Equilibrium Modeling of Astaxanthin Extraction from *Haematococcus pluvialis*

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Abstract: *Astaxanthin is a natural antioxidant, and the highest content of this compound is found in* Haematococcus pluvialis *microalgae. Microwave-assisted extraction (MAE) is one of the environmentally friendly extraction methods and has many advantages. This study aims to investigate the extraction of astaxanthin through the MAE method using various solvents. Several equilibrium models were proposed to describe this solid-liquid equilibrium. The solid-liquid extraction equilibrium parameters were determined by minimizing the sum of squares of errors (SSE), in which equilibrium constants were needed for scaling up purposes. Previously, the microalgae were pretreated with HCl to soften their cell walls thus improving the extraction recovery. In this study, dichloromethane, acetone, methanol, and ethanol were used as the solvents for the extraction process. The astaxanthin concentration was determined by high-performance liquid chromatography (HPLC) and spectrophotometry. Astaxanthin was found to attain equilibrium at 57.42% recovery in a single-step extraction. Thus, several steps were required in sequence to obtain optimum recovery. The experimental data were fitted to three equilibrium models, namely, Henry, Freundlich, and Langmuir models. The experimental data were well fitted to all the models for the extraction in dichloromethane, methanol, ethanol and acetone, as evident from a similar SSE value for each model.*

Keywords: *equilibrium constant; mass transfer coefficient; astaxanthin; extraction;* Haematococcus pluvialis

■ INTRODUCTION

Astaxanthin, with a molecular formula of $C_4OH_{52}O_4$ and molecular weight of 596.84 g/mol, is an antioxidant from the carotenoid group. Its antioxidant capacity is 100–500 times higher than those of vitamin E [1-2] and vitamin C [3], and also 40–600 times higher than that of α-tocopherol [4]. Astaxanthin has an essential role in maintaining liver function and preventing inflammation, oxidation due to UV rays, and cell damage due to the aging process. Astaxanthin is also known for its ability to prevent heart cancer disease by enhancing the immune system, thereby increasing life expectancy, and has several other important functions in the physiological system. Astaxanthin is widely used in the pharmaceutical, cosmetic, and food industries [1,3,5-8].

Astaxanthin can be obtained from *Haematococcus pluvialis* (*H. pluvialis*) microalgae in up to 0.5–5% of dry weight content [9-11]. There are several methods, categorized as conventional and non-conventional methods, for extracting astaxanthin from *H. pluvialis*. Maceration or solvent extraction is a conventional method, while microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE), etc., are non-conventional methods. Based on the astaxanthin recovery in these methods, SFE and MAE have been reported to provide a high yield of astaxanthin [6].

Besides the aspect of astaxanthin recovery, the MAE method is an interesting technique. It has several advantages, such as the requirement of simple equipment and small solvent volumes, high extraction efficiency, short extraction time, and minimum solvent waste [6,12-13]. The short extraction time is extremely beneficial for minimizing the degradation of astaxanthin [14]. Although astaxanthin extraction using the MAE method has been studied previously [13-16], the studies were limited to determining the optimum extraction conditions.

An equilibrium model is required to represent the extraction equilibrium and it is extremely beneficial for the design and scaling up of laboratory processes to the pilot and industrial scales [17-18]. To the best of our knowledge, the equilibrium for astaxanthin extraction from *H. pluvialis* microalgae using MAE has not been studied before. This study aims to determine the equilibrium constant and mass transfer coefficient through minimization of the sum of squares of errors (SSE) between the experimental data and simulated data of the equilibrium model. Three equilibrium models, namely, Henry, Freundlich, and Langmuir models, were used for describing the solid-liquid extraction process. Evaluation of the extraction equilibrium constants was based on the unsteady-state process.

Fig. 1 depicts the theoretical scheme of the astaxanthin mass transfer process. In this figure, N_A is the mass transfer rate, C_{AP} is the astaxanthin concentration on solid microalgae surface, C_{AS} is astaxanthin concentration at equilibrium, and C_A is the astaxanthin concentration in the solvent. Astaxanthin was assumed to transfer from the microalgae through the boundary layer lying between the solid microalgae surface and solvent.

