สำนักหอสมุดกลาง พระจอมเกล้าลาดกระบัง

PREPARATION OF n-BUTYL-CHITOSAN CROSSLINKED MICROSPHERES AS A STATIONARY PHASE FOR HIGH PERFORMANCE HYDROPHOBIC INTERATION CHROMATOGRAPHY

SUWIMON KWUNSIRIWONG

เลขหมู่.......50177
เลขทะเบียน..........
วัน,เดือน,ปี....2 3 พ.ค. 2551

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ชื่อนักศึกษา	นางสาวสุวิมล ขวัญศิริวงศ์
รหัสประจำตัว	46068809
ปริญญา	วิทยาศาสตรมหาบัณฑิต
สาขาวิชา	เคมี (เคมีวิเคราะห์)
อาจารย์ที่ปรึกษาวิทยานิพนธ์	ผศ.คณิตา ตังคณานุรักษ์

บทคัดย่อ

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Thesis Title	Preparation of n-butyl-chitosan crosslinked microspheres as a stationary phase for high performance hydrophobic interaction chromatography
Student	Miss Suwimon kwunsiriwong
Student ID.	46068809
Degree	Master of Science
Program	Chemistry (Analytical Chemistry)
Thesis Advisor	Asst. Prof. Kanita Tangkananurak

ABSTRACT

The main purpose of this research work was to study the potential and limitation of using the chitosan microspheres particles from shrimp shell and immobilized hydrophobic ligand, butyl group, to form an hydrophobic adsorbent. The optimized condition for preparation of chitosan microspheres as stationary phase was found that using 250 mL of 0.5% (w/v) chitosan, 3 mL of 50% (v/v) glutaraldehyde and atomizer speed 35,000 rpm. The FTIR spectrum analysis showed a butyl group was linked to $-NH_2$ by a C-N bond. The average particle size of n-butyl chitosan crosslinked microspheres were 5.5 μm, average surface area of 3.02 m^2/g, major pore size of 43.72 Å, amorphous form, spherical shape and smooth surface. The HPHIC column (i.d. 4.6 x 150 mm.) packing was performed using slurry packing technique. This HPHIC columns were employed for separation of a mixture of two protein models (lysozyme and bovin serum albumin) and a gradient elution system (0 - 4 min, 5 m*M* phosphate buffer containing 10% sodium sulfate; 4 - 6 min, 5 m*M* phosphate buffer containing 8% sodium sulfate; 6 - 9 min, 5 m*M* phosphate buffer containing 5% sodium sulfate; 9 - 14 min, 5 m*M* phosphate buffer) at the flow rate of 0.3 mL/min. Peak area detection was performed with a UV detection at 280 nm gave resolution at 1.03. The influence of ionic strength and temperature on the adsorption of protein on n-butyl chitosan crosslinked microspheres were studied, and it was found that the behavior of adsorption met with the theory of hydrophobic interaction.

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CHAPTER 1

INTRODUCTION

1.1 PROBLEM STATEMENT

Of all the protein purification techniques currently used, hydrophobic interaction chromatography is considered to be an ideal one, since it can preserve the biological activity of the isolated protein, it can also isolate protein from extreme values and less denaturing environment. In hydrophobic interaction chromatography, the main hydrophobic interaction chromatography support materials which are commercially available are crosslinked agarose and synthetic organic polymer.

Chitosan is an important natural polymer prepared by the deacetylation of chitin mainly obtained from crab and shrimp shell. Because of the presence of many free amino groups, the applications of chitosan to bio-medical, pharmaceutical and membrane materials have been investigated widely. Chitosan is a useful support of immobilized enzymes and cells [1], porous chitosan beads have been used as a protease immobilized support [2], but the application of chitosan in hydrophobic interaction chromatography has not been frequently explored, and chitosan has many of the excellent chemical properties of agarose, such as chemical stability and compatibility with organic compounds. Therefore, the main purpose of this research work was to study a procedure for the preparation of chitosan microspheres particles with an hydrophobic interaction ligand immo-bilized on it to form an hydrophobic interaction chromatography adsorbent. The use of this adsorbent for the hydrophobic interaction chromatography of α – amylase is studied.

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1.2 OBJECTIVE OF THE STUDY

- Produce the chitosan microspheres particles as a support of stationary phase for hydrophobic interaction chromatography column.
- Study of immobilized ligand on chitosan microspheres particles to form an hydrophobic interaction chromatography adsorbent.
- Produce the hydrophobic interaction chromatography low cost column.

1.3 SCOPE OF THE STUDY

- Study of chitosan production process from shrimp shell.
- Preparation of crosslinked chitosan microspheres particles.
- Study of immobilized ligand on crosslinked chitosan microspheres particles for hydrophobic interaction chromatography application.
- Study of characterization of butyl-chitosan crosslinked microspheres.
- Study of hydrophobic interaction chromatography column packing techniques
- Evaluate of hydrophobic interaction chromatography column efficiencies.

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CHAPTER 2

THEORY AND LITERATURE REVIEWS

2.1 CHITIN AND CHITOSAN

2.1.1 STRUCTURE OF CHITIN AND CHITOSAN

Chitin is one of the most abundant organic materials, beings second only to cellulose in the amount produced annually by biosynthesis. It occurs in animals, particularly in crustacean, mollusks and insects, where it is a major constituent of the exoskeleton, and in certain fungi, where it is a major constituent of the exoskeleton, and in certain fungi, where it is the principal fibrillar polymer in the cell wall.

Chitin is occurs naturally in three polymorphic forms, α-chitin is the most abundant, crystalline, tightly compacted and stable form in which the chains are arranged in an antiparallel fashion. In β-chitin the chains are arranged in parallel whereas in γ-chitin there are two <up> chains to every <down> chain. α-chitin is found where extra hardness proves essential whereas β and γ-chitin provide toughness, flexibility and mobility together with control electrolytes and polyanionic characteristics [3].

Chitin is poly[β(1$\longrightarrow$4)-2-acetamido-2-deoxy-D-glucopolynose], and its idealized structure is shown in Figure 2.1

Figure 2.1 The structure of chitin [4].

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The principal derivative of chitin is chitosan, produced by alkaline deacetylation of chitin. Chisan is poly [β(1⟶4)-2-amino-2-deoxy-D-glucopyranose] and its idealized structure is shown in Figure 2.2

Figure 2.2 The structure of chitosan [4].

Thus the terms chitin and chitosan describe a continuum of copolymers of N-acetyl-D-glucosamine and D-glucosamine residues, the two being distinguished by insolubility or solubility in dilute aqueous acid solutions. Their properties are frequently dependent on the relative proportions of N-acetyl-D-glucosamine and D-glucosamine residues, as are their biodegradability and their biological role.

2.1.2 PREPARATION OF CHITOSAN

(1) PURIFICATION OF CHITIN

Crustacean shell waste From crab, shrimp, lobster, or crawfish, is the richest source of chitin as well as being the only chitinons source presently arailable in quantities sufficient to support a commercial chitin / chitosan industry

Crustacean shell waste mainly consists of protein (30-40%), calcium carbonate (30-50%), and chitin (20-30%) These proportions rary witth species and with the season (Table 2.1)

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Table 2.1 Chemical composition of various crustacean wastes [4]

Source	Chemical composition (%)				
	Moisture	Protein	Ash	Lipid	Chitin
Lobster (Linuparus trigonus)					
	13.5	17.0	54.7	-	-
Crab					
1) Callinectes sapidus					
	-	30-35	50	-	13-15
	4.5	2.40[a]	56.0	2.0	12.9
2) Chinonecetes opilio					
	-	29.19	40.60	1.35	26.65
3) Portunus trituberculatus					
	12.9	10.3	57.9	0.3	17.1
Shrimp					
1) Penaeus monodon					
(Carapace)	9.1	26.8	29.3	0.5	34.9[b]
(Shell)	9.7	42.8	20.8	1.2	36.5[b]
2) Pandalus borealis					
	-	23.5	33.9	14.7	30.0
3) Penaeus sp.					
(Head)	3.2	55.8	22.6	8.9	11.9[c]
(Hull)	4.0	45.0	31.7	0.4	27.2[c]
Prawn	9.24	61.6*	26.67	1.40*	30.00
Crawfish (Procambarus clarkia)					
	-	16.9	63.6	0.6	23.5
	5.7	28.1[a]	44.0	4.4	12.5
Krill (Euphausio superba)					
	-	41.0	23.0	11.6	24.0
	-	44.6[a]	24.7	1.8	19.9

*dry basis.

[a]Corrected for chitin.

[b]Based on 6.9% N present in chitin.

[c]Expressed as fiber.

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Preparation of chitin from crustacean shell waste consists of three basis steps.

Step 1 Deproteinization

Crustacean shell waste usually is ground and treated with dilute sodium hydroxide solution (1-10%) at elevated temperature (65-100 ^{o}C) to dissolve the protein present (Table 2.2)

Step 2 Demineralization

Demineralization is conventionally accomplished by extraction with dilute hydrochloric acid at room temperature to dissolve the calcium carbonate as calcium chloride (Table 2.3)

Step 3 Decoloration

Acid and alkali treatments alone produce a colored chitin product. When bleached chitin is desired, pigments can be removed with reagents as shown in table 2.4 [5].

(2) DEACETYLATION OF CHITIN

Conversion of chitin to chitosan is generally achieved by treatment with aqueous alkali to remove some or all of the acetyl group from the polymer. The most frequently used alkali is NaOH. The extent of deacetylation is governed mainly by the alkali concentration, temperature, time of reaction, atmosphere, ratio of chitin to alkali solution, and particle size (table 2.5). Completely deacetylation is rarely achieved. However, it is normally necessary since solubility of chitosan in dilute aqueous acids is obtained at an extent of deacetylation of ~ 60% or above. The higher the concentration of alkali used the lower the temperature, and or the shorter the time of treatment required [5].

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Table 2.2 Conditions used for deproteinization in preparation of various chitins[4]

Source	Deproteinization				
	Alkali Conc.		Temp. (°C)	Time (hr)	Ratio (w/v)
Lobster	1N	NaOH	100	12x5*	1:5.5
	10%	NaOH	room	72[a]	-
	10%	NaOH	100	2.5	1:50
	15%	NaOH	65	3	1:10
Krill	3.5%	NaOH	90-95	2	1:10
	3.5%	NaOH	25	2	-
	3%	KOH	95	2	1:10
	4%	NaOH	98	2.5	1:25
Shrimp	3%	NaOH	100	1	-
	1%	NaOH	65	1	1:10
	1%	KOH	90	2	1:20
	4%	NaOH	boiling	1	-
	pH	11.5	30	1	1:30
	1N	NaOH	100	1	1:6
	5N	NaOH	100	1	-
Prawn	5%	NaOH	Reflux	2	1:15-20
	5%	NaOH	100	0.5	1:1
	0.5%	NaOH	boiling	0.5	2:3
	15%	NaOH	65	3	1:10
Crwafish	3.5%	NaOH	65	2	1:10
	15%	NaOH	65	3	1:10
Crab	1N	NaOH	80	3x2*	-
	2%	KOH	90	2	1:20
	1N	NaOH	50	6	-
	5%	NaOH	90	2	-
	1N	NaOH	50	5	-
	5%	NaOH	65	1	1:15

*times of repeated alkali treatment.

[a]freah NaOH solution was used for soaking each day.

[b]when chitosan is the final product.

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Table 2.3 Conditions used for demineralization in preparation of various chitins[4]

Source	Demineralization			
	HCl conc.	Temp. (°C)	Time (hr)	Ratio (w/v)
Lobster	2N	room	5	1:9
	& 2N	cold	48	1:5.5
	37%	-20	4	-
	90% formic	room	18	1:10
	1N	room	2	1:15
Krill	0.6N	room	2	1:22
	3.5%	20	1.5	1:4
	22%	room	2	1:10
	2N	15.25	0.5	1:25
Shrimp	1N	room	0.5	3x***
	0.5N	room	-	1:11
	2.5%	20	1	1:10
	5%	room	-	-
	8%	30	8	1:10
	0.75N	room	0.5	1:12
	1.25N	room	0.5	1:12
	1.75N acetic	25	12	1:15
	6N	room	1	-
Prawn	5%	room	2	1:15-20
	5%	room	1	1:2
	1.25N	room	1	-
	1N	room	2	1:15
Crawfish	1N	room	0.5	1:15
	1N	room	2	1:15
Crab	1N	room	12x2*	-
	2.5%	20	1	1:10
	1N	20	3	-
	5%	room	24	-
	0.1N	room	-	excess
	1N	room	0.5	1:15

*times of repeated acid treatment.

**second extraction.

***three fold excess to the stoichiometric amount of calcium carbonate in the dry hulls.

[a]for shell. [b]for carapace. [c]deproteinized with proteolytic enzymes.

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Table 2.4 Conditions used for decoloration in preparation of chitins[4]

Source	Decoloration			
	Reagent	Temp. (°C)	Time (hr)	Ratio (w/v)
Lobster	ethanol+ether	room	washing	-
	NaOCl	cold	-	-
	ethanol+acetone+ether	room	washing	-
	Acetone+NaOCl	room	15	1:10
Krill	chloroform	-	-	-
	ethyl acetate	30	30	1:4
	acetone	-	-	-
Shrimp	acetone	-	-	-
	1% K_2MnO_4	room	60	-
Prawn	acetone	reflux	45	1:15-20
	0.5% H_2O_2	room	overnight	-
	NaOCl	cold	30	-
	acetone+NaOCl	room	15	1:10
Crawfish	acetone+0.315% NaOCl	room	5	1:10
	acetone+NaOCl	room	15	1:10
Crab	Acetone	reflux	-	-
	Acetone	-	-	-
	3% H_2O_2	-	-	-
	acidic H_2O_2	cold	360	1:20
	3% H_2O_2	60	120	-
	0.32% NaOCl	room	3	1:10

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Table 2.5 Deacetylation conditions in chitosan production[4]

Source	Deacetylation				
	Atmos-phere	Alkali Conc.	Temp. (°C)	Time (hr)	Ratio (w/v)
Lobster	A/N	55% KOH	100-140	0.5-1.5	1:100
Crab	N	47% NaOH	60,110	1,2x1-4*	-
	N	40% NaOH	reflux	10	-
Shrimp	N	50% NaOH	100	0.5-5	-
	A/N	50% NaOH	145-150	1/12,1/4	1:10
	A	50% NaOH	60	2**	1:4
	V	50% NaOH	100	0.5x2*	1:15
	V	50% NaOH	100	0.75x2*	1;15
	A	50% NaOH	95	3	-
Prawn	A	50% NaOH	30	24-144	1:14-56
	A	60% KOH	100	1	1:65
	A	50% NaOH	100	2	-
Krill	A	50% NaOH	80-96	1/3	-
	A	39% KOH	reflux	20	1;17

[a]A = air, N = nitrogen, V = vacuum.

[b]dissolved in 95 % ethanol + ethylene glycol.

*times of separate alkali treatment.

**dry heating.

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2.1.3 PHYSICAL AND CHEMICAL PROPERTIES OF CHITOSAN

2.1.3.1 Degree of deacetylation

Chitosan is characterized by either the degree of acetylation (DA), which corresponds to the degree of deacetylation DDA (DDA = 1.00-DA) D-glucosamine groups. In general, the degree of deacetylation is more than 50% for chitosan.

The degree of acetylation has an influence on all the physicochemical properties, (molecular weight, viscosity, solubility, etc) so, it is one of the most important parameters. Varions techniques were used for determination of the degree of deacetylation such as IR spectroscopy, H-NMR spectroscopy and colloidal titration. The most appropriatc technique for rapid characterization seems to be the IR spectroscopy

2.1.3.2 Molecular weight [6]

The molecular weight of native chitin is usually larger than $1x10^6$ while that of the commercial chitosan product is between $2x10^5$ to $1.2x10^6$ Several methods are used to determine molecular weight of chitosan ; for examples, light scattering, gelpermeation chromatography (GPC), high performance liquid chromatography (HPLC), and viscometry method is one of the simplest and most rapid methods, or which are based on the well-know Mark-Houwink equation

$$[\eta] = K * \overline{M}_W^a \qquad (2.1)$$

where ; a and K are the constant

$\overline{M}_w$ is the weight-average molecular weight

Since 1974, several reports have dealt with the determination of the values of K and a for chitosan. However, each of these results was different although the determining conditions, such as solvents, and temperature, were very similar.

Wang et al., (1991) obtained the following equation to determine a and K.

$$K = 1.69 * 10^{-30} * DD^{14.0} \qquad (r = 0.996)$$

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$$a = -1.02 * 10^{-2} * DD + 1.82 \quad (r = 0.998)$$

2.1.4 CROSSLINKED CHITOSAN

Chitosan is stable in neutral condition due to the fact that the amine and hydroxyl groups on glucosamine unit can form strong inter- and intra-molecular hydrogen bond to crystallize. The crystal structure was broken down in acidic condition due to the electrostatic repulsion between protonated amine groups on chitosan.To preserve the stability of chitosan under HIC media, its amine groups on polymeric chains have to be fixed by crosslinking.

Chemical crosslinking of chitosan chains with the biofunctional reagent glutaraldehyde occurs by a Schiff `s reaction of aldyhyde group on glutaraldehyed with amino groups on the chitosan biopolymer chain.(Figure 2.3)

Figure 2.3 The structure of crosslinked chitosan. [7]

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2.2 HYDROPHOBIC INTERACTION CHROMATOGRAPHY [8]

Hydrophobic interaction chromatography (HIC) is a well established bioseparation technique in laboratory-scale as well as industrial-scale purification of proteins. In industry, HIC is usually employed in the intermediate purification steps, with typical particle sizes for the sorbents of 30–100 mm [2]. In analytical separations, particle sizes of 5–30 mm are more common. Popular hydrophobic ligands include butyl, octyl and phenyl groups. The proteins are generally adsorbed to the hydrophobic stationary phase at high salt concentration, with the driving force for adsorption being a displacement of ordered water molecules around the proteins and the ligands which leads to an increase in entropy. The adsorbed proteins are then usually eluted with a decreasing salt gradient which weakens the hydrophobic interactions.[16]

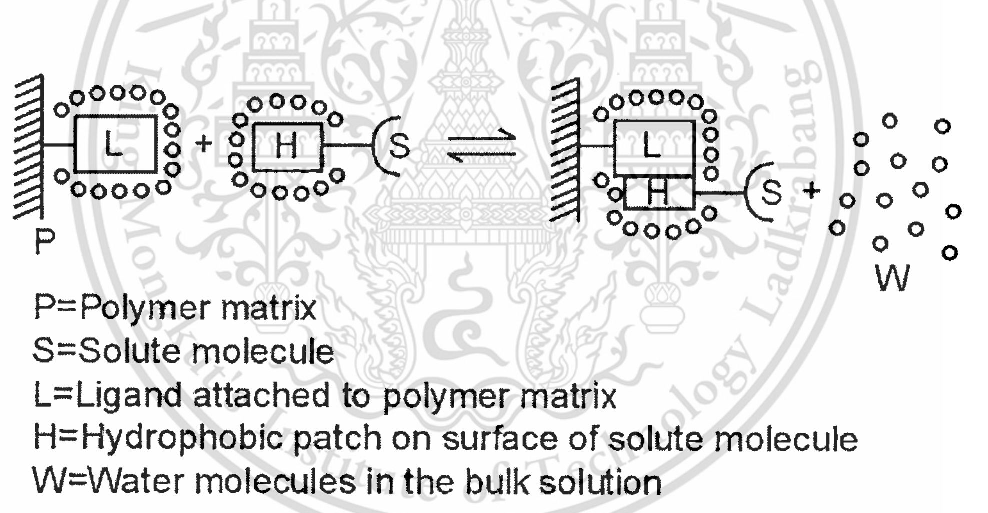

Figure 2.3 Close to the surface of the hydrophobic ligand and solute(L and H), the water molecules are more highly ordered than in the bulk water and appear to ''shield off'' the hydrophobic ligand and solute molecules. Added salt interacts strongly with the water molecules leaving less water available for the ''shielding off'' effect, which is the driving force for Land H to interact with each other.

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2.2.1 HIC vs RPC

In theory, HIC and reverse-phase chromatography (RPC) are closely related LC techniques. Both are based upon interactions between solvent-accessible non-polar groups (hydrophobic patches) on the surface of biomolecules and the hydrophobic ligands (alkyl or aryl groups) covalently attached to the gel matrix. In practice, however, they are different. Adsorbents for RPC are more highly substituted with hydrophobic ligands than HIC adsorbents. The degree of substitution of HIC adsorbents is usually in the range of 10–50 μmoles/ ml gel of C_2–C_8 alkyl or simple aryl ligands, compared with several hundred μmoles/ml gel of C_4–C_{18} alkyl ligands usually used for RPC adsorbents. Consequently, protein binding to RPC adsorbents is usually very strong, which requires the use of non-polar solvents for their elution. RPC has found extensive applications in analytical and preparative separations of mainly peptides and low molecular weight proteins that are stable in aqueous-organic solvents.

In summary, HIC is an alternative way of exploiting the hydrophobic properties of proteins, working in a more polar and less denaturing environment.

Compared with RPC, the polarity of the complete system of HIC is increased by decreased ligand density on the stationary phase and by adding salt to the mobile phase.

2.2.2 FACTORS AFFECTING HIC

The main parameters to consider when selecting HIC media and optimizing separation processes on HIC media are:

- Ligand type and degree of substitution
- Type of base matrix
- Type and concentration of salt
- pH
- Temperature
- Additives

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2.2.3 TYPE OF LIGAND

The type of immobilized ligand (alkyl or aryl) determines primarily the protein adsorption selectivity of the HIC adsorbent . In general, straight chain alkyl (hydrocarbon) ligands show ''pure'' hydrophobic character while aryl ligands show a mixed mode behaviour where both aromatic and hydrophobic interactions are possible . It is also established that, at a constant degree of substitution, the protein binding capacities of HIC adsorbents increase with increased alkyl chain length (Figure 2.4) . The charged type HIC adsorbents show an additional mode of interaction, which will not be discussed here. The choice between alkyl or aryl ligands is empirical and must be established by screening experiments for each individual separation problem.

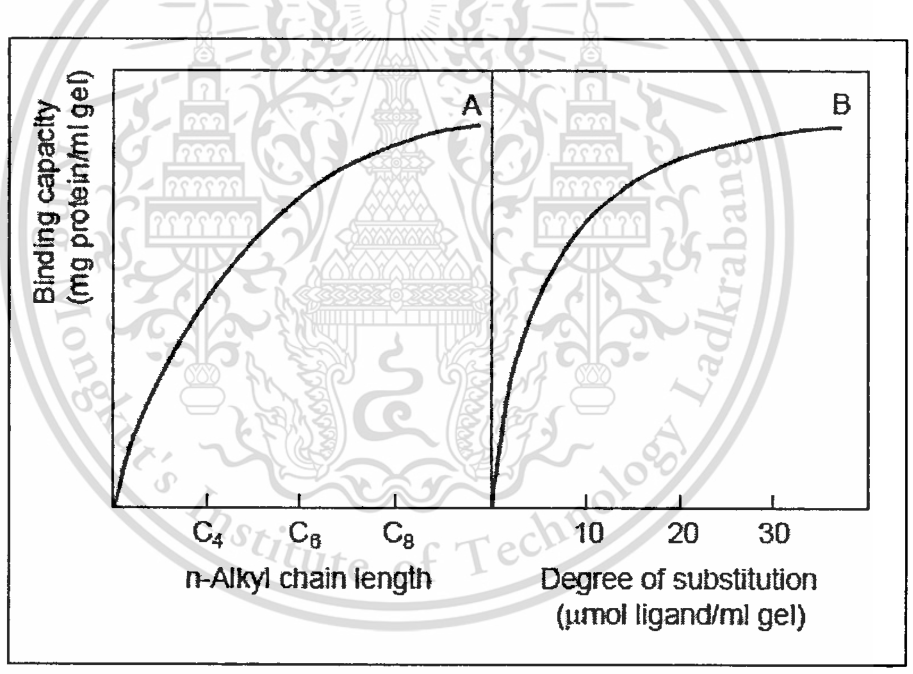

Figure 2.5 The effect of alkyl chain length and degree of substitution on binding capacity in HIC. In Fig. 2A it is assumed that the degree of substitution is the same for each alkyl chain length shown.

