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Assessment of Solvent Extraction using Sonication to Recover Tryptophan from *Kappaphycus alvarezii* (Doty) Doty ex Silva: Experimental and Modelling

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Abstract

The application of *Kappaphycus alvarezii* in the food system has attracted researchers due to its bioactive compounds, including tryptophan. The present study was conducted to extract tryptophan from *K. alvarezii* with the aid of sonication. A reduced multilevel factorial design was conducted to evaluate the effect of solvent, ultrasound power, duty-pulse cycle, time and temperature on the recovery of tryptophan. Analysis of variance suggested that the type of solvent and combination setting of power and duty-pulse cycle significantly influenced the extraction ($p < 0.05$). In contrast, extraction time and temperature did not alter the extraction ($p > 0.05$). The optimum was using ethanol, ultrasound power 80 %, pulse duty-cycle 0.8 s^{-1}, extraction time 10 min, and temperature 25 °C and tryptophan concentration was 56.41 ± 2.42 mg L^{-1}. Subsequently, a COnducto-like Screening MOdel for Real Solvent (COSMO-RS) was performed to clarify the impact of solvent affinity and polarity on the tryptophan extraction from *K. alvarezii.*

Keywords: COSMOS-RS, Multilevel factorial design, Ultrasound-assisted extraction, Polarity, Affinity, Ethanol, HPLC-DAD

Introduction

Indonesia is an archipelagic country that consists of approximately 17,500 islands with 81,000 km of coastline, making the nation rich in marine resources [1,2]. One of the leading marine products with great economic value is macroalgae [3]. In Asia, Indonesia is the highest macroalgae-producing country, along with China [4]. The most valuable cultivated macroalgae are red algae grown in Celebes, Moluccas, Lesser Sunda, Borneo and East Java. Numerous research has described the benefits of red algae on human health

and disclosed the chemical compounds contributing to the effect, specifically by *K. alvarezii*. This red alga is also known commercially as *Eucheuma cottoni*, a source of carrageenan and antioxidant compounds [5].

In addition, *K. alvarezii* contains some amino acids essentials, including tryptophan [6,7] approximately 0.59 mg g^{-1} [8]. The amino acid tryptophan is a precursor of the biosynthesis of essential metabolites. Various studies have investigated the benefits of tryptophan, especially for infant growth in the 1st month of life [9]. In line with research conducted by Amaya-Guerra *et al*. [10] soybean fortification of corn tortillas with high-tryptophan soy flour could improve rats' brain development. The consumption of a certain dose of tryptophan diet can reduce depressive symptoms and anxiety [11]. Moreover, tryptophan can reduce inflammatory bowel disease [12] control social behavior in individuals having disorders [13] and plays an essential role in enhancing active components of food [14].

In order to take advantage of tryptophan, an effective procedure is required to isolate and determine the level of this compound in *K. alvarezii*. Extraction is the 1st step to separating the desired natural products from the raw material in solid and semi-solid matrices [15,16]. A reliable determination method will help to establish the levels of tryptophan in macroalgae produced in different areas or under different conditions. Additionally, an optimal extraction method allows the production of high-level tryptophan extracts that will provide the right conditions to develop new functional foods.

Green extraction techniques are currently proposed as alternatives to conventional extraction procedures due to less time and solvent consumption. Besides, specific alternative methods showed a high sensitivity for the isolation of the desired compound and can reduce the formation of by-products and unwanted reactions during the extraction [5,17]. The extraction techniques in question include Supercritical Fluid Extraction (SFE), Microwave-Assisted Extraction (MAE), Subcritical Water Extraction (SWE) and Ultrasound-Assisted Extraction (UAE) [18]. Among these techniques, UAE has successfully extracted tryptophan and its derivatives from a food matrix [19]. Moreover, UAE demonstrated a high extraction efficiency of tryptophan [20].

UAE can improve extraction efficiency by promoting the rupture of vegetable cells [21] using acoustic waves in the range of 20 to 100 kHz and a power of 50 - 500 W, which vibrate through a solvent-producing cavitation bubble. When the bubbles collapse and burst at the surface of a complex matrix, they damage the cell wall and release the analyte leach from the matrix [21,22]. The efficiency of UAE can be affected by some factors, i.e., extraction temperature, time, sonication power, solvent-to-solid ratio and solvent type [23-25].

A higher temperature for the extraction can increase the mass transfer and solvent diffusion rate [21]. Moreover, a higher ultrasound power can provide more significant damage to the cell wall; thus, the solvent can easily penetrate the solid material and increase the extraction of bioactive compounds [23]. Furthermore, the type of solvent must be considered to maximize the recovery of the desired compound from macroalgae [23]. The solvent type must be selected according to the solubility of the target compound. Besides, the toxicity, cost and availability of the solvents must be considered [26]. Various solvents were used to extract the tryptophan, such as ethanol [19], acetonitrile and water [27] and methanol [28].

