Research Article

Received: 18 January 2023 Revised: 12 April 2023 Accepted article published: 22 April 2023 Published online in Wiley Online Library: 3 May 2023

(wileyonlinelibrary.com) DOI 10.1002/jctb.7407

Menthol-based deep eutectic solvents as green extractants for the isolation of omega-3 polyunsaturated fatty acids from *Perna canaliculus*

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Abstract

BACKGROUND: Marine-derived omega-3 polyunsaturated fatty acids (PUFA) are used globally as food supplements for their anti-inflammatory properties and to reduce the risk of obesity, cardiovascular diseases, and cancer. New Zealand green-lipped mussels (*Perna canaliculus*) contain diverse valuable compounds, including omega-3 PUFA. The most abundant omega-3 PUFA in *Perna canaliculus* are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), both of which account for many of the health benefits accredited to these mussels. This research investigates the feasibility of using deep eutectic solvents (DESs) to isolate EPA and DHA from *Perna canaliculus*.

RESULTS: Twenty-two hydrophobic DESs were screened using COSMO-RS software to predict their affinity for EPA and DHA. Nine top-ranked DESs were used for experimental extraction validation. The results exposed that the extraction capacity of the menthol: lidocaine DES (1:1 M ratio) for EPA and DHA was superior (172.04 μg EPA g^{-1} dry weight (DW) of biomass and 602.79 μg DHA g^{-1} DW) amongst other DESs. This was a considerably higher extraction yield than that observed using ethanol (95.65 μg EPA g^{-1} DW and 311.49 μg DHA g^{-1} DW). The optimisation of the menthol: lidocaine ratio identified a 1:2 M ratio as the optimal composition for this DES. The highest yields of EPA (267.59 μg g^{-1} DW) and DHA (1014.84 μg g^{-1} DW) were obtained by this optimised DES ratio at optimum extraction conditions (extraction temperature 65 °C, extraction time 2.12 h, and biomass loading 5 w/v%). The EPA and DHA showed good stability in the menthol: lidocaine DES over 7 days. This DES also showed good reusability, with a comparable extraction efficiency being observed after five extraction cycles.

CONCLUSION: This research revealed that menthol-based DES can be a potential green solvent for the extraction of EPA and DHA from New Zealand green-lipped mussels and it may be applicable for extracting other bioactive compounds with similar characteristics.

Keywords: eicosapentaenoic acid; docosahexaenoic acid; extraction; COSMO-RS; deep eutectic solvents; *Perna canaliculus*

INTRODUCTION

New Zealand's green-lipped mussels (NZGLM), *Perna canaliculus*, are a bivalve mollusc species that are endemic to the region and are exported to 77 countries, with the mussel export markets totalling over 336 million NZD in 2020.[1] Due to their unique marine environment, NZGLM contain a variety of fats, proteins, and carbohydrates. Most importantly, NZGLM contain a high concentration of lipids, including omega-3 (n-3) polyunsaturated fatty acids (PUFAs). Previous studies have discovered that marine-derived n-3 PUFAs have significant health benefits, including combating obesity and reducing the risks of cardiovascular diseases and cancer.[2-4] Amongst the array of n-3 PUFAs in NZGLM,

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the most abundant are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), both of which account for more than 80% of all n-3 PUFAs.[5] EPA and DHA hold the highest value amongst these compounds, as they are predominantly accountable for the associated health benefits.[6] Because of this, fish oil supplement manufacturers often use EPA and DHA contents as a direct point of advertisement. Amongst New Zealanders, fish oil supplements are consumed daily by ~12% of the country's population.[7] The substantial economic value and relative abundance of biomass containing these fatty acids have led to several methods being developed to extract fatty acids from wet or dry biomass. This has accelerated in recent years due to the escalating cost of fish oil, which increased by ~904% between the years of 2000 and 2015.[8]

The most utilised method for extracting n-3 PUFAs is supercritical fluid extraction, which utilises compressed carbon dioxide as the solvent. Supercritical fluid extraction can be controlled by altering the temperature, pressure, and carbon dioxide flow rate. However, the equipment used in supercritical processes often comes at a high cost. Furthermore, these processes have high power requirements for higher extraction yields, making them significantly unsustainable.[9] Basic solid–liquid or liquid–liquid solvent extraction processes often produce low yields, making them impractical in industrial settings. Because of this, these processes are often used in conjunction with alternative methods to increase extraction efficiency. The main methods used include microwave-assisted extraction, ultra-sound assisted extraction, pulsed electric field assisted extraction, and enzyme assisted extraction.[10-13] Each method has unique capabilities to promote mass transfer between the solute and solvent. Usually, these methods operate by attacking the cell walls of biomass through rapid heating, friction, or by promoting cavitation. This, in turn, promotes penetration of the solvent into the biomass matrix. Although it can be of economic relevance to optimise the extraction yield, there is often a trade-off when using these extraction methods. Many of these methods incur high capital costs as well as scale-up challenges.[14] Furthermore, each method relies on the properties of the biomass to be effective. This could include the conductivity of the biomass, the potential for unwanted side reactions, the polarity of the biomass source, and its resistance to thermal degradation. In addition to this, the solvents involved in these processes are often volatile, toxic, and/or flammable.[15]

With the constant world-wide push for sustainability and with global leaders committing to initiatives, such as the Paris Climate Accords, unsustainable industrial processes are regularly being re-assessed for their environmental impact. This has led to an almost exponential increase of academic research into novel processes with an emphasis on sustainability. With this current industry trend, it is more important now than ever to find a sustainable alternative for fatty acid extraction from marine organisms. Ionic liquids (ILs) are a novel class of green solvents that possess high ionic conductivity, good chemical stability, negligible vapour pressure, and high heat capacity.[16] Additionally, the properties of ILs can be easily modified by their anionic or cationic components.[17] The culmination of these factors makes ILs ideal solvents for extracting bioactive compounds. DESs are closely related to ILs due to their similar characteristics.[18] DESs are often more sustainable than ILs because they can more easily utilise inherently less hazardous components and can typically be prepared in a simpler and more energy- and material-efficient manner.[19] DESs are prepared by mixing Lewis acids and bases at moderate temperatures, with one component typically referred to as a hydrogen bond acceptor (HBA) and another as a hydrogen bond donor (HBD) due to the role hydrogen-bonding plays in suppressing the melting points of many DES mixtures.[20,21] DESs can be easily tailored for a range of applications by altering the HBA and HBD compounds. DESs can be low-cost because of their simple preparation and the inexpensive nature of their components. DESs have been utilised across various sectors, including biomass and biodiesel processes,[22-24] metal electrodeposition and electropolishing,[25] nanotechnology,[26,27] and separation and purification.[28-31] In recent years, many research papers have been published illustrating the ability of DESs to replace toxic solvents in the extraction of bioactive compounds, such as flavonoids,[32,33] terpenoids,[34,35] phenolic acids,[36-38] polyphenols,[39] tanshinones,[40] rosiglitazones,[41] saponins,[42] and anthraquinones.[43]

Although DESs have been proven to be effective in the direct extraction of many bioactive compounds, there has been no research into PUFAs extraction from marine organisms using DESs. This study aims to address this shortcoming by investigating DES selection and optimising extraction conditions (temperature, time, and biomass percentage) for the direct extraction of EPA and DHA from NZGLM to promote a new avenue for more sustainable extraction methods in the marine industry.