2.2.4 DEGREE OF SUBSTITUTION

The protein binding capacities of HIC adsorbents increase with increased degree of substitution of immobilized ligand. At a sufficiently high degree of ligand

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substitution, the apparent binding capacity of the adsorbent remains constant (plateau is reached) but the strength of the interaction increases (Figure 2.5 B). Solutes bound under such circumstances are difficult to elute due to multi-point attachment .

2.2.5 TYPE OF BASE MATRIX

It is important not to overlook the contribution of the base matrix. The two most widely used types of support are strongly hydrophilic carbohydrates, e.g. cross-linked agarose, or synthetic copolymer materials. The selectivity of a copolymer support will not be exactly the same as for an agarose based support substituted with the same type of ligand.

To achieve the same type of results on an agarose-based matrix as on a copolymer support, it may be necessary to modify adsorption and elution conditions.

2.2.6 TYPE AND CONCENTRATION OF SALT

The addition of various structure-forming (''salting out'') salts to the equilibration buffer and sample solution promotes ligand-protein interactions in HIC . As the concentration of such salts is increased, the amount of proteins bound also increases almost linearly up to a specific salt concentration and continues to increase in an exponential manner at still higher concentrations.

This latter phenomenon is demonstrated in figure 2.6 where total binding capacity of Phenyl Sepharose High Performance for α-chymotrypsinogen and RNAse was examined at gradually increasing salt concentrations.

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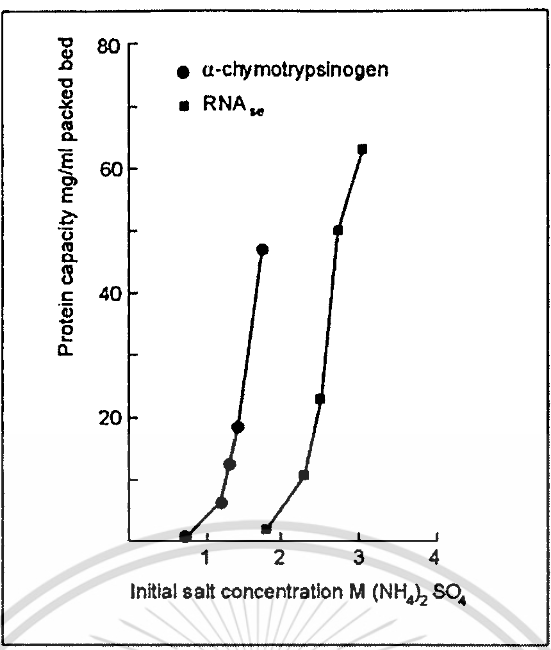

Figure 2.6 Protein binding capacity on Phenyl Sepharose High Performance as a function of salt concentration in the column equilibration buffer.

A significant increase in adsorption capacity can be seen when the salt concentration is increased above the precipitation point

This phenomenon is probably due to the precipitation of proteins on the column. It has a concomitant negative effect on the selectivity of the HIC adsorbent.

The effects of salts in HIC can be accounted for by reference to the Hofmeister series for the precipitation of proteins or for their positive influence in increasing the molal surface tension of water . These effects are summarized in Tables 2.6 and 2.7

Table 2.6 The Hofmeister series on the effect of some anions and cations in precipitating proteins.

← Increasing precipitation ("salting -out") effect

Anions: PO_4^{3-}, SO_4^{2-}, $CH_3 \cdot COO^-$, Cl^-, Br^-, NO_3^-, ClO_4^-, I^-, SCN^-

Cations: NH_4^+, Rb^+, K^+, Na^+, Cs^+, Li^+, Mg^{2+}, Ca^{2+}, Ba^{2+}

Increasing chaotropic ("salting-in") effect →

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Table 2.7 Relative effects of some salts on the molal surface tension of water.

$Na_2SO_4 > K_2SO_4 > (NH_4)_2SO_4 > Na_2HPO_4 > NaCl > LiCl \ldots > KSCN$

In both instances, sodium, potassium or ammonium sulphates produce relatively higher ''salting-out'' (precipitation) or molal surface tension increment effects. It is also these salts that effectively promote ligand-protein interactions in HIC. Most of the bound proteins are effectively desorbed by simply washing the HIC adsorbent with water or dilute buffer solutions at near neutral pH.

2.2.7 EFFECT OF pH

The effect of pH in HIC is also not straightforward. In general, an increase in pH weakens hydrophobic interactions , probably as a result of increased titration of charged groups, thereby leading to an increase in the hydrophilicity of the proteins. On the other hand, a decrease in pH results in an apparent increase in hydrophobic interactions. Thus, proteins which do not bind to a HIC adsorbent at neutral pH bind at acidic pH . Hjertén *et al.*found that the retention of proteins changed more dras-tically at pH values above 8.5 and/or below 5 than in the range pH 5–8.5 (figure 2.7).

These findings suggest that pH is an important separation parameter in the optimization of hydrophobic interaction chromatography and it is advisable to check the applicability of these observations to the particular separation problem at hand.

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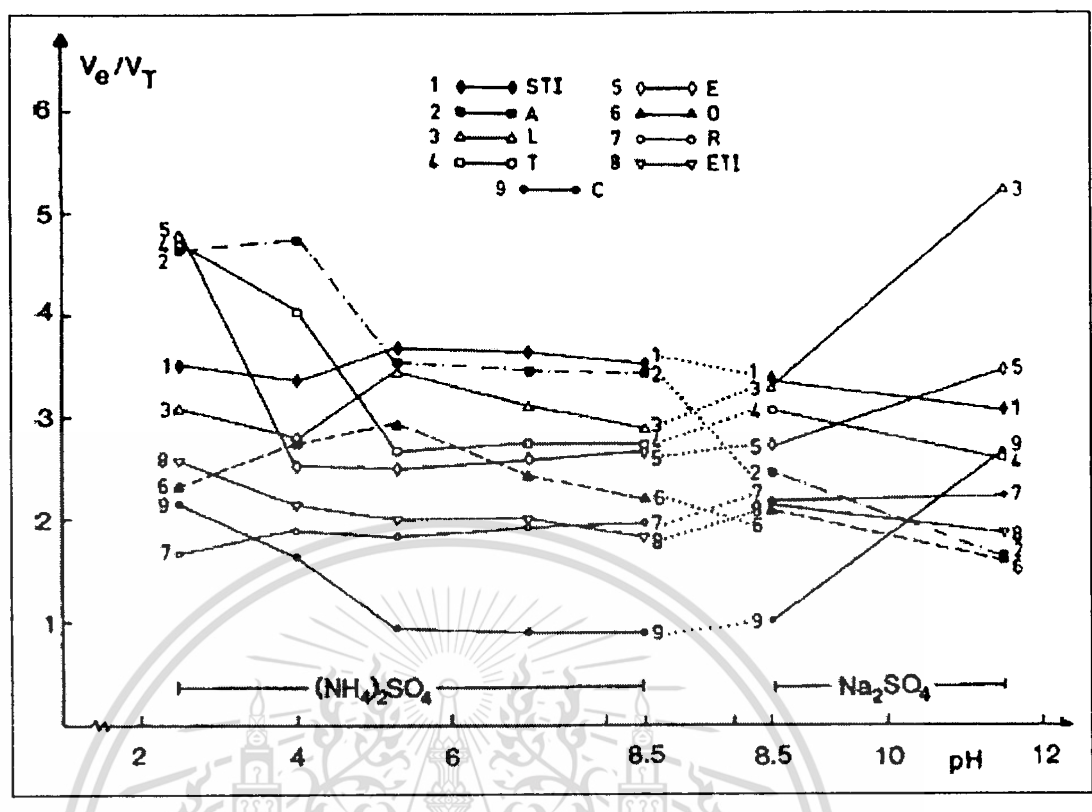

Figure 2.7 The pH dependence of the interaction between proteins and an octyl agarose gel expressed as V_e/V_T (V_e is the elution volume of the different proteins and V_T is the elution volume of a non-retarded solute). Elution was by a negative linear gradient of salt. The model proteins used were STI=soy trypsin inhibitor, A=human serum albumin, L=lysozyme, T=transferrin, E=enolase, O=ovalbumin, R=ribonuclease, ETI=egg trypsin inhibitor and C=cytochrome c. (Reproduced with permission, from

2.2.8 EFFECT OF TEMPERATURE

Based on theories developed for the interaction of hydrophobic solutes in water , Hjertén proposed that the binding of proteins to HIC adsorbents is entropy driven [$\Delta G = (\Delta H - T\Delta S) \sim -T\Delta S$], which implies that the interaction increases with an increase in temperature. Experimental evidence to this effect has been presented by Hjertén and Jennissen . It is interesting to note that the van der Waals attraction forces, which operate in hydrophobic interactions , also increase with increase in temperature . However, an opposite effect was reported by Visser & Strating indicating that the role

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of temperature in HIC is of a complex nature. This apparent discrepancy is probably due to the differential effects exerted by temperature on the conformational state of different proteins and their solubilities in aqueous solutions.

In practical terms, one should thus be aware that a downstream purification process developed at room temperature might not be reproduced in the cold room, or *vice versa*.

2.2.9 ADDITIVES

Low concentrations of water-miscible alcohols, detergents and aqueous solutions of chaotropic (''salting-in'') salts result in a weakening of the protein-ligand interactions in HIC leading to the desorption of the bound solutes. The non-polar parts of alcohols and detergents compete effectively with the bound proteins for the adsorption sites on the HIC media resulting in the displacement of the latter. Chaotropic salts affect the ordered structure of water and/or that of the bound proteins. Both types of additives also decrease the surface tension of water (see Table 2.8) thus weakening the hydrophobic interactions to give a subsequent dissociation of the ligand-solute complex. Although additives can be used in the elution buffer to affect selectivity during desorption, there is a risk that proteins could be denatured or inactivated by exposure to high concentrations of such chemicals. However, additives can be very effective in cleaning up HIC columns that have strongly hydrophobic proteins bound to the gel medium.

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Table 2.8 Physical properties of some solvents used in HIC (data at 25 °C).

Solvent	Viscosity (centipoise)	Dielectric constant	Surface tension (dynes/cm)
Water	0.89	78.3	72
Ethylene glycol	16.9	40.7	46.7
Dimethyl Sulphoxide	1.96	46.7	43.54
Dimethyl Formamide	0.796	36.7	36.76
n-propanol	2	20.33	23.71

2.2.10 HYDROPHOBICITY OF PROTEINS

It is estimated that as much as 40–50% of the accessible surface area of proteins is non-polar. These areas are responsible for the binding of proteins to HIC adsorbents in the presence of moderate to high concentrations of salting-out salts. The strength of this salt-promoted interaction may be predicted from the close relationship between precipitation data for proteins and their relative retention on HIC adsorbents. Since such retention data are not readily available for the large majority of proteins, they must be established from case to case for the protein(s) of interest in a biological sample.

2.2.11 CHOICE OF HIC MEDIA

The type of immobilized ligand, the degree of substitution and the type and concentration of salt and pH used during the adsorption stage have a profound effect on the overall performance (i.e., selectivity and capacity) of a HIC medium. Moreover, the type of matrix used and the coupling chemistry can also influence to a variable degree the binding and elution behaviour of many proteins. The practical implications of these effects are that different HIC media must be compared much more rigorously

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than ion exchange or affinity media, especially when the HIC step is part of a downstream purification process intended for an industrial scale operation.

General considerations

i.) The HIC medium should bind the protein of interest at a reasonably low concentration of salt. This is often dependent on the type of salt chosen, e.g. up to four times higher concentration of NaCl might be necessary to obtain a binding effect comparable to that obtained with ammonium or sodium sulphate. The salt concentration should be below the concentration that causes precipitation of different proteins in the crude feed stock. 1 M ammonium sulphate is a good starting point for screening experiments. If the substance does not bind in e.g. 1 M ammonium sulphate, then choose a more hydrophobic medium. The right choice of a suitable HIC medium can often lead to a lower consumption of salt in the binding buffer. This in turn has a direct bearing on the economic and environmental aspects of the purification process, especially for large-scale HIC applications.

ii.) The bound protein should be eluted from the column with salt-free buffer and with high recovery (75% or higher). If non-polar solvents are required for its elution, try a less hydrophobic medium.

iii.)The pH of the start buffer and the type of salt to use are both parameters that can be exploited to maximize selectivity during the adsorption phase. This is done by checking the adsorption properties of the media at different pH-values and with different types of salts during the screening of different ligands.

iv.) Since hydrophobic interaction is dependent on temperature, it is important that method development work is performed at the intended final working temperature.

2.2.12 OPTIMIZING A HIC STEP

The main purpose of optimizing a chromatographic step is to reach the predefined purity level with highest possible recovery by choosing the most suitable combination of the critical chromatographic parameters. In process applications there

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is also a need to reach the highest possible throughput. The screening experiments outlined previously will mainly help in establishing the most suitable medium to use. The sections below will deal with some important guidelines for optimizing the critical operational parameters which affect the maximum utilization of the HIC step. These parameters include: type of buffer salt, salt concentration, buffer pH, temperature, bed height, flow rate, gradient shape and gradient slope.

2.2.13 THE SOLUTE

As in other adsorption chromatography techniques, the way HIC is used depends on the size of the solute molecule.

Small molecules such as small peptides interact with the medium by single point attachment. Their migration velocity depends directly on the binding constant of a single bond and can vary over a wide interval depending on the ionic strength of the mobile phase. Larger molecules such as proteins and nucleic acids interact with the medium by multi-point attachment. Their migration velocity depends on the sum of several bonds. Thus their velocity is extremely low at all ionic strengths over a certain value. The protein is more or less stuck to the column. Below this ionic strength, the protein is practically not retarded at all .

The interval of eluting strength where a large molecule is partly retarded on the column is thus much smaller than for a small molecule. This means that purifying large molecules on HIC is a typical on-off technique where the difference in retention for the molecules to be separated can be substantial at any specific ionic strength. In other words, separation of large molecules on HIC is a high selectivity technique. The separation should be optimized by manipulating the parameters affecting the selectivity of the system, i.e. optimizing the chemistry of the system by means of salt concentration, type of salt, pH, gradient slopes or stepwise elution schemes. By effecting relatively small changes in selectivity, large changes in resolution can occur.

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When purifying small molecules on the other hand, the selectivity of the system is usually much lower and the requirements for purity might not be met by working on the selectivity alone.

The efficiency parameters such as bed height, bead size, theoretical plates, linear flow rate and sample volume may also have to be optimized.

In conclusion, when purifying large molecules such as proteins, relatively short columns can be used if the selectivity of the adsorbent is exploited in an optimal way. The linear flow rate should, if required, be sufficiently reduced in order to optimize the kinetics of the adsorption and desorption process. This can also be further enhanced by choosing a smaller bead size. Smaller beads will also provide the necessary increace in efficiency when more difficult separation problems are encountered.

2.2.14 THE SOLVENT

This is one of the most important parameters to have a significant influence on the binding capacity and selectivity of a HIC medium. In general, the adsorption process is often more selective than the desorption process and it is therefore important to optimize the starting ''binding'' buffer conditions with respect to critical parameters such as pH, type of salt, concentration of salt and temperature. The combination of salt and pH can be manipulated to give optimum selectivity during purification by HIC. Optimal conditions differ from application to application and are best established by running linear gradients and varying the parameters in a controlled way (for example by using Factorial design). Changes of temperature and pH are sometimes restricted by the stability of the substance of interest or by system constraints etc. but may often be of interest to evaluate. The Hofmeister series (Table 2.6) gives important guidelines in choosing the type of salt to use. The most efficient salts are normally ammonium sulphate and sodium sulphate but also ''weaker'' salts such as sodium chloride should be considered. In an ideal situation, the correct choice of salt and salt concentration will result in the selective binding of the protein of interest while the majority of the

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impurities pass through the column unretarded. If the protein of interest binds weakly to the column, an alternative approach is to choose the starting buffer conditions which will result in the maximum binding of a large proportion of the contaminating proteins but allowing the protein of interest to pass through unretarded. An extension of this strategy is to increase the salt concentration in the unbound fraction to such an extent that the protein of interest binds to the same column in a second run while most of the impurities pass through the column unretarded.

The effect of varying the concentration of salt in the binding buffer on the purification of a monoclonal antibody (IgG1) from mouse ascites fluid is shown in figure 2.6. The column of Alkyl Superose was equilibrated with varying concentrations of ammonium sulphate (2 M to 0.8 M) and its selectivity for the IgG1 investigated. The results show that high selectivity for IgG1 is obtained using 1 M ammonium sulphate in the binding buffer.

It should be pointed out that the higher the salt concentration in the equilibration buffer, the greater the risk that some of the proteins in the sample will precipitate. Since such precipitates can clog tubings and column filters, the sample must be filtered or centrifuged. This extra step can be avoided by equilibrating the sample in a lower salt concentration than is required for its precipitation and then applying it to a column which is equilibrated with a higher salt concentration . Some of the proteins will precipitate on the column (zone precipitation) but they redissolve upon reduction of the salt concentration during stepwise or gradient elution.

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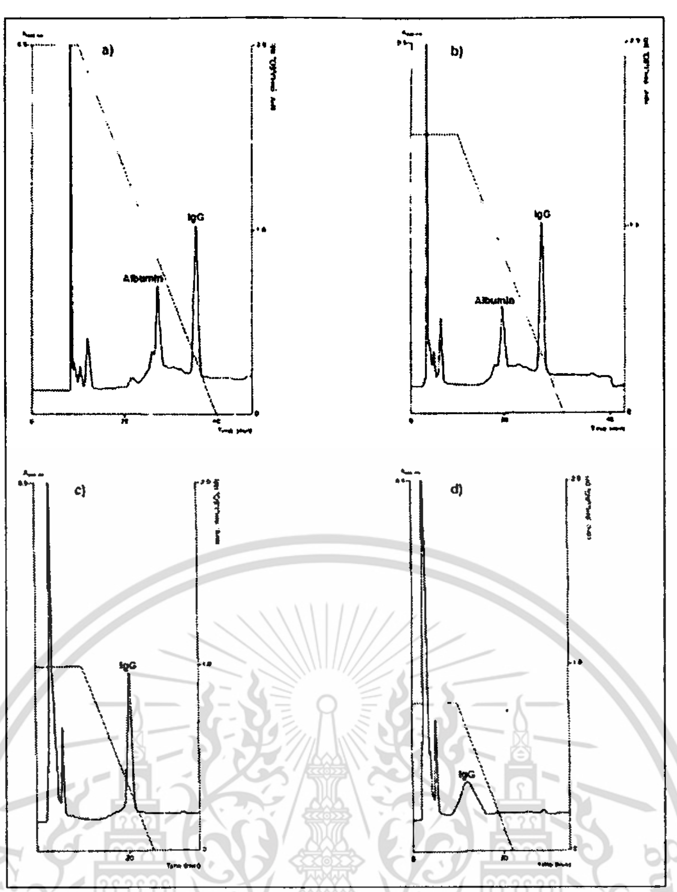

Figure 2.8 The effect of starting conditions in HIC. Sample, 100 µl anti - CEA MAB (-IgG_1) from mouse ascites fluid in 0.8 M $(NH_4)_2SO_4$ (corresponding to 20 µl ascites); column. Alkyl Superose HR 5/5; flow rate, 0.5 ml min -1; buffer A, 0.1 M sodium phosphate, pH 7.0, $(NH_4)_2SO_4$. (a) Sample applied in 2 M $(NH_4)_2SO_4$: both albumin and IgG are absorbed. (b) Sample applied in 1.5 M $(NH_4)_2SO_4$: less albumin binds and IgG elutes earlier in the gradient. (c) Sample applied in 1.0 M $(NH_4)_2SO_4$: albumin does not bind and, therefore, the column has a greater capacity for binding IgG. (d) Sample applied in 0.8 M $(NH_4)_2SO_4$: albumin does not bind; IgG is retarded, but elutes in a broad peak.

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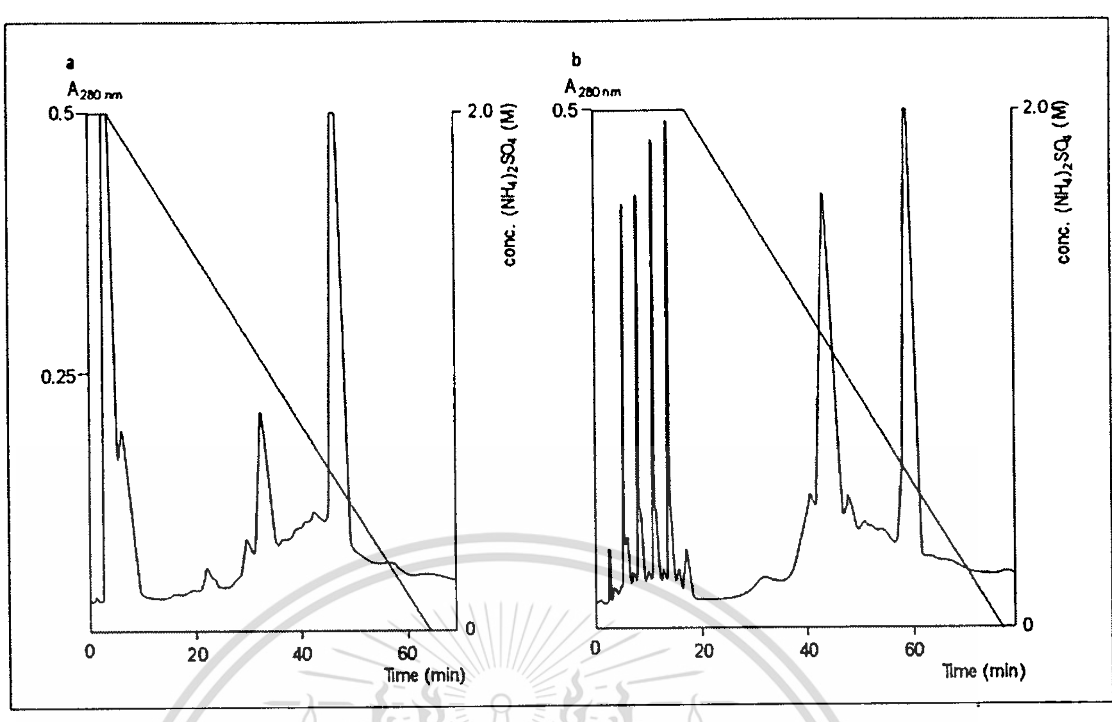

Figure 2.9 The effect of loading conditions in HIC. Column, Alkyl Superose HR 5/5; flow rate, 0.5 ml min-1; buffer A, 0.1 M sodium phosphate, pH 7.0, 2 M $(NH_4)_2SO_4$. (a) Sample (500 µl anti-CEA MAB (IgG_1) from mouse ascites fluid in 0.9 M (NH4)2SO4 (corresponding to 115 µl ascites) applied in one injection. (b) Sample as (a) applied in five 100 µl injections with 1.3 ml 2.0 M $(NH_4)_2SO_4$after each portion.

