Effect of different operating conditions in cloud point assisted extraction of thymol from Ajwain (*Trachyspermum Ammi* L.) seeds and recovery using solvent

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Abstract Cloud point assisted extraction of thymol from water extract of Ajwain (*Trachyspermum Ammi* L.) seeds has been reported. Effects of different operating conditions, i.e., concentration of surfactant, heating time and temperature in extraction efficiency were investigated. It was observed that maximum extraction efficiency of thymol was achieved with 30% (v/v) of SPAN 80 surfactant, 45 min of heating at 65 °C. Recovery of thymol from the surfactant complex was optimal at 1:3 coacervate phase to solvent (acetone) volume ratio. A semi-empirical correlation was proposed at the optimum time to predict the concentration of surfactant and temperature required for a desired yield.

Keywords Ajwain · Thymol · Cloud point extraction · SPAN 80 · Acetone

List of symbols

B	Constant (dimensionless)
C	Constant (dimensionless)
C_d	Concentration of thymol in dilute phase (mg/L)
C_f	Concentration of thymol in feed (mg/L)
C_s	Concentration of thymol in surfactant phase (mg/L)
D	Constant (dimensionless)
E	Extraction efficiency (%)
E_0	Constant (dimensionless)

F	Constant (dimensionless)
F_1	Constant (dimensionless)
F_2	Constant (dimensionless)
F_c	Coacervate phase volume ratio (dimensionless)
G	Constant (dimensionless)
K_p	Partition coefficient (dimensionless)
T	Operating temperature (dimensionless)
S_0	Surfactant concentration (dimensionless)
V_d	Volume of dilute phase (mL)
V_T	Volume of feed (mL)
V_s	Volume of surfactant (mL)
Z_0	Constant (dimensionless)

Introduction

Identification and prevention of complex diseases in human body are active areas of research. Most of the abnormalities in metabolic systems are caused by pathogen-carried infections. For example, bacteria residing in the oral cavity, like, *Actinomyces*, *Lactobacillus*, *Peptococcus* and *Treponema* adhere in biofilms on the tooth surface and gingival epithelium of the tongue, resulting in dental carries and periodontal diseases (Aas et al. 2008; Wade 2013). Such oral diseases can be prevented through restricting bacterial growth, which is performed by rinsing with mouth wash, containing anti-bacterial monoterpenes (a class of organic compounds having two isoprene units) (Ncube et al. 2008). Examples of monoterpenes include thymol, eucalyptol, menthol and borneol (Oz et al. 2015). Among these, thymol is present naturally in the seeds of *Trachyspermum ammi* L., commonly known as Ajwain (Gujar et al. 2010). Besides, restricting the growth of bacterial film on the mouth epithelium, it also adds to

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generation of adenosine tri phosphate for increased metabolic rate (Gujar et al. 2010).

Such monoterpenes are extracted by solid–liquid, liquid–liquid extraction and membrane separation techniques (Azmir et al. 2013; Roldán-Gutiérrez et al. 2008; Dupuy et al. 2011). Previously, thymol has been extracted from Ajwain seeds by solid phase and microwave assisted extraction (Gujar et al. 2010). However, liquid–liquid extraction shows higher efficiency, but it involves the use of expensive, inflammable and toxic organic solvents. Also, complete removal of these solvents from extract phase cannot be achieved (Sarafraz-Yazdi and Amiri 2010). To circumvent the limitations of organic solvents, cloud point extraction (CPE) can be a viable alternative to separate thymol from aqueous extract of Ajwain seeds (Demirhan and Tuzen 2010). In this technique, non-ionic surfactant is added to the feed and it is heated beyond cloud point temperature. The solution becomes cloudy and separates out in two phases, i.e., aqueous phase and surfactant rich coacervate phase. Distribution of solute is more in surfactant rich phase due to solubilisation in the micelles, thereby leading to its extraction. Extraction efficiency can be affected by temperature of heating, time of exposure, electrolyte concentration and pH of the medium (Shunping et al. 2010). Phase separation in cloud point extraction occurs due to dehydration on outer micelle surface or increase in surfactant aggregation number and size, at higher temperature (Mukherjee et al. 2011; Heidarizadi and Tabaraki 2016; Mittal and Fendler 2012). Advantages of CPE include less energy consumption, high extraction efficiency and inexpensive nontoxic solvent, like water (Zain and Nadhirah 2015). CPE techniques have been used in separation of various dyes, like rhodamine 6G (Duran et al. 2011), malachite green (Pourreza and Elhami 2010) and other persistent organic pollutants (Xie et al. 2010). Recently, studies have also been conducted to explore the applicability of CPE in pre-concentration of tracer in feed for accuracy in measurements (Ojeda and Rojas 2012), e.g., microwave assisted CPE for determination of drugs and bioactive compounds (Madej 2009), determination of triazine herbicides in milk (Liu et al. 2014) and phenolic antioxidants in perfumes (Li et al. 2014).