MATERIALS AND METHODS

Materials

The freeze-dried NZGLM powder from whole *Perna canaliculus* (200 µm particle size) was produced by Sanford Ltd (ENZAQ facility, Blenheim, New Zealand). All chemicals used in this research are listed in Table 1. The EPA and DHA analytical standards and methyl laurate were purchased from Sigma-Aldrich, Inc, USA, and additional chemicals were supplied from AK Scientific Inc, USA. All chemicals were used as received without further purification.

COSMO-RS screening

COSMO-RS is an efficient quantum chemistry-based method for the prediction of the chemical and thermodynamic properties of fluid mixtures.[44] COSMO-RS was used to calculate the capacity

Table 1. List of chemicals used in this research

Name	Assay	Supplier
DL-Menthol	99.0%	AK Scientific Inc, USA
Decanoic acid	98.0%	AK Scientific Inc, USA
Dodecanoic acid	98.0%	AK Scientific Inc, USA
Octanoic acid	95.0%	AK Scientific Inc, USA
Oleic acid	95.0%	AK Scientific Inc, USA
1-Dodecanol	98.0%	AK Scientific Inc, USA
1-Tetradecanol	98.0%	AK Scientific Inc, USA
Lidocaine	98.0%	AK Scientific Inc USA
Ethylene glycol	98.0%	AK Scientific Inc, USA
Tetrabutylammonium chloride (TBAC)	99.5%	AK Scientific Inc, USA
Ethanol	95.0%	AK Scientific Inc, USA
Methyl laurate	≥98.0%	Sigma–Aldrich, Inc, USA
EPA standard	≥99.0%	Sigma–Aldrich, Inc, USA
DHA standard	≥98.0%	Sigma–Aldrich, Inc, USA

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of each DES towards the solvation of EPA and DHA to screen for the best performing solvents. Twenty two hydrophobic DESs were selected and screened in this research. The methodology for predicting the surface charge density of EPA, DHA, and all DESs, and for optimising the molecular geometry of all compounds was performed as detailed in the previous work.[45] DESs are represented in COSMO-RS according to the mole composition of their constituents.[46,47] The activity coefficients of EPA and DHA were estimated at an infinite dilution of each DES via COSMO-RS and were used for calculating the capacity of DESs. The capacity of each DES for EPA and DHA was calculated as the inverse of the EPA and DHA activity coefficients.

Experimental extraction of EPA and DHA

The studied DESs were prepared by mixing the constituents at the desired molar ratio at 80 °C and 500 rpm agitation for 2 h. The extraction of EPA and DHA using DESs and the benchmark solvent (ethanol) was accomplished at 25 °C and 1000 rpm in a closed vial for 1 h with a mussel powder: DES ratio of 1:20 (w/v) for all samples. After extraction, the mixture was centrifuged at 10 000 rpm for 10 min at 25 °C. The extract was then filtered by a PTFE microfilter (0.45 μm). The filtrate was diluted with ethanol for the quantification of EPA and DHA by HPLC. Each test was performed in duplicate.

Optimisation of extraction variables

Design-Expert software version 7.1 (Stat-Ease Inc., USA) was for the experimental design and optimisation of the EPA and DHA extraction process from NZGLM. The three selected conditions, extraction time (A), temperature (B), and biomass percentage (C), were investigated using the Box–Behnken design (BBD) coupled with response surface methodology (RSM). Table 2 presents the independent variables at three levels. Biomass loadings of 5, 10, and 15 w/v% were used at temperatures within the range of 35–65 °C for 1–3 h. Two responses were taken: the amount of EPA extracted and the amount of DHA extracted. Both EPA and DHA are sensitive to elevated temperatures. Hence, the temperature range of 35–65 °C was chosen to prevent the thermal degradation of EPA and DHA.

Stability and reusability study

The stability study of EPA and DHA in the DES was performed at the optimum extraction conditions. The sample was kept at 35 °C in a laboratory incubator (Sanyo, MIR 253). The EPA and DHA concentrations were determined on days 1, 3, 5, and 7 by taking a known amount of sample and diluting it with ethanol for HPLC analysis. The reusability of the DES was explored by performing the extraction under optimal extraction conditions for 5 cycles. After each extraction cycle, the mixture was centrifuged at 10 000 rpm for 10 min and the biomass was removed. The EPA and DHA contents of the supernatant containing DES was quantified, and then the volume of the supernatant was measured. The supernatant was re-employed for extraction by mixing it with the appropriate biomass percentage. The DES was reused for a total of 5 cycles.

Table 2. Independent variables and levels used for optimisation

		Level		
Factor	Variable	−1	0	1
A	Time (h)	1	2	3
B	Temperature (°C)	35	50	65
C	Biomass (w/v%)	5	10	15

HPLC analysis

A HPLC method was developed to determine EPA and DHA directly from the sample without the need to esterify the sample. Current analytical methods require the methyl esterification of EPA and DHA,[48] which can cause inaccuracies in quantification due to incomplete conversion of EPA and DHA. The HPLC method was performed using a Shimadzu LC-20AT pump, ELSD-LT II detector, and a C18 column (4.6 × 150 mm, 5 μm). The mobile phase was acetonitrile/Milli-Q water/trifluoroacetic acid (89.9:10:0.1). The column temperature was set to 25 °C. The flow rate, ultraviolet wavelength, and injection volume were set to 0.5 mL min^{-1}, 500 nm, and 5.0 μL, respectively. Prior to analysis, samples were diluted with ethanol at a dilution factor of 3, thoroughly mixed, filtered through a 0.22 mm PTFE micro filter, and stored in a freezer. The results were expressed as μg EPA/DHA per gram dry weight of the freeze-dried biomass. EPA and DHA standards were purchased from Sigma Aldrich and mixed with ethanol to create stock solutions of 1000 ppm. The stock solutions of EPA and DHA were diluted to 5, 10, 25, 50, 100, and 200 ppm for calibration. The EPA content was obtained using a calibration curve with a regression equation: $y = 1.657E\text{-}05x - 5.0202$, $R^2 = 0.9998$ (where y is the concentration of standard EPA in dichloromethane [ppm] and x is the peak area [mAU]). The DHA content was calculated using a calibration curve with a regression equation: $y = 4.613E\text{-}05x - 1.884E+00$, $R^2 = 0.999$. Each sample was analysed using HPLC with duplicate measurements.

RESULTS AND DISCUSSION

COSMO-RS screening results

Capacity relates to the solubility and, hence, the extraction efficiency of a solvent. This is considered an essential parameter for DES selection in this research since the extraction yield has a more significant impact on production costs than selectivity. Since EPA and DHA molecules are non-polar and contain long-chain alkenes, DESs with varied alkyl chains were selected based on those reported in the literature. Table 3 shows all studied DESs and their calculated activity coefficient and capacity. These are compared with the benchmark solvents methanol and ethanol for both EPA and DHA.

