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RESEARCH ARTICLE

Optimisation of sulfate polysaccharide extraction from *Ulva lactuca* using natural deep eutectic solvent with ultrasound assistance

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Abstract

Background: *Ulva lactuca*, or sea lettuce, is a green macroalgae found in the Indonesian Sea, containing the active component Ulvan sulfated polysaccharide. **Objective:** This study aims to investigate the impact of extraction-free variables on ulvan yield and its corresponding metabolomic profile. **Methods:** Ulvan was extracted using three choline chloride-based NADES (KKG, KKL, KKT) with ultrasonic waves, optimising variables such as temperature, solution-to-solid ratio, and power. Total polysaccharides were measured using the phenol-sulfuric acid method, while total sulfates were measured by BaCl₂ absorption turbidimetry at 490 nm and 420 nm. Ulvan metabolomic profiles were assessed via FTIR, XRD, and LC-HRMS. **Results:** The independent variables consisted of 3 factors and 3 levels, resulting in 51 test points with optimum parameters: temperature at 78.27°C, L/P ratio at 39.62, and ultrasonic power at 139.23 watts. KKG achieved the highest yield at 11.7% ± 0.706, with sulfate levels ranging from 6.37% to 6.92% across all NADES. Extraction results were influenced by both single and combined independent variables. FTIR metabolomics revealed -CH, -S=O, -C=O, and -SO₄ groups, while LC-HRMS identified Rhamnosa 3-sulfate and DI-Xylosa compounds. UHD contains the Xylose-Rhamnosa 3-sulfate chain, part of Ulvanobiosis U3s. **Conclusion:** Choline chloride-based NADES, with ultrasound assistance, shows potential as a method for isolating active compounds from marine biota.

Introduction

Ulva lactuca, or sea lettuce, is a macroalga characterised by high adaptability, rapid growth (Olsson *et al.*, 2020), and relatively simple cultivation techniques (Hung *et al.*, 2021), as well as possessing potential bioactive compounds known as Ulvan (Olsson *et al.*, 2020). The main components of ulvan are sulfate group, glucuronic acid, iduronic acid, xylose, and rhamnose (Sari-Chmayssem *et al.*, 2019). Several physiological and biological properties have been studied, including anti-cancer (Thanh *et al.*, 2016), anticoagulant (Dhahri *et al.*, 2020), antioxidant (Chakraborty *et al.*, 2015), antifungal (Carvalho *et al.*,

2020), and anti-tumour (Figuroa *et al.*, 2022). The polysaccharide structure of ulvan has unique characteristics and functions (Andryukov *et al.*, 2020). The dried products of green macroalgae are consumed as a dietary supplement known as Nori (Manikandan & Lens, 2022).

Conventional extraction of natural materials using organic compounds requires a high solvent-to-sample ratio, a slow extraction process, and low selectivity (Georgiopoulou *et al.*, 2023). Additionally, the use of organic compounds as the primary solvent can lead to issues related to low biodegradability, biocompatibility, and sustainability (Ling & Hadinoto, 2022). The use of organic solvents in specific clinical applications can lead

to poor pharmacokinetics, off-target toxicity, and ineffective therapies. (Karaosmanoglu et al., 2021). Safe and efficient extraction techniques using solvents can reduce the cost and degradation of active ingredients (Sun et al., 2020).

Natural Deep Eutectic Solvent (NADES) is an eutectic mixture whose eutectic point is lower than that of an ideal liquid mixture, and it is composed of primary metabolites (Jeliński et al., 2019). Choline chloride-based NADES are environmentally friendly, biocompatible solvents that are highly effective for the extraction of phenolic compounds (Lu & Liu, 2022). NADES-based Ultrasonic-Assisted Extraction (UAE) can significantly reduce solvent usage compared to other extraction methods, such as solid-liquid extraction, motion-assisted extraction, and enzyme-assisted extraction with organic solutions (Menezes Maciel Bindaes et al., 2019).