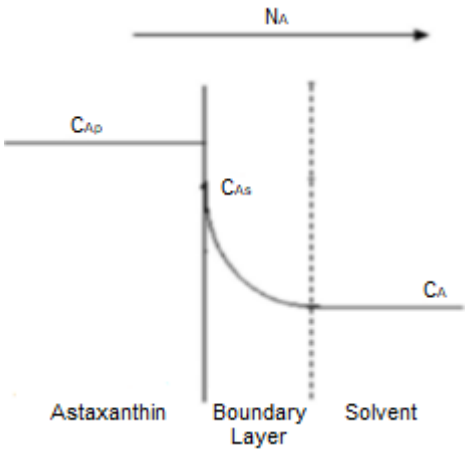

Fig 1. Scheme of astaxanthin mass transfer process

Astaxanthin was transferred continuously until the equilibrium condition has been reached. Upon the attainment of equilibrium, astaxanthin from microalgae did not dissolve any further in the solvent [19].

In a solid-liquid extraction process, the mass transfer of a solute from the solid to liquid phase occurs in two stages. The first stage involves the internal diffusion from the interior of the solid microalgae to its surface, and the second stage involves mass transfer from the surface to the solvent [20].

Due to the small size of microalgae, as for the case of astaxanthin extraction, the internal diffusion was considered to occur very fast. The astaxanthin concentration in the microalgae (C_{Ap}) could then be assumed to be homogeneous, i.e., no concentration gradient existed within the solid. Therefore, the overall mass transfer between the two phases would be entirely controlled by the mass transfer process.

The rate of astaxanthin mass transfer and its coefficient (k_c) can be derived from its mass balance equation, as given by Eq. (1).

$$-M\frac{dC_{Ap}}{dt} = A_s.k_c.(C_{As} - C_A) \quad (1)$$

Another important parameter in solid-liquid extraction is the equilibrium constant [19]. In this case, the distribution of a solute between the solid and liquid phases can be determined from the adsorption isotherm, which can be explained using several equilibrium models such as the Henry, Freundlich, and Langmuir models, as given by Eq. (2), (3), and (4).

Henry equilibrium model:

The Henry model is represented by a simple linear equation, as given by Eq. (2).

$$C_{As} = H.C_{Ap} \quad (2)$$

H, the Henry constant, represents the equilibrium constant; a high H value indicates stronger interaction between the solute and solid, while a low H value indicates a weaker solute-solid interaction [18]. This model is suitable for low concentrations of liquid [21].

Freundlich equilibrium model:

The Freundlich model is given by Eq. (3).

$$C_{Ap} = K_F.C_{As}^{(1/n)} \quad (3)$$

This model is suitable for heterogeneous, monolayered porous solid surfaces [21]. In Equation (3), K_F is the equilibrium constant. 1/n is always positive and generally not an integer. It can be determined by plotting log C_{AP} vs. log C_{AS} [19].

Langmuir equilibrium model

Eq. (4) represents the Langmuir model. This model is suitable for homogeneous, monolayered flat solid surfaces.

$$C_{Ap} = \frac{K_L.q_m.C_{As}}{1+K_L.C_{As}} \quad (4)$$

Here, k_L is the equilibrium constant, and q_m is the monolayer capacity approached at large concentrations. These can be determined by plotting $1/C_{Ap}$ vs. $1/C_{AS}$ [19].

The experimental data were fit to the above equilibrium models using MATLAB. The equilibrium constant and mass transfer coefficient were determined from the fitting parameters. The accuracy of the fit was optimized by minimizing the SSE, which is defined by Eq. (5).

$$SSE = \sum (C_{experiment} - C_{calculation})^2 \quad (5)$$

■ EXPERIMENTAL SECTION

Materials

In this study, astaxanthin was extracted from *H. pluvialis* microalgae obtained from Xi'an Saiyang Bio-Technology Co., Ltd, China. EMSURE grade of acetone, methanol, ethanol, and dichloromethane were purchased from Merck and used as the solvents. An astaxanthin standard with a purity of 98%, CAS 472-61-7, was purchased from Sigma Aldrich, Singapore, as the reference standard for HPLC.