The capacity of the top nine DESs from Table 3 were found to be as follows – menthol: lidocaine (1:1 M ratio) > lidocaine: decanoic acid (1:1 M ratio) > lidocaine: dodecanoic acid (1:1 M ratio) > lidocaine: oleic acid (1:1 M ratio) > TBAC: 1-dodecanol (1:2 M ratio) > TBAC: 1-tetradecanol (1:2 M ratio) > menthol: octanoic acid (1.5:1 M ratio) > menthol: decanoic acid (1.5:1 M ratio) > menthol: dodecanoic acid (3:1 M ratio). The COSMO-RS results exposed that the predicted capacity increases with the decreasing carbon chain length of the HBD. The results also revealed that the DESs containing lidocaine have the most significant predicted potential for extraction of EPA and DHA, as these presented the largest capacities, which were substantially higher than those of DESs containing other classes of HBD. The menthol: lidocaine DES (1:1 M ratio) presented a superior capacity for extracting n-3 PUFAs based on its high capacity values of 135.01 for EPA and 106.87 for DHA. Methanol and ethanol offered a lower capacity

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Table 3. Activity coefficients and capacities of screened DESs and benchmark solvents

HBA	HBD	Molar ratio	γ EPA	γ DHA	Capacity EPA	Capacity DHA
TBAC	1-Dodecanol	1:2	0.2528	0.1985	3.9551	5.0386
	1-Tetradecanol	1:2	0.2651	0.2038	3.7717	4.9066
	Octanoic acid	1:2	1.1448	1.0317	0.8735	0.9693
	Decanoic acid	1:2	1.1883	1.0503	0.8416	0.9521
	Dodecanoic acid	1:2	1.2118	1.0613	0.8252	0.9422
Menthol	Octanoic acid	1.5:1	0.3542	0.2520	2.8231	3.9687
	Decanoic acid	1.5:1	0.3878	0.2736	2.5785	3.6544
	Dodecanoic acid	1.5:1	0.4136	0.2883	2.4178	3.4685
	Lidocaine	1:1	0.0074	0.0094	135.0109	106.8780
Thymol	Octanoic acid	1:1.38	1.1069	1.2852	0.9034	0.7781
	Decanoic acid	1:1	0.9797	1.1800	1.0207	0.8474
	Dodecanoic acid	1:2.5	1.3444	1.3885	0.7438	0.7202
TOAC	Hexanoic acid	1:2	1.1165	0.8771	0.8957	1.1402
	Octanoic acid	1:2	1.1668	0.9667	0.8570	1.0344
	Decanoic acid	1:2	1.2528	0.9834	0.7982	1.0169
	Dodecanoic acid	1:2	1.3430	1.0379	0.7446	0.9635
Lidocaine	Decanoic acid	1:1	0.1644	0.1985	6.0838	5.0380
	Dodecanoic acid	1:1	0.1700	0.2018	5.8824	4.9549
	Oleic acid	1:1	0.1773	0.2036	5.6390	4.9117
Dodecanoic acid	Octanoic acid	1:3	1.4601	1.3663	0.6849	0.7319
	Nonanoic acid	1:3	1.5060	1.4586	0.6640	0.6856
	Decanoic acid	1:2	1.5256	1.4140	0.6555	0.7072
Benchmark solvents	Methanol		2.4807	3.0705	0.40310	0.3256
	Ethanol		0.7381	0.7383	1.3549	1.3544

for EPA and DHA compared to the nine top-ranked DESs, implying that many of these DESs may become suitable alternative solvents for this extraction.

The σ-profile and σ-potential analysis were used to clarify why the menthol: lidocaine DES performance was predicted to be superior for the extraction of EPA and DHA compared to the use of the other DESs screened or conventional solvents, such as ethanol. The σ-profiles of EPA, DHA, the menthol: lidocaine DES, and ethanol are shown in Fig. 1. The charge density for both EPA and DHA mainly results from two peaks within the range of ± 0.0085 eA^{-2} and another small peak at 0.01 eA^{-2}. This indicates, perhaps surprisingly, that these long-chain fatty acids have a poor formal hydrogen bond donating ability despite the carboxylic acid group, with a moderate hydrogen bond accepting ability due to the oxygen atoms in the carboxylic acid group acting as hydrogen-bond acceptors. However, the largest peaks for both DHA and EPA occur around -0.005 eA^{-2}, which points to a large proportion of weakly positively charged surface. Nonetheless, these fatty acids can be classified as mainly non-polar compounds. Since the peak areas of EPA and DHA are incredibly similar, the extraction selectively of these two compounds should be very similar regardless of capacity. To achieve a high extraction efficiency for both EPA and DHA, a DES with a similar charge density but with the opposite sign is required to attain favourable interactions within the non-polar region. The menthol: lidocaine DES has one peak at -0.003 eA^{-2}, which would be expected to result in a favourable interaction with the peak of EPA/DHA at 0.002 eA^{-2}. The second peak in the DES's σ-profile at 0.002 eA^{-2} would result in a favourable interaction with the peak of EPA/DHA at -0.004 eA^{-2}. The location of these two peaks indicates that the DES has a high extraction capacity for the target n-3 PUFAs. The high capacity of the DES compared to ethanol can be attributed to the higher peak areas in these non-polar regions when compared to ethanol arising from the larger non-polar surface area.

The σ-potential plots indicate a component's affinity toward other components in a mixture. In the σ-potential plot, a more negative value of the σ-potential indicates a greater interaction of one compound in a fluid mixture with another, and vice versa, a more positive value indicates a stronger repulsive behaviour. Similarly to the σ-profile plot, values outside of ± 0.0085 eA^{-2} reflect the preference for interaction with HBDs (leftmost region) and HBAs (rightmost region), whereas values within ± 0.0085 eA^{-2} are indicative of non-polar interactions. EPA and DHA both have significant negative values in the HBA region, as shown in Fig. 2. However, looking at the non-polar region, a more positive value was observed between a potential of 0 and the formal HBA region. On the other hand, there was less repulsion observed for both EPA and DHA in the HBD region, even though negative values were not observed until more negative surface potentials. Only EPA had a considerable negative value in the HBD region. This indicates that EPA and DHA both interact favourably with strong HBA, although they are more strongly repelled by compounds that possess a weak negative surface charge than those with regions containing a weak positive surface charge. This suggests that a favourable solvent for these compounds would possess a combination of strong HBA ability in conjunction with predominant HBD ability. In terms of σ-potential, this would imply large negative values in the HBD region with negative or near zero values in the non-polar region near the HBD region. The menthol: lidocaine DES had a substantial negative value (-0.73) in the HBD region, meaning it has excellent interactions with hydrogen bond donating compounds and that it displays σ-potential close to zero in the non-polar region nearest

