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Original Research Article

A green method for separation of betaine from beet molasses based on cloud point extraction methodology using polyethylene glycol as a food grade surfactant

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ABSTRACT

In this study, an experimentally efficient and green-scalable procedure was designed using a sustainable surfactant to extract betaine from the beet waste (molasses) of sugar industry. This procedure was extended based upon the cloud point extraction (CPE) technique. An optimization framework was developed using the response surface methodology (RSM) to achieve an optimal value for the factors affecting the extraction efficiency of the experimental procedure. The main operational factors were surfactant concentrations, electrolyte concentration, pH, and the incubation temperature. Under the measured and optimal conditions, an extraction efficiency of as high as 88% was obtained for the betaine recovery. The polyethylene glycol (PEG) was used as a food grade surfactant that is a renewable substance approved by the U.S. food and drug administration (FDA) used in the food and drink industry. In the last step, the extracted betaine was freeze-dried at -56 °C for 16 h under 0.5 bar ambient pressure. The results revealed that, the final betaine powder product can be directly used as a supplement in livestock feed supply since PEG is an edible surfactant. Thus, the proposed experimental procedure for betaine extraction from molasses is regarded as a scalable, cost-effective, sustainable and eco-friendly approach.

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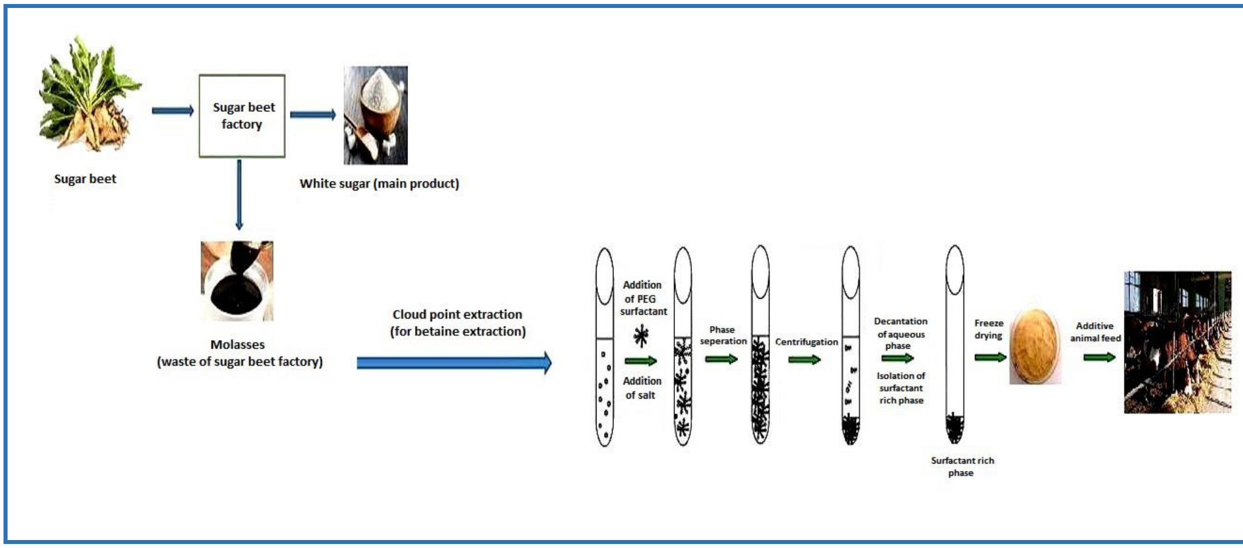
Graphical Abstract



Introduction

The food and drink industry have experienced many decades on utilizing different types of surfactants. Within the most popular food products such as mayonnaise, sour creams, dressings, deserts, and the natural surfactant, lecithin found in egg yolks and various proteins of milk plays an important role. Some polar liquids such as monoglycerides could also be used as emulsifier in the food preparing processes. Nowadays, synthetic surfactants such as sorbitan esters, ethoxylates, and sucrose esters are also commonly used in food manufacturing industries [1, 2]. Recently, parallel to the emulsifying role, aqueous solutions of surfactant are widely utilized in the field of green separation techniques (cloud point extraction, CPE) due to the advances in membrane technologies, liquid-liquid extraction, and solid-liquid extraction [3, 4].

