

The Selective Supercritical Extraction of High-value Fatty Acids from *Tetraselmis suecica* using the Hansen Solubility Theory

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The aim of this work was to test the utility of the Hansen theory to predict the best cosolvent for supercritical carbon dioxide (sc-CO₂) to reach the selective extraction of fatty acids from *Tetraselmis suecica*. The order in the cosolvent power was established with five organic solvents used in food production: acetone, diethyl ether, ethanol, n-hexane and methanol. Predictions focused on the selective extraction of oleic, linoleic and α -linolenic acid. The cosolvent power depended on the fatty acid, but in general, the best cosolvent for the three target compounds was ethanol. Predictions were validated through equilibrium data and extraction yields from *T. suecica*. Operating at 305.15 K and 20 MPa, the extracted oil with the sc-CO₂-ethanol (5 % mass fraction) mixture significantly improved the content of the target fatty acids compared with pure sc-CO₂; e.g. the α -linolenic acid content was 16 % in the oil obtained with pure sc-CO₂ while it was 25 % in the oil obtained with sc-CO₂ + 5 % ethanol. However, the Hansen theory predicted that the miscibility enhancement of the fatty acids caused by increasing ethanol concentrations in the supercritical solvent mixture was not progressive. In fact, at high pressures and high ethanol concentrations, it was predicted up to less than half the miscibility enhancement.

1. Introduction

Microalgae have received widespread attention in the world population due to the high amounts of bioactive compounds within their structure. Some of these valuable compounds include mono (MUFAs) and polyunsaturated fatty acids (PUFAs) (Viso and Marty, 1993). *Tetraselmis suecica*, for example, is a marine green microalga extensively used in aquaculture, which has already demonstrated its potential as a feedstock for the production of bioactive compounds such as MUFAs and PUFAs (Pérez-López *et al.*, 2014). There is a special interest in the extraction of these fatty acids from natural matrices since functional roles and effects on human health are attributed to them (Calder, 2015). Regarding this, the supercritical carbon dioxide (sc-CO₂) extraction seems to be a suitable technology to achieve the extraction of high-value fatty acids from natural sources (Martínez and Aguiar, 2013). The use of sc-CO₂ has become an alternative solvent for oil extraction from microalgae and plants, because it can achieve good oil yield with respect to the conventional organic solvent extraction, with better product quality and offering the possibility of obtaining a product without the presence of traces of a residual solvent in the final product, which makes the process attractive from the health and environmental point of view. Moreover, CO₂ has easy achievable supercritical conditions (304.13 K and 7.38 MPa are the critical temperature and pressure, respectively) (Mendes *et al.*, 1995). On the other hand, in order to increase the solubility of specific compounds in sc-CO₂ and therefore improve their extraction, a cosolvent is added in the fluid to increase the separation factor and/or reduce operating pressure and CO₂ consumption. However, the selection of the best cosolvent requires long and expensive experimental effort. For that reason, theoretical estimations should be implemented and the Hansen solubility theory (HST) has already demonstrated to be a useful tool for this sort of evaluations (Tirado *et al.*, 2018). In this theory, the total solubility parameter (δ_T) of a substance is divided into three components: dispersion (δ_d), polar (δ_p) and hydrogen-bonding (H-bonding, δ_h) forces, later on, known as Hansen solubility parameters

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(HSP) (Hansen, 1967). Thus, δ_T is calculated as the square root of a sum of each Hansen component squared. On the other hand, the Hansen theory provides a numerical estimation of the degree of interaction among materials and solvents through the R_a parameter, which can be a good indicator of their miscibility. This last represents the distance between the three-dimensional coordinates of a target compound (δ_{d1} , δ_{p1} and δ_{h1}) and those of the solvent (δ_{d2} , δ_{p2} and δ_{h2}) and can be calculated by Eq. (1). The smaller the value of R_a , the larger the miscibility between the compound and the solvent. Consequently, and according to the Hansen theory, supercritical systems with the lowest R_a value would represent the most suitable one.

$$R_a = \sqrt{4(\delta_{d1} - \delta_{d2})^2 + (\delta_{p1} - \delta_{p2})^2 + (\delta_{h1} - \delta_{h2})^2} \quad (1)$$

In previous work, Tirado *et al.*, (2018) demonstrated the suitability of the HST to predict the best cosolvent for sc-CO₂ in the solubilization of fatty acids within a specific interval of operating conditions. Moreover, the same research group proved that this theoretical approach was also useful to predict the best cosolvent for the extraction of carotenoids from *Dunaliella salina* (Tirado and Calvo, 2019). However, the extension of the Hansen theory to the supercritical extraction of fatty acids from natural matrices has not yet been demonstrated. Therefore, this study aimed to validate the Hansen approach to predict the best supercritical mixture to reach the selective SFE of oleic, linoleic and α -linolenic acid from *T. suecica*. Emphasis was placed on these fatty acids because of their interest in the food industry. In addition, the initial content of these fatty acids in the microalgae was enough to notice the impact of cosolvent power after the SFE.

