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Evaluation of Deep Eutectic Solvents for the Extraction of Fucoxanthin from the Alga *Tisochrysis lutea* - COSMO-RS Screening and Experimental Validation

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ABSTRACT

Fucoxanthin is a carotenoid in algae with purported beneficial health-related properties including antioxidant, anti-photoaging, anti-metastasis, anti-hypertensive activity and more. These properties give fucoxanthin the potential to be used in cosmetic, dietary, and medicinal applications. This study evaluates the use of deep eutectic solvents (DESs) to extract fucoxanthin from the microalgae *Tisochrysis lutea*. Conductor-like Screening Model for Real Solvents (COSMO-RS) was used to screen the performance of 24 different types of DESs in the extraction of fucoxanthin based on their calculated capacities. Experimental extraction validation was then carried out using the 6 top-ranked DESs. The experimental results revealed that the extraction capacity of the thymol: dodecanoic acid DES (1.25: 1 molar ratio) for fucoxanthin was the most efficient (7.69 mg/g dry biomass weight (DW)) among the DESs explored under the screening conditions and was higher than the capacity of the conventional solvents methanol (6.29 mg/g DW) and ethanol (6.75 mg/g DW). This corresponded with COSMO-RS screening results. Then, the optimisation of extraction conditions for fucoxanthin using thymol: dodecanoic acid DES was further investigated, revealing that the highest yield of fucoxanthin (22.03 mg/g DW) was extracted at optimum experimental conditions at a temperature of 36.2 °C, stirring time of 2.58 h, and the biomass percentage of 11.36%. Additionally, fucoxanthin showed good stability in thymol: dodecanoic acid DES over eleven days of storage. After seven extraction cycles, the final fucoxanthin concentration (13.06 mg/mL DES) resulted in a good reusability of the terpene-based food safe DES.

KEYWORDS: Fucoxanthin; Extraction; COSMO-RS; Deep eutectic solvents; Microalgae; *Tisochrysis lutea*; Food safe extraction.

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1. INTRODUCTION

Fucoxanthin is a major carotenoid found in many algae, especially in brown algae, with potentially valuable bio-functional properties [1]. Fucoxanthin has a molecular structure ($C_{42}H_{58}O_6$) that consists of an allelic bond, a 5,6-monoepoxide, and nine conjugated double bonds in a polyene chain, which provides strong antioxidant activity [2]. Numerous beneficial properties and biological functions of fucoxanthin have been investigated, and include antioxidant, anticancer, antidiabetic, and anti-inflammatory effects [2-5]. An essential characteristic of fucoxanthin is its antioxidant activity, which is characterised by the ability of substances to block the propagation stage in oxidative chain reactions to reduce reactive oxygen species (ROS). Fucoxanthin has been identified as an effective radical scavenger [2]. Sangeetha et al. have investigated the effect of fucoxanthin on oxidative stress indicators, which showed that fucoxanthin's antioxidant activity helps protect cell membranes against lipid peroxidation caused by retinol deficiency [3]. Hence, fucoxanthin could prevent some diseases related to oxidative stress. Furthermore, researchers have revealed that the apoptosis-inducing effect of fucoxanthin could inhibit the growth of tumour cells. Hosokawa *et al.* reported that fucoxanthin is able to induce apoptosis of HL-60 cells due to its antiproliferative activity [1]. Ganesan et al. report that fucoxanthin has greater cytotoxic effects against cultured HL-60 cells, MCF-7 breast cancer cells, and Caco-2 colon cancer cells, than similar carotenoids astaxanthin and β-carotene [5]. Therefore, it suggests that fucoxanthin has potential for new anticancer product developments in the food or biopharmaceuticals and cosmeceutical industries.

Fucoxanthin represents approximately 10% of the estimated total carotenoid production in the marine environment [1]. Therefore, fucoxanthin has received considerable attention from the functional food, nutraceutical, biopharmaceutical, and cosmetic/cosmeceutical industries for its potential applications in human health. Although it is expected that the fucoxanthin market size will expand to reach $123 million by 2025 [6], there are still many challenges to fucoxanthin production. For example, it can be produced chemically, but its chemical synthesis is complex and inefficient [7]. In contrast, extracting fucoxanthin from natural sources such as its extraction from various algal sources is considered more environmentally sustainable and cost-effective, but suitable extraction methods of fucoxanthin have not been identified and standardised yet.

Seaweeds are traditionally consumed as food in South East Asian countries such as China, Japan, and Indonesia. However, their popularity is growing due to their high nutritional values as foods, and as they represent a possible source of compounds with bioactive potential like fucoxanthin. Fucoxanthin can be

extracted in higher quantities from brown macroalgae compared to red or green macroalgae [8]. Fucoxanthin is an abundant pigment in many brown macroalgae [9]. Fucoxanthin has been studied in brown macroalgae groups including *Undaria* spp. [10], *Sargassum* spp. [6], *Ascophyllum* spp., *Laminaria* spp. [11], and it is found in some related microalgae also such as *Tisochrysis lutea* (previously referred to as *Isochrysis* affinis *galbana* and still sometimes referred to in the aquaculture industry as *T-Iso* or the 'Tahitian strain') [12, 13]. *Tisochrysis lutea* is a flagellated golden-yellow haptophyte marine microalga that is evolutionarily related to the brown seaweeds. It is a common microalgal source of animal food and is often used to feed mollusc, shrimp and other aquaculture species larvae [14] and it has a comparatively high content of fucoxanthin and dietarily important polyunsaturated fatty acids [15-18].

Processing methods for the extraction of fucoxanthin from microalgae are determined by the cost of operation, the yield and quality of products, and the demand for quality. Currently, conventional extraction methods, including maceration, Soxhlet, or vortex extractions, are currently the most used methods to extract fucoxanthin [19, 20]. Although these extraction methods have easy operation and can be relatively low cost, they are time-consuming and have potential toxicity dependent on the nature of the solvents used for the extraction. Common solvents used to extract fucoxanthin from microalgae are methanol, ethanol, ethyl acetate and acetone [15]. However, these solvents can not satisfy both environmental and economic concerns. Supercritical carbon dioxide extraction is one of the current greener technologies used to extract fucoxanthin from brown algae, where carbon dioxide at high pressure (74-300 bar) and a specific temperature (31-60 °C) is used as the solvent. Despite its good performance, the extraction system is complicated with high capital and operating costs, its need for co-solvents and poor selectivity for fucoxanthin [21]. Several studies have found that using co-solvents such as ethanol, vegetable oils, and water in supercritical carbon dioxide extraction slightly improved the yield of fucoxanthin from microalgae [22-24]. Green solvent extraction using environmentally benign alternative solvents can be more selective, faster, more sustainable, lower cost and operate at lower temperatures, which avoids the risk of thermal degradation of heat-labile components.