Surfactants are amphipathic macromolecules (polymers) with a polar hydrophilic head and lipophilic tail, a linear or branched aromatic or non-aromatic hydrocarbon chain. Surfactants aggregate in aqueous solutions to form micelles under definite conditions. A homogeneous surfactant solution, at a defined temperature and concentration beyond the critical micellar concentration (CMC), becomes cloudy and separates into a dilute aqueous phase and a surfactant rich phase [5, 6]. Heat weakens the hydrogen bonds between a water molecule and the hydrophilic head of the surfactant that results in solubility reduction of the surfactant in aqueous medium, and consequently, the clouding phenomenon occurs (micelle formation). If the solution is allowed to settle at a temperature beyond its cloud point, phase separation occurs in which most of the surfactant remains in the smaller phase called surfactant phase. Every analyte solubilized in the hydrophobic core of the micelles will be separated from the solution [7, 8].

The cloud point extraction (CPE) technique can be stated as environmental friendliness due to its non-solvent, nonflammable, nonvolatile, less toxic surfactant, gentle extraction conditions, simplicity, and low-cost equipment [9–11]. Since the volumetric ratio of micellar phase is 10 to 100 fold smaller than the volume of an aqueous phase, analyte is concentrated at much higher by this extraction technique compared to the other conventional techniques [12–16]. Considering these, the cloud point extraction is in agreement with the green chemistry principles [17–19].

The following items indicate the wide application of the CPE method in food industries. In the extraction of proteins and amino acids [20], casein from cow milk [21], antioxidant from olive mill wastewater [22], carotenoids from orange juice [23], polyphenols (antioxidant) from wine sludge [14], licopene from tomato pomace [24] and determination of food dyes in food samples [25]. The U.S. food and drug administration (FDA) has announced that the nonionic surfactants, mainly with branched aliphatic chains or aromatic tails are edible [26]. PEGs are known edible surfactants that are used in many biological, biochemical, and cosmetic applications. Precipitating of molecules, stabilizing, or aggregating particles or as carrier for drug delivery and edible films for food coating are also other applications of PEG. Due to some beneficial features including, low toxicity, water solubility, and ease of handling, the application of this polymer has been considerably increased. *Cavalcanti* et al. [27], used from PEG 8000 for separation of mycotoxin and *Cisneros* et al. [28], recovered lutein from a two-phase systems of carotene production *via* microalgae. Using these surfactants, the process and devices to separate analyte from the surfactant is not necessary; therefore, it bears an economic advantage compared to other surfactants since the cost of the separation process is ignored [5].

Betaine is an excellent source of methyl group that can reduce the risk of heart disease by converting the homocysteine to methionine in the human blood. Since human body does not synthesize methyl group, a permanent dietary stream of methyl group is indispensable [29]. In animals, betaine is converted to acetate in rumen which is used for milk fat synthesis. Therefore, milk production yield has increased using betaine supplementation in Holstein dairy cows [30]. Not onlybetaine boosts the digestibility of special nutrients, particularly fiber and minerals but also it helps in protein and energy metabolism process. Advantages of supplemental betaine on growth efficiency in pigs and poultry have also been discussed in many publications [31].

In this research study, the extraction and recovery of betaine from beet molasses (sugar factory final run off) was optimized through a cloud point extraction (CPE) procedure. The overall goal of this experimental study is to establish a framework to facilitate the CPE application in food sciences using food grade surfactants such as PEG in the extraction of betaine from beet molasses.

Experimental

Materials and methods

The molasses was supplied by a giant sugar factory, Hegmatan, located in Hamedan, a city in western part of Iran. They were kept in a refrigerator at 4 °C. Our analysis showed that their molasses contained the following substances by mass, sucrose, at 50%; ash at 14.3%; betaine 6% and water 20%. A solution of 25% (w/v) was acquired by dissolving 25 gr of the molasses in water diluted to 100 mL in a volumetric flask. 15% (w/v) of PEG 8000 was prepared by dissolving 15 g polyethylene glycol 8000 (Merck) in water diluted to 100 mL volumetric flask. For the pH adjustment of the samples, hydrochloric acid and sodium hydroxide solutions (0.1 mol/L^{-1}) were used. An electrolyte of 20% (w/v) sodium sulfate was prepared by dissolving 20 gr of sodium sulfate (Merck) in water diluted to 100 mL in a volumetric flask.

