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PREPARATION OF WALL-BONDED, GEL FILLED CAPILLARIES VIA HYDRIDE-MODIFIED QUARTZ GLASS SURFACES

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ABSTRACT

PREPARATION OF WALL-BONDED, GEL FILLED CAPILLARIES
VIA HYDRIDE-MODIFIED QUARTZ GLASS SURFACES
by Seema Agarwal

Gel-filled capillaries were prepared by modifying their inner surfaces via a hydride intermediate. A bifunctional olefin was used as a coupling agent. One end of the coupling agent was bonded to the hydrided capillary surface and the other end was bonded to the polyacrylamide gel. There resulted a direct ≡Si-C bonding on the capillary surface which is more stable as compared to ≡Si-O-C or ≡Si-O-Si-C linkages. The effect of reaction time and amount of catalyst on bonding of olefin to the hydrided silica surface were studied. The stability of two cross-linkers bisacrylamide and dihydroxyethylene bisacrylamide was tested. The temperature was varied from $0 - 20°$ and its consequences were reported. A gel was filled in the capillary at both atmospheric and high pressure (6200 psi) and their effects were studied. Four proteins, α-lactalbumin, β-lactoglobulin, trypsinogen, and pepsin were separated on the basis of molecular weight ranging between 14-35 K. Karger and his coworkers' work was tested with slight changes in the procedures. Evidence was presented to show that the time required for the detection of proteins using the hydrided capillary was much less when compared to Karger's work.

Acknowledgements

I was extremely fortunate to have Dr. Joseph J. Pesek as my M.S. advisor. He was not only my research advisor but also the source of my confidence and motivation. I also acknowledge Dr. Junior E. Sandoval who guided me during the initial phases of experimental investigations. I appreciate careful reading of this thesis by Dr. Gerald Selter and Dr. Sam Perone. I am extremely thankful to my husband Sunil who helped me in writing this thesis.

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Chapter 1

Introduction

1.1 Electrophoresis

Electrophoresis is the process of migration of ions in a solution under the influence of an electric field. The ions move toward the electrodes of opposite polarity through a buffer solution at a constant speed. The velocity of an ion depends on its size, its charge, and on the magnitude of the electric field strength. This phenomenon is modelled mathematically by the following equation of electrophoresis:

$$v = \mu E \tag{1.1}$$

In equation (1.1), the proportionality constant μ is called the electrophoretic mobility, v is the velocity of the ion, and E is the applied electric field. When a mixture is injected into a migration column and an electric field is applied across the ends, the mixture separates into various zones that migrate at different rates depending on their electrophoretic mobility [1].

During electrophoresis, Joule heat is generated by the passage of electricity through the buffer medium. This heat can dissipate only at the edges of the migration column. This results in a temperature gradient along the column. The viscosity of the gel is low at high temperatures, therefore, due to non-uniform heat dissipation, gels in some regions are more viscous than others. This results in zone broadening as shown in Figure 1.1 [2].

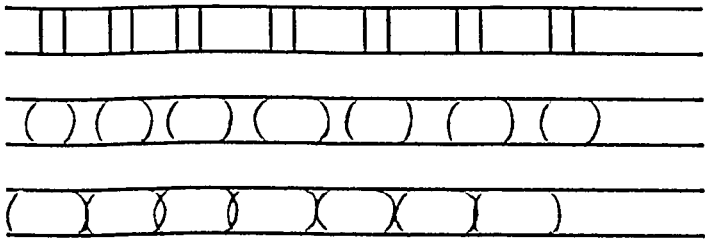


Figure 1.1: The zone broadening in capillaries of large diameters.

1.2 Capillary Electrophoresis

When electrophoresis is performed in a capillary, it is known as Capillary Electrophoresis or Capillary Zone Electrophoresis (CZE). More commonly, it is referred to as High Performance Capillary Electrophoresis (HPCE). The walls of a capillary are made of quartz glass coated with a polyimide film.

In earlier times, capillary electrophoresis was carried out in a tube of 3-4 mm diameter. The potential was often applied for only a short period of time and then stopped. The sample would separate into various zones and would move toward the respective electrodes. One of the drawbacks of this process was that due to large diameter of the tube, the sample would remain stationary between the potential pulses and get stuck in between the flow. When a potential field is applied, Joule heat is released due to high resistance of the capillary. The larger the diameter of the tube, the higher is the temperature gradient between the center and the surface of the tube. This results in variation of the bandwidth of the separated mixture. Also, thermally sensitive components may decompose. As shown in Figure 1.1, the bands bulge out due to diffusion which lowers the possibility of their separation. Thus due to joule heat, there may result density gradients and subsequent convection and temperature gradients leading to zone broadening. To summarize, tubes with large diameters have problems of Joule heating, sample decomposition, band overlapping resulting in poor detection. Soon, it was realized that the diameter of tubes for electrophoresis must be smaller. This reduces the amount of sample needed, avoids overheating of the tube and improves the resolution of detection.

A capillary has a high surface-to-volume ratio (e.g., for a capillary with an O.D.

of 75 μm, an I.D. of as little as 30 μm is possible) which allows Joule heat to dissipate through walls very efficiently. Thus, voltages as high as 30 KV can be applied for separation of molecules. The velocity of ions is faster at high voltages, as evident from equation (1.1), and this results in faster analysis. The velocity of migration is given by equation (1.1), where

$$\mu = \frac{l/V}{t/L} \tag{1.2}$$

l is the length of the migration column from injection to detector, L is the total length of the column, V is the applied voltage, and t is the retention time [3]. The height equivalent to theoretical plates (H) is defined by the following equation:

$$H = \frac{L}{N} \tag{1.3}$$

where N is the number of theoretical plates.

The efficiency of the separation is given by the number of theoretical plates, defined by the following equation:

$$N = \frac{\mu V}{2D} \tag{1.4}$$

where V is the applied voltage and D is the diffusion coefficient of the solute with electrophoretic mobility μ. The narrower the band, the greater is the number of theoretical plates.

Since length has no direct relationship with the efficiency of separation, usually the trend in experimentation is to use high voltage across short capillaries to obtain good separations in short periods of time. Capillaries of 40-100 cm length with internal diameters 50-100 μm are useful in giving efficient separations with $N > 10^5$ in a short analysis time of 30 minutes. The resolution of separation is given by the following equation:

$$R_s = \frac{\mu_1 - \mu_2}{4\sqrt{2}}\left[\frac{V}{D(\bar{\mu} + \mu_{osm})}\right]^{1/2} \tag{1.5}$$

where R_s is the resolution, μ_1 and μ_2 are the electrophoretic mobilities of the two components, $\bar{\mu}$ is their average mobility, and μ_{osm} is the electroosmotic flow coefficient, where electroosmosis is the flow of the solvent in a capillary under a potential field. If the electroosmotic flow is in the same direction as the electrophoretic migration, the resolution is not good. However, very high resolution can be obtained by balancing

the electroosmotic flow against electrophoretic flow, but this results in long analysis time according to the following equation:

$$t = \frac{l^2}{(\mu_e + \mu_o)V} \tag{1.6}$$

where, t is the analysis time, μ_e is the electrophoretic mobility, μ_o is the electroosmotic coefficient, and V is the applied electric field [4]. An optimum length of the capillary (usually, 40-100 cm) must be used because as l, the length of the capillary, increases the analysis time increases even though the heat dissipation is high. On the other hand, if l is short there is less heat dissipation even though the analysis time is short. High resolution can be obtained if the components have different electrophoretic mobilities.

Due to its small volume, a capillary enables the use of nano to pico liter levels of samples. The polyimide coating on the capillary can be burnt off over a small portion which can act as a cell for UV detection. Hence, by using quartz capillaries, on-line detection can be performed. The detector is stationary and the sample passes the window and is detected.

1.3 Polyacrylamide Gel Electrophoresis

The inner surface of a capillary can be either untreated (i.e. it contains silanols on the surface) or modified by attatching some organic species to it. Capillaries are of two types: (a) an open capillary and (b) a gel filled capillary. An open capillary tube contains only buffer without any stabilizer. On the other hand, a gel filled capillary contains both the buffer and the stabilizing medium, e.g., polyacrylamide, starch, or agarose gel. Gels are useful for electrophoretic separations for the following reasons: (i) They provide an anticonvective media which helps in dissipating the Joule heat efficiently through the walls of the capillary. (ii) They minimize the solute diffusion that contributes to zone broadening. (iii) Due to their viscous nature, gels prevent solute adsorption to the capillary walls [5].

Electrophoresis carried out in a polyacrylamide gel is known as polyacrylamide gel electrophoresis (PAGE) [6]. A polyacrylamide gel is most commonly used as the

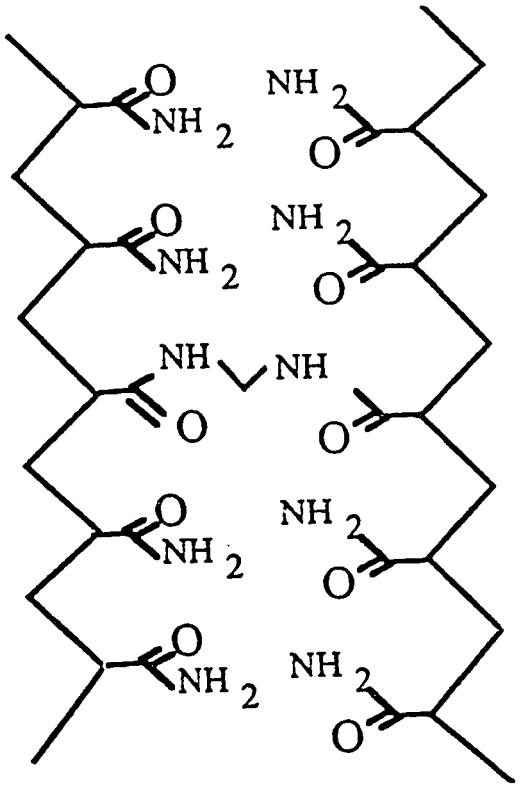


Figure 1.2: The structure of the polyacrylamide gel.

stabilizing medium because it has uniform properties and can be easily prepared with high reproducibility. Polyacrylamide gel is prepared by free radical polymerization of acrylamide monomers $CH_2 = CH - CO - NH_2$ to form polyacrylamide chains. These chains are crosslinked using a bifunctional monomer, e.g., N, N'-methylene-bis-acrylamide ($CH_2 = CH - CO - NH - CH_2 - NH - CO - CH = CH_2$) or dihydroxyethylene bisacrylamide (DHEBA). This results in a 3-dimensional network of cross linked chain as shown in Figure 1.2.

The polymerization is initiated by addition of ammonium persulphate as the initiator and N, N, N', N'-tetramethylenediamine (TEMED) as the catalyst. The rate of polymerization is optimized by varying the amounts of the initiator and the catalyst. The gel composition may be changed by varying the amounts of acrylamide and

bisacrylamide or DHEBA used. The concentrations of both acrylamide and bisacrylamide or DHEBA determine the physical properties of the gel in terms of density, elasticity, mechanical strength, and pore size.

Separation of molecules can be done on the basis of either molecular size or charge. PAGE is very useful in carrying out the separation on the basis of molecular size and is also referred to as molecular sieving by gels. This depends on the pore size of the gel which can be altered by changing the total concentration of monomer, $\%T$ and the concentration of crosslinking agent, $\%C$ [7].

$$\%T = \frac{gms\ of\ acrylamide + gms\ of\ bisacrylamide}{100\ ml\ of\ water} \tag{1.7}$$

$$\%C = \frac{gms\ of\ bisacrylamide \times 100}{gms\ of\ bisacrylamide + gms\ of\ acrylamide} \tag{1.8}$$

1.4 Sodium dodecyl sulphate PAGE

High performance capillary Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is a very powerful analytical tool in biotechnology. It is a very efficient method for separation and molecular weight determination of biomolecules including peptides, proteins, amino acids, nucleotides, and oligonucleotides. The procedure involves heating a protein mixture in an excess of an anionic surfactant, SDS and a reducing agent, e.g., β-mercaptoethanol, that results in disulphide S-S cleavage. The protein becomes denatured and the molecules take a common shape. A constant amount of SDS is adsorbed per unit weight of the protein and molecules of constant charge density are obtained. This allows molecular sieving and molecules can be separated on the basis of their molecular weights [8].

1.5 Conventional Methods

The conventional methods involve a gel slab apparatus or an open capillary tube. In the gel slab method, the solutions have to be prepared and cast into gels and the gels have to be stained or destained to see the protein bands. The separations are

run at low voltages (5-10 V/cm) for long periods of time (8-10 hours). Thus it is a labor intensive and time consuming method. In the gel slab apparatus, the gel is not bonded to the wall of the slab and thus when a high electric field is applied, the gel extrudes out of the system. Also, the Joule heat can be dissipated only at the ends of the gel compartment and this increases the convection current and results in a temperature gradient followed by zone broadening. In open capillary tubes, there is no stabilizing medium which contributes to solute diffusion and adsorption to the capillary walls. These factors limit gel slab and open capillary methods to low voltages and separations have poor resolution [1].

Karger and his coworkers have recently developed an improved method by covalently binding the gel to the wall of the capillary and using SDS-PAGE. The procedure involves treating the capillary wall, made of quartz silica containing silanol groups, with a bifunctional compound 3-methacryloxypropyltrimethoxysilane, of which one end binds to the silanol group and the other binds to the polyacrylamide gel. Since the gel is bonded to the capillary wall, high voltages can be applied without the gel extruding out of the capillary [8]. This method gives a ≡Si-O-Si-C bonding which is hydrolytically more stable as compared to the conventional ones, where there is no capillary modification. Voltages up to 30 KV can be applied easily without any gel extrusion which contributes to fast analysis and high resolution.

1.6 Proposed Method

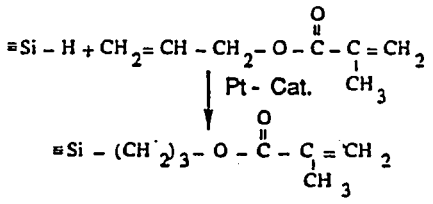
The purpose of our study is to prepare wall bonded gel filled capillaries via a hydride intermediate and carry out SDS-PAGE to separate proteins. This new method involves the hydride derivatization of the capillary wall to obtain hydrided capillary with a $Si-H$ bond. This can be obtained by a two step procedure: (i) chlorination of the capillary using thionyl chloride in dry toluene as the chlorinating agent

$$\equiv Si-OH \xrightarrow{SOCl_2 / \text{ Toluene}} \equiv Si-Cl + HCl \tag{1.9}$$

and (ii) reduction of the chlorinated capillary using lithium aluminium hydride in dry ether as the reducing agent [10].

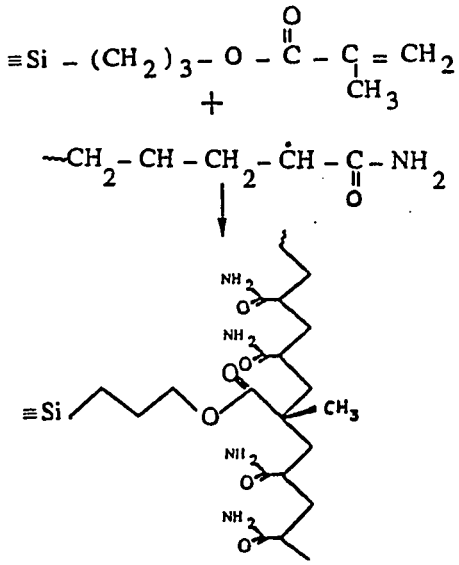
$$\equiv Si - Cl \xrightarrow{LiAlH_4 \text{ / Dry ether}} \equiv Si - H \tag{1.10}$$

The next step is to bond a bifunctional olefin allylmethacrylate to the hydrided silica. One end of the olefin will bond to the hydrided capillary surface to obtain a ≡Si-C linkage via hydrosilylation and the other end to the polyacrylamide gel through free radical polymerization.