Interest in a new generation of solvents called deep eutectic solvents (DESs) has increased recently due to their potential application as green solvents [25-28]. These DESs possess excellent physicochemical properties, such as negligible volatility, adjustable viscosity, a wide range of polarity and a high degree of solubilisation strength for different compounds [29]. The tunability of DESs enables their selectivity to be optimised for a bioactive compound of interest. The advantages of using DESs for extraction include their enhanced thermal stability, often low toxicity, biodegradability, low production cost, easy production

procedure, reduced adverse effects on the environment, and ability to be used for large-scale applications [30].

To the best of our knowledge, no reported studies exist for DES extraction of fucoxanthin from algae. In this study, the extraction of fucoxanthin from *T. lutea* was conducted using different types of DESs. Conductor-like Screening Model for Real Solvents (COSMO-RS) was used as an initial screening tool to select the most promising solvents, with solvents that had favourable predicted extraction capacities experimentally validated. furthermore, the extraction parameters of the most favourable DES identified were optimised based on response surface methodology combined with BBD (Box-Behnken design).

2. MATERIALS AND METHODS

2.1. Materials

Tisochrysis lutea algae were from stocks originally obtained from the Scottish Association of Science (SAMS), Culture Collection of Algae and Protists (CCAP) as CCAP927/14. Algae stocks were maintained in 500 mL Erlenmeyer flasks containing F/2 culture media [31], prepared by addition of F/2 culture medium nutrients (supplied by Varicon Aqua Ltd. as their product 'F2P') to autoclaved seawater (salinity of 35-36 ppt) followed by filter sterilisation (0.35 µm). These were grown with continuous white fluorescent light at ~100 µmol photons m^{-2} s^{-1} (measured at the edge of the growing vessel) at 21ºC [32]. Algal biomass for this work was from large-scale continuous cultures in the same growth medium, temperature and light conditions, but produced in 38 L plastic bag bioreactors (custom made from 250 µm thick polyethylene film supplied by Amcor Flexibles (NZ) Ltd.) receiving continuous new media addition at a flow rate of 10 mL min^{-1}, supplied by a pneumatic processing pump. The bioreactors also had continuous aeration (3 L min^{-1}) with CO_2 added (0.3% v/v) to the airstream. Algae culture was collected from an outflow from the bioreactors at the same flow rate. Algae (104 L at ~0.64 g wet weight per L) were harvested by centrifugation in 1 L batches at 4250 x *g* for 6 min at room temperature, then shell frozen at -80 ºC and freeze dried. A food-grade stainless steel grain miller milled the dried algae to make a powder with the particle size of 200 µm, which was then collected by a Vibratory Sieve Shaker AS 200. *Tisochrysis lutea* biomass is light- and temperature-sensitive, so the dry samples were kept in plastic containers with wrapped aluminium foil in the freezer at -20 °C.

The chemicals used for this study are listed in Table 1. The fucoxanthin analytical standard was obtained from Sigma-Aldrich, Inc, USA, and the rest of the chemicals were purchased from AK Scientific Inc.

Table 1. List of chemicals used in this work.

Name	Purity	Supplier
Thymol	95.0%	AK Scientific Inc.
Decanoic acid	98.0%	AK Scientific Inc.
Dodecanoic acid	98.0%	AK Scientific Inc.
Octanoic acid	95.0%	AK Scientific Inc.
Acetic acid	95.0%	AK Scientific Inc.
Tetrabutylammonium chloride (TBAC)	99.5%	AK Scientific Inc.
Methanol	99.85%	AK Scientific Inc.
Ethanol	95.0%	AK Scientific Inc.
Fucoxanthin Standard	99.3%	Sigma–Aldrich, Inc, USA

2.2. COSMO-RS Screening

The COSMO-RS (Conductor-like Screening Model for Real Solvents) software is a helpful tool for predicting the thermodynamic properties of fluid mixtures [33]. COSMO-RS was used to screen the DESs performance for extracting fucoxanthin by calculating their predicted capacity in this study. Twenty four DESs with different compositions were screened in this work. The surface charge density of fucoxanthin and all DES constituents was predicted using COSMO-RS (Software for Chemistry and Materials BV (SCM), Amsterdam, The Netherlands) within the Amsterdam density functional (ADF) package. The molecular geometry of all compounds was pre-optimised with a universal force field (UFF) that was then optimised using density functional GGA: BP86 and basis set TZP for COSMO-RS calculations using the ADF input Version 2018.104. The resulting COSMO-RS files were used as an input in ADF COSMO-RS Version 2018.104 to compute σ-profiles and σ-potentials of the DESs and fucoxanthin. DESs are represented in COSMO-RS according to the mole composition of their constituents [34, 35]. The activity coefficient of fucoxanthin at infinite dilution in each DES was calculated via COSMO-RS and used for evaluating the capacity of DESs. The activity coefficient value describes the interaction between fucoxanthin and DES at an infinitesimal concentration of fucoxanthin. The capacity of each DES for fucoxanthin, which indicates the maximum amount of fucoxanthin that can be dissolved in the DES, was calculated as the inverse of the activity coefficient for fucoxanthin (Equation 1):

$$Capacity_{Fucoxanthin} = \frac{1}{\gamma_{Fucoxanthin}} \quad \text{(Eq.1)}$$

where γ is the activity coefficient of fucoxanthin at infinite dilution in solution.

2.3. Preparation of DESs

DESs were prepared by mixing the different hydrogen bonding acceptors (HBAs) and hydrogen bonding donors (HBDs) at the desired molar ratio at 80 °C with constant stirring of 1000 rpm using a heating magnetic stirrer, as has been described previously [36]. The DESs are defined as prepared when the mixtures turn into a homogeneous liquid phase without any solid precipitation. For the purpose of preventing moisture absorption, the DES bottles were sealed with Parafilm.

2.4. Extraction of Fucoxanthin using DESs and Benchmark Solvents

All prepared DESs were mixed with ground dried *T. lutea* biomass at a solid to liquid ratio of 1:25 (w/v). The extraction was performed at 25 °C, stirring at 1000 rpm, in a closed vessel wrapped with aluminium foil for 1 h. Following extraction, the mixture was centrifuged at 14534 x *g* for 10 min at room temperature, and then the resulting extract was filtered by a PTFE microfilter (0.45 μm). The filtrate was diluted with ethanol and fucoxanthin was quantified using HPLC. Each sample was performed in duplicate. The same procedure was repeated for screening the extraction performance of benchmark solvents (i.e., ethanol and methanol).