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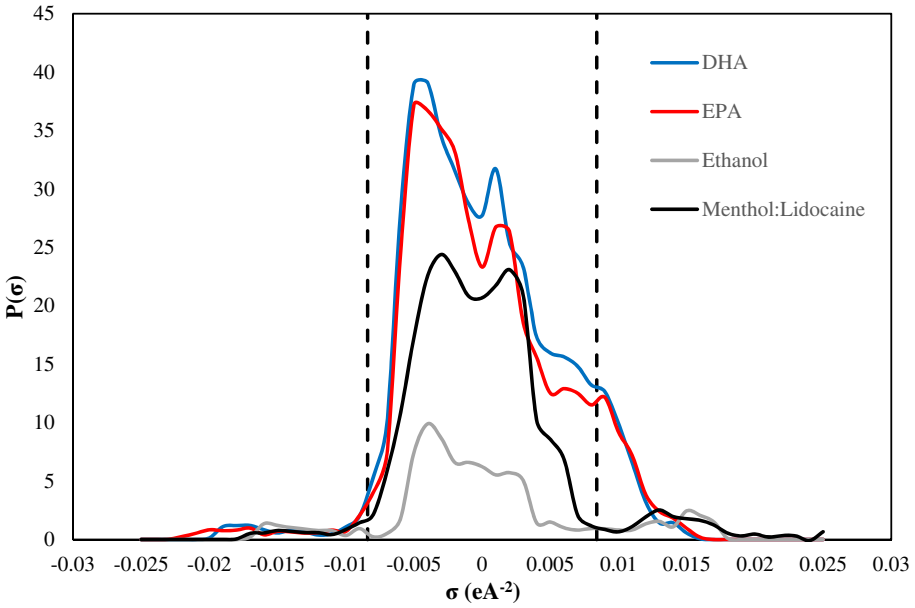

Figure 1. σ-profiles of DHA, EPA, ethanol and menthol: lidocaine DES. Dashed lines indicate σ-potential thresholds assigned to hydrogen-bonding interactions: hydrogen bond donor region ($\sigma < -0.0085$ eA^{-2}), hydrogen bond acceptor region ($\sigma > 0.0085$ eA^{-2}), and non-polar region (0.0085 eA^{-2} < σ < 0.0085 eA^{-2}).

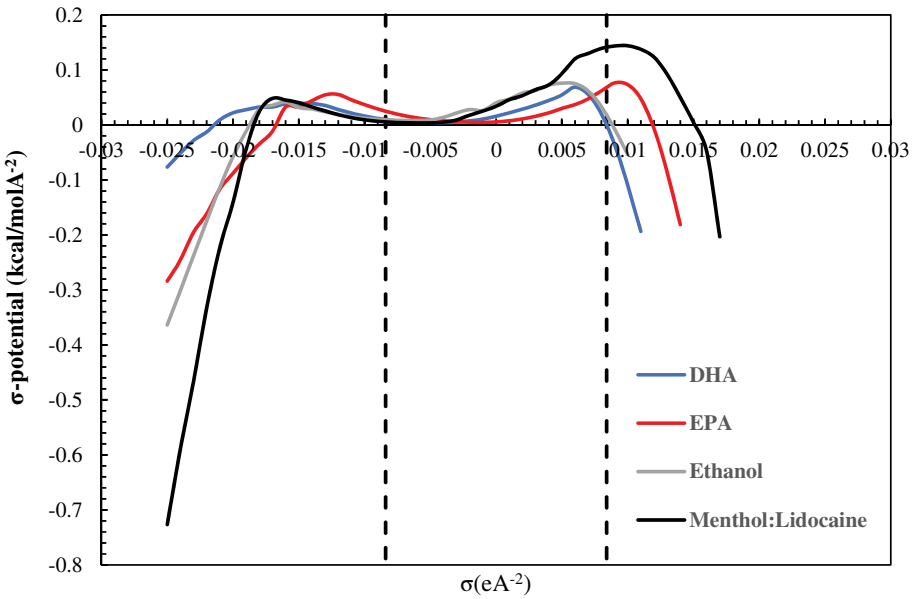

Figure 2. σ-potential of DHA, EPA, ethanol and menthol: lidocaine DES. Dashed lines indicate σ-potential thresholds assigned to hydrogen-bonding interactions: hydrogen bond donor region ($\sigma < -0.0085$ eA^{-2}), hydrogen bond acceptor region ($\sigma > 0.0085$ eA^{-2}), and non-polar region (0.0085 eA^{-2} < σ < 0.0085 eA^{-2}).

the formal HBD line. This implies that the menthol: lidocaine DES has a high affinity toward both EPA and DHA due to the hydrogen bond accepting abilities of the DES, which are accentuated by its proportionally greater surface area with a small partial negative (rather than positive charge).

Experimental screening results

The top nine DESs from COSMO-RS screening were selected and prepared. These were then used to extract EPA and DHA from *P. canaliculus* mussel powder. The extraction was performed at 25 °C and at 1000 rpm for 1 h by mixing the mussel powder and the prepared DESs at 5 w/v% of biomass loading. The choice of these conditions was based on those used previously to benchmark the extraction of fucoxanthin from algae.[45] Figure 3 presents the experimental results for the extraction of EPA and DHA using these DESs. The experimental screening results generally agreed qualitatively with predicted values using COSMO-RS in terms of the relative order of the DESs' ability to extract EPA and DHA. Both COSMO-RS and the experimental results showed a decrease in the solvent's ability to

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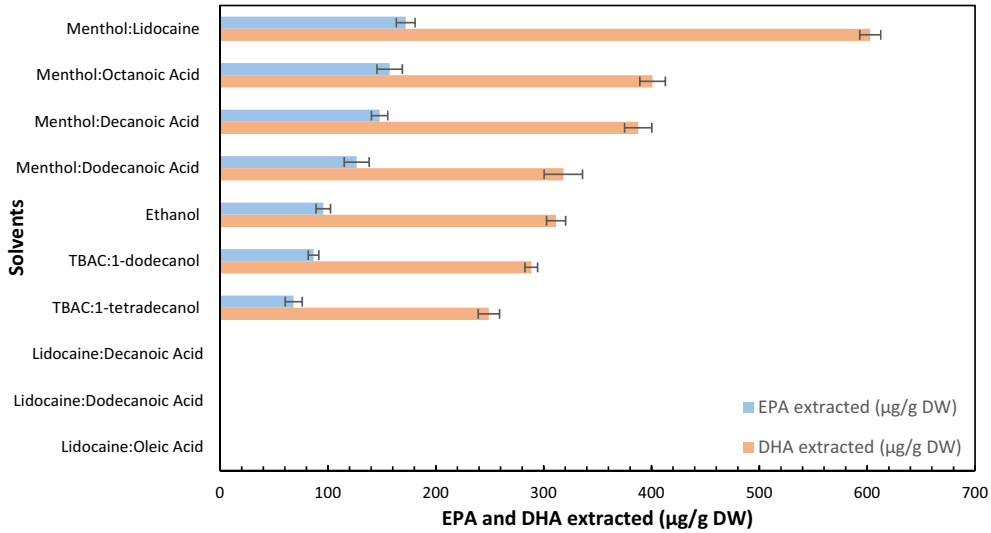

Figure 3. EPA and DHA extracted from mussel powder using the top nine DESs and ethanol.

extract EPA and DHA when the alkyl chain length of the HBD increased. This can be seen by comparing the extraction efficiency of the menthol-based DESs, menthol: octanoic acid (C8) (157.14 μg EPA g^{-1} DW and 400.95 μg DHA g^{-1} DW) and the longer chained menthol: dodecanoic acid (C12) (126.58 μg EPA g^{-1} DW and 318.18 μg DHA g^{-1} DW). Unlike the COSMO-RS predictions, lidocaine: fatty acid DESs did not lead to any EPA or DHA extraction. Compared with the other DESs, it was noted that these solvents had a very high viscosity at room temperature, which may have been the cause of this result rather than the absolute solubility of the acids.