When sample is applied at a salt concentration lower than that used for equilibration of the column, the sample volume becomes important. This is demonstrated in figure 2.9 a When a 500 ml sample of ascites fluid was applied to a 1 ml column of Alkyl Superose, albumin, the weakest interacting substance, started to elute during sample application (figure 2.9 a). Dividing the sample into portions, e.g. five 100 ml samples and adding equilibration buffer (1.3 ml) after each sample application to enhance the hydrophobic interaction prevented early elution of albumin (Figure 2.9 b).

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2.2.15 ELUTION

This can be achieved by:

(i.) A linear or step-wise decrease of the concentration of salt.

(ii.) Adding various proportions of organic solvents to the elution buffer provided that the protein of interest is stable upon exposure to such solvents. These additives decrease the polarity or surface tension of the eluent resulting in a reduction in the binding strength and the elution of the bound proteins from the column. Usually, 40% ethylene glycol or 30% iso-propanol, dissolved in salt-free buffer, is used. In some applications, it can be advantageous to linearly increase the concentration of such additives as the salt concentration of the elution buffer is simultaneously decreased by a linear gradient. The latter procedure can sometimes lead to increased resolution of the bound proteins.

(iii.) Adding neutral detergents (usually 1%) to the elution buffer. However, some detergents are bound so strongly that they are difficult to wash out completely with common organic solvents (e.g. ethanol). In the worst case, this might lead to a decrease in the capacity of the HIC medium for subsequent applications. These additives must therefore be used with care.

The preferred method of elution is a linear or step-wise decrease of the salt concentation in the elution buffer. Some typical examples are presented below.

2.2.16 GRADIENT ELUTION

Simple linear gradients are the first choice for screening experiments, but when more experience is at hand it might be advantageous to make a gradient more shallow in areas where resolution is inadequate. Consequently, areas where resolution is good can be covered by a steep gradient (Figure 2.9). Such complex gradients offer

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maximum flexibility in terms of combining resolution with speed during the same separation. :

By increasing the total gradient volume (i.e. decreasing gradient slope) of a linear gradient, resolution will be improved in all parts of the chromatogram (Figure 2.10). This is usually not the best approach in preparative mode where the prime issue is not to resolve as many peaks as possible but to separate the compound of interest from the rest of the compounds in the feed material. Increased gradient volume will also give increased cycle time and the separated fractions will also be more diluted.

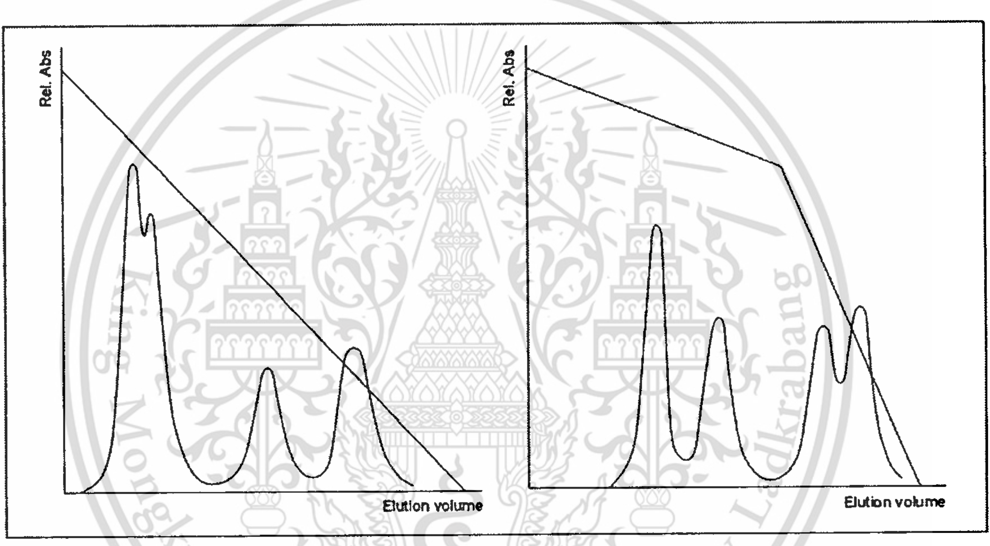

Figure 2.10 Effect of a complex gradient on resolution.

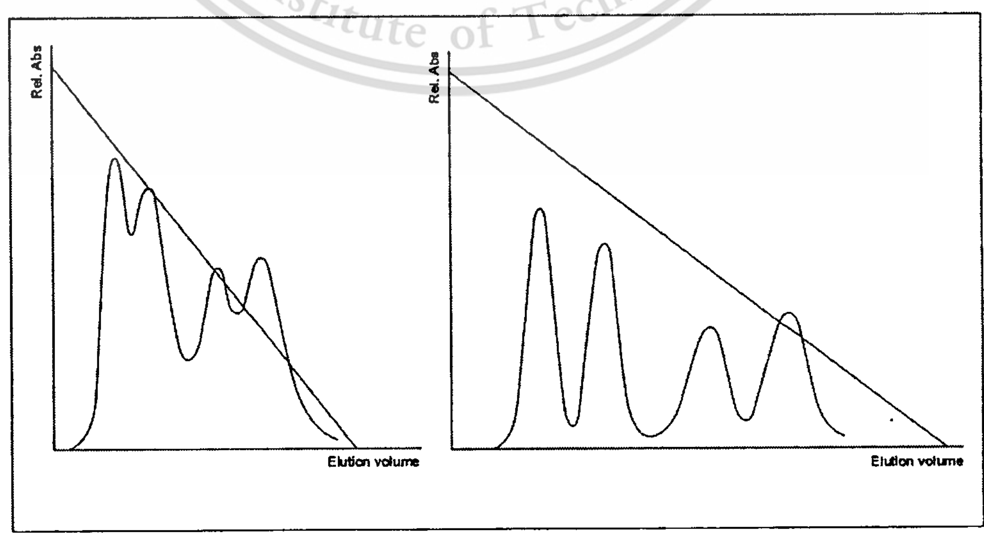

Figure 2.11 Effect of gradient slope on resolution.

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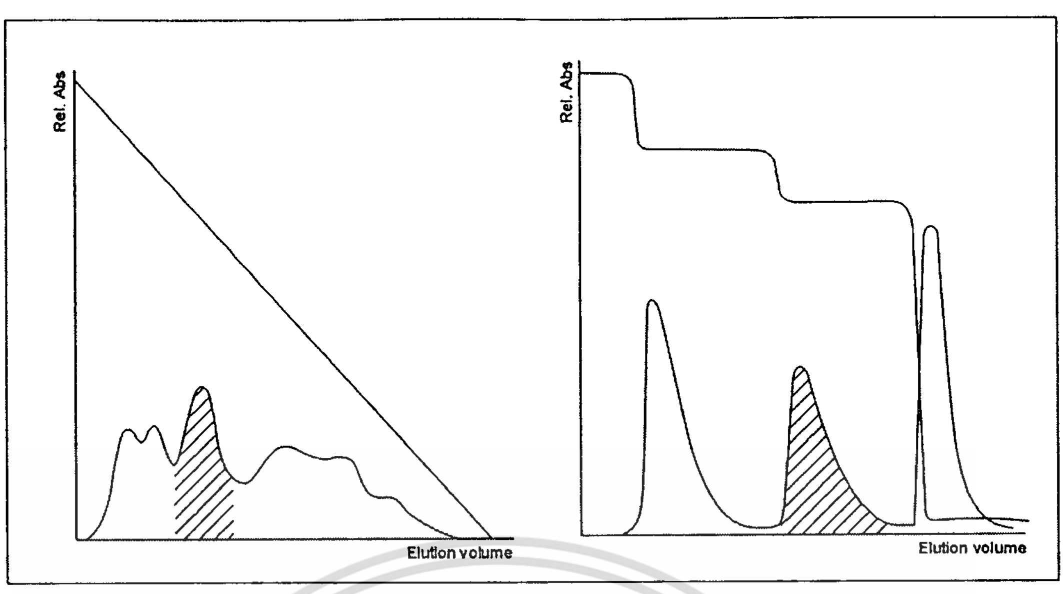

Figure 2.12 Switching from a continuous gradient to step-wise elution.

2.2.17 STEP-WISE ELUTION

Step-wise elution is often preferred in large scale preparative applications since it is technically more simple and reproducible than gradient elution.

Step-wise elution can sometimes be advantageous also in small scale applications since the compound of interest can be eluted in a more concentrated form if the eluting strength of the buffer can be kept high enough without causing co-elution of more strongly bound compounds.

The principle of step-wise elution is to increase resolution in the area where the peak of interest elutes. Figure 2.11 illustrates how a three step increase in eluting strength can be used to obtain maximum resolution of the fraction of interest (shaded peak).

In the first step, the strength and the volume of the elution buffer is optimized to elute all compounds binding less strongly to the gel than the compound of interest. The elution strength and volume of buffer should be large enough to elute these contaminating weaker binding substances, but it must not exceed that level where the peak of interest starts to co-elute with the contaminating compounds.

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In the second step the elution strength is increased to the point where the compound of interest elutes. The elution strength should be large enough to elute the compound of interest without excessive dilution, but must be kept below the level where the more strongly bound contaminating compounds start to co-elute.

In the final step, the elution strength is further increased to elute all of the remaining contaminating compounds. This step can be a very short one with high elution strength.

When step-wise elution is applied, one has to keep in mind the danger of getting artefact peaks when a subsequent step is administered too early after a tailing peak. For this reason it is recommended to use continuous gradients in the initial experiments to characterize the sample and its chromatographic behaviour.

2.2.18 REGENERATION

After each cycle, bound substances must be washed out from the column to restore the original function of the medium. HIC adsorbents can normally be regenerated by washing with distilled water after each run. To prevent a slow build up of contaminants on the column over time, more rigorous cleaning protocols may have to be applied on a regular basis. (See''Cleaning, sanitization and sterilization procedures'').

2.2.19 CLEANING, SANITIZATION AND STERILIZATION PROCEDURES

Cleaning-in-place (CIP) is the removal from the purification system of very tightly bound, precipitated or denatured substances generated in previous purification cycles. In some applications, substances such as lipids or denatured proteins may remain in the column bed instead being eluted by the regeneration procedure. If such contaminants accumulate on the column for a number of purification cycles, they may affect the chromatographic properties of the column. If the fouling is severe, it may also block the column, increasing back pressure and reducing flow rate.

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A specific CIP protocol should be designed according to the type of contaminants that are known to be present in the feed stream.

NaOH is a very efficient cleaning agent that can be used for solubilizing irreversibly precipitated protein and lipid material and in HIC it can be effectively combined with solvent or detergent based cleaning methods.

Sanitization is the inactivation of microbial populations. When a packed column is washed with a sanitizing agent, the risk of contaminating the purified product with viable microorganisms is reduced. The most commonly used sanitization method in chromatography today is to wash the column with NaOH. NaOH has a very good sanitizing effect and also has the additional advantage of cleaning the column.

. Sterilization, which is not synonymous with sanitization, is the destruction or elimination of all forms of microbial life in the system.

Suggested protocols for cleaning-in-place (CIP), santization-in-place (SIP) and sterilization that can be applied to the full range of HIC products outlined in Chapter 3, ''Product Guide'', are summarized in Table 2.9.

The CIP protocols should be used as guidelines to formulate a cleaning protocol specific for the raw material to be applied. The frequency of use will depend on the raw material applied to the column but it is recommended to use a CIP procedure at least every 5 cycles during normal use. Depending on the nature of the contaminants, different protocols may have to be used in combination. If fouling is severe the protocols may have to be further optimized. During CIP the flow direction through the column should be reversed.

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Table 2.9 Suggested CIP, SIP and sterilization protocols for HIC media from Amersham Biosciences.

Purpose	**Procesure**
Removal of precipitated proteins	4 bes volumes of 0.5-1.0 M NaOH at 40 cm/h followed by 2-3 bed volumes of water
Removal of strongly bound hydrophobic proteins, lipoproteins and lipids	4-10 bed volumes of up to 70% ethanol or 30 % isopropanol followed by 3-4 bed volumes of water.(Removal of 20% ethanol from Phenyl Sepharose Fast Flow (high sub) is show in figure 2.11 or 1-2 bed volumes of 0.5% non-ionic detergent (e.g. in 1 M acetic acid) followed by 5 bed volumes of 70% ethanol,to remove the detergent, and 3-4 bed volumes of water
Sanitization	0.5-1.0 M NaOH with a contact time of 30-60 min
sterilization	autoclave the medium at 120°C for 20 min

Note: Detergents should be used with care since they work as displacers and may sometimes bind so hard to the gel that it affects the binding capacity during subsequent purification cycles.

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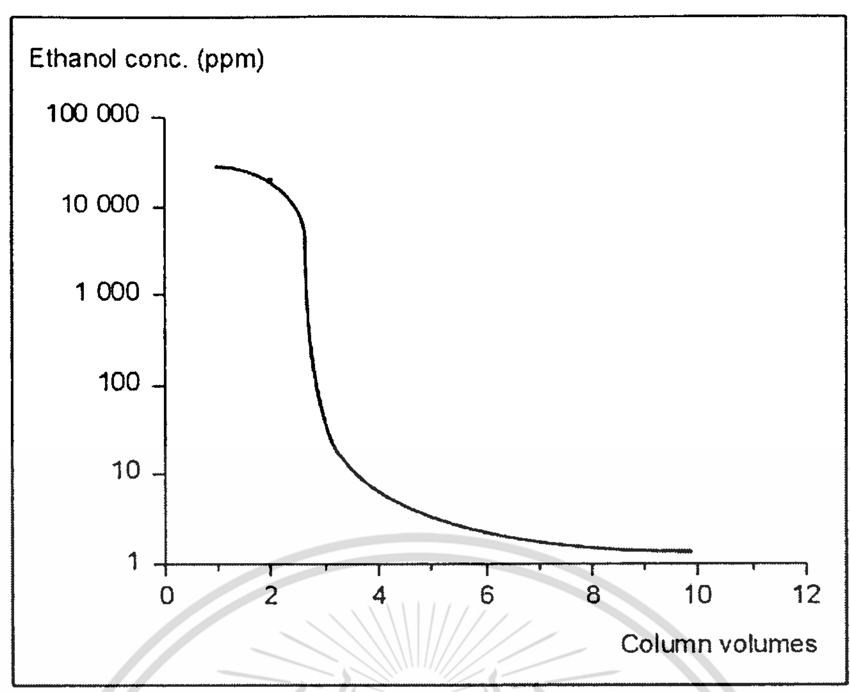

Figure 2.12 Removal of 20% ethanol from Phenyl Sepharose 6 Fast Flow (high sub) in an HR 10/10 Column, bed volume 8 ml; mobile phase H_2O; flow rate 1 ml/min.

2.2.20 STORAGE OF GELS AND COLUMNS

Prevention of microbial growth

Steps should always be taken to prevent bacterial growth in columns during storage. Microbial growth can seriously interfere with the chromatographic properties of the column and contaminate the purified product with microorganisms and endotoxins or other pyrogenic material. During storage, an antimicrobial agent should always be added to the chromatographic media. Antimicrobial agents may be eluted from columns before chromatographic runs or they may be present in the eluent during chromatography. Antimicrobial agents which interact with sample substances must be avoided if they are to be used in eluents, otherwise any agent which does not interact with the gel may be used. Some of the more commonly used antimicrobial agents are described below.

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Antimicrobial agents

i.) Sodium hydroxide

Sodium hydroxide, 0.01 M, is an effective bacteriostatic agent and is, besides 20% ethanol, the main recommendation for storage of HIC media from Amersham Biosciences. At higher concentrations (0.5–1.0 M) it is an effective sanitizer for contaminated columns. For the most frequent contaminants in chromatographic systems, such as gram-negative bacteria, a good bactericidal effect is reached even at such low concentrations as 0.01 M NaOH

NaOH is a widely accepted agent for maintaining chromatographic columns and systems since it not only gives efficient sanitization but also effectively destroys endotoxin (LPS) and solubilizes precipitated and denatured substances that have accumulated on the column. An additional advantage is the lack of toxicity as a contaminant in the end product.

ii.) Ethanol 20%

Chromatography media from Amersham Biosciences are supplied as a suspendsion containing 20% ethanol. 20% ethanol can also be used as an alternative to NaOH for storing chromatography media under bacteriostatic conditions.

iii.) Chlorhexidine

Chlorhexidine (e.g. Hibitane™) is a very efficient bacteriostatic agent that inhibits the growth of many bacteria at a concentration of 0.002%. The effect against fungi is less pronounced, but the growth of many types can be inhibited by concentrations between 0.01% and 0.1%. Hibitane is incompatible with only a very few substances.Precipitation may occur on storage of Hibitane in solutions with appreciable concentrations of chloride or sulphate ions.

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iv.) Phenyl mercuric salts

Phenyl mercuric salts (acetate, nitrate, borate) are most efficient as bacteriostatics in weakly alkaline solutions. Concentrations recommended are from 0.001% to 0.01%. (The use of mercury containing antimicrobial agents is on the decline because of their toxicity. When used in a manufacturing process they may have to be proved absent in the end product.)

v.) Thimerosal

Thimerosal (ethylmercuric thiosalicylate e.g. Merthiolate™) is a bacteriostatic most efficient in weakly acidic solutions. Concentrations recommended are from 0.005% to 0.01%. It is bound to and inactivated by substances containing thiol groups. (The use of mercury containing antimicrobial agents is on the decline because of their toxicity. When used in a manufacturing process they may have to be proved absent in the end product.)

vi.) Trichlorobutanol

Trichlorobutanol (e.g. Chloretone™) is another bacteriostatic showing highest efficiency in weakly acidic solutions. Concentrations recommended are from 0.01% to 0.05 %

vii.) Sodium azide

Sodium azide is a very widely used bacteriostatic agent giving a high bacteriostatic effect at a concentration of 0.02%–0.05%.

Note: The use of sodium azide is discouraged in many countries since it forms explosive insoluble salts with heavy metals and it is believed to be a mutagen.

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Storage of unused media

Unused media should be stored in closed containers at a temperature of +4°C to +25°C. Note that it is important that the media are not allowed to freeze as the structure of the beads may be disrupted by ice crystals. This disruption will generate fines.

Storage of used media

Used media should be stored at a temperature of +4°C to +8°C in the presence of a suitable bacteriostatic agent, e.g. 0.01 M NaOH or 20% ethanol. Note that it is important that the media are not allowed to freeze as the structure of the beads may be disrupted by ice crystals. This disruption will generate fines.

Storage of packed columns

Packed columns should be stored at a temperature of +4°C to +8°C in the presence of a suitable bacterostatic agent, e.g. 0.01 M NaOH or 20% ethanol.

For long-term storage, the packed column should be thoroughly cleaned (CIP) before equilibration with the storage solution.

Recycling the storage solution through the column or flushing the column once a week with fresh storage solution is recommended to prevent bacterial growth.

2.3 BAND BROADENING AND COLUMN EFFICIENCY [9]

To obtain optimal separations, sharp, symmetrical chromatographic peaks must be obtained. This means that band broadening must be limited. It is also beneficial to measure the efficiency of the column.

2.3.1 THE THEORETICAL PLATE MODEL OFCHROMATOGRAPHY

The plate model supposes that the chromatographic column is contains a large number of separate layers, called *theoretical plates*. Separate equilibrations of the sample between the stationary and mobile phase occur in these "plates". The analyte

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moves down the column by transfer of equilibrated mobile phase from one plate to the next.

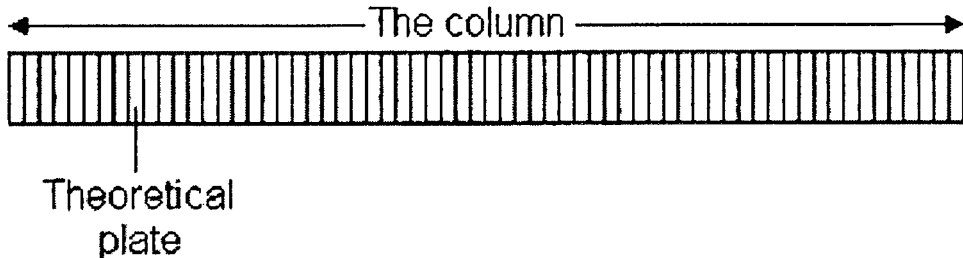

Figure 2.14 Theoretical plate model.

It is important to remember that the plates do not really exist; they are a figment of the imagination that helps us understand the processes at work in the column.They also serve as a way of measuring column efficiency, either by stating the number of theoretical plates in a column, N (the more plates the better), or by stating the plate height; the *Height Equivalent to a Theoretical Plate* (the smaller the better).

If the length of the column is L, then the HETP is

$$\text{HETP} = L / N \quad (2.2)$$

The number of theoretical plates that a real column possesses can be found by examining a chromatographic peak after elution;

$$N = \frac{5.55\, t_R^{\,2}}{w_{1/2}^{2}} \quad (2.3)$$

where $w_{1/2}$ is the peak width at half-height.

As can be seen from this equation, columns behave as if they have different numbers of plates for different solutes in a mixture.

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2.3.2 THE RATE THEORY OF CHROMATOGRAPHY

A more realistic description of the processes at work inside a column takes account of the time taken for the solute to equilibrate between the stationary and mobile phase (unlike the plate model, which assumes that equilibration is infinitely fast). The resulting band shape of a chromatographic peak is therefore affected by the rate of elution. It is also affected by the different paths available to solute molecules as they travel between particles of stationary phase. If we consider the various mechanisms which contribute to band broadening, we arrive at the Van Deemter equation for plate height;

$$\mathrm{HETP} = A + B/u + Cu \tag{2.4}$$

where u is the average velocity of the mobile phase. A, B, and C are factors which contribute to band broadening.

***A* - Eddy diffusion**

The mobile phase moves through the column which is packed with stationary phase. Solute molecules will take different paths through the stationary phase at random. This will cause broadening of the solute band, because different paths are of different lengths.

***B* - Longitudinal diffusion**

The concentration of analyte is less at the edges of the band than at the center. Analyte diffuses out from the center to the edges. This causes band broadening. If the velocity of the mobile phase is high then the analyte spends less time on the column, which decreases the effects of longitudinal diffusion.

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C **- Resistance to mass transfer**

The analyte takes a certain amount of time to equilibrate between the stationary and mobile phase. If the velocity of the mobile phase is high, and the analyte has a strong affinity for the stationary phase, then the analyte in the mobile phase will move ahead of the analyte in the stationary phase. The band of analyte is broadened. The higher the velocity of mobile phase, the worse the broadening becomes.

2.3.3 VAN DEEMTER PLOTS

A plot of plate height vs. average linear velocity of mobile phase.