2.5. Optimisation and Extraction Conditions

Design-Expert 7.1 software (Stat-Ease Inc., U.S.A) was used to design the optimisation of the fucoxanthin extraction process from *T. lutea* in this work. Optimisation of the extraction process is essential as it can maximise the yield of the target, explore the effect of independent variables, and minimise cost and time. Response surface methodology (RSM) combined with Box-Behnken design (BBD) was used to investigate the impact of three extraction parameters at three levels on the yield of fucoxanthin. These parameters were the extraction temperature (A), time (B), and biomass percentage (% w/v) (C). Box-Behnken design is an efficient and economical design for optimising processes as it helps select a central point from a three-level arrangement and estimate the first and second-order coefficients [37]. Thus, BBD can fit quadratic models that require three levels of each factor. The three levels of extraction time were 1, 2, and 3 hours, and the algae biomass percentages used were 5, 10, and 15%. To avoid thermal degradation of the fucoxanthin at elevated temperatures, a temperature range of 25-55 ℃ was selected. The experiment consisted of 15 runs with three replicates of the centre points.

2.6. Stability Study

The stability of fucoxanthin in the DES was explored for the sample obtained by extraction at the optimum extraction conditions. The sample was kept at ambient temperature in a dark and dry place in a closed vessel wrapped with aluminium foil. Fucoxanthin quantification was performed on days 1, 3, 5, 7, 9 and 11 by HPLC following the dilution of a known amount of sample with ethanol.

2.7. Reusability Study

The reusability study was carried out at the optimum extraction conditions. After each extraction cycle, the mixture was centrifuged at 14534 x *g* for 10 min and then the biomass was removed. The fucoxanthin content of the remaining supernatant containing DES was quantified and then residual DES re-employed for extraction by mixing it with a fresh biomass batch. The DES was reused for a total of seven cycles.

2.8. High Performance Liquid Chromatography Analysis

High Performance Liquid Chromatography (HPLC) analysis was used to quantify fucoxanthin. This was performed using a commercial C18 column (4.6 x 150 mm, 5 μm) with an injection volume of 10.0 μL. The mobile phase, which consisted of acetonitrile/water (80/20, v/v), was used for isocratic elution with a flow rate of 1.0 mL min^{-1}. The column temperature was controlled at 40°C and a detection wavelength of 445 nm was used. HPLC calibration was performed using 5 ppm, 10 ppm, 25 ppm, 50 ppm, 75 ppm, and 100 ppm standards of fucoxanthin. Based on the results, the content of fucoxanthin was determined using a calibration curve with a regression equation: $y = 62508.2x - 69824.0$, $R^2 = 0.999$, (where y is the concentration of standard fucoxanthin in ethanol [ppm], and x is the peak area [mAU]), factoring in the standard used was 99.3% pure. Each sample was analysed using HPLC with duplicate measurements. During HPLC analysis, it was observed that the presence of DESs in the samples did not affect the chromatograms obtained.

3. RESULTS AND DISCUSSION

3.1. DES Ranking from COSMO-RS Screening Results

The results of DES screening from COSMO-RS are explained in terms of capacity at infinite dilution. Capacity is calculated as the reciprocal activity coefficient at infinite dilution. Capacity would reflect the maximum amount of fucoxanthin that could be dissolved in the DES, which is a critical parameter in terms of the viability of extraction processes. It is also more straightforward to model than selectivity given the complex nature of the *T. lutea* substrate. Twenty four DESs with different polarities were selected and

screened in this work. These DESs were selected to screen a range of hydrophobic and hydrophilic DESs with varied polarity and balance of alkyl chains to polar functional groups based on those commonly reported in the literature. The mole ratios selected for these DES were based on their reported eutectic points or ratios that have been commonly employed in their use as solvents. Table 2 presents the list of screened DESs along with the calculated activity coefficient and capacity of fucoxanthin in the DESs and benchmark solvents.

Table 2. Capacities of screened DESs and benchmark solvents.

HBA	HBD	Molar ratio	Activity coefficient	Capacity
Choline Chloride	Glycerol	1:2	2.10×10^{2}	4.77×10^{-3}
Choline Chloride	1,2-Ethanediol	1:2	3.29×10^{2}	3.04×10^{-5}
Choline Chloride	1,4-butanediol	1:2	3.86×10^{1}	2.59×10^{-2}
Choline Chloride	Lactic acid	1:2	5.74×10^{1}	1.74×10^{-2}
Choline Chloride	Urea	1:2	4.41×10^{4}	2.27×10^{-5}
Choline Chloride	Acetic acid	1:2	5.56	1.80×10^{-1}
TBAC	Glycerol	1:2	2.66	3.76×10^{-1}
TBAC	1,2-Ethanediol	1:2	1.67	6.00×10^{-1}
TBAC	1,4-Butanediol	1:2	2.08	4.81×10^{-1}
TBAC	Lactic acid	1:2	1.13×10^{-1}	8.86
TBAC	Acetic acid	1:2	8.50×10^{-3}	1.18×10^{2}
TBAC	Decanoic acid	1:2	5.74×10^{-3}	1.74×10^{2}
TBAC	1-dodecanol	1:2	8.88×10^{-1}	1.13
TBAC	1-tetradecanol	1:2	9.49×10^{-1}	1.05
TBAC	Octanoic acid	1:2	8.43×10^{-3}	1.19×10^{2}
Menthol	Lactic acid	1:2	5.61×10^{-1}	1.78
Menthol	Acetic acid	1:2	6.49×10^{-2}	1.54×10^{1}
Menthol	Octanoic acid	1:2	9.79×10^{-1}	1.02
Menthol	Decanoic acid	1.5:1	1.04	9.59×10^{-1}
Menthol	Dodecanoic acid	3:1	1.81	5.52×10^{-1}
Thymol	Octanoic acid	1:1.38	5.87×10^{-5}	1.7×10^{4}
Thymol	Decanoic acid	1:1	9.93×10^{-6}	1.01×10^{5}
Thymol	Dodecanoic acid	1.25:1	5.26×10^{-6}	1.9×10^{5}
Lidocaine	Dodecanoic acid	1:2	1.83×10^{-1}	5.47
Benchmark solvents				
	Methanol		9.92	1.01×10^{-1}
	Ethanol		3.32	3.01×10^{-1}
	Ethyl acetate		5.59×10^{-1}	1.78

From Table 2, the capacity of the top 6 DESs were ranked in the order of – thymol: dodecanoic acid (DES 1) (1.9×10^{5}) > thymol: decanoic acid (DES 2) (1.01×10^{5}) > thymol: octanoic acid (DES 3) (1.7×10^{4}) >

TBAC: decanoic acid (DES 4) (1.74×10^2) > TBAC: octanoic acid (DES 5) (1.19×10^2) > TBAC: acetic acid (DES 6) (1.18×10^2). The results showed that the predicted capacity increases by increasing carboxylic acid-based HBD's carbon chain length for thymol-based DESs and TBAC-based DESs, although the opposite trend was seen for the menthol series. The results also showed that DES containing thymol as a HBA have the greatest predicted potential for fucoxanthin extraction as these displayed the largest capacities. Carboxylic acid-based HBDs were found to possess the highest predicted solubilities of the HBDs screened. All 6 top-ranked DESs presented a superior capacity for fucoxanthin compared to methanol, ethanol and ethyl acetate.

