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Optimized ultrasound-assisted tryptophan extraction from *Kappaphycus* spp.: Enhancing bioactive compound profiling and cultivation strategies

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ABSTRACT

Kappaphycus spp., a widely cultivated seaweed, is valued for its diverse bioactive compounds, including tryptophan. This study aimed to optimize and validate an ultrasound-assisted extraction (UAE) method to quantify tryptophan using a Box-Behnken design integrated with response surface methodology. The factors evaluated included solvent composition (50–90 % methanol in water), pH (2–8), ultrasound amplitude (25–75 %), and pulse duty cycle (0.2–1.0 s^{-1}). The optimized extraction variables—73 % methanol in water, pH 8, 58 % ultrasound amplitude, and a 0.8 s^{-1} pulse duty cycle at 60 °C for 10 min—achieved high recovery (>90 %) and precision (CV <5 %). This method was applied to 36 samples of *Kappaphycus alvarezii* and *Kappaphycus striatum*, revealing significant variations in tryptophan content based on species, environmental factors, and farming methods. Notably, *K. striatum* from Sekotong (Lombok) cultivated via the longline method exhibited the highest tryptophan yield. The findings underscore the potential of optimized UAE for supporting seaweed biotechnology and enhancing bioactive compound production through improved cultivation strategies.

1. Introduction

Indonesia's vast and biodiverse waters hold immense potential for seaweed production, with *Kappaphycus* spp. being the most extensively cultivated genus. In 2022, this genus alone accounted for 9.29 million tonnes of seaweed production (KKP, 2022). Traditionally, *Kappaphycus* spp. primarily utilized for hydrocolloid production, particularly agar and carrageenan (FAO, 2023). However, emerging research highlights its potential as a valuable source of bioactive compounds, including phenolic compounds (flavonoids and phenolic acids), sulfated polysaccharides, carotenoids, and amino acids, which exhibit promising therapeutic applications in the prevention and management of chronic diseases (Qiu et al., 2022; Ummat et al., 2020).

Among these bioactive compounds, amino acids—particularly tryptophan—have gained increasing attention due to their significant physiological and biochemical roles in both human health and seaweed metabolism. Tryptophan is an essential precursor for serotonin and melatonin, influencing mood regulation, sleep, and cognitive function (Kałużna-Czaplińska et al., 2019). In seaweed cultivation, tryptophan functions as a key growth hormone, promoting thallus development and productivity, which correlates with increased carrageenan yield (Plaimo et al., 2021). These multifaceted roles suggest that tryptophan could serve as a potential biomarker for optimizing cultivation strategies in *Kappaphycus* spp.. However, research on tryptophan extraction from *Kappaphycus* spp. remains limited, particularly regarding its efficient recovery, quantification, and application, warranting further investigation.

The extraction of tryptophan from *Kappaphycus* spp. presents significant challenges due to its dense cell matrix, which hinders compound release. Conventional extraction methods, such as maceration and Soxhlet extraction, are inefficient in breaking down thick cell walls, resulting in low bioactive compound yields and limited extraction

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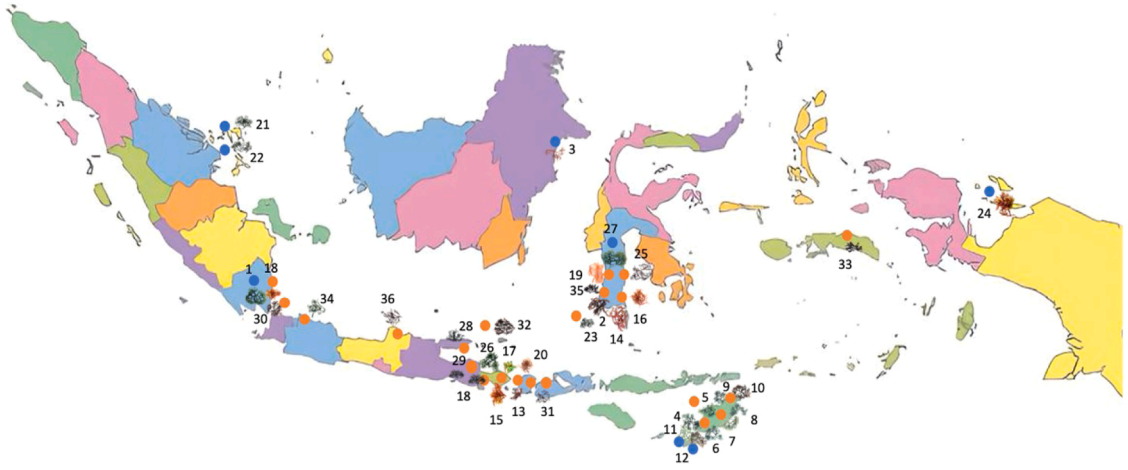


Fig. 1. Distribution map of 36 *Kappaphycus* samples collected from various locations in Indonesia using two different cultivation methods: bottom-off (●) and longline (●). The sampling site: (1) Teluk Pandan, (2) Puntondo, (3) Kepulauan Melahing, (4) Desa Akle, (5) Desa Sulamu, (6) Desa Tablolong, (7) Desa Oenaek, (8) Desa Tesabela, (9) Desa Lifuleo Green, (10) Desa Lifuleo Brown, (11) Desa Naikean Green, (12) Desa Naikean Brown, (13) Sekotong, (14) Djene Ponto, (15) Takalar, (16) Djene Ponto *alvarezii*, (17) Lembongan Island, (18) Teluk Pandan, (19) Puntondo, (20) Lombok, (21) Geranting Island (short thallus), (22) Geranting Island (long thallus), (23) Tenakeke Island, (24) Serui, (25) Bantaeng, (26) Nusa Penida, (27) Tamalaba, (28) Sumenep, (29) Bali, (30) Ketapang, (31) Sekotong, (32) Situbondo, (33) Moluccas, (34) Thousand Islands, (35) Takalar, and (36) Jepara.

efficiency. Additionally, these techniques are time-consuming, require large volumes of organic solvents, and consume high amounts of energy, making them environmentally unsustainable (Chemat et al., 2011; Mandal et al., 2015).

To address these limitations, green extraction techniques such as Supercritical Fluid Extraction (SFE), Pressurized-Liquid Extraction (PLE), Ultrasound-Assisted Extraction (UAE), Microwave-Assisted Extraction (MAE), Enzyme-Assisted Extraction (EAE), Ultrasound-Microwave-Assisted Extraction (UMAE), and Liquefied Gas Extraction (LGE) have been developed. These methods have demonstrated higher extraction efficiency, reduced solvent usage, and improved reproducibility, making them more sustainable alternatives (Quitério et al., 2022; Ummat et al., 2021). Among these, UAE is particularly advantageous for extracting bioactive compounds from *Kappaphycus* spp. due to its ability to deliver high-intensity ultrasound through acoustic cavitation, effectively disrupting rigid cell walls and enhancing mass transfer.