Methanol and ethanol are less polar than water, supporting the solubility and diffusion of tryptophan which is classified as non-polar amino acid [29,30]. According to a study by Maharany *et al*. [3] methanol recovered the highest bioactive compounds from *K. alvarezii*. Due to the differences in extraction efficiency, it is essential to find the most appropriate solvent to extract the selected organic compounds of *K. alvarezii*.

In this study, the effect of different solvents as well as UAE factors, on the recovery of tryptophan from *K. alvarezii* was evaluated. Methanol, ethanol, water and ethyl acetate were studied in addition to

extraction temperature, time, duty pulse cycle and ultrasound power. In order to explain the impact of solvent and temperature on the experimental data, a COSMO-RS modeling was performed.

COSMO-RS is a model based on statistical thermodynamics and quantum chemistry. It can predict individual compounds' chemical potential in liquid mixtures [31-33]. Therefore, it can be used to optimize bio-based solvents, such as dimethyl carbonate, isopropanol, ethanol and ethyl acetate. The approach for optimization is based on relative solubility [34]. COSMO-RS successfully predicted the solubility of compounds in various ionic liquids with over 1800 available structures, which were investigated based on the COSMO-RS computation [35].

Hence, this study aimed to determine the best solvent and UAE condition for tryptophan extraction from *K. alvarezii*. The optimization was also proposed by COSMO-RS modeling that could simplify the solvent selection extraction procedure to extract the target compound. Combining experimental and computational methods could reduce the number of experiments necessary to develop a process for obtaining a tryptophan-rich extract from *K. alvarezii* using UAE.

Materials and methods

Chemicals

The chemicals used were methanol (CAS-No. 67-56-1), ethanol (CAS-No. 64-17-5), ethyl acetate (CAS-No. 141-78-6), acetic acid (CAS-No. 64-19-7), sodium carbonate (CAS-No. 497-19-8) and Folin-Ciocalteu's phenol reagent. All chemicals were analytical grade and obtained from Merck KGaA (Darmstadt, Germany). Distilled water obtained from PT. Ikapharmindo Putramas (Indonesia). The standard compound used in this study was tryptophan, purchased from Merck KGaA (Darmstadt, Germany).

Sample collection and preparation

The macroalgae used in this study were fresh *K. alvarezii* collected from Jepara, Indonesia. Firstly, fresh samples were cleaned with water to remove physical impurities. Subsequently, the samples were freeze-dried for 48 h. The dried samples were then ground into powder and stored using vacuum plastic in a refrigerator (4 °C) until further analysis.

Ultrasound-assisted extraction

Extraction was performed using an UAE (UAE, Hielscher UP200St, Teltow, Germany), evaluating solvent type, extraction time, temperature, ultrasound power and pulse duty cycle. The studied solvents were ethanol, methanol and ethyl acetate. The levels combinations of the ultrasound power-duty pulse cycle and combination of extraction time-temperature are presented in **Table 1**. A multi-level factorial consisting of 27 experiments was conducted in triplicate.

Table 1 Selected factors and their levels for multilevel factorial design.

Factors	Coded		
	1	2	3
Solvent	Methanol	Ethanol	Ethyl acetate
Power + Duty pulse-cycle	20 %, 0.2 s^{-1}	80 %, 0.8 s^{-1}	N/A
Time + Temperature	10 min, 25 °C	20 min, 50 °C	N/A

K. alvarezii powder was weighed (1 g) and mixed with 20 mL of solvent (methanol, ethanol, or ethyl acetate). The mixture was then extracted using UAE with different conditions according to the design of the experiment in **Table 2**. Later, the extract was centrifuged (Thermo Fisher, Osterode, Germany), and the supernatant was followed by further evaporation (IKA-Werke Gmbh & Co. Kg, Stauten, Germany) up to 5 mL.

Table 2 Multilevel Categorical Design (MCD) and its responses.

D.O.E	Solvent	Power + Duty pulse cycle	Time + Temperature	Tryptophan (mg L^{-1})
1	1	2	1	33.31
2	1	2	2	41.03
3	1	2	1	69.03
4	1	1	1	33.17
5	1	2	2	58.74
6	1	2	1	49.17
7	1	1	1	36.46
8	1	2	2	52.46
9	1	2	1	40.89
10	2	2	1	77.03
11	2	2	2	87.74
12	2	2	1	87.03
13	2	1	1	40.31
14	2	2	2	62.46
15	2	2	1	65.31
16	2	1	1	41.74
17	2	2	2	58.03
18	2	2	1	56.89
19	3	2	1	19.31
20	3	2	2	34.31
21	3	2	1	38.01
22	3	1	1	16.46
23	3	2	2	31.31
24	3	2	1	28.17
25	3	1	1	20.60
26	3	2	2	25.60
27	3	2	1	33.17