3.2. Molecular Interactions

The affinity of an individual component in a mixture towards other components can be investigated through their σ-profile and σ-potential computed during the COSMO-RS screening. The σ-profile represents a histogram of charge density distribution over the molecular surface. The σ-potential is a measure of the affinity of a component in a mixture towards another. Hence, the COSMO-RS σ-profile and σ-potential analysis were used to explore why the performance of thymol-based DESs was better for the extraction of fucoxanthin compared to TBAC-based DESs and conventional solvents.

The σ-profiles of fucoxanthin, thymol-based DESs (DES1 to DES3), methanol and ethanol are shown in Fig.1. The molecular surface is considered too non-polar to participate in hydrogen bonding when the screening charge density is in the range of $\pm\ 0.0085\ eA^{-2}$[34]. The hydrogen bond donor region ($\sigma < -\ 0.0085\ eA^{-2}$), and the hydrogen bond acceptor region ($\sigma > 0.0085\ eA^{-2}$) indicate the hydrogen bond donor ability and hydrogen bond acceptor ability, respectively. The compound has a greater HBA or HBD ability if a larger proportion of its σ profile lies within the HBA or HBD region.

The σ-profile of fucoxanthin clearly shows that it is mainly distributed in the non-polar region, with three peaks formed. This indicates that a substantial proportion of the fucoxanthin molecule can be classified as non-polar, consistent with the substantial conjugated alkene region in the fucoxanthin molecule. The σ-profile also emphasises that fucoxanthin contains very few HBD groups, with its HBA ability being stronger due to the presence of ketone, ester and epoxide functionalities but remaining a relatively minor component. Therefore, it is expected that fucoxanthin will be more soluble in less polar DESs that contain HBD groups. The σ-profiles of thymol-based DESs are also mainly distributed in the non-polar region, which indicates their comparable polarity to fucoxanthin. Given the preferred HBA rather than HBD character of fucoxanthin, ideal solvents would display better HBD character in addition to be being

predominantly non-polar. In this regard, thymol-based DESs have a higher peak value than methanol or ethanol, which implies that thymol-based DESs will interact more strongly with fucoxanthin. Fig.1 also reveals that by increasing the carbon chain length of HBD in thymol-based DESs, the DES becomes less polar and has a higher peak value in the non-polar region. These trends accord with the high predicted solubility of fucoxanthin in thymol-based DES compared to the other solvents screened.

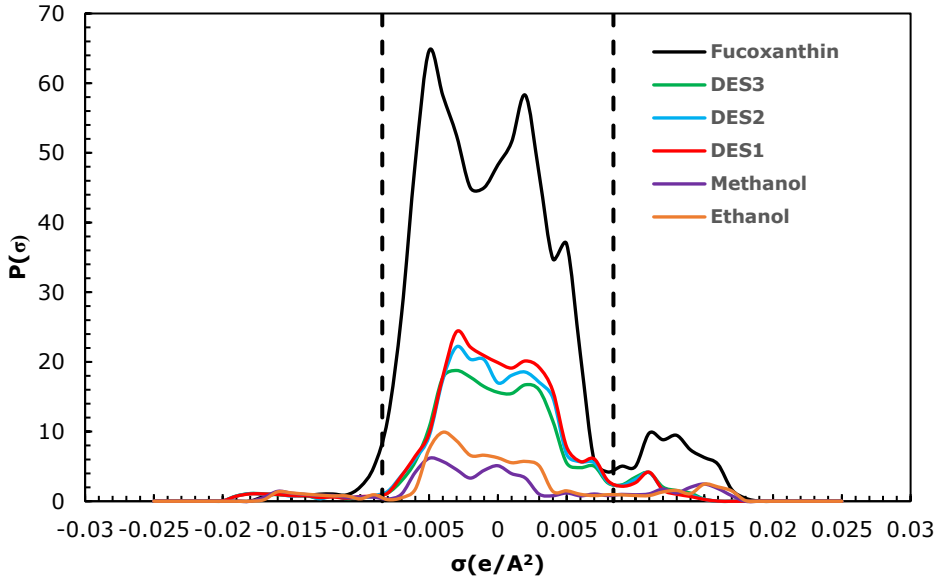


Fig.1. σ-profiles of fucoxanthin, thymol-based DESs, methanol and ethanol. Dashed lines indicate σ-profile thresholds assigned to hydrogen-bonding interactions.

σ-potential plots can be used to identify the affinity of a component in a mixture towards another. The more negative the value of the σ-potential, the stronger the interaction between components, while a more elevated positive value indicates an increase in repulsive behaviour. Similar to the σ-profile plot, there are three regions divided by the hydrogen bond threshold. The σ-potential of fucoxanthin (Fig.2a) presents a high negative value in the HBD region, positive value in the non-polar areas, and positive value in the HBA region. This indicates that fucoxanthin is likely to be best solvated by solvents containing effective HBD groups. Therefore, to maximise the extracting ability of DESs for fucoxanthin, the DES should have the most negative value in the HBA region which would emphasise their complementary interactions. The σ-potentials of all thymol-based DESs show a high negative value in the HBA region, suggesting their strong affinity for HBA molecules. On the contrary, their low negative value in the HBD region indicates that they have less affinity with HBD molecules, allowing them to more effective solvate fucoxanthin by reducing self-association between the solvent's HBA and HBD groups. For methanol and ethanol, their σ-potential plots (Fig.2b) are synchronised with the curve of fucoxanthin. This indicates that they do not

have complementary affinities to fucoxanthin which would improve their efficacy as solvents, unlike the thymol DESs. Therefore, thymol-based DESs are predicted by COSMO-RS to be better performing extraction solvents for fucoxanthin over methanol or ethanol.