The experimental result showed that the best DES for EPA and DHA extraction was the menthol: lidocaine DES (1:1 M ratio), as predicted by COSMO-RS. Overall, the results showed that the amounts of EPA and DHA extracted using menthol-based DESs were higher than those of other studied DESs. In addition, the extraction yield obtained from menthol: lidocaine DES (172.04 μg EPA g^{-1} DW, 602.79 μg DHA g^{-1} DW) was higher than that when using ethanol (95.65 μg EPA g^{-1} DW, 311.49 μg DHA g^{-1} DW).

The physicochemical properties of DESs can be influenced by DES components and the molar ratio. Hence, the effect of the molar ratio of menthol: lidocaine DES on the extraction efficiency of EPA and DHA was also investigated. Menthol: lidocaine DESs at different molar ratios (1:0.5, 1:1, 1:1.5, and 1:2 M ratios) were prepared and examined for EPA and DHA extraction.

The melting points of menthol: lidocaine DESs were measured using differential scanning calorimetry (PerkinElmer DSC 8500) and the results were presented in Table 4. The DSC measurements were carried out at a heating rate of 5 °C min^{-1}. The uncertainty in melting point measurements was ±0.05 °C. The melting point of the menthol: lidocaine DES at a 1:1 M ratio was 32.46 °C. However, the melted DES remained a homogeneous liquid at 25 °C.[49] In contrast, the melting points of the menthol: lidocaine DESs at 1:1.5 and 1:2 M ratios were 32.51 °C and 33.82 °C, respectively, and they were crystalline at 25 °C. At a ratio of 1:0.5, the melting point was reduced further to 24.12 °C, suggesting that the eutectic composition of this mixture is likely to be around 1:0.5 (i.e., 2:1 menthol: lidocaine). The water content of menthol: lidocaine DES at different molar ratios was also shown in Table 4. The water content of DESs was measured using Karl Fischer Moisture Analyzer (T5, Mettler Toledo). The measurement accuracy of the Karl Fisher coulometer was checked with the HYDRANAL water standard 10.00 mg g^{-1}. This standard was supplied in glass ampoules from Merck Pty Limited.

The extraction of EPA and DHA using the menthol: lidocaine DESs was performed at 35 °C to ensure all DESs were molten. Extractions were performed for 1 h by mixing each DES with mussel powder at 5 w/v%. The extraction results revealed that decreasing the lidocaine mole fraction decreased the extraction efficiency (Table 4), indicating that lidocaine has a high affinity for EPA and DHA. This was also in accordance with COSMO-RS screening results in Table 4. Therefore, the menthol: lidocaine DES at 1:2 M ratio, with extraction yields of 219 μg EPA g^{-1} DW and 910 μg DHA g^{-1} DW, was selected to optimise extraction parameters. Given the results for the other lidocaine DES, the presence of menthol is crucial for ensuring the low viscosity of the mixture[50] and enabling a high capacity of both EPA and DHA, alongside the appropriate physical properties to ensure the extraction remains feasible.

Optimisation of extraction variables

Optimisation of the extraction process can help enhance the target's yield, reduce the number of experiments needed, investigate the interactions between the studied variables, and minimise cost and time. Design-Expert software version 7.1 (Stat-Ease Inc., USA) was used to perform the experimental design and optimisation of the EPA and DHA extraction process from *P. canaliculus*. The software was used to create different extraction conditions and investigate the effects of these conditions on the DESs extraction efficiency. The three selected conditions, extraction time (A), temperature (B), and biomass w/v% (C), were investigated using BBD coupled with RSM. Biomass percentages of 5, 10, and 15% were used at temperatures within 35–65 °C for 1–3 h. Two responses were taken: EPA extracted and DHA extracted. Table 5 shows the experimental data of the three factors at different levels and the experimental response values for EPA and DHA. Since no

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Table 4. Predicted capacities of menthol: lidocaine DES for EPA and DHA at different molar ratios and experimental extraction results

HBA	HBD	Molar Ratio	Melting temperature (°C)	Water content (%)	γ EPA	γ DHA	Capacity EPA	Capacity DHA	EPA extracted (μg EPA g^{-1} DW)	DHA extracted (μg DHA g^{-1} DW)
Menthol	Lidocaine	1:0.5	24.12	0.1220	0.0100	0.0120	100.0000	83.3333	176	584
		1:1	32.46	0.1401	0.0074	0.0094	135.0109	106.8780	183	690
		1:1.5	32.51	0.1791	0.0064	0.0083	156.2500	120.4819	198	760
		1:2	33.82	0.2267	0.0059	0.0077	170.2713	129.3566	219	910

Table 5. Experimental runs and obtained response values for extracting EPA and DHA using menthol: lidocaine DES (1:2 M ratio)

Run	A: Time (h)	B: Temperature (°C)	C: Biomass (%)	Response: (μg EPA g^{-1} DW)	Response: (μg DHA g^{-1} DW)
1	3	65	10	222.92	886.54
2	3	50	15	206.88	856.18
3	2	50	10	241.99	989.1
4	2	65	15	224.12	963.18
5	1	35	10	191.44	750.59
6	2	50	10	240.22	988.67
7	3	35	10	204.34	807.35
8	2	65	5	261.95	1003.79
9	2	35	15	220.21	905.05
10	1	50	5	212.11	836.51
11	3	50	5	220.27	875.61
12	2	50	10	238.6	985.15
13	2	35	5	241.08	926.76
14	1	65	10	197.38	823.93
15	1	50	15	181.28	775.33

previous studies have been conducted on extracting EPA and DHA from green-lipped mussels, a moderate level of variables was used for optimisation. The main goal was to explore the effects of time, temperature, and biomass percentage on extracting EPA and DHA.

Using the experimental data from BBD, quadratic polynomial equations, Eqns. (1) and (2), were derived to describe the interactions between the responses and extraction variables to optimise EPA and DHA yields:

$$EPA = \begin{aligned} &+240.27+9.02A+6.16B-12.87C+3.16AB \\ &+4.36AC-4.24BC-33.98A^2-2.27B^2-1.16C^2 \end{aligned} \quad (1)$$

$$DHA = \begin{aligned} &+987.64+29.91A+35.96B-17.87C+1.46AB \\ &+10.44AC-4.72BC-142.16A^2-28.37B^2-9.57C^2 \end{aligned} \quad (2)$$

The determined coefficient (R^2), adjusted coefficient (R^2), and predicted coefficient (R^2) for the EPA model and the DHA model are shown in Table 6. The EPA and DHA models had the correlation coefficients of 0.9949 and 0.9991, respectively. The predicted R^2 for both models is close to the adjusted R^2, meaning that the models can adequately represent the data and are expected to be reliable in predicting responses for new experiments.