Identification and quantification of tryptophan

The extract was analyzed using a high-performance liquid chromatography instrument (Shimadzu, Kyoto, Japan) coupled with a diode array detector (HPLC-DAD). The separation to identify tryptophan in the extract was carried out on a Shimadzu Shim-pack GIST Column 5 µm C18 4.6×150 mm^2. The injection volume was 10 µL. The dual mobile phases program was as follows A (2 % acetic acid, 5 % methanol in

water) and B (2 % acetic acid, 88 % methanol in water). The solvent flow rate was 1 mL min^{-1}. The gradient was: 18.3 % B (0 - 0.02 min), 100 % B (0.02 - 10 min), 100 % B (10 - 13 min).

Quantification of tryptophan was conducted by using a UV-Vis spectrophotometer (Genesys 10S UV-Vis, Thermo Fisher, Tianjin, China) according to Subbaraju *et al*. [37] with modifications. The extract (0.1 mL) was briefly mixed with 1 mL of Folin-Ciocalteau reagent (1:2) and allowed to stand for 5 min. Then 1 mL of sodium carbonate was added and incubated in a dark room for 25 min. Each sample was measured using a spectrophotometer at 750 nm standing against the blank. Five-point calibrations were strictly linear ($R^2 > 0.9989$) in the concentration range of 5 - 100 mg L^{-1} with tryptophan as the standard. The regression equation was $y = 0.0035x + 0.0219$. The standard deviation of the calibration curve and slope were obtained from the calibration curve and were used to calculate the Limit of Detection (LOD, 4.17 mg L^{-1}) and Limit of Quantification (LOQ, 12.64 mg L^{-1}).

Stability test

One g *Kappaphycus* spp. was mixed into 25 mL methanol in water 70 %, temperature 60 °C, pH 8, amplitude 50 % and pulse duty cycle 1 s^{-1} and the mixture was extracted 10 min. Stability test was evaluated during 11 weeks with 3 different temperature storage (4, 25 and 30 °C) in 3 replicates. All experiments were evaluated with non-factorial completely randomized design and ANOVA.

Statistical analysis

The experimental design and the resulting data were constructed and analyzed using Statsgraphic Centurion XVII (Statpoint. Technologies, Inc.: Warrenton, VA, USA). Analysis of Variance (ANOVA) was used to determine the significance of the studied factors.

Computation method using COSMO-RS

The computational method used to understand the mechanism of solvent to extract tryptophan contents was COSMO-RS calculations. This modeling was conducted by COSMOtherm software. The computational method consisted of 2 stages: 1) Selecting the most efficient solvent (ethanol, ethyl acetate and methanol) in the biphasic region and 2) Evaluating tryptophan extraction performance using the L.L.E. platform by reuse of solvent in up to 3 consecutive stages for 2 different strategies (precipitation with cold-acetone and sequential liquid-liquid extraction) [36].

Results and discussion

Identification of tryptophan in *K. alvarezii*

The identification of tryptophan presented in *K. alvarezii* was priorly performed. For this purpose, a qualitative screening was conducted by extracting and concentrating the extract to reach a reliable signal analyzed by a high-performance liquid chromatography tandem with a diode array detector (HPLC-DAD). The resulting chromatograms of the tryptophan standard compound in the extraction solvent and *K. alvarezii* extract are compiled in **Figure 1**.

The identification of tryptophan was confirmed by comparing the collected spectra from 200 to 400 nm and the retention time found in the sample to the data obtained from the analysis of a solution of the commercial standard. The finding is supported by Suantika *et al*. [6], who reported that *K. alvarezii* contains some amino acid essentials, including tryptophan.