This work involves extraction of thymol from water extract of Ajwain seeds by non ionic surfactant assisted cloud point extraction followed by subsequent recovery of thymol from the surfactant complex by the use of solvents. Effects of different non-ionic surfactants, their concentration, temperature and time on the extraction efficiency were studied. Following this, effect of different organic solvents and their concentration on the recovery efficiency was investigated. Detailed route for CPE of thymol from water extract of Ajwain seeds and subsequent recovery from the surfactant by solvent is shown in Fig. 1. A semi

empirical correlation was proposed to quantify the efficiency of CPE.

Experimental procedures

Material

Thymol (Isopropyl-m-cresol) crystals (99% extra pure), non ionic surfactants, like Triton X-100 (4-Octyl phenol polyethoxylate), SPAN 80 (Sorbitan monooleate) and TWEEN 80 (Polyoxyethylene (20) sorbitan monooleate) were supplied by M/s, Loba Chemie Pvt. Ltd., Mumbai, India. The cloud point temperature of Triton X-100, SPAN 80 and TWEEN 80 is 65, 45 and 90 °C, respectively. Details of thymol and surfactants used in this study are presented in Tables S1 and S2, respectively, in the supplementary section. Organic solvents used (without further purification) for dissolution of surfactant–solute complex were acetone, ethanol and acetonitrile. These reagents were of analytical grades and purchased from M/s, Merck (India) Ltd., Mumbai, India. Ajwain seeds were purchased locally.

Methods

Water extraction of Ajwain seeds

60 g of Ajwain seeds was washed and put in a beaker, containing 180 mL of distilled water. The beaker was placed in hot water bath (supplied by REMI laboratory instruments, Pvt. Ltd., Mumbai India) at 52 °C for 16 min, to obtain the maximum thymol concentration in aqueous extract. The extract was stored in a container and used for future experiments.

Cloud point extraction experiments of water extract

A known volume of the Ajwain extract was taken in a measuring cylinder (capacity 50 mL) followed by addition of non ionic surfactant solution and distilled water. After heating the constituents in a water bath above the cloud point temperature (mentioned in Sect. 2.1) for 1 hour, it was allowed to cool. The cloudy suspension separated into two phases, a dilute aqueous phase and a surfactant rich coacervate phase. Samples from aqueous phase were obtained by the help of long needle syringe and its thymol concentration was measured. The surfactant was selected among Triton X-100, SPAN 80 and TWEEN 80, based on maximum extraction efficiency. Effect of different operating conditions, like, surfactant concentration (10–90% by volume), temperature (45–95 °C) and time (5–120 min) of heating was studied.

Fig. 1 Steps for extraction of thymol from Ajwain water by non-ionic surfactant using cloud point extraction and subsequent recovery of thymol from coacervate phase by solvents

Dissolution of surfactant–thymol complex in solvents

Known amount of surfactant rich phase was taken in a measuring cylinder and spiked with different solvents (ethanol, acetone and acetonitrile) and stirred gently. Concentration of thymol in the surfactant dissolved solvent was measured using HPLC and reported. Solvent was selected based on the maximum concentration of thymol recovered from the surfactant phase. Following this, variation in thymol concentration was observed with increasing ratio of surfactant to solvent volume. Detailed experimental procedure has been shown in Fig. 1.