This study focuses on the development of various variables that can affect the extraction results. Response surface methodology (RSM) is used to investigate interactions between variables. The Box-Behnken Design (BBD) is run for the efficiency of experimental research (Ahmad et al., 2020). RSM-BBD is expected to be effective in determining the optimal parameters for extracting sulfate polysaccharide (SP) from *Ulva lactuca* using NADES in the UAE. Metabolomics analysis uses the Fourier Transform Infrared (FTIR) spectrum to determine the functional groups of bioactive compounds.

Methods

Materials and reagents

Fresh *Ulva lactuca* from the coast of Takalar Regency and Barru in South Sulawesi Province was dried by aeration. Choline chloride, lactic acid, glycerol, triethylene glycol, absolute ethanol, 2-propanol, phenols, concentrated sulfuric acid, and standard D-glucose were derived from Sigma-Aldrich Chemical Co., Ltd. Aquadest was derived from Labiomed Puskesmas.

Sample preparation

Samples of *Ulva lactuca* were prepared as described by Barakat et al. (2022), with slight changes. In summary, dried *Ulva lactuca* was ground and then sifted through a 40-mesh sieve to obtain a green powder. Then, 20 grams of green powder was washed with 200 mL of 80% cold ethanol, gently shaken at 25°C for 12 hours, to eliminate phenolic compounds, free sugars, colour pigments, amino acids, and fats from the sample. The sample was centrifuged at 4500 rpm at 15°C for 15

minutes. Supernatants were discarded to obtain the biomass of *Ulva lactuca*. The addition of an n-hexane solvent removed the colour filaments in the biomass. The solvent was evaporated under a fume hood to obtain the Research Sample (RS). The moisture content of RS was analysed with the Moisture analyser VPB-10 Henk Maas®.

NADES preparation

The preparation of NADES was carried out following the method described by Fanali et al. (2021). In short, choline chloride was dried in an oven at 90 °C for 12 hours due to its hygroscopic properties. NADES was an eutectic mixture of compounds that are hydrogen bond donors (HBD) and hydrogen bond acceptors (HBA). Choline chloride-glycerol was mixed at a molar ratio (1:2) as done by J.-Z. Liu et al. (2024) and placed in an Erlenmeyer. The mixture was heated at 80 °C with constant shaking until it formed a thick and clear solution. To reduce the viscosity of the solution, 35% v/v aquadest was added, and the mixture was shaken again until a clear solution was formed.

Extraction of polysaccharides

The UAE procedure was conducted as described by Nie et al. (2020) with modifications to the independent variables. In summary, 1 g of SP was weighed, and the prepared NADES solvent was added. The independent variables used in the extraction were temperature (60, 70, and 80°C), liquid-to-solid ratio (20:1, 30:1, and 40:1), and ultrasonic power (50, 100, and 150 Watts). A single-factor experiment was conducted to evaluate the yield data. The sonicator's amplitude level was set at 40%, and the extraction time was 30 minutes. The extracted SP was centrifuged at 4,500 x g for 15 minutes at 25 °C. Then the residue was removed, and the supernatant was collected. The supernatant was mixed with 3 times the volume of 2-propanol and kept at 4°C overnight. The resulting precipitate was then separated from the solution by centrifugation at 4500xg for 15 min at room temperature. The crude extract obtained was washed with absolute ethanol and dried until raw polysaccharides were obtained. The coarse polysaccharides were dissolved again with distilled water and then dialysed with Aquadest to obtain Ulvan Dialysis Results (UDR). The high-purity SP was then freeze-dried for further analysis. Total polysaccharides were measured by the phenol-sulfuric acid method, and total sulfates were measured by the BaCl₂ absorption turbidimetry method at wavelengths of 490 nm and 420 nm, respectively. The percentage SP yield (%) was calculated as follows:

$$\text{Yield (\%)} = \frac{\text{Conc.SP (mg)} \times \text{NADES Vol. (mL)}}{\text{Dry Weight SP (mg)}} \times 100\% \quad (\text{Eq.1})$$