(1.11)

It is claimed that the direct ≡Si-C bond obtained is more stable hydrolytically compared to either a ≡Si-O-C bonds (obtained by treating silanols with an alkyl group) or ≡Si-O-Si-C bond (obtained by treating silanols with a bifunctional compound). The reduced capillary should be treated with allylmethacrylate to obtain a ≡Si-C bond followed by in situ polymerization of the acrylamide under optimized conditions of tempertaure and gel concentration.



The pore size of the gel is influenced by the $\%T$ and $\%C$. The higher the $\%C$,

the higher is the pore size allowing the molecules of higher molecular weight to pass through the gel and thus separation of high molecular weight species is possible [11].

The normal gel concentrations are 10%T, 7.5%T and 5%T with 5%C, 3.3%C, etc [see equations (1.7 and 1.8)]. The optimum temperature is 12-15 °C with a polymerization time between 25-45 min. We have used the gel composition of 5%T and 3.3%C which is a good percentage for high molecular weight determinations. The gel filled capillaries are first rinsed with buffer followed by injection of protein sample. A constant high voltage of about 10KV is applied for a capillary of length 50-53 cm and UV detection is made. The separation is completed within 30 min. giving sharp peaks and high resolution. Detection is also made on capillaries filled by Karger's method involving treatment of capillaries in a non-aqueous method and our proposed method. Karger's method involves surface modification of the capillary by using 3-methacryloxypropyltrimethoxysilane (MPTMS) as the olefin and pyridine as the catalyst.

$$\equiv Si - OH + X_3Si(CH_2)_3OC(=O) - C(CH_3) = CH_2 \xrightarrow{\text{Pyridine}} \equiv Si - O - Si(CH_2)_3 - O - C(=O) - C(CH_3) = CH_2 \qquad (1.12)$$

A $\equiv Si - O - Si - C$ bond is obtained by this method. The modified capillary surface in Karger's method is shown in Figure 1.3. This figure shows the bonding between the capillary wall and carbon from the olefin through a $-O - Si-$ bridge. Due to the presence of lone pair of electrons on oxygen, the $\equiv Si - O - Si - C$ bond has less hydrolytic stability as compared to the direct $\equiv Si - C$ bond of the surface shown in Figure 1.4. This figure shows the direct $\equiv Si - C$ bond claimed by the proposed method. Here, Si on the surface is directly attatched to the carbon without any bridge. Silicon and carbon are in the same group IV A and therefore the bond energy in Si-C bond is very high, making this bond very stable. A direct $\equiv Si - C$ bond provides more surface coverage [12]. The comparisons between the two synthetic approaches are made in terms of resolution and stability with respect to time.

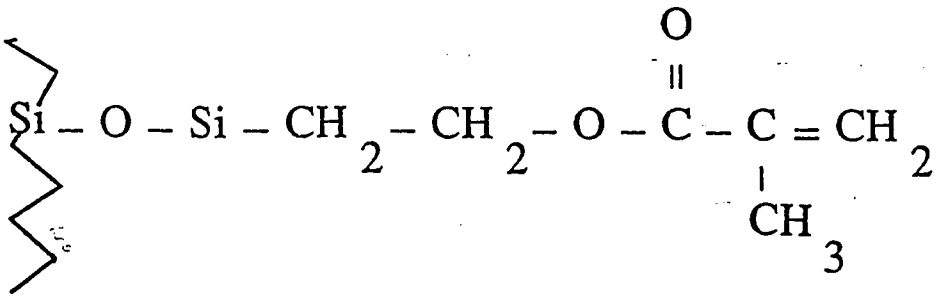


Figure 1.3: The modified capillary surface in Karger's method.

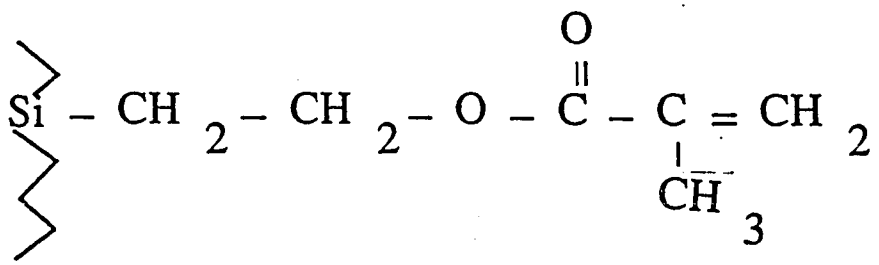


Figure 1.4: The modified capillary surface by proposed method.

Chapter 2

Experimental Details

2.1 Materials

All chemicals that were obtained used in the experimentation were of electrophoresis grade. The chemicals were procured from the following sources: acrylamide and trizma base from Aldrich Chemical Company, N N', bisacrylamide and mercapta acetic acid from Eastman Company, 2-mercaptoethanol from Bio-Rad Company, sodium dodecyl sulphate (SDS), polyethylene glycol, and dihydroxyethylene bisacrylamide from Sigma Chemical Company, urea from J.T. Baker Chemical Company, and ammoiun persulphate from Fischer Scientific Company. The silica used was partisil-40 (Whatman, Inc., Clifton, N.J.) with a 40 μm mean particle size, 85 A° mean pore size, and 315.3 m^2 per gram surface area. All proteins were purchased from Sigma. The buffer solutions were filtered through a Nylon 66 membrane (Alltech Company) of 0.2 μm pore size. The capillary was purchased from Poly Micro Technologies,Inc. with 365 μm O.D. and 75 μm I.D.

2.2 Apparatus

Fourier Transform Infra Red (FTIR) spectra were obtained on a Perkin-Elmer Model No. 1800 in our laboratory at San Jose State University and the carbon analysis were performed by the Micro Lab, University of California, Department of Chemistry,

Berkeley, CA 94720.

The capillary electrophoresis apparatus used was designed and built at San Jose State University. A high voltage D.C. power supply, (Model Series 230, Bertan Associates, Inc.) which could deliver high voltages up to 30 KV was used. The U.V. detector used was Spectra-Physics, Model Spectra 200. An analog to digital interface (Hewlett Packard 35900) was connected to a computer (HP VC/Q50) to record the results.

2.3 Procedure

Silica and quartz glass have similar chemistry except that silica is porous while quartz glass is non-porous. Due to their similar chemistry, their surfaces undergo similar reactions. Since it is easier to do experiments with silica as opposed to a small diameter capillary made of quartz, the preliminary tests were performed on samples of silica. The purpose of the preliminary tests was to obtain the optimum conditions of reaction. Once the optimum conditions were obtained, further reactions were performed on the capillary. The overall experimental procedure can be summarized under the following main headings:

2.3.1 Preparation of chlorinated silica (Partisil-40)

- **Chlorination of silica:** Silica (10 g) was weighed in a 3-necked flask and dried in an oven at 110°C under vacuum. Dry distilled toluene (140 mL, predried with CaH_2) was transferred through a canula to the 3-necked flask containing silica. Dry nitrogen was passed through the flask to ensure moisture-free conditions. Thionyl chloride, $SOCl_2$, (7 mL) was added through a silicon septum. Nitrogen flow was stopped and the flask was heated at the reflux temperature. The flask was then wrapped with a thin rope to avoid heat loss. Chlorination was allowed to occur for 48 hours.

- **Washing of chlorinated silica:** The sample was washed with dry toluene to remove the unreacted thionyl chloride. The silica was allowed to settle down

and its top layer was aspirated under vacuum. Dry toluene (60-70 mL) was added while stirring for about 10 minutes. The sample was then allowed to settle down for an hour until the separation between the solid and the liquid was seen properly. The top layer was aspirated and the washing was done for 7-8 times. The washed silica was dried in a heating mantel after tying a heating tape around the flask.

2.3.2 Reduction of chlorinated silica

The chlorinated silica was reduced by lithium aluminium hydride in dry ether as the reducing agent. This reduction consists of the following steps:

- **Preparation of lithium aluminium hydride:** Lithium aluminium hydride (1.6 g) was transferred to a 500 mL brown bottle. Dry distilled ether (400 mL) was transferred to the bottle through a canula. Both weighing and transferring of $LiAlH_4$ were done in a glove bag under dry nitrogen.

- **Reduction of chlorinated silica:** $LiAlH_4$/ether (120 mL) was added to a 3-necked flask containing a condenser filled with isopropanol and dry ice. The dried silica was transferred in small lots to the flask by using an addition funnel while stirring constantly. Reduction was carried out for about 3 hours.

- **Washing of reduced silica:** The reduced silica was washed with 0.5M HCl five times in a fritted disk crucible. This was followed by washings with $HPLC$ water, water/methanol, methanol/acetone, acetone/dichloromethane, and dichloromethane. The product was then dried and weighed.

- **Another reducing agent:** Partisil-40 was also reduced by using another reducing agent, disodium aluminium hydride (Na_2LiAlH_2). The steps were the same as with the reducing agent lithium aluminium hydride.

Silica	Partisil 40	0.5 gms
Olefin	Allylmethacrylate	6.5 ml
Solvent	Dry tolouene	1.5 ml
Inhibitor	DBHQ	200.0 mg
Catalyst	DCPtCl	350.0 μl, 62.5 mM
Reaction time	---	48.0 hrs
Induction time	---	60.0 min
Reaction temp.	---	104 +/- 1°C

Table 2.1: The data for bonding of allylmethacrylate on reduced silica.

2.3.3 Bonding of allylmethacrylate on reduced silica

- **Bonding on reduced silica:** Reduced silica (0.5 g) was weighed and dried in a vacuum oven at 110°C for an hour. Dry toluene (1.5 mL) was transferred to a 15 mL round bottom flask fitted with a condenser. 2,5 Di-tert-butyl hydroquinone (DBHQ, 80 mg) was added. Allylmethacrylate (6.5 mL) and 350 μl of 62.5 mM of dicyclopentadenyl platinum chloride(DCPtCl) was added to the mixture. After an hour of induction time at 70°C, reduced silica was added. The reaction was continued for 48 hours by maintaining the temperature at 105-106°C in a silicone oil bath. Figure 2.1 shows this reaction setup.

- **Washing of the product:** The product was washed with 5 mL of toluene (5 times), 5 mL of ether (3 times), and 5 mL of dichloromethane (2 times). The washed product was first dried at room temperature and then in the oven at 110°C. The product was cooled and weighed. Carbon analysis and FTIR spectra were taken of the sample.

- **Effect of catalyst:** The effect of catalyst on the bonding of allylmethacrylate with the reduced silica was studied by changing the concentrations of DCPtCl.

- **Effect of reaction time:** The effect of the reaction time on the bonding of allylmethacrylate on reduced silica was studied by carrying out six reactions with reaction times of 12, 24, 36, 48, 60, and 72 hours.

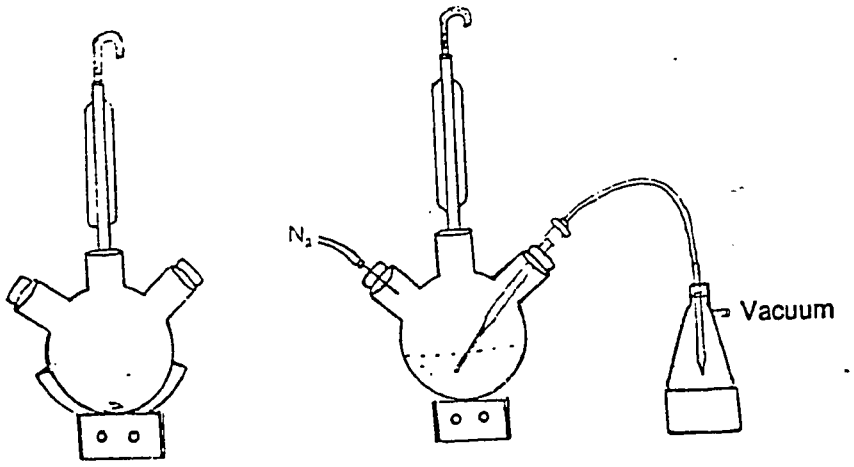


Figure 2.1: The schematic of the reaction setup for reactions on silica.

2.3.4 Bonding of 3-methacryloxypropyltrimethoxysilane on untreated silica

- **Bonding on untreated silica:** Dry toluene (75 mL) was added to a 100 ml three-necked round bottom flask containing 1.00 g of dry native partisil-40. 3-MPTMS (39 mL) was added followed by 45 μL of dry pyridine as the catalyst. The mixture was refluxed at 105°C with magnetic stirring for about 20 hours.

- **Washing and drying of product:** The product was washed using 15 mL portions of toluene (3 times), dichloromethane (2 times), and ether (1 time). The product was dried at room temperature until all the ether was evaporated and then overnight under vacuum at 110°C. The FTIR spectrum was taken and the sample was sent for elemental analysis.

2.3.5 Reactions on the capillary

- **Pretreatment of capillary:** A 5 meter length of capillary was taken with 365 μm O.D. and 75μm I.D. The capillary was pretreated by flushing with the following liquids: HCl (25 mM) for 15 min, deionized water for 15 min, KOH (20mM) for 20 min, and then deionized water for 20 minutes. It was dried overnight by nitrogen at 110°C in a silicone oil bath.

- **Reduction of Capillary:** The reduction of the capillary was done in two steps: (i) chlorination using thionyl chloride in dry ether as the chlorinating agent, and (ii) reduction of the chlorinated capillary by using Na_2LiAlH_2 as the reducing agent.

- **Bonding of MPTMS by Karger's method:** Toluene (12 mL) was taken in a dry reservoir. MPTMS (624 μL) was transferred to the reservoir followed by 7.2 μL of pyridine. One end of the capillary was immersed in this reservoir and the other end to another reservoir for collecting the liquid coming out of the capillary.

- **Olefin bonding to the capillary by proposed method:** Toluene (1.5 mL) was taken in the reservoir and 80 mg of inhibitor was added while stirring using a magnetic stirbar. Olefin (allylmethacrylate, 6.5 mL) was added followed by 650 μL of 62.5mM dicyclopentadenyl platinumchloride in chloroform as the catalyst. One hour of induction period was given at 70 $\pm$2°C. The capillary end was kept above the liquid with the nitrogen flowing through the capillary during the induction period. The capillary end was dipped into the liquid after the induction period and the temperature of the oil bath was increased to 104 $\pm$2°C. The reaction was continued for 72 hours. The bonded capillary was washed with dry toulene for 10 hours followed by ether and dichloromethane for an hour each. It was dried overnight under nitrogen at 110 °C.

The procedure for the above three steps on the capillary was the same as that followed for preparing reduced silica. One end of the capillary was immersed in the reservoir containing the reaction mixture and the other end was left in the reservoir

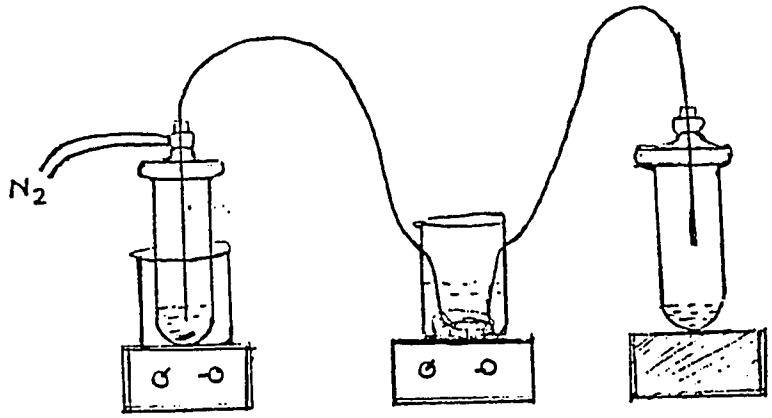


Figure 2.2: The schematic of the reaction setup for reduction of capillary.

containing a drying tube. The rest of the capillary was coiled and immersed in a silicone oil bath to maintain the reaction temperature, as shown in Figure 2.2. The solution was passed through the capillary under dry nitrogen at a pressure of 10-40 psi.

