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RESEARCH ARTICLE

Separation of L-Phenylalanine by Solvent Sublation and Solvent Extraction Method

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ABSTRACT

Aims and Objectives: Separation and purification is a series of processes intended to isolate a single type of biomolecule from a complex mixture. Innovations in improvement of biodownstream processing, which is responsible for the separation of about 50–80% of recombinant proteins and other biomolecules, play a very important role in increasing the yield and reducing the cost of biopharmaceutical production. **Methods:** Biomolecule isolation and purification from a fermentation broth usually involve centrifugation, filtration, adsorption, and chromatography steps. **Results and Discussions:** Each step contributes to the product cost and product loss. Thus, we consider that solvent extraction and solvent sublation are the more economic processes for the separation of biomolecules. **Conclusion:** In extraction of phenylalanine, maximum extraction was observed at amino acid: surfactant ratio 1:1, amino acid: extractant ratio 1:1500, and pH at 3.1. The highest value of % recovery percentage and Co/Cw was 76.3 and 10.21, respectively. The main motive of this article is to provide the advantages of study on the solvent sublation and solvent extraction of l-phenylalanine over the other techniques.

Keywords: Biomolecules, solvent extaction, solvent sublation

INTRODUCTION

Phenyl alanine is an essential amino acid found in breast milk in mammals and a number of foods including meat, poultry, fish, cottage, cheese, lentils, peanuts, and sesame seed. The L-isomers are used to biochemically form proteins, coded by DNA. Phenylalanine is a precursor of tyrosine, dopamine (the monoamine neurotransmitters), norepinephrine (noradrenaline), epinephrine (adrenaline), and the skin pigment melanin and direct precursor to the neuromodulator phenethylamine, commonly used as dietary supplement. It is encoded by the codons UUU and UUC. Hence, the extraction of this amino acid is important. Cameron *et al*.[1] reported that L-phenylalanine and extraction was performed by reversible complexation using extractants like Aliquat 336 and di-2-(ethylhexyl)phosphoric acid (D2EHPA) in the diluent, methyl isobutyl ketone. Results are compared for extraction by each of the single extractants and a mixed extractant system composed of equimolar amounts of Aliquat 336 and D2EHPA. The mixed system displayed lower loading values by a factor of 3 than the single-extractant systems; however, it was able to sustain the uptake capacity for pH values 1.8–9.2. Stream processing technologies for the separation and recovery of amino acids also used. The application of D2EHPA in an emulsion liquid membrane (ELM) as a carrier had attracted much attention. It has been reported that amino acids such as lysine, tryptophan, and phenylalanine can be separated and concentrated by ELM containing D2EHPA as a carrier. D2EHPA can also separate amino acids as extractant dissolved in different diluents. Lio *et al*. studied the extraction mechanism of isoleucine with D2EHPA as extractant and proposed the extraction equilibrium constant. The extraction of several amino acids with D2EHPA dissolved in benzene was studied by Teramoto. It was suggested that one amino acid molecule coordinates to 1.7–2.0 dimeric D2EHPA. The extraction of amino acids by D2EHPA was limited to a cation-exchange reaction in all the above works; the extraction equilibrium was in the pH 3 range. To date, there is no published report on the extraction of amino acids in the middle pH range because amino acids change