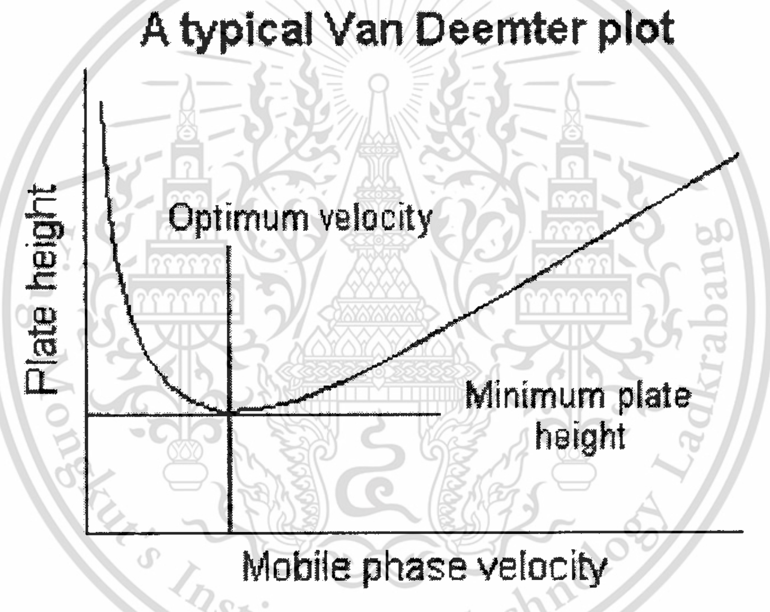

Figure 2.15 A typical of Van Deemter plot.

Such plots are of considerable use in determining the optimum mobile phase flow rate.

2.3.4 RESOLUTION

Although the selectivity factor, a, describes the separation of band centres, it does not take into account peak widths. Another measure of how well species have been separated is provided by measurement of the *resolution*. The resolution of two species, A and B, is defined as

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$$R = \frac{2[(t_R)_B - (t_R)_A]}{W_A + W_B} \tag{2.5}$$

Baseline resolution is achieved when $R = 1.5$

It is useful to relate the resolution to the number of plates in the column, the selectivity factor and the retention factors of the two solutes;

$$R = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{1 + k'_B}{k'_B}\right) \tag{2.6}$$

To obtain high resolution, the three terms must be maximised. An increase in N, the number of theoretical plates, by lengthening the column leads to an increase in retention time and increased band broadening - which may not be desirable. Instead, to increase the number of plates, the height equivalent to a theoretical plate can be reduced by reducing the size of the stationary phase particles.

It is often found that by controlling the capacity factor, k', separations can be greatly improved. This can be achieved by changing the temperature (in Gas Chromatography) or the composition of the mobile phase (in Liquid Chromatography).

The selectivity factor, a, can also be manipulated to improve separations. When a is close to unity, optimising k' and increasing N is not sufficient to give good separation in a reasonable time. In these cases, k' is optimised first, and then a is increased by one of the following procedures:

1. Changing mobile phase composition
2. Changing column temperature
3. Changing composition of stationary phase
4. Using special chemical effects (such as incorporating a species which complexes with one of the solutes into the stationary phase)

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2.4 LITERATURE REVIEWS

P. He, S.S. Devis and L. Illum (1999) studied non-crosslinked and crosslinked chitosan microspheres were prepared by a spray drying method. The microspheres so prepared had a good sphericity and a smooth but distorted surface morphology. They were positively charged. The particle size ranged from 2 to 10m. The size and seta potential of the particles were influenced by the crosslinking level. With decreasing amount of crosslinking agent, both particle size and zeta potential were increased. Preparation conditions also had some influence on the particle size.

R. Agarwal and M N. Gupta. (1995) studied gluteraldehyde modified chitosan was investigated as matrix for hydrophobic interaction chromatography, by studying binding of six different enzymes/proteins to the matrix. In all cases, except one, there was a substantial increase in the binding of enzyme activity to chitosan after modification with gluteraldehyde. Thus modified chitosan described here can be used for hydrophobic interaction chromatography as successfully illustrated with alkaline phosphatase.

Y. Wu, Y. Dong, L. Chen, J. Huang and J. Li. 2002. studied a series of derivatives of chitosan - *N*-alkyl(methyl, ethyl, propyl and butyl) chitosans - were synthesized from completely deacetylated chitosan. The degree of substitution (from 0.15 to 0.81) of the *N*-ethyl chitosan were obtained by controlling the molar ratio of the reactants. All the products showed lyotropic liquid-crystalline properties regardless of the length of the side chains and the degree of substitution.

Y. C. Shi, Y. M. Jiang, D. X. Sui, Y. L. Li, T. Chen, L. Ma. 1996. studied chitosan beads were prepared for use as affinity adsorbent carrier. The affinity ligand, chicken ovomucoid, was immobilized on the chitosan via a cross-linker, glutaraldehyde.The procedure for preparing the chitosan-based affinity adsorbents was much safer and simpler than when a Sepharose-based matrix was the support.The

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experimental results revealed that the affinity adsorbents possessed good mechanical strength and storage stability and could be also operated repeatedly. Chitosan was suitable for use as an affinity adsorbent support for laboratory-scale and large-scale purification.

B. Krajewska. (2004) studied functional materials,chitin and chitosan offer a unique set of characteristics : biocompatibility, biodegradability to harmless products, nontoxicity, physiological inertness, antibacterial properties, heavy metal ions chelation, gel forming properties and hydrophilicity, and remarkable affinity to proteins. Owing to these characteristics, chitin-and chitosan-based materials,as yet underutilized, are predicted to be widely exploited in the near future especially in environmentally benign applications in system sworking in biological environments, among others as enzyme immobilization supports.

Y. Wang, M. Guo and Y. Jiang (2002) studied n-valeraldehyde modified Chitosan (pentyl-Chitosan CL) was prepared by Schiff-base formation and hydrogenation.By studying the IR spectra of Chitosan and pentyl-Chitosan CL, it is suggested that a pentyl group is linked to $2'$-NH_2 by a C–N bond. The influence of temperature and ionic strength on the adsorption of protein on pentyl-Chitosan CL were studied, and it was found that the behavior of adsorption met with the theory of hydrophobic interaction.

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CHAPTER 3

EXPERIMENTAL

3.1 CHEMICALS

1. Sodiumhydroxide (NaOH), >99.0 % (w/w), A.R. grade, ANALYTICAL SCIENCES (LAB_SCAN).
2. Hydrochloric acid (HCl), 37% (w/v), A.R. grade, ANALYTICAL SCIENCES (LAB_SCAN).
3. Glutaraldehyde ($C_5H_8O_2$), 50% in water, A.R. grade, Fluka Chemika.
4. Acetic acid (CH_3COOH), A.R. grade, ANALYTICAL SCIENCES (LAB_SCAN).
5. Acetone (CH_3COCH_3), A.R. grade, CARLO ERBA REAGENTI.
6. Acetronitrile (C_2H_3N), HPLC grade, ANALYTICAL SCIENCES (LAB_SCAN).
7. Ethanol (C_2H_6O), HPLC grade, CARLO ERBA REAGENTI.
8. n-Butyraldehyde (C_4H_8O), >= 99 %, A.R. grade, Mearck KGaA.
9. Sodium boro hydride ($NaBH_4$), >=95%, CARLO ERBA REAGENTI.
10. Bovine serum albumin, Sigma-Aldrich.
11. lysozyme, Sigma-Aldrich.
12. Benzamide (C_7H_7NO), A.R. grade, Sigma-Aldrich.
13. Sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$), >=98.0%, CARLO ERBA REAGENTI.
14. Di-sodium dihydrogen phosphate heptahydrate ($Na_2HPO_4.7H_2O$), A.R. grade, Mearck KGaA.
15. Sodium sulphate crystals (Na_2SO_4), A.R. grade, CARLO ERBA REAGENTI.

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3.2 EQUIPMENTS

1.	Fourier transforms infrared spectrometer	: FTIR spectrum GX, Perkin Element.
2.	Spray dryer	: L-8, Ohkawara Kakohki Co., Ltd.
3.	Scanning Electrone Microscope	:1455VP/EDAX, LEO
4.	Surface area analyzer	: Autosorb-1,Quana chrome
5.	Particle size analyzer	: Masterzizer X,MaL Y≡RN
6.	HPLC empty column i.d. 4.6 x 150 mm	: 316 stainless steel
7.	HPLC packing pump	: Model CP Lab Alliance
8.	High Performance Liquid Chromatography	: Waters 510 HPLC pump / Waters 486 Tunable absorbance detector, MILLIPORE.
9.	High Performance Liquid Chromatography	: Shimadzu C-R7A plus chromato-pac / Shimadzu LC-10 AD vp solvent delivery module.
10.	X-ray Diffractometer; XRD	: 08 Advance,Bruker AG

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3.3 RESEARCH METHODOLOGY

Step 1 : Preparation of chitosan microspheres from shrimp shell.

- Preparation chitosan from shrimp shell.
- Chitosan characterization (e.g., % DD, IR spectrum).
- Optimized condition for preparation of chitosan crosslinked microspheres by spray drying technic.
- Chitosan microspheres characterization (e.g., partical size, pore size and surface area).

Step 2 : Synthesis of n-butyl - chitosan crosslinked microspheres.

- Study of synthesis process of n-butyl - chitosan crosslinked microspheres for HPHIC.
- Characterization of n-butyl-chitosan crosslinked microspheres that produced.

Step 3 : Column packing process.

- Packing of HPHIC column.

Step 4 : Chromatographic evaluation.

- The column efficiency testing.
- The linearity (or dynamic range) testing.
- The separation efficiency testing.

Step 5 : Adsorption of protein on n-butyl-chitosan crosslinked microspheres.

- The effect of salt concentration on the adsorption of protein.
- The effect of temperature on the adsorption of protein.

Step 6 : Storage stability of n-butyl-chitosan crosslinked microspheres.

- Study of n-butyl-chitosan microspheres was stored under different condition.

Step 7 : Conclusion.

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3.4 PROCEDURES

3.4.1 PREPARATION OF CHITOSAN

3.4.1.1 Purification of chitin

Step 1 Deproteinization

Frozen shrimp shells were deproteinization with 1% NaOH in the ratio 1:10 (w/v) at 65° C for 1 hours. The deproteinization shell were washed will deionized water until the filtrate was neutral.

Step 2 Demineralization

The deproteinization shell were then demineralization with 0.75N HCl solution in the ratio 1:12 (w/v) at room temparature for 0.5 hours. The demineralization shell were washed will deionized water until the filtrate was neutral.

Step 3 Decoloration

Pigments can be removed with acetone at room temperature overnight.

3.4.1.2 Deacetylation of chitin

Chitin was deacetylated with 50% (w/v) NaOH in the ratio 1:10 (w/v) at 95° C for 3 hours. After reaching the reaction time chitosan was washed with deionized water until the filtrate was neutral and dried in a 60 ^{o}C oven.

3.4.2 PHYSICOCHEMICAL CHARACTERIZATION

3.4.2.1 Infrared spectroscopy

Infrared spectra were examined by using a Fourier transform infrared spectro-photometer (FTIR spectrum GX, Perkin Elment). The spectrum was recorded with a KBr pressed disk.

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3.4.2.2 Degree of deacetylation of chitosan determination

The degree of deacetylation of chitosan determination by infrared spectroscopy method. The degree of deacetylation was calculated using the equation

$$\text{Degree of deacetylation(\%)} = 100 - [(A_{1655}/A_{3450}) \times 100/1.33]$$

3.4.3 PREPARATION OF CHITOSAN CROSSLINKED MICROSPHERES

The required volume (usually 250 mL) of a 0.1-0.5 % aqueous solution of chitosan. Chitosan flakes were dissolved in acetic acid (2%, v/v) and then filtered with filter paper (Whatman No. 541) to remove insoluble materials. Variable amounts of glutaraldehyde were added to the chitosan solution (table 3.1) and stirred at room temperature for 0.5 hours. The crosslinked chitosan thus obtained was then introduced into the spray dryer by using peristaltic pump. The spray drier condition were

Inlet temperature : 130 ^{o}C
Exhaust air temperature : 80 ^{o}C
Pump rate : 20 mL/min.

Study of optimized condition for preparation of chitosan microspheres as a stationary phase are summarized as a table and showed in table 3.1.

3.4.4 PREPARATION OF n-BUTYL-CHITOSAN CROSSLINKED MICROSPHERES FOR HPHIC COLUMN

About 10 g of chitosan crosslinked microspheres was treated with 50 mL 5M $NaBH_4$ overnight. After extensive washing, the chitosan crosslinked microspheres was suspended in 100 mL water, and variable amounts of n-butyraldehyde was then added and stirred at room temperature to obtain the butyl-chitosan crosslinked microspheres. The n-butyl-chitosan crosslinked microspheres was treated with 50 mL $NaBH_4$. After 12 hours, the butyl-chitosan crosslinked microsphere was dried at 60 ^{o}C before packing into HIC column.

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In this study, factors that could effect the change of hydrophobicity were also investigated. They are choices of reaction time and amounts of n-butyraldehyde (Table 3.2).

Table 3.1 Preparation parameters of chitosan microspheres

Preparation parameters	Concentration of chitosan (%(w/v))	Crosslinking agent (mL)	Atomizer speed set (rpm)
1. Concentration of chitosan (%(w/v))	0.2	Glutaraldehyde(25%(v/v)) 3	35000
	0.5	Glutaraldehyde(25%(v/v))3	35000
	1	Glutaraldehyde(25%(v/v))3	35000
	2	Glutaraldehyde(25%(v/v))3	35000
2. Concentration of glutaraldehyde	0.5	Glutaraldehyde(5%(v/v))3	35000
	0.5	Glutaraldehyde(10%(v/v))3	35000
	0.5	Glutaraldehyde(25%(v/v))3	35000
	0.5	Glutaraldehyde(50%(v/v))3	35000
3. Amounts of glutaraldehyde 50%(v/v)	0.5	1	35000
	0.5	2	35000
	0.5	3	35000
	0.5	4	35000
4. Atomizer speed	0.5	3	25000
	0.5	3	30000
	0.5	3	35000
	0.5	3	40000

Table 3.2 Synthesis parameters of n-butychitosan crosslinked.

Factors	Various of studies
Reaction time	4,8,12,16 and 20 hours
Amount of n-butyraldehyde	2,4,6,8,10,12 and 14 mL

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3.3.5 CHARACTERIZATION OF n-BUTYL-CHITOSAN CROSSLINKED MICROSPHERES

The functional group and chemical structure of n-butyl-chitosan crosslinked microspheres were characterized using FTIR. The morphology of n-butyl-chitosan crosslinked particle was determined using SEM. Particle size distribution was measured using light scattering analysis technique. Surface area and pore size distribution were measured using AUTOSORB-1. Crystal structure of n-butyl-chitosan crosslinked microspheres were characterized using XRD.

3.4.6 PACKING OF CHROMATOGRAPHIC COLUMN

Preparation of the gel

The n-butyl-chitosan crosslinked microspheres was pre-swollen in 20% ethanol. Prepare a slurry by decanting the 20% ethanol solution and replace it with deionize water (packing solution) in a ratio of 10% settled gel to 90% packing solution. The gel was packed into a 4.6 mm diameter stainless steel column to a bed height of 150 mm.

Packing

This can be achieved by

(1) Equilibrate all materials to the temperature at which the chromatography will be performed.

(2) De-gas the gel slurry to minimize the risk of air bubbles in the packed bed.

(3) Pour the slurry into the reservoir of the packing system in one continuous motion (minimize the introduction of air bubbles) and connect the column to pump. Immediately fill the remainder of the column with packing solution and the system was topped off.

(4) Pack with a flow rate of 10 mL/min using a pneumatic HPLC packing pump for 20-60 min.

(5) After packing, a few minutes were allowed to reduce flow rate to return to atmospheric pressure.

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(6) The packed column was disconnected from the packing system, the excess of stationary phase on the top of the column was carefully removed and finally the inlet frit and end-fritting were installed and the ends plugged.

(7) The column was conditioned for 5 hours with mobile phase at a flow rate of 0.5 mL/min.

3.4.7 CHROMATOGRAPHIC EVALUATION

3.4.7.1. The chromatographic tests were performed

The chromatographic tests were performed using a modular HPLC system with a Waters 510 pump and a Water 486 tunable wavelength absorbance detector. All experiments were carried out at room temperature, with detection at 254 nm. The injection volume was 10 μL. All solvents were fillered and degassed before use.The Van Deemter's plot was tested with standard benzamide (100 ppm). The linearity testing was tested by varying the concentration of standard benzamide (40 to 200 ppm).

3.4.7.2. Separation of model proteins

The separation of model proteins were performed using a Shimadzu C-R7A plus chromatopac / shimadzu LC-10 AD vp solvent delivery module. The hydrophobic inteaction chromatography column was equilibrated with 5 m*M* phosphate buffer (pH 8.1) containing 10% sodium sulfate. The protein mixture was dissolved with equilibrated buffer to obtain 0.25 mg/mL lysozyme and 0.25 mg/mL bovine serum albumin. A 1 μL volume of protein mixture was loaded. Gradient elution : 0 - 6 min, buffer A; 6 - 9 min, buffer B; 8 - 11 min, buffer C; 11 - 16 min, buffer D. Buffer A : 5 m*M* phosphate buffer containing 10% sodium sulfate; buffer B : 5 m*M* phosphate buffer containing 8% sodium sulfate; buffer C : 5 m*M* phosphate buffer containing 5% sodium sulfate; buffer D : 5 m*M* phosphate buffer containing.The proteins were detected spectrophotometrically at 280 nm.

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3.4.8 ADSORPTION OF PROTEIN ON n-BUTYL-CHITOSAN CROSSLINKED MICROSPHERES

Lysozyme 1 mg was added to 4 mL of n-butyl-chitosan microspheres in phosphate buffer (20 mM , pH 7), under different condition (i.e.,different temperature and salt concentration) and the total volume was made to 8 ml by the same buffer. The mixture was incubated at 30 °C for 2 h and then centrifuged at 35000 rmp for 5 min. The protein in the supernatant was measured to calculate the total bound protein. (Measure the absorbance of the solution at 562 nm by UV-VIS spectroscopy). The amount of protein was determined by comparison of absorbance of the samples to a calibration curve.

Study of effect of salt concentration and effect of temperature on the adsorption of protein on n-butyl-chitosan crosslinked are summarized as a table and showed in table 3.3.

Table 3.3 Condition for study of effect of salt concentration and effect of temperature on the adsorption of protein on n-butyl-chitosan crosslinked.

Factors	Various of studies
Salt concentration (sodium sulphate)	2.5,5,7.5,10,12.5, and 15 (%w/w)
Temperature	30, 45, 60, 75 and 90 (°C)

3.4.9 STORAGE STABILITY OF n-BUTYL CHITOSAN CROSSLINKED MICROSPHERES

n-Butyl-Chitosan crosslinked microspheres was stored under two different conditions, after 100 day storage in 20% ethanol at 4°C and stored in distilled water. Lysozyme 1 mg was added to 4 mL of n-butyl-chitosan microspheres in phosphate buffer (20 mM , pH 7), stored under different condition and the total volume was made

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to 8 ml by the same buffer. The mixture was incubated at 30 °C for 2 h and then centrifuged at 35000 rmp for 5 min. The protein in the supernatant was measured to calculate the total bound protein (measure the absorbance of the solution at 562 nm by UV-VIS spectroscopy). The amount of protein was determined by comparison of absorbance of the samples to a calibration curve.

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CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 CHITOSAN PREPARATION

Chitosan preparation from chitin according to the methods outline in section 3.4.1. The chitin was deacetylated in 50% (w/v) NaOH solution at 120°C for 3 hours. The functional group of chitosan were determined by using FTIR.

4.1.1 INFRARED SPECTROSCOPY

The infrared spectrum of chitosan are demonstrated in figure 4.1. The spectrum show a peak at about 1655 cm^{-1} for C=O stretching and 1559 cm^{-1} for N-H bending. From this observation, the adsorbtion peak at about 1655 cm^{-1} in chitosan associated with the degree of deacetylated was determined according to the method outlined section 3.4.2.1. The lower the 1655 cm^{-1} peak (The lower the amount of carbonyl group), the higher the degree of deacetylation.

4.1.2 DEGREE OF DEACETYLATION

Degree of deacetylation of chitosan was determined by infrared spectroscopy method (outlined section 3.4.2.1). The degrees of deacetylation of chitosan were 79.74 percent (Degree of deacetylation(%) $= 100 - [(A_{1655}/A_{3450}) \times 100/1.33]$).

4.2 OPTIMIZED CONDITION FOR PREPARATION OF CHITOSAN MICROSPHERES

Chitosan crosslinked microspheres prepared by glutaraldehyde crosslinking reaction and a spray drying method (outline in section 3.4.3). The morphology of the chitosan microspheres were determined using scanning electron microscope (1455 VP/ EDAX LEO). In order to selects the optimum particle size that have high efficiency for HPHIC using, the particle size should be uniform shape in range of 3-10 μm.

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Scanning electron micrographs of chitosan crosslinked microspheres and their surface morphology are shown below in table 4.1 to table 4.4.

The optimum preparation condition of chitosan microspheres were 0.5 % (w/v) chitosan solution, 50 % (v/v) aqueous solution of glutaraldehyde 3 mL and atomizer speed of spray dryer was 35000 rpm. The chitosan crosslinked particle were average size of microspheres was 2 to 10 μm (in diameter), smooth surface and spherical in shape.

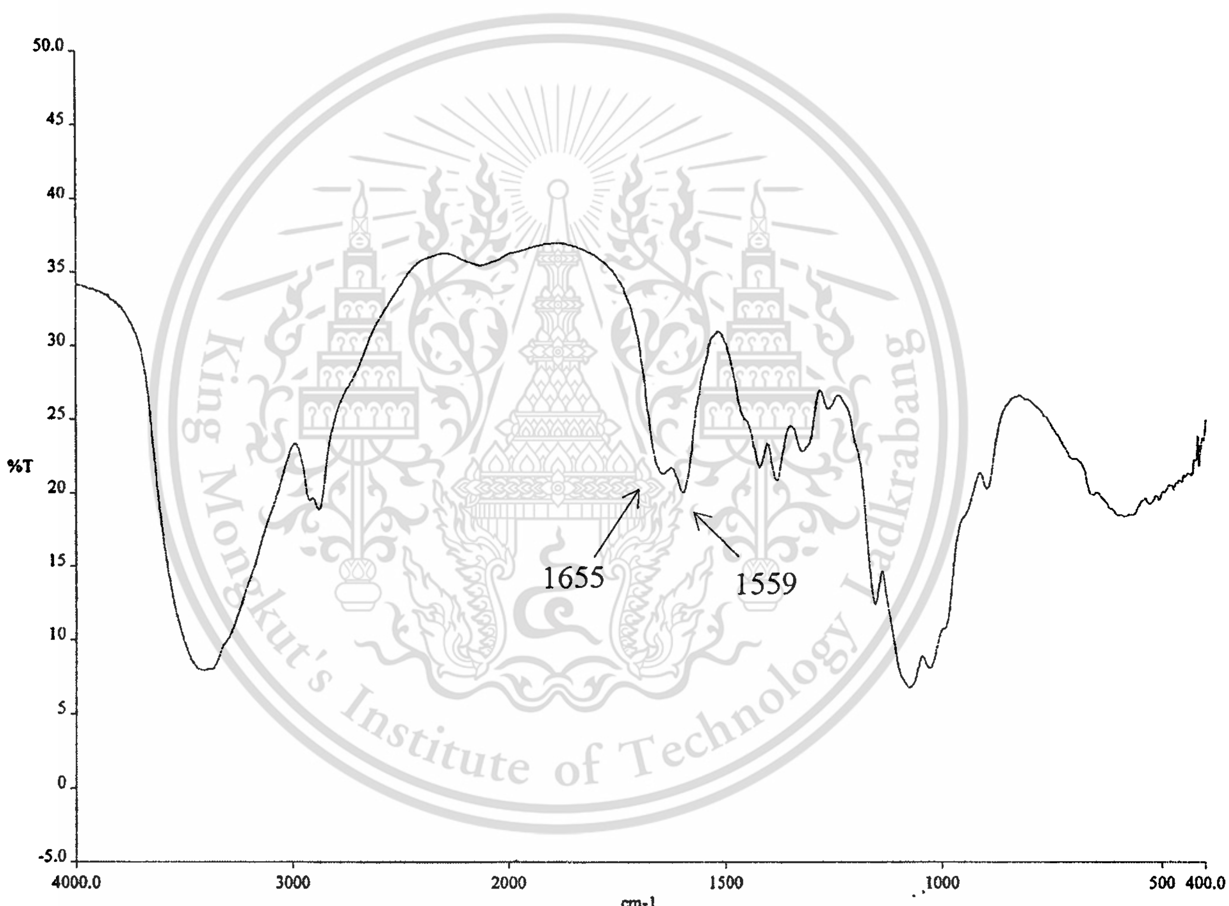

Figure 4.1 Infrared spectrum of chitosan from shrimp shells.