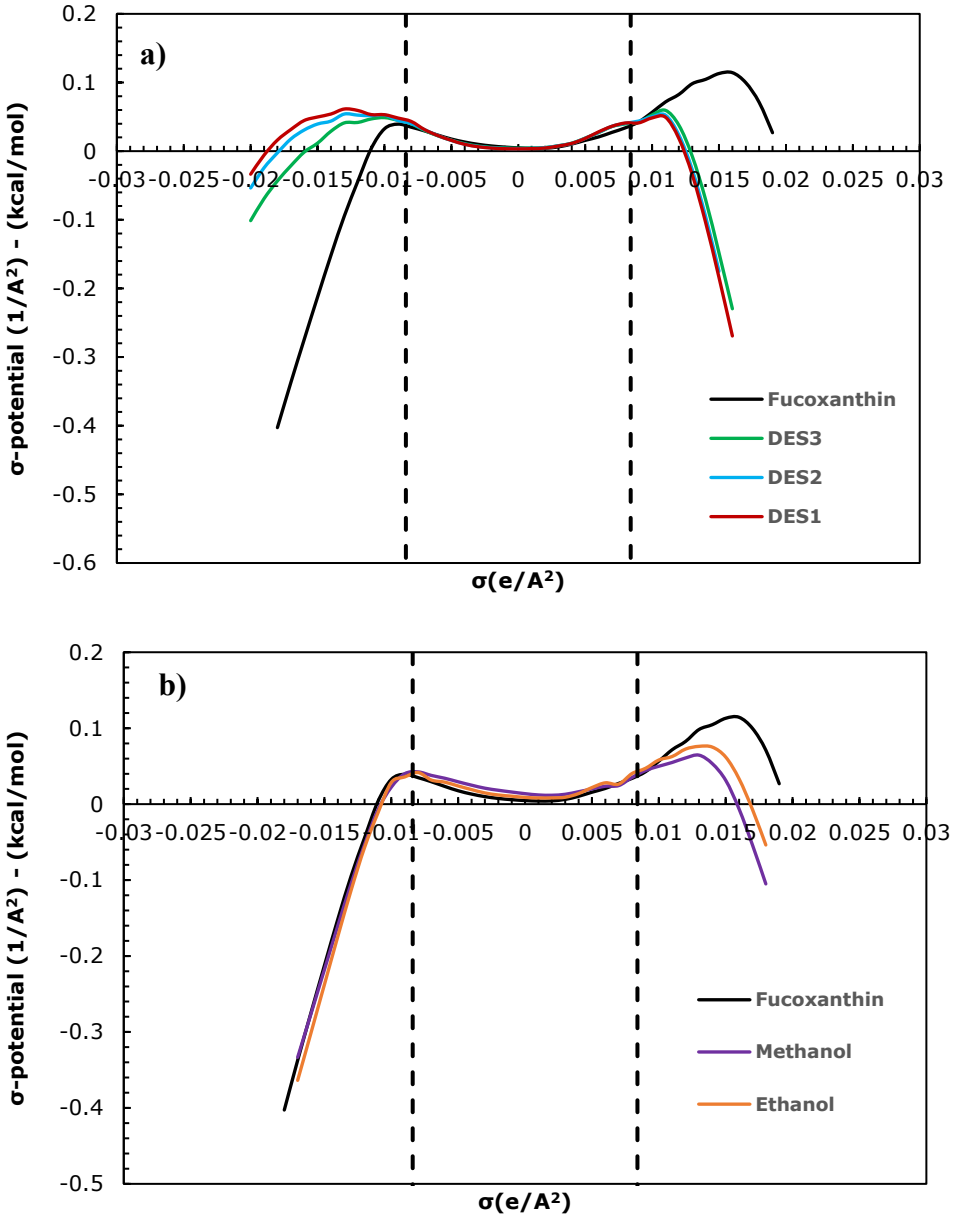


Fig.2. a) σ-potential plot of fucoxanthin and thymol-based DESs b) σ-potential plot of fucoxanthin, methanol and ethanol. Dashed lines indicate σ-potential thresholds assigned to hydrogen-bonding interactions.

3.3. Experimental Screening

Experimental screening was performed on the top 6 DES candidates identified from the COSMO-RS results in terms of their predicted capacity (Table 3), to explore their efficiency for the extraction of fucoxanthin from *T. lutea* microalgae powder. These DESs were prepared and the extraction performed at 25 ºC with 1000 rpm stirring by mixing each DES with microalgae powder at a solid to liquid ratio of 1:25 (w/v).

Table 3. Selected DESs for experimental fucoxanthin extraction and their abbreviation.

Abbreviation	HBA	HBD	Molar Ratio	Melting temperature (ºC)	References
DES1	Thymol	Dodecanoic acid	1.25:1	24.83	[38]
DES2	Thymol	Decanoic acid	1:1	18.86	[38]
DES3	Thymol	Octanoic acid	1:1.38	6.68	[38]
DES4	TBAC	Decanoic acid	1:2	-11.95	[39]
DES5	TBAC	Octanoic acid	1:2	-	[40]
DES6	TBAC	Acetic acid	1:2	-	[40]

Fig.3 presents the extraction yield (in mg/g dry biomass weight (DW)) for the extraction of fucoxanthin using the selected DESs. The experimental screening results followed the capacity trend predicted by COSMO-RS for the DESs. As predicted by COSMO-RS, extraction yields improved with the length of the alkyl chain on the carboxylic acid HBDs within both TBAC and thymol series and the thymol DESs were more effective than all TBAC DESs explored. Hence, the experimental screening results confirmed that, of the DES tested, the best DES for fucoxanthin extraction is DES1 (thymol: dodecanoic acid), with an extraction efficiency of 7.69 mg/g DW. This DES was chosen for subsequent experiments. In addition, the extraction yields obtained from DES1 were higher than the conventional solvents ethanol and methanol.

Biodegradable fatty acids and terpenes have very low solubility in water and are relatively inexpensive, making them good candidates to prepare sustainable and cost-effective hydrophobic solvents [38, 41]. Thymol and its eutectic mixtures have been investigated in the pharmaceutical field as analgesics, antimicrobial agents, and anti-inflammatory drug delivery vehicles [42, 43]. In addition, a sustainable thymol: dodecanoic acid DES has been evaluated for the extraction and separation of metals [38].

Therefore, a DES composed of bio-based compounds like terpenes and fatty acids is an attractive alternative to conventional solvents for many applications including for bioactive compound extraction.