The experimental range for the RSM-BBD variable in the early stages used a single-factor test. One variable was examined individually for each one-factor experiment, with the other two variables remaining constant. Using a square polynomial equation, the relationship between the variables was encoded as follows: temperature (X_1), liquid/solid ratio (X_2), and ultrasonic power (X_3). The association between code variables and response variables used a quadratic polynomial model expressed in the following equation:

$$Y = -\beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (\text{Eq.2})$$

Where Y is the codified response corresponding to the sulfate polysaccharide extraction output (%); X_1 , X_2 , and X_3 are the codified variables for extraction temperature ($^{\circ}\text{C}$), L/S ratio (mL/g), and ultrasonic power (Watts), respectively. β_0 is the intercept coefficient, while β_1 , β_2 , and β_3 are linear coefficients. β_{11} , β_{22} , and β_{33} are the coefficients of square, while β_{12} , β_{13} , and β_{23} are the coefficients of interaction. Design-Expert 13 from StatEase was utilised to conduct an Analysis of Variance (ANOVA), develop a regression model, and determine the optimal extraction conditions to optimise the response. The BBD employs three factors and three levels with three centre points per block. Seventeen random test points are obtained for each solvent used.

Fourier transform infrared spectroscopy

A Fourier transform-infrared (FT-IR) spectrometer (IR Spirit, Shimadzu, Japan), combined with a Dynamic Alignment system, Sealed interferometer, was used to investigate the SP functional cluster. Single geranium crystal and the dried specimens (~ 1 mg) were placed on a trough plate. The range of the spectrum was 4.000 – 400 cm^{-1} .

Liquid Chromatography - High-Resolution Mass Spectrometry (LC-HRMS)

LC-HRMS testing followed the procedure described by Windarsih et al. (2022) (Windarsih et al., 2022). In summary, 17.8 mg of Sample UHD was dissolved in 1 mL of HPLC-grade methanol and vortexed for 30 seconds. The sample was then sonicated for 30 minutes at 25 $^{\circ}\text{C}$, followed by centrifugation at 1400 rpm for 5 minutes. The resultant supernatant was filtered through a 0.22 μm nylon membrane filter. A total of 5 μL of filtrate was injected into the column and diluted with solution A (0.1% formic acid in water) and solution B (0.1% formic acid in acetonitrile). Elution was started with a 5% solution B, gradually increased to 90% over 16 minutes, held at 90% for 4 minutes, and then returned to 5% B for 25 minutes. The elution rate was set at 0.3 mL/min.

Statistical analysis

In the single factor test for NADES, 3 factors and 3 levels were used, namely: X_1 : (Extraction temperature: 60, 70, and 80 $^{\circ}\text{C}$), X_2 (L/S ratio: 20:1, 30:1, and 40:1) and X_3 (ultrasonic power: 50, 100, and 150 Watts) have been set for the analysis of the response surface. The data was analysed with Design-Expert 13 from StatEase, and BBD was applied to the experimental design of this study. The encoding and actual values are presented in Table I, and the extraction rate corresponds to the response value. The output data were used to create response surface plots, contour plots, and interference plots. The average value is declared significant if the p-value is less than 0.05.

Results

Research sample and NADES

The research sample used was in the form of a pale white powder with a moisture of 18.87%, and the NADES produced was a clear, viscous, and odourless liquid.

Analysis of experiments with a single-factor

Effect of NADES on extract yield

The effect of the type of NADES solvent used in the extraction is shown in Figure 1a, where the extraction yield from KKG yields better results compared to CCT and CCL. CCG may have pH and viscosity values that are closer to the optimum value of extraction. The solvent will more easily penetrate into the cell, dissolve the target compound, and carry it out of the matrix. Additionally, KKG has a greater capacity for hydrogen bonding and exhibits more electrostatic contact with UHD than any other material, as explained by Khezeli et al. (2016).