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Table 4.1 Effect of concentration of chitosan for preparation of chitosan crosslinked microspheres.

Concentration of chitosan(% w/v)	Scanning electron micrographs
0.2	
0.5	
1.0	The chitosan solution was changed in gel, so it can not introduced into the spray dryer.
2.0	The chitosan solution was changed in gel, so it can not introduced into the spray dryer.

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Table 4.2 Effect of glutaraldehyde (%v/v) for preparation of chitosan crosslinked microspheres.

Glutaraldehyde (% v/v)	Scanning electron micrographs
5%	
10%	
25%	
50%	

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Table 4.3 Effect of amount of glutaraldehyde for preparation of chitosan crosslinked microspheres.

Amount of glutaral-dehyde 50 (% v/v)	Scanning electron micrographs
1 mL	
2 mL	
3 mL	
4 mL	

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Table 4.4 Effect of atomizer speed for preparation of chitosan crosslinked microspheres.

Atomizer speed (rpm)	Scanning electron micrographs
25000	
30000	
35000	
40000	

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4.3. PREPARATION OF n-BUTYL-CHITOSAN CROSSLINKED MICROSPHERES FOR HPHIC COLUMN

In this study, n-butyraldehyde were selected to react with the amino group of chitosan in order to modify the hydrophobicity of the chitosan crosslinked microspheres (Figure 4.2).

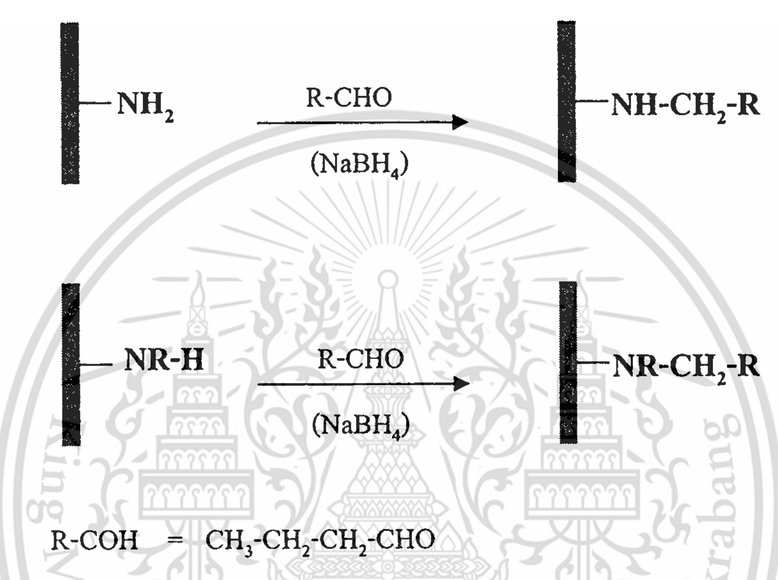

Figure 4.2 Reaction between amino groups on chitosan crosslinked microspheres and n-butyraldehyde.

Hydrophobicity modification of chitosan microspheres was studied by attaching n-butyraldehyde on amino group. The functional groups of n-butyl-chitosan crosslinked microspheres were determined by using ATR-FTIR. In this study, factors that could effect the change of hydrophobicity were also investigated. The reaction time and amounts of n-butylraldehyde were choosed for study (outline section 3.4.3). The results are shown in figure 4.3 and figure 4.4. The optimum preparation condition of n-butyl-chitosan crosslinked were 12 hours of reaction time and 12 mL of n-butyraldehyde .

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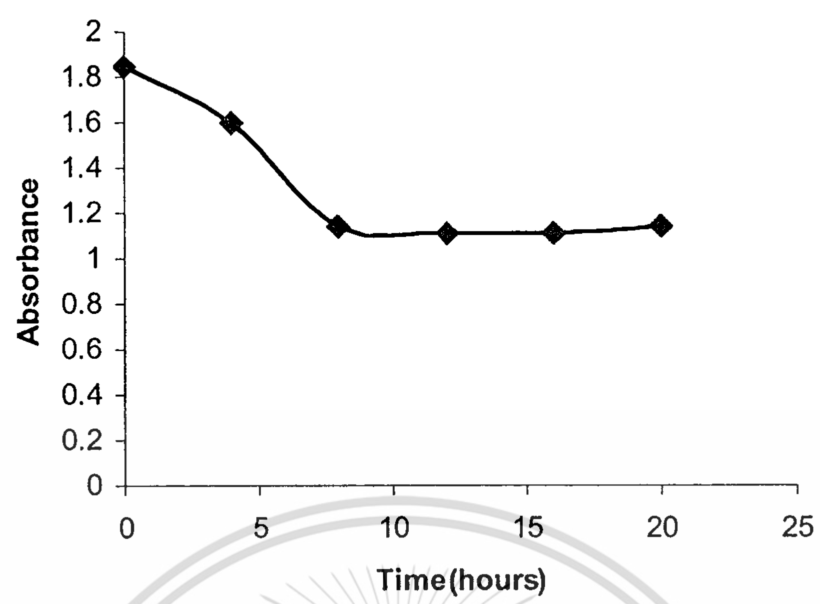

Figure 4.3 The effect of reaction time on the preparation of n-butyl-chitosan crosslinked microspheres.

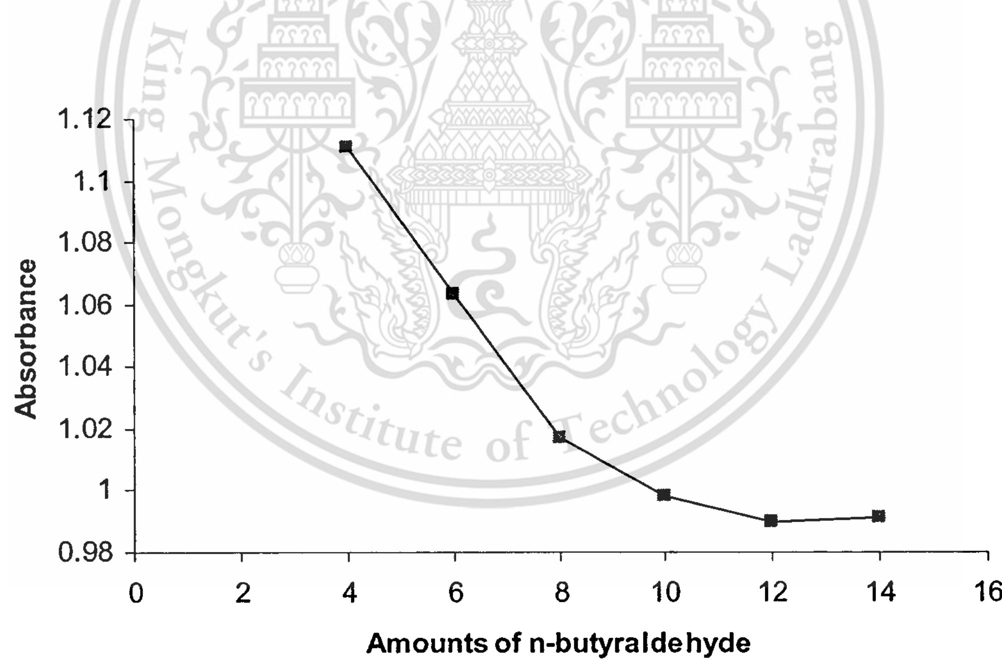

Figure 4.4 The effect of amounts of n-butyraldehyde on the preparation of n-butyl-chitosan crosslinked microspheres.

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A comparison of the infrared spectrum for n-butyl-chitosan crosslinked and chitosan and chitosan crosslinked is thus presented in figure 4.5. The results indicate that an obvious change takes place after modification with n-butylraldehyde. Two strengthening adsorption bands at about 2930 cm^{-1} (ν_{C-H} of CH_2 group) and 1467 cm^{-1} (δ_{C-H} of CH_2 group) were observed. That means an alkyl group was linked to chitosan crosslinked during the modification with n- butylraldehyde. A weakened band in the region of 1600 cm^{-1} (δ_{N-H} of NH group) was also observed.

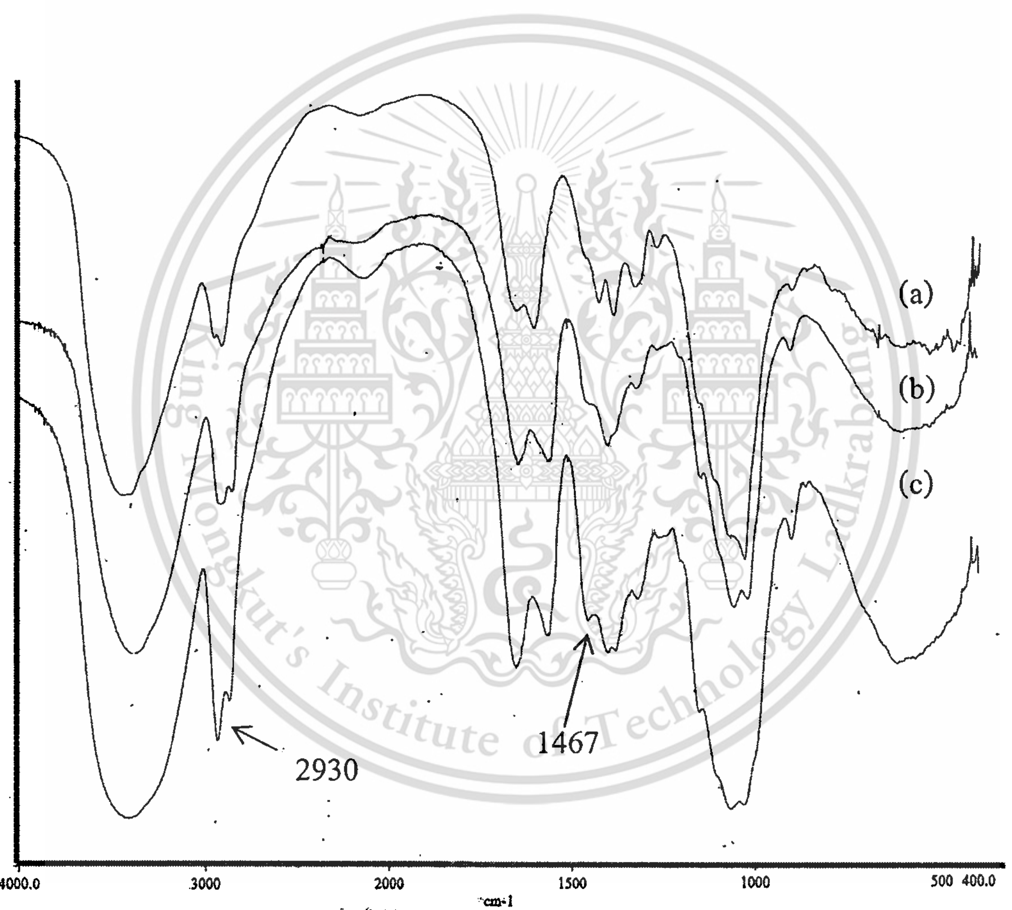

Figure 4.5 Infrared spectrum of three types of chitosan : a = chitosan; b = chitosan crosslinked; c = n-butyl-chitosan crosslinked microspheres.

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4.4. CHARACTERIZATION OF n-BUTYL-CHITOSAN CROSSLINKED MICROSPHERES FOR HPHIC COLUMN

The particle size distribution and the particle shape of n-butyl-chitosan crosslinked microspheres were determined using light scattering analysis technique (Mastersizer X) and scanning electron microscope (SEM) as shown in figure 4.6 and figure 4.7, respectively.

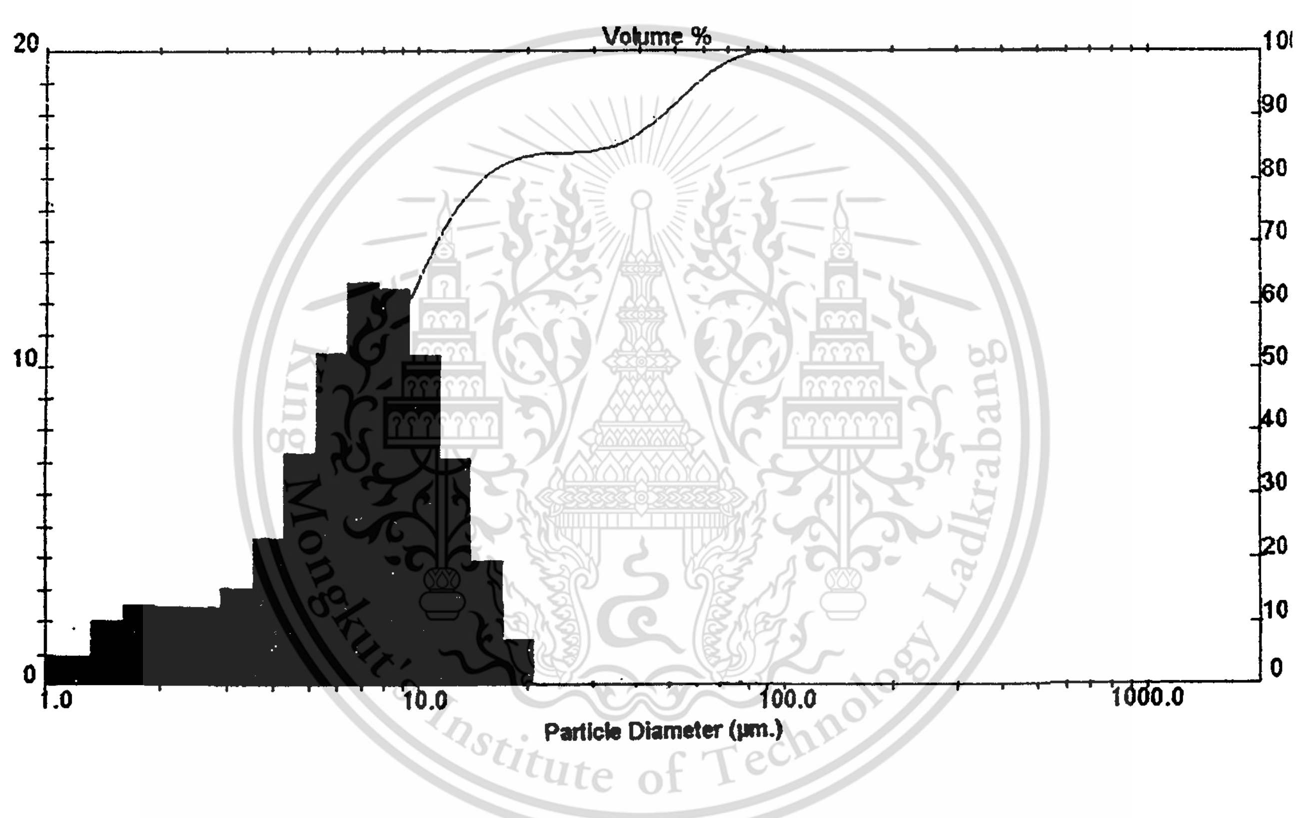

Figure 4.6 Particle size distribution of chitosan particle after modification with n- butylraldehyde.

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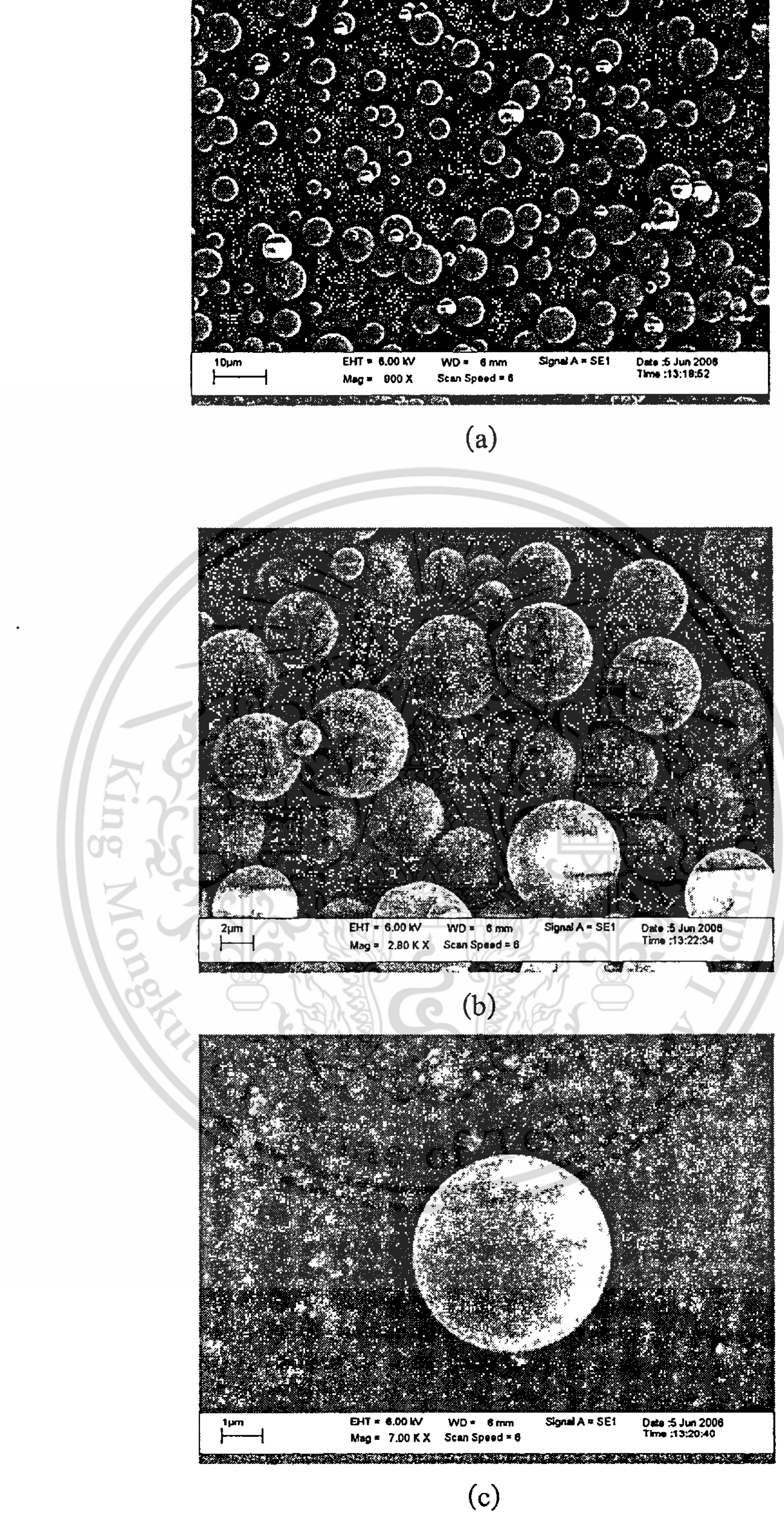

Figure 4.7 SEM photographs of butyl-chitosan crosslinked microspheres. (a) 900 X magnification: scale bar is 10 µm. (b) 2.8 K X magnification: scale bar is 2 µm. (c) 7.0 K X magnification: scale bar is 1 µm.

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From the results for particle size distribution and morphology, the average particle size of n-butyl chitosan crosslinked microspheres was 5.5 μm and spherical shape and smooth surface.

Surface area and pore size distribution of n-butyl-chitosan crosslinked microspheres were measured by Brunauer – Emmett – Teller (BET) method (Autosorb-1) as show in figure 4.8. The n-butyl-chitosan crosslinked microspheres had average surface area of 3.02 m^2/g and major pore size of 43.72 Å.

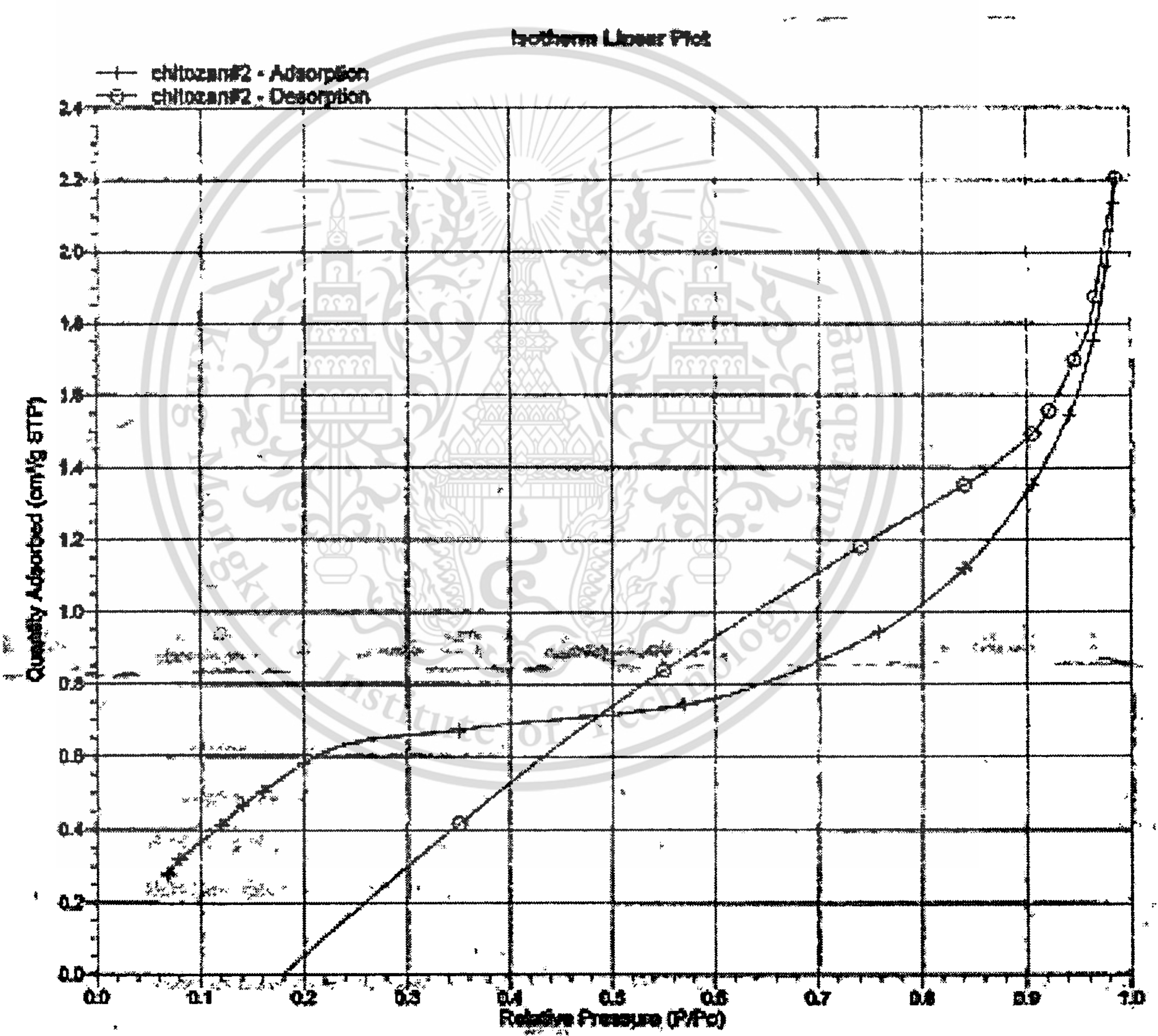

Figure 4.8 Brunauer – Emmett – Teller photographs of n-butyl-chitosan crosslinked microspheres.