Instruments

pH was measured using a Metrohm Swiss made pH-meter. A heidolph unimax 2010 shaker was utilized for mixing the samples. Heating the solutions performed in a wisebath thermostatic water bath made by Korean daihan scientific company. A centrifuge with 10 mL calibrated centrifuge tubes (Hettich, Germany) wasused to accelerate the phase separation process. Measurement of betaine concentration in samples was carried using a (Uicam-Crystal-200) HPLC with diode-array spectrophotometer and (Phenomenex, Germany) ODS2 C18 column (250 mm× 4.6 mm internal diameter). The mobile phase was a mixture of 13 mM sodium heptane sulfonate and 5 mM Na_2SO_4 in distilled water that was set to pH 3.7 using diluted H_2SO_4 solution. The HPLC mobile phase was delivered by an isocratic pump at a flow rate of 0.8 mL/min^{-1}.

CPE procedure

1 mL of molasses solution [25% (w/v)] was transferred into a 10 mL centrifuge test tube. Following, based on experimental design and according to Table 1, a certain volume of 15% (w/v) of PEG 8000 is used to keep the final concentration in the range of 1 to 6% (w/v) and some certain volume of 20% (w/v) of sodium sulfate solution to form its final concentration in the range of 1 to 12% (w/v) were also added. The solution was diluted to approximately 8 mL with distilled water. In the next step, the pH of solution for each run was adjusted from 2 to 10 by sodium hydroxide or hydrochloric acid per experimental design Table 1. At this step, the final volume was diluted to the mark with distilled water. The solution was placed in a shaker at 150 rpm for 30 min. After this, the tube was heated and kept in a temperature-controlled water-bath for 30 min at 25-65 °C until the

clouding phenomenon were observed and equilibrated. After clouding of solution, separation of aqueous phase from surfactant rich phase was reached using a centrifuge device for 5 min at 3500 rpm. Following the separation, to increase the viscosity of surfactant rich phase, the tube was cooled in an ice bath. The stripped aqueous phase samples were taken and injected into the HPLC to measure the amount of residual betaine. This CPE procedure was repeated three times under the same conditions for the aqueous phase that hold some un-extracted betaine. The following equation was used to calculate the extracted betaine in surfactant rich phase:

$$\%ER = \frac{C^0 - C}{C^0} \times 100 \quad \text{(Eq. 1)}$$

Where %ER represents the efficiency of betaine recovery, C^0 refers to the initial concentration of betaine in sample and C stands for the concentration of betaine residual in aqueous phase. Finally, the collected surfactant rich phase containing betaine was freeze-dried at -56 °C during 16 hours at 0.5 bar ambient pressure to prepare the supplementary powder product.

Table 1. Experimental design matrix and responses

Runs	The factors Surfactant Concentration% (w/v) (A)	Electrolyte concentration% (w/v) (B)	pH (C)	Incubation temperature (°C) (D)	Response (%ER)
1	5	9.15	2	65	25
2	0.5	6	8.4	25	0
3	3.34	12	2	44.2	33
4	2.82	9.4	4.81	25.8	32
5	3.4	6	5.92	65	0
6	0.5	9.66	6.88	49.8	20
7	5	12	6.14	25	28
8	0.5	6	2	58	0
9	2.38	8.79	3.28	56	28
10	0.5	10.83	2	25	25
11	4.55	9.57	6.96	47.6	28
12	1.74	12	10	65	25
13	3.34	12	2	44.2	30
14	3.36	8.85	10	25	33
15	3.36	8.85	10	25	34

16	5	6	6.88	25	0
17	0.5	12	5.92	65	24
18	0.5	6	6.96	65	0
19	0.5	9.66	10	49.8	25
20	3.4	6	10	65	0
21	4.55	9.57	10	47.6	30
22	5	6	5.76	45.2	0
23	5	12	10	65	27
24	0.5	12	4	25	24
25	3.34	6	4	38.2	0

Experimental procedure

The response surface methodology (RSM) is an effective statistical technique for the optimization of experimental and theoretical studies with many variables. In this method, a minimum quantity of runs is used to determine the optimum values for the variables to maximum an objective function (dependent variable). The factor interactions are also considered in response surface optimization. Therefore, it offers several advantages over the conventional single parameter testing, in which the effect of only one factor at a time (OFAT) on the objective function is performed [32].