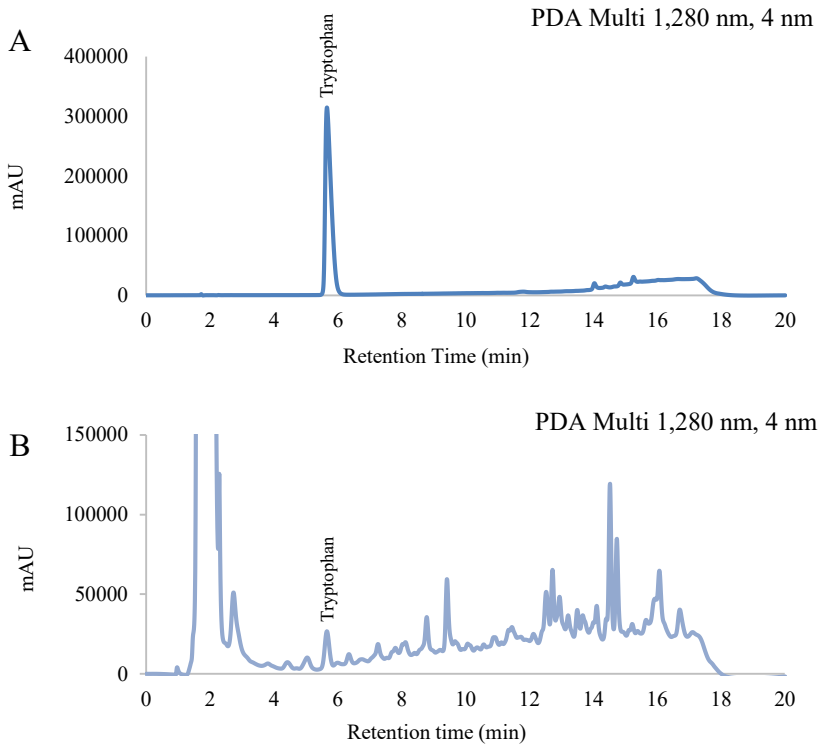

Figure 1 Chromatogram of tryptophan in standard solution (A) and *K. alvarezii* extract (B).

Evaluation of the factors that influence the tryptophan recovery by UAE

The effect of extraction factors on extraction performance was evaluated using a MCD, consisting of 27 experiments. The studied factors were the type of solvent (methanol, ethanol and ethyl acetate), ultrasound power (20 and 80 %), pulse duty cycle (0.2 and 0.8), extraction time (10 and 20 min), and temperature (25 and 50 °C). The level of each factor was defined based on the former reports related to the extraction of bioactive compounds, including tryptophan, using UAE [19,28]. The highest temperature level was 50 °C because some degradation of tryptophan appeared at high temperatures [37]. The combination of power and pulse duty cycle was evaluated simultaneously, as well as the combination of time and temperature. The response measured was the tryptophan content (mg L^{-1}) in *K. alvarezii* extract using the same amount of solid sample. The results are presented in **Table 2**.

Multifactor ANOVA suggested that the type of solvent and combination of power and pulse duty cycle were the most influencing factors ($p < 0.05$) in extracting the tryptophan, while the combination of time and temperature did not significantly affect the recovery (**Figure 2**). Furthermore, the combination of power and pulse duty cycle has a positive influence on recovery of tryptophan. It must be noted that, increasing both duty cycle and power, the ultrasonic power is increased, therefore the cavitation effects of the ultrasonics are also increased. Consequently, a larger amount of cavitation bubbles appears. The implosion of the bubbles produced solvent flows and microjets that resulted in cell rupture and increased mass transfer of organic compounds from the matrix into the solvent [28].

The combination of time and temperature had no statistically significant influence on the recovery of tryptophan from *K. alvarezii*. According to Zhang *et al*. [16], the extraction efficiency increases in line with the increase in extraction time within a specific time range. Extraction time will not influence the extraction after the solute's balance is reached inside and outside the solid material. A study by Setyaningsih *et al*.

[39] showed the optimal UAE conditions for extraction time to extract tryptophan from rice grain was 5 min, and non-significant higher recoveries were found even after 20 min of extraction.

As the type of solvent and the combination of ultrasound power and pulse duty cycle significantly affected the tryptophan recovery, the analysis was continued using the least significant difference (LSD) to compare among the means and thus could determine the optimum condition to extract tryptophan from *K. alvarezii* (**Figure 2**).