Instrumentation

HPLC and spectrophotometry techniques were used to determine the astaxanthin concentration. UV-visible spectroscopy was performed on a UV Mini 1240 Shimadzu UV/Vis spectrophotometer. The wavelength at 476, 475, 477, and 480 nm were detected when ethanol, methanol, acetone, and dichloromethane, respectively, were used as the extraction solvents. For comparison, HPLC Shimadzu LC-2010CHT with photodiode array detector SPD-M10A$_{VP}$ was performed on a C18 column, with 0.05% trifluoroacetic acid/methanol (3:97 (v/v)) as the mobile phase. The retention time was 5.5 min.

Procedure

Pretreatment process

The pretreatment process was conducted to soften the thick cell wall of the microalgae, as hard cell walls can hinder the extraction of astaxanthin. Based on a previous study [22], the microalgae used in this study were pretreated by mixing them with 4 M HCl at 70 °C for 2 min. Afterward, the mixture was cooled to ambient temperature and washed with distilled water until the pH of the mixture was 7. Then, the wet microalgae were dried in a refrigerator at ~4 °C for 24 h. The sample was used for the next stage of the extraction process.

Astaxanthin extraction

The extraction was performed using the MAE method. Microwaves (Electrolux type MM823AB4-POOC) with 800-W power and frequency of up to 2450 MHz were used. The microwave treatment leads to the resonance of the water molecules in the microalgae cells, producing heat due to molecular friction. This results in the disruption of cell walls due to increased internal pressure. As the cell wall disrupts, the rate of mass transfer of astaxanthin from the solid microalgae to the solvent increases remarkably [16]. During the extraction, the astaxanthin concentration in the solution continues to increase till equilibrium is attained [17]. The microalgae to solvent ratio was maintained at 10:1 (w/v), following the previously reported procedures [5,22]. The extraction temperature was 5 °C at the boiling point of each solvent.

Hydrolysis

In the microalgae extract, astaxanthin is present in the form of free astaxanthin (5%), monoester astaxanthin (70%), and diester astaxanthin (25%) [15,23]. The complexity of the ester form renders the analysis difficult. Thus, the ester bond is cleaved by hydrolysis to form free astaxanthin, thereby rendering the analysis easier. The effectiveness of hydrolysis is

evident in the Fourier transform infrared (FTIR) spectra Thermo Nicolet IS 10, wherein the change in the absorbance ratio of the alternating C–O–R to C–O–H bonds on the hydrolyzed sample can be clearly identified. Additionally, the spectrophotometric analysis confirmed the enhancement of free astaxanthin recovery after this process [26].

Hydrolysis was conducted by mixing the microalgae extract with 0.02 M alcoholic NaOH, according to previously reported studies [24-25]. Alcoholic NaOH was prepared by mixing NaOH with methanol. It was reported that a 1:2 (v/v) ratio of the sample to alcoholic NaOH was effective for hydrolysis [26].

■ RESULTS AND DISCUSSION

Effect of Pretreatment on Astaxanthin Recovery

Fig. 2 shows a comparison of the astaxanthin concentration recovered from the extraction process with and without the initial pretreatment, using acetone and ethanol as the solvents (Fig. 2(a) and 2(b), respectively).

In both figures, a sharp increase in the astaxanthin recovery is observed for the pretreated samples in the first two minutes, before reaching equilibrium, as shown by the steady concentration. In contrast, the release of astaxanthin during extraction seems difficult without pretreatment, and a longer time is required to attain the equilibrium. In Fig. 2(a), the extraction is shown to increase by 74% upon pretreatment compared to the case without pretreatment. Similar behavior is observed for the case of ethanol (Fig. 2(b)), where 79% of the astaxanthin could be extracted after the pretreatment. Thus, pretreatment not only shortens the extraction time but also improves the maximum astaxanthin recovery. This suggests that pretreatment can successfully soften the cell walls of the microalgae, thereby facilitating the extraction process; this phenomenon is similar to that reported previously [22,27].