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A comparison of the X-ray diffraction spectra for pure chitosan and n-butyl-chitosan crosslinked is thus presented in figure 4.9. Two intense diffraction peaks for pure chitosan were clearly located at 11.4° and 20.2 °, respectively. However, no peak for crystallization regions compared with pure chitosan were found for n-butyl-chitosan crosslinked, indicating significant decrease of crystallization. This attributes to that glutaraldehyde (crosslinked materials) disturbed the arrangement of chitosan chains, leads to the crystallinity decrease in of n-butyl-chitosan crosslinked.

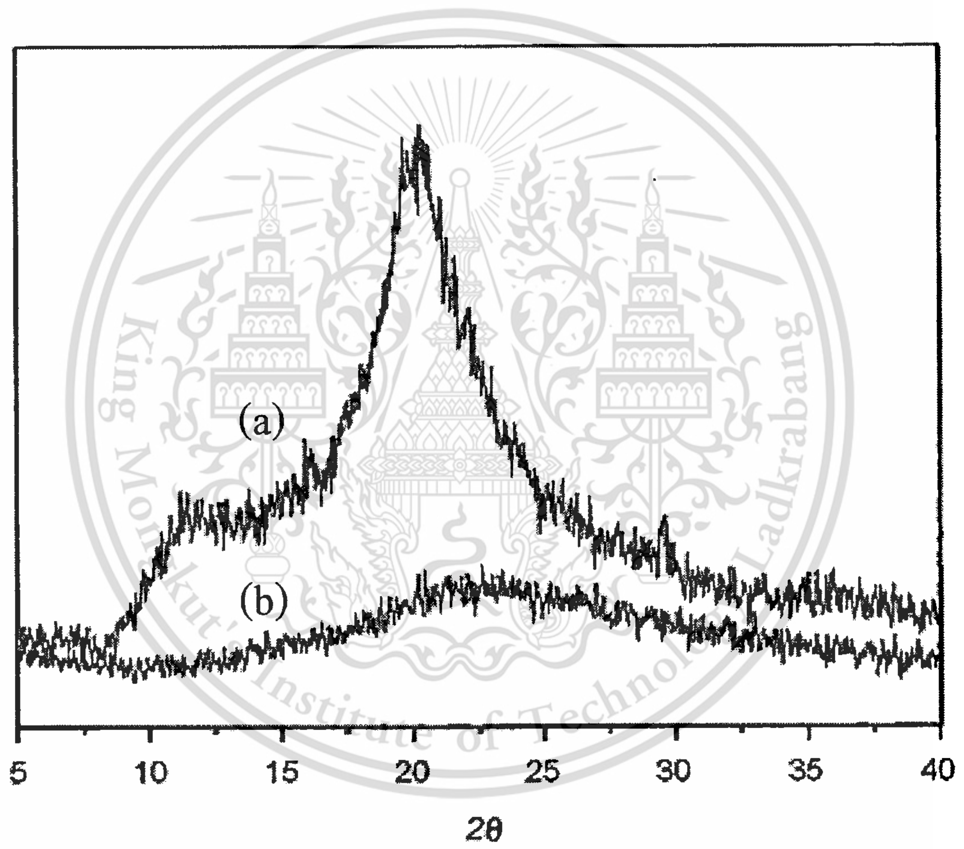

Figure 4.9 X-ray diffraction patterns of pure chitosan (a) and n-butyl-chitosan crosslinked microspheres (b).

4.5. DETERMINATION OF CHROMATOGRAPHIC PARAMETERS

The first chromatographic tests were performed using a standard benzamide for compared Van Deemter's plot testing of n-butyl-chitosan crosslinked microspheres column (particle size 5.5 μm, I.D. 4.6 x 150 mm). Van Deemter curve of n-butyl-

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chitosan crosslinked microspheres column was shown in figure 4.10. The optimum flow rate for n-butyl-chitosan crosslinked microspheres column are 0.3 ml/min. Linearity testing of n-butyl-chitosan crosslinked microspheres column was tested by varying the concentration of standard benzamide (40 to 200 ppm). Figure 4.11 shows the linearity testing of n-butyl-chitosan crosslinked microspheres column.

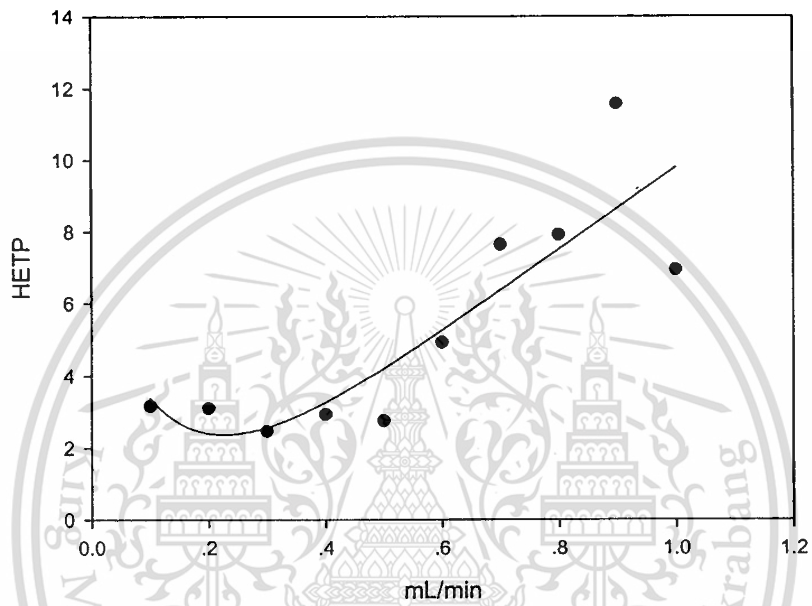

Figure 4.10 Plots of *H* (plate height) for benzamide 100 ppm : 10 µl at different flow-rate of 75% acetronitrile for n-butyl-chitosan crosslinked microspheres column.

For linearity testing, the n-butyl-chitosan crosslinked microspheres column was exhibited the R^2 of 0.9955 that shown good characteristic for quantitative analysis used. For separation of model proteins testing of n-butyl-chitosan crosslinked microspheres column was performed using protein mixture (lysozyme and bovine serum albumin) at 0.3 ml/min, using gradient elution : 0 - 6 min, buffer A; 6 - 9 min, buffer B; 8 - 11 min, buffer C; 11 - 16 min, buffer D.

Buffer A : 5 m*M* phosphate buffer containing 10% sodium sulfate

Buffer B : 5 m*M* phosphate buffer containing 8% sodium sulfate

Buffer C : 5 m*M* phosphate buffer containing 5% sodium sulfate

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Buffer D : 5 m*M* phosphate buffer.

The proteins were detected spectrophotometrically at 280 nm. This protein mixture can separated using n-butyl-chitosan crosslinked microspheres column as shown in figure 4.12. The selectivity factor were 2.61 and resolution were 1.03

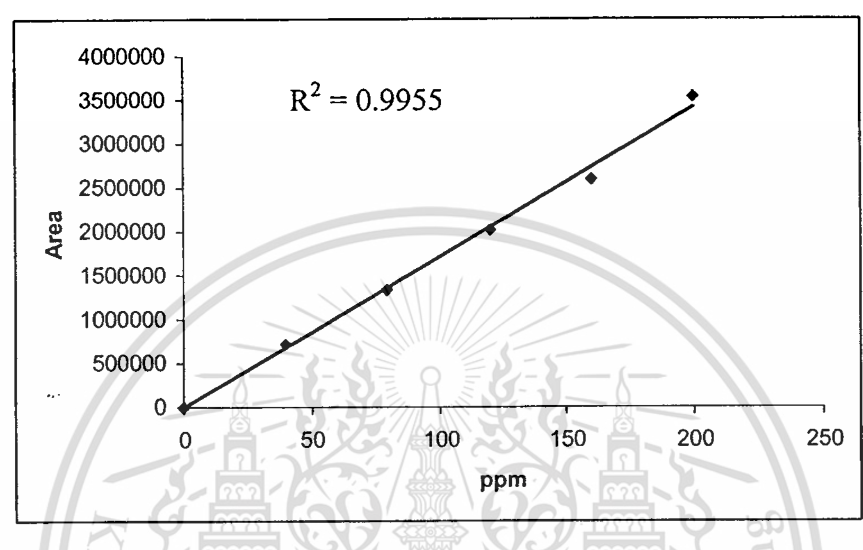

Figure 4.11 Plots of linearity for benzamide (40-200 ppm) : 10 μL at 0.3ml/min of 75% acetronitrile.

***Note :** For linearity testing was performed with 3-times for each concentration.

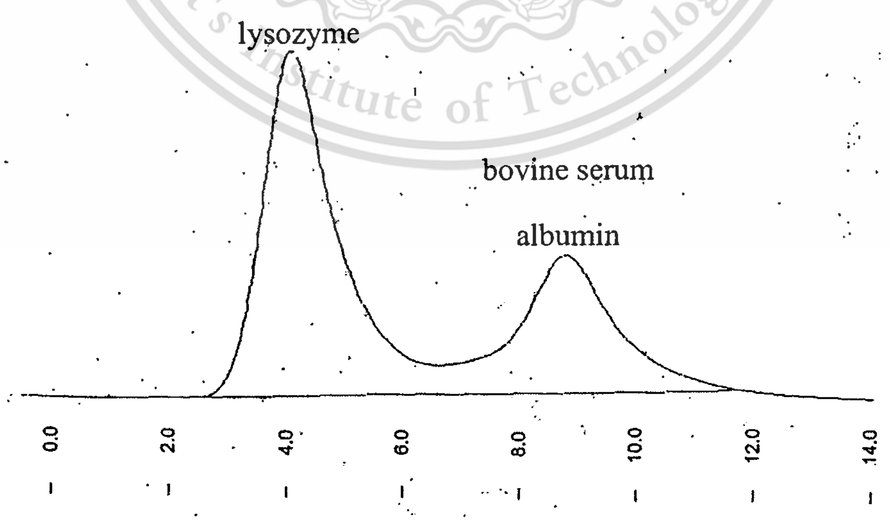

Figure 4.12 The separation of model proteins testing of n-butyl-chitosan crosslinked microspheres column. Column size: 4.6x150 mm i.d. Sample: 5 μL of

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protein mixture containing 0.25 mg/mL lysozyme and 0.25 mg/mL bovine serum albumin in equilibrated buffer. gradient elution : 0 - 4 min, buffer A; 4 - 6 min, buffer B; 6 - 9 min, buffer C; 9 - 14 min, buffer D. Buffer A : 5 m*M* phosphate buffer containing 10% sodium sulfate; buffer B : 5 m*M* phosphate buffer containing 8% sodium sulfate; buffer C : 5 m*M* phosphate buffer containing 5% sodium sulfate; buffer D : 5 m*M* phosphate buffer.

4.6. ADSORPTION OF PROTEIN ON n-BUTYL-CHITOSAN CROSSLINKED MICROSPHERES

4.6.1. THE EFFECT OF SALT CONCENTRATION ON THE ADSORPTION OF PROTEIN

It is well known from other studies, that the hydrophobic interaction is promoted by high salt concentration[17]. The effect of salt concentration on the adsorption of protein on n-butyl-chitosan crosslinked microspheres was determined. Results are shown in figure 4.13. Here, with increasing salt concentration, the adsorption of protein on n-butyl-chitosan crosslinked microspheres increased dramatically. The results show the binding of protein to n-butyl-chitosan crosslinked microspheres was mostly due to hydrophobic interaction.

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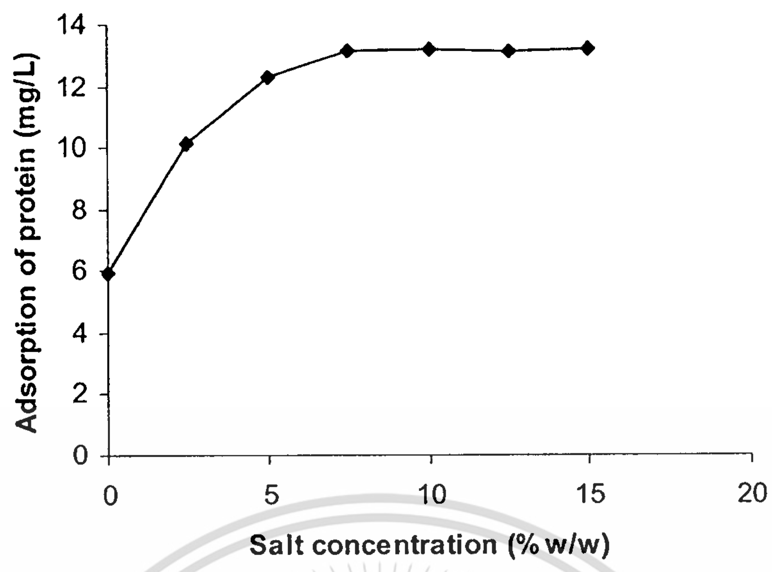

Figure 4.13 The effect of salt concentration on the adsorption of protein on n-butyl chitosan crosslinked microspheres.

4.6.2. THE EFFECT OF TEMPERATURE ON THE ADSORPTION OF PROTEIN

The previous studies show that the temperature is one of the most important parameters for retention in HPHIC[18]. In order to investigate the effect of temperature on hydrophobic interaction, the experiment was carried out at different temperatures (figure 4.14) The results indicate that the nature of temperature plays multiple roles in the adsorption of protein. Increasing temperature enhanced not only the hydrophobic interaction (caused adsorption) but also the molecular motion (caused desorption). During 25 to 75 ^{o}C, with increasing temperature, the enhancement of hydrophobic interaction is higher than that of molecular motion and the adsorption of protein increased. When the temperature is above 75 ^{o}C, the increasing temperature enhanced molecular motion far more than hydrophobic interaction and the adsorption of protein decreased.

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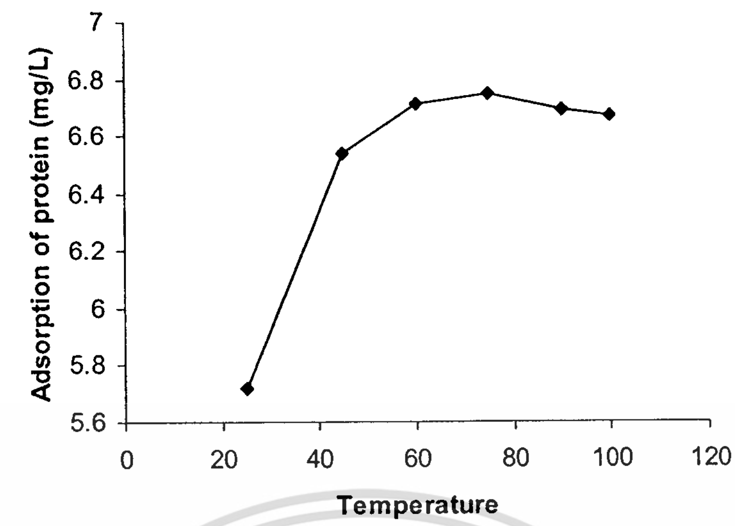

Figure 4.14 The effect of temperature on the adsorption of protein on n-butyl-chitosan crosslinked microspheres.

4.7 STORAGE STABILITY OF n-BUTYL CHITOSAN CROSSLINKED MICROSPHERES

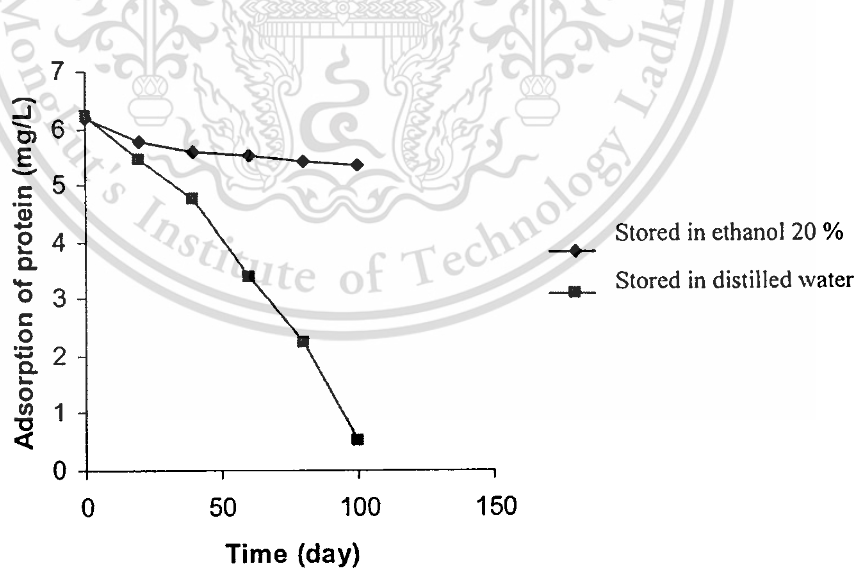

Figure 4.15 Storage stability n-Butyl-Chitosan crosslinked microspheres.

n-Butyl-Chitosan crosslinked microspheres was stored under two different conditions. Figure 4.15 shows that compared two storage conditions, after 100 day

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storage in 20% ethanol at 4°C, the adsorption quantity decreased a little. The most possible reason was the ethanol and low temperature restraining the microbiological activity. So 20% ethanol and 4 ^{o}C was the most suitable condition.

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CHAPTER 5

CONCLUSIONS

Over about the last decade, hydrophobic interaction chromatography has been widely employed for the separation of bioactive compounds. Unlike other chromatographic methods, fewer separation media are available for this chromatography and it is largely dominated by matrices modified by attaching alkyl or aryl groups.

This research was aimed to study the potential and limitation of using shrimp shell to produced the chitosan microspheres particles and hydrophobic ligand, butyl group, immobilized on it to form an hydrophobic adsorbent.

From results obtained, optimized condition for preparation of chitosan microspheres were found that was using 250 mL of 0.5% (w/v) chitosan, 3 mL of 50% (v/v) glutaraldehyde and atomizer speed 35,000 rpm. The average particle size of n-butyl chitosan crosslinked microspheres were 5.5 μm, average surface area of 3.02 m^2/g, major pore size of 43.72 Å, amorphous form, spherical shape and smooth surface. The FTIR spectrum analysis showed a butyl group was linked to $-NH_2$ by a C-N bond. The behavior of the binding protein to n-butyl-chitosan crosslinked microspheres was investigated. The binding was clearly mediated by hydrophobic interaction; this was indicated by the observation that increasing salt concentration increased the binding. The HPHIC columns packed with the n-butyl chitosan crosslinked microspheres were employed for separation of a mixture of two model protein (lysozyme and bovin serum albumin). Thus, n-butyl-chitosan crosslinked microspheres described here appears to be a useful packing material for hydrophobic interaction chromatogaphy.

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APPENDICES

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APPENDIX A

CHITOSAN PREPARATION

Table A-1 The degree of deacetylation of chitosan from shrimp shells.

Batch	Degree of deacetylation (%)
1	81.07
2	80.09
3	78.11
4	79.67
Average	79.74

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APPENDIX B

PREPARATION OF n-BUTYL-CHITOSAN CROSSLINKED MICROSPHERES FOR HPHIC COLUMN

Table B-1 The effect reaction time on the preparation of n-butyl-chitosan crosslinked microspheres.

Reaction time (hours)	Absorbance of A_{1559}/A_{3450} absorbtion in ATR-FTIR spectrogram
0	1.850
4	1.602
8	1.142
12	1.107
16	1.106
20	1.139

Table B-2 The effect of amount of n-butyraldehyde on the preparation of n-butyl-chitosan crosslinked microspheres.

Amount of n-butyraldehyde(mL)	Absorbance of A_{1559}/A_{3450} absorbtion in ATR-FTIR spectrogram
4	1.111
6	1.063
8	1.017
10	0.998
12	0.990
14	0.991

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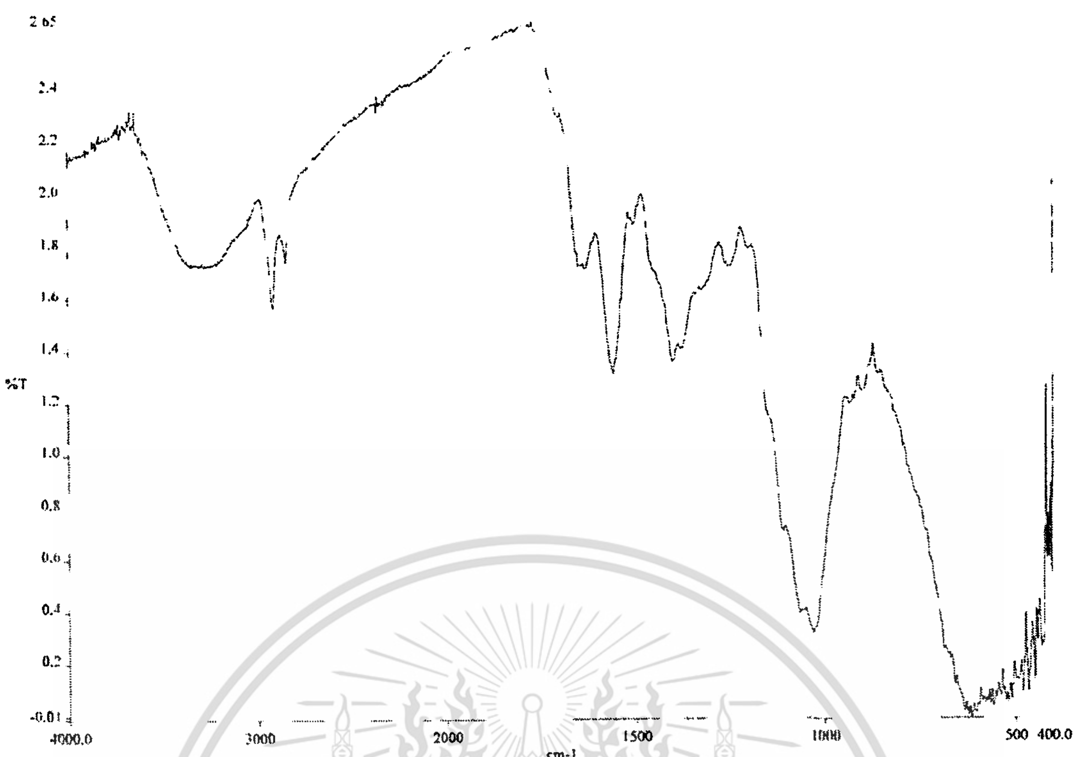

Figure B-1 ATR-FTIR spectrum of n-butyl-chitosan crosslinked microspheres (Reaction time 0 hours).