Analytical measurements using HPLC

Sample was injected to an Agilent Zorbax C-18 reverse phase HPLC column (4.6 mm I.D; 250 mm length and 5 μ m particle size), supplied by M/s, Perkin Elmer Co., Shelton, Connecticut, USA. Acetonitrile and water mixture (65:35, v/v) were used as mobile phase at flow rate of 0.5 mL/min. Injection volume was 30 μ L and the wavelength was set at 280 nm for thymol (Gujar et al. 2010). Experimental procedure for obtaining thymol concentration from chromatograms was as follows: initially a series of standard solution of thymol (10–1000 mg/L) was prepared in acetonitrile and filtered through 0.2 μ m syringe filter (whatman), before injecting to the column. Area of the chromatogram peaks was noted for known standards to obtain the calibration curve. Concentration of thymol in the feed and aqueous phase was measured by interpolating peak area of unknown sample with that of the calibration curve (see Figure S1). Fourier Transform Infrared Spectroscopy (FTIR) was performed in a spectrophotometer, supplied by M/s, Perkin Elmer, CT; model: Spectrum 100.

Results and discussion

Characteristics of Ajwain seeds and thymol

FTIR spectra of Ajwain seeds and pure thymol are presented in Fig. 2a. Elementary observation shows that the molecules are aromatic as the recorded transmittance peaks were above 3000 cm^{-1} (Alpert et al. 2012). The broad stretch of transmittance peaks from 3250 to 3500 cm^{-1} refer to the presence of hydroxy group (H bonded OH stretch). Stretch from 2900 to 2850 cm^{-1} corresponds to symmetric methyl group in the compounds (Alpert et al. 2012). Presence of dimethyl elements can also be indicated from the presence of peaks at 1370 cm^{-1} (structure of thymol is given in Table S1). Peaks at 1750 , 1640 and 1460 cm^{-1} refer to alcohol group in the chain (Sanchez-Garcia et al. 2008). Phenolic components are confirmed by the peaks at 720 , 1030 and 1230 cm^{-1} (Sanchez-Garcia et al. 2008). Ring vibration of thymol is typically recorded at 720 cm^{-1} . It can be observed that peaks recorded in case of pure thymol at 3250 , 1230 , 1030 and 720 cm^{-1} are in common with the one recorded for Ajwain seeds. These peaks are intense and represent different forms of aromatic ring substitution. Similarity in recorded vibration at these wavelengths confirms the existence of purest form of thymol in Ajwain seeds. However, there are some differences between the spectra of seeds and pure thymol. For example, additional stretching frequencies are observed at peaks around 1443 , 630 and 570 cm^{-1} , in case of pure thymol. These peaks have very low transmittance and are not present in FTIR spectra of Ajwain seeds. Similarly, the pattern of stretching between the wavelength 3250 and 1230 cm^{-1} (for Ajwain seeds) does not match with pure thymol. This might be due to the presence of additional non thymol

Fig. 2 **a** FTIR study of thymol and Ajwain seeds. **b** Sample chromatogram of pure thymol and Ajwain

components, like, paracymene, gamma-terpinene, alpha-pinene, betapinene, α -terpinene, styrene, delta-3-carene, betaphyllanderene, terpinene-4-ol and carvacrol (Zarshenas et al. 2014). Typical chromatograms of pure thymol and Ajwain water extract are shown in Fig. 2b. Characteristics retention time of pure thymol (under the experimental conditions described in Sect. 2.3) as observed from its chromatogram is 12.4 min. It can be observed that characteristics retention time of thymol also appears in the chromatogram of Ajwain seeds. However, the broad stretch from 2.5 to 6 min in the chromatogram of Ajwain seeds may represent any impurities, formed during extraction of bioactive components from plant products (El-Semary 2012).

