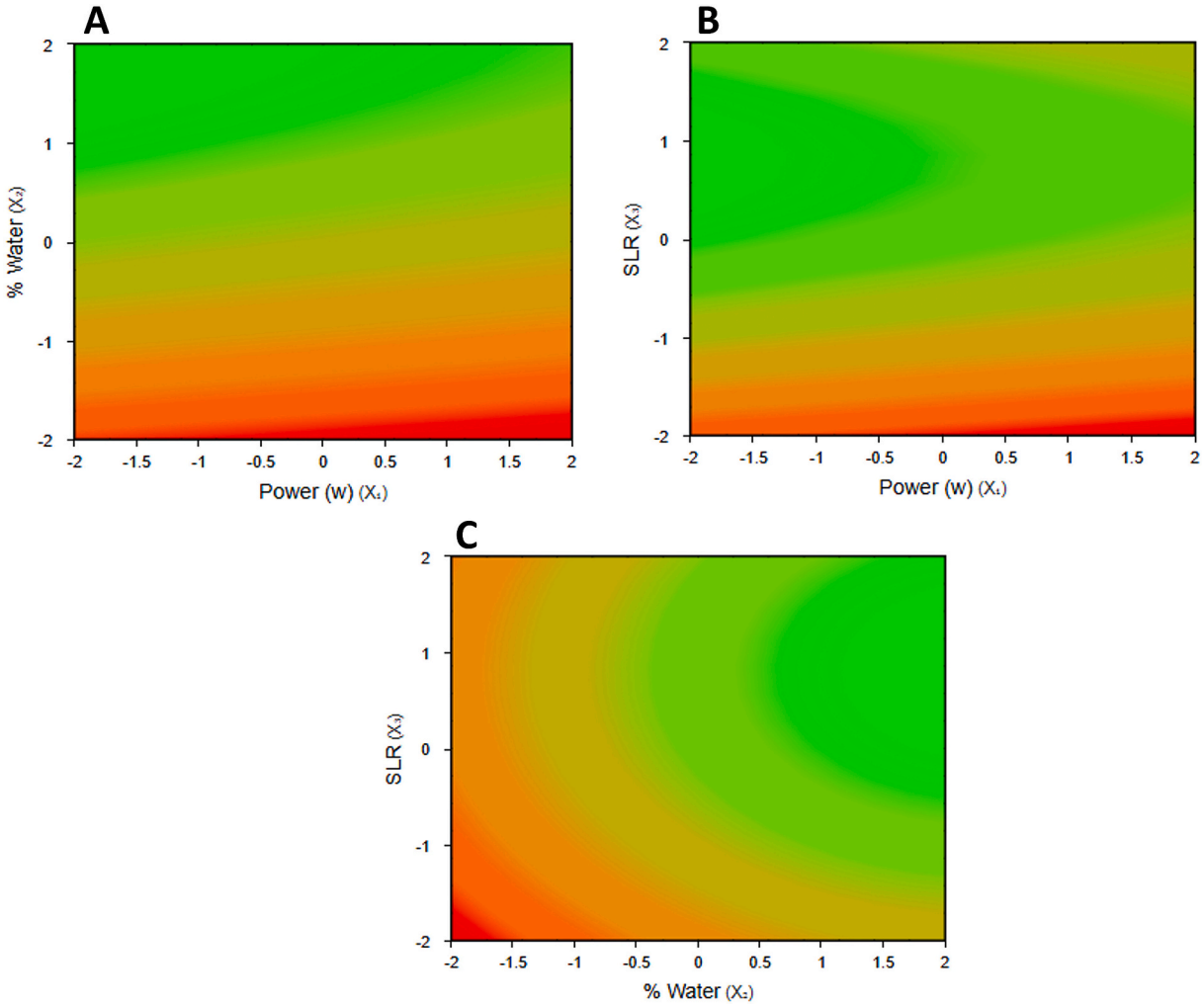

Fig. 4. Response contour plots obtained from the CCRD predictive model (2^3+ axial points) for the yield of r-phycoerythrin recovered from *Amansia* sp. from C:CA (1:6 mol/mol) in 5 min considering solid liquid ratio (SLR mg.mL $^{-1}$) cavitation power (W) and DES water content (%) as independent variables. A combination of independent variables was performed 2 by 2 as follows: %w vs. Pw (A); SLR vs. Pw (B) and (C) SLR vs. %w. The colors represent the amount of recovered phycoerythrin, with a stronger pink indicating a higher amount. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