Based on the ANOVA results for EPA (Table 7) and DHA (Table 8), the high F values (107.86 and 601.80, respectively) and low *P*-values (<0.0001) indicate that both models were significant. Furthermore, the lack of fit (0.2379 and 0.1541, respectively) was insignificant, indicating that the models accurately described EPA and DHA yield. Regarding the EPA model, the *P*-values revealed that all single variables (A, B, and C) were significant. However, for the quadratic terms, only the quadratic term coefficient for temperature (A^2) was significant, as indicated by the *P*-value. The *P*-values from the DHA model indicated that all single variables and their quadratic term coefficients (A^2, B^2, and C^2) were significant. The interaction between temperature and time (AB) was found to be insignificant for both models. In addition, for the DHA model, the other insignificant interaction was that between time and biomass (BC).

The interactions between variables and the effects of these interactions on EPA and DHA extraction yields are shown on the surface response plots in Figs. 4 and 5. The figures show the interaction between two independent variables, with the third variable fixed at the centre point. Figure 4(a) represents the surface response of EPA yield due to the interaction between extraction time and temperature. From the plot, it can be established that increasing the temperature had little effect on the EPA yield. Conversely, extraction time had a significant effect, with a notable maximum being observed after 2 h. The ANOVA analysis revealed that the interaction between temperature and extraction time was statistically insignificant due to the high *P*-value (0.0617). However, the effect of temperature was more significant (F value = 93.88) than that of extraction time (F value = 43.77). This may be due to improved mass transfer properties resulting from lower viscosity at higher temperatures, as well as to potentially higher solubility values. Figure 4(b) presents the surface response of EPA yield due to the interaction between

Table 6. Analysis of variance for the fitted quadratic polynomial model of EPA and DHA using menthol: lidocaine DES (1:2 M ratio)

	EPA	DHA		EPA	DHA
Std.dev.	2.63	4.22	R^2	0.9949	0.9991
Mean	220.32	891.58	Adjusted R^2	0.9857	0.9974
C.V.%	1.20	0.47	Predicted R^2	0.9297	0.9866
PRESS	476.22	1294.29	Adequate precision	37.785	74.210

Table 7. ANOVA for response surface quadratic model of EPA extraction using menthol: lidocaine DES (1:2 M ratio)

Source	Sum of squares	Degree of freedom	Mean square	*F* value	*P*-value prob > *F*	Remarks
Model	6738.12	9	748.68	107.86	<0.0001	Significant
A:Temperature	651.60	1	651.60	93.88	0.0002	Significant
B:Time	303.81	1	303.81	43.77	0.0012	Significant
C:Biomass	1324.07	1	1324.07	190.76	<0.0001	Significant
AB	39.94	1	39.94	5.75	0.0617	Not significant
AC	76.04	1	76.04	10.96	0.0212	Significant
BC	71.91	1	71.91	10.36	0.0235	Significant
A^2	4262.66	1	4262.66	614.13	<0.0001	Significant
B^2	19.07	1	19.07	2.75	0.1583	Not significant
C^2	4.95	1	4.95	0.71	0.4371	Not significant
Residual	34.70	5	6.94			
Lack of fit	28.96	3	9.65	3.36	0.2379	Not significant
Pure error	5.75	2	2.87			
Cor total	6772.82	14				

Table 8. ANOVA for response surface quadratic model of DHA extraction using menthol: lidocaine DES (1:2 M ratio)

Source	Sum of squares	Degree of freedom	Mean square	*F* value	*P*-value prob > *F*	Remarks
Model	96 369.48	9	10 707.72	601.80	<0.0001	Significant
A:Temp	7159.26	1	7159.26	402.36	<0.0001	Significant
B:Time	10 345.69	1	10 345.69	581.45	<0.0001	Significant
C:Biomass%	2553.62	1	2553.62	143.52	<0.0001	Significant
AB	8.56	1	8.56	0.48	0.5189	Not significant
AC	435.77	1	435.77	24.49	0.0043	Significant
BC	89.30	1	89.30	5.02	0.0752	Not significant
A^2	74 622.19	1	74 622.19	4193.92	<0.0001	Significant
B^2	2972.83	1	2972.83	167.08	<0.0001	Significant
C^2	338.16	1	338.16	19.01	0.0073	Significant
Residual	88.96	5	17.79			
Lack of fit	79.57	3	26.52	5.65	0.1541	Not significant
Pure error	9.39	2	4.70			
Cor total	96 458.45	14				

extraction time and biomass loading. It was found that increasing the biomass percentage from 5% to 15% decreased the amount of EPA extracted. The ANOVA analysis also showed that the interaction between extraction time and biomass percentage was statistically significant due to the low *P*-value (0.0212). However, the effect of biomass was the most significant of all variables (F value = 190.76). Decreasing the biomass percentage in a solid–liquid extraction increases the volume of liquid within the extraction vessel, and this increase in the liquid ratio may allow for better penetration of the biomass' solid matrix. The solubility of the target solute can also become a limiting factor in the presence of high biomass loadings, leading to the formation of saturated solutions, which reduce the extraction efficiency per mass of biomass. Figure 4(c) represents the surface response of EPA yield due to the interaction between temperature and biomass loading. It was observed that increasing the temperature while decreasing the biomass loading will result in a significant increase in EPA yield.

Figure 5(a) represents the surface response of DHA yield due to the interaction between extraction time and temperature. The

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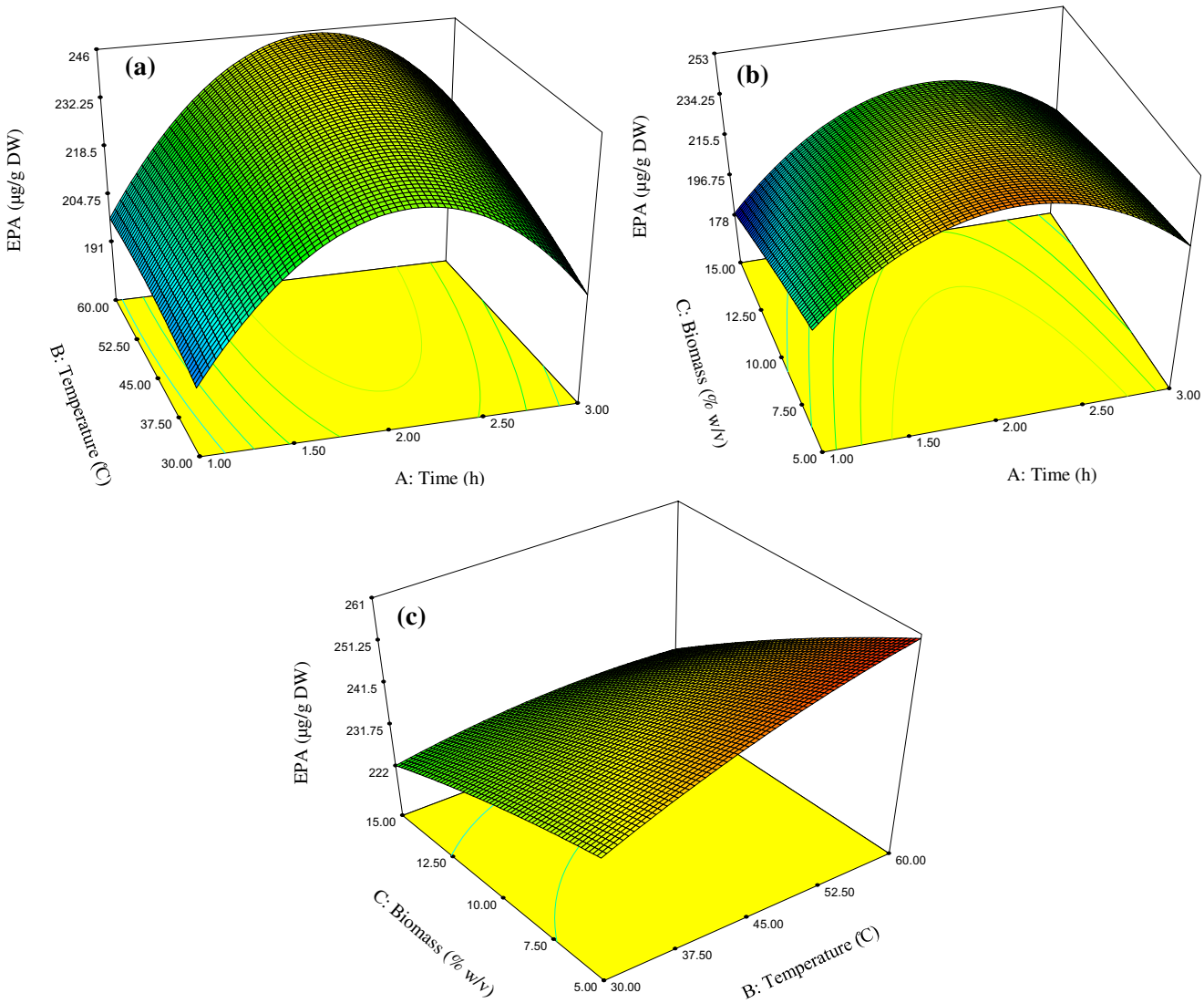