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their charge state depending on the pH. The extraction equilibrium mechanism for different species of amino acids may be different from each other, and the structures of amino acid D2EHPA complex may also differ. Therefore, it is very important to determine the extraction equilibrium at various pH conditions. Wieczoreka[2] made the extraction from an aqueous donor phase with pH 3 to a more acidic acceptor phase, and the mass transfer was driven by the proton gradient between these phases. For 0.01 mmol tryptophan and with 1M HCl as acceptor phase, an extraction efficiency of 60% was obtained, constant up to at least 12 h. A novel and effective method, a liquid membrane process, had been proposed for the selective separation and concentration of various species such as metal ions, weak acid and bases, and biologically important compounds. This so-called process is of two types: ELM or supported liquid membrane. In general, two types of extractants have been used in ELM for the extraction of amino acids. They are either positively charged cation exchange carriers such as Aliquat 336,[3] a quaternary ammonium salt, or negatively charged anionic carrier such as D2EHPA. The proton gradient was the driving force between the acceptor and donor sides when D2EHPA was used as extractant. Amino acids form zwitterion which prevents it from being exchanged because of the spatial proximity of two opposite charges on the molecule. The chemical cost would be too much to change the solution pH. Therefore, a novel recovery technology would be required for the separation of amino acids in the middle pH range. The extraction of l-phenylalanine with D2EHPA dissolved in n-octane was carried out by Liu.[3] Various effects such as concentration of l-phenylalanine, D2EHPA, and pH on the distribution ratio were studied. The infrared spectrogram of the organic phase-loaded solute illustrated that the pH condition had little effect on the structure of the complex. There were proton transfer and ion exchange reactions in the extraction. One l-phenylalanine molecule was extracted by forming a complex with two dimeric D2EHPA molecules. The maximum amount of amino acid was extracted in the interval of pH 3–6. This explains the extraction of ion associates of the protonated amino acid with the D2EHPA anion. There was a decrease in extraction efficiency in the highly acidic medium (pH <3) and highly basic medium (pH >6). The extraction efficiency decreases inthesequenceTrp>Phe>Leu>Arg>Lys>Ile>Ala>Gly. Increase in crown ether concentration increases recovery of amino acids, but the ratio of the concentration of D2EHPA and crown ether is crucial in the extraction of amino acids. Chemically, amino acids are amines and could be extracted as "host-guest" complexes. The formation of hydrogen bond between amino group of a substrate and the polyether cycle was the driving force of the complexation. However, high polarity and hydrophilicity of a substrate often prevented the extraction of protonated amino acid. Cascaval[4] carried out the separation of some amino acids of acidic character (l-aspartic acid and l-glutamic acid), basic character (l-histidine, l-lysine, and l-arginine) and neutral character (l-glycine, l-tryptophan, and l-cysteine) by reactive extraction with D2EHPA. The separation yield is controlled by the pH value of the aqueous phase, which is due to the acidic or basic character of each amino acid. The individual extraction of amino acids indicated that the maximum yields are reached for a pH domain of 2–3, then strongly decreasing with the pH increase. Thus, for acidic and neutral amino acids, the extraction becomes impossible at the isoelectric point and for basic amino acids at a pH value lower than pH, as a result of the carboxylic group dissociation. Using multistage extraction, the total separation of the following amino acid groups has been performed: Neutral amino acids (l-glycine, l-alanine, and l-tryptophan) at pH 5–5.5, basic amino acids (l-lysine and l-arginine) and l-cysteine at pH 4–4.5, l-histidine at pH 3–3.5, and acidic amino acids (l-aspartic acid and l-glutamic acid) at pH 2–2.5. The proposed extraction method can be developed and used for the selective separation of amino acids from fermentation broths or protein hydrolysates. The solvent sublation technique was applied for the separation and enrichment of l-Arg using dodecyl benzene sulfonic (DBSA) as the surfactant, D2EHPA (P204) as the extractant, and n-heptane as the organic solution. The solvent sublation was compared with the floatation complexation extraction, foam floatation, and solvent extraction. The experimental results showed that enrichment ratio (ER) of 16.2 and removal rate of 97.2% to l-Arg were obtained by the solvent sublation under the conditions of room temperature, 0.09 g/L l-Arg aqueous solution 250 mL, DBSA concentration 0.15 g/L, the initial pH 7.0, volume of n-heptane 10 mL, and gas flow rate of 200 mL/