5, 9, 13, 17, and 21 min) were assessed under controlled conditions, with real-time temperature monitoring to correlate thermal profiles with pigment stability. As shown in Fig. 5, this analysis revealed a non-linear relationship between extraction time and yield, where r-PE

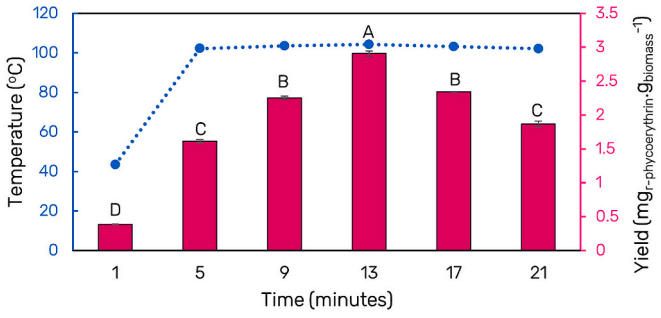

Fig. 5. Effect of extraction time and temperature on r-phycoerythrin yield from *Amansia* sp. Under ultrasound-assisted conditions. The bars represent pigment yield recovery $mg_{r\text{-phycoerythrin}} \cdot g_{biomass}^{-1}$ with statistical groupings indicated by letters (A–D). The purple dotted line shows the temperature profile. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

recovery increased rapidly during the initial 9 min before plateauing, with no significant degradation observed up to 21 min, further confirming the eutectic's thermal protective capacity. Critically, the peak yield (2.90 ± 0.04 $mg_{r\text{-phycoerythrin}} \cdot g_{biomass}^{-1}$), was achieved at 13 min, representing an improvement over conventional 30 min protocols based on UAE_{bath} while minimizing energy input and thermal exposure. This optimized duration aligns with the eutectic's kinetic profile: sufficient time for hydrogen-bond-mediated pigment solubilization without inducing heat-induced degradation, a limitation observed in aqueous systems under prolonged sonication. The temperature remained below 40 °C only for 1-min extraction, underscoring the DES's role in dissipating cavitation energy and preserving r-PE integrity.

Critical temperature monitoring revealed a rapid initial rise from 43.5 °C to 102.2 °C within 5 min, followed by a stable plateau throughout the 21 min extraction (102.1 °C) (Fig. 5). This moderated thermal profile, attributed to the DES's hydrogen-bond network dissipating cavitation energy, contrasts sharply with conventional aqueous systems where uncontrolled heating occurs. The r-PE yield exhibited a biphasic trend: a steep increase up to 13 min (peak yield: 2.90 ± 0.04 $mg_{r\text{-phycoerythrin}} \cdot g_{biomass}^{-1}$), statistically superior to all other time points ($p < 0.05$), followed by a decline at 21 min (peak yield 1.86 $mg \cdot g^{-1}$). The initial ascent reflects ultrasound-enhanced mass transfer and cell disruption, facilitating efficient pigment release from *Amansia* sp.