2.3.6 In situ gel formation in the bonded capillary

- **Preparation of stock monomer solution:** A monomer solution of acrylamide/bisacrylamide (30% w/v) was prepared by weighing 29.000 g of acrylamide and 1.000 g of bisacrylamide and 28.720 g of acrylamide and 128.00 g of DHEBA respectively in a weighing tube. The solids were transferred separately to two 100 mL volumetric flasks and dissolved using double filtered deionized water. The volume was made up to the mark.

- **Preparation of stock buffers:** (a) Phosphate buffer (100 mM): 69.3417 g of sodium dihydrogen phosphate, NaH_2PO_4 was transferred to 1 liter volumetric flask. Double filtered deionized water (800 mL) was added, the pH was adjusted

Stock Solution	10% T	7.5% T	5 % T w/AA /bis	5% T w/AA/DHEBA
AA/Bis (ml)	2.0	1.5	1.0	1.0
Buffer (ml)	1.2	1.2	1.2	1.2
1.5% APS (μl)	300.0	200.0	130.0	60.0
water (ml)	2.5	3.0	3.5	3.5
TEMED (μl)	3.0	3.0	3.0	4.0

Table 2.2: The data for gel preparation.

to 7.32 with 2 M $NaOH$, and the volume was completed with water. (b) Phospate-Tris buffer (100 mM): Concentrated phosporic acid (34 mL) was added to a 1 liter volumetric flask containing 800 ml of double filtered water. The pH was adjusted to 7.34 with Tris base and then water was added up to the mark.

- **Preparation of polyacrylamide gel with 5%T and 3.3%C:** Urea (2.788 g, 8M) was weighed in a test tube and 3.5 mL of water was added to it, followed by 1.2 mL of buffer. Urea was dissolved in the solution and 1 mL of stock acrylamide/bisacrylamide was added followed by 130 μL of ammonium persulphate. The solution was degassed ian ultrasonicator under vacuum for 10-15 min and cooled for 5 min in a cold bath. Tetraethylenediamine (TEMED, 3 μL) was added and the mixture was stirred and degassed for a minute. This mixture was filled in the capillary by using a gas and liquid tight syringe. The capillary was rinsed with buffer before filling the gel. Gel formed in about 20-45 min. at

room temperature. The acrylamide/bisacrylamide mixture was polymerized to form a gel with 10%T, 7.5%T, and 5.0%T with 3.3%C, but finally the capillaries were filled with 5%T and 3.3%C gels. Table 2.2 shows the conditions used to obtain gel with the desired characteristics. The polymerization temperature was varied from 0 °C to 25 °C. The polymerization was finally tried at room temperature under a high pressure of 6200 psi for about an hour. All solutions were degassed in an ultrasonicator under vauum for at least 20 min.

- **Preparation of 10% SDS solution:** SDS (10.000 g) was dissolved in double filtered deionized water in a 100 mL volumetric flask and water was made up to the mark. The solution was clear and colorless.

- **Preparation of buffer for separation by SDS-PAGE:** (a) For 100 mM phosphate-tris buffer, 0.1 w/v SDS, 8 M urea: Stock H_3PO_4 tris buffer (10 mL) was transferred to a 50 mL volumetric flask, followed by 0.5 mL of 10% w/v SDS and 24.04 g of urea. The volume was made up to the mark by water and the solution was filtered through a nylon membrane, 0.2μm pore size, and degassed for 20 min. before use.

2.3.7 Preparation of the protein sample

To 5 and 20 mg of standard protein, 50 μL of 10% SDS solution was added. This was followed by 100 μL of 0.5M phosphate buffer and 50 μL of 2-mercaptoethanol. The volume was completed to 1 mL with double filtered water and the mixture was boiled for 3-5 min. It was cooled at room temperature and stored in the refrigerator in small alliquotes of 200 μL.

Chapter 3

Results and Discussion

3.1 Reduction of Silica

Silica surfaces are covered with hydroxyl groups that are attatched to silicon atoms. The hydroxyl groups are very polar in nature and make Si-O bond in $\equiv Si-OH$ susceptible to hydrolysis. The $Si-O$ bond in silanols is highly susceptible to hydrolysis. Studies have been made to improve the hydrolytic stability of the silica surface. One of the methods used is to reduce the silanols to hydrides which are less susceptible to hydrolysis under acid conditions [10]. According to this method silanols are reduced to give $\equiv Si-H$ on the silica surface and then an olefin is bonded to the surface resulting in a direct $\equiv Si-C$ bond. The $\equiv Si-C$ bond obtained is claimed to be less susceptible to hydrolysis as compared to $\equiv Si-OH$ and $\equiv Si-O-C$ [12].

FTIR Data: The silica was first chlorinated by using thionyl chloride under moisture-free conditions followed by reduction by with lithium aluminium hydride as the reducing agent. The FTIR spectrum was taken for 1:1 ratio of the sample and powdered potassium bromide. This spectrum is shown in Figure 3.1. As from the spectrum, prominent peaks at 2200 and 2250 cm^{-1} are seen for reduced silica before and after HCl treatment, respectively, which clearly indicate Si-H bond stretching. The peaks at 910 and 830 cm^{-1} indicate the Si-H bond bending. When silica was reduced with lithium aluminium hydride as the reducing agent, no Si-C bond stretching was observed. On the other hand, some C-H bonding was observed when disodium

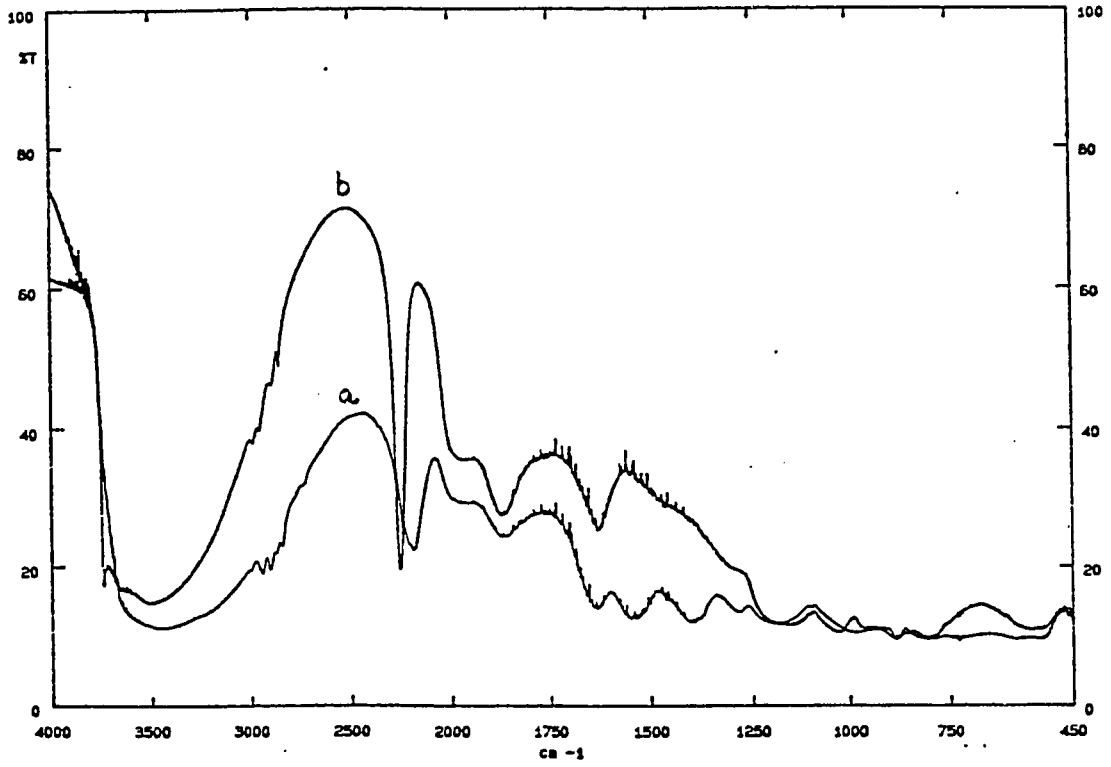


Figure 3.1: The FTIR spectra for reduced silica (a) before HCl treatment, (b) after HCl treatment.

lithium aluminium hydride, Na_2LiAlH_2 was used . This may be due to some soluble organic species from the washing solvents. The reduced silica was washed with 0.5 M HCl and FTIR spectrum showed an intense Si-H peak as compared to the one for reduced silica before HCl treatment. This was due to complete removal of unreacted lithium aluminium hydride.

Carbon Analysis Data: A carbon percent of 0.51% was obtained for reduced silica prepared by using Na_2LiAlH_2 as the reducing agent. This is the amount of carbon that has to be substracted from the %C obtained for the olefin bonded silica.

Reaction time (hours)	Temp (^{0}C)	C %	Corrected C %	α (μmol/m)
11	102	1.925	1.426	0.55
25	103	2.115	1.615	0.62
35	103	2.130	1.633	0.63
48	103	2.280	1.784	0.69
60	103	2.360	1.864	0.72
72	103.5	2.580	2.080	0.81

Table 3.1: The effect of reaction time on bonding of allylmethylacrylate on reduced silica.

3.2 Bonding of the olefin on reduced Silica

- **Bisacrylamide as the crosslinking agent:** Bisacrylamide was tried as the crosslinking agent between the hydrided silica (obtained by chlorination followed by reduction of silica as described earlier) and the polyacrylamide gel. The monomer is a mixture of acrylamide and bisacrylamide, so it was thought that it would act as the natural crosslinker but the reactions were not successful as the products polymerized. This polymerization was observed by gain in weight and formation of a gel-like substance.

- **Allylmethacrylate as the crosslinking agent:** Due to the failure of bisacrylamide as the crosslinker, allylmethacrylate was tried as the cross linking agent between the hydrided silica and the polyacrylamide gel. This procedure was then repeated on hydrided capillary surface. Reaction time of 72 hrs was given and the product was found to be unpolymerized. Optimum conditions were obtained in terms of reaction time and amount of catalyst by studying the effect of reaction time and catalyst concentration on the bonding of allylmethacrylate on reduced silica.

  Effect of reaction time: Reaction times of 11, 25, 35, 48, 60, and 72 hours were tried. It was observed that the carbon percent for C-H bonding increases

with increase in reaction time and reaches a plateau after 72 hours. Thus, it was concluded that 72 hours of reaction time was optimum. Table 3.1 shows the effects of reaction time on %C and α_R. The surface coverage α_R of bonded olefin can be calculated from the carbon content of the modified silica, the molecular weight of the olefin, and the specific surface area of (S_o) of the starting material according to the following formula:

$$\alpha_R = \frac{10^4 P_2'}{M_c n_c S_o (1 - \frac{1 - P_2' M R}{100 M_c n_c})} \tag{3.1}$$

and

$$P_2' = \frac{P_2 - P_1}{1 - \frac{P_1 M_R}{100 n_c M_c}} \tag{3.2}$$

where P_2' is the corrected %C for the bonded silica, M_c is the molecular weight of carbon, n_c (= 7) is the number of carbons in the olefin, S_o (= 315.3 m^2/gm) is the surface area of silica per gram, M_R (= 128.16) is the molecular weight of allylmethacrylate, P_2 is the %C of bonded silica and P_1 is the %C of reduced silica (0.51%). Figure 3.2 shows the effect of reaction time on %C for the olefin bonded silica. The surface coverage was found to increase with increase in reaction time. A surface coverage of 0.81 μmol/m was obtained for olefin bonded silica in 72 hours of reaction time.

FTIR Data: FTIR spectra of 1:1 samples of bonded silica and potassium bromide were taken. Five peaks for C-H bond stretch in CH_2 were observed in the region 3085-2900 cm^{-1}. C-H bond bending was identified in the region 1450-1375 cm^{-1}. Figure 3.3 indicates the spectrum of the sample obtained after 72 hours of reaction time. Comparing Figure 3.1 and Figure 3.3, it is noticed that the Si-H peak is reduced and the C-H peak is increased in Figure 3.3. This shows that the olefin has bonded to the hydrided silica. The surface coverage shows the amount of carbon bonding on hydrided silica.

Figure 3.4 indicates the detailed spectrum of the bonded silica in the region 3085-2900 cm^{-1}. Five sharp peaks at 3070 (due to H-C=C), 2990, 2940, and 2900 cm^{-1} are seen in this region due to CH_2 stretching of propyl chain in bonded silica. Figure 3.5 indicates the detailed spectrum at 1450 and 1375 cm^{-1}.

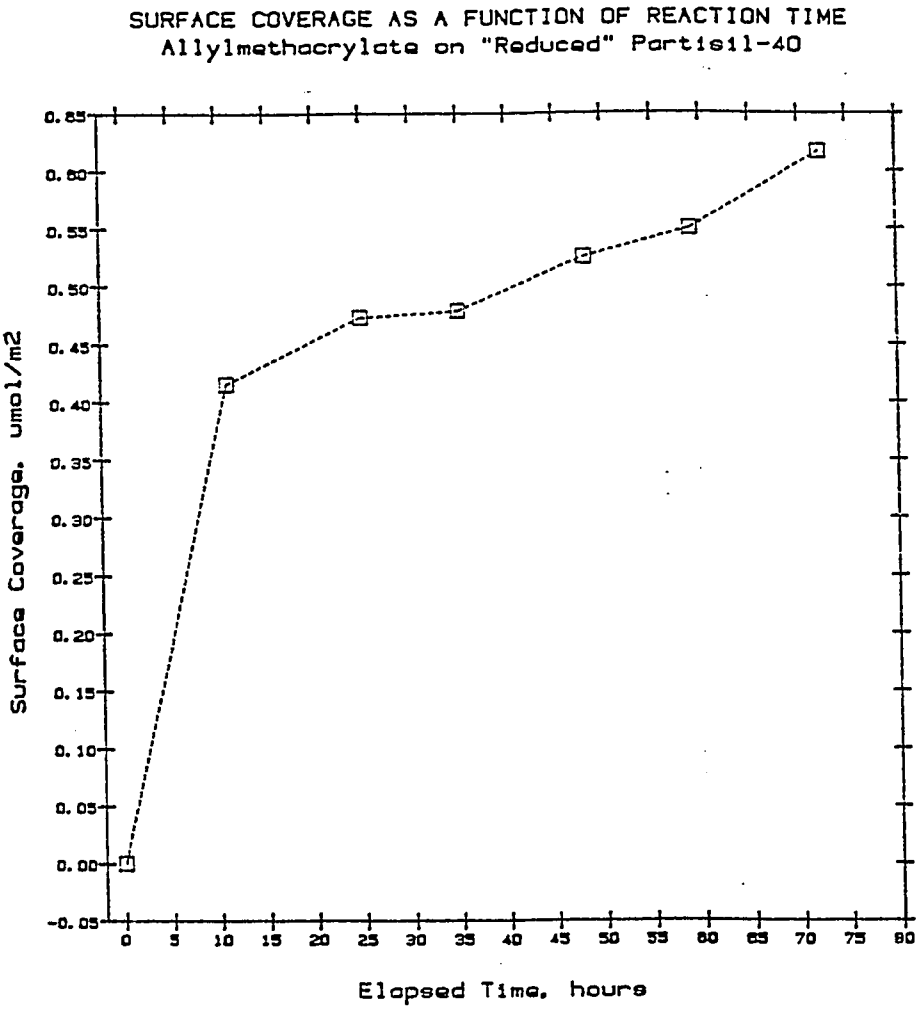


Figure 3.2: The effect of reaction time on %C in bonded silica.