Effect of operating conditions

Effect of different operating conditions, e.g., surfactant concentration, temperature and time of heating on the extraction efficiency was observed. Concentration of

thymol (calibration curve for thymol measurement is provided in Fig. S1, in the supplementary section) in the surfactant rich coacervate phase was calculated by simple mass balance,

$$C_f V_T = C_d V_d + C_s V_s \quad (1)$$

where, C_f , C_d and C_s are the concentration of thymol in the feed, dilute aqueous phase and coacervate phase, respectively; V_T , V_d and V_s are the total volume (feed volume before CPE), aqueous phase and coacervate phase, respectively after CPE. The extraction efficiency (E) is calculated as,

$$E = \left(1 - \frac{C_d}{C_f}\right) \times 100\% \quad (2)$$

Coacervate phase volume ratio (F_c) is defined as the ratio of surfactant volume to that of total volume (V_T). It can be estimated as,

$$F_c = \frac{V_s}{V_T} \quad (3)$$

Partition coefficient (K_p) can be calculated as the ratio of concentration of thymol in coacervate phase to that in dilute phase. High partition coefficient indicates higher concentration of thymol in surfactant rich phase than in dilute phase. It can be denoted as,

$$K_p = \frac{C_s}{C_d} \quad (4)$$

Effect of different surfactants (for information on properties of the surfactants, see Table S2) on the extraction efficiency of thymol is presented in Fig. 3a. Feed concentration was 45 mg/L. The extraction efficiency is observed to be 25% for TWEEN 80 ($C_d = 33.75$ mg/L), whereas it is 90% for Triton X-100 ($C_d = 4.5$ mg/L) and 96% for SPAN 80 ($C_d = 1.8$ mg/L). Dehydration of hydroxyl group and dissociation of hydrogen ion in the feed solution occur at high temperature (Demirhan and Tuzen 2010). The pairing up of this free hydrogen with the hydroxyl group in the thymol chain results in the formation of thymol-surfactant complex. This is only possible for the non ionic surfactants which are mostly alkyl derivatives of organic acids (Walcarius and Mercier 2010). However, in case of TWEEN 80, the cloud point temperature is 90 °C (Chawla and Mahajan 2011) and most of the food products start denaturing at this temperature (Campus 2010). Due to this, extraction efficiency of thymol from water extract is low for this surfactant. Hence, SPAN 80 surfactant is selected for further experiments as its extraction efficiency is the highest and the cloud point is lower than other two surfactants. The coacervate phase volume ratio and partition coefficient for SPAN 80 assisted separation are 0.25 and 97, respectively (see Table 1).

Fig. 3 Effect of different parameters on extraction efficiency, i.e., **a** different non ionic surfactants; **b** surfactant concentration; **c** time; **d** temperature

Concentration of surfactants also plays an important role in the extraction of thymol from Ajwain water extract. Variation of extraction efficiency with surfactant concentration is shown in Fig. 3b. The extraction efficiency was 82.2% ($C_d = 8$ mg/L) for a surfactant concentration of 10% (v/v). Extraction efficiency increased with addition of surfactants due to enhanced solubilisation of thymol in the micelles (Mukherjee et al. 2011; Heidarizadi and Tabaraki 2016; Mittal and Fendler 2012). Maximum efficiency of 96% ($C_d = 1.7$ mg/L) was observed for 30% (v/v). The coacervate phase volume ratio and the distribution coefficient are 0.15 and 171, respectively, for this surfactant concentration.

Effect of time determines the amount of thermal energy supplied to the system to raise the temperature above the cloud point of the surfactant. The effect of time on the extraction efficiency is presented in Fig. 3c. Enhanced

heating time results in increased solubilisation of surfactant (Mukherjee et al. 2011; Heidarizadi and Tabaraki 2016; Mittal and Fendler 2012). The extraction efficiency increases to 92.5% ($C_d = 3.4$ mg/L) after 5 min, when the temperature of the heating bath is kept above the cloud point. Marginal increase is observed till 45 min, when the extraction efficiency is 96.7% ($C_d = 1.5$ mg/L) and beyond that, it remains almost invariant. This is due to solubility saturation of thymol in the surfactant micelles.