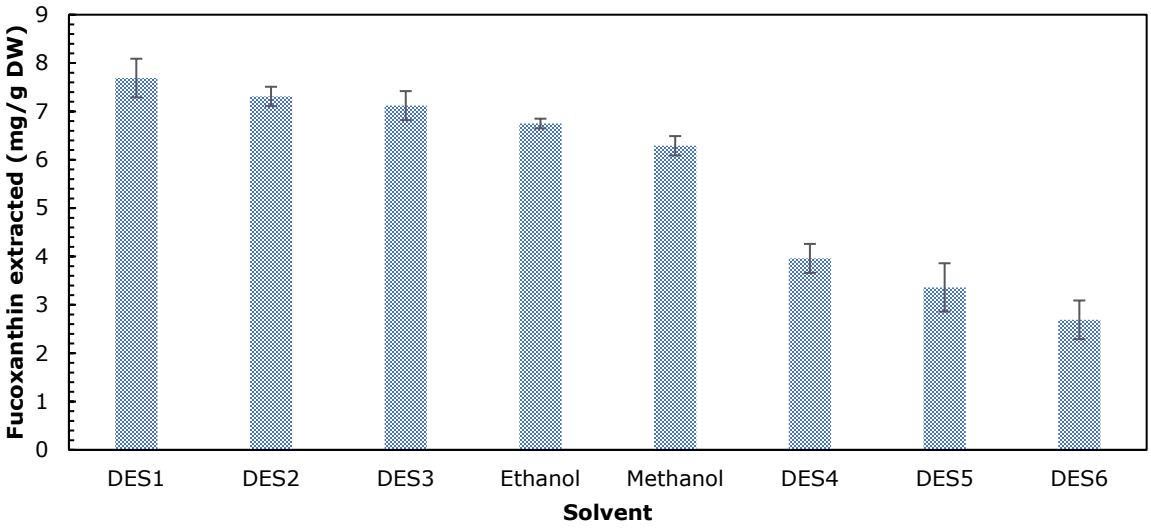


Fig.3. The results of fucoxanthin extraction from *T. lutea* using selected DESs and benchmark solvent.

3.4. Optimisation of Fucoxanthin Extraction

Design-Expert software was used to optimise the key factors that affect the extraction of fucoxanthin from *T. lutea* algal biomass using DES1 (thymol: dodecanoic acid). The key factors explored were extraction temperature (25-55°C), extraction time (1-3 h) and biomass percentage (5-15%). The experiment consisted of 15 runs of BBD with three replicates at the centre point, with the yield of fucoxanthin (in mg/g DW) used as a response. Table 4 shows the independent variables at three levels, and Table 5 displays the experimental data obtained.

Table 4. Independent variables and levels used for BBD experiments

Factor	Variable	Level		
		-1	0	1
A	Temperature (°C)	25	40	55
B	Time (h)	1	2	3
C	Biomass (% w/v)	5	10	15

The experimental data were analysed using multiple regression to develop a quadratic polynomial equation to describe the interaction between the extraction yield of fucoxanthin and extraction variables. The following regression model was established, Equation 2:

$$FX = +19.48 - 2.28A + 3.68B + 0.77C - 0.87AB - 0.35AC - 0.45BC - 5.52A^2 - 1.44B^2 - 1.93C^2 \quad \text{(Eq.2)}$$

Table 5. Experimental BBD matrix and obtained response values for the extraction of fucoxanthin using DES1.

Run	A: Temperature (°C)	B: Time (h)	C: Biomass (% w/v)	Response: (mg FX/g DW)
1	55.00	2.00	15.00	10.22
2	40.00	1.00	15.00	13.34
3	25.00	1.00	10.00	10.53
4	40.00	1.00	5.00	10.98
5	40.00	2.00	10.00	19.55
6	25.00	2.00	15.00	15.44
7	55.00	1.00	10.00	7.66
8	40.00	2.00	10.00	19.63
9	40.00	3.00	5.00	19.76
10	25.00	3.00	10.00	19.11
11	40.00	3.00	15.00	20.33
12	55.00	3.00	10.00	12.75
13	40.00	2.00	10.00	19.25
14	25.00	2.00	5.00	13.11
15	55.00	2.00	5.00	9.3

Analysis of variance (ANOVA) was used to validate the model by comparing between-group variation to within-group variation to assess the differences in population means of the data sets. The determined coefficient adjusted coefficient and predicted coefficient are shown in Table 6. The coefficient of determination of 0.9977 indicates the model can justify 99.77% of the variance. In order to assess whether the quadratic polynomial equation can effectively predict Fucoxanthin extraction yield, the predicted coefficient needs to be compared to the adjusted coefficient. In this case, the adjusted coefficient (0.9936) and the predicted coefficient (0.9673) in conjunction verify the validity of the model.

Table 6. Analysis of the variance for the fitted quadratic polynomial model of fucoxanthin extraction using DES1.

Std.dev.	0.36	R^2	0.9977
Mean	14.73	Adjusted R^2	0.9936
C.V.%	2.45	Predicted R^2	0.9673
PRESS	9.29	Adequate precision	43.111

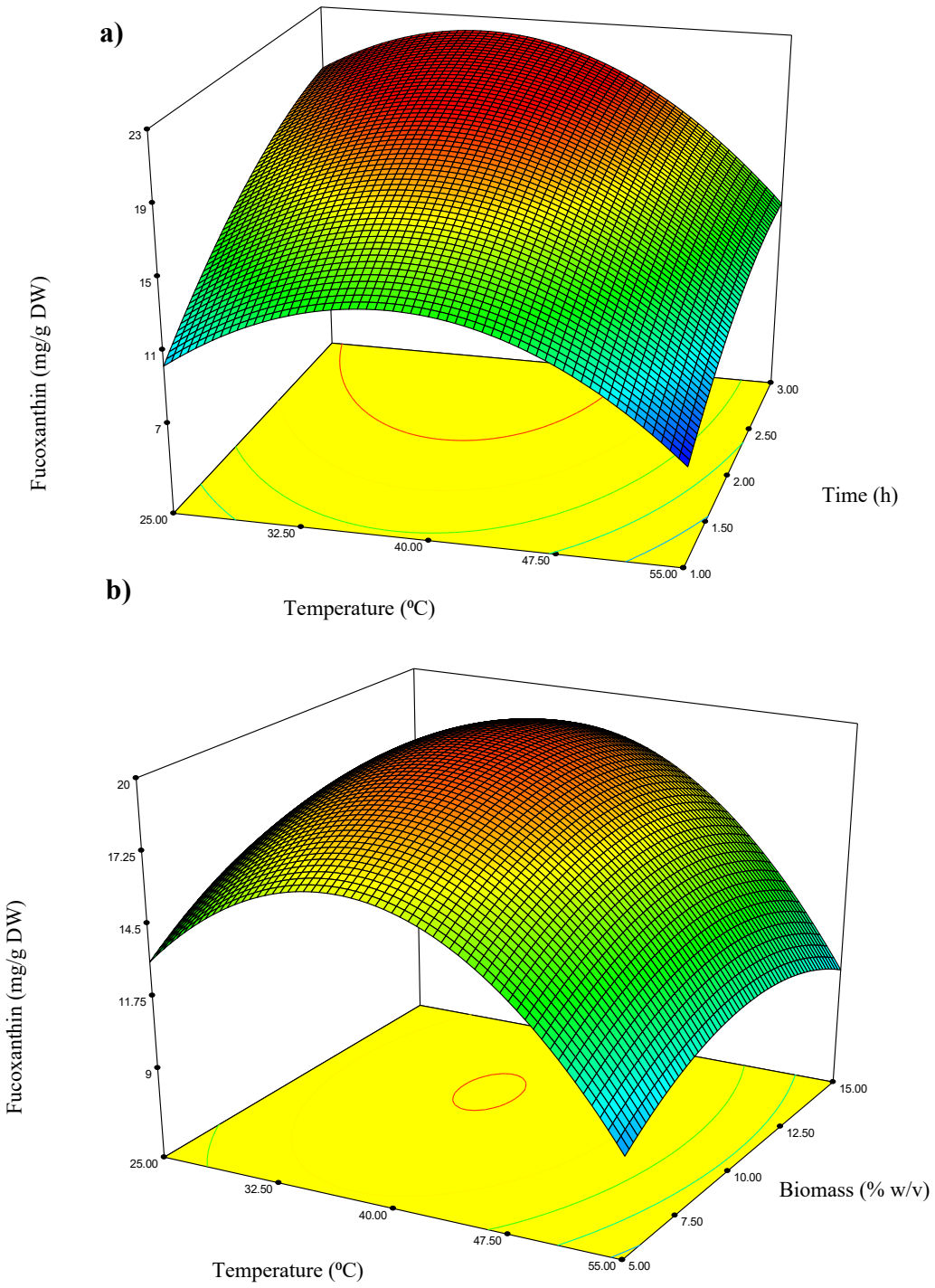
The F and p-value were used to verify the significance of the calculated model. Based on the ANOVA results from Table 7, the high F value (242.44) and low p-value (<0.0001) indicated the model was significant. Additionally, the lack of fit (0.1796) was not significant. The p-values indicated that all variables (A, B and C) were significant. Furthermore, the p-values indicated that the interaction between temperature and time (AB) was significant, although the interaction between temperature and biomass percentage (AC) as well as the interaction between time and biomass (BC) was not. Finally, the p-values indicated that the quadratic term coefficients (A^2, B^2 and C^2) were significant.