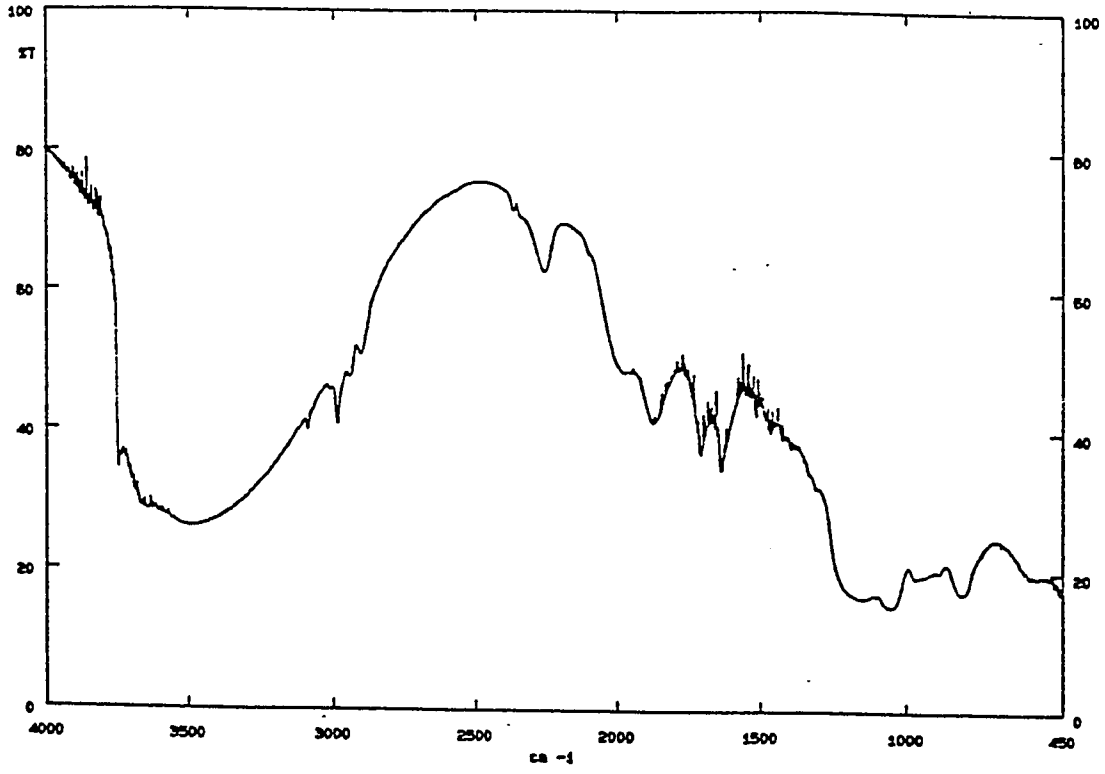


Figure 3.3: The bonding of allylmethacrylate on reduced silica for 72 hours of reaction time.

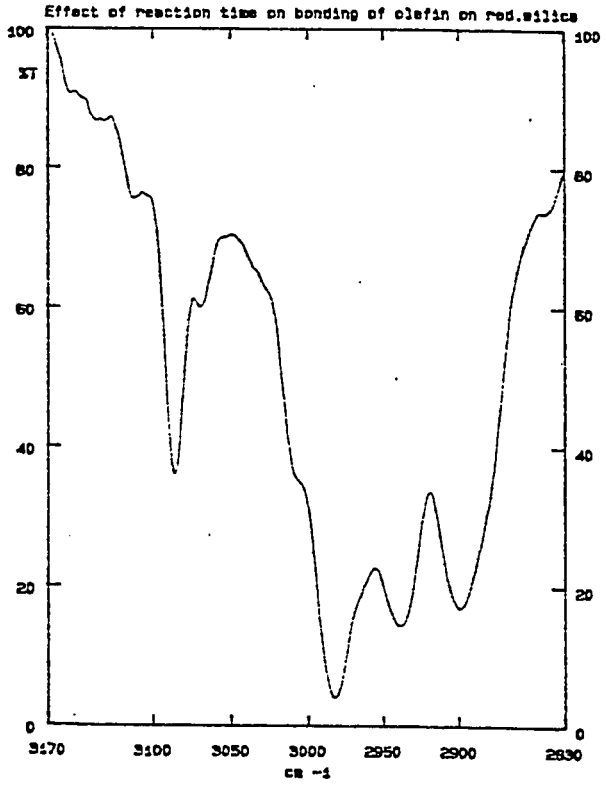


Figure 3.4: The spectrum of bonded silica in the region 3085-2900 cm^{-1}.

The peak at 1640 cm^{-1} is due to monosubstituited olefin, C=C stretch of olefin bonded silica. The carbonyl peak is shown at 1700 cm^{-1}. Symmetric bending vibrations for CH can be seen around 1375 cm^{-1} and asymmetric vibrations at 1450 cm^{-1}, which is almost overlapping with CH bending vibrations.

Effect of catalyst: Dicyclopentadenyl platinumchloride was used as the catalyst and various concentrations of the catalyst were tried. It was observed that the amount of C-H bonding increased with an increase in catalyst concentration. The observed data is listed in Table 3.2. Figure 3.6 indicates the effect of catalyst concentration on %C of the bonded silica. From Figure 3.6, it is observed that an increase in catalyst concentration leads to increase in bonding and reaches a plateau after using 40 μl of 65 mM catalyst.

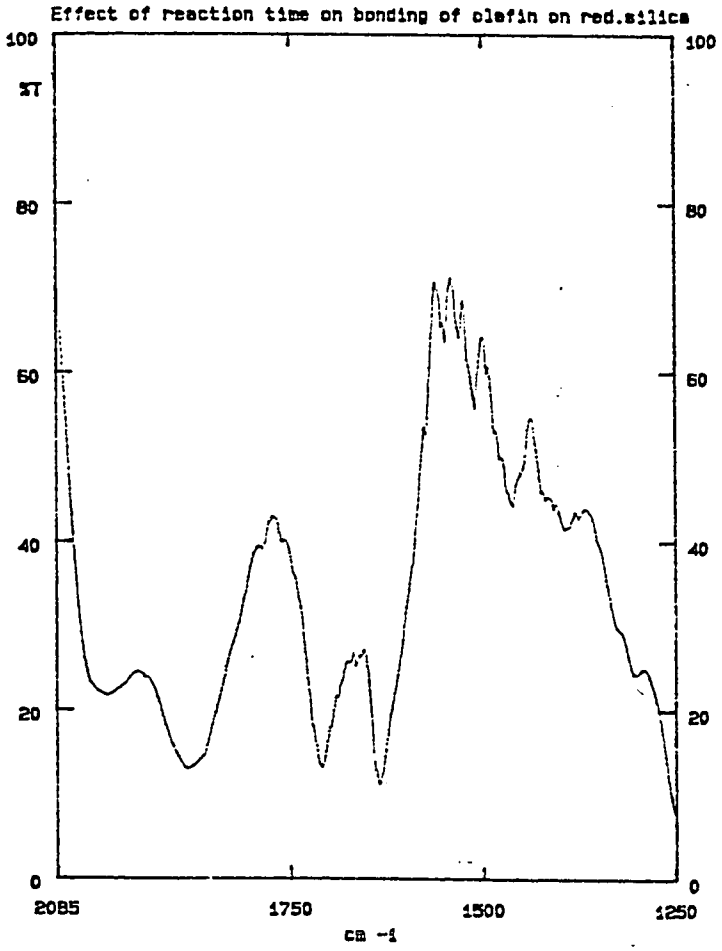


Figure 3.5: The spectrum of bonded silica in the region 2050-1450 cm^{-1}.

Concentration of catalyst (mM)	Amount of catalyst (μl)	C %	Corrected C %	α (μmol/m)
62.50	350	2.74	2.248	0.88
62.50	150	2.64	2.147	0.84
62.50	50	2.58	2.087	0.81
6.25	150	2.30	1.804	0.72
6.25	40	2.22	1.724	0.61

Table 3.2: The effect of concentration of catalyst on bonding of allylmethacrylate on reduced silica.

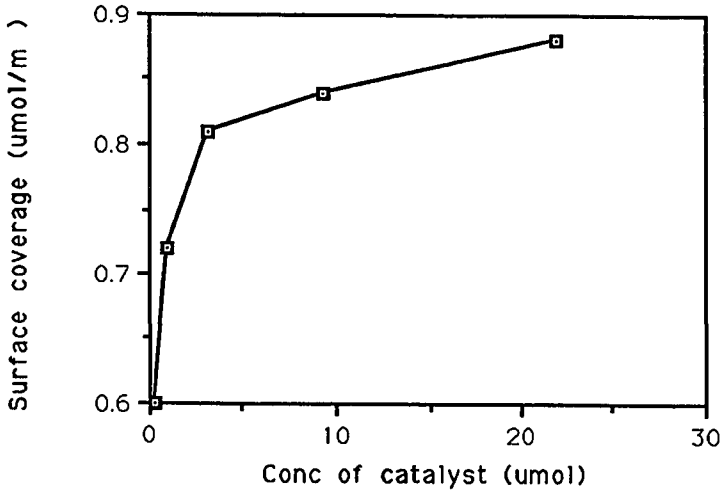


Figure 3.6: The effect of concentration of the catalyst on %C of the bonded silica.

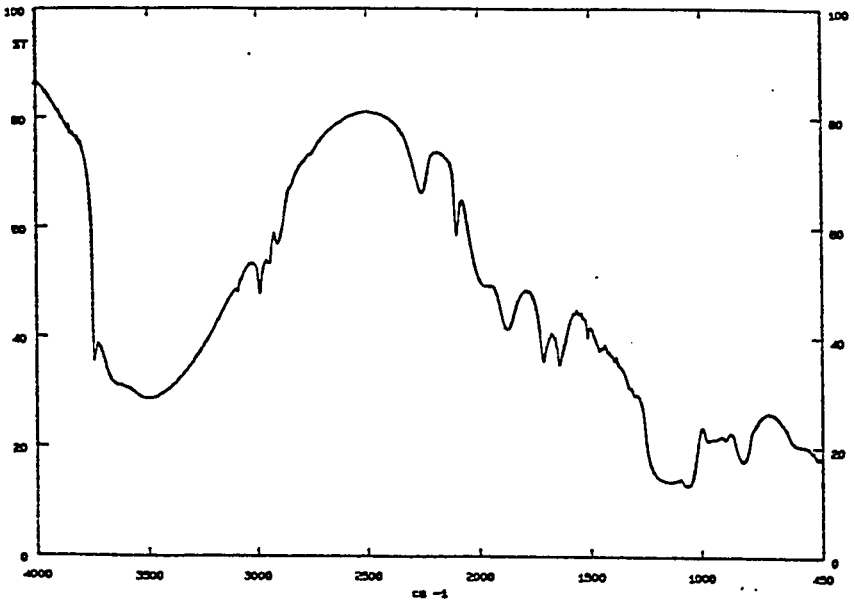


Figure 3.7: The bonding of allylmethacrylate on reduced silica using 21.9 μmol of catalyst.

FTIR data: FTIR spectra for the samples were taken using 1:1 ratio of the sample and powdered potassium bromide. Five sharp peaks for C-H bond stretching were identified in the region 3100-2850 cm^{-1}. The C-H bending peaks were observed around 1450 cm^{-1}. Figure 3.7 indicates the spectrum with 21.9 μmol of DCPtCl catalyst. Figure 3.8 indicates four peaks at 3085, 2900, 2935, and 2900 cm for bonded silica in the region 3110-2850 cm^{-1}, which shows CH bond stretch of propyl chain of olefin bonded silica. Figure 3.9 indicates the spectrum in the region 2150-1360 cm^{-1}. The peak at 1635 cm is due to C=C olefenic bond stretch of bonded silica. The C=O peak is observed at 1720 cm^{-1}. The CH bending peak can be seen around 1450 cm^{-1}. These spectra indicate that the olefin has bonded to the hydrided silica.

Hexachloroplatinic acid (H_2PtCl_6) was also tried as a catalyst but the catalyst blackened during the induction period. This indicated the reduction of platinum

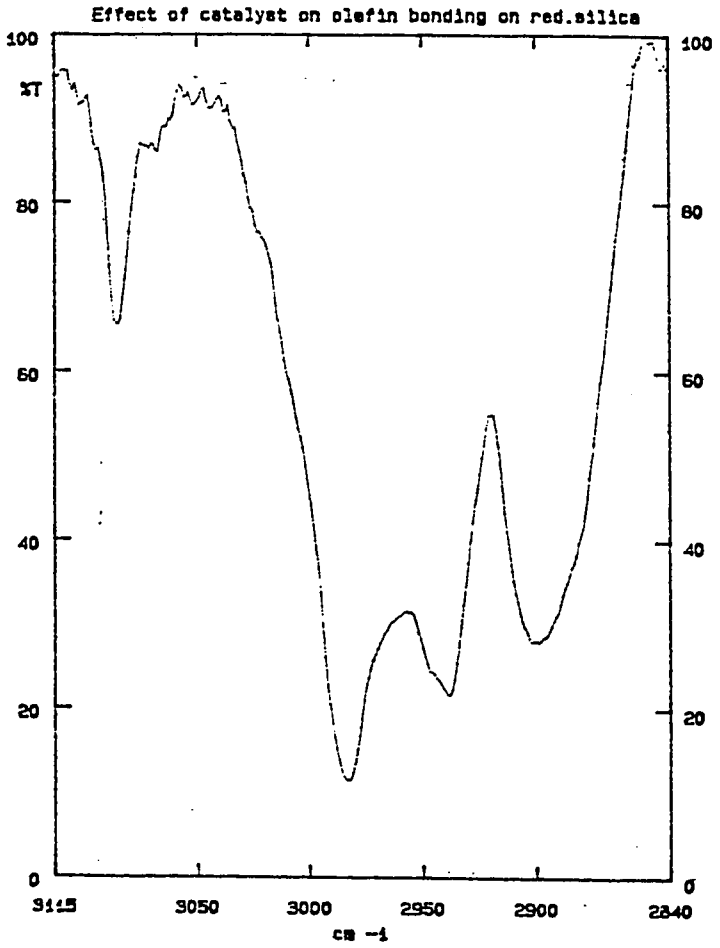


Figure 3.8: The spectrum of bonded silica in the region 3110-2850 cm^{-1}.

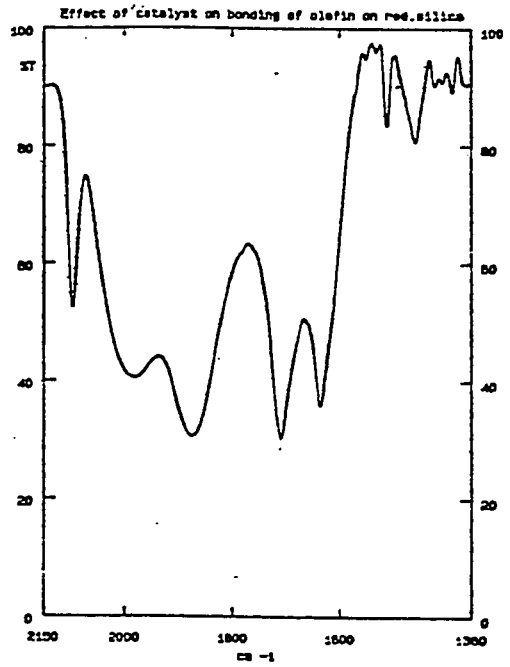


Figure 3.9: The spectrum of bonded silica in the region 2150-1360 cm^{-1}.