With an aim to observe the morphology, the microalgae were visualized under the focused beam of electrons of a scanning electron microscope (SEM). The images are shown in Fig. 3. At 1000 times magnification, the differences between the pretreated and non-pretreated samples were fairly obvious—much darker spots were observed for the cell walls of the microalgae that were pretreated. The openings were deeper in this sample, probably because of the damage on its surface as a consequence of the cell wall disruption.

Determination of Astaxanthin Concentration by Spectrophotometry

Fig. 4 depicts the spectrophotometrically determined astaxanthin concentration [28-30] upon extraction using different solvents.

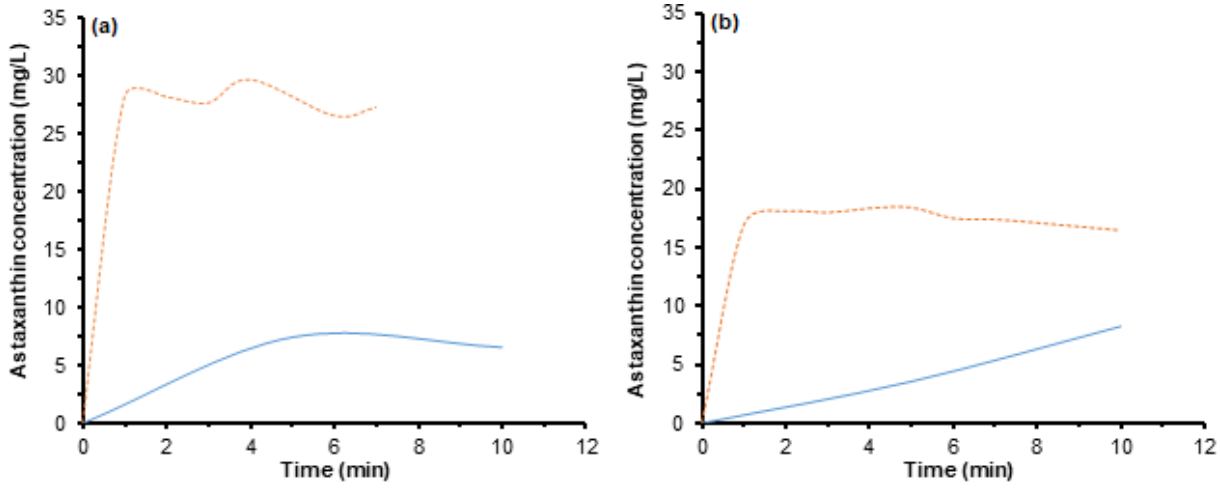

Fig 2. Comparison of the outcome of pretreated and non-pretreated samples on astaxanthin extract using (a) acetone and (b) ethanol as the extraction solvent. Blue line shows the astaxanthin concentration without a pretreatment process while orange dotted line shows the astaxanthin concentration with a pretreatment process