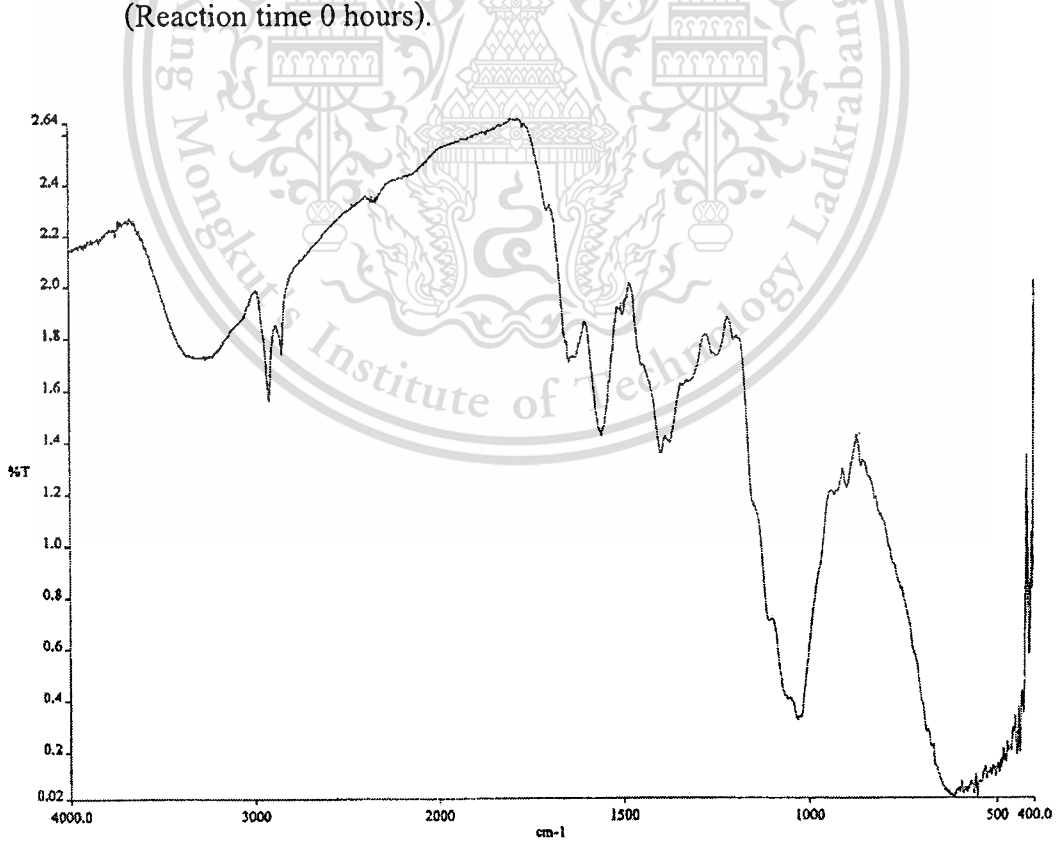

Figure B-2 ATR-FTIR spectrum of n-butyl-chitosan crosslinked microspheres (Reaction time 4 hours).

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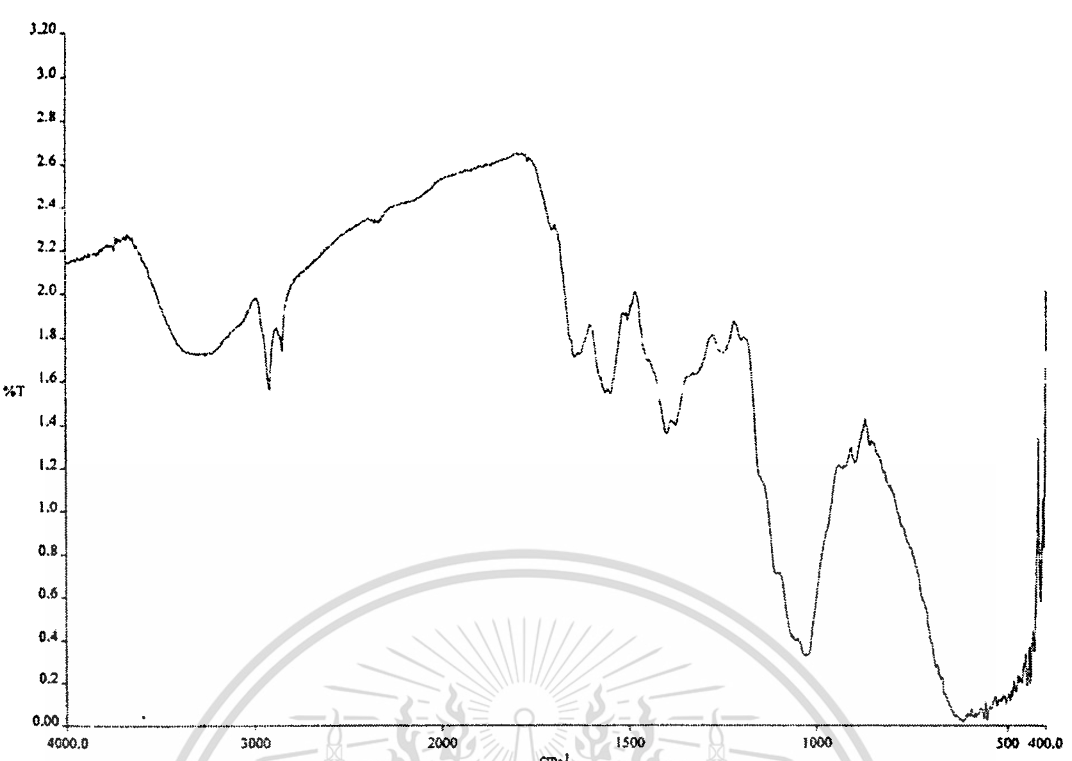

Figure B-3 ATR-FTIR spectrum of n-butyl-chitosan crosslinked microspheres (Reaction time 8 hours).

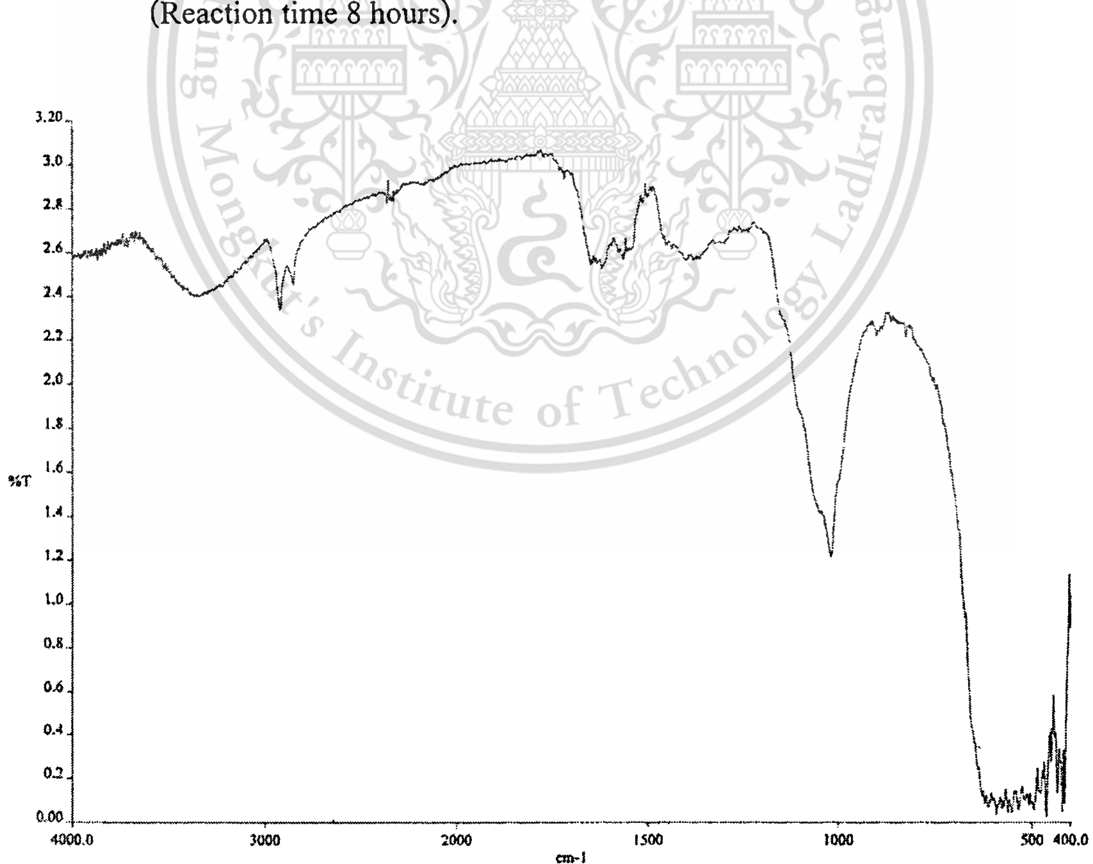

Figure B-4 ATR-FTIR spectrum of n-butyl-chitosan crosslinked microspheres (Reaction time 12 hours).

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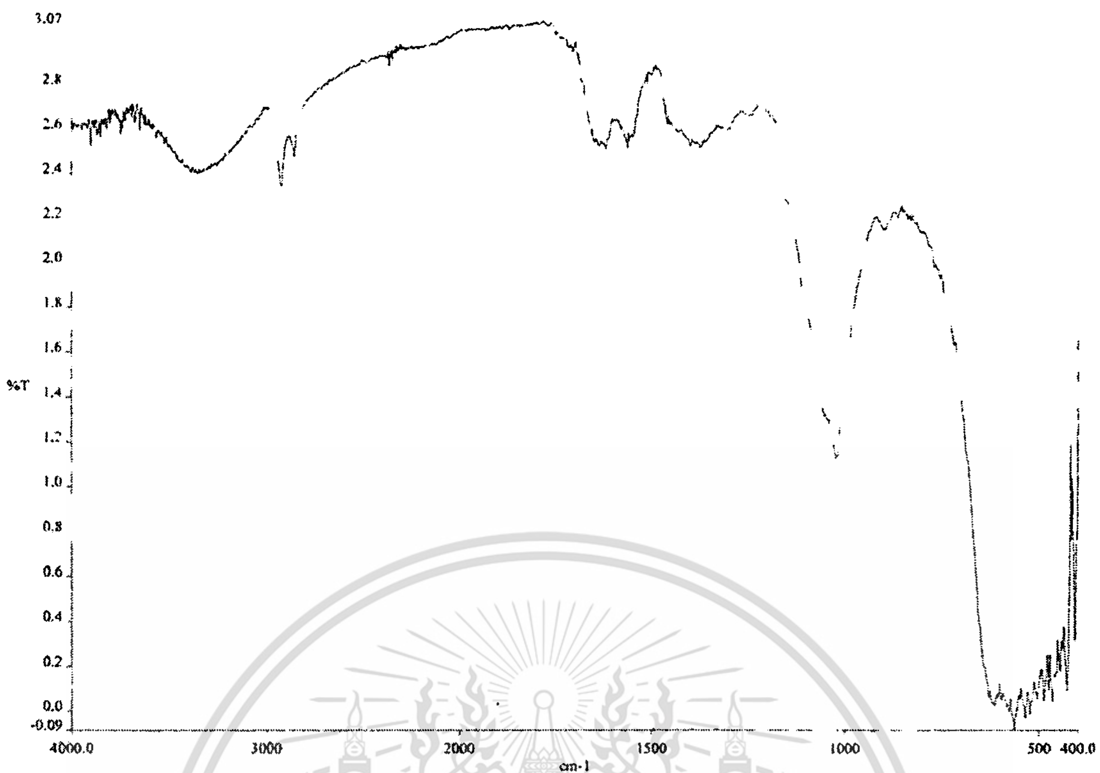

Figure B-5 ATR-FTIR spectrum of n-butyl-chitosan crosslinked microspheres (Reaction time 16 hours).

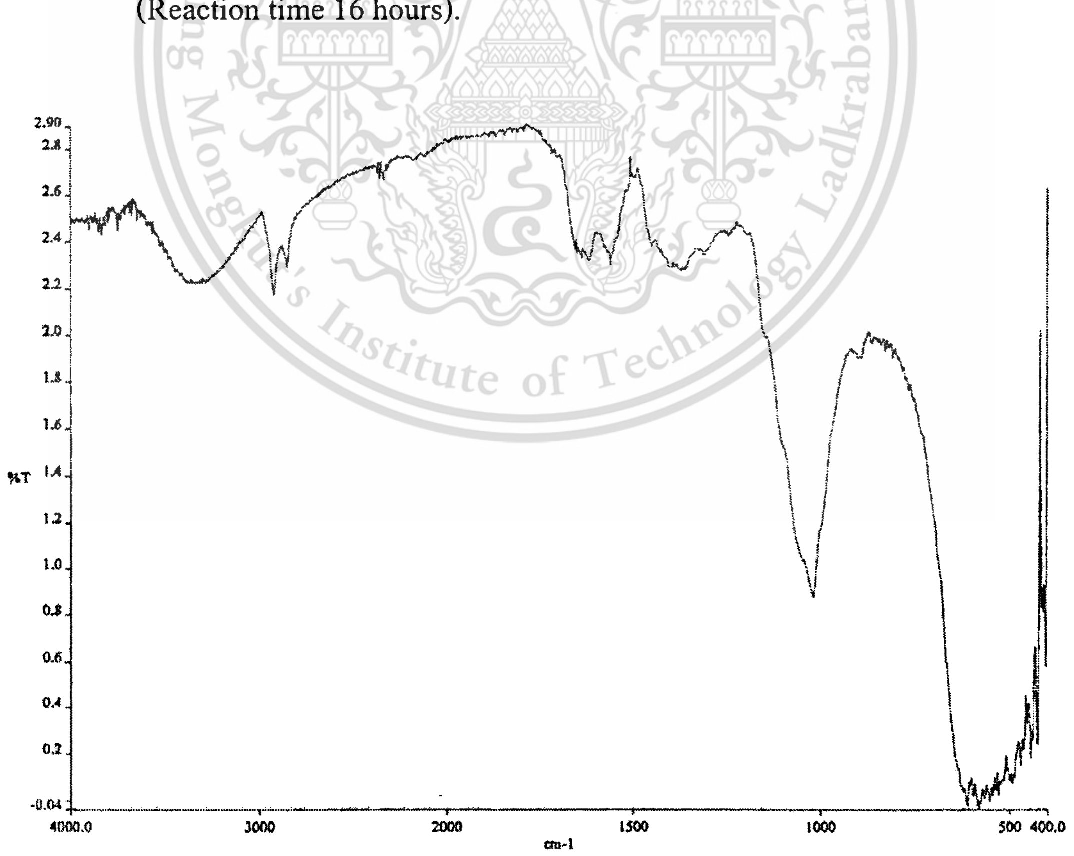

Figure B-6 ATR-FTIR spectrum of n-butyl-chitosan crosslinked microspheres (Reaction time 20 hours).

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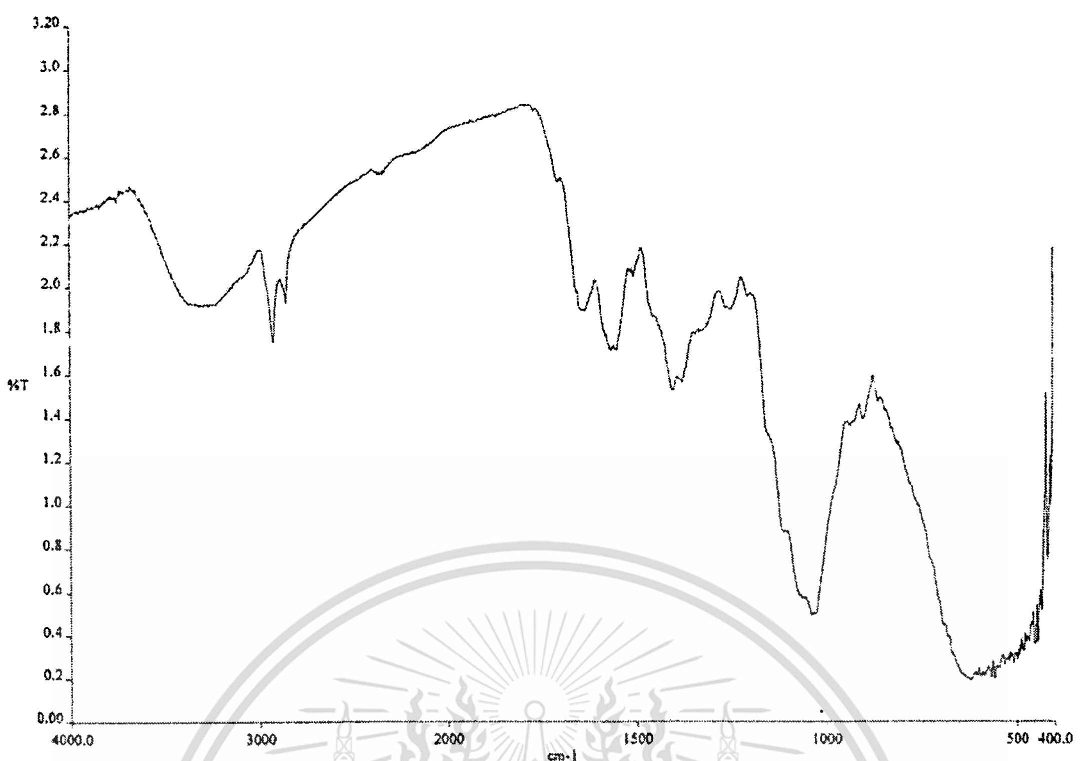

Figure B-7 ATR-FTIR spectrum of n-butyl-chitosan crosslinked microspheres (Amount of n-butyraldehyde 4 mL).

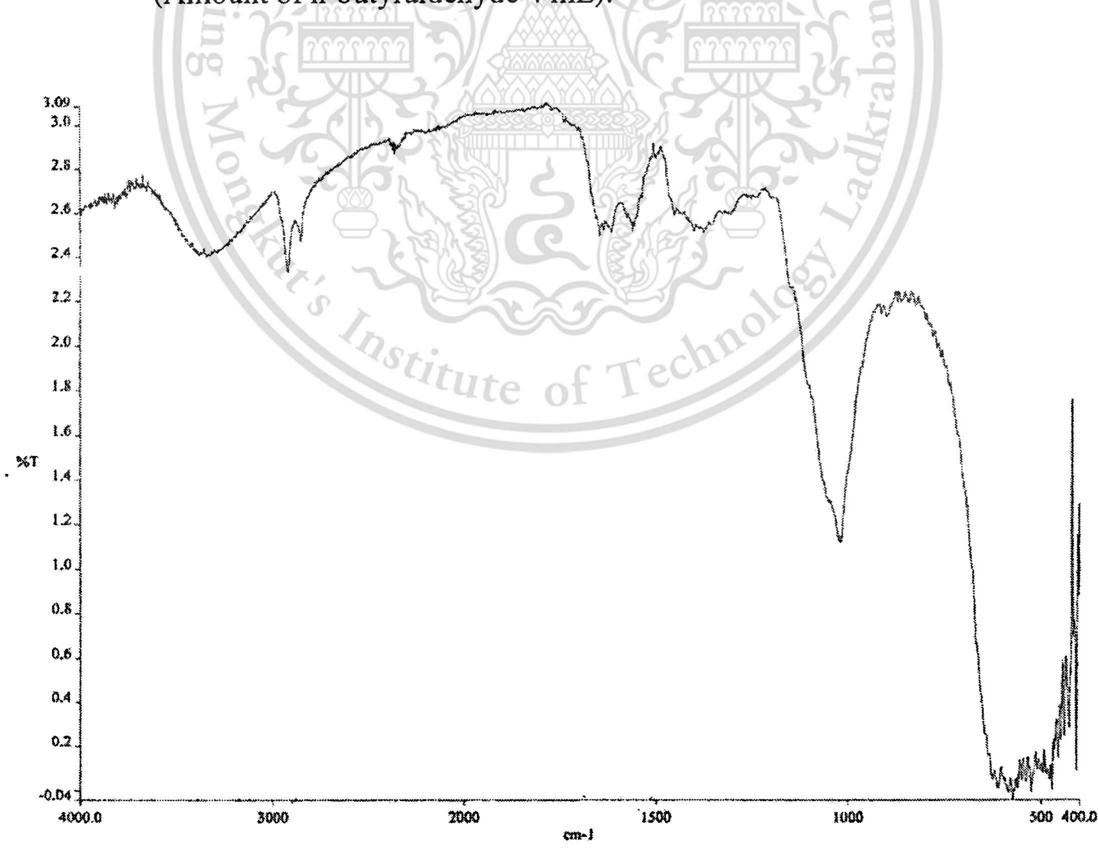

Figure B-8 ATR-FTIR spectrum of n-butyl-chitosan crosslinked microspheres (Amount of n-butyraldehyde 6 mL).

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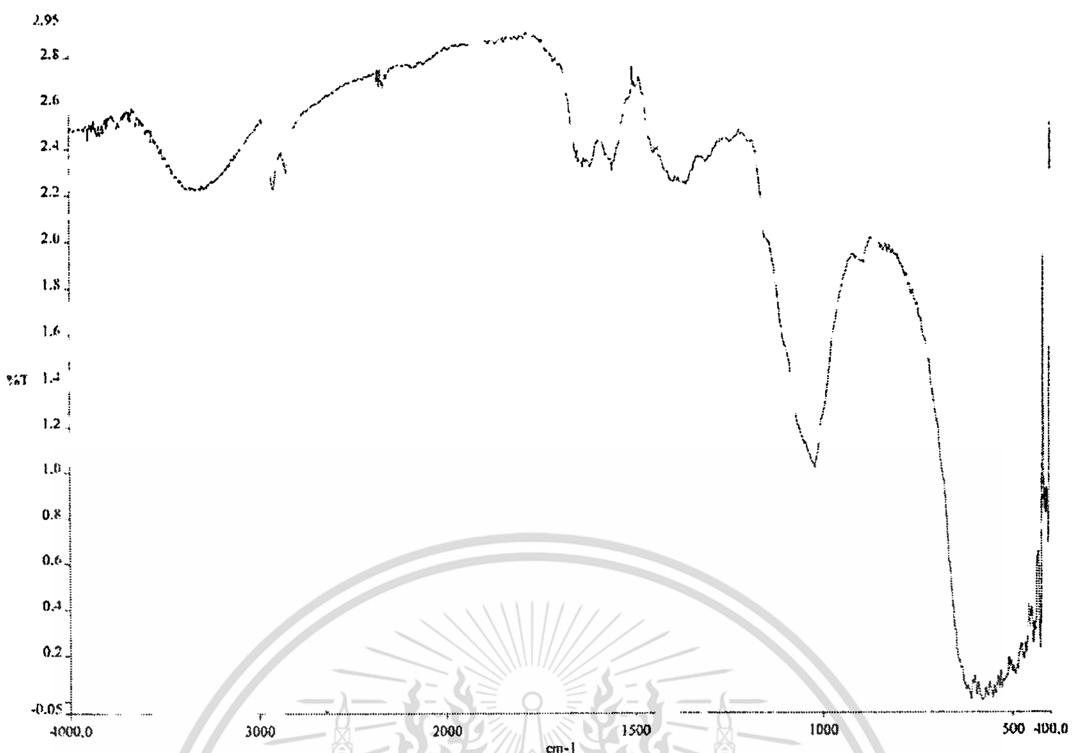

Figure B-9 ATR-FTIR spectrum of n-butyl-chitosan crosslinked microspheres (Amount of n-butyraldehyde 8 mL).