biomass. The UAE operates through the conversion of electrical energy into mechanical energy by transducers, generating high-frequency vibrations (>20 kHz). When applied to liquids, ultrasound can induce acoustic streaming, and if gas nuclei are present in the medium, they undergo cycles of compression and rarefaction that may culminate in collapse. This collapse of microbubbles, known as "acoustic cavitation," can occur either symmetrically or asymmetrically. In the case of symmetric collapse, shock waves are produced, enhancing agitation and energy transfer within the medium and thereby facilitating the disruption of intermolecular interactions between target compounds and the surrounding matrix [32]. Crucially, the 13 min optimum represents the critical equilibrium point where cavitation-driven solubilization maximizes before degradation mechanisms begin to outweigh extraction gains. Although the bulk temperature remained relatively stable after 13 min, the prolonged exposure of r-phycoerythrin to elevated temperatures, combined with the localized hot spots, shear forces, and microjets generated during ultrasonic probe operation, may contribute to protein denaturation and chromophore degradation [33,34]. Consequently, the reduction in yield observed after 13 min is likely the result of cumulative thermal and mechanical stresses rather than temperature alone. This finding underscores the DES's dual functionality: (i) enabling rapid extraction kinetics through optimized hydrogen-bond interactions, and (ii) delaying degradation via thermal buffering, validating its superiority over water/PBS where unmitigated heating occurs. These results establish a precise, energy-efficient extraction protocol (13 min, 450 W) that maximizes yield while preserving pigment quality—essential for food-grade applications were thermal degradation compromises colour stability and bioactivity. A control experiment was conducted using an aqueous citric acid solution prepared at the same effective concentration as present in the DES system (3.9 mol L^{-1}), corresponding to the citric acid content derived from the 1:6 creatine: citric acid molar ratio with 30% water (Fig. S5- Supplementary Material). The extraction was performed under optimized conditions to those applied for the DES. The results demonstrated that the aqueous citric acid solution resulted in a significantly lower r-PE extraction yield (1.675 ± 0.11 mg $_{\text{r-phycoerythrin}}.g^{-1}_{\text{biomass}}$) compared to the DES system (2.90 ± 0.04 mg $_{\text{r-phycoerythrin}}.g^{-1}_{\text{biomass}}$). These findings confirm that, although citric acid is the dominant component in the DES formulation, the eutectic medium and the specific interactions between creatine and citric acid play a crucial role in enhancing extraction performance beyond the effect of citric acid alone.

3.4.1. Applicability of the extraction process to other red macroalgae

To evaluate the applicability of the proposed phycoerythrin extraction process additional experiments were carried out with different red macroalgae species, including *Amansia* sp., *Hypnea* sp., *Halymenia* sp., *Gracilaria* sp., and *Kappaphycus* sp. These species were selected to represent distinct taxonomic groups within the Rhodophyta, allowing us to assess whether the extraction protocol previously optimized for *Amansia* sp. could be successfully extended to other algal matrices.

Phycoerythrin-containing extracts were successfully obtained from all species evaluated, with significant extraction yields, demonstrating that the proposed method is not restricted to the biomass used during the optimization stage. These findings indicate the broad applicability of the process and highlight its potential as a versatile platform for phycoerythrin extraction from different red macroalgae. The extraction yields obtained for each species are presented in Fig. S6- Supplementary material. As expected, the extraction performance varied among the biomasses, which can be attributed to intrinsic differences in the biochemical composition of each species, particularly regarding the initial phycoerythrin content, its intracellular distribution, and its overall bioavailability. Furthermore, structural characteristics of the algal matrix, such as cell wall composition and pigment organization, may also influence extraction efficiency. Therefore, although the final yield depends on the specific characteristics of each biomass, the results demonstrate that the creatine-based DES extraction process exhibits considerable potential for application across a wide range of red macroalgae, reinforcing its technological robustness and its relevance as a sustainable approach for the production of natural pigments.

Also, the r-PE yield obtained in the present study (2.90 ± 0.04 $mg_{\text{r-phycoerythrin}}.g^{-1}_{\text{biomass}}$), compares favorably with values reported for several red macroalgae extracted using conventional aqueous media. For example, r-PE recoveries ranging from approximately 1.2 to 1.7 $mg_{\text{phycoerythrin}}.g^{-1}_{\text{biomass}}$ biomass have been reported for species such as *Gracilaria birdiae* and *Palmaria palmata* using water- or buffer-based extraction systems [35,36]. Also, the comparison of the extraction yield obtained in the present study with previous DES-based approaches is challenging due to the scarcity of studies in this field. [37] employed a choline chloride–urea deep eutectic solvent in an aqueous two-phase system for the purification of r-PE from *Porphyra yezoensis*. However, the study focused on protein purification, preventing direct quantitative comparison with the present results. Therefore, the results presented here not only demonstrate the effectiveness of the citric acid–creatine DES as an extraction medium but also contribute novel insights into the application of sustainable solvents for the recovery of high-value phycobiliproteins from marine biomass.