Effect of temperature also determines the extent of extraction. It is observed from Fig. 3d that extraction efficiency is 82.2% ($C_d = 8$ mg/L) when the temperature is 45 °C, whereas it is 96.4% ($C_d = 1.6$ mg/L) when the temperature is 75 °C. Maximum extraction efficiency of 99.5% ($C_d = 0.22$ mg/L) is observed at 65 °C. Non ionic surfactants are hydrophobic at higher temperature, thereby promoting dehydration leading to higher solubilisation of

Table 1 Calculation of concentration factor and partition coefficient for different operating conditions

Effect	Variation	Coacervate phase volume ratio (F_C)	Partition coefficient (K_P)
Surfactant type (time: 60 min; temperature: 75 °C; surfactant concentration: 50%)	Triton X-100	0.43	22.2
	TWEEN 80	0.45	1.7
	SPAN 80	0.25	97.0
Surfactant concentration (v/v %) (surfactant: SPAN 80; time: 60 min; temperature: 75 °C)	10	0.05	93.5
	20	0.10	108.2
	30	0.15	170.8
	40	0.20	114.4
	70	0.35	69.6
Time (min) (surfactant: SPAN 80; temperature: 75 °C; surfactant concentration: 30%)	5	0.20	83.2
	15	0.20	105.4
	30	0.20	127.7
	45	0.20	194.3
	90	0.20	184.8
	120	0.20	180.4
Temperature (°C) (surfactant: SPAN 80; time: 45 min; surfactant concentration: 30%)	45	0.20	31.8
	55	0.20	44.3
	65	0.20	1358.0
	85	0.20	69.3
	95	0.20	54.3

Bold indicates the optimum operating conditions at which maximum extraction efficiency is obtained

thymol in the micelles, increasing extraction efficiency. However, extraction efficiency decreases to 89% ($C_d = 5$ mg/L) at 95 °C, due to denaturing of thymol at higher temperatures (Campus 2010).

Therefore, the optimum process conditions selected for maximum extraction efficiency are 30% surfactant concentration, 45 min of heating at 65 °C.

Empirical relation between dilute phase concentration and operating parameters

A generalized relationship has been established between the dilute phase concentration and operating parameters (i.e., surfactant concentration and temperature of extraction). For this, concentration of dilute phase was noted for different sets of temperature and surfactant concentration (10, 30, 40 and 70%). Concentration of dilute phase was measured, whereas concentration of coacervate phase was calculated (according to Eq. 1) after each experiment. Following this, partition coefficient (calculated according to Eq. 4) was correlated with dimensionless surfactant concentration (S_0) and temperature (T) by the following equation,

$$K_P = Z_0 + B \exp(F_1) + E_0 \exp(F_2) + H \exp(F_1 + F_2) \quad (5)$$

where, F_1 and F_2 are represented as,

$$F_1 = \frac{-(\ln(\frac{S_0}{C}))^2}{2D^2} \quad (6)$$

$$F_2 = \frac{-(\ln(\frac{T}{F}))^2}{2G^2} \quad (7)$$

Z_0 , B , E_0 , H , C , F , D and G are constants and their values were determined by non linear regression method, i.e., by minimizing the error (S) involved as,

$$S = \sum_{i=1}^n \left(\frac{K_{p,\text{exp}}^i - K_{p,\text{Cal}}^i}{K_{p,\text{exp}}^i} \right)^2 \quad (8)$$

where, $K_{p,\text{exp}}^i$ and $K_{p,\text{Cal}}^i$ are the experimental and calculated partition coefficients for i th experiments, n is the maximum number of experiments performed. The values of constants are $Z_0 = 31.4$; $B = 18.1$; $E_0 = 210.2$; $H = 2281.7$; $C = 0.3$; $F = 1.2$; $D = 0.4$ and $G = -0.008$. Dimensionless temperature is defined as,

$$T = \frac{\text{Operating Temperature} + 273}{273} \quad (9)$$

Equations (1), (3) and (4) can be rearranged to correlate concentration of thymol in the dilute phase with a given feed concentration (C_F), targeted coacervate phase volume ratio, temperature and partition coefficient by the following equation,

$$C_d = \frac{C_F}{(1 - F_C) \left[1 + \left\{ \left(\frac{F_C}{1 - F_C} \right) \times K_p \right\} \right]} \quad (10)$$