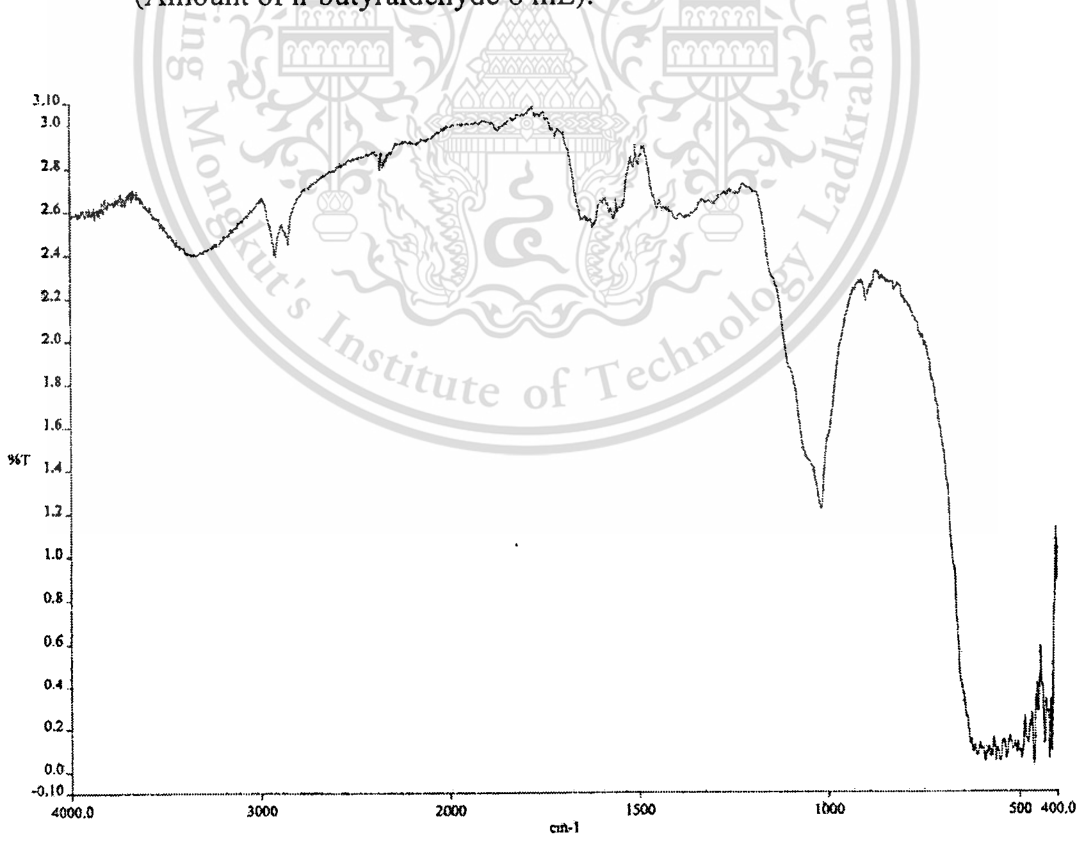

Figure B-10 ATR-FTIR spectrum of n-butyl-chitosan crosslinked microspheres (Amount of n-butyraldehyde 10 mL).

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Figure B-11 ATR-FTIR spectrum of n-butyl-chitosan crosslinked microspheres (Amount of n-butyraldehyde 12 mL).

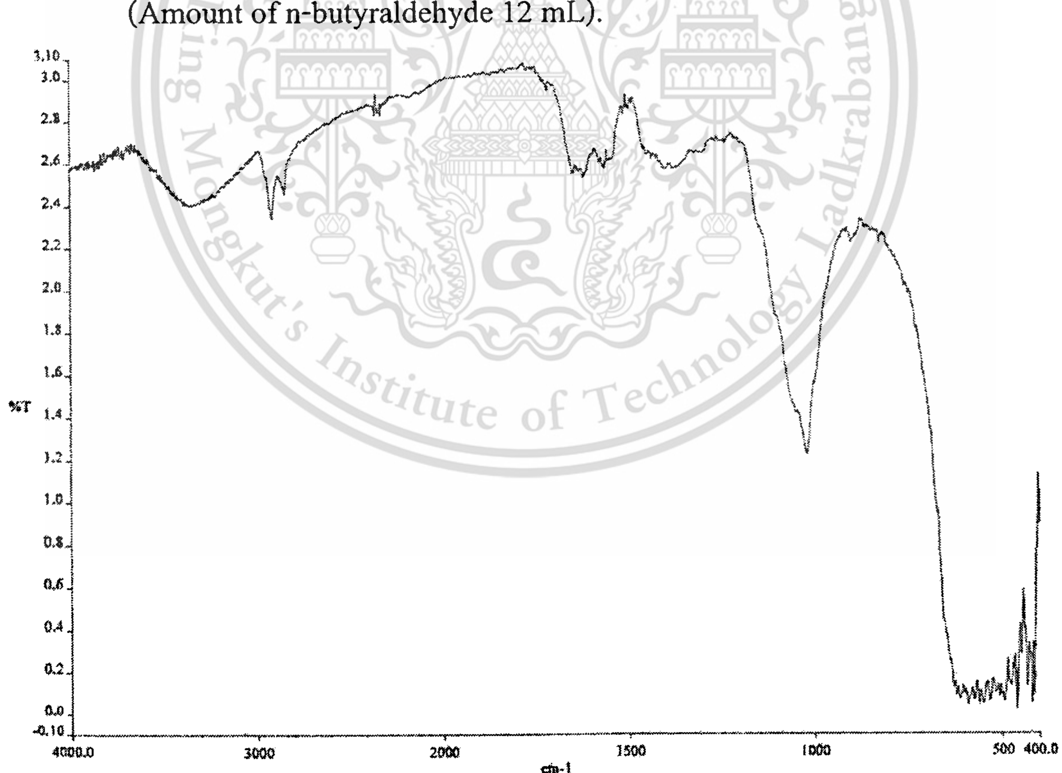

Figure B-12 ATR-FTIR spectrum of n-butyl-chitosan crosslinked microspheres (Amount of n-butyraldehyde 14 mL).

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APPENDIX C

CHROMATOGRAPHIC EVALUATION

Table C-1 Chromatographic parameter calculation for n-butyl-chitosan crosslinked microspheres column

Flow rate (mL/min)	Retention time(min)	$W_{(0.5)}$	N^*	H (cm)
0.1	8.476	2.9	47.333	3.169
0.2	7.092	2.4	48.375	3.101
0.3	6.629	2.0	60.862	2.465
0.4	6.081	2.0	51.215	2.929
0.5	4.703	1.5	54.459	2.754
0.6	4.688	2.0	30.438	4.928
0.7	3.767	2.0	19.654	7.632
0.8	2.775	1.5	18.960	7.911
0.9	2.295	1.5	12.969	11.566
1.0	1.978	1.0	21.675	6.920

Note : N^* wree calculate from eqution $N = 5.55\ (t_R\ /\ w_{1/2})^2$

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Table C-2 Data for linearity plot of standard benzamide (40-200 ppm) :10 μL at 0.3 mL/min of 75 % acetronitrile (n-butyl-chitosan crosslinked microspheres column)

Concentration (ppm)	Peak area	Retention time (min)	Average peak area
40	704063	6.63	717,828
	730179	6.628	
	719243	6.705	
80	1338687	6.81	1,345,060
	1357645	6.615	
	1338847	6.921	
120	2020694	6.533	2,013,386
	2015078	6.712	
	2004387	6.734	
160	2627363	6.557	2,596,911
	2625579	6.88	
	2537791	6.517	
200	3574473	6.598	3,543,363
	3425806	6.612	
	3629810	6.771	

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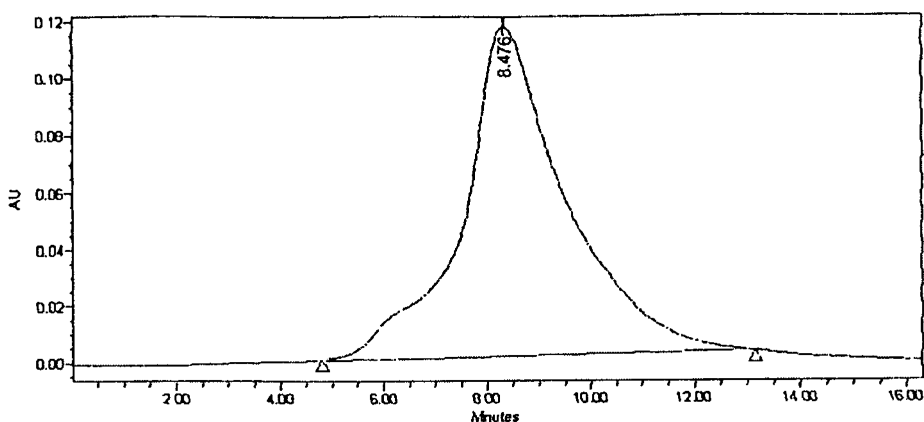

Figure C-1 Chromatogram of standard benzamide 100 ppm ; 10 μL ; at 0.1 mL / min of 75% acetronitrile (n-butyl-chitosan crosslinked microspheres column).

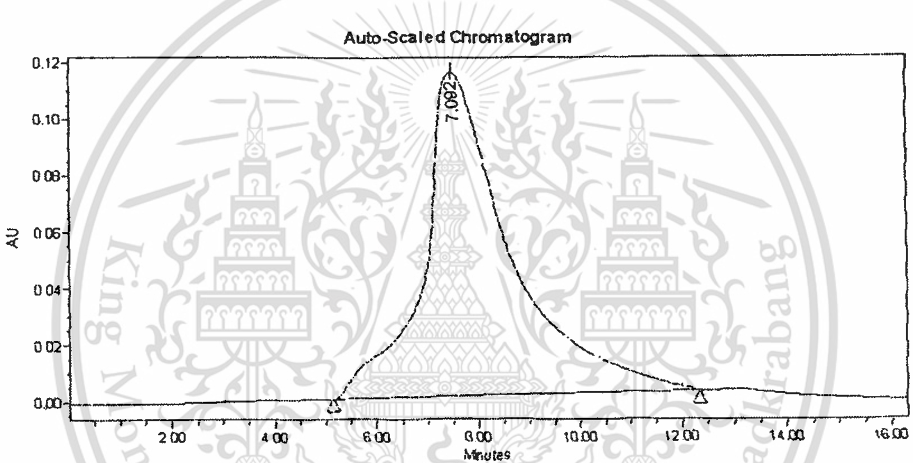

Figure C-2 Chromatogram of standard benzamide 100 ppm ; 10 μL ; at 0.2 mL / min of 75% acetronitrile (n-butyl-chitosan crosslinked microspheres column).

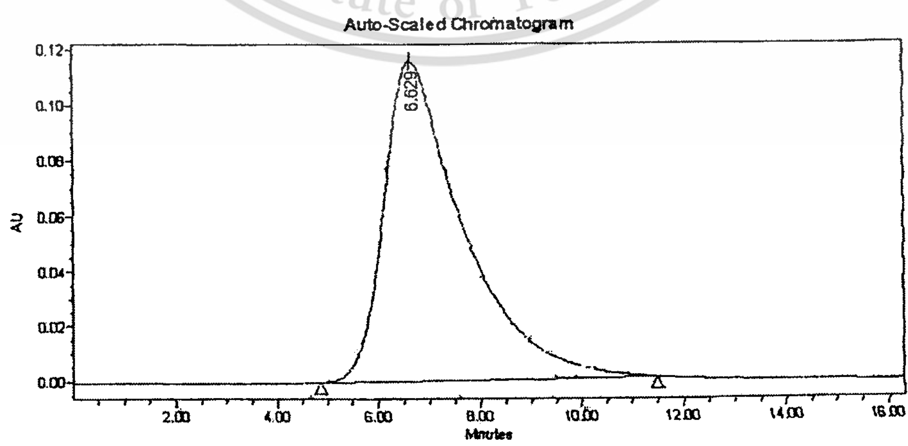

Figure C-3 Chromatogram of standard benzamide 100 ppm ; 10 μL ; at 0.3 mL / min of 75% acetronitrile (n-butyl-chitosan crosslinked microspheres column).

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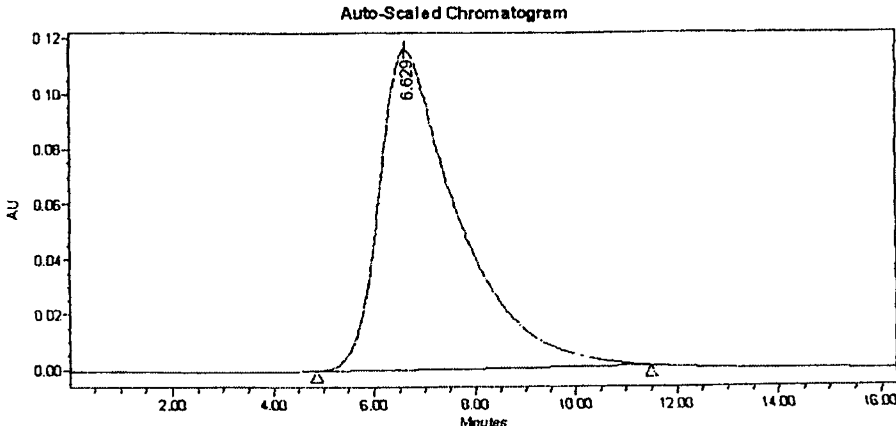

Figure C-4 Chromatogram of standard benzamide 100 ppm ; 10 μL ; at 0.4 mL / min of 75% acetronitrile (n-butyl-chitosan crosslinked microspheres column).

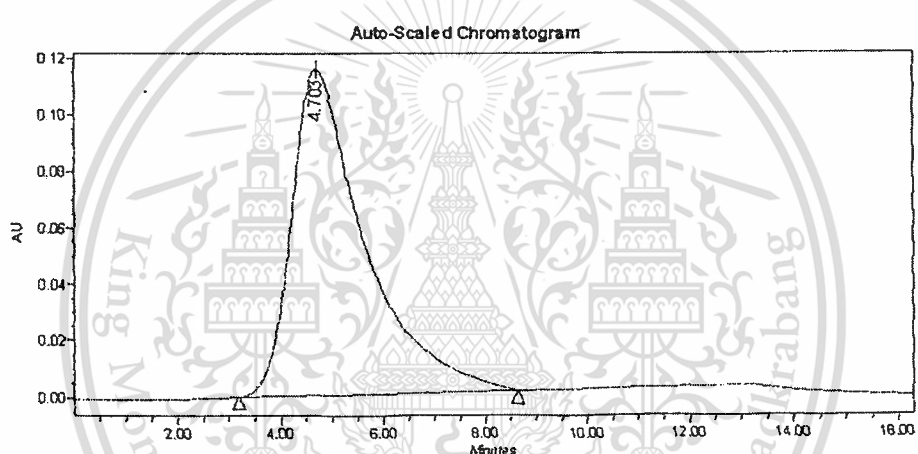

Figure C-5 Chromatogram of standard benzamide 100 ppm ; 10 μL ; at 0.5 mL / min of 75% acetronitrile (n-butyl-chitosan crosslinked microspheres column).

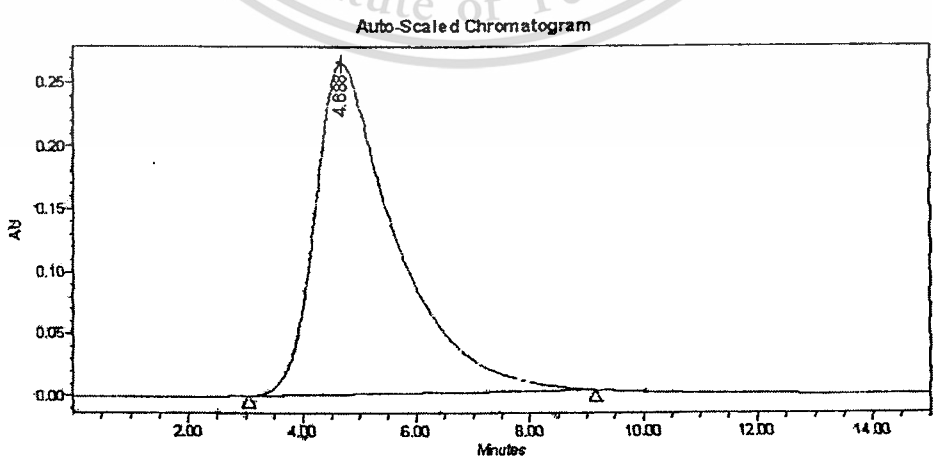

Figure C-6 Chromatogram of standard benzamide 100 ppm ; 10 μL ; at 0.6 mL / min of 75% acetronitrile (n-butyl-chitosan crosslinked microspheres column).

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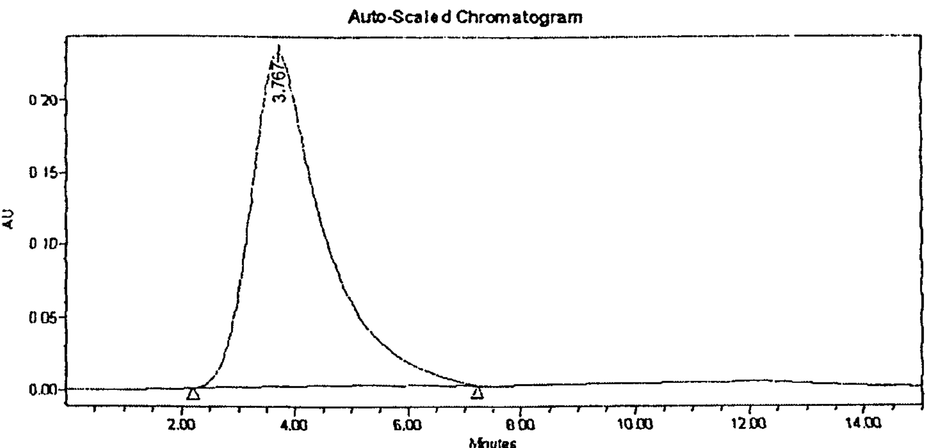

Figure C-7 Chromatogram of standard benzamide 100 ppm ; 10 μL ; at 0.7 mL / min of 75% acetronitrile (n-butyl-chitosan crosslinked microspheres column).

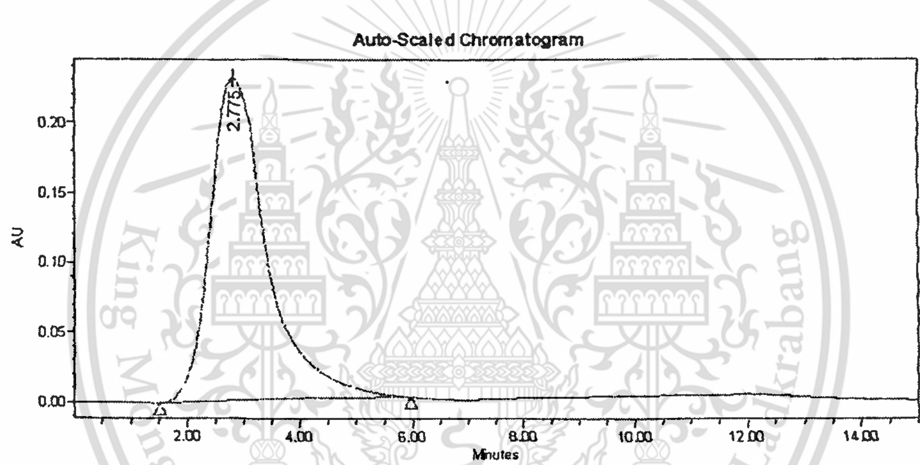

Figure C-8 Chromatogram of standard benzamide 100 ppm ; 10 μL ; at 0.8 mL / min of 75% acetronitrile (n-butyl-chitosan crosslinked microspheres column).

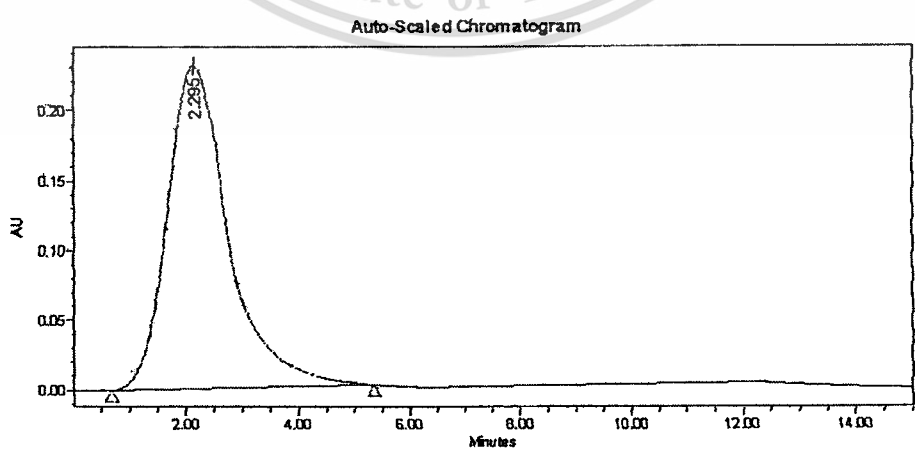

Figure C-9 Chromatogram of standard benzamide 100 ppm ; 10 μL ; at 0.9 mL / min of 75% acetronitrile (n-butyl-chitosan crosslinked microspheres column).

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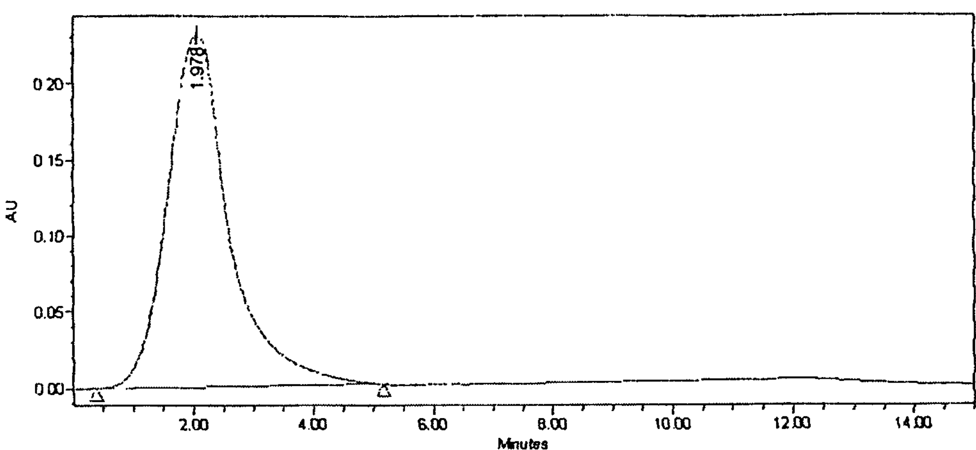

Figure C-10 Chromatogram of standard benzamide 100 ppm ; 10 μL ; at 1.0 mL / min of 75% acetronitrile (n-butyl-chitosan crosslinked microspheres column).

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BIOGRAPHY

The author wad born on December 13, 1980 in Bangkok, Thailand. She graduated with a Bachelor of Science in chemistry at Thaksin University in 2002. She entered King Mongkut's Institute of Technology Ladkrabang since 2004. She graduated with a Master of Science in Analytical Chemistry at King Mongkut's Institute of Technology Ladkrabang in 2007.

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