Table 7. ANOVA for response surface quadratic model fucoxanthin extraction using DES1.

Source	Sum of	Degree	Mean square	*F* value	*P*-value	Remarks
Model	283.36	9	31.48	242.44	< 0.0001	significant
A-Temperature	41.68	1	41.68	320.94	< 0.0001	significant
B-Time	108.34	1	108.34	834.26	< 0.0001	significant
C-Biomass	4.77	1	4.77	36.76	0.0018	significant
AB	3.05	1	3.05	23.45	0.0047	significant
AC	0.50	1	0.50	3.83	0.1078	
BC	0.80	1	0.80	6.17	0.0556	
A^2	112.69	1	112.69	867.78	< 0.0001	significant
B^2	7.65	1	7.65	58.92	0.0006	significant
C^2	13.82	1	13.82	106.41	0.0001	significant
Residual	0.65	5	0.13			
Lack of Fit	0.57	3	0.19	4.73	0.1796	not significant
Pure Error	0.080	2	0.040			
Cor Total	284.01	14				

Three-dimensional RSM plots in Fig.4 analysed the interactions between independent variables on the extraction yield of fucoxanthin. Fig.4a shows the interaction between time and extraction temperature. It can be observed that by increasing the time from 1 to 3 hours and temperature from 25 ℃ to 36 ℃ is predicted to lead to the maximum extraction yield of fucoxanthin. The predicted decrease in the amount of extracted fucoxanthin beyond the temperature of 36 ℃ is likely due to the thermal degradation of fucoxanthin within the biomass [44]. Fig.4b presents the interaction between the extraction temperature

and the biomass percentage on the yield of fucoxanthin. At a temperature of 36 ℃, the fucoxanthin yield increased with increasing biomass percentage. Fig.4c shows the interaction between time and biomass percentage, which revealed that the highest yield of fucoxanthin could be observed by increasing the extraction time to 3 h and the biomass percentage up to 11%. Higher biomass percentages likely result in reduced yields due to mass transfer limitations.


a)
Fucoxanthin (mg/g DW)
23
19
15
11
7
25.00
32.50
40.00
47.50
55.00
Temperature (ºC)
1.00
1.50
2.00
2.50
3.00
Time (h)
b)
Fucoxanthin (mg/g DW)
20
17.25
14.5
11.75
9
25.00
32.50
40.00
47.50
55.00
Temperature (ºC)
5.00
7.50
10.00
12.50
15.00
Biomass (% w/v)