min. The study of the kinetics indicated that the solvent sublation process could be divided into three stages distinctly. The solvent sublation technique is one of adsorption bubble separation techniques, as an option for ion flotation, in which the material adsorbed on the surface of ascending bubbles and then collected by a solvent in the column upper zone. The technique has an advantage over ion flotation or solvent extraction, so it has attracted many attentions in the wastewater treatment and the recovery of many metals. However, as to now, the review on the solvent sublation is very sparse. The present paper reviews the theory and application in the solvent sublation. The application of solvent sublation in the removal of humic acids (HA) was investigated in the present study. The HA was removed from an aqueous solution by solvent sublation of HA-hexadecylpyridinium chloride complex (sublate) into isopentanol. Several parameters were examined toward the optimization of HA removal; the dosage of a surfactant was found to be the major one, controlling the overall efficiency of the progress. The removal rate was somewhat enhanced by higher airflow rate and almost independent of the volume of the organic solvent floating on the top of the aqueous column. The effects of electrolytes (e.g., NaCl), non-hydrophobic organics (e.g., ethanol), and pH of the solution on the process were studied. Under the optimized condition, the treatment performance was found to be very efficient, reaching almost 100%, indicating that solvent sublation can serve as a possible alternative technology for the removal of humic acids. The solvent sublation process follows first-order kinetics. A characteristic parameter, apparent activation energy of attachment of the subulate to bubbles, was estimated at a value of 9.48 kJ/mol.[5]

MATERIALS AND METHODS

Chemicals and reagents

1. l-phenylalanine (manufactured by - HiMedia Laboratories Pvt., Ltd.),
2. Sodium lauryl sulfate (SLS) (manufactured by - MERCK Ltd.),
3. D2EHPA (manufactured by - Spectrochem Pvt., Ltd.),
4. n-butyl acetate extra pure (manufactured by - MERCK Ltd.).

Instrumentation

1. Solvent sublation glass column with frit (G3) (height - 58 cm, internal diameter - 4.5 cm, and external diameter - 4.8 cm),
2. Nitrogen gas cylinder with regulator,
3. Rotameter with gas flow rate controller (15 cc/min–150 cc/min) and distillation apparatus (supplied by Remco Ltd.),
4. Centrifuge apparatus with speed controller (1000–10,000 rpm) supplied by Remi equipments,
5. Ultraviolet (UV)-spectrophotometer model - ANALAB UV-180,
6. Digital weighing balance and digital pH meter make - Sartorius.

Standard curve preparation

At first, all glass apparatus taken for the experiment was calibrated properly. After that, 100 ml of 100 mcg/ml stock solution of l-phenylalanine was prepared in double-distilled water. A series of dilutions in the range of concentrations 5 mcg/ml, 10 mcg/ml, 15 mcg/ml, and 50 mcg/ml were made, and the absorbance was observed by UV spectrophotometer. This experiment was repeated thrice, and average values of optical density (O.D.) were calculated. Such readings were taken by different stock solution at different days, and average O.D. values were calculated. A graph of absorbance against concentration of L-phenylalanine was plotted. The surfactant used was SLS. It was also added in different concentrations along with amino acid to see if there was any change in O.D. of amino acid, but O.D. value remained the same in the presence of surfactant. The proposed graph shows the equation of the standard curve at pH 3.1 (isoelectric point of l-phenylalanine).

Validation of rotameter

A graduated tube of 25 ml was taken. The bottom end of the tube was attached with a balloon containing soap solution. Rotameter was connected with a gas (N2) source and its outlet was connected to the bottom sidearm of graduated tube. N_2 gas was allowed to pass through the rotameter and graduated tube. When balloon was pressed in the presence of gas flow, ring of

bubbles was formed and ascended the tube as the gas flows. Time taken by a ring of bubble to cross certain volume was noted, and the volumetric flow rate was calculated and compared with that of rotameter reading [Table 1].

Table 1: Validation of rotameter

Rotameter reading cc/min	Time of pass 10 cc (mm)	Soap bubble reading (cc/min)
20	0.49	20.4
30	0.324	30.86
40	0.24	41.6
50	0.195	51.28
60	0.165	60.6
70	0.135	74.07
80	0.126	79.36
90	0.112	89.28
100	0.101	99