In this study, the response surface method (RSM) tool in a design expert software was conducted specify the optimal processing condition to obtain a maximum betaine recovery in this experiment. To solve for the dependent variable, betaine recovery (%ER), four independent variables (also called operational factors) were examined as the following: surfactant concentration (A), electrolyte concentration (B), pH (C) and incubation temperature (D). The independent variables were assigned a range that span as for surfactant concentration (A) between a 0.5 to 5% (w/v), electrolyte concentration (B) in a 6 to 12% (w/v), pH (C) in 2 to 10% and for the incubation temperature (D) in the range of 25 to 65 °C. These values were used to prepare 25 different experiments that are given in Table 1 with their respective recorded results. RSM is an optimal design and in this study there are four independent factors which have low and high limits. According to the model considered, the middle points in the specified intervals are applied to maintain the consistency of measurement levels. So the points considered in Table 1 are not plan levels. Based upon the observed values after initial experiments, the final values for the independent variables were carefully selected. Using the current experimental design setup, any unnecessary extraction experiential trial was removed. Hence, less noisy data were acquired, and an accurate interpretation was performed.

Results and Discussion

Application of the aqueous micellar solutions in separation sciences, especially in using a biodegradable and food grade nonionic surfactant has gained more attention over the last few years. The present work introduces a framework to apply the cloud point extraction (CPE) as a recovery procedure to extract betaine from beet molasses. Often, in CPE procedure, the factors affecting recovery and their interactions are the most complex and have shown plurality. The conventional one factor at a time technique is analyzed in most of CPE experiments in the literature to optimize recovery. However, it shows many shortcomings in considering the nonlinear relationship within the interacting factors.

In this experimental study, all operational factors have varied at the same time and their combined effects on the efficiency of betaine recovery were examined for each specific run. The effect of these four factors in CPE method of betaine from beet molasses has been further investigated. A total of 25 tests were performed and the analysis of variance (ANOVA) for response reduced quadratic model (RRQM) is presented in Table 2.

To analyze the effects and to eliminate the irrelevant factors on the extraction of betaine, *p*-values were used that also help identify the degree of importance of each parameter. *P*-values also helped find the interaction effect between each independent variable. As shown in Table 2, values of Prob> F (*p*–value) which are less than 0.01, represents the model and shows considerable model terms (variants). In this case, A (surfactant concentration) and B (electrolyte concentration) were found significant in model terms. The pH and incubation temperature were found insignificant.

The model *p*-value <0.0001 points out that the model is significant for explanation of the process. The model residual variance is found to have contributions from two factors, lack of a good fit and pure experimental error. The poor quality of fit variance is not resulted from the contributions of first order terms while the pure experimental error variance is computed by the variation difference between observations at equivalent experimental conditions of runs in a random sequence. The poor quality of fit in this model was not significant (*p*-value>0.01). Regression analysis certified that a second order multinomial equation fits the response variable, %ER, in terms of four independent variables. The entire data set can be modeled and used by this multinomial equation (Eq. 2).

$$ER = -129.12922 + 4.28203\,A + 30.26421\,B - 1.37752\,C - 5.925\,D + 0.30663\,AB - 1.03290\,A2 - 1.47496\,B2 + 0.19340\,C2 + 7.71616\,D2 \quad (R^2=0.89) \qquad \text{(Eq. 2)}$$

Table 2. Analysis of variance for the response surface reduced quadratic model for betaine extraction