3.5. Thermal stability

The thermal stability of r-PE represents a critical bottleneck for its incorporation into food products, where thermal processing steps such as pasteurization (70–95 °C) and sterilization are routinely applied [27]. During extraction, particularly under ultrasound irradiation, localized heating, oxidative stress, and prolonged exposure to aqueous environments can accelerate chromophore dissociation, protein denaturation, and irreversible colour loss [38]. Consequently, strategies to enhance r-PE stability under thermal stress are essential for commercial viability. Organic acids, notably citric acid, have demonstrated protective effects by chelating metal ions that catalyze oxidation and by modulating solution pH to preserve the pigment's native conformation [38,39]. Given that the optimized DES comprises creatine and citric acid at a 1:6 M ratio, this section evaluates whether the DES matrix itself confers intrinsic thermal protection to r-PE beyond the stabilizing effect of citric acid alone. Comparative stability assays were conducted in the DES versus conventional media (water and PBS) under controlled heating to determine whether the hydrogen-bonded eutectic network mitigates degradation kinetics and extends the pigment's functional shelf life in thermally demanding food applications [38,39].

However, there are still no studies directly addressing the role of creatine-based solvents in modulating the thermal stability of natural compounds, like r-PE. Evidence from other natural phytochemicals suggests that solvent choice can be decisive in this subject. For instance, recent studies have demonstrated that alternative solvents, particularly eutectic mixtures, can significantly increase the stability of bioactive molecules during extraction. Anthocyanins obtained from Brazilian berry wastes [17] and flavonoids extracted from lemon peel [16] showed markedly higher stability when recovered with DES compared to conventional systems (based on ethanol, methanol and water). These findings highlight the potential of solvent engineering as a promising strategy to preserve sensitive biomolecules. In this context, Table 1 shows the results regarding the thermal stability of the r-PE considered in this study extracted from the *Amansia* sp. with C:CA (1:6 mol:mol) solvent compared to water.

The comparative evaluation of the thermal stability of r-PE extracted with two different solvents reveals distinct patterns of degradation kinetics. At 55 °C, the protein extracted in water exhibited a lower degradation constant (Kd = 0.0011 min^{-1}) and consequently a longer half-life (651 min) compared to the C:CA (Kd = 0.0015 min^{-1}; $T_{1/2}$ = 467 min). This suggests that under mild thermal stress, water provides a favorable environment for maintaining pigment stability. However, as the temperature increases, the protective effect of water decreases. At 75 °C, the C:CA solvent exhibited a substantially lower degradation

Table 1
Influence of the extraction solvent (creatine:citric acid and water) on the r-phycoerythrin thermal stability extracted from *Amansia* sp. (55 °C, 75 °C, and 95 °C).

Extraction solvent	55 °C		75 °C		95 °C		Ea (KJ/Mol)	R^2
	Kd (min^{-1})	$T_{(1/2)}$ (min)	Kd (min^{-1})	$T_{(1/2)}$ (min)	Kd(min^{-1})	$T_{(1/2)}$ (min)		
Creatine: Citric acid*	0.0015	467.47	0.006	105.38	0.0168	41.22	61.10	0.9905
Water	0.0011	651.00	0.02	35.55	0.1940	3.57	130.74	0.9988

Kd = Thermal degradation constant; $T_{(1/2)}$ = Half-life time (min); Ea = Thermal activation energy. *Extract obtained under the optimum operational conditions (1:6 mol:mol; 30% water w:w).

constant (Kd = 0.006 min^{-1}) than water (Kd = 0.0200 min^{-1}), resulting in a markedly longer half-life (105 min versus 35 min). This trend becomes even more pronounced at 95 °C, where the pigment in water undergoes rapid degradation (Kd = 0.1940 min^{-1}; $T_{1/2}$ = 3.57 min), while the C:CA still sustains a half-life of 41 min, highlighting its superior stabilizing capacity under severe thermal stress.