(II) to platinum (0) which may not be effective in catalyzing the olefin bonding on hydrided silica.

3.3 Bonding of methacryloxypropyltrimethoxysilane on untreated Silica

The aim of this project was to prepare an olefin bonded capillary via a hydride intermediate, fill gel into it and carry out separation of biomolecules. Since it is believed that our method has substantial advantages in terms of hydrolytic stability of capillaries and separation of biomolecules, the capillaries have been filled using Karger's method in order to make comparisons. Karger and coworkers used methacryloxypropyltrimethoxysilane (MPTMS) as the crosslinking agent between the untreated capillary wall and polyacrylamide gel. The gel was filled in a modified capillary.

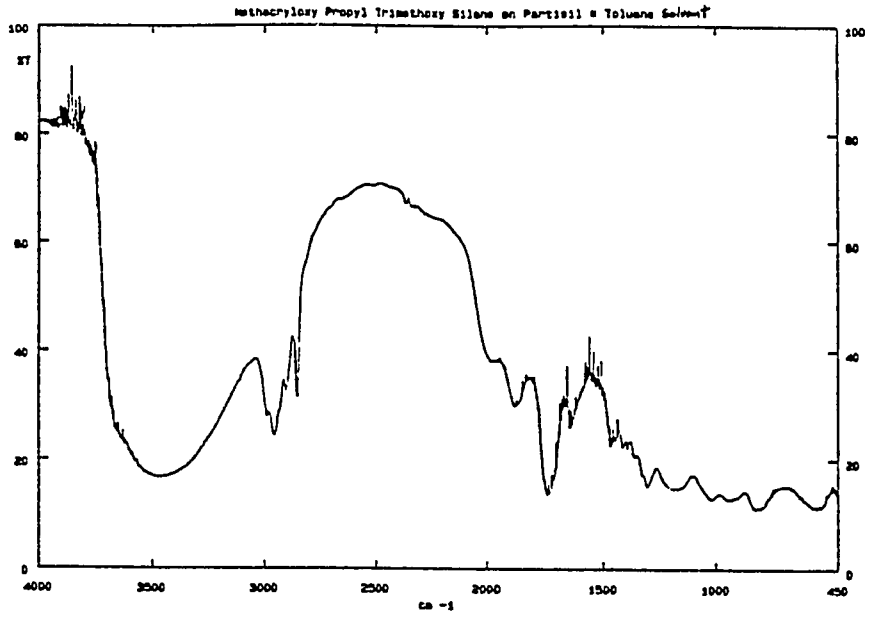


Figure 3.10: The bonding of methacryloxypropyltrimethoxysilane on untreated silica.

FTIR Data: The FTIR spectrum was taken by using 1:1 ratio of sample and powdered potassium bromide (Figure.3.10). The C-H bond stretch peaks were observed in the region 3035-2830 cm^{-1}. Figure 3.11 indicates the detailed spectrum in the region 3035-2830 cm. The peaks at 2960 and 2850 cm^{-1} are due to the CH stretching modes of the propyl chain. This area confirms the bonding of MPTMS to the silica. Figure 3.12 indicates the detailed spectrum in the region 2100-1210 cm^{-1}. Usually the carbonyl peak of MPTMS appears at 1720 cm^{-1} but it can shift to higher wavelength upon polymerization of the C=C functional group. The C-H bond bending was identified at 1440 cm^{-1}. The shoulder at 2050 cm^{-1} is due to silica lattice vibrations. The peaks at 930 and 850 cm^{-1} represent Si-O stretch in $Si-O-Si-C$. The Si-O stretch peak can be identified at 850 cm^{-1}.

Carbon Analysis Data: The elemental analysis showed a carbon % of 8.13 for the bonded silica prepared by this procedure. This reflects the olefin bonding on the silica. The surface coverage was calculated to be 3.3 μmol/m of silanol groups which is a good surface coverage.

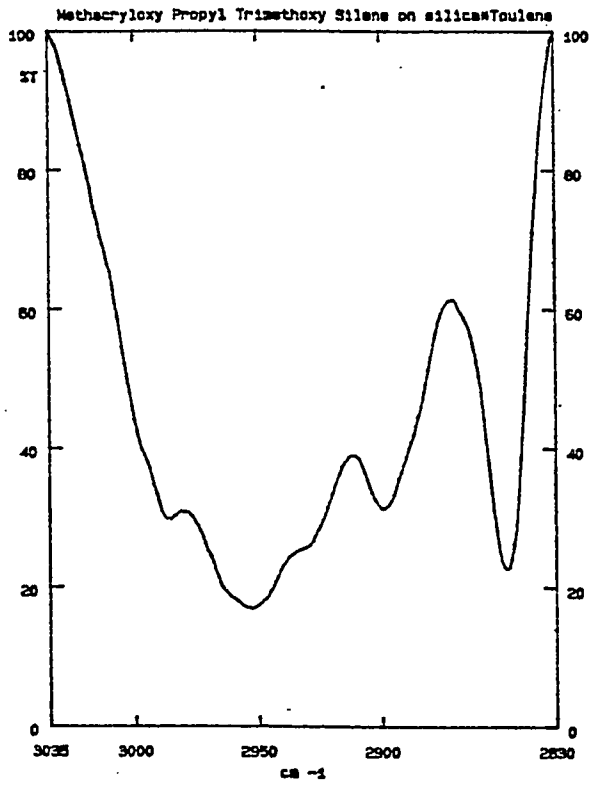


Figure 3.11: The spectrum of bonded silica using MPTMS in the region 3035-2830 cm^{-1}.

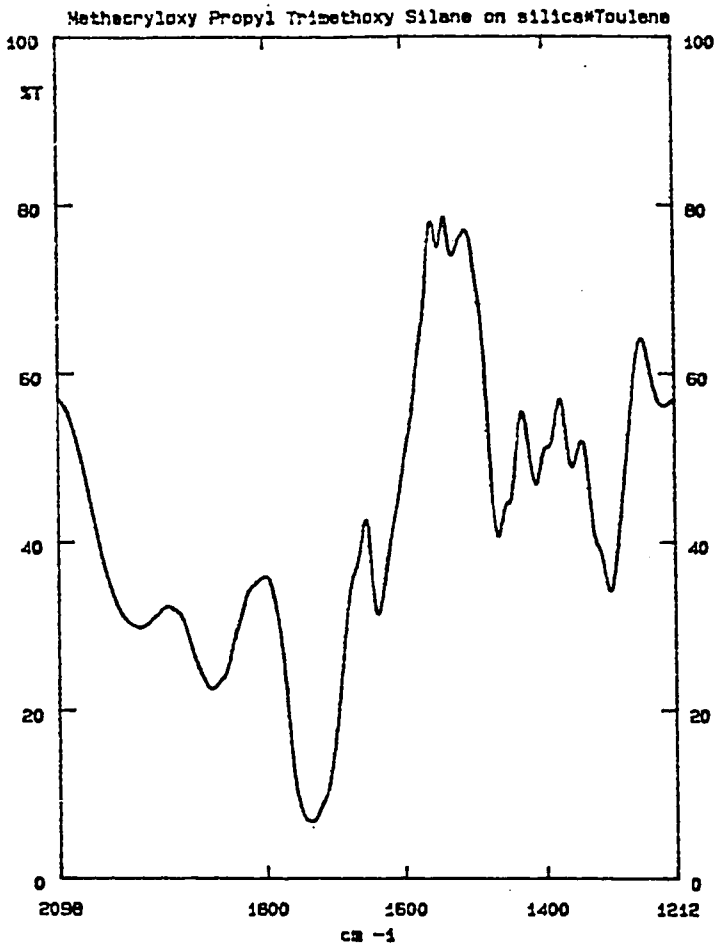


Figure 3.12: The spectrum of bonded silica using MPTMS in the region 2100-1210 cm^{-1}.

3.4 Reactions on Capillary Surfaces

As mentioned earlier, the chemistry of silica and capillary surfaces is similar. In the previous sections, the results of the experiments with silica have been described. The best conditions that were obtained with silica were used for reactions on the capillary.

3.4.1 Reduction and olefin bonding on the capillary

The hydrided capillary was obtained upon chlorination followed by reduction, which was then bonded to allymethacrylate, but certain problems were encountered during this whole procedure. During the reduction of capillary, although appropriate care was taken while transferring lithium aluminium hydride into the reservoir and immersing the end of the capillary into it, the capillary got clogged at various places. This was probably due to the exposure of the reducing agent to the moisture. The capillary had to be cut several times to remove the clogged portions.

During the olefin bonding on hydrided capillary, a reaction time of 72 hours was given. The flow of the reaction mixture was observed to decrease with time and it almost stopped after 72 hours. The capillary did not show any clogging when observed under microscope. This may be due to increase in density of the reaction mixture with time.

3.4.2 Gel preparation and gel filling in the capillary

The gel was prepared by polymerizing acrylamide/bisacrylamide and acrylamide/-DHEBA solutions using ammonium persulphate as the initiator and TEMED as the catalyst. It was important to optimize the time required in the formation of the gel. Gels which form in a short time (within 10 minutes) or in a long time (more than an hour) are not good in terms of mechanical strength [7]. They do not have much elasticity and tend to break quickly. We chose the optimal time in the formation of the gel to be 25-45 minutes. The temperature plays an important role during gel formation. When the gel is formed, the volume shrinks, giving rise to voids. At cold temperatures, the shrinkage is minimal, which avoids voids. The pore size of the gel

APS (μl)	TEMED (μl)	Gel formation time (min.)
300	4	10
250	4	17
250	3	22
200	3	27
200	3	27-30
200	3	27-30

Table 3.3: The gel formation time under different conditions without using a stabilizer.

increases with an increase of %T. Gels with %T of 10, 7.5, and 5 with %C of 3.3 were tried but the capillaries were finally filled with 5%T and 3.3%C gel concentration. This was done so that proteins (oligonucleotides, etc.) with high molecular weights could be separated. The pore size increases with decrease in %T. Gels with %T of 5 are good for separation of biomolecules with molecular weights between 20000-200,000.

The following are the different cases under which the gel preparation and the gel filling of the capillary were tried.

- **Without stabilizer:** Amounts of ammonium persulphate (APS) and TEMED were varied in order to obtain the gel formation time of 25-45 minutes. Table 3.3 gives the effect of amounts of APS and TEMED on the gel formation time. All trials were attempted at 14-17 °C.

  Five capillaries were filled by using 200 μL of APS and 3 μL of TEMED. Two of these contained bubbles and voids. Although all the solutions were degassed prior to the experiment for at least 20 minutes in an ultrasonicator under vacuum, bubbles appeared in the filled capillary.

  Bubbles are formed due to the presence of air in the gel mixture. They can be avoided by degassing the solution before filling the capillary. Voids are formed due to the shrinkage of gel. Due to the fact that the volume of monomer mixture

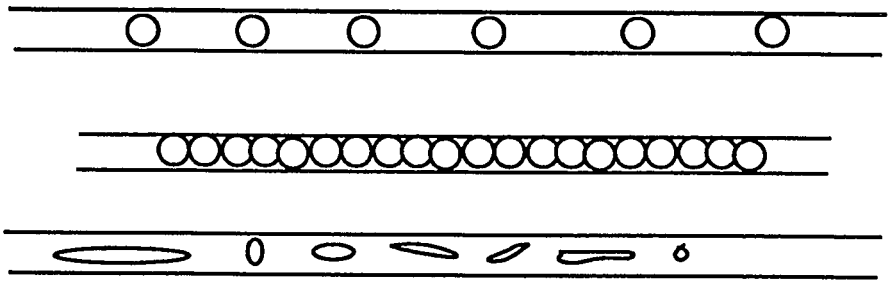


Figure 3.13: (a) Bubbles and (b) Voids formed in a capillary during gel formation.

APS (μl)	TEMED (μl)	Gel formation time (min.)
250	3	20
200	3	30-35
200	3	30

Table 3.4: The gel formation time with urea at 14-17 °C.

is larger than the polymer, there is a contraction of volume during gel formation, which leads to voids. Bubbles are regular in shape, whereas voids are irregular and twisted in shape.

- **With urea:** As voids appeared in the previous case, 8 M urea was tried as a stabilizer while preparing the gel. Urea is a hydrogen bond breaker and was expected to help in avoiding voids. Optimum conditions in terms of polymerization time (25-45 minutes) and amount of initiator were tried in order to obtain good gel preparation time. Table 3.4 shows the various trials. Three capillaries were filled and all of these were found to be bubble and void free. As the capillaries were obtained without voids and bubbles, a detection window was made on the capillary. Polyimide coating (about 0.5 cm) on the capillary was burnt using fuming sulphuric acid dropwise and heating that portion with an electric heater. The capillary was then observed under the microscope. Voids were observed near the window. This happened because when the window was

APS (μl)	TEMED (μl)	Gel formation time (min.)
200	3	15
130	3	25
130	3	27
130	3	27-28

Table 3.5: The gel formation time with urea at room temperature.

made on the capillary by heating, the gel broke from the nearby portion, giving rise to bubbles. Also, voids were seen in the capillary.

- **With urea at room temperature:** Due to the formation of bubbles and voids in the last procedure, it was decided to make the window before filling the capillary with gel. As the capillary becomes very fragile at its window, due to the removal of polyimide coating, it could not be coiled and placed in the cold trap during the gel formation. So, it was decided to fill the capillary at the room temperature. Table 3.5 shows the conditions and required time for gel formation. Three capillaries were filled and all of them contained voids.

- **With urea at 3-5 °C:** Since the capillary could not be coiled in the cold trap and the attempt to fill the capillary at room temperature was unsuccessful, the capillary was filled in the refrigerator room at 3-5 °C. Table 3.6 shows the conditions and the gel formation times to obtain the gel. Two capillaries were prepared using this procedure. Both were free of bubbles and voids. Later during detection, however, it was found that these could stand an electric field of 200 V/cm for only 6-7 hours.

- **With urea under high pressure:** The stability period of 6-7 hours was not considered sufficient, so another procedure was tried. The capillary was filled under high pressure (6000 psi) at room temperature. The same conditions as listed in Table 3.5 were used.

APS (μl)	TEMED (μl)	Gel formation time (min.)
500	4	10
450	4	13
500	3	15
450	3	20
400	3	28
400	3	30-32
400	3	30

Table 3.6: The gel formation time with urea at 3-5 °C.

Two capillaries were filled and both were free of bubbles and voids. Later, on application of electric field, it was found that they could stand an electric field of 200 V/cm for only 6-7 hours.

- **With polyethylene glycol (PEG) and urea:** Karger and coworkers used polyethylene glycol as a stabilizer in gel preparation [9]. To improve the stability of the gel, 20 % w/v PEG was included in the gel mixture. The gel formation times are listed in Table 3.7. Two capillaries were tried. The gel formed with PEG and urea was turbid. The capillaries were without bubbles and voids. One of them contained some precipitate and the gel was not smooth when seen under the microscope. No current was observed when an electric field was applied. The other capillary did not show any precipitate but could stand the electric field of 200 V/cm for only 2 hours.

- **With polyethylene glycol:** Because polyethylene glycol formed a turbid gel with urea, polyethylene glycol was tried without urea in the preparation of the gel according to the conditions listed in Table 3.8. Two capillaries were tried by this procedure [9]. One of these contained voids and the other was bubble and void free. On application of electric field, it was observed that it could stand the electric field of 150 V/cm for only 25 minutes and then the current dropped

APS (μl)	TEMED (μl)	Gel formation time (min.)
200	3	10
150	3	15-18
125	3	25
100	3	30
50	3	38-42
50	3	35-40

Table 3.7: The gel formation time with PEG and urea under high pressure.