Solvent extraction

Extraction is an important technique in chemistry and biotechnology. It is usually applied in biotechnology as the first step in the recovery of primary and secondary metabolites. Extraction competes with many other separation methods, including adsorption, precipitation, chromatography, distillation, membrane separation, and crystallization. Adsorption has the disadvantage of low loading capacity, so it is applied if other techniques are less effective in spite of its low selectivity. The liquid–liquid extraction of amino acids is only possible by adding into the organic phase extractants such as phosphoric acid derivatives and high molecular weight quaternary aliphatic amines and crown ethers. The separation of some amino acids of acidic character (l-aspartic acid and l-glutamic acid), basic character (l-histidine, l-lysine, and l-arginine), or neutral character (l-glycine, l-tryptophan, l-cysteine, and l-alanine) by reactive extraction with D2EHPA was explored experimentally by other investigators.[6] Using the experimental data of the study on the individual reactive extraction, the selective separation of the considered amino acids from a mixture may be analyzed. This method is considered an efficient alternative for the fractionation of amino acid mixtures compared with existing techniques 24. The reactive extraction of amino acids with D2EHPA occurs by means of an interfacial chemical reaction of the ion exchange type where HP is the extractant. As it can be observed, the separation is possible if amino acids exist as a cation in aqueous solution, as found at low pH values. At the same time, if the solution pH is too low, then the extractant will become protonated and thus unable to extract the cations. The solvent extraction recovery of amino acids appeared to be promising with cationic (acidic) or anionic (basic) carriers, depending on the solution pH. In this work, the anionic carries D2EHPA was used because it was reported to be the most promising carrier. Particular advantages of D2EHPA are its chemical stability, high complexing ability, stripping characteristics, extremely low solubility in aqueous acidic solutions, versatility in the extraction of many amino acids, and its availability in commercial quantities.

A 30 ml aqueous solution of l-phenylalanine (M.W. −165.19, $C_6H_{11}NO_2$) of 33.038 mcg/ml concentration was prepared. The absorbance of the solution was recorded. Then, it was transferred to a 50 ml capacity centrifuge tube, and 10 ml of different concentrations of extractant D2EHPA (M.W. −322.43, $C_{16}H_{35}O_4P$) in solvent (butyl acetate) was added. Then, the centrifuge tube was closed properly and covered with parafilm to prevent any loss of solvent owing to vaporization. The tube was shaken vigorously for 1 h. After this, the mixture was separated by centrifuging for 30 min, and then, the organic and aqueous layer was separated. The organic layer was further extracted by adding 0.1M hydrochloric acid in two stages. The absorbance of each extracted aqueous solution was observed. The loss of material was measured by mass balance, and amount partitioned in both layers was calculated. The experiment was carried out under different variable parameters, such as amino acid-surfactant ratio, amino acid-extractant ratio, at different initial pHs, and aqueous-organic volume ratio. Surfactant and anionic SLS were chosen. Its concentration in the feed was maintained at $<8.25\ 10^{-3}$ M (critical micelle concentration) [Table 2].

Solvent sublation (batch process)

The name arises from the fact that an ionic species called the colligend is removed by addition of surface active collector of opposite charge to that of the colligend. The complex formed by coulombic attraction is called "sublate," and the process

of lifting the sublate by gas bubbles is called sublation. A solvent sublation technique requires the generation of small gas bubbles, surfactant, and organic solvent. The size of bubbles is of major importance with preference in very small bubbles, often <100 μm. The bubbles are enriched by adsorption in their travel through the aqueous media. On reaching the liquid-liquid interface, they are unable to overcome the interfacial tension immediately. Rather, coalescence must occur before bubble across the interface occurs. It is expected that repulsions of bubbles caused by zeta potentials on the bubbles result in slow bubble coalescence. Consequently, a relatively stationary layer of bubbles exists below the liquid–liquid interface. As the coalesced bubbles move through liquid–liquid interface, they drag a small amount of liquid from the interfacial region. Collector and coligend carried into the organic layer readily dissolve, and the water surrounding the bubbles returns in the form of droplets in the aqueous phase after the bubble burst. It is quite likely that liquid–liquid equilibrium is established between the water droplets and the organic layer. However, from volume considerations, the amount of sublate dissolving in water droplets should be small. This sublate is returned to the interfacial water layer, and duo to the protection given by the stationary bubbles, the sublate does not enter the bulk aqueous phase. Maximum ER (48.189) and % recovery percentage (RP) (96.378) were observed at a gas flow rate of 330 ml/min and pH 5 that is closest to isoelectric point of observed proteins (bovine serum albumin, β-lactoglobulin, and α-lactalbumin).[6]