The activation energy (Ea) values further illustrate the contrasting behavior of the two solvents. The Arrhenius plots (Fig. S7- Supplemetary Material) further highlight the different temperature dependence of r-PE degradation in the two solvents. Although the activation energy of the C:CA DES (61.10 kJ mol^{-1}) was lower than that of water (130.74 kJ mol^{-1}), the degradation kinetics cannot be interpreted based on Ea alone, since both the activation energy and the pre-exponential factor determine the temperature dependence of the degradation constant. While water showed a slightly lower degradation rate at 55 °C, its degradation constant increased much more sharply with temperature. In contrast, the DES exhibited a more moderate increase in Kd, resulting in substantially greater pigment stability at 75 and 95 °C.

This apparent paradox may be explained by the stabilizing interactions between creatine, citric acid, and the protein structure, which mitigate conformational changes and aggregation typically induced by heat. Taken together, these findings indicate that provides a more robust protective effect under higher-temperature conditions (besides higher extraction yield). This enhanced stability is particularly relevant for industrial and biotechnological applications where thermal processing is unavoidable, such as in food formulation, pharmaceuticals, and fluorescence-based technologies [40].

3.6. Antioxidant analysis

Beyond its role as an extraction medium and thermal stabilizer, the C:CA DES may confer additional functional benefits by mitigating oxidative stress during pigment recovery, a critical factor given that r-PE degradation proceeds via both thermal denaturation and oxidation of its phycobilin chromophores. To evaluate whether the DES matrix actively preserves or enhances the antioxidant capacity of the extracted pigment, three complementary assays (DPPH, ABTS and FRAP) were employed to capture distinct redox mechanisms: hydrogen atom transfer, single electron transfer, and ferric ion reduction, respectively. Extracts obtained under optimized DES conditions (1:6C:CA, 30%w 13 min UAE) were compared against conventional aqueous extracts while the DES itself was independently assayed to quantify any intrinsic radical-scavenging contribution from its components. This dual assessment, pigment integrity and solvent antioxidant potential, addresses whether the DES functions merely as a passive carrier or actively participates in stabilizing r-PE redox-active structure, a distinction with direct implications for the shelf-life and functional performance of algal pigments in oxidizing food matrices.

The three antioxidant assays revealed that solvent selection critically modulates the redox capacity of r-PE extracts (Fig. 6). In the DPPH assay (Fig. 6A), the DES-extracted pigment exhibited the highest radical-scavenging activity (5.39 ± 0.25 $mg_{Trolox\ equivalent}.mL^{-1}_{extract}$), surpassing both the aqueous extract (2.20 ± 0.38 $mg_{Trolox\ equivalent}.mL^{-1}_{extract}$) and the DES control (1.04 ± 0.48 $mg_{Trolox\ equivalent}.mL^{-1}_{extract}$). This hierarchy indicates that the DES not only extracts r-PE more efficiently but also preserves the structural integrity of its phycobilin chromophores,