The use of a probe in UAE provides greater extraction control, as it delivers high-intensity ultrasound energy through a small surface area, leading to more efficient cell wall disruption compared to other green extraction techniques (Maia et al., 2023). This makes UAE particularly suitable for maximizing tryptophan recovery while maintaining high selectivity and reproducibility. Additionally, UAE offers several advantages over conventional methods, including faster extraction times, lower solvent consumption, and reduced thermal degradation, ensuring that tryptophan remain intact throughout the extraction process. However, the effectiveness of UAE depends on optimizing key extraction variable, such as solvent composition, pulse duty cycle, pH, temperature, liquid-to-solid ratio, and particle size (Tomšik et al., 2016). Despite its potential, previous studies have not comprehensively evaluated these critical UAE variables for tryptophan extraction from *Kappaphycus* spp., and the influence of different cultivation sites and farming methods on tryptophan content remains unexplored. Addressing these gaps is crucial for developing a standardized, efficient, and reproducible method for bioactive compound extraction in seaweed matrices.

This study aimed to optimize UAE conditions for tryptophan extraction from *Kappaphycus* spp. using a Box-Behnken Design (BBD) in conjunction with Response Surface Methodology (RSM). The use of BBD is particularly advantageous as it allows for efficient optimization of quadratic model variables and accurately predicts optimal extraction conditions (Ferreira et al., 2007). The findings of this study provide a validated and reproducible UAE method for tryptophan quantification, ensuring maximum recovery while minimizing degradation, thereby enhancing analytical accuracy and reliability. Additionally, this study offers insights into how different *Kappaphycus* spp. cultivation methods influence tryptophan content, contributing to bioactive compound profiling and potential improvements in seaweed farming strategies. Furthermore, this research establishes a standardized UAE protocol that can be applied in future studies on the extraction of other nitrogen-containing bioactive compounds from marine sources, expanding its applicability in the field of seaweed biotechnology and functional ingredient development. These insights are crucial for advancing sustainable seaweed utilization, improving biotechnological applications, and optimizing cultivation strategies to enhance bioactive compound production. By addressing this research gap, our study provides a scientifically robust foundation for further exploration of *Kappaphycus* spp. as a valuable functional bioresource with applications in nutraceuticals, functional foods, and marine-based bioactive research.

2. Materials and methods

2.1. *Kappaphycus* samples

This study utilized two species of *Kappaphycus*—*Kappaphycus alvarezii* and *Kappaphycus striatum*—to assess the tryptophan content across different environmental and cultivation conditions. A total of 36 fresh *Kappaphycus* samples (Fig. 1) were obtained from local farmers across eight islands in Indonesia, namely Sumatra, Java, Bali, Lombok, Celebes, Borneo, Moluccas, and Papua. These islands were chosen to provide diverse environmental and cultivation conditions (bottom-off and longline techniques), forming a comprehensive dataset for evaluating the variability of tryptophan content based on geographic and agricultural factors.

Following collection, the seaweed samples were immediately subjected to lyophilization using a Labcoco FreeZone series benchtop freeze dryer (MO, USA). Lyophilization was conducted at −88 °C and 0.099 bar for 36 h to preserve the integrity of bioactive compounds by minimizing thermal degradation. Once freeze-dried, the samples were ground into a fine powder using a KLAZ grinder (Guangdong, China). The grinding process involved an on/off cycle of 30 s, repeated until all particles could pass through a 60-mesh sieve, ensuring uniform particle size for subsequent analyses.

The powdered samples were then stored in vacuum-sealed plastic

bags at −4 °C to prevent moisture absorption and potential degradation of bioactive compounds, particularly tryptophan. This standardized preparation ensured the stability and reproducibility of the samples during analysis.

2.2. Chemical and reagents

Chromatography-grade solvents including ethanol, methanol, ethyl acetate, and acetonitrile were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained using a Milli-Q water purification system (EMD Millipore Corporation, Bedford, MA, USA). The standard tryptophan compound (>98 % purity) was acquired from Sigma Aldrich (St. Louis, MO, USA).

2.3. Ultrasound-assisted extraction (UAE)

The *Kappaphycus* samples were extracted using an ultrasonic system (Sonopuls HD 4200, 20 Hz, 200 W, BANDELIN electronic GmbH & Co KG, Heinrichstrabe, Berlin, Germany) equipped with a TS 104 probe (4.5 mm diameter). The ultrasonic system was temperature-controlled using a water bath (J. P. Selecta, Barcelona, Spain) to maintain stable extraction conditions. To ensure consistency in ultrasound energy dispersion and reproducibility, the sample-to-probe distance was standardized at 1.5 cm from the bottom of the extraction vessel across all replicates. This distance was determined based on preliminary trials, which demonstrated that 1.5 cm provided optimal cavitation efficiency, uniform acoustic wave propagation, and enhanced mass transfer, facilitating the effective release of tryptophan from *Kappaphycus* spp.

The study optimized ultrasound-assisted extraction (UAE) conditions for tryptophan recovery using a Box-Behnken design with three independent extraction conditions: solvent concentration, solid-to-solvent ratio, and extraction time: solvent composition (50–90 % methanol in water), pulse duty cycle (0.2–1 s^{-1}), pH (2–8), and ultrasonic power (25–75 %). A solvent-to-solid ratio of 25:1 and an extraction temperature of 60 °C were selected based on preliminary screening experiments, which indicated effective tryptophan recovery while minimizing degradation. Following extraction, the mixture was centrifuged (Sorval ST-8R, Thermo Fisher Scientific, Langenselbold, Germany) at 4000 rpm for 10 min to separate the supernatant. The resulting supernatant was then diluted to 25 mL with fresh methanol, transferred to sealed vials, and stored at −4 °C to preserve stability before further analysis. To ensure the integrity of tryptophan during storage, a stability assessment was conducted by monitoring tryptophan levels over an 11-week period at three different temperatures: 4, 25, and 30 °C. The results demonstrated that tryptophan remained stable at 4 °C, with no significant variations detected throughout the storage duration. However, a progressive decline in tryptophan concentration was observed at 25 °C and 30 °C, likely due to oxidative and thermal degradation.

To further minimize degradation risks and ensure stability, all extracts were stored in tightly sealed amber glass vials to prevent light exposure and maintained under refrigeration at −4 °C. These measures were implemented to ensure that the extracted tryptophan remained chemically stable prior to subsequent analytical procedures, thereby enhancing the reliability of the quantification process.

2.4. Determination of tryptophan using UPLC-FD

Tryptophan in the extracted samples was quantified using an ultrahigh-performance liquid chromatography system coupled with a fluorescence detector (UPLC-FD). The analysis was performed on a C18 solid-core reverse-phase column (1.7 μm, 2.1 × 100 mm, CORTECS UPLC, Waters Corporation, Ireland) maintained at a constant temperature of 47 °C. The mobile phase consisted of phase A (2 % acetic acid in ultrapure water) and phase B (2 % acetic acid in acetonitrile), using a gradient elution program: 0 min, 0 % B; 1 min, 0 % B; 3 min, 5 % B; 4 min, 10 % B; 4.5 min, 10 % B; 5 min, 20 % B; 7 min, 20 % B; 8 min, 30 % B; 9 min, 100 % B; 12 min, 100 % B; and 13 min, 0 % B. The flow rate was set at 0.55 mL min^{-1}. Prior to injection, the extracts were filtered through a 0.22 μm nylon syringe filter (Filter-Lab, Barcelona, Spain).

Chromatographic data were processed using the ACQUITY UPLC H—Class system in conjunction with Empower 3 software (Waters Corporation, Milford, MA, USA). Identification of tryptophan was based on comparing the retention time and fluorescence spectra of the sample peaks (excitation at 280 nm and emission at 300–400 nm) with those of a tryptophan standard. To confirm the identification, a spiking technique was employed to verify peak alignment. Quantification was performed using fluorescence detection with excitation at 280 nm and emission at 340 nm.