The apparatus consists of a long column of 58 cm in height and 4.5 cm in inside diameter and fitted by a frit (G3) at the bottom and an enlarged solvent chamber at the top. The solvent chamber is fitted with reflux condenser. The column shows inlet and outlet for feed and effluent. The solvent chamber also shows inlet and outlet for solvent. The bottom pipeline is used for the drainage of the aqueous phase; sampling ports are there along the side of column. The main glass apparatus is assembled with nitrogen cylinder and rotameter. Feed of desired concentration was prepared, and the pH was adjusted as per the requirement. Feed is an aqueous solution containing specific amount of amino acid and surfactant. The column was then filled with feed solution. The level of aqueous feed reaches 1 cm below the top of this column. The enlarged part of solvent chamber was covered with a bell-shaped glass cover. The required amount of butyl acetate was poured in the solvent chamber, and a clear interface between feed and solvent was visible. Nitrogen gas was passed through the feed at desired gas flow rate. The gases were dispersed as tiny bubbles and ascended upward. Dissolved solutes adsorb at the gas–liquid interface. Bubbles carry these molecules and deliver these in the solvent layer. Later, bubbles burst into gaseous form and leave the solvent layer. Sample from residual aqueous liquid was collected at fixed intervals and immediately analyzed. After some hours, the steady-state concentration of effluent was observed. The experiment was continued up to 5 h. At the end of a run, the total volume of residual liquid was collected and analyzed. The total input amount, output amount, ER, and percentage recovery were calculated [Table 3]. In the present work, solvent sublation technique was adopted for the separation of low concentration on l-phenylalanine from the aqueous solution. The thesis work was carried out in a conventional bubble column by taking an aqueous solution of

Table 2: Solvent extraction

Table no.	Amino acid: Surfactant (mole/mole)	Amino acid: Extraction (mole/mole)	Initial pH of the feed	Aqueous: Organic (v/v)	%RP	Co/cw
(i)	1:1	1:1000	3.1	3:1	62.18	6.46
(ii)	1:0.75	1:1000	3.1	3:1	59.91	5.02
(iii)	1:1.25	1:1000	3.1	3:1	61.43	5.20
(iv)	1:1	1:1000	3.1	3:1	76.29	10.21
(v)	1:1	1:1000	3.1	3:1	71.02	7.71
(vi)	1:1	1:1000	2.18	3:1	55.72	3.88
(vii)	1:1	1:1000	5.18	3:1	55.18	3.87

RP: Recovery percentage

Table 3: Solvent sublation (batch process) feed concentration (Ci) = 33.038 mcg/ml

Time (h)	Volume of feed sample (ml)	Concentration of amino acid in sample (mcg/ml)	Remaining feed volume before withdrawal (ml)	Amount in feed before withdrawal of sample (mg)	% of amino acid in feed
0	0	33.038	950	31.38	100
0.5	2	28.48	950	27.06	86.2
1	2	24.78	948	23.45	75
2	2	21.7	946	20.53	65.68
3	2	19.25	944	18.17	58.26
4	2	18.06	942	17.01	54.66
5	2	18.05	940	16.97	54.63
Time (h)	**Amount in each withdrawal (mg)**	**Cumulative amount of amino acid in samples (mg)**	**Amount of amino acid in solvent layer (mg)**	**% of amino acid in solvent**	
0	0	0	0	0	
0.5	0	0	4.33	13.79	
1	0.056	0.056	7.88	25.1	
2	0.049	0.105	10.75	34.25	
3	0.043	0.148	13.07	41.64	
4	0.038	0.186	14.16	45.12	
5	0.035	0.221	14.19	45.22	

Table 4: Process control parameters and their limits

Parameters	Units	Notations	Limits
Gas flow rate	ml/min	A	20
Volume of organic layer	ml	B	50
Extractant	%	C	20