APS (μl)	TEMED (μl)	Gel formation time (min.)
50	3	25
40	3	30
30	3	40-45
30	3	40-45

Table 3.8: The gel formation time with PEG under high pressure.

APS (μl)	TEMED (μl)	Gel formation time (min.)
100	3	70
150	4	12
75	4	20
60	4	38-42
60	4	40-45

Table 3.9: The gel formation time with DHEBA under high pressure.

to zero.

- **With dihydroxyethylene bisacrylamide (DHEBA):** With the above mentioned problems encountered in using bisacrylamide as the crosslinker and polyethylene glycol as the stabilizer, another crosslinker dihydroxyethylene bisacrylamide was tried with urea as the stabilizer. Table 3.9 shows the conditions for the gel preparation by this method. The gel obtained had more elasticity as compared to the one formed with bisacrylamide. The capillary was stable for 20 hours under an electric field of 180 V/cm. The current varied from 32-38 μamp.

3.5 Detection of proteins

The capillary prepared by Karger's method filled with AA/bisacrylamide gel containing urea at 3-5 °C was mounted. The two ends were immersed in the phosphate buffer (pH=7.34). An electric field of about 200 V/cm was applied. It took roughly 4 hours for the capillary to equilibrate with the buffer and the current to become constant (42 μamps) under a fixed voltage. This was done to obtain a smooth base line. A protein sample of 0.1% egg albumin was injected under an electric field of 100 V/cm for about 20 seconds. Wavelengths of 210, 220, 230, 240, 254, 260, and 280 nm

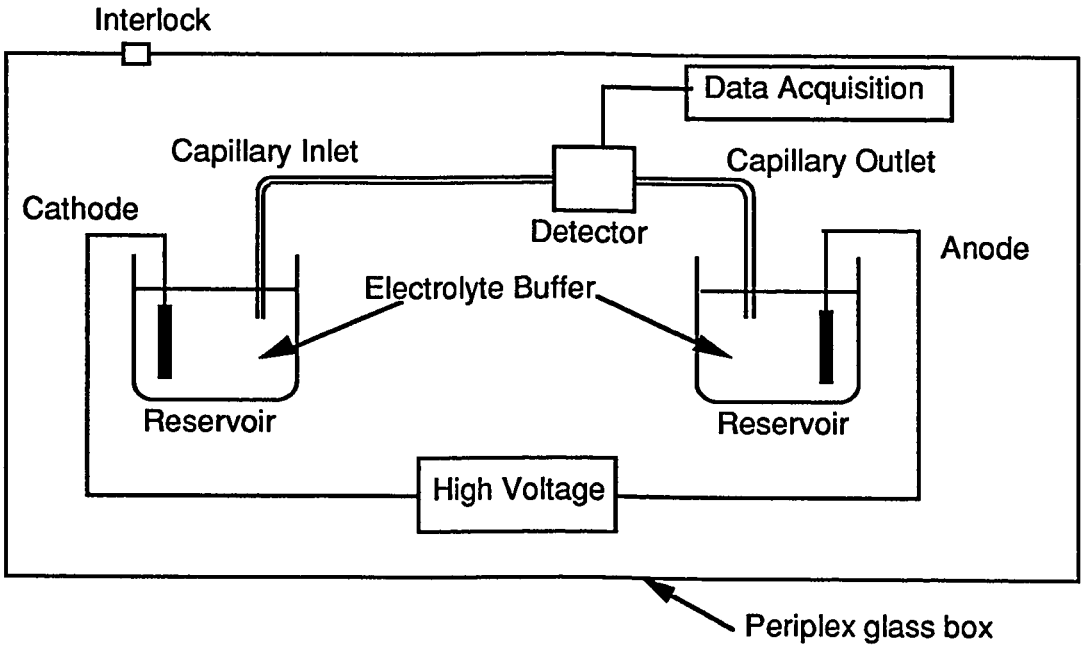


Figure 3.14: The schematic diagram of a capillary electrophoresis system.

were tried but no detection of protein was obtained. The gel in the capillary broke after 6-7 hrs and the capillary was found to contain bubbles when observed under the microsope.

The capillary filled with gel containing urea under high pressure was mounted. The capillary was allowed to equilibriate with the buffer for about 5 hours to obtain a constant current of 40 μamps under the electric field of 200 V/cm. The current was stable upto 6 hrs and then dropped down to zero. The capillary was checked under the microscope and was found to contain bubbles and small voids.

Similarly, the capillary with gel containing polyethylene glycol and urea was tried under an electric field of 200 V/cm but the current dropped to zero after 2 hours.

When an electric current of 150 V/cm was applied through the capillary containing polyethylene glycol, the current dropped to zero only after 25 minutes. Finally, the capillary with gel containing DHEBA as a cross linking agent with urea was tried under an electric field of 180 V/cm. The current increased from 32-38 μamps in about 20 hours. The buffer containing urea and SDS was passed through the capillary for

Stabilizer	Temp (C)	Pressure	Electric field(V/cm)	Stability (hours)
Urea	4	Atmospheric	290	6-7
Urea	25-27	6000 psi	290	6-7
PEG+Urea	25-27	6000 psi	200	2
PEG	25-27	6000 psi	150	0.4

Table 3.10: Stability of capillaries (in hours) for different experiments.

about two hours till a steady base line was obtained.

By Modified Karger's Method: The following four protein samples were tried under a constant electric field of 180 V/cm on a 46 cm long capillary. The wave length for detection was set at 230 nm. The sample (5 mg/mL) was injected through electromigration by using 100 V/cm of electric field for 25 seconds. The retention time was observed to decrease with increase in current and extended use of the capillary.

- **α-Lactalbumin from bovine milk (m.w. 14200):** Three injections were made with the protein. Sharp peaks were obtained at retention times of 11.63, 11.5, and 11.52 min. respectively [Figure 3.15-3.17].

- **β-Lactoglobulin from bovine milk (m.w.18,400):** Three injections were made with the proteins. Sharp peaks were obtained at retention times of 14.63, 14.31, and 14.08 min. respectively [Figure 3.18-3.20].

- **Trypsinogen from bovine pancreas (m.w. 24,000):** Three injections were made with the proteins. Doublets were obtained at retention times of 22.0, 21.5, and 21.0 min. respectively [Figure 3.21-3.23]. The doublets may be due to some impurity in the protein.

- **Pepsin from hog stomach mucosa (m.w. 34,700):** Three injections were made with the proteins. Sharp peaks were obtained at retention times of 29.56, 29.44, and 29.11 min. respectively.

Protein	Retention time (min)		
	Run 1	Run 2	Run 3
α- Lactalbumin	11.63	11.52	11.50
β- Lactoglobulin	14.63	14.31	14.08
Trypsinogen	22.00	21.50	21.00
Pepsin	29.56	29.44	29.11

Table 3.11: Retention time of the proteins by Karger's method.

- **α-Lactalbumin and pepsin (1:1):** Two injections were made with the proteins. Sharp peaks were obtained at retention times of (11.96, 29.46) and (11.78, 28.79), respectively. The peaks around 11 min. are for α-lactalbumin and around 29 min. are for pepsin [Figure 3.25-3.26].

- **α-Lactalbumin, β-lactoglobulin, and trypsinogen (1:1:1):** One injection was made with the proteins. Sharp peaks were obtained at retention times of (11.9, 14.5, 27.5). The peak around 11 min. is for α-lactalbumin, 14.5 for β-lactoglobulin, and 27 min. for trypsinogen [Figure 3.24].

Table 3.11 shows the proteins and their migration times when performed by Karger and his coworkers by using 5%T and 3.3%C..

By Proposed Method: The proteins α-lactalbumin and β-lactoglobulin were tried on the hydrided capillary under a constant electric field of 100V/cm. The length of the capillary was 44 cm with 22 cm of migration distance. Each sample (20 mg/mL) was injected through electomigration by using 100 V/cm for 60 seconds.

- **α-Lactalbumin from bovine milk (m.w. 14200):** Two injections were made with the protein. Sharp peaks were obtained at retention times of 17.9 and 20.7 min., respectively [Figure 3.27-28].

Protein	Retention time (min)
α- Lactalbumin	17.9
β- Lactoglobulin	61.2

Table 3.12: Retention time of the proteins by the proposed method.

- **β-Lactoglobulin from bovine milk (m.w. 18,400):** Two injections were made with the sample. Sharp peaks were obtained at retention times of 61.2 and 61.03 min., respectively [Figure 3.29-30].

Table 3.12 lists the proteins and their retention times when performed by the proposed by the method.

Comparison of retention times: Table 3.13 shows the retention times by Karger's reported papers (at 400 V/cm), Karger's modified method, and the proposed method (at 110 V/cm). It can be clearly seen from the table that the analysis of α-lactalbumin and β-lactoglobulin is much faster as compared to Karger's method.

Table 3.14 shows the normal data for retention time at 200 V/cm with migration distance 20 cm for the different methods used. Table 3.15 shows the resolution between different proteins. The order of resolution between α-lactalbumin and β-lactoglobulin can be given as follows: Proposed method > Karger's modified method > Karger's reported method.

Protein	Retention time (min)		
	Karger's rep. 400 V/cm	Karger's mod. 180 V/cm	Proposed 110 V/cm
α- Lactalbumin	21.5	11.5	17.9
β- Lactoglobulin	23.5	14.3	61.2
Trypsinogen	26.0	21.5	-
Pepsin	30.0	29.4	-

Table 3.13: Comparison of retention time of the proteins by different methods used.

Protein	Retention time (min)		
	Karger's rep. 200 V/cm	Karger's mod. 200 V/cm	Proposed 200 V/cm
α- Lactalbumin	47.3	10.3	9.8
β- Lactoglobulin	51.7	12.8	33.6
Trypsinogen	57.2	19.3	-
Pepsin	66.0	26.4	-

Table 3.14: Normalized data of retention time of the proteins by different methods used.

Protein	Resolution		
	Karger's rep.	Karger's mod.	Proposed
A+B	1.06	1.24	3.42
B+T	1.10	1.50	-
T+P	1.15	1.34	-

Where:

α- Lactalbumin = A
β- Lactoglobulin = B
Trypsinogen = T
Pepsin = P

Table 3.15: Resolution between different proteins.

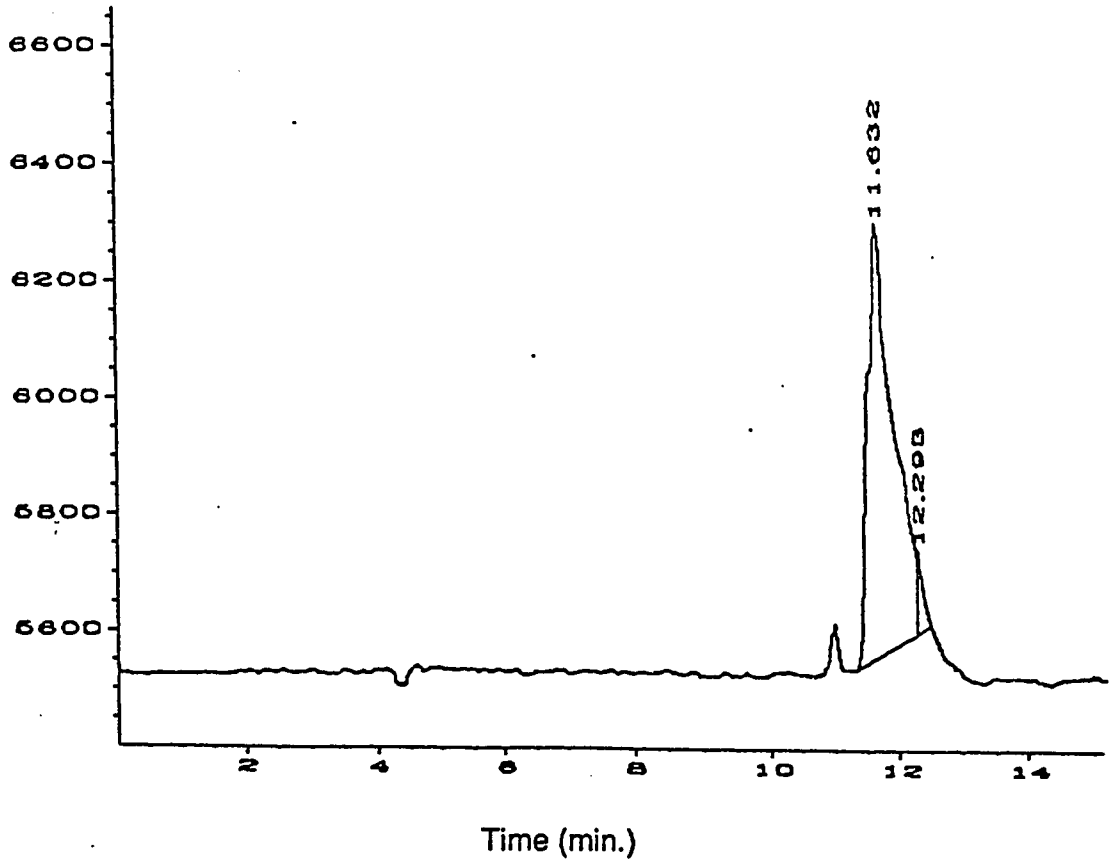


Figure 3.15: Electropherogram of α-lactalbumin at 180 V/cm, injection 1 (Karger's method).

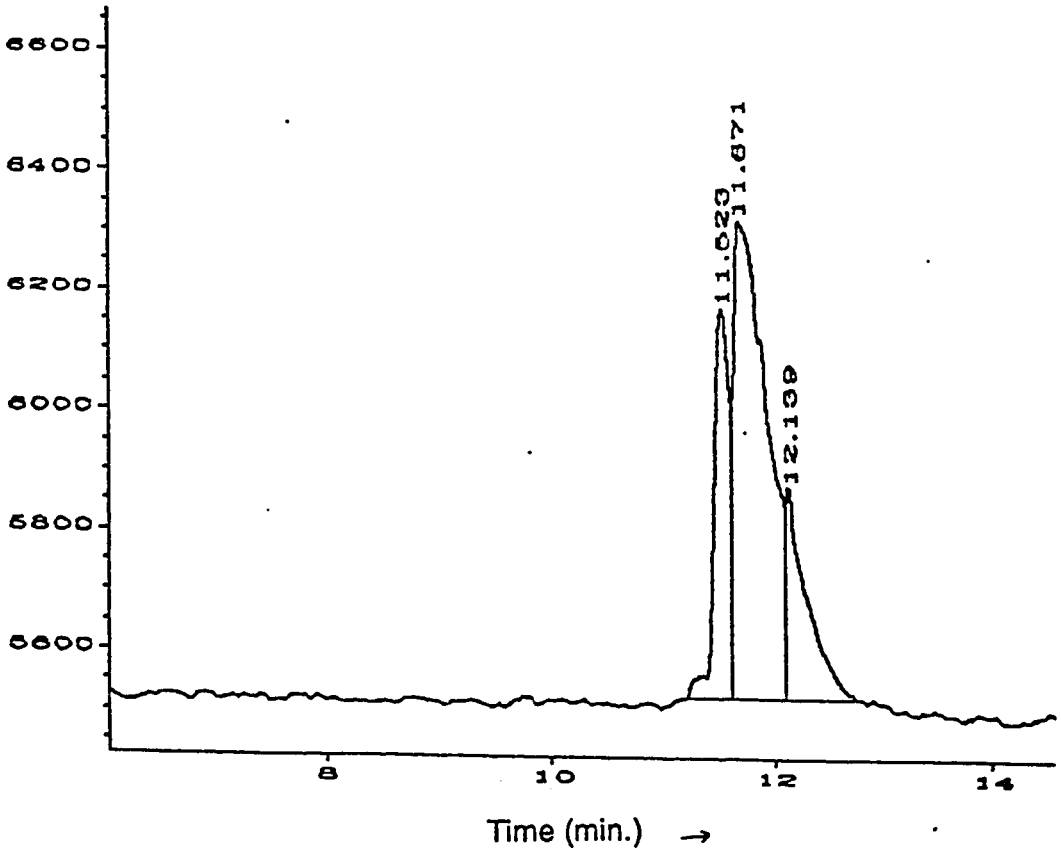


Figure 3.16: Electropherogram of α-Lactalbumin at 180 V/cm, injection 2 (Karger's method).