Effect of extraction temperature on extract yield

The results showed that the optimum temperature was reached at 79.6 $^{\circ}\text{C}$, as shown in Figure 1b. Higher extraction temperatures may increase yields, but they may not be able to withstand polysaccharide thermolysis (Nipornram et al., 2017).

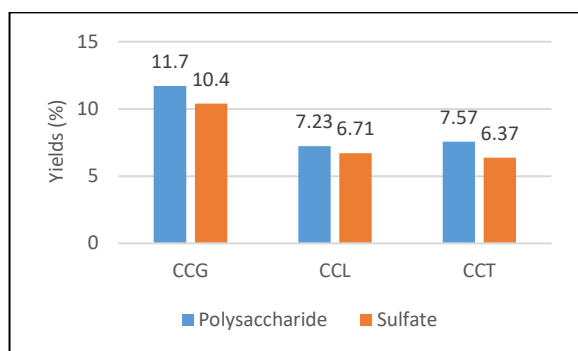
Effect of liquid-solid ratio on extract yield

Three Nades-SP variations were prepared with L/S (mL/g) ratios of 40:1, 30:1, and 20:1, studied, and the results are shown in Figure 1c. The ideal proportion of liquid to solid affects the extraction results, with the optimum ratio at 39.4 mL/g.

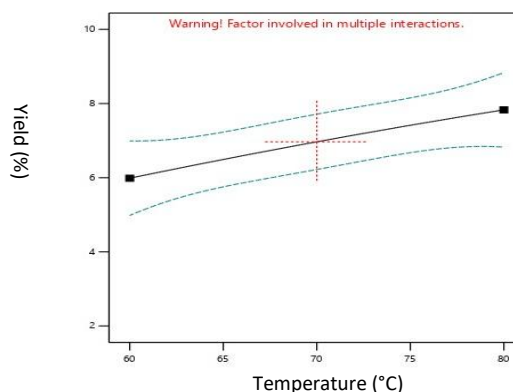
Effect of ultrasonic power on extract yield

The optimum ultrasonic power during the study was obtained at 143.9 Watts, as shown in the Figure. 1d.

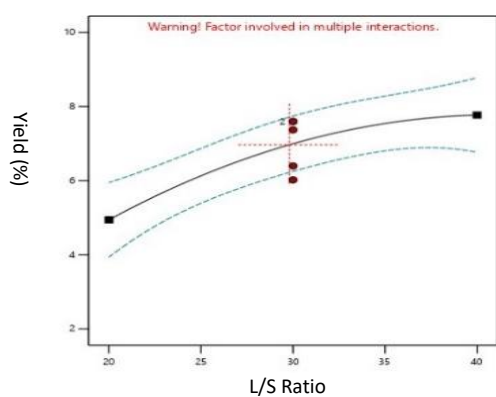
Meanwhile, the time of use of ultrasonic waves was set for 30 minutes during the study to avoid the possibility of bioactive compound damage.



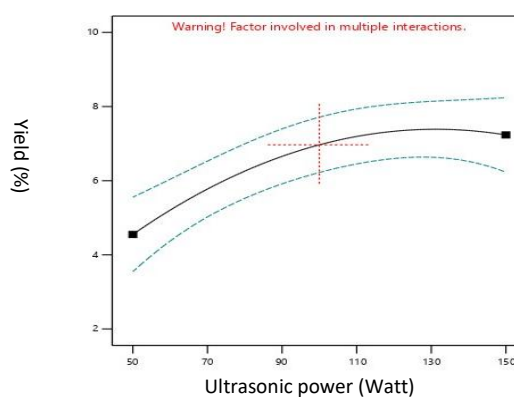
(a)



(b)



(c)



(d)

Figure 1: Impact of a single variable on extract yield

Response surface methodology

The effects of three independent variables — namely, temperature, L/S ratio, and ultrasonic power — have been examined using Response Surface Methodology (RSM) to determine the optimal ultrasound extraction conditions. This study uses three single factors with three levels as independent variables. Meanwhile, SP yield is used as a bound variable. To further optimise the sulfate polysaccharide extraction conditions, data were collected from 17 test points based on a BBD. Among these, the 14th run yielded the highest SP extraction output, reaching 11.70%.