2. Materials and Methods

2.1 Reagents

Acetone ($\geq 99.9\%$, Sigma-Aldrich), anhydrous sodium sulphate ($\geq 99\%$, Sigma-Aldrich), boron trifluoride (1.5 M in methanol, ACROS Organics™), butylated hydroxytoluene ($\geq 99\%$, Sigma-Aldrich), ethanol ($\geq 99.8\%$, Fisher Chemical), heptadecanoic acid ($\geq 98\%$, Sigma-Aldrich), n-hexane ($\geq 99\%$, Fisher Chemical), methanol ($\geq 99.9\%$, Fisher Chemical), fatty acid methyl esters (FAME Mix C8 - C22, Sigma-Aldrich), sodium hydroxide ($\geq 98\%$, Sigma-Aldrich), sodium chloride ($\geq 99.5\%$, Sigma-Aldrich), α -linolenic acid (99%, ACROS Organics™), helium ($\geq 99.99\%$, Air Liquide), nitrogen ($\geq 99.99\%$, Air Liquide), hydrogen ($\geq 99.99\%$, Air Liquide) and CO₂ ($\geq 99.98\%$, Air Products) were used in this work. All substances were acquired in Spain and used without any pre-treatment.

2.2 Computational methods

The modelling of the Hansen theory implemented in this work was carried out such as it was described in previous works (Tirado *et al.*, 2018; Tirado and Calvo, 2019) using Microsoft Excel.

2.2.1 Hansen solubility parameters of fatty acids

The HSP at 298.15 K and 0.1 MPa of the oleic (C18:1 *cis*-9), linoleic (C18:2 *cis*-9,12) and α -linolenic (C18:3 *cis*-9,12,15) acids were calculated by using the group contribution method (GCM) proposed by Hansen (Hansen, 2007). The influence of temperature on the HSP values was calculated by the Jayasri and Yaseen method (Jayasri and Yaseen, 1980). Working below the critical points of the target compounds, pressure does not exert a considerable influence on their HSP (Tirado *et al.*, 2018), therefore, this influence was not considered. The critical temperatures were calculated by the GCM proposed by Joback (Joback and Reid, 1987).

2.2.2 Hansen solubility parameters of solvents

The mixtures sc-CO₂ + cosolvent should be in the supercritical state (completely miscible) at the operating conditions to reach the desired cosolvent effect (Tirado *et al.*, 2018). Therefore, calculations were performed at a constant cosolvent volume fraction of 5% (0.05 m³ m⁻³) to ensure the selection of the best cosolvent at supercritical conditions. The existence of a single homogeneous phase for all the mixtures at the explored conditions was corroborated by visual inspection in a high-pressure variable volume view cell (see Section 2.3). The reference solubility parameters values for CO₂ and the cosolvents at 298.15 K and 0.1 MPa were obtained from the Hansen Handbook (Hansen, 2007); while the molar volume as function of the temperature and pressure studied (20 MPa and 305.15 K) were calculated by using the Reference Fluid Properties (REFPROP) model from the National Institute of Standards and Technology (NIST), with help of the Aspen Plus V 10.0 software. Finally, for the mixtures consisting of sc-CO₂ and a volume fraction of 5% cosolvent, the solubility parameters were determined with a linear blend rule. The total HSP value of the mixture was equal to the sum of the product of the respective volume fractions of the components present in the mixture, and their corresponding HSP value.