Figure 4. 3D plots of EPA extraction using menthol: lidocaine DES (1:2 M ratio) on the interactions between the considered factors: (a) temperature and time, (b) time and biomass w/v%, and (c) temperature and biomass w/v%.

plot shows that increasing the extraction time had little influence on the amount of DHA extracted, like EPA. Figure 5(b) shows that, by increasing the extraction time from 1 to 2 h, the DHA yield increased considerably. However, increasing the biomass percentage from 5% to 15% had little effect on DHA yield. This is reflected in each variable's statistical significance, with extraction time (F value = 581.45) having a much greater significance than biomass percentage (F value = 143.52) in terms of DHA yield. The ANOVA analysis indicated that the interaction between extraction time and biomass percentage was significant in terms of DHA yield. Figure 5(c) shows that increasing the temperature from 35 to 65 °C substantially influenced the DHA yield. This is clearly reflected in the ANOVA analysis, where the temperature was of high statistical significance (F value = 402.36). However, the ANOVA analysis indicated that the interaction between temperature and biomass percentage was not statically significant in terms of DHA yield. It is worth noting that the surface response of DHA yield (>1000 µg g^{-1}) could be achieved between temperatures of 50 to 65 °C and biomass percentages of 5% to 9.5%.

The optimum conditions calculated by the DOE software for the extraction of both EPA and DHA from NZGLM are displayed in Table 9. To verify the model, an extraction was carried out at optimum conditions and the extraction yield of EPA and DHA were compared to their respective predicted values. The experimental yield of EPA was found to be 267 µg g^{-1} DW, which was similar but slightly larger than the predicted value of 260.55 µg g^{-1} DW. Furthermore, the experimental value for DHA yield of 1014 µg g^{-1} DW was also similar but slightly larger than the predicted value of 1008.78 µg g^{-1} DW. The experimental results provided validation of the predicted models (error < 5%). This indicates that, to increase the extraction yield of EPA and DHA, the temperature needs to be increased towards the upper limit of the tested range at 65 °C, the biomass loading needs to be decreased towards the lower limit of the tested range of 5 wt%, and the extraction time needs to be 2.12 h, which is the intermediate between the values explored. From a safety perspective, the use of this DES will be less problematic than using ethanol at these elevated temperatures with respect to the lower volatility and flammability of the DES.

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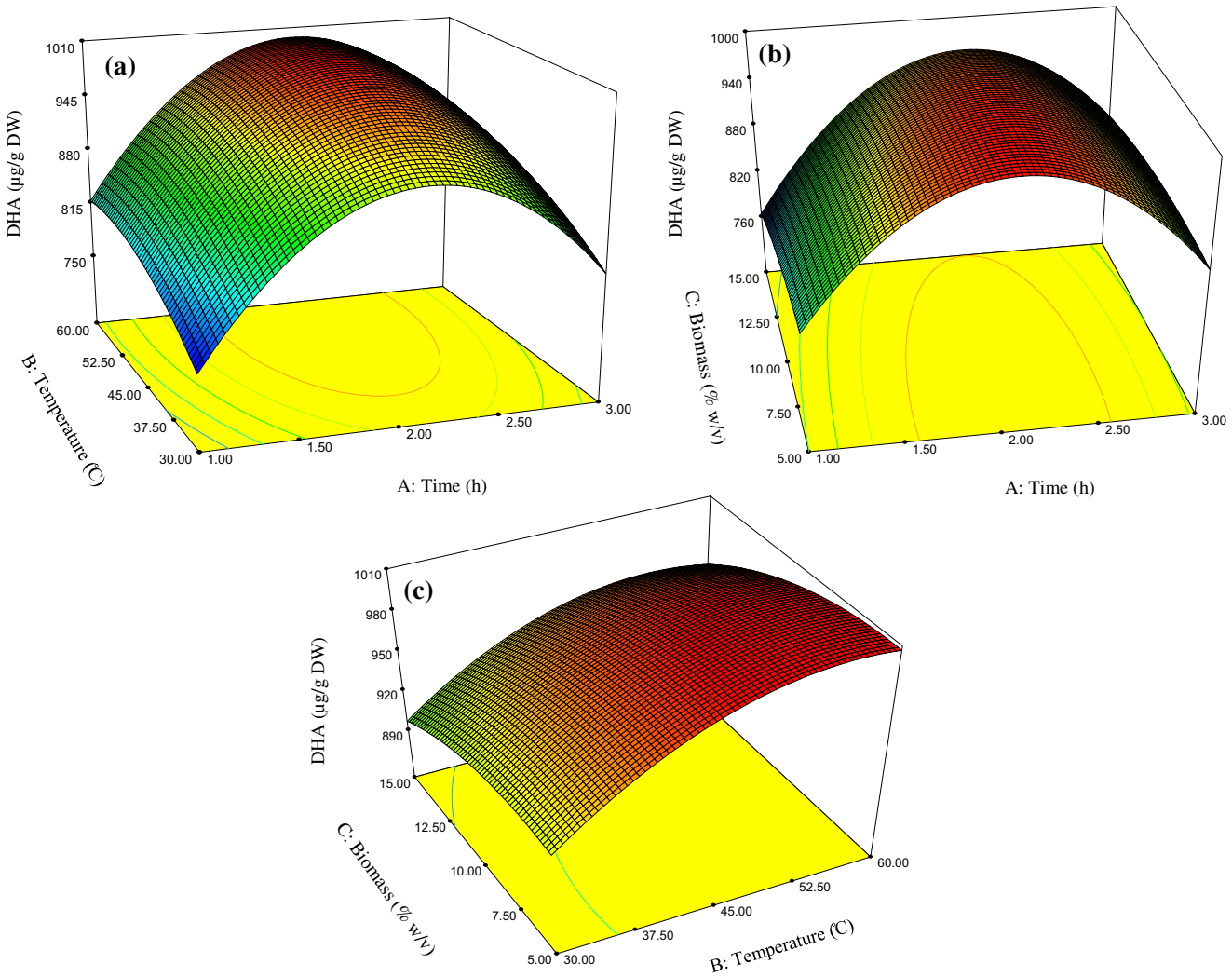