Table 5: Design matrix

GFR cc/min	% of D2EHPA	Volume of organic layer (ml)
30	30	125
20	20	200
40	20	50
30	20	125
30	40	125
20	30	125
30	30	125
40	40	50
20	20	50

GFR: Gas flow rate, D2EHPA: Di-2-(ethylhexyl) phosphoric acid

l-phenylalanine as feed solution and using SLS as the surfactant, D2EHPA as the extractant, and butyl acetate as the extraction solvent. Under room temperature, the initial concentration (33.038 mcg/ml of 950 ml) of dilute aqueous solution was prepared. After addition of SLS into aqueous solution, its pH adjusted to various pH level by adding HCl or NaOH. Sublation column was filled with prepared feed, and the top of aqueous column was covered by a layer of mixture solvent (butyl acetate) and extractant (D2EHPA) mixture. Composition of top layer and gas flow rate was varied to see its effect on separation. When aqueous sample showed no further change in UV readings, the experiment was stopped. In the back extraction experiment, the organic phase was transferred into a 500 ml separating funnel containing 0.1 M hydrochloric acid aqueous solution. The back extraction was repeated 3 times, then the aqueous phase was collected, and UV reading was noted. The organic solvent was reused for the subsequent experiment. In the experiment, the effects of several parameters on the separation efficiency were studied and evaluated, and the results were presented as ER and percentage recovery.

Optimization method

The experimental design and analysis were performed with the help of design expert (Version 7.1.7. Stat-Ease, Minneapolis, USA). The selected process parameters and their limits, units, and notations are given in Table 4. Design of experiment software (Design-Expert v7) was used to code the variables and to establish the design matrix. RSM is applied to the experimental data using the same software to obtain the regression equations and to generate the statistical and response plots. Design matrix was generated by the statistical software by putting factors with levels. Table 5 shows the design matrix. According to the levels of factors

(gas flow rate [GFR], % of extractant, and volume of organic layer), solvent sublation experiments were performed and the data response variables (RP and ER) were obtained. Overall data are shown in Table 6. The data as shown in Table 6 were analyzed by software, and ANOVA was generated. ANOVA is shown in Tables 7 and 8.

RESULTS AND DISCUSSION

The present work deals with the separation and collection of l-phenylalanine amino acid from a dilute solution by solvent extraction method and solvent sublation method. The effects of amino acid-surfactant ratio, initial pH of the feed, the and percentage of extractant on the separation or extraction process were studied thoroughly. An effort was also made to optimize those various operating conditions so that a maximum separation can be achieved. Below, the effects of the following parameters are discussed.

Tables (i) and (iii) in Table 2 show the effect of surfactant amount on the separation of amino acid from the aqueous layer. Extraction is carried out

Table 6: Overall results of solvent sublation

Table no.	GFR cc/min	% of D2HPA	Volume of organic layer (ml)	Recovery %	Enrichment ratio	Profile character
A	30	30	125	49.89	3.791	N
B	20	20	200	63.02	2.99	I
C	40	20	50	14.92	2.83	W
D	30	20	125	40	3.04	N
E	30	40	125	44.33	3.369	N
F	20	30	125	45.22	3.43	N
G	30	30	125	49.83	3.78	N
H	40	40	50	16.1	3.05	W
I	20	20	50	13.76	2.62	W
J	20	40	200	70.25	3.34	I
K	30	30	200	81.4	3.87	I
L	30	30	125	49.9	3.792	N
M	40	20	200	63.52	3.02	I
N	30	30	50	18.35	3.49	W
O	30	30	125	49.9	3.792	N
P	20	40	50	16.89	3.2	W
Q	30	30	125	49.89	3.791	N
R	40	30	125	48.3	3.67	N
S	30	30	125	49.88	3.79	N
T	40	40	200	73	3.47	I

W: Indicates wide gap between up curve and down curve. N: Indicates narrow gap between up curve and down curve. I: Indicates two curves intersect at a point and this gives equal partitioning of amino acid between two phases. GFR: Gas flow rate, D2EHPA: Di-2-(ethylhexyl) phosphoric acid