2.2.3 Prediction of miscibility enhancement

The miscibility enhancement was defined by Eq. (2) as the percentage reduction of the R_a values of the mixture sc-CO₂ – cosolvent in relation to pure sc-CO₂ as a function of the cosolvent volume fraction at different pressures (Tirado *et al.*, 2018).

$$\text{Miscibility enhancement (\%)} = \left[1 - \left(\frac{R_{a \text{ sc-CO}_2 + \text{cosolvent}}}{R_{a \text{ pure sc-CO}_2}} \right) \right] \times 100 \% \quad (2)$$

2.3 Apparatus and experimental procedure of the assays performed at supercritical conditions

SFE of fatty acid rich-oil from *T. suecica* was carried out at 20 MPa and 305.15 K using sc-CO₂ and its mixtures with a mass fraction of 5 % (0.05 kg kg⁻¹) cosolvent. The procedures to fill up the raw material, start-up and stop the equipment could be consulted in previous work (Tirado and Calvo, 2019). Additionally, the predictions were validated for α -linolenic acid with equilibrium data obtained in a high-pressure variable volume view cell, following the static synthetic method. Bubble pressures at 305.15 K using a molar fraction of 1.5 10⁻³ α -linolenic acid in sc-CO₂ and the supercritical mixture formed by sc-CO₂ + 5 % mass fraction of cosolvent were measured. The bubble pressures of oleic and linoleic acids were already reported (Tirado *et al.*, 2018). For both validation methods, mass fraction and not volume fraction was used due to practical limitations. e.g., during SFE, the flow rate in the experimental set up was measured in mass. However, considering the low amount of cosolvent, the variation with respect to the volume fraction was insignificant (Tirado and Calvo, 2019).

2.4 Fatty acids analysis

First, the supercritical extracts were derivatized following the procedure described by the AOAC Official Method 969.33 (AOAC, 1995). Subsequently, the samples were analysed by gas chromatography (GC) with a Shimadzu 2010 Plus gas chromatograph (Shimadzu Corporation, Japan) equipped with a Flame Ionization Detector (FID) and a Zebron ZB-1HT capillary column (20 m × 0.18 mm i.d. × 0.18 μ m film thickness). The separation was carried out with helium (1.8 10⁻⁶ m³ min⁻¹) as a carrier gas. The column temperature was programmed starting at a constant value of 393.15 K during 3 min, heated to 458.15 K at 3 K min⁻¹, held at 458.15 K during 3 min, heated again to 523.15 K at 15 K min⁻¹ and finally held at 523.15 K for 5 min. The equilibration time was 5 min. A split injector (50:1) at 523.15 K was used. The FID was also heated at 553.15 K. The injection volume was 1 10⁻⁹ m³. The fatty acid methyl esters (FAMES) were identified by comparison of their retention times with those of chromatographic standards. The compounds were quantified related to the area of the internal standard.

2.5 Statistical analysis

The experiments were repeated at least three times. Analyses were performed in duplicate for each replicate (n = 3 × 2). Means and standard deviations were calculated for all data. The experimental error was deduced from selected tests that were repeated six times. The maximum standard deviation for the fatty acid concentration was 0.5 % mass fraction of the fatty acid in the extract and the standard deviations in the bubble pressures were on average 0.1 MPa.

3. Results and Discussion

First, the miscibility of the fatty acids with sc-CO₂ and its mixtures with a volume fraction of 5 % cosolvent was estimated using the HST. Five organic solvents approved for the manufacture of food products (European Parliament and Council of the European Union, 2009) were used for the predictions: acetone, diethyl ether, ethanol, n-hexane and methanol. The miscibility predictions were compared with the concentration of the fatty acids in the oil extracted by SFE from *T. suecica* by using pure sc-CO₂, sc-CO₂ + ethanol and sc-CO₂ + n-hexane. Also, equilibrium data (bubble pressures) of α -linolenic acid in the supercritical mixtures was compared. To match the conditions used in the experimental validation, 20 MPa and 305.15 K were selected for the HST estimations.

3.1. Hansen solubility parameters of fatty acids

Table 1 shows the HSP for oleic, linoleic and α -linolenic acids at 298.15 K and 0.1 MPa, and at the selected conditions for the supercritical extraction. In the three fatty acids, the dispersion force (δ_d) had the highest contribution, due to the presence of a long straight-chain hydrocarbon in their structures. Furthermore, these fatty acids contain unsaturations (olefin groups) and one carboxylic group, which gives polar (δ_p) and H-bonding contributions (δ_h), respectively. However, the carboxylic group has a bigger impact than the olefin

group, and for that reason, the H-bonding forces are higher. On the other hand, the HSP of the three fatty acids varied only slightly within the explored temperature interval, as it was before described (Tirado *et al.*, 2018).

Table 1: Hansen solubility parameters of the fatty acids studied in this work at room and supercritical conditions.