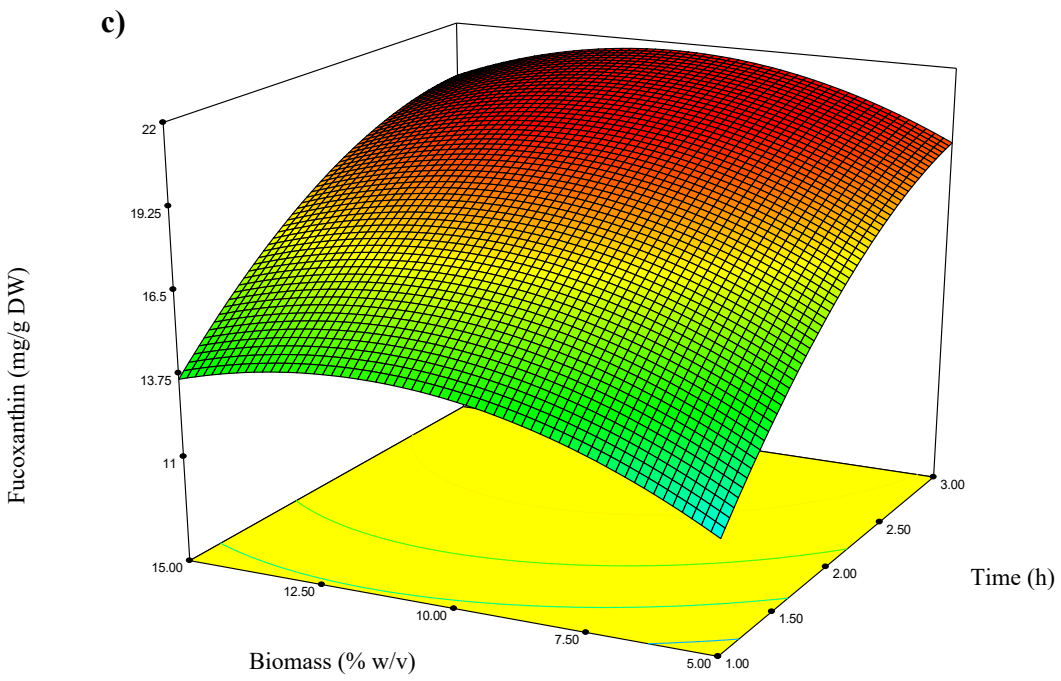
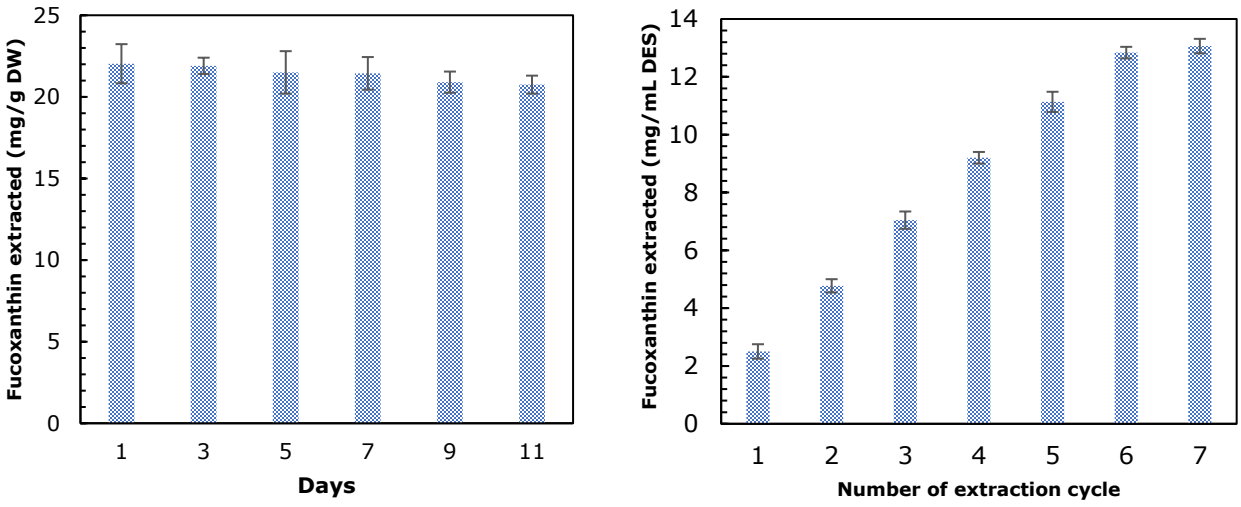


Fig.4. 3D plots of fucoxanthin extraction using DES1 on the interactions between the considered factors: (a) temperature and time, (b) temperature and % w/v biomass, and (c) time and % w/v biomass.

Fucoxanthin extraction from *T. lutea* microalgal biomass at the optimal conditions was conducted and repeated in triplicate to verify the experimental value with the predicted one. A value of 22.03 mg/g DW (SD 0.276 mg/g DW) was obtained when the extraction temperature, time and biomass percentage were 36.2 ℃, 2.58 h and 11.36% respectively, which is in close agreement with the yield predicted by the model (21.50 mg/g DW) and was within the confidence intervals of the model (20.45–22.55 mg/g DW).

3.5. Stability and Reusability Results

Fig.5a shows the stability of fucoxanthin in thymol: dodecanoic acid DES1 over 11 days of storage in a dry and dark place at 25 ºC. Good stability of fucoxanthin in the DES1 was demonstrated with no significant change in the fucoxanthin concentration at the end of day 11. This shows that the DES would be suitable for short term storage of the fucoxanthin extract without the need for cooling. As thymol and dodecanoic acid are recognised as safe food additives [45, 46], the extract could potentially be used without needing to isolate fucoxanthin, increasing the viability of this approach. The thymol: dodecanoic acid DES1 has negligible volatility, which is an essential trait for solvents containing bioactive products in the food, pharmaceuticals, and cosmetic sectors.



a) b)

Fig.5. a) Fucoxanthin concentration on prolonged storage at 25 ºC, b) Fucoxanthin concentration after seven extraction cycles.

The concentration of fucoxanthin over seven successive extraction cycles is presented in Fig.5b. The fucoxanthin concentration progressively increased for six extraction cycles, with the increase in fucoxanthin concentration notably decreasing after the 4th extraction cycle. The fucoxanthin concentration remained constant after the 6th cycle with a final concentration of 13.06 mg/mL DES. This highlights the high fucoxanthin extraction yields that can be obtained by reusing the DES, allowing solvent use to be minimised in the generation of a concentrated fucoxanthin extract. We note that the final concentration of fucoxanthin achieved after 7 extraction cycles is substantially higher than the loading of 0.1-0.3 mg/mL proposed for therapeutic applications of fucoxanthin [47]. Given the DES components are safe food additives, this highlights the potential to use these extracts as concentrates that could be diluted to appropriate concentrations for therapeutic use. While each individual component of this solution is recognised as safe, the toxicology of the mixture itself would need to be assessed before further application to ensure no synergistic toxicological impacts were observed. The reusability and high ultimate concentration of fucoxanthin in this DES is promising attributes for commercial scale-up and process sustainability.

4. CONCLUSION

Fucoxanthin extraction from *Tisochrysis lutea* microalgae using DES has been studied. COSMO-RS screened 24 DESs according to their predicted capacity for fucoxanthin and the 6 top-ranked DESs were

validated experimentally as extractants for fucoxanthin from *Tisochrysis lutea* algal biomass. The experimental results revealed that the thymol: dodecanoic acid DES (1.25:1 molar ratio) showed the highest extraction efficiency and greater extraction efficiency than methanol and ethanol, which corresponded with COSMO-RS screening results. Moreover, the extraction of fucoxanthin using the thymol: dodecanoic acid DES was further optimised using RSM combined with BBD. The results showed that the optimum extraction conditions were at a temperature of 36.2 ℃, stirring time of 2.58 h, and biomass loading of 11.36% (w/v). The thymol: dodecanoic acid DES optimised yield was 22.03 mg/g DW, which was close to the predicted value of 21.50 mg/g DW based on the optimisation results. Furthermore, fucoxanthin was stable in the thymol: dodecanoic acid DES over 11 days of storage. Reusing the DES led to a maximum fucoxanthin concentration of 13.06 mg/mL DES being achieved after seven extraction cycles which was far in excess of that required for therapeutic use. As thymol and dodecanoic acid are both recognised as safe food additives, the thymol: dodecanoic acid DES has good potential to act as a sustainable replacement for conventional solvents for fucoxanthin extraction from algae. Further assessment of the bioactivity of this formulation and the toxicological profile of the solution is required before this process can be implemented more widely. The DES and extraction approach undertaken here may prove suitable for extracting similar bioactive compounds from natural sources or more complex separation processes such as the separation of co-extracted components, as has been observed previously for fucoxanthin and docosahexaenoic acid [16, 17], by leveraging the hydrophobicity of the DES.

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