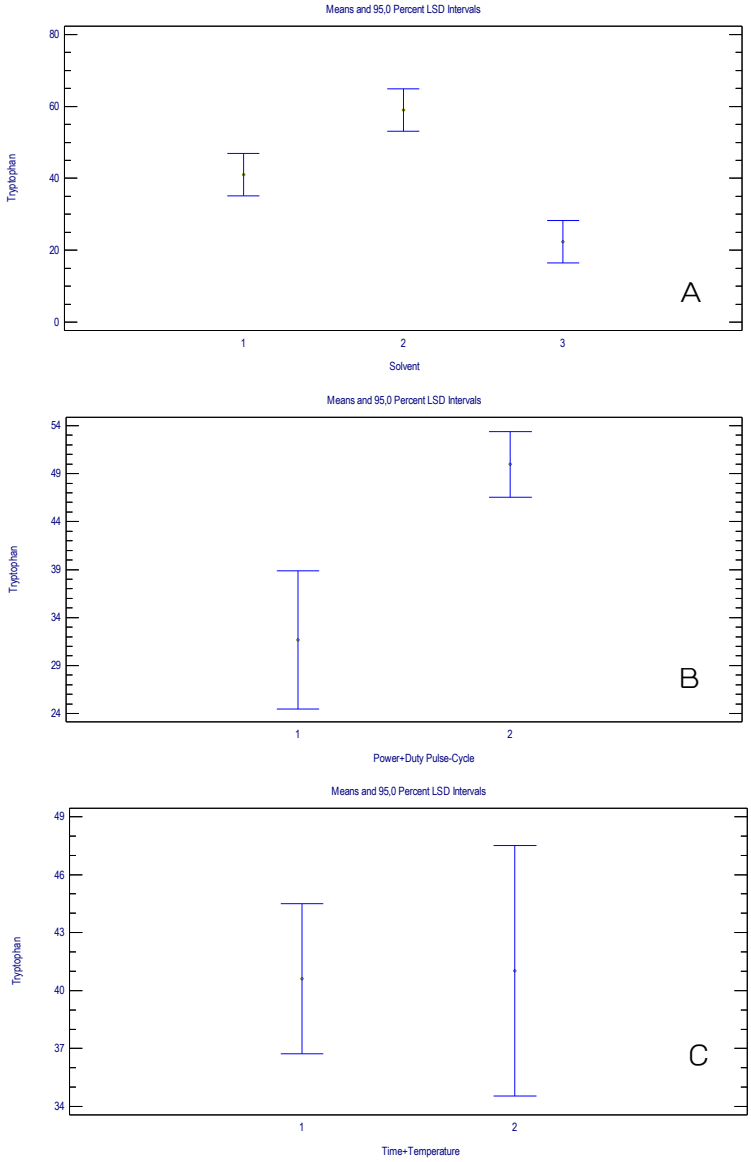

Figure 2 A LSD among the (A) solvent: 1) Methanol, 2) Ethanol and 3) Ethyl acetate), (B) ultrasound power + pulse duty cycle (1. 20 %, 0.2 s^{-1}; 2. 80 %, 0.8 s^{-1}), (C) time + temperature (1. 10 min, 25 °C, 2. 20 min, 50 °C).

The result shows that the highest amount of tryptophan extracted was obtained using ethanol. This result agrees with the data reported by Garcia-Castello *et al*. [29] and Cotas *et al*. [18] that the appropriate solvents for the extraction of bioactive compounds were methanol and ethanol because both have a much lower polarity than water. According to Horovitz and Paşca [30], tryptophan is classified as a non-polar

amino acid. Since ethanol is less polar than methanol, it could extract a higher amount of tryptophan. Although ethyl acetate is less polar than ethanol, it recovered the smallest amount of tryptophan from *K. alvarezii*. Therefore, the factors conditioning the recovery are not only by the solvent's polarity, but also the size and properties of the molecule of the solvent that condition the diffusion capabilities into the solid material. Ethyl acetate molecule is a much larger than both the methanol and ethanol molecules.

Vargas *et al*. [40] found that intracellular compounds accumulated inside the biomass cells, a very hydrophilic environment, making the solvent polarity crucial due to this circumstance, only the polar solvent could penetrate through the hydrophilic environment that surrounds the active compound. Thus, a non-polar solvent, like ethyl acetate, is not able to penetrate the hydrophilic cell.

In order to obtain a high recovery of tryptophan from *K. alvarezii* by UAE, the optimized condition must be defined. Optimization can be done by increasing the value of the most influential factor and positively affecting the response. In this study, solvent and temperature were also further optimized by Computational Method Using COSMO-RS.

Modeling

The previous section showed that the increase in the extraction of tryptophan from *K. alvarezii* could be enhanced by decreasing the polarity of the solvent, from methanol to ethanol. However, ethyl acetate, with lower polarity than ethanol, could not improve the extraction process. Therefore, the purpose of this COSMO-RS is to clarify the impact of solvent properties on the extraction of tryptophan from *K. alvarezii*. The main advantage of using the COSMO-RS approach is that the model could estimate the thermodynamic properties of the chemical compound in the pure and mixture states. In this context, the molecular interactions in the system of interest (methanol, ethanol and ethyl acetate) result from the polarity (sigma surfaces) with the solute (tryptophan).

Figure 3 shows the sigma profile of the studied solvents and solutes. As a rule of thumb, the sigma profile is divided into 3 main regions, namely: 1) $\sigma > 1.0$ e·nm^{-2} correspond to the polar region due to their ability to form hydrogen-bond acceptor, 2) $-1.0 < \sigma < 1.0$ that indicates non-polar nature of the compound and 3) $\sigma < -1.0$ e·nm^{-2} designate to polar region due to their ability to act as a hydrogen-bond donor. The studied solute (tryptophan) presents a series of peaks throughout the polar and non-polar regions. The high intensity peaks within the nonpolar region further indicate that tryptophan is a mainly non-polar compound. The studied solvents also present a similar peak with tryptophan. For the studied solvent, the peak area in the non-polar region can be ranked as follows: Methanol < ethanol < ethyl acetate. This rank also specifies their non-polar character rank.