The calculated and experimental values of dilute phase concentration are plotted with surfactant concentration and temperature in Fig. 4. The experimental values are in close agreement with the calculated one from Eq. (10). It is observed from Fig. 4 that the dilute phase concentration of thymol initially decreases with the temperature in the range from 45 to 65 °C, for all surfactant concentrations. For example, the calculated concentration is 17.7 mg/L at 45 °C and decreases to 4.83 mg/L at 65 °C at a surfactant concentration of 10%. However, it starts increasing as the temperature is further increased, beyond 65 °C probably due to denaturing of thymol, as mentioned earlier. Thus, 65 °C was selected as the optimum temperature. Decrease in dilute phase concentration at 30% surfactant concentration is minimal. It is noted that extraction efficiency is not affected at higher surfactant concentration, due to saturation in solubilisation of micelles. Therefore, one can effectively calculate the dilute phase concentration with the input of feed concentration, coacervate phase volume ratio and partition coefficient, by Eq. (10).

Fig. 4 Variation of concentration of thymol in dilute phase with surfactant concentration calculated from the empirical correlation and experimental value with temperature

Effect of solvent on recovery of thymol from coacervate phase

Recovery of concentrated thymol from coacervate has been conducted by treating surfactant–thymol complex with different solvents (see Fig. 5). It has been observed from Fig. 5a that type of solvent plays an important role in thymol recovery from surfactant phase. Concentration of thymol in coacervate phase (calculated by Eq. 1) is 298 mg/L (for feed concentration of 45 mg/L). However, the concentration of recovered thymol from the surfactant phase is measured at 212 mg/L, when the coacervate phase is dissolved in acetone. Thymol concentration is 170 and 165 mg/L when it is dissolved in acetonitrile and ethanol, respectively. Carbonyl group of acetone determines the extent of solubilisation of surfactant in it, thereby leading to increased recovery (Mittal and Fendler 2012). Acetone is

Fig. 5 Effect of **a** different organic solvents and **b** concentration of solvent for recovery of thymol from surfactant rich phase

selected for further experiments, based on the highest recovery of thymol.

Concentration of thymol in the solution after solvent extraction is also affected by solvent to surfactant volume ratio, as shown in Fig. 5b. The viscosity of 1:2 (surfactant to solvent volume ratio) is too high for injecting to HPLC column. Thereby, 1:3 (surfactant to solvent volume ratio) is selected as starting point for these experiments. Concentration of thymol decreases successively as the ratio of surfactant to solvent ratio is increased. For example, the concentration of thymol after solvent treatment of surfactant (3 mL solvent to 1 mL surfactant) is 250 mg/L, whereas it decreases to 70 mg/L, as the volume fraction is altered (1 mL of surfactant dissolved in 30 mL of solvent). Following this, methods like distillation or any other fractionation techniques can be employed to separate pure thymol from the selected solvent, i.e., acetone.

Conclusion

In this study, thymol concentration was increased from its feed in water extract of Ajwain (*Trachyspermum ammi* L.) seeds by using cloud point extraction. The highest partition coefficient and coacervate phase volume ratio was observed for SPAN 80 surfactant, in comparison to other surfactants used in this study. Maximum extraction efficiency was observed at 30% (v/v) of surfactant, 45 min of heating at 65 °C. Following cloud point experiments, surfactant–thymol complex was dissolved in three solvents, i.e., acetone, acetonitrile and ethanol. Maximum recovered thymol concentration was found for 1:3 coacervate phase to acetone volume ratio. A correlation was obtained between surfactant concentration, temperature and partition coefficient to predict the concentration of thymol in the dilute phase for known input parameters, i.e., feed concentration, coacervate phase volume ratio and partition coefficient.

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