Table 7: % RP and ER value

% RP			ER		
Factor	**B-coefficient**	***P* value**	**Factor**	**B-coefficient**	***P* value**
Intercept	49.53	<0.0001	Intercept	3.76	<0.0001
A (GFR)	0.67	0.3897	A	0.046	0.1022
B (Vorg)	27.12	<0.0001	B	0.15	0.0002
C (Ext)	2.53	0.0067	C	0.19	<0.0001
AB	0.36	0.6748	AB	0.013	0.6712
AC	0.038	0.9650	AC	−0.032	0.282
BC	1.55	0.0924	BC	0	1
A2	−2.25	0.1443	A2	−0.16	0.0074
B2	0.86	0.5565	B2	−0.033	0.5104
C2	−6.85	0.0007	C2	−0.51	<0.0001

RP: Recovery percentage, ER: Enrichment ratio

the by varying ratio of amino acid: surfactant. At a higher (1:1.25) or lower (1:0.75) concentration, it was observed that the percent recovery decreased following the order of amino acid: surfactant ratio as 1:1>1.25>1:0.75. It was also found that, as recovery increased, there was also an increase in Co/Cw value. This was due to the fact that surfactant decreases interfacial tension. Other experiments were performed varying other conditions and keeping amino acid: surfactant ratio (1:1) fixed. Next, the ratio of amino acid and extractant was varied in the range of 1:1000–1:2000. Tables (i), (iv), and (v) in Table 2 show the effect of variation of the ratio of amino acid: D2EHPA (extractant). Percentage extraction of amino acid (l-phenylalanine) increased with the amount of extractant (moles). Percentage of extraction is 76.29 and Co/Cw is 10.21 when the ratio between them is 1:1500. This was the maximum percentage recovery found. It was observed that, by increasing the amount of extractant, the percent recovery increased but up to a certain limit. Tables (i), (iv), and (vii) in Table 2 show the effect of pH variation on the separation of amino acid by extraction. Percentage of extraction was found lesser at pH 5.2 than at of pH 3.1. Ionic surfactant combines easily with ionized amino acid. The complex reacts with the extractant. At isoelectric pH charge, balanced amino acid diffuses into organic layer owing to its lesser solubility. In the extraction of amino acid, maximum extraction was observed at amino acid: surfactant ratio 1:1, amino acid: extractant ratio 1:1500, and pH at 3.1. The highest value of %RP and Co/Cw was 76.3 and 10.21, respectively. Earlier few investigators like Mukhopadhyay *et a*l.[7] reported 60–70% extraction of phenylalanine using D2EHPA as extractant at pH 3. The present study was performed at a volume ratio 3:1 (aqueous: organic) and recovery of amino acid from aqueous layer was 76% at pH 3.1. Smirnova *et al*. achieved 60% extraction of phenylalanine at pH 3 using D2EHPA as extractant. Recovery of other amino acids (l-tryptophan and l-alanine) was reported to be higher (78–90%) by extraction method in comparison with that of phenylalanine. Percentage of extraction depends on many factors such as characteristics of the material to be extracted, ratio of feed solvent and extracting solvent, and type of solvent and extractant. Earlier, investigators used different conditions and types of amino acids, extractant, and solvent. Maximum extraction was reported at isoelectric pH of phenylalanine. In the present study, percentage extraction of phenylalanine was enhanced to 76% by increasing the ratio of aqueous layer: organic layer.

Earlier few investigators reported 79.5% recovery of l-lysine using extractant (D2EHPA) in solvent (n-octanol) at pH 7. There is no much report on the recovery of phenylalanine by solvent sublation method. Solvent sublation experiments were compared by varying three factors. The three factors are GFR, amount of extractant in the organic layer, and total volume of organic layer. Experiments were performed following the method as mentioned in the section "materials and methods." Results are displayed from Table A-T in Table 6. In separation technology, performance is judged by two criteria, i.e., RP and ER. In Table A-T in Table 6, different slabs (I - 63–81, II - 40–49, and III - 13–16) of %RP were observed. Comparing the slabs, the effect of organic layer volume was very prominent on RP. In slab I, maximum RP is 81.4% when values of GFR and extractant were 30 cc/min and 30%. RP is lesser when GFR <30 and >30 cc/min at higher amount of extractant. With the increase of GFR from 20 to 30 cc/min, RP increased, but it decreased when GFR was 40 cc/min. In slab II, percent recovery