Table 1
UAE variables and their coded levels for Box-Behnken design.

Independent variables	Symbols	Coded levels		
		−1	0	1
Solvent composition (% methanol in water)	x_1	50	70	90
pH	x_2	2	5	8
Amplitude (%)	x_3	25	50	75
Pulse duty-cycle (s^{-1})	x_4	0.2	0.6	1

Table 2
Matrix of Box-Behnken design with observed responses and prediction error.

Run	Variables				Responses*		
	x_1	x_2	x_3	x_4	Observed ($n = 3$)	Predicted	Error (%)
1	−1	+1	0	0	376,710 ± 1 466.30	396,838	5.07
2	−1	0	0	−1	288,794 ± 2947.43	267,920	7.79
3	+1	−1	0	0	491,692 ± 3894.77	463,589	6.06
4	0	+1	0	+1	667,607 ± 5760.63	621,362	7.44
5	+1	0	+1	0	516,724 ± 8667.37	506,557	2.01
6	0	0	−1	−1	415,672 ± 5621.38	409,353	1.54
7	0	0	0	0	638,920 ± 8647.18	577,007	10.73
8	0	+1	+1	0	553,030 ± 3612.62	570,042	2.98
9	+1	0	0	−1	403,484 ± 5988.04	429,234	6.00
10	+1	0	0	+1	402,329 ± 3701.42	434,222	7.34
11	−1	−1	0	0	215,006 ± 6661.11	197,868	8.66
12	0	+1	0	−1	530,488 ± 4580.47	526,153	0.82
13	0	0	+1	−1	528,386 ± 9389.19	490,962	7.62
14	−1	0	0	+1	273,496 ± 4598.74	258,765	5.69
15	0	−1	−1	0	361,782 ± 2277.49	355,789	1.68
16	+1	+1	0	0	458,723 ± 9770.48	467,887	1.96
17	+1	0	−1	0	305,808 ± 3037.75	277,271	10.29
18	0	0	0	0	565,062 ± 8302.40	577,007	2.07
19	0	−1	0	+1	421,145 ± 1978.55	422,435	0.31
20	0	0	+1	+1	521,544 ± 2459.78	519,888	0.32
21	−1	0	+1	0	196,012 ± 7148.99	221,504	11.51
22	0	+1	−1	0	493,567 ± 4091.52	497,844	0.86
23	0	0	0	0	527,038 ± 6387.44	577,007	8.66
24	0	−1	+1	0	502,086 ± 1213.53	508,829	1.33
25	−1	0	−1	0	218,430 ± 8027.15	225,553	3.16
26	0	−1	0	−1	478,611 ± 1590.56	521,812	8.28
27	0	0	−1	+1	346,811 ± 6767.33	376,260	7.83

* Observed response indicates the peak area (mAU × *s*) of tryptophan in the chromatogram, representing raw UPLC-FD data used for method optimization. These values were later converted to concentration in the final quantification step using a calibration curve.

2.5. Screening design

A screening design was conducted to evaluate the statistical significance of independent factors affecting the extraction process and to establish the optimal range for each variables. The factors assessed included solvent composition, and temperature, which were analyzed using a non-factorial, completely randomized design. The study utilized four solvents (water, ethanol, methanol, and ethyl acetate), 12 solvent compositions (50, 60, 70, 80, 90, and 96 % methanol in water, and 50, 60, 70, 80, 90, and 96 % ethanol in water), and seven temperature levels

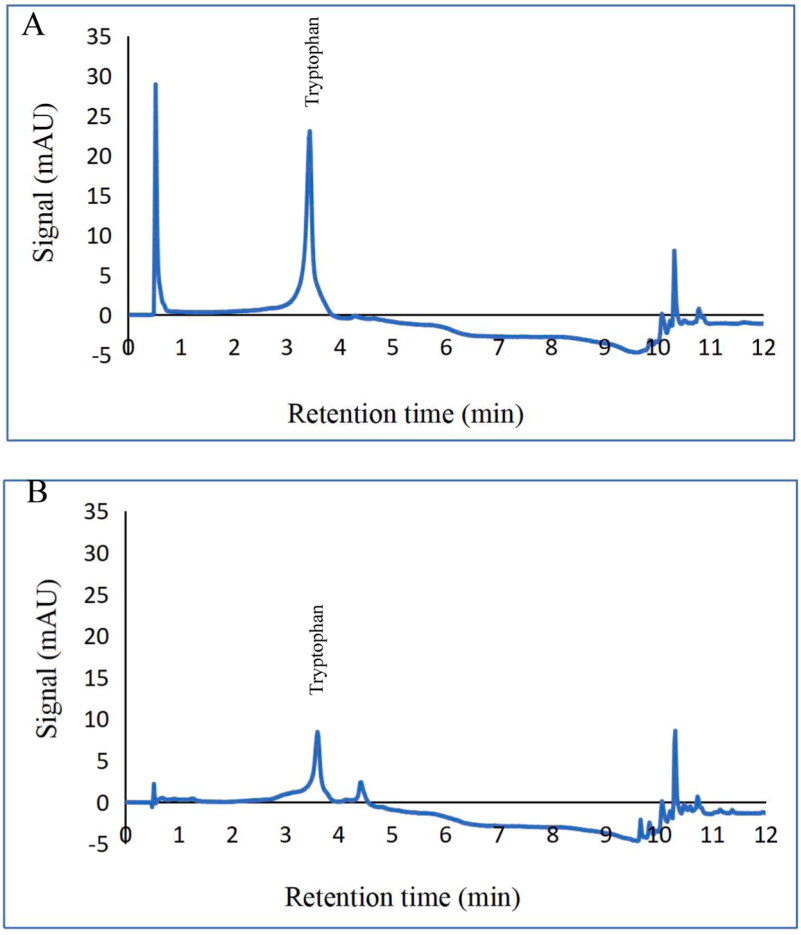


Fig. 2. UPLC-FD chromatograms of tryptophan at excitation wavelength of 280 nm and emission wavelength of 340 nm in (A) standard solution and (B) *Kappaphycus* extract.

(10, 20, 30, 40, 50, 60, and 70 °C). Extractions were performed in triplicate using 1 g of ground sample with 25 mL of solvent, a pulse duty cycle of 1 s^{-1}, and an amplitude of 50 %.

2.6. Optimization using Box-Behnken design

The effect of ultrasound-assisted extraction (UAE) variables on tryptophan recovery was simultaneously evaluated using a Box-Behnken design (BBD) with three levels (−1, 0, and 1). The actual values of the coded independent variables are presented in Table 1. A total of 27 experiments (Table 2) were conducted in random order, including three repetitions of the central point to ensure accuracy and reproducibility.

The response surface methodology (RSM) was used to analyze the main effects, interactions, and quadratic terms, and these were incorporated into a regression model to obtain the predictive equation (Eq. (1)).

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_1^2 + \sum_{i=1}^{k} \sum_{j=1,\ j\neq i}^{k} \beta_{ij} x_i x_j + \quad (1)$$

where Y is the predicted response (tryptophan recovery), x_1, x_2, …, x_k represent the UAE factors affecting the extraction efficiency, and β_0 is the intercept term. The coefficients $\beta_i (i = 1, 2, \ldots, k)$ represent the linear effects, $\beta_{ii} (i = 1, 2, \ldots, k)$ are the quadratic effects, and $\beta_{ii} (i = 1, 2, \ldots, k; j = 1, 2, 3, \ldots, k; j \neq i)$ are the interaction effects. Finally, ε is the random error term.