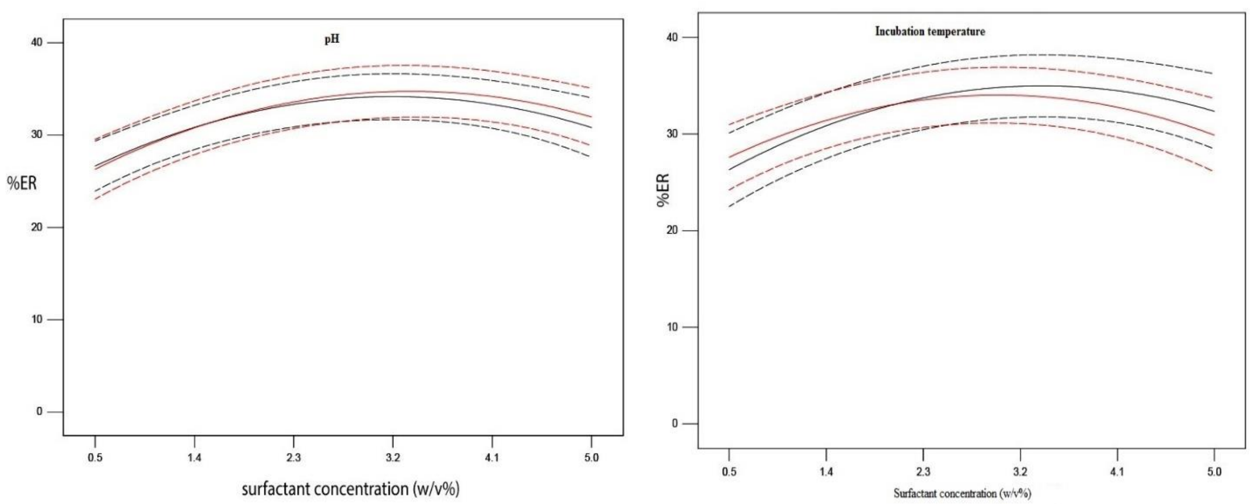
Source	Sum of squares	df	Mean square	*F*-value	*p*-value Prob>F
Model	4374.88	14	312.49	93.12	< 0.0001
A	30.45	1	30.45	9.07	0.0131
B	2325.38	1	2325.38	692.96	< 0.0001
C	2.63	1	2.63	0.78	0.3970
D	25.26	1	25.26	7.53	0.0207
AB	40.59	1	40.59	12.10	0.0059
A^2	117.52	1	117.52	35.02	0.0001
B^2	760.80	1	760.80	226.72	<0.0001
C^2	36.51	1	36.51	10.88	0.0080
D^2	0.37	1	0.37	0.11	0.7467
Residual	33.56	10	3.36		
Lack of fit	14.06	5	2.81	0.72	0.6359 not significant
Pure error	19.50	5	3.90		
Cor total	4408.44	24			

Where ER shows efficiency of betaine recovery. This *R*-square indicates the effectiveness (goodness of fit) of the model to data points that predicts a response value. In this study the pred *R*-squared value of 0.8902 locates in sensible settlement with "Adj *R*-squared" of 0.8981. "Adeq precision" measures the signal to noise ratio and favorable value is greater than 4. In this model, this signal is 24.343 which is a sufficient signal. R^2 (*R*-squared) defines the overall performance of the model as a degree of correlation between the observed and predicted values [32]. For this model R^2 and "Adj R^2" were equal 0.9924 and 0.89817, respectively. This approves that the model is suitable to predict the process behavior at the design space.

Effect of operational factors and process optimization

Three-dimensional surface graphs were used to evaluate the effect of operational factors on the experimental extraction process and the maximum recovery. The influence of four factors on the efficiency of betaine recovery was examined using the response surface reduced quadratic model. The effect of incubation temperature and pH on the betaine recovery was not observed in this study. Three important factors that affect the experiment include the following, surfactant concentration, electrolyte concentration and surfactant concentration versus electrolyte concentration on betaine recovery. The maximum recovery of betaine was obtained when the surfactant concentration was approximately 3.3% (w/v) at the pH from 2 to 10 as shown in Figure 1**a**. Any deviation of surfactant concentration from this condition resulted in a reduction of the betaine recovery.

In all surfactant concentrations, it was found that changing the pH has no effect on the betaine recovery. The result in Figure 1**b** for the recovery of betaine at all incubation temperatures (25-65 °C) is repeated. For all incubation temperatures and at surfactant concentration of 3.3% the maximum recovery of betaine is achieved. In Figures 1**a** and 1**b**, three curves for each pH and temperature are graphed. The middle curve is the main and two others are the upper and lower bounds that shows the confidence limits.