Statistical data

The F value for the Quadratic model, according to ANOVA, is 12.01, and the *p*-value is 0.0017 or < 0.005. This indicates that the model employed is significant, whereas the Lack of Fit F value is 0.81, which is not significant. Simulation on test data with Design Expert 13

software can predict the yield according to the following equation:

$$\text{Yield} = -9.68294 + 0.108408 X_1 - 0.230618 X_2 + 0.191596 X_3 + 0.008407 X_1 X_2 - 0.001836 X_1 X_3 + 0.001671 X_2 X_3 + 0.000594 X_1^2 - 0.006395 X_2^2 - 0.000430 X_3^2 \quad (\text{Eq.3})$$

Eq. 3 is a mathematical equation resulting from a test that uses Nades KKG solvent as the solvent that produces the highest yield. By using the equation model above for each solvent, prediction data of extraction results and the variables that affect it are obtained. The comparison of actual data with predictions shows results that are almost identical. This demonstrates that the statistical model, utilising the RSM approach, yields accurate and efficient results by focusing on the optimal parameters of its supporters. Currently, the RSM approach has been widely used in extraction research with a high degree of accuracy. The response surface plot and contour plot from Figure 2 show how the variables affect the total polysaccharide

yield of SP. The use of extraction temperature and solution-solids ratio in this study follows the data of previous research. The low-power use of ultrasonics is

intended to reduce damage to target compounds, which can hinder the development of extraction efficiency, as mentioned by Şahin et al. (2013).

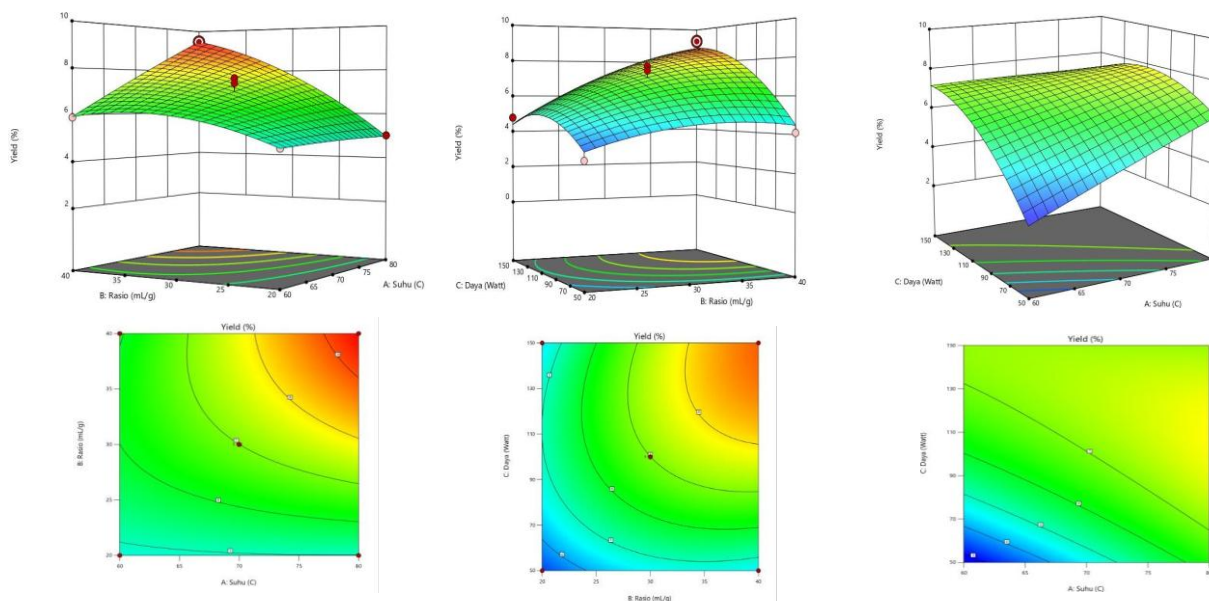


Figure 2: 3D and surface response contours on free-variable interactions (a: temperature, b: liquid/solid ratio, and c: ultrasonic power) concerning the yield of the whole extract of polysaccharides.