2.7. Determination of the optimal extraction time

The optimal extraction time for tryptophan recovery was determined by evaluating its concentration at various extraction durations (5, 10, 15, 20, 25, and 30 min) under the conditions optimized by the RSM. Each extraction time was tested in triplicate using a completely randomized design.

2.8. Method validation

The performance of the UPLC-FD method was validated prior to tryptophan detection and quantification, considering key parameters such as linearity, limit of detection (LOD), and limit of quantification (LOQ). The optimized method was validated according to the International Council for Harmonisation guidelines (ICH, 2005), covering accuracy and precision.

Precision was assessed at two levels, intraday and interday, expressed as the coefficient of variation (CV, %). For intraday precision, nine extractions were performed on the same day ($n = 9$). Interday precision was evaluated over three consecutive days with three extractions per day ($n = 3 \times 3$). According to the Association of Official Analytical Chemists guidelines, a maximum CV of ± 10 % is acceptable for the Peer-Verified Method program (AOAC, 2016).

Accuracy was determined by measuring the recovery rate (R, %) of tryptophan using a spiking technique. Additionally, the applicability of the developed analytical method was verified by extracting tryptophan from 36 samples of *Kappaphycus* spp. using the optimized UAE conditions.

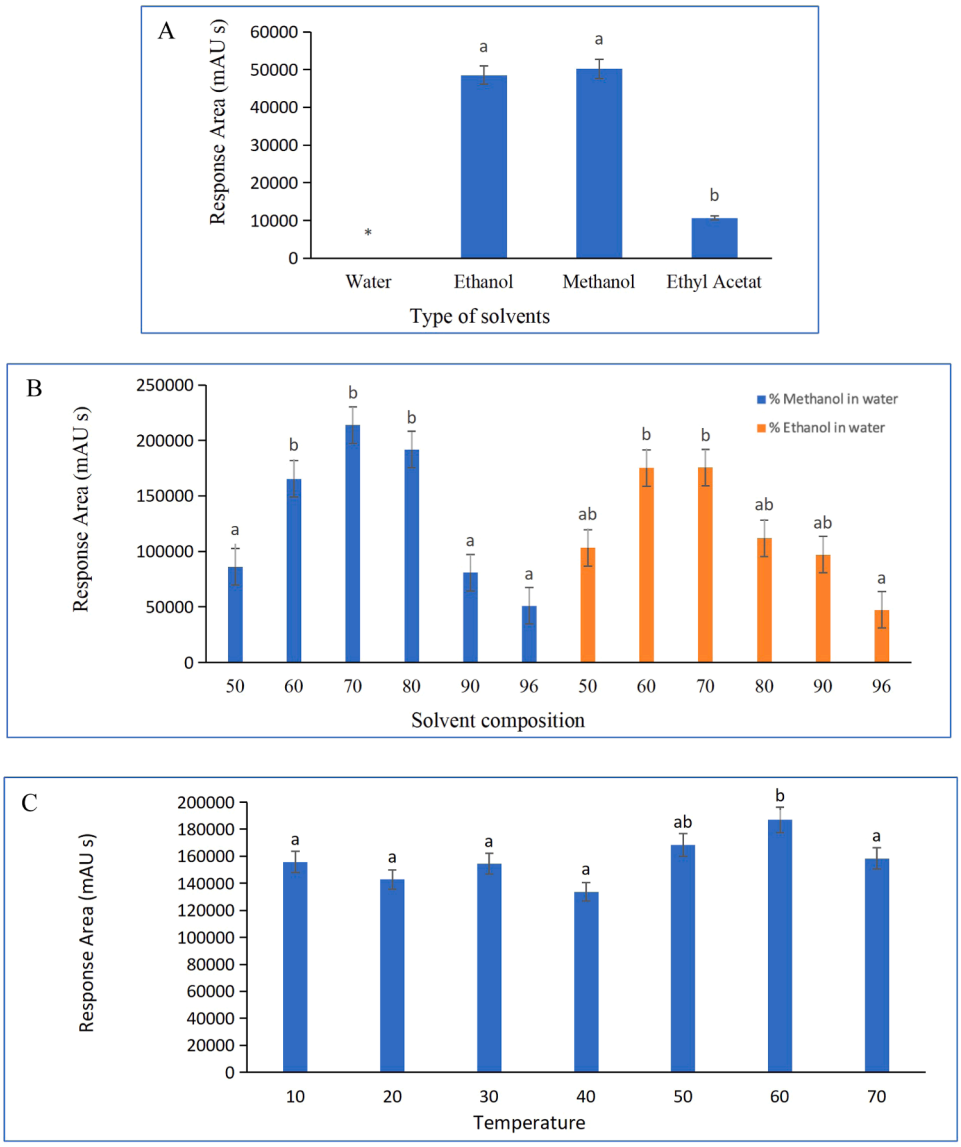


Fig. 3. Screening of UAE factors: Type of solvents (A), solvent compositions (B), and temperature (C). Bars followed by the same letters indicate values that are not significantly different ($p < 0.05$), while * indicates values below the detection limit.

2.9. Data analysis

The non-factorial experiment, employing a one-factor-at-a-time (OFAT) approach, was analyzed using Microsoft Excel 2024 (Redmond, Washington, USA). Statistical significance was determined through analysis of variance (ANOVA) with a threshold significance level set at $p = 0.05$. Pairwise comparisons between means were conducted using the Least Significant Difference (LSD) test, also with $p = 0.05$, to identify statistically significant differences. For the Box-Behnken Design (BBD) and Response Surface Methodology (RSM) analyses, specialized statistical software was utilized. Statgraphics Centurion 18, developed by Statgraphics Technologies, Inc. (The Plains, Virginia, USA), was used to design experiments and analyze data.

3. Results and discussion

3.1. Performance of UPLC-FD method

The UPLC-FD method was validated according to ICH Guideline Q2 before its application in identifying and quantifying tryptophan in the samples. During UPLC-FD analysis, the tryptophan peak was observed at a retention time of 3.4 min in both the standard solution and *Kappaphycus* extract (Fig. 2).

The UPLC-FD method used in this study demonstrated superior performance compared to conventional HPLC and other advanced techniques for tryptophan quantification in *Kappaphycus* spp. matrices. UPLC-FD offers higher sensitivity and resolution than HPLC-UV, as fluorescence detection provides greater specificity for tryptophan, reducing matrix interference and improving peak clarity (Liu et al., 2004). Additionally, UPLC operates at higher pressures (up to 1000 bar) and utilizes smaller particle sizes (≤1.7 μm), enabling faster separation with analysis times reduced by 10–20 % compared to traditional HPLC methods. Furthermore, solvent consumption is reduced by approximately 80 %, making UPLC a more efficient and cost-effective alternative. Compared to LC-MS/MS, which provides the highest specificity and sensitivity, UPLC-FD remains a more practical and accessible option for routine quantification due to its simpler sample preparation and lower operational costs (Nováková & Vlčková, 2009).