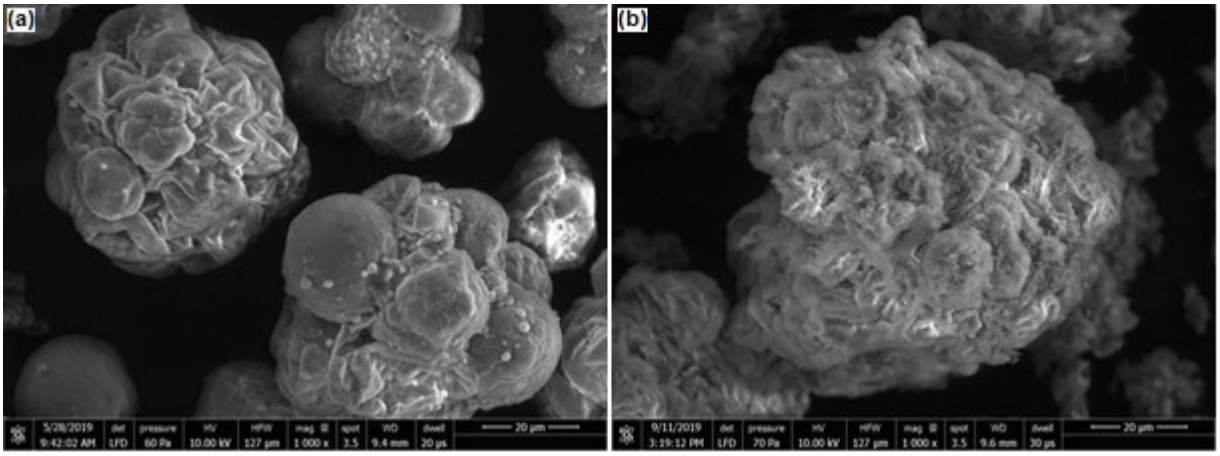

Fig 3. SEM images of microalgae (a) without and (b) with pretreatment

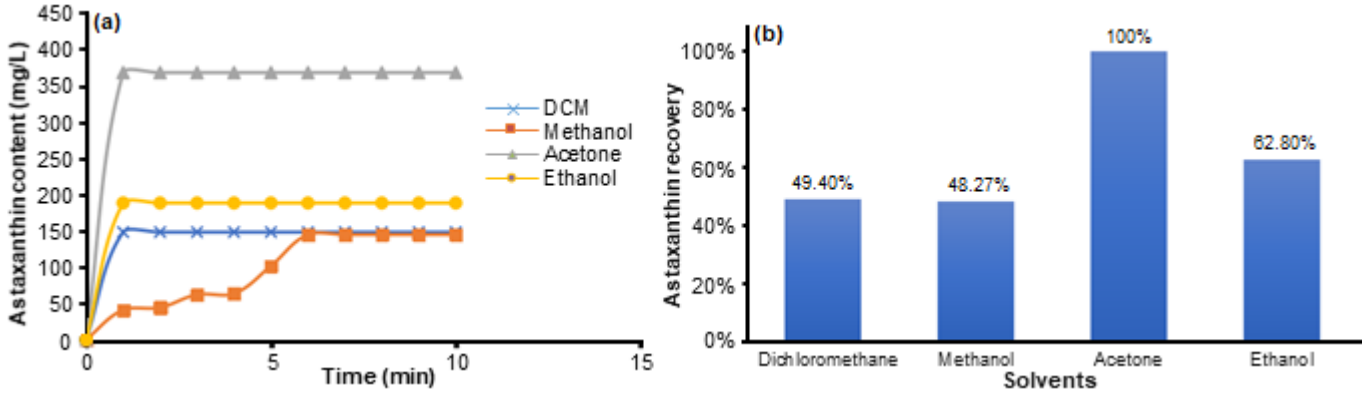

Fig 4. Astaxanthin concentration analyzed by spectrophotometry: (a) evolution of concentration with extraction time and (b) recovery percentage in various solvents

Fig. 4(a) suggests that all the solvents tested can be used to extract astaxanthin from microalgae, and an equilibrium is attained in the process. The extraction in acetone, dichloromethane, and ethanol reaches equilibrium within a minute. For methanol, a slightly slower mass transfer rate seems to be responsible for the longer time required to reach the equilibrium condition (Fig. 4(b)) shows that the extraction of astaxanthin from dry powder microalgae proceeds with 100% recovery in acetone, followed by 62.80, 49.40, and 48.27% recovery in ethanol, dichloromethane, and methanol, respectively. Thus, spectrophotometric analysis suggests that the compatibility between astaxanthin and the solvent plays a significant role in determining the extraction kinetics and total recovery, with acetone being the best choice among the tested solvents for extracting astaxanthin from microalgae.