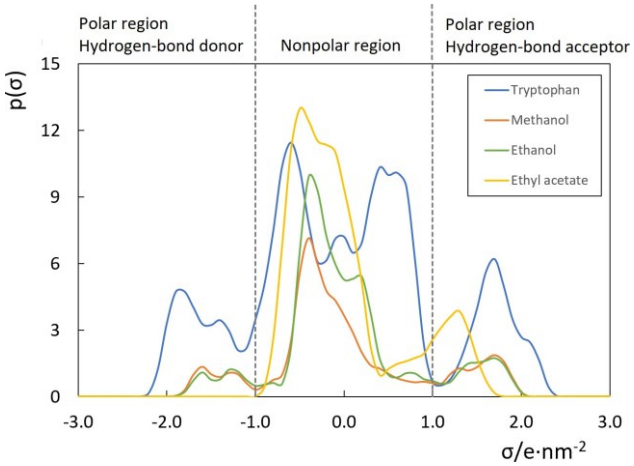

Figure 3 Sigma profile of the studied solvents and solute.

Furthermore, it showed that the studied solvents and solutes present highly overlapping peaks in the non-polar region of the sigma profile. Thus, it can be projected that the interaction of tryptophan with the solvents mainly occurs within the non-polar fragments. As proven experimentally, ethanol (less polar) could extract tryptophan higher than methanol (more polar). This finding further supports the result from [41]. **Table 3** lists the predicted solvent affinity toward tryptophan and water molecules. In this context, the solvent affinity represents their hydrophilicity. Methanol is the most hydrophilic, followed by ethanol and the least hydrophilic is ethyl acetate. The rank of solvent hydrophilicity does not follow their ability to extract tryptophan from the biomass.

Table 3 Solvent polarity and hydrophilicity were predicted using COSMO-RS at 298.15 K.

Solvent	$\ln \gamma_{Tryptophan}$		$\ln \gamma_{H2O}$	
	25 °C	**50 °C**	**25 °C**	**50 °C**
Methanol	1.83	1.82	0.86	0.71
Ethanol	0.44	0.42	2.07	1.95
Ethylacetate	–0.25	–0.30	4.56	4.26

However, it is interesting to see the impact of combining the solvent affinity toward tryptophan and a water molecule, as depicted in **Figure 4**. It seems that the solvent affinity toward tryptophan (representing the polarity) and water (representing the hydrophilicity) plays an essential role in the extraction of solute from *K. alvarezii*. In this scenario, it seems that the active compound, such as tryptophan, from *K. alvarezii* is located inside the hydrophilic cell. Even though ethyl acetate has the highest affinity toward tryptophan, it does not seem hydrophilic enough to penetrate the cell wall of *K. alvarezii*. On the opposite, methanol can penetrate the cell wall, but it has the lowest ability to dissolve tryptophan. Therefore, the best solvent should be hydrophilic enough to penetrate the cell wall and have a high affinity toward tryptophan. Under this scenario, it appears that ethanol is the best solvent to extract tryptophan from *K. alvarezii*.

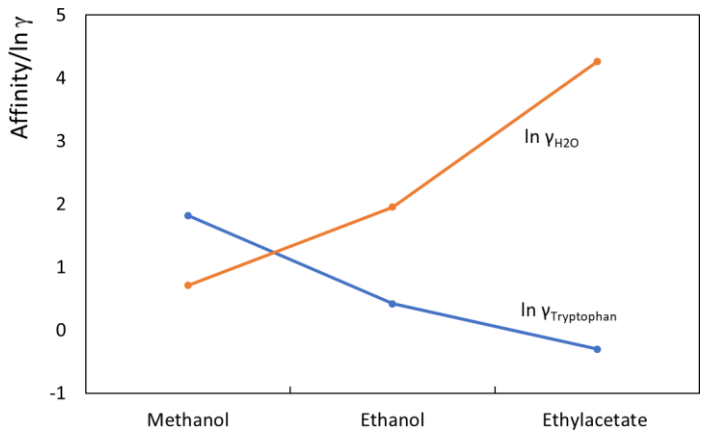

Figure 4 Solvent affinity toward tryptophan and water molecules predicted using COSMO-RS at 298.15 K.

The interaction effects between temperature and the solvents on the affinity among either tryptophan and water and the 3 solvents were also calculated, it can be seen in **Figure 5**. It can be seen that there were no differences between the affinities at different temperatures (25 or 50 °C) for any solvent. Therefore, the

temperature has no significant effect on the affinity of the solvent for tryptophan and the hydrophilicity of the 3 solvents. This finding supports the results of the experiments that have been carried out. In the experiments in the experimental design, no effects of the temperature on the extraction of tryptophan from *K. alvarezii* was found.