The 3D response surface plot and contour plot in Figure 2 illustrate how the variable affects the total polysaccharide yield of the SP. The use of extraction temperature and solution-solids ratio in this study is consistent with findings from previous studies. In contrast, the use of low ultrasonic power in this study was intentional to minimise damage to the target compounds, which can hinder the development of extraction efficiency. From the data in Table I, it is

evident that the optimum parameters for ulvan extraction from *Ulva lactuca* using a choline chloride-based NADES in the UAE are: a temperature of 78.27°C, a solution-to-solids ratio of 39.62, and an ultrasonic power of 139.23 Watts. This demonstrates that the statistical model, utilising the RSM approach, yields accurate and efficient results by focusing on the optimal parameters of its supporters.

Table I: Optimum parameters of extraction

Factor	Name	Level		Optimum	Std.Dev.	Code
		High	Low			
A	Temperature (°C)	80	60	78.27	0.000	Actual
B	l/s Ratio	40	20	39.62	0.000	Actual
C	Ultrasonic power (Watt)	150	50	139.23	0.000	Actual
D	NADES	CCG	CCL	CCT	N/A	Actual

The UDR FTIR spectrum shows a unique and wide absorption band at 3317 cm⁻¹ (J.-Z. Liu et al., 2024). It shows the stretching vibrations of the OH group in UHD, which result from intermolecular and intramolecular hydrogen bonds (Robic et al., 2009), while the slight peak at 2938 cm⁻¹ refers to the kinetic

stretching vibrations of the CH aliphatic bonds of the methyl group, typical for polysaccharides (Barakat et al., 2022). The highest point in the spectrum appears around 1038.88 cm⁻¹, reflecting the stretching of the CO bonds in the main rhamnose sugar. The characteristics of sulfate polysaccharides are validated

by an absorption band in the exact region of 860.95 cm⁻¹, indicating a sulfate group, and the spectrum at 954

cm⁻¹ is predicted to be an ester group (S=O) as described by Abdallah et al. (2022).

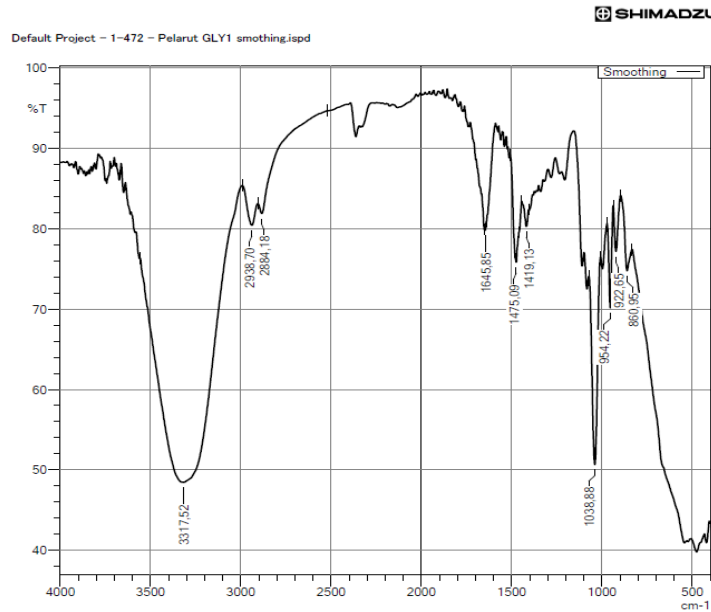
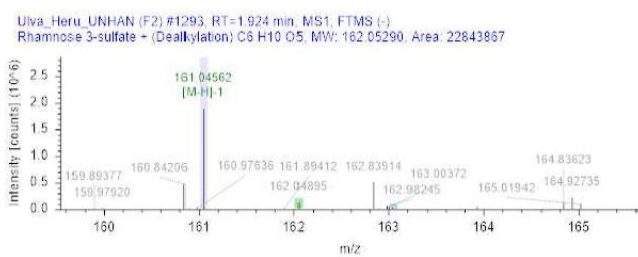
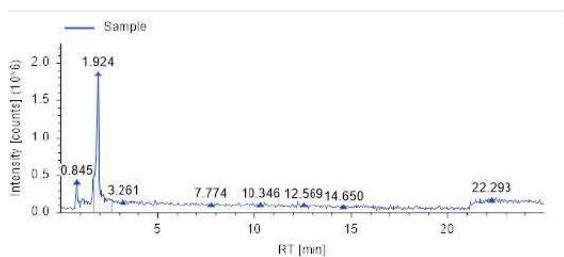