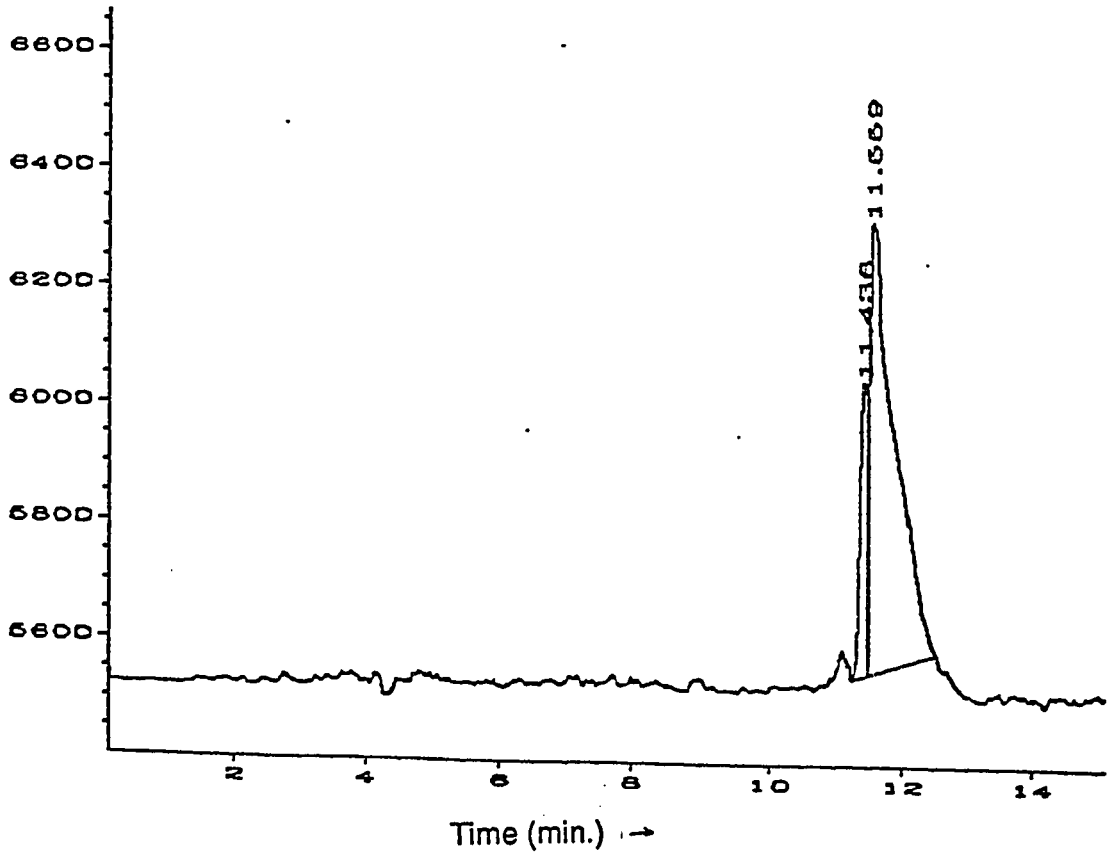


Figure 3.17: Electropherogram of α-Lactalbumin at 180 V/cm, injection 3 (Karger's method).

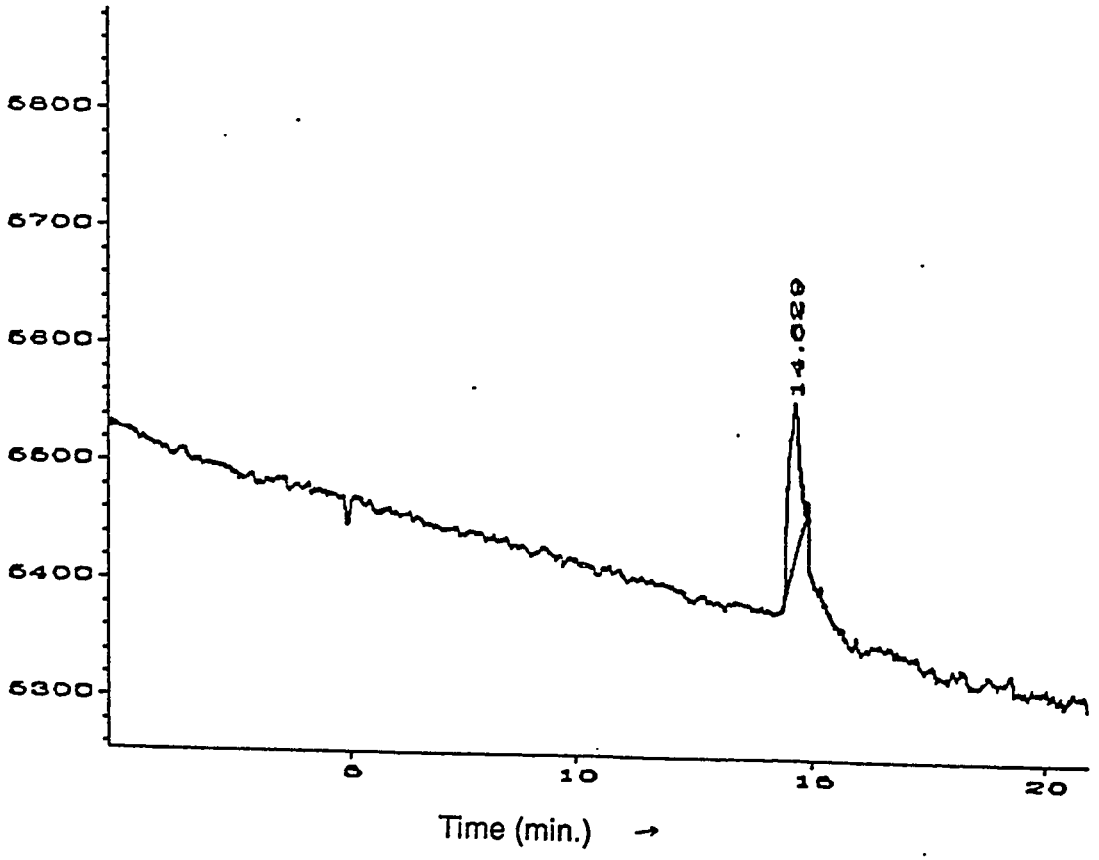


Figure 3.18: Electropherogram of β-Lactoglobulin at 180 V/cm, injection 1 (Karger's method).

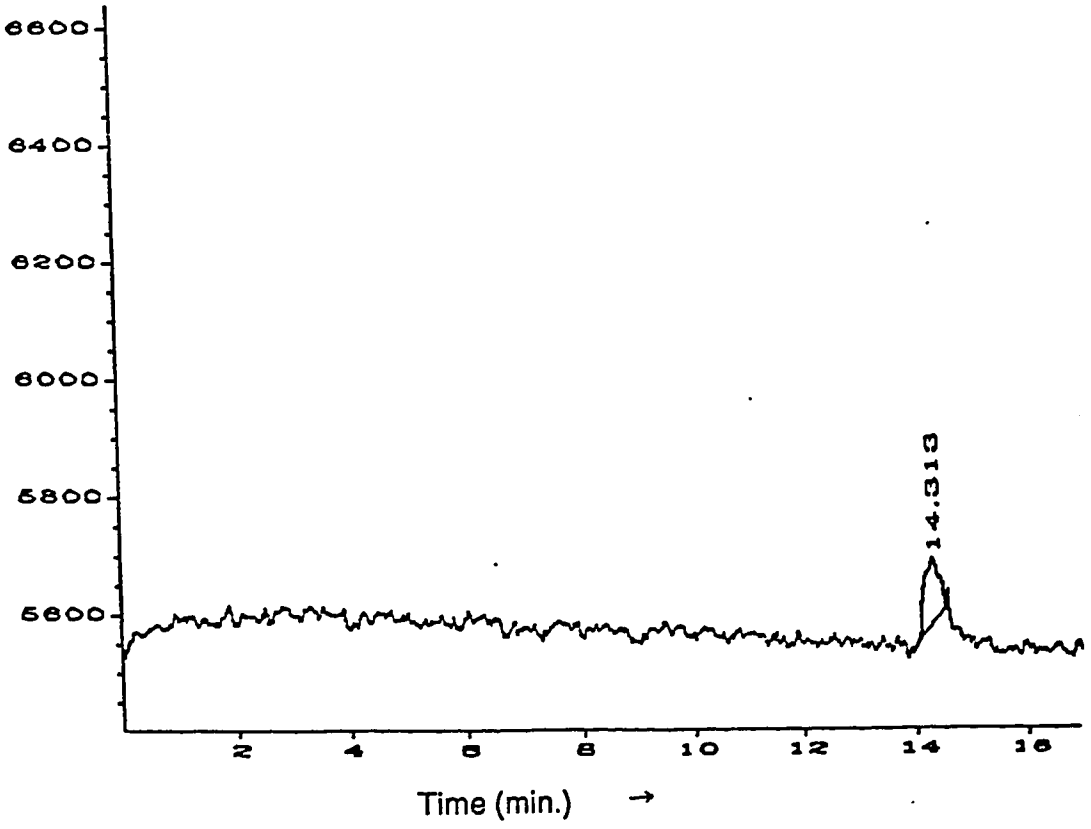


Figure 3.19: Electropherogram of β-Lactoglobulin at 180 V/cm, injection 2 (Karger's method).

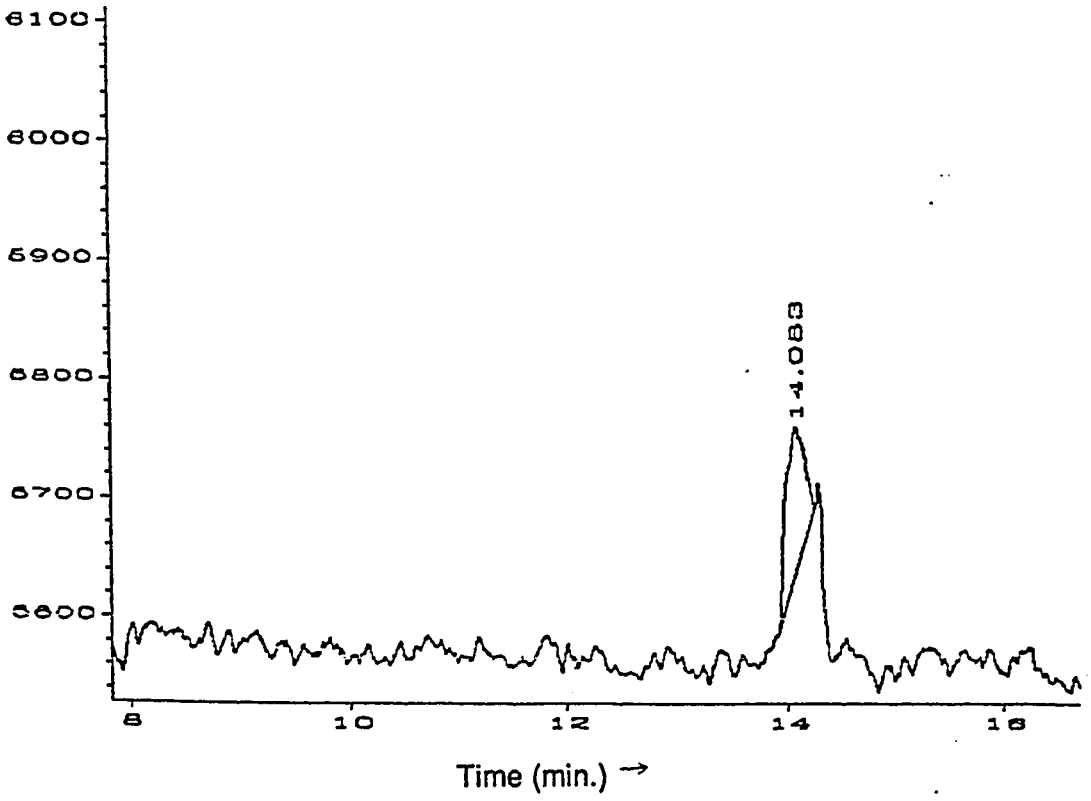


Figure 3.20: Electropherogram of β-Lactoglobulin at 180 V/cm, injection 3 (Karger's method).

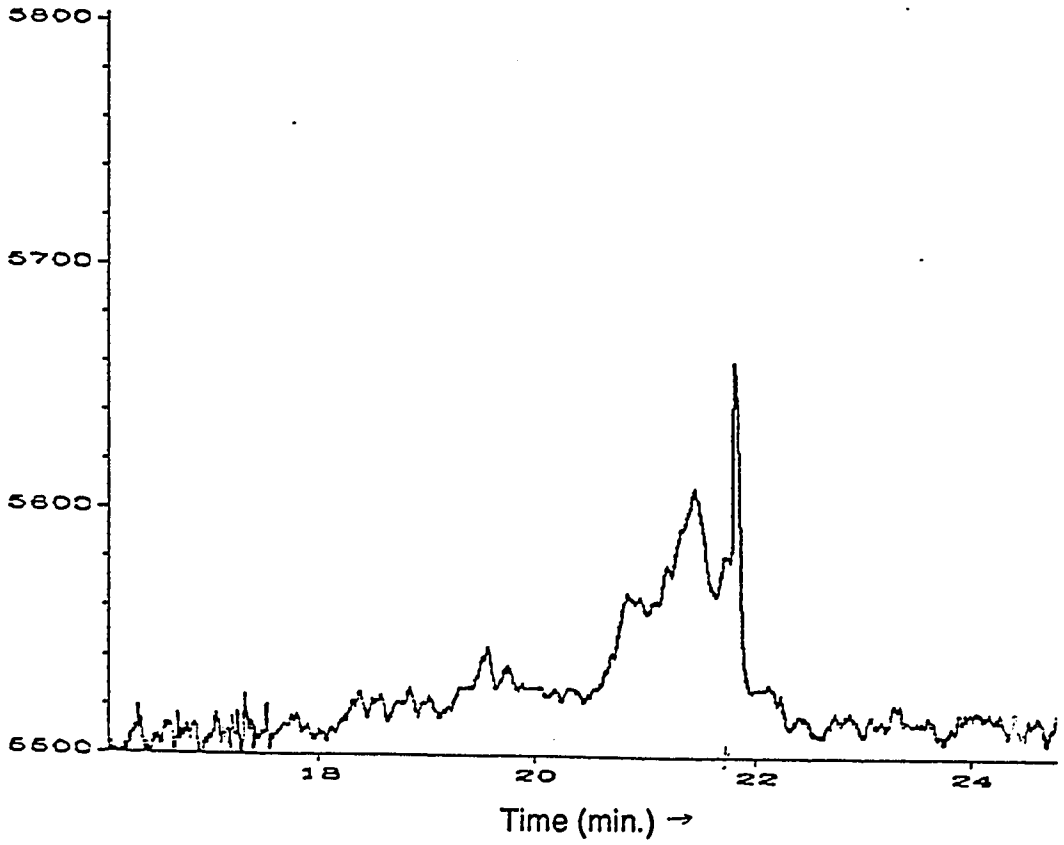


Figure 3.21: Electropherogram of Trypsinogen at 180 V/cm, injection 1 (Karger's method).