To ensure the reliability of the method, a calibration curve was constructed using tryptophan standard solutions ranging from 0.5 to 10 ppm, exhibiting excellent linearity ($R^2 = 0.99$). The strong linear correlation indicates high predictive capability across the tested

concentration range, supporting the robustness of the method. Furthermore, sensitivity was assessed by determining the limit of detection (LOD) and limit of quantification (LOQ) which were found to be 0.34 ppm and 1.02 ppm, respectively. These values are significantly lower than those reported in previous studies, highlighting the superior sensitivity of the UPLC-FD method for tryptophan quantification in *Kappaphycus* spp. Compared to spectroscopy-based methods, which typically exhibit higher LOD/LOQ values due to lower detector sensitivity and greater matrix interference, the UPLC-FD approach enhances precision and reduces detection limits. The low LOD/LOQ values obtained in this study enable the detection of trace amounts of tryptophan, ensuring more accurate quantification and a deeper understanding of its variability across *Kappaphycus* samples.

The low LOD (0.34 ppm) and LOQ (1.02 ppm) values achieved in this study are critical for ensuring the sensitivity and precision of the analytical method. These values allow for the detection of subtle differences in tryptophan content across various extraction conditions and cultivation practices. For instance, the ability to quantify tryptophan at low concentrations is particularly important when comparing samples from different environmental conditions or cultivation methods, as it enables the identification of optimal extraction conditions and provides insights into how cultivation practices influence the production of bioactive compounds in *Kappaphycus* spp. This level of sensitivity is essential for supporting the development of more efficient and sustainable seaweed cultivation strategies aimed at maximizing bioactive compound yields (Shalvina et al., 2022).

The precision and reproducibility of the method were further evaluated through intra-day and inter-day variability assessments. Intra-day precision analysis resulted in a mean tryptophan concentration of 5.47 $\pm$ 0.14 ppm, with a coefficient of variation (CV %) of 2.29 %, indicating minimal variation within a single day. Inter-day precision analysis yielded a mean concentration of 5.68 $\pm$ 0.28 ppm, with a CV % of 4.52 %, reflecting slightly higher but still acceptable variability across multiple days. Both values are well within the AOAC acceptance threshold of 10 % (AOAC, 2016), confirming the method's robustness and reproducibility. Additionally, statistical validation using one-way ANOVA demonstrated that extraction conditions, including solvent composition, pH, and ultrasonic amplitude, significantly influenced tryptophan recovery ($p < 0.05$, $F >$ critical value). These findings underscore the importance of optimizing extraction conditions to maximize efficiency and accuracy in tryptophan quantification.

3.2. Screening of solvent composition, and temperature

Water, methanol, ethanol, and ethyl acetate were selected as solvents for the extraction of *Kappaphycus* spp., based on their varying polarities relative to the target molecule, tryptophan. Among these solvents, water has the highest polarity, followed by methanol, ethanol, and ethyl acetate (Zhu et al., 2019). The results showed that water led to a significantly lower tryptophan recovery (Fig. 3A). This inefficiency is likely due to the gelling effect of water at high extraction temperatures, which hinders the release of tryptophan from the seaweed matrix (Putra et al., 2022). In contrast, methanol and ethanol achieved significantly higher tryptophan recoveries, with values of 45.89 % and 44.38 %, respectively. Their lower polarities compared to water promote the solubility and diffusion of tryptophan, which is classified as a non-polar amino acid (Garcia-Castello et al., 2015; Horovitz & Paşca, 2017). Ethyl acetate demonstrated a tryptophan recovery of only 9.74 %, which can be attributed to its relatively non-polar nature. Despite its partial polarity, the limited hydrophilicity of ethyl acetate hinders its ability to effectively penetrate the cell walls of *Kappaphycus* spp. This physical limitation restricts the solvent's interaction with intracellular compounds, resulting in a reduced extraction efficiency.

Following the selection of methanol and ethanol as suitable solvents, further screening was conducted to identify the optimal solvent composition (Fig. 3B). The efficiency of tryptophan extraction from *Kappaphycus* spp. is strongly influenced by solvent composition, as differences in polarity, solubility, and matrix interaction affect compound recovery. In this study, various methanol-water and ethanol-water mixtures were tested to determine the optimal solvent concentration UAE. The results demonstrated that 70 % methanol in water provided the highest tryptophan recovery, which was set as the reference maximum (100 %) for comparison.

Among the methanol-based extractions, 60 % methanol achieved 77.3 % relative recovery, while 80 % methanol reached 89.6 %, indicating that solvent polarity plays a key role in maximizing tryptophan solubility and diffusion. However, at higher methanol concentrations ($\geq$90 %), a sharp decline in extraction efficiency was observed, with 90 % and 96 % methanol yielding only 37.8 % and 23.8 % of the maximum recovery, respectively. This reduction suggests that excessive methanol disrupts the solvent-matrix balance, potentially reducing tryptophan solubility or limiting cell wall penetration.

For ethanol-based extractions, the highest tryptophan recovery was achieved with 70 % ethanol, yielding 82.1 % relative to 70 % methanol, followed closely by 60 % ethanol at 81.9 %. However, higher ethanol concentrations ($\geq$80 %) led to reduced recoveries, with 96 % ethanol extracting only 22.1 % of the maximum yield. The lower performance of ethanol compared to methanol suggests that methanol's stronger solvating power enhances tryptophan release from the seaweed matrix, while ethanol's lower polarity may limit solubility and extraction efficiency.

These findings highlight that solvent selection is a critical factor in optimizing UAE conditions for tryptophan extraction, with 70 % methanol in water providing the best balance between solubility, cell wall penetration, and overall recovery efficiency. The observed decline at higher solvent concentrations underscores the importance of maintaining an optimal solvent polarity range to prevent solubility limitations and reduced diffusion efficiency. This finding aligns with the suggestion by Waszkowiak et al. (2016) that binary solvent systems (water–methanol and water-ethanol) are more effective at extracting bioactive compounds than pure solvents.

Since this study focuses on analytical method development rather than industrial-scale extract production, the selection of 70 % methanol in water was based on analytical precision rather than industrial application. While methanol is not suitable for large-scale food or nutraceutical applications due to its toxicity and regulatory limitations, its use in controlled laboratory conditions ensures complete analyte recovery with minimal variability, which is critical for accurate quantification. Furthermore, the small solvent volumes used in this study reduce environmental concerns related to large-scale solvent consumption and waste management.

In addition to solvent type and composition, the influence of temperature on extraction efficiency was also assessed (Fig. 3C) as it influences the solubility of compounds, reduces solvent viscosity, and enhances the diffusivity of target metabolites within the solvent (Assunção et al., 2022). However, the ideal temperature must be selected based on the stability of the analyte being extracted to prevent degradation or loss of bioactivity. Higher temperatures typically improve extraction efficiency by increasing the solubility of compounds in the solvent and facilitating their release from the matrix. However, tryptophan recovery between 60 and 70 °C did not follow the expected trend. Statistical analysis ($p < 0.05$) confirms that recovery at 60 °C is significantly higher than at 70 °C, reinforcing the likelihood that degradation processes influence tryptophan stability at elevated temperatures. These findings suggest that UAE should be conducted at moderate temperatures ($\leq$60 °C) to maximize recovery while preventing compound degradation.