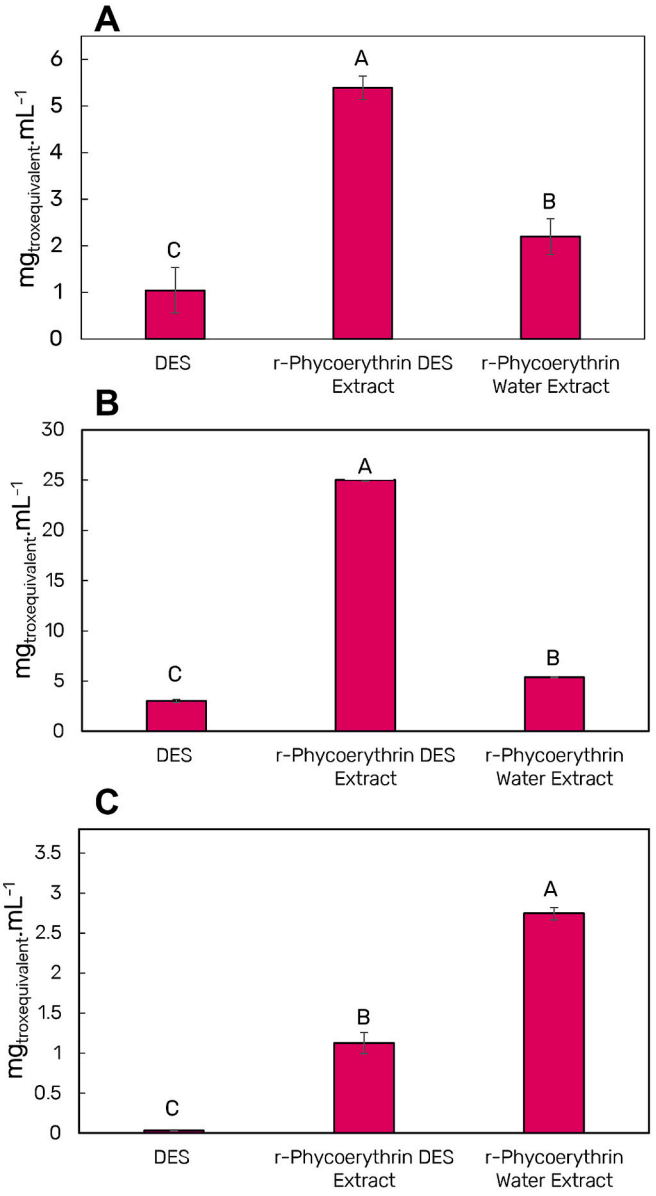

Fig. 6. Antioxidant capacity evaluation of the extracts by (A) radical scavenger DPPH assay; (B) radical scavenger ABTS assay; and (C) ferric reducing FRAP assay. Different letters above bars indicate statistically significant differences ($p < 0.05$).

key sites for hydrogen atom donation, consequently enhancing intrinsic antioxidant function. Creatine itself plays multiple roles in human metabolism, including the suppression of pro-inflammatory signaling and mitigation of oxidative stress associated with the senescence-associated secretory phenotype. These properties align with the antioxidant results, highlighting that the solvent system, together with r-PE, may contribute to human health benefits [41].

The modest activity of the DES control reflects citric acid's metal-chelating capacity, which contributes minimally to direct radical quenching but may indirectly protect the pigment during extraction by sequestering pro-oxidant metal ions. Similar trends were observed in ABTS assay (Figs. 6B), where DES extracts consistently outperformed aqueous counterparts by 5×, confirming that the eutectic environment mitigates oxidative damage during recovery. These findings align with recent evidence that DES formulations stabilize redox-sensitive phytochemicals by forming protective hydrogen-bond networks around labile functional groups [17].

The contrasting FRAP results (Fig. 6C), where the aqueous extract exhibited higher reducing capacity than the DES extract, reflect fundamental differences in assay mechanisms and solvent selectivity rather than inferior extract quality. FRAP quantifies direct ferric ion (Fe^{3+}) reduction under acidic conditions (pH 3.6), a reaction dominated by small, highly hydrophilic reducing agents such as ascorbate derivatives, free phenolics, or exposed thiol groups. Water, as a pure polar solvent, efficiently partitions these low-molecular-weight reductants into the extract phase. In contrast, the creatine–citric acid DES (30 w%) maintains an extensive hydrogen-bond network that may retain such hydrophilic species within the solvent matrix or alter their redox potential through solvation effects, thereby diminishing their contribution to FRAP activity. To investigate this hypothesis, an additional experiment was performed using ascorbic acid as a model hydrophilic antioxidant. At the same concentration (0.2 mg. mL), ascorbic acid dissolved in water exhibited a FRAP value of 44.39 ± 2.74 μg_{TE}. mL^{-1}, whereas the corresponding solution prepared in the DES showed a significantly lower value of 15.48 ± 0.90 $\mu g_{TE.}$ mL^{-1} (Fig. S8- Supplementary Material). This nearly threefold reduction supports the hypothesis that interactions between antioxidant molecules and the DES hydrogen-bond network can decrease the apparent reducing capacity measured by the FRAP assay. Therefore, the lower FRAP activity observed for the DES extract may not necessarily indicate a lower antioxidant content, but rather a reduced accessibility of reducing compounds to the ferric complex under the assay conditions. Moreover, it is well established that the antioxidant activity of DES depends on their specific formulation, the amount of water present, and the nature of the compounds extracted. These factors explain the differences observed among the antioxidant assay results [42].