Determination of Astaxanthin Concentration by HPLC

The recovery of astaxanthin extraction, as determined using HPLC [13-14,22,31-33], is shown in Fig. 5. Fig. 5 suggests that both the rate and recovery percentage are affected by the solvent. Rate analysis suggests that the extraction takes the maximum time to attain the equilibrium in methanol, and astaxanthin can no longer diffuse into the solvent in this state [19]. A fraction of astaxanthin (13 mg/L) dissolves into the methanol in less than a minute. The equilibrium was reached directly in the first minute in acetone, in the second minute in dichloromethane, and ethanol (Fig. 5(a)). However, when methanol was used for the extraction, equilibrium was attained in 5 min. Acetone gives the highest astaxanthin recovery percentage, as

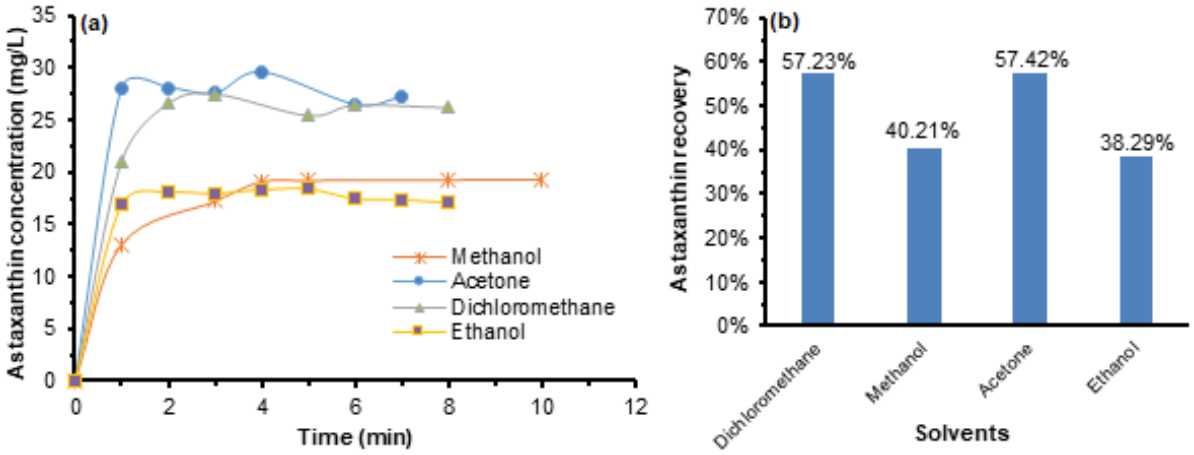

Fig 5. Astaxanthin concentration analyzed by HPLC: (a) evolution of concentration with extraction time and (b) percentage recovery in various solvents

evident from Fig. 5(b). Compared to other organic solvents such as methanol, ethanol, and acetonitrile [14], the highest astaxanthin recovery was obtained in acetone (44 ± 1%). Acetone was the most appropriate solvent because its structure is very similar to that of astaxanthin, which contains many carbonyl groups. Since astaxanthin extraction reached equilibrium with 100% recovery, the extraction process must be conducted in a stepwise manner.

Equilibrium of Astaxanthin Extraction

Three equilibrium models were investigated in this study—the Henry, Freundlich, and Langmuir models. Fig. 6(a) depicts the plot of astaxanthin concentration vs. extraction time and its fit using the Henry equilibrium model. The Henry constant (H) is obtained by plotting C_{Ap} and C_{As}. Meanwhile, k_c is derived from the mass balance. Their values are summarized in Table 1. For the Freundlich model, the Freundlich constants (n and k_L) are obtained by plotting log C_{Ap} vs. log C_{As} and solving the mass balance equation, respectively. Table 1 and Fig. 6(b) show the corresponding values and the fitting curves for the various solvents used.

Fig. 6(c) shows the fit of the experimental data to the Langmuir model. Parameters k_L and q_m derived from the plot of $1/C_{Ap}$ vs. $1/C_{As}$ are listed in Table 1. Besides, k_c is determined by solving the mass balance equation and is given in the same table. From Table 1 H is the Henry constant, k_F and 1/n are the Freundlich constants, and k_L and q_m are the Langmuir constants. The k_c value gives an estimate of the extraction rate, wherein higher kc values correspond to a faster mass transfer rate and hence, a shorter extraction time.