A plausible explanation for this trend is that tryptophan remains stable and exhibits enhanced solubility up to 60 °C, allowing for efficient extraction under these conditions. However, at higher temperatures ($\geq$70 °C), thermal degradation may occur, leading to a reduction in extraction yield. This observation aligns with previous studies reporting

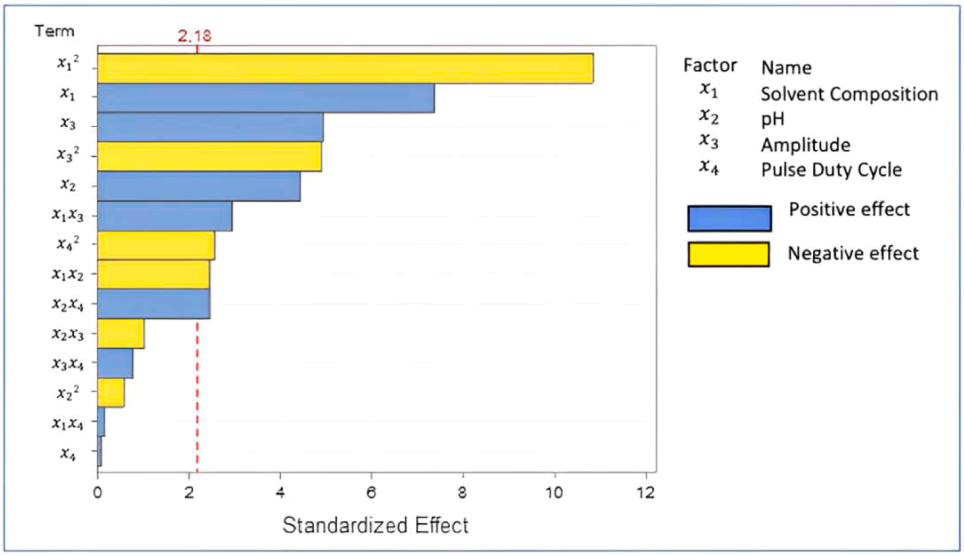


Fig. 4. The main, interaction, and quadratic effects of the UAE variables on tryptophan levels in the extracts. The red dotted line across the bars demonstrates that the corresponding factor has a positive ( ) or negative ( ) impact ($p < 0.05$).

that prolonged exposure to elevated temperatures can induce thermal degradation (Bellmaine et al., 2020).

3.3. Effect of UAE variables

Twenty-seven experiments were conducted using a Box-Behnken design, including triplicates of the central point to ensure robustness. The study covered four independent variables: variables (solvent composition (x_1), pH (x_2), amplitude (x_3), and pulse duty cycle (x_4). Analysis of variance (ANOVA) was applied to evaluate the main effects, interaction effects, and quadratic effects of the UAE variables, determining their statistical significance. The significance of each effect was assessed by calculating the mean square variance derived from the regression model and dividing it by the mean square residual error (Fig. 4). This approach facilitated the identification of critical factors influencing tryptophan recovery from *Kappaphycus* spp. and helped optimize the extraction process.

In this study, solvent composition (x_1) was identified as the most influential variable, followed by pH (x_2) and amplitude (x_3). In contrast, the pulse duty cycle (x_4) did not significantly affect the extraction yield. The solvent composition (x_1) had a significant positive effect, indicating that increasing the proportion of methanol in the solvent mixture resulted in higher tryptophan levels in the extract. However, the quadratic effect of solvent composition was negative, suggesting that an excessively high methanol concentration could reduce tryptophan recovery.

Although tryptophan is generally considered a non-polar amino acid, the nitrogen atom in its indole ring can participate in hydrogen bonding, enabling it to form strong hydrogen bonds with polar solvents like water and alcohol (Khemaissa et al., 2021). Consequently, a mixed solvent system of methanol and water is most suitable for extracting tryptophan, providing an optimal balance of polarity to achieve high recovery rates.

Previous research by Setyaningsih et al. (2017) demonstrated that tryptophan could be successfully extracted from the rice matrix using a solvent mixture containing a higher proportion of water (92 % water in methanol). However, in this study, increasing the water content in the solvent led to a gelling reaction of polysaccharides present in *Kappaphycus* spp. at the studied temperature of 60 °C. This phenomenon likely occurs because *Kappaphycus* spp. contain high levels of polysaccharides, which can form gels at elevated temperatures. As reported by Barbarino and Lourenço (2005), such gels can trap extracted proteins and bioactive compounds, resulting in lower yields during spectrophotometric measurements. Additionally, decreased protein solubility during extraction may be caused by the presence of anionic polysaccharides in the cell walls, which interact with proteins and reduce their release into the solvent (Fleurence, 1999). On the other hand, using a higher proportion of methanol creates a greater polarity difference between the solvent and the target analyte, which reduces the solubility of tryptophan and subsequently lowers the extraction yield (Nurkhasanah et al., 2023). Hence, a balanced composition of methanol and water is crucial to optimize the extraction process by maintaining adequate solvent polarity while preventing gelling or precipitation of polysaccharides.

The variable pH (x_2) positively influenced tryptophan yield, indicating that an increase in alkalinity made tryptophan more accessible for extraction. This result is consistent with the findings of Parniakov et al. (2015), who observed that protein recovery from *Nannochloropsis* sp. increased at pH values between 8.5 and 11. Alkaline solvents are known to disrupt the cell wall structure by breaking hemicellulose linkages in algae (Safi et al., 2014), thereby enhancing the extraction of intracellular compounds. Under these conditions, water-insoluble hydrophobic amino acids, such as tryptophan, become more soluble. Alkaline conditions are commonly used for protein extraction from algae, as they can facilitate the solubilization of proteins, followed by precipitation at their isoelectric points (Parimi et al., 2015; Safi et al., 2014; Ursu et al., 2014).

Conversely, the amplitude (x_3) had a significant positive effect on tryptophan extraction, while the pulse duty cycle (x_4) showed no significant influence on the extraction process. This finding is supported by Kumar et al. (2021), who reported that the yield from UAE typically increases with higher ultrasonic power and pulse duty cycles, but then declines once the optimal yield is reached. However, the quadratic effects of ultrasonic power (x_3x_3) and pulse duty cycle (x_4x_4) had a negative impact on tryptophan recovery. This suggests that excessively high power or prolonged sonication can lead to the destruction or degradation of tryptophan in the sample, ultimately reducing the yield of the target analyte. High ultrasonic power levels can cause cavitation, which generates intense localized heat and shear forces that may break down the chemical structure of sensitive compounds like tryptophan. As a result, it is critical to maintain a balance in ultrasonic power and pulse duty cycle to achieve maximum extraction efficiency without compromising the integrity of the analyte.

The interaction between solvent composition and pulse duty cycle (x_1x_4) had a significant and positive impact on tryptophan yield. As the pulse duty cycle increased, ultrasonic vibrations intensified, promoting greater cell disintegration (Cárcel et al., 2012). This allowed the solvent