Figure 3: FTIR spectra of sulfate polysaccharides

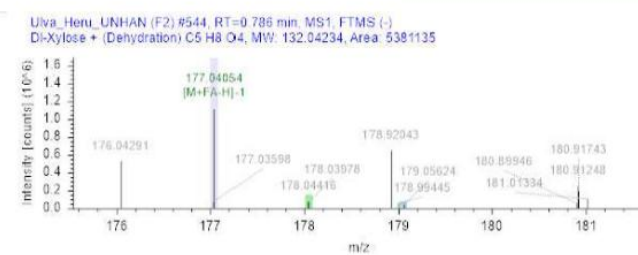
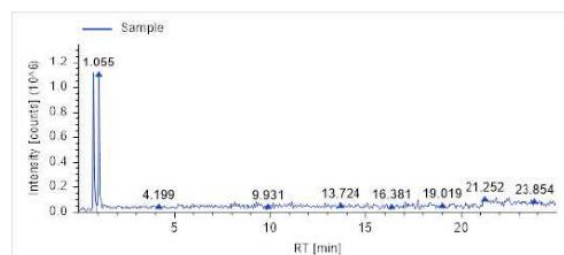
Liquid chromatography high-resolution mass spectrometry (LC-HRMS) is an advanced analytical technique that combines the separation capabilities of

liquid chromatography (LC) with high-resolution, accurate mass measurement capabilities (Figure 4).

Parent Compound	Formula	Calc. MW	RT [min]	Dealkylated	Transformations	Composition Change	FISH Coverage	Group Areas
Rhamnose 3-sulfate	C6 H10 O5	162.05290	1.92	X		-(H2 O3 S)	n/a	2.29e7



Parent Compound	Formula	Calc. MW	RT [min]	Dealkylated	Transformations	Composition Change	FISH Coverage	Group Areas
Di-Xylose	C5 H8 O4	132.04234	0.79		Dehydration	-(H2 O)	n/a	5.38e6



Ulva_Heru_UNHAN (F2) #567, RT=0.847 min, MS2, FTMS (-), (HCD, DDA, 177.0405@ (30.50.70), -1)
Di-Xylose + (Dehydration) C5 H8 O4, MW: 132.04234, Area: 5381135

Figure 4: LC-HRMS spectra

UDR analysis revealed the presence of a target compound, namely Rhamnosa 3-sulfate, which was detected at an RT of 1.924 minutes and a molecular weight of 162.05290 in the 22843867 area. While the

peak at 0.79 minutes, the molecular weight of 132.04234 is a DI-Xyloso compound, so UDR contains the Xylose Rhamnose 3-sulfate chain, classified as Ulvanobiosis U3s, as shown in Figure 5.