Comparison of the Henry, Freundlich, and Langmuir Models

Comparison of the Henry, Freundlich, and Langmuir models based on the SSE in each solvent is presented in Table 2. The smallest SSE value corresponding to each solvent represents the best fit. For acetone, methanol, ethanol, and dichloromethane, the experimental data were well fitted to all models, as the SSE values are almost equal regardless of the model used. For acetone, H and kc values from the Henry model were 13.505 g microalgae/dm^3 and 20.405 dm/min, respectively. In the Freundlich model, 1/n, k_F, and k_c were 0093, 1.516 dm^3/g microalgae, and 3.200 dm/min, respectively. In the Langmuir model, k_L, q_m, and k_c were 0.021 dm^3/mg astaxanthin, 5.549 mg astaxanthin/g microalgae, and 15.847 dm/min, respectively.

For methanol, H and k_c values from the Henry model were 6.675 g microalgae/dm^3 and 5.600 dm/min, respectively. In the Freundlich model, 1/n, k_F, and k_c were 1.052, 0.129 dm^3/g microalgae, and 5.735 dm/min, respectively. In the Langmuir model, k_L, q_m, and k_c were 0.005 dm^3/mg astaxanthin, 33.454 mg astaxanthin/g microalgae, and 5.305 dm/min, respectively. For ethanol, H and k_c values from the Henry model were 5.922 g microalgae/dm^3 and 14.563 dm/min, respectively. In the