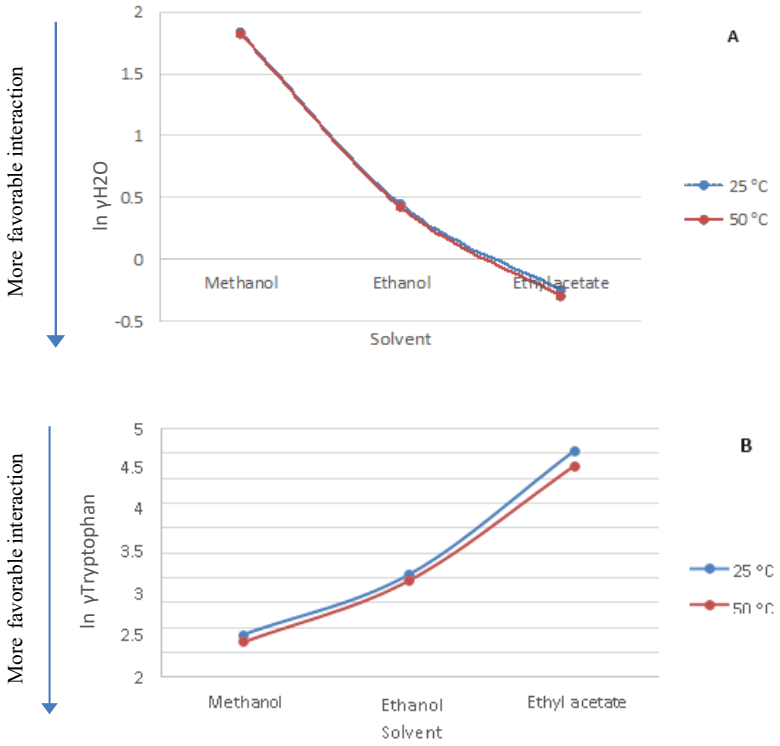

Figure 5 Effect of temperature on the affinity of solvent toward tryptophan and its hydrophilic properties: (A) ln γTryptophan stands for the affinity of solvent to tryptophan and (B) ln γH2O hydrophilicity of solvent.

Comparison of experimental data to modeling and optimization of the extraction of tryptophan in *K. alvarezii* using the optimum conditions

From the experimental data, the best solvent to extract tryptophan was ethanol. These findings were in line with the COSMO-RS modeling results, where ethanol was also the best solvent because it has sufficient hydrophilicity and affinity to tryptophan to extract high recovery of tryptophan from *K. alvarezii*. The combination of time and temperature did not affect the extraction of tryptophan from *K. alvarezii* experimentally and increasing the temperature from 25 to 50 °C did not have a significant impact on increasing extraction efficiency in modeling. Therefore, experimentally and in modeling, the temperature had no effect, so the lowest temperature, i.e., 25 °C was used. The optimum extraction conditions were solvent ethanol, 10 min of time, 25 °C of temperature, 80 % of power and 0.8 s^{-1} pulse duty cycle. The optimum conditions could extract 56.41 mg L^{-1} tryptophan from *K. alvarezii* extract.

Stability test

Through adopting the conditions obtained from the results of this study and using selected solvents. This method was used to extract tryptophan from *K. alvarezii* in order to determine whether the level

remained stable during storage. The results of the stability assessment showed that tryptophan levels were relatively stable throughout the storage period. Statistical analysis did not show significant variations, thus confirming the robustness of the conditions used and the solvent chosen. This indicates that the tryptophan extracted from *K. alvarezii* remains unaffected by storage conditions and maintains its integrity. In this study, sample *kappaphycus* spp. powder was storaged at 3 difference temperatures (4, 25 and 30 °C) and stored for 11 weeks (**Figure 6)**.