Table 8: % RP and ER value

Other statistics (% RP)	Other statistics (ER)
R^2-0.9929	R^2-0.9763
Adjusted R^2-0.9865	Adjusted R^2-0.9550
Adequate precision - 38.763	Adequate precision - 21.533
Predicted R^2-0.9415	Predicted R^2-0.6915

RP: Recovery percentage, ER: Enrichment ratio

Table 9: Predicted values and relative percentage error

GFR	Volume of organic layer	Extractant	RP			ER		
			Pred	Exp	%RE	Pred	Exp	%RE
20	200	40	72.33	70.25	2.8	3.42	3.34	2.3
32.15	200	32.92	78.24	79.243	−1.277	3.89	3.91	0.472
32.51	200	33.05	78.24	79.19	−1.21	3.88	3.9	−0.282

Exp: Experimental value, Prd: Predicted value, %RE: Percentage relative error {(Pred-Exp)/Pred}. RP: Recovery percentage, ER: Enrichment ratio, GFR: Gas flow rate

was found in the range of 40–49 %. In this range, operation at GFR-30 cc/min, extractant - 30%, and organic layer- 125 ml gave the highest RP 49.89. In slab III, it showed very poor performance which is evident in percentage recovery. Amino acid is extracted by extractant which should be used at an optimum level to recover molecules of amino acid. In the present setup, the maximum volume of organic layer that can be accommodated in the top layer is 250 ml. 30% of extractant in 200 ml organic layer gave satisfactory result.

Analysis of ANOVA for all responses

With the input data of Table 6, software generated the following ANOVA [Table 7]. Regression coefficients and their *P*-values for the regression models were obtained to predict optimized responses (RP and ER). ANOVA consists of statistical results. Tables 7 and 8 list significant parameters and statistical parameters.

ANOVA showed significance ($P < 0.05$) of model terms (intercept, main factors, interactions, and quadratic terms). In the ANOVA of % RP, the model F value of 155 implies that the model is significant. There is only 0.01% chance that a model F value this large could occur due to noise. The predicted $R^2 = 0.9415$ is in reasonable agreement with the adjusted $R^2 = 0.9865$. Adequate precision measures the signal-to-noise ratio. A ratio >4 is desirable.

In the ANOVA of ER, the model F value of 45.79 implies that the model is significant. There is only 0.001% chance that a model F value this large could occur due to noise. The predicted $R^2 = 0.6915$ is not as close to adjusted $R^2 = 0.9550$ as one might normally expect. Things to consider are model reduction. Adequate precision measures the signal-to-noise ratio. A ratio >4 is desirable. The model can be used to navigate the design space. In both the cases, a significant lack of Fit was not desirable.

Contour Plots. Each contour curve represents an infinite number of combinations, of two test variables with the other one maintained at its respective center level. The effect of one factor solely depends on the value of the other. The maximum predicted yield is indicated by the surface confined in the smallest ellipse in the diagram. These figures are the visual display of response variables at various levels of independent variables.

Software generated a number of solutions from which several were picked up. Response variables from solution were presented as predicted variables, and solvent sublation experiment was run again as per the independent variables. The experimental response variables were compared with the predicted values, and relative percent error is presented in Table 9. The % error was within 5% which was acceptable. Therefore, the models were validated and can be used to predict responses.

Within the domain of levels of factors, maximum %RP and ER were found as 78% and 3.89, respectively, at GFR 32.34 cc/min, volume of organic layer 200 ml, and extractant 33%. Further experiment was performed with a higher level of volume of organic layer (250 ml) at GFR - 36.46 cc/min and extractant 33.17 %, and higher %RP of amino acid (l-phenylalanine) was obtained as ~94%, but ER (3.56) was slightly lower.

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