Critically, the DES extract outperformed aqueous counterparts in both DPPH (Fig. 6A) and ABTS (Fig. 6C) assays, which are mechanistically distinct tests that evaluate hydrogen atom transfer and cationic radical neutralization, respectively. This superiority arises because the DES preserves the native conformation of r-phycoerythrin's phycobilin chromophores (as evidenced in thermal stability assays) and co-extracts amphiphilic co-antioxidants that synergistically quench diverse radical species. Thus, the FRAP anomaly does not indicate DES inadequacy but rather underscores assay-specific biases, meaning that FRAP favors small hydrophilic reductants, whereas DPPH and ABTS respond to structurally complex radical scavengers better preserved in eutectic media.

3.7. Greennes assessment by the Path2Green metric

The sustainability of the process was assessed through the *Path2-Green* metric, which relies on twelve guiding principles of green extraction processes[43]. The evaluation, performed using the *Path2-Green* mobile app, is presented in Fig. 7, while the detailed scores for each principle are provided in Table S5 of the supplementary material. The proposed method achieved a *Path2Green* score of 0.142, indicating a positive pathway toward sustainability according to the principles evaluated by this metric. Based on the correlation established within the *Path2Green* framework, this score corresponds to an estimated carbon-footprint indicator of approximately 568 $g_{CO_2} \cdot g_{biomass}^{-1}$. This value should not be interpreted as a direct greenhouse gas emission measurement or as the result of a complete life-cycle assessment (LCA).

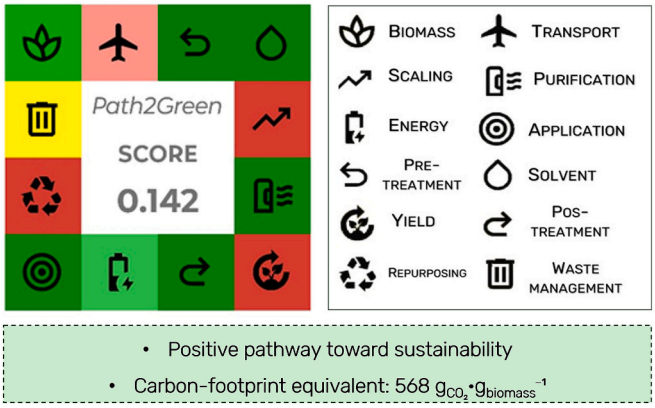

Fig. 7. Pictogram illustrating the final score of the *Path2Green* metric, which is based on the 12 principles of green extraction. The principles are depicted around the pictogram in varying shades of green, yellow, and red: green represents high adherence to the principle, yellow indicates a neutral score, and red signifies poor adherence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Instead, it represents a comparative sustainability indicator derived from the Path2Green methodology (that convert score into $g_{CO_2} \cdot g_{biomass}^{-1}$). It is important to highlight that Path2Green was validated by comparing sustainability scores from different extraction studies with carbon-footprint values obtained from LCA-based assessments, expressed in g $g_{CO_2} \cdot g_{biomass}^{-1}$. Thus, the value reported here is based on an established correlation between the Path2Green score and LCA-derived carbon-footprint data from the literature. Accordingly, the indicator provides a comparative estimation of the sustainability profile of the extraction pathway, rather than an absolute process-specific emission factor. Furthermore, the carbon-footprint indicator is not associated with whether the biomass was used on a fresh or dry basis. Rather, it reflects the overall sustainability performance of the proposed extraction process within the Path2Green framework. The positive score observed in this study is mainly explained by the high scores obtained for Principles 3, 4, 6, 8, and 10, highlighting the advantages of the method regarding solvent selection, process efficiency, safety, energy demand, and applicability.

Principle 3 (Pre-treatment: optimization to avoid unnecessary steps and adoption of cost-effective strategies) achieved the highest sustainability score (1.000), as no preliminary treatment of *Amansia* sp. was required. This result is attributed to the absence of interfering compounds that could compromise pigment integrity, allowing the direct extraction of target pigments without the need for pre-treatment and without affecting extraction selectivity. Additionally, Principle 4 (Solvents) received the highest score due to the use of natural, non-toxic, and biodegradable solvents. Creatine is a naturally occurring nitrogen-containing compound that plays an integral role in cellular metabolism [44] and Citric acid, is found in most fruits, especially citrus [45]. These solvents contributed to the process sustainability due to its biological origin, biodegradability, and, in the case of DES formulation, non-volatility.