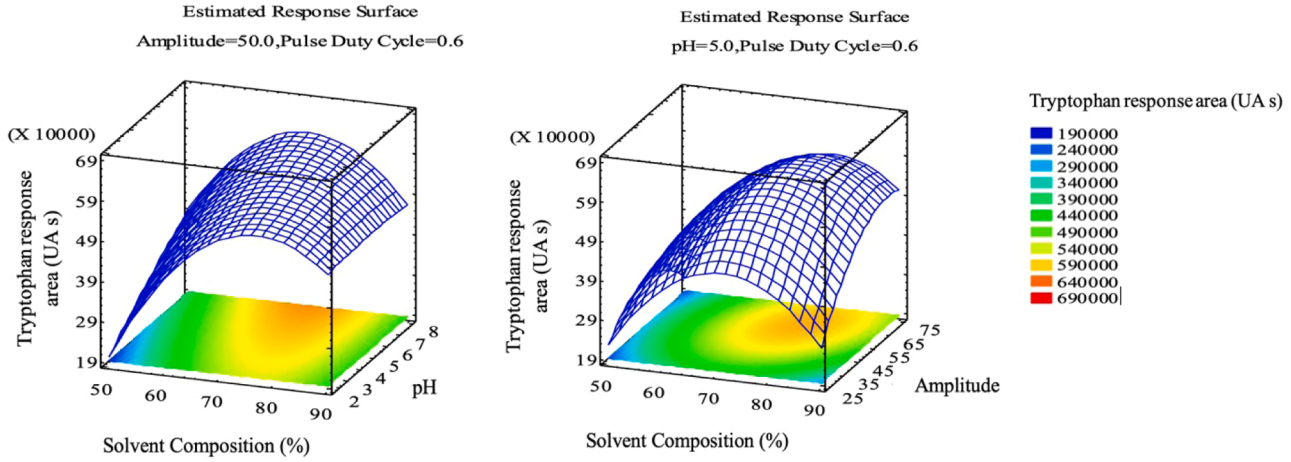


Fig. 5. Response surface 3D depicting the effects of UAE factors on tryptophan levels in the extract of *Kappaphycus* spp.

to penetrate the cell walls more effectively, enhancing the solubility and release of the target analyte into the extraction solvent. Consequently, UAE led to an increased yield of tryptophan.

In contrast, the interaction between pH and amplitude (x_2x_3) negatively influenced the extraction yield. The increase in ultrasonic power due to higher amplitude caused intensified cavitation, disrupting the cell walls and facilitating the entry of a significant amount of solvent into the cellular material. This enhanced mass transfer and released more cell content, which initially improved extraction efficiency (Wang et al., 2021). However, after reaching an optimal point, further increases in ultrasonic power resulted in a decline in yield. This reduction is attributed to protein hydrolysis, denaturation, and aggregation caused by excessive ultrasonic power (Cui et al., 2018; Wang et al., 2021). Moreover, alkaline extraction conditions generally improve the solubility of amino acids, leading to higher nitrogen content in the final extract (Juul et al., 2023). However, when the pH exceeded 12, the tryptophan content in the extracts decreased, likely due to the instability of tryptophan under strongly alkaline conditions, which causes its degradation and reduces overall extraction efficiency.

3.4. Response optimization and verification

Response surface analysis was applied to optimize the UAE conditions for tryptophan extraction. Three-dimensional surface plots (Fig. 5) were generated to visualize the interactions between variables and predict the response based on varying levels of independent factors. The relationship between the independent variables and tryptophan yield was modeled using the second-order polynomial (Eq. (2)).

$$y = -2403564 + 67068x_1 + 74011x_2 + 7919x_3 + 16527x_4 - 463.6x_1^2 - 1114x_2^2 - 134.2x_3^2 - 275239x_4^2 - 811x_1x_2 + 116.7x_1x_3 + 442x_1x_4 - 269x_2x_3 + 40539x_2x_4 + 1550\ x_3x_4 \quad (2)$$

where y represents the tryptophan yield, and x_1, x_2, x_3, and x_4 are the UAE factors (solvent composition (x_1), pH (x_2), amplitude (x_3), and pulse duty cycle (x_4)).

A lack-of-fit test was conducted to assess whether the selected model adequately described the observed data or if a more complex model was required. The lack-of-fit test compares the variability of the observations at different factor levels with that of the current model. The resulting ANOVA p-value for lack of fit was 0.88, which was non-significant, indicating that the model was appropriate for describing the data at the 95 % confidence level. This suggests that no significant unexplained variation remained, and the current model was sufficient to predict the tryptophan yield under the tested conditions.

The experimental results demonstrated that the mean absolute error between the predicted and actual values was 5.11 %, indicating a close match between the model and observed data. Furthermore, the high coefficient of determination (R^2) value of 0.955 confirmed the strong predictive capability of the model (Table 2). This high R^2 value suggests that the model accurately represents the relationship between the independent variables and tryptophan yield, with 95.5 % of the variation in extraction yield being accounted for by the model. Consequently, the model can reliably predict the response under different UAE conditions and be used to determine the optimal extraction variables for maximizing tryptophan yield from *Kappaphycus* spp.

The RSM analysis suggested that the optimal UAE conditions for maximizing tryptophan recovery were as follows: 73 % methanol in water as the extraction solvent, pH 8, extraction duration of 10 min at a temperature of 60 °C, amplitude of 58 %, pulse duty cycle of 0.8 s^{-1}, and a solvent-to-sample ratio of 25:1. The optimized UAE method was verified by performing tryptophan extractions three times under these conditions. The average tryptophan concentration obtained was 136.70 ± 2.31 μg g^{-1}, with a deviation of only 1.17 % from the predicted value, indicating a high degree of accuracy and validation of the model. This result confirms the compatibility of the BBD with RSM for optimizing tryptophan extraction from *Kappaphycus* spp.

3.5. Optimal extraction time

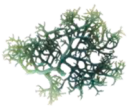
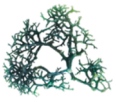
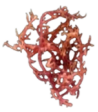
Using the optimized values for the extraction variables, the extraction time was further evaluated by analyzing the tryptophan content at different durations over a 30-minute period. The tryptophan concentration reached a steady state at 10 min and did not show significant differences at longer extraction times ($p > 0.05$). Therefore, 10 min was selected as the optimal extraction time for the UAE process, ensuring efficient tryptophan recovery without extending the extraction period unnecessarily.

3.6. Method validation

The optimal extraction method was validated according to the ICH guidelines to ensure its reliability for determining tryptophan levels. The accuracy of the method was evaluated by calculating the UAE recovery percentage (R %). The developed extraction method successfully recovered 101.69 % of tryptophan, which falls within the acceptable range of 80–110 %, indicating a high level of accuracy.

Precision was assessed by measuring both repeatability and intermediate precision. The coefficient of variation (CV) for repeatability was 2.29 % ($n = 9$), while the intermediate precision CV was 4.52 % ($n = 3 \times 3$), both of which are well below the acceptable limit of 10 % set by the

Table 3
Tryptophan concentration in *Kappaphycus* spp. Samples.

Species	Cultivation site	Island	Cultivation method	Picture	Tryptophan (μg g^{-1})
Kappaphycus striatum	Teluk Pandan	Sumatera	Bottom-off		57.81 ± 1.59[a]
	Puntondo	Celebes	Longline		14.71 ± 0.11[b]
	Kepulauan Melahing	Borneo	Bottom-off		20.90 ± 0.23[c]
	Desa Akle	Lombok	Longline		101.50 ± 1.31[d]
	Sulamu	Lombok	Longline		137.08 ± 2.10[e]
	Tablolong	Lombok	Longline		113.23 ± 1.12[f]
	Oenaek	Lombok	Longline		137.07 ± 2.92[e]
	Tesabela	Lombok	Longline		95.46 ± 2.73 g
	Lifuleo	Lombok	Longline		130.63 ± 2.22[h]
Kappaphycus striatum	Lifuleo	Lombok	Longline		130.64 ± 1.66[h]
	Naikean	Lombok	Bottom-off		64.88 ± 2.49[i]
	Naikean	Lombok	Bottom-off		65.44 ± 1.60[i]
	Sekotong	Lombok	Longline		209.82 ± 3.72[j]
	Djene Ponto	Celebes	Longline		21.26 ± 1.36[k]