(a) **(b)**

Figure 1. Response surface plots illustrating the effect of surfactant concentration versus pH a) and surfactant concentration versus incubation temperature b) on betaine recovery yield

Figure 2**a** shows that the maximum betaine recovery at pH of 2-10 occurred at about 10.5 % (w/v) of electrolyte (salt) concentration. Increasing or decreasing the amount of pH in this range, the betaine recovery remained unchanged. Figure 2**b** indicates the same result obtained for the recovery of betaine for all incubation temperatures. The maximum recovery of betaine for all incubation temperatures occurred at approximately 10% (w/v) of electrolyte concentration, the same trend as before.

A related point to consider is that pH is often an effective parameter in cloud point processes. At certain pH levels, analyte becomes more hydrophobe that is solubilized in surfactants. In this experiment, it was seen that pH had no effect on CPE which is may be due to the hydrogen bonds between carboxyl group of betaine and oxygen of ethylene oxide groups of polyethylene glycol. It was

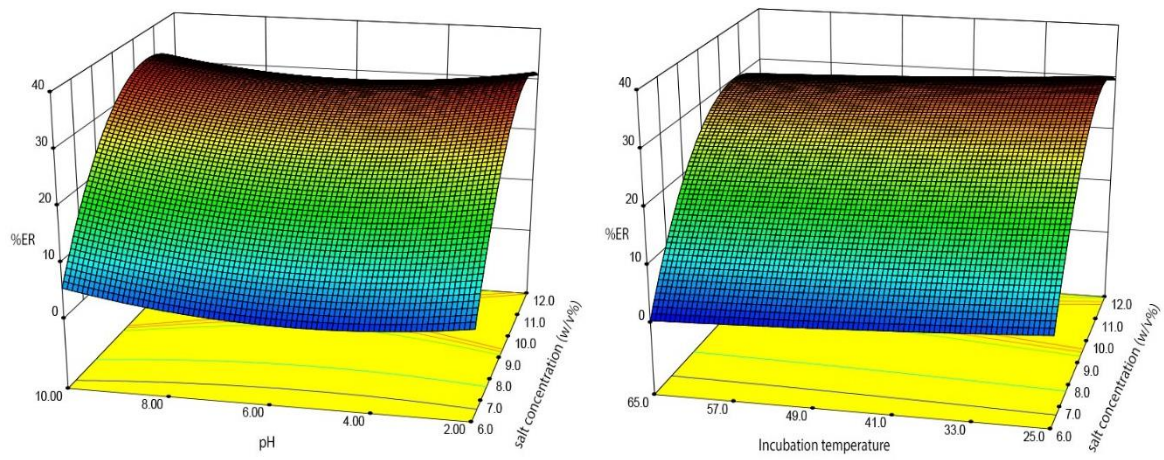


Figure 2. Response surface 3D plots showing the effect of salt (electrolyte) concentration versus pH a) and salt (electrolyte) concentration versus incubation temperature b) on betaine recovery efficiency

observed that in this cloud point method, hydrogen bond between an analyte with a surfactant is more effective than the hydrophobicity of the analyte in its extraction.

The interaction effect of surfactant concentration with electrolyte concentration on betaine recovery is shown in Figure 3. It shows that betaine recovery reaches to a maximum level of 31% while the surfactant concentration is increased to 3.3 and electrolyte concentration up to 10.5% (w/v). As shown, in case of increasing the surfactant concentration above 3.3% (w/v), betaine recovery was decreased.

Through using an applied software and also based on a reduced mathematical model build in this study, the operation parameters were optimized. To achieve the goal of this study, all four variables and the model response, %ER were set in the range to maximize the objective. In first extraction and under the settings, software predicted %ER equal to 34 percent. The optimal values of the surfactant concentration, electrolyte concentration, pH, and incubation temperature were calculated as following, 3.06% (w/v), 10.49% (w/v), 9.85, and 48.24 °C. Extra-experiments to verify the precision of the model prediction were performed, and at the optimum conditions, a recovery of 38% was reached. A three-step CPE was employed under the optimum conditions in water-phase containing the un-extracted betaine to maximize the overall recovery. The efficiency of the recovery was increased up to 66% by the second extraction and as high as 88% after the third extraction.