Principles 6 (Purification) and 8 (Post-treatment) achieved the maximum score (1.000), indicating that the extracts can be used directly without additional purification or modification. The purity of r-PE was estimated using the A_{565}/A_{280} ratio. A purity index of 0.235 was obtained, indicating the co-extraction of other proteins and water-soluble compounds from the algal biomass. This result was expected, as the objective of the present study was to maximize r-PE recovery using a sustainable DES rather than to perform protein purification. In applications such as colorants, crude extracts are often acceptable and may even be advantageous due to synergistic effects among their constituents (such as annatto colorants and betacyanins from beetroots) [13,46].

Principle 10 (Application) achieved the maximum score (1.000), reflecting the production of safe, natural extracts with antioxidant properties and broad applicability across food, nutraceutical, pharmaceutical, and biomaterial sectors. Principle 1 (Biomass selection) received a high score (0.750) due to the use of naturally abundant *Amansia* sp., while Principle 2 (Transport) showed a negative score (−0.44) because of the long-distance transport (1920 km) between the collection site and processing facility, highlighting logistics as a key sustainability challenge.

Principles 5 and 9 received penalties due to limitations inherent to the extraction method. Principle 5 (Scaling) scored −1.000 because the process is conducted in batch mode, which limits continuous operation. However, batch processing is advantageous in exploratory and small-scale studies, as it allows better control of extraction parameters and safer evaluation of novel solvents such as eutectic mixtures [47,48]. Principle 9 (Energy) scored −0.50. Although renewable energy was used, the relatively high energy demand of the ultrasound system negatively affected the score, indicating the need for improved energy efficiency to better align with sustainability goals.

Principles 7, 11, and 12 obtained lower scores, indicating aspects that require further improvement. Principle 7 (Yield) scored −0.5 due to the semi-exhaustive extraction, which efficiently recovers r-PE but leaves other compounds unvalorized. Principles 11 (Repurposing) and 12 (Waste management) also showed limitations related to waste generation. From a biorefinery perspective, the residual biomass may represent a valuable feedstock for the recovery of sulfated polysaccharides, proteins, minerals, and other bioactive compounds, contributing to the development of integrated valorization strategies with minimal waste generation. Despite this, the method introduces creatine-based eutectic solvents as an innovative and bio-based approach, and the *Path2Green* assessment highlights opportunities to improve sustainability through future integration of biomass valorization and closed-loop biorefinery strategies.

4. Conclusion

This study demonstrates that creatine-based deep eutectic solvents are a promising and sustainable approach for extracting r-phycoerythrin from *Amansia* sp., overcoming common challenges such as pigment instability and low yields. The combination of creatine and citric acid (1:6 M ratio) formed stable eutectic systems that enhanced pigment solubilization while preserving stability and antioxidant activity. Coupling these solvents with ultrasound-assisted extraction improved efficiency and reduced extraction time. Under the optimized extraction conditions (solid-to-liquid ratio of 0.08 $g_{biomass} \cdot mL^{-1}_{solvent}$, ultrasonic power of 450 W, 30% water in the DES, and an extraction time of 13 min). Notably, creatine acts both as a solvent component and a bioactive ingredient, enabling the direct use of the extract without purification and supporting circular bioeconomy principles. Future studies should focus on the comprehensive chemical characterization of the extracts obtained with the DES in order to identify potential co-extracted bioactive compounds and to better understand their contribution to the overall antioxidant activity of the extract. Although the DES components (citric acid and creatine) are generally regarded as low-toxicity compounds, further studies evaluating the cytotoxicity and biocompatibility of the resulting extracts are necessary to confirm their safety for cosmetic, pharmaceutical, or biomedical applications.

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 data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.seppur.2026.139534.

Data availability

No data was used for the research described in the article.

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