Figure 5. 3D plots of DHA extraction using menthol: lidocaine DES (1:2 M ratio) on the interactions between the considered factors: (a) temperature and time, (b) time and biomass w/v%, and (c) temperature and biomass w/v%.

Table 9. Experimental and predicted results of EPA and DHA extraction using menthol: lidocaine DES (1:2 M ratio) at optimum conditions

			EPA yield (µg g^{-1} DW)		
Time (h)	Temperature (°C)	Biomass w/v%	Predicted	Measured	Error (%)
2.12	65	5	260.55	267	2.47
			DHA yield (µg g^{-1} DW)		
			Predicted	Measured	Error (%)
			1008.78	1014	0.51

Stability and reusability results

The stability of both EPA and DHA over a seven-day period of storage in a dry place at 35 °C is shown in Fig. 6(a). The EPA and DHA concentrations after 7 days were within the experimental error of concentration day 1. The menthol: lidocaine DES had negligible volatility, which is an important trait for solvents containing bioactive products in food, pharmaceuticals, and cosmetics.

A solvent's reusability can be accredited to the degree of valuable compounds extracted over several regeneration cycles. Figure 6(b) shows the concentrations of EPA and DHA in the menthol: lidocaine DES that was recycled for five successive extraction

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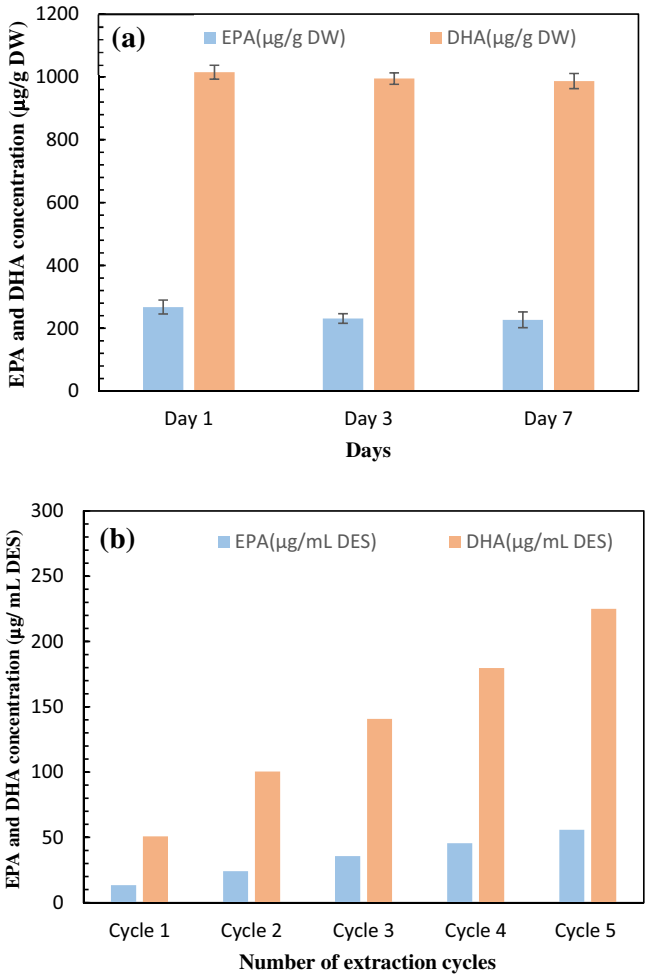

Figure 6. (a) EPA and DHA concentration at 35 °C over 7 days; (b) EPA and DHA concentration over five extraction cycles.

cycles, with a final EPA concertation of 55.83 μg mL^{-1} DES and DHA concentration of 225 μg mL^{-1} DES. Both EPA and DHA concentrations steadily increased over five extraction cycles. It can be expected that, after a certain number of cycles, no further ability to extract EPA and DHA will be observed. However, the DES's clear ability for reusability is a promising attribute for commercial scale-up and process sustainability.

CONCLUSIONS

COSMO-RS software was used to screen 22 hydrophobic DESs for the extraction of DHA and EPA. The DESs with the largest predicted capacities were selected for the experimental screening of these solvents. The experimental screening results confirmed that all menthol-based DESs outperformed ethanol and the other DESs studied. The screening results also showed that the best DES for EPA and DHA extraction was the menthol: lidocaine DES (1:1 M ratio), which agreed with the COSMO-RS predictions and gave extraction yields of 172.04 μg EPA g^{-1} DW and 602.79 μg DHA g^{-1} DW. The effect of the molar ratio of menthol: lidocaine DES on the extraction efficiency of EPA and DHA was also investigated and the results revealed that increasing the lidocaine mole fraction increased the extraction efficiency. Therefore, the menthol: lidocaine DES at 1:2 M ratio with an extraction yield of 219.82 μg EPA g^{-1} DW and 910.34 μg DHA g^{-1} DW under the screening conditions was selected to optimise extraction parameters. The process parameters (time, temperature, and biomass percentage) were optimised using BBD coupled with RSM. The analysis of variance results and response surface plots indicated that extraction time, temperature, and biomass loading were essential process parameters. The optimal process parameters were calculated to be 2.12 h, 65 °C, and 5 w/v% biomass. An experimental run at optimal conditions verified the model, with extraction yields of 267.59 μg EPA g^{-1} DW and 1014.84 μg DHA g^{-1} DW. Furthermore, the efficiency of the solvent after multiple extraction cycles and the stability of the solutes in the solvent over long periods of time were investigated. The recyclability results showed that the extraction efficiency of the menthol: lidocaine DES does not change significantly after five extraction cycles and that both EPA and DHA exhibited excellent stability over 7 days. While these extractions were performed using freeze-dried samples of NZGLM powder, the hydrophobicity of the optimal DES offers the potential to explore similar extractions using wet biomass, which is an approach currently under investigation in our laboratory.

ACKNOWLEDGEMENTS

The authors acknowledge the New Zealand Product Accelerator (NZPA) for providing master student scholarships for this work. The authors thank Sanford. Ltd, New Zealand, for providing the *Perna cannliculus* mussel powder. The authors would also like to thank Dr Matthew Sidford in the Department of Chemical and Materials Engineering at the University of Auckland for his support in conducting the HPLC analysis. Open access publishing facilitated by The University of Auckland, as part of the Wiley - The University of Auckland agreement via the Council of Australian University Librarians.

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