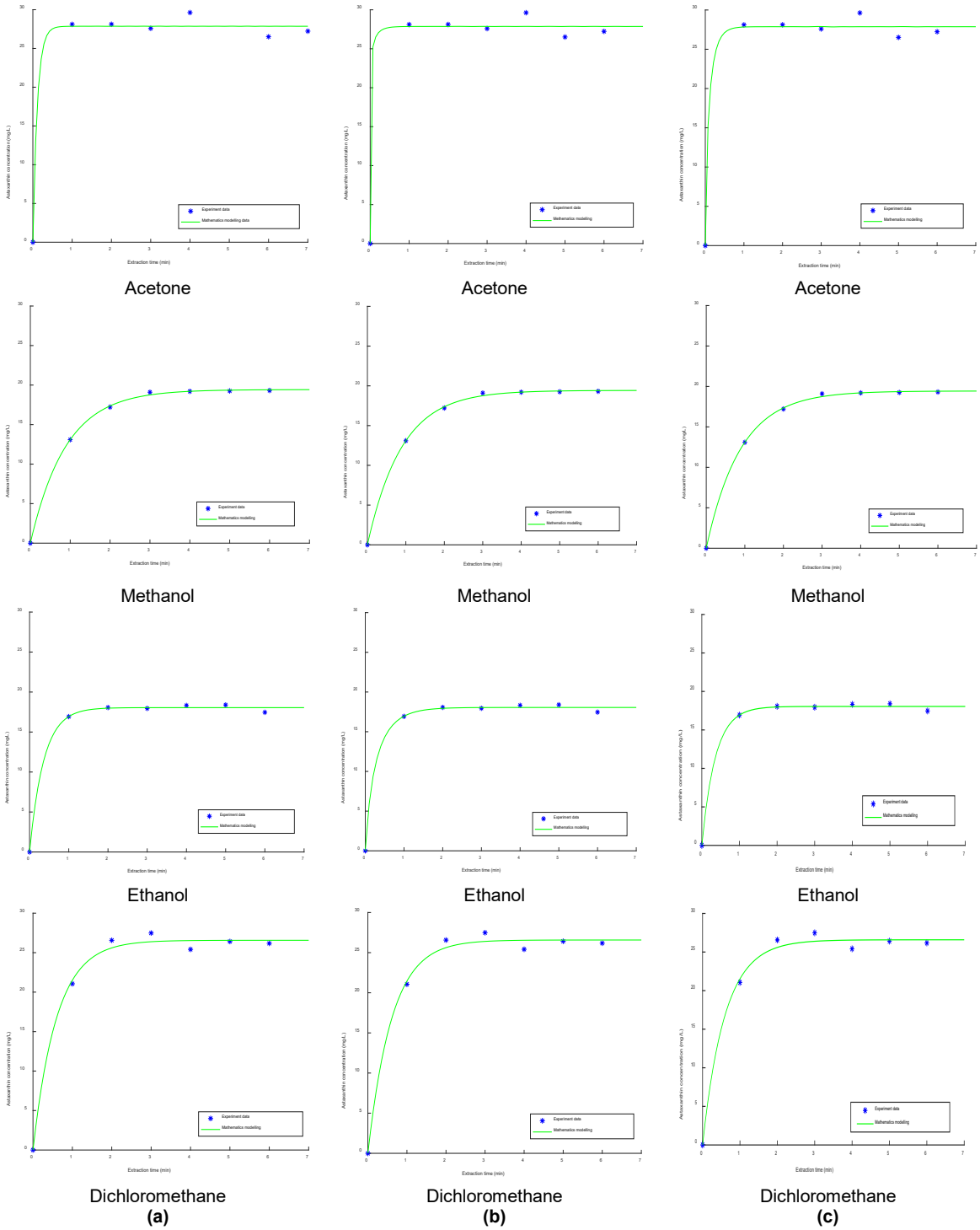

Fig 6. Equilibrium models for the extraction in different solvents. (a) Henry model, (b) Freundlich model, (c) Langmuir model

Freundlich model, 1/n, k_F, and k_c were 0.245, 1.498 dm^3/g microalgae, and 5.544 dm/min, respectively. In the Langmuir model, k_L, $q_{m,}$ and k_c were 0.005 dm^3/mg astaxanthin, 38.396 mg astaxanthin/g microalgae, and 0.549 dm/min, respectively.

For dichloromethane, H and k_c values from the

Table 1. Model constants for the tested solvents

Solvents	Henry Model H = g microalgae/dm^3 k_c = dm/min	Freundlich Model k_F = dm^3/g microalgae k_c = dm/min	Langmuir Model k_L = dm^3/mg astaxanthin q_m = mg astaxanthin/g microalgae k_c = dm/min
Acetone	H = 13.485 k_c = 73.895	1/n = 0.093 k_F = 1.516 k_c = 3.200	k_L = 0.021 q_m = 5.549 k_c = 15.847
Methanol	H = 6.675 k_c = 5.600	1/n = 1.052 k_F = 0.129 k_c = 5.735	k_L = 0.005 q_m = 33.454 k_c = 5.305
Ethanol	H = 5.922 k_c = 14.563	1/n = 0.245 k_F = 1.498 k_c = 5.544	k_L = 0.005 q_m = 38.396 k_c = 14.009
Dichloromethane	H = 12.097 k_c = 6.254	1/n = 1.336 k_F = 0.027 k_c = 7.412	k_L = 0.002 q_m = 44.004 k_c = 5.969

Table 2. SSE of the fit of the Henry, Freundlich, and Langmuir models to the experimental data

SSE	Henry Model	Freundlich Model	Langmuir Model
Acetone	5.515	5.505	5.511
Methanol	0.157	0.156	0.162
Ethanol	0.549	0.557	0.549
Dichloromethane	3.773	3.684	3.834

Henry model were 12.097 g microalgae/dm^3 and 6.254 dm/min, respectively. In the Freundlich model, 1/n, k_F, and k_c were 1.336, 0.027dm^3/g microalgae, and 7.412 dm/min, respectively. In the Langmuir model, k_L, q_m, and k_c were 0.002 dm^3/mg astaxanthin, 44.004 mg astaxanthin/g microalgae, and 5.969 dm/min, respectively.

■ CONCLUSION

Astaxanthin can be extracted from microalgae using the microwave-assisted extraction method. Acetone, methanol, ethanol, and dichloromethane were examined for extraction. Among these solvents, acetone was found as the best solvent for obtaining the highest recovery. The experimental data were fit to three equilibrium models, namely, Henry, Freundlich, and Langmuir models, using MATLAB. The experimental data were well fitted to all the three models for the extraction in acetone, methanol, ethanol, and dichloromethane, as evident from the almost same SSE value for each model.

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