(*continued on next page*)

Table 3 (*continued*)

Species	Cultivation site	Island	Cultivation method	Picture	Tryptophan (μg g^{-1})
	Takalar	Celebes	Longline		42.21 ± 0.71^{lm}
Kappaphycus alvarezii	Djene Ponto	Celebes	Longline		29.77 ± 2.46^{ld}
	Lembongan Island	Bali	Longline		8.40 ± 1.90^{ef}
	Teluk Pandan	Sumatera	Longline		21.90 ± 2.47^{k}
	Puntondo	Celebes	Longline		115.76 ± 1.35^{f}
Kappaphycus alvarezii	Lombok	Lombok	Longline		45.20 ± 1.10^{l}
	Geranting Island (short thallus)	Sumatera	Bottom-off		6.54 ± 1.01^{p}
	Geranting Island (long thallus)	Sumatera	Bottom-off		5.83 ± 0.77^{p}
	Tenakeke Island	Celebes	Longline		15.11 ± 0.50^{b}
	Serui	Papua	Bottom-off		26.74 ± 0.69^{o}
	Bantaeng	Celebes	Longline		55.23 ± 2.54^{aq}
	Nusa Penida	Bali	Longline		51.91 ± 1.99^{c}
	Tamalaba	Celebes	Bottom-off		43.50 ± 1.37^{lm}
	Sumenep	Java	Longline		20.01 ± 3.34^{k}
	Bali	Bali	Longline		10.24 ± 0.77^{c}

(*continued on next page*)

Table 3 (*continued*)

Species	Cultivation site	Island	Cultivation method	Picture	Tryptophan (μg g^{-1})
Kappaphycus alvarezii	Ketapang	Sumatera	Longline		70.69 ± 1.48^{r}
	Sekotong	Lombok	Longline		64.20 ± 0.24^{i}
	Situbondo	Java	Longline		10.52 ± 1.50^{c}
	Moluccas	Moluccas	Longline		15.81 ± 0.45^{b}
	Thousand Island	Java	Longline		40.70 ± 1.38^{m}
	Takalar	Celebes	Longline		72.38 ± 0.30^{r}
	Jepara	Java	Longline		30.88 ± 0.23^{n}

[a-r] The same letter in the same column shows an insignificant difference ($p > 0.05$).

AOAC. These results demonstrate that the optimized extraction method is not only accurate but also highly precise, making it suitable for the consistent quantification of tryptophan in *Kappaphycus* spp. samples.

3.7. Real sample application

The applicability of the optimized extraction method was evaluated by quantifying tryptophan in 36 *Kappaphycus* spp. samples collected from different cultivation sites and grown using various techniques (Table 3). Among the samples analyzed, *Kappaphycus striatum* from Sekotong (Lombok) had the highest tryptophan concentration at 209.82 ± 1.71 μg g^{-1}, while *Kappaphycus alvarezii* from Geranti Island exhibited the lowest concentration at 5.83 ± 0.77 μg g^{-1}. Statistical analysis confirmed that the tryptophan content in *K. striatum* from Sekotong was significantly higher than all other samples, as indicated by the distinct superscript letters in Table 4 ($p < 0.05$). This variation in tryptophan levels could be attributed to differences in environmental factors at the growing sites, such as salinity, pH, light penetration, and wave exposure (Widyartini et al., 2017).

Simatupang et al. (2021) and Mutiarahma et al. (2021) reported that Lombok provides an ideal environment for *Kappaphycus* spp. growth, promoting the production of bioactive compounds. However, cultivation techniques also play a significant role in the synthesis of secondary metabolites. In Indonesia, the commonly used cultivation methods for *Kappaphycus* spp. are longline and bottom-off. The study found that the longline method was superior to the bottom-off method in terms of tryptophan yield. Seaweeds grown near the surface using the longline method are frequently exposed to environmental stressors such as UV radiation and fluctuating temperatures, which can induce the production of unique biologically active secondary metabolites not typically synthesized under less stressful conditions (Rodríguez-Meizoso et al., 2010). As a result, seaweeds cultivated near the sea surface generate higher concentrations of secondary metabolites than those grown in deeper water (Nugroho et al., 2021). This highlights the importance of both environmental conditions and cultivation techniques in optimizing the production of bioactive compounds in *Kappaphycus* spp.

The results further indicated that *K. striatum* contained significantly higher tryptophan levels (89.51 ± 3.24 μg g^{-1}) compared to *K. alvarezii* (37.74 ± 2.89 μg g^{-1}). Statistical analysis confirmed that this difference was significant, as indicated by the *t*-test and ANOVA results ($p = 0.0009$, $p < 0.05$). These findings align with previous studies reporting that *K. striatum* generally exhibits a higher concentration of bioactive compounds than *K. alvarezii* (Diyana et al., 2015; Putra et al., 2022). The observed differences in tryptophan levels between the two species can be attributed to a combination of internal genetic factors and external environmental influences, such as variations in nutrient availability, water quality, and stress exposure at different cultivation sites. These factors collectively contribute to the metabolic profiles of the two species, influencing their capacity to synthesize and accumulate bioactive compounds like tryptophan.

4. Conclusions

This study successfully optimized ultrasound-assisted extraction (UAE) conditions using the Box-Behnken design and response surface methodology to enhance the recovery of tryptophan from *Kappaphycus* spp. The optimal conditions—73 % methanol in water, pH 8, 10 min extraction at 60 °C, 58 % amplitude, pulse duty cycle of 0.8 s^{-1}, and a solvent-to-sample ratio of 25:1—ensured high extraction efficiency and reproducibility (CV <10 %). The highest tryptophan yield (209.82 ± 3.72 μg/g) was observed in *K. striatum* cultivated using the longline method in Sekotong (Lombok), while the lowest (5.83 ± 0.77 μg/g) was found in Geranting Island samples cultivated with the bottom-off method. The higher tryptophan accumulation in longline cultivation may be attributed to increased sunlight exposure, improved water circulation, and enhanced nutrient availability, which collectively support photosynthesis and nitrogen-containing compound biosynthesis.

Despite the successful optimization, a key challenge was the

naturally low tryptophan abundance in *Kappaphycus* spp., necessitating the use of a highly sensitive UPLC-FD method for reliable quantification. Future studies should explore environmental factors such as seasonal variations, nutrient dynamics, and genetic influences to better understand tryptophan accumulation mechanisms. Additionally, the scalability of UAE should be assessed in terms of environmental impact, cost-effectiveness, solvent recovery strategies, and its integration into existing seaweed processing industries. Beyond seaweed cultivation, this study contributes to sustainable development by promoting eco-friendly extraction methods that minimize solvent use, energy consumption, and processing time. In biotechnology and functional food research, the optimized UAE method provides a standardized approach for bioactive compound profiling, supporting advancements in nutraceutical and pharmaceutical applications. These findings highlight the potential of marine resources in bioeconomy development and health-related industries.

CRediT authorship contribution statement

Nuzulia Izmi: Writing – original draft, Visualization, Software, Resources, Investigation, Formal analysis. **Manikharda:** Writing – review & editing, Validation, Resources, Project administration. **Miguel Palma:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Data curation, Conceptualization. **Lideman:** Writing – review & editing, Validation, Resources. **Mohamad Rafi:** Writing – review & editing, Validation, Software. **Nandian Mareta:** Writing – review & editing, Resources. **Widiastuti Setyaningsih:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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