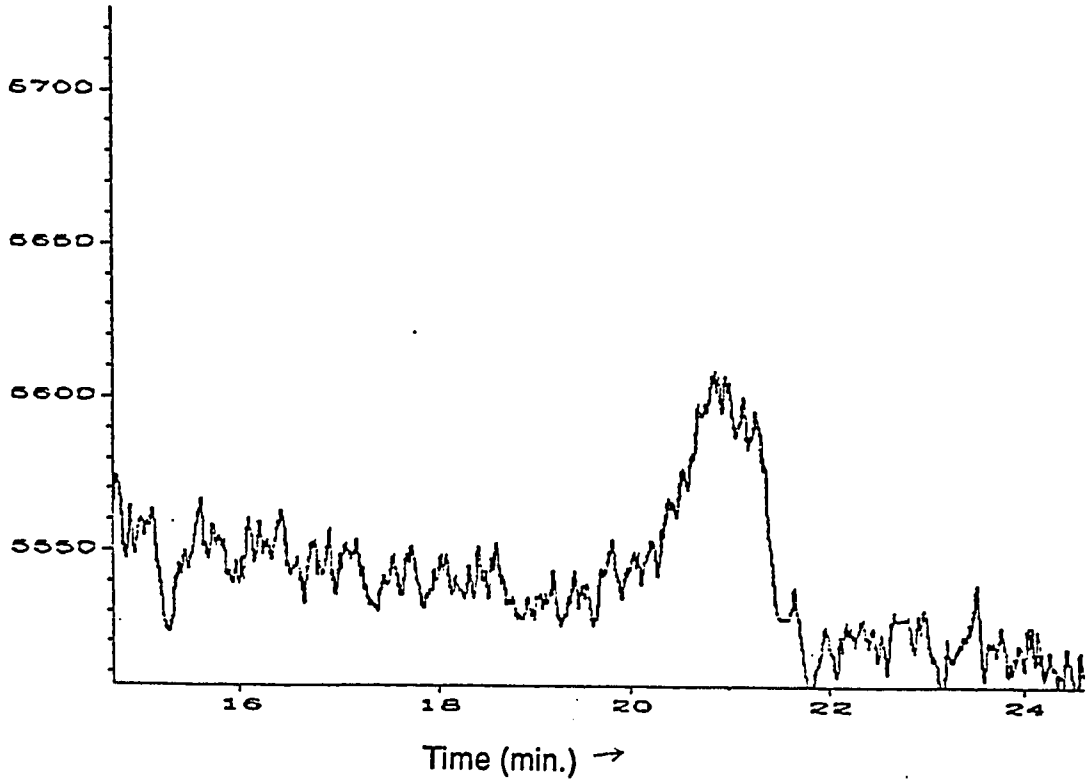


Figure 3.22: Electropherogram of Trypsinogen at 180 V/cm, injection 2 (Karger's method).

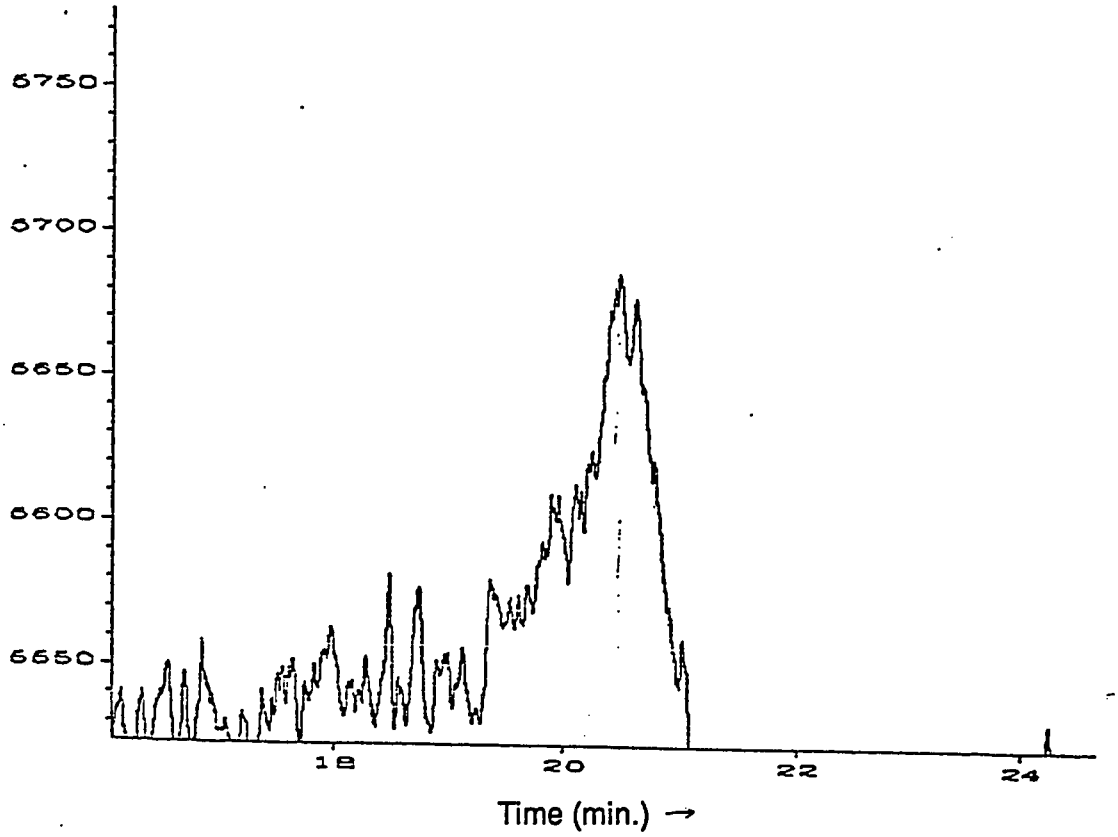


Figure 3.23: Electropherogram of Trypsinogen at 180 V/cm, injection 3 (Karger's method).

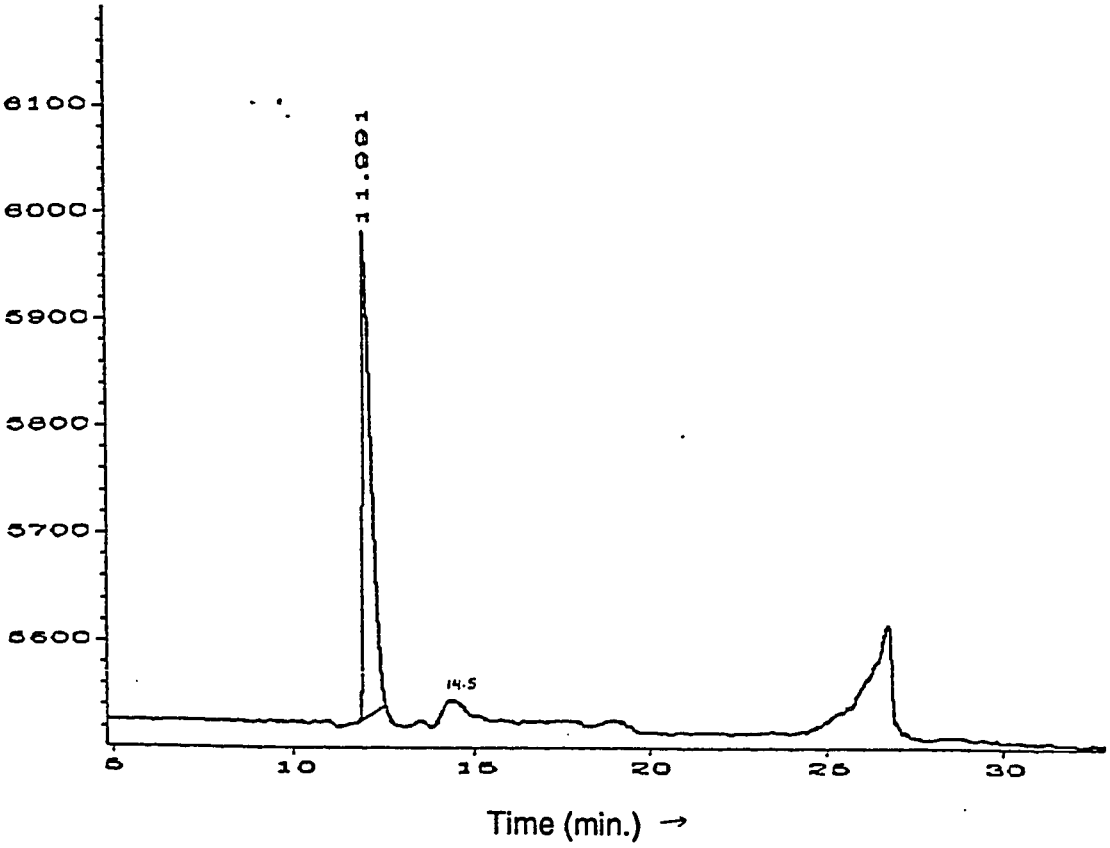


Figure 3.24: Electrophoretic separation of α-Lactalbumin, β-Lactoglobulin, and Trypsinogen at 180 V/cm (Karger's method).

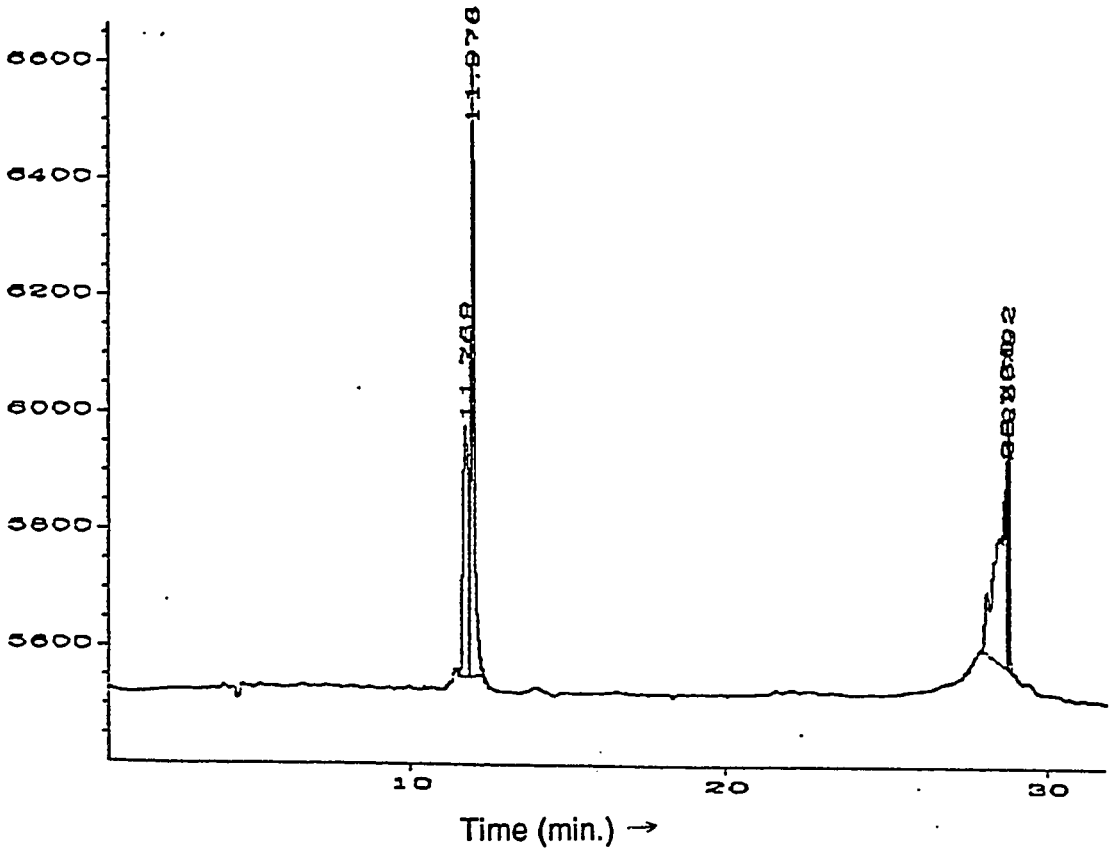


Figure 3.25: Electrophoretic separation of α-Lactalbumin and Pepsin at 180 V/cm, injection 1 (Karger's method).

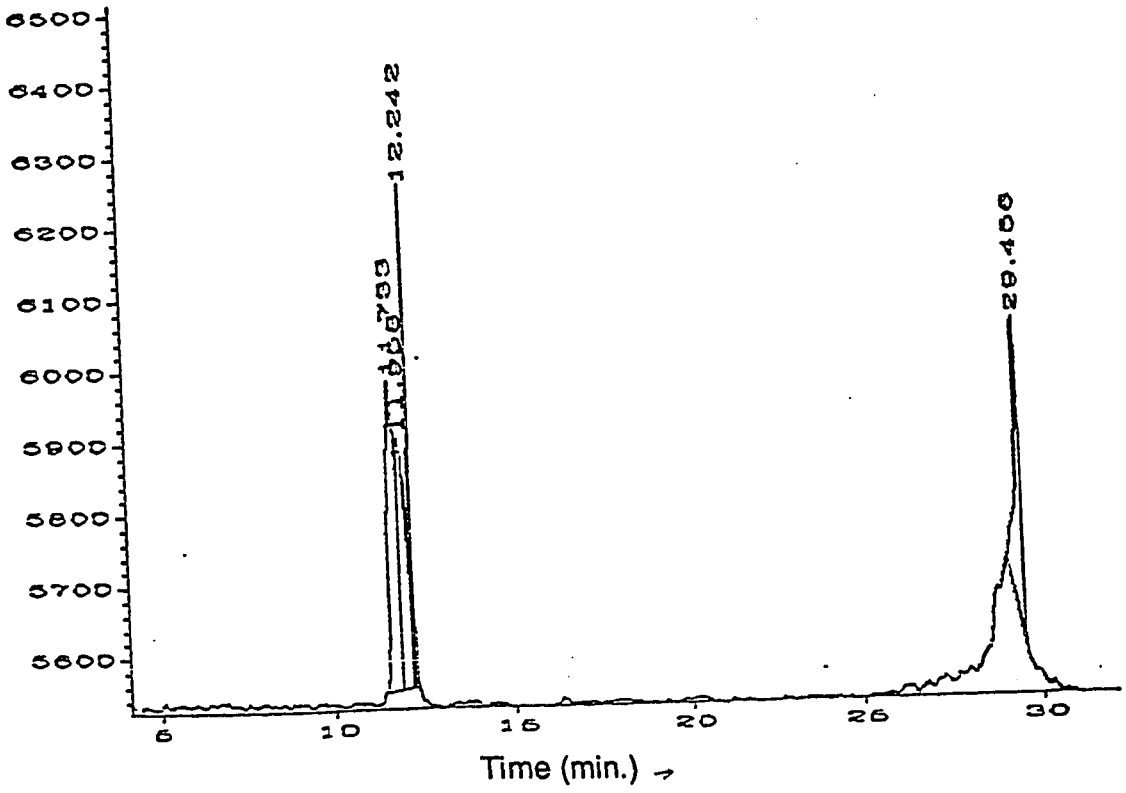


Figure 3.26: Electrophoretic separation of α-Lactalbumin and Pepsin at 180 V/cm, injection 2 (Karger's method).