Fatty acid	298.15 K and 0.1 MPa			305.15 K and 20 MPa		
	δ_d MPa ^{1/2}	δ_p MPa ^{1/2}	δ_h MPa ^{1/2}	δ_d MPa ^{1/2}	δ_p MPa ^{1/2}	δ_h MPa ^{1/2}
Oleic acid	17.40	2.67	6.43	17.35	2.66	6.42
Linoleic acid	18.29	2.94	7.24	18.24	2.93	7.22
α -Linolenic acid	18.03	3.03	7.61	17.99	3.02	7.59

3.2 Hansen solubility parameters of the supercritical solvent mixtures

Table 2 shows the HSP of sc-CO₂ and its mixtures with a volume fraction of 5 % cosolvent. The addition of cosolvents to sc-CO₂ mostly increased δ_d . The highest value of δ_d happened in the sc-CO₂-ethanol mixture.

Table 2: Hansen solubility parameters of sc-CO₂ and its supercritical mixtures with several cosolvents (5 % volume fraction) at 305.15 K and 20 MPa.

Supercritical fluid	δ_d MPa ^{1/2}	δ_p MPa ^{1/2}	δ_h MPa ^{1/2}
sc-CO ₂	11.30	5.52	4.97
sc-CO ₂ + acetone	11.53	5.77	5.07
sc-CO ₂ + ethanol	11.54	5.69	5.69
sc-CO ₂ + diethyl ether	11.49	5.40	4.97
sc-CO ₂ + n-hexane	11.51	5.25	4.72
sc-CO ₂ + methanol	11.51	5.87	5.83

3.3 Prediction of the best cosolvent

Table 3 shows the R_a values between all the studied fatty acids and the supercritical mixture at 305.15 K and 20 MPa. The cosolvent order depended on the fatty acid. For oleic acid, the most adequate cosolvents by increasing miscibility (minor R_a), at the tested operating conditions, were ethanol and n-hexane. In the case of linoleic and α -linolenic acids, the best cosolvents were both alcohols. These differences in the cosolvent order could be explained by the different affinities of the fatty acids (due mainly to their structural differences) with the supercritical mixture since the cosolvent effect is obtained when there are specific intermolecular interactions between the pair cosolvent – solute (Walsh *et al.*, 1987). In any case, the best cosolvent power for all the studied fatty acids was given by ethanol, as the supercritical mixture containing this alcohol provoked the highest reduction in R_a in relation to pure sc-CO₂.

Table 3: R_a values between fatty acids and supercritical solvents at 305.15 K, 20 MPa and a cosolvent volume fraction of 5 %.

Supercritical fluid	R_a between the fatty acid		
	Oleic acid	Linoleic acid	α -Linolenic acid
sc-CO ₂	12.514	13.845	14.286
sc-CO ₂ + acetone	12.127	13.438	13.881
sc-CO ₂ + ethanol	12.031	13.297	13.759
sc-CO ₂ + diethyl ether	12.126	13.463	13.902
sc-CO ₂ + n-hexane	12.093	13.456	13.887
sc-CO ₂ + methanol	12.132	13.376	13.843

3.4. Validation of the predictions

Validations were performed using ethanol (as better) and n-hexane (as worse) cosolvent. The R_a values provided by these cosolvents differed sufficiently enough to find experimental variations on the equilibrium pressures and on the fatty acid content in the supercritical extracts. Table 4 shows the fatty acid content of the extracts obtained with pure sc-CO₂ and with its mixture with an ethanol and n-hexane mass fractions of 5 % at 305.15 K and 20 MPa. The use of both cosolvents improved the selective extraction of fatty acids from the microalgae, with the largest increase occurring in the mixture made up of CO₂ + ethanol, which agreed with the predictions based on the R_a values. The obtained extract with pure sc-CO₂ contained 14.9 %, 3.7 % and 15.7 % of oleic, linoleic and α -linolenic acid, respectively; while the obtained extract with the sc-CO₂-ethanol mixture contained roughly 53 %, 54 % and 61 % more.

Table 4: Fatty acids content in the oil extracts obtained from *Tetraselmis suecica* at 305.15 K, 20 MPa with a cosolvent mass fraction of 5 % in sc-CO₂. Bubble pressures for α -linolenic acid at 305.15 K.