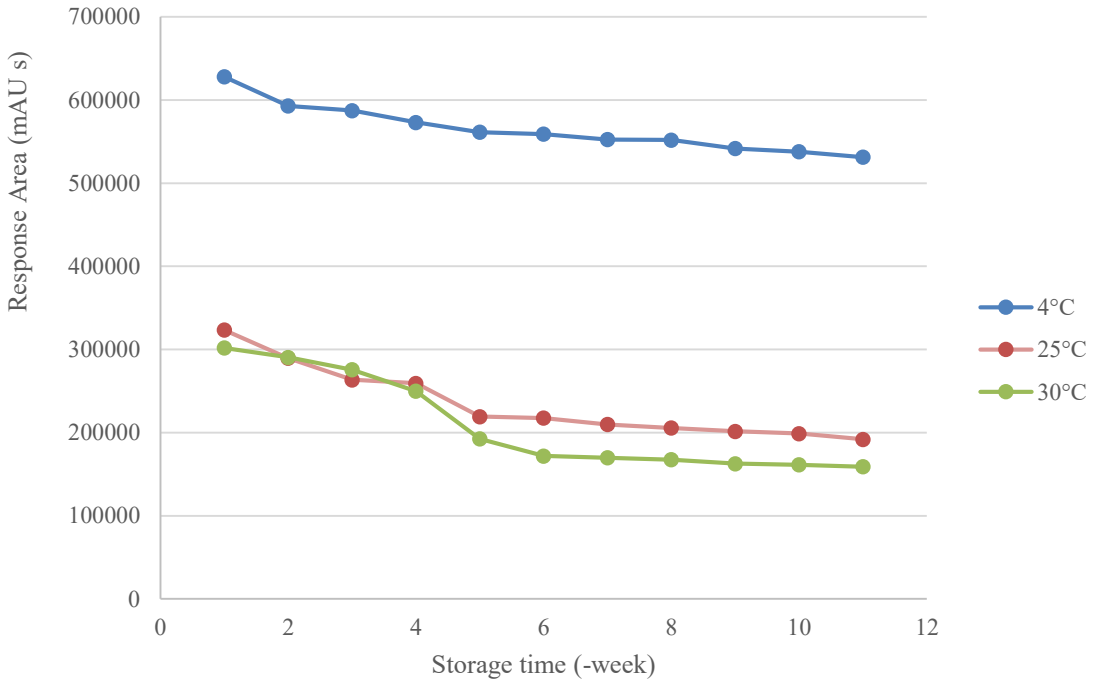

Figure 6 Stability test during 11 weeks in different temperatures.

Ultrasound impact in enhancing the extraction

An optical microscope was used to investigate the impact of the extraction using UAE on solid sample of *K. alvarezii* (**Figure 7**). Medina-Torres *et al*. [42] stated the bubble from the vibration of ultrasound could extract the desired material from the plant. In the 1st step, the bubbles will break down the cell wall, making the bubbles collapse. Therefore, the solvent will diffuse into the cell and cause cellular disruption and release the desired compounds. **Figure 7** shows the image of the cells before and after the extraction process. It is clear that cells were damaged after the extraction process.

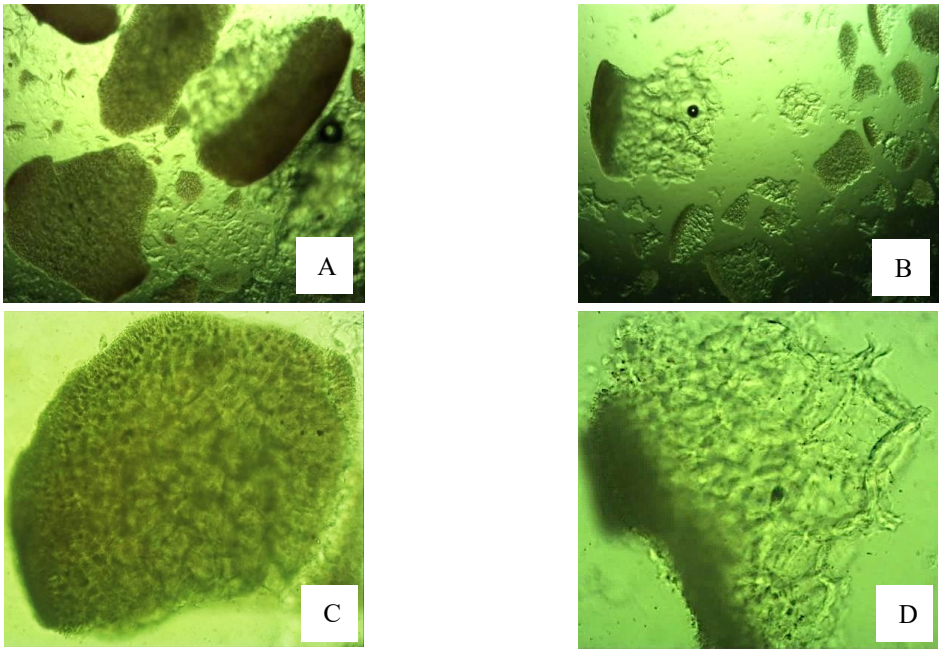

Figure 7 Cell damage by UAE: (A) Before extraction (magnification 52×), (B) After extraction (magnification 52×), (C) before extraction (magnification 130×) and (D) after extraction (magnification 130×).

Conclusions

The most important extraction variables during the UAE from *K. alvarezii* were the type of solvent and the combination of ultrasound power and pulse duty cycle. In contrast, the combination of temperature and time had no effect. There is an agreement between experimental and COSMO-RS modeling on the best solvent and temperature for the extraction of tryptophan from *K. alvarezii*, it is ethanol. It has been demonstrated that not only the affinity to the solvent, but both the interaction solvent-tryptophan and solvent-hydrophilic matrix are important for the extraction process.

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