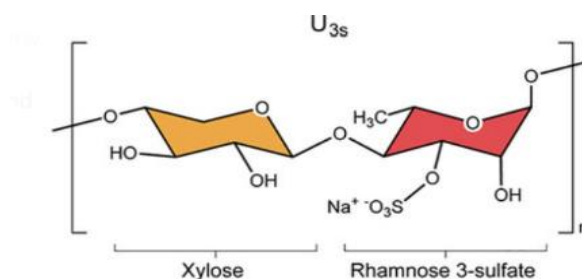


Figure 5: Ulvanobiosis U3s

Discussion

Analysis of experiments with a single-factor

During the extraction process, an interaction occurs between NADES and the target compound. The solvent is expected to extract the target compound from the sample matrix. The characteristics of NADES can directly affect the penetration of the solvent into the sample matrix and the mass transport of the target compound from the sample cell to the solution. Additionally, the interaction between PUL and chloride anions can be significantly reduced by the steric hindrance of the three hydroxyl groups on glycerol, as explained by Chi et al. (2020).

One crucial aspect that affects the extraction yield is the temperature. The target chemical is absorbed into the sample matrix through chemical and physical adsorption. Both physical and chemical adsorption interactions frequently decrease at high temperatures. This results in an increased flow of the target compound from the solvent. (Qi et al., 2015). In addition, as the extraction temperature increases, mass transport is enhanced and solvent diffusion improves, while the solvent's viscosity decreases significantly.

The extraction results are affected by the solution-solids ratio. As the liquid-to-solid ratio increases, the extraction yield first increases and then remains stable. This occurs because the target compound is only partially extracted at the extraction equilibrium, which is easily achieved with a minimal volume of extraction solvent. Although increasing the solvent volume can accelerate the release of the target compound, excessive solvent use may complicate the extraction process and result in unnecessary solvent waste, as noted by Wang et al. (2016).

The tissue structure of plant cells can be destroyed by the ultrasonic waves generated by an ultrasonic generator. The intense cavitation, constant mechanical vibrations, and associated disruptive effects facilitate the rapid release of polysaccharide compounds from the target cells (Manukovskaya et al., 2021). However, increasing ultrasonic power does not necessarily enhance the extraction effect, as intense ultrasound energy can break the bonding structure of SP compounds, thereby reducing the amount of SP extracted, as explained by Liu et al. (2023).

Response surface methodology

The data demonstrate a substantial correlation between the impacts of the extraction parameters and the extraction outputs. Single-factor investigations revealed that the total polysaccharide content varied based on the type of NADES, extraction temperature, solvent-solids ratio, and ultrasonic intensity. The optimal polysaccharide extraction findings generated from the BBD matrix experiment closely match those of the previous single-factor experiments, indicating the efficacy of the proposed ultrasonic extraction parameters: a temperature of 79.6 °C, a solvent-to-solids ratio of 39.4 mL/g, and an ultrasonic power of 143.9 Watts. According to Lahaye & Robic (2007), the composition of sulfates in RS is relatively the same at 6.37–6.92%. Additionally, sulfated polysaccharides (SP) account for approximately 9–36% of the dry weight in *Ulva* species.

Conclusion

In summary, NADES is used to extract sulfate polysaccharides from *Ulva lactuca* with the help of

ultrasound. RSM-BBD is employed to optimise extraction conditions based on the findings of single-factor experiments and multi-factor studies. The test results revealed that several parameters significantly influence the extraction outcome. These findings support the conclusion that the NADES-based, ultrasound-assisted extraction method is a rapid, efficient, and environmentally friendly approach for isolating sulfated polysaccharides from the sample matrix. Extraction optimisation with UAE-based NADES is designed to increase the yield of extraction from the *Ulva lactuca* cell matrix. The optimum value conditions can be estimated with the highest confidence level at 79.9 °C, a liquid/solid ratio of 39.47 mL/g, and 129,83 Watts of ultrasonic power. The results of the model validation measurement indicate consistency with the predicted value. Sulfate polysaccharide extraction from *Ulva Lactuca* using NADES with ultrasound assistance shows a potential method for isolating active compounds from marine biota.

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