Figure 4 demonstrates the typical HPLC chromatograms of the betaine. The retention time of 4.9 min for chromatogram of betaine in beet molasses is appeared. The chromatogram of stripped phase

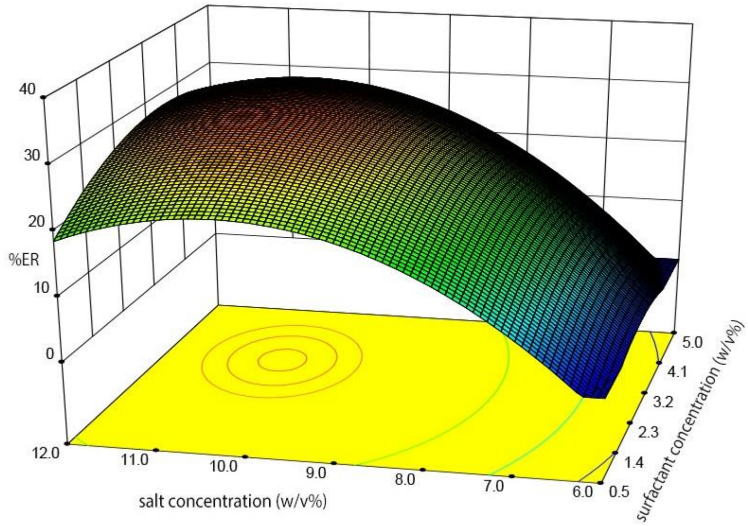


Figure 3. Response surface 3D plots showing the interaction effect of surfactant concentration with salt (electrolyte) concentration on betaine yield

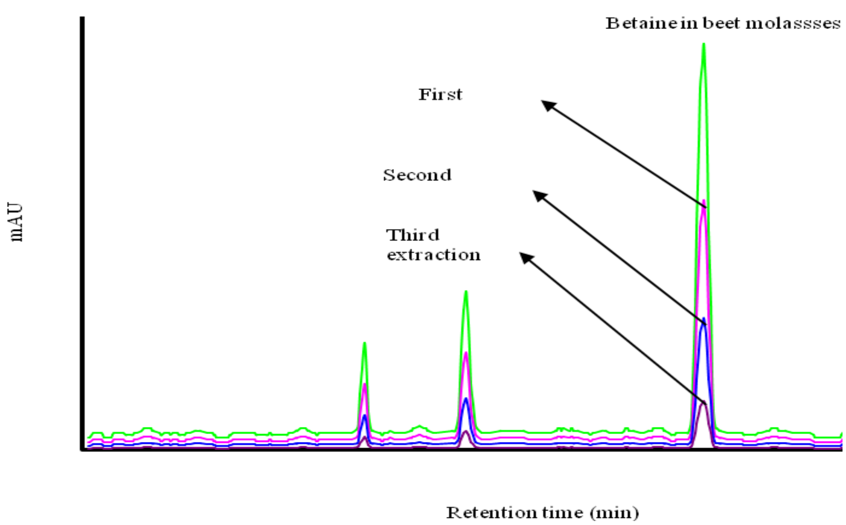


Figure 4. Typical chromatograms of betaine inbeet molasses sample before cloud point extraction, and same sample after cloud-point extraction

of beet molasses after cloud-point extraction in three steps were noticed and shown. The separation effect of cloud-point extraction was also clearly shown.

Conclusions

It is known that betaine is an anti-stress and anti-cardiac disease agent. This highly valuable substance is found in beet waste abundant in sugar industry. In present study, the CPE method using food grade surfactant, as a green and environmentally-friendly method was used for the extraction of the betaine from molasses. Some features such as the simplicity, least time, labor-consuming, and the minimal equipment requirements are the benefits that makes CPE an attractive alternative to conventional techniques for betaine extraction at large-scale. Applying RSM and a second order multinominal model guide predict the recovery of betaine in an applied CPE experimental settings with high accuracy. The model allowed for a straightforward optimizaion of factors affecting betaine recovery by CPE. Optimal conditions for maximum recovery was obtained under a surfactant concentration of 3.06% (w/v), incubation temperature of 48.24 °C, pH of 9.85, and electrolyte concentration of 10.49% (w/v). The results showed that, implementing a two to three-step CPE procedure in water-phase withun-extracted betaine resulted in the recovery rate of 66%, and 88%, respectively. Also it was found that, by a freeze-dried process, a supplementary powder of betaine can be directly used as a nutrient in livestock feed supply.

Disclosure Statement

No potential conflict of interest was reported by the authors.

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