Supercritical fluid	Mass fraction of the fatty acid in the extract (%)			Bubble pressure (MPa) for α -linolenic acid
	Oleic acid	Linoleic acid	α -Linolenic acid	
sc-CO ₂	14.9	3.7	15.7	13.0
sc-CO ₂ + n-hexane	15.7	3.9	16.1	12.8
sc-CO ₂ + ethanol	22.7	5.7	25.3	12.1

Additionally, Table 4 shows the bubble pressures obtained by the static synthetic method at 305.15 K for a molar fraction of $1.5 \cdot 10^{-3}$ α -linolenic acid in sc-CO₂ and sc-CO₂ + 5 % mass fraction of ethanol or n-hexane. Lower bubble pressure values indicated higher miscibility. Therefore, the best cosolvent for the solubilization of α -linolenic in sc-CO₂ was ethanol followed by n-hexane, which was consistent with the predictions made using the HST. Similar results were obtained for oleic and linoleic acids. For example, the bubble pressure of a 0.003 molar fraction of linoleic acid at 313.2 K was 9.6 MPa in the mixture CO₂-ethanol, 15.5 MPa in the mixture CO₂-hexane and 22.2 MPa in pure sc-CO₂ (Tirado *et al.*, 2018).

3.5. The impact of the amount of cosolvent on the miscibility enhancement

Figure 1 shows the miscibility enhancement predicted by the Hansen theory of α -linolenic with raising ethanol concentrations at increasing pressures. The percentage reduction of the R_a values of the mixture sc-CO₂ + ethanol in relation to pure sc-CO₂ was plotted as a function of increasing volume fraction of ethanol. In Figure 1, a non-linear relationship between the miscibility enhancement and the cosolvent concentration was observed, mainly at the highest pressures. Thus, at ethanol concentrations in the supercritical solvent mixture higher than 25 %, the miscibility enhancement curves were not linear. Above 50 MPa, the lines even curved down. Similar behaviour was previously predicted for oleic acid in the same supercritical solvent mixture; however, it happened at a lower ethanol concentration of 10 % (Tirado *et al.*, 2018). It was also experimentally detected for different systems (Brunner, 1994; Ekart *et al.*, 1993; Güçlü-Üstündağ and Temelli, 2005).

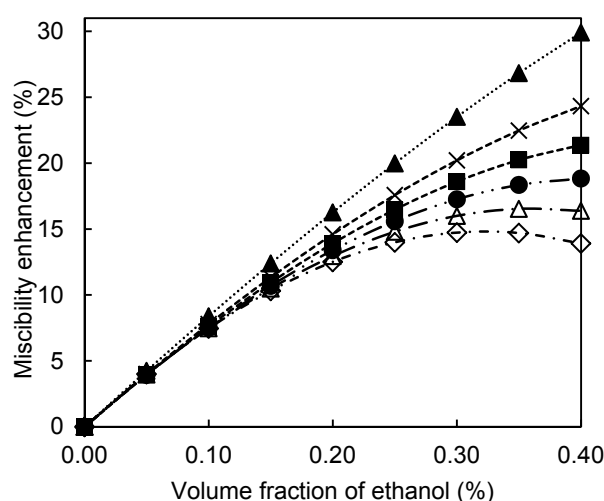


Figure 1: Predicted miscibility enhancement of α -linolenic acid in sc-CO₂ + ethanol at 313.15 K and 10 MPa (\blacktriangle), 20 MPa (\times), 30 MPa (\blacksquare), 40 MPa (\bullet), 50 MPa (\triangle) and 60 MPa (\diamond) by the Hansen theory.

Theoretically, this behaviour was explained due to the reduction of the molar volume of the solvent mixture with the increase of pressure and the cosolvent fraction. Besides, Güçlü-Üstündağ and Temelli (2005) related this conduct to the self-association of the cosolvent. Although these cannot be the only explanations, given that for the same cosolvent, the reduction in the miscibility enhancement does not always occur at the same pressure and concentration. It must also be related to the target solute. Based on the predictions we have made with Hansen's theory, the lower the miscibility of the compound of interest in sc-CO₂ alone, the higher the concentration of cosolvent and the pressure needed to observe the bending phenomenon of the miscibility enhancement curves.

4. Conclusions

In this study, the Hansen solubility theory was validated to choose the best supercritical solvent mixture to reach the selective extraction of oleic, linoleic and α -linolenic acids. According to this theory, the best cosolvent for supercritical carbon dioxide (sc-CO₂) in the extraction of the three fatty acids from *Tetraselmis suecica* was ethanol, as it was experimentally corroborated. Consequently, the Hansen solubility parameters could be a suitable tool to at least reduce the number of experiments for the selection of the best cosolvent for sc-CO₂ in the extraction of a target compound from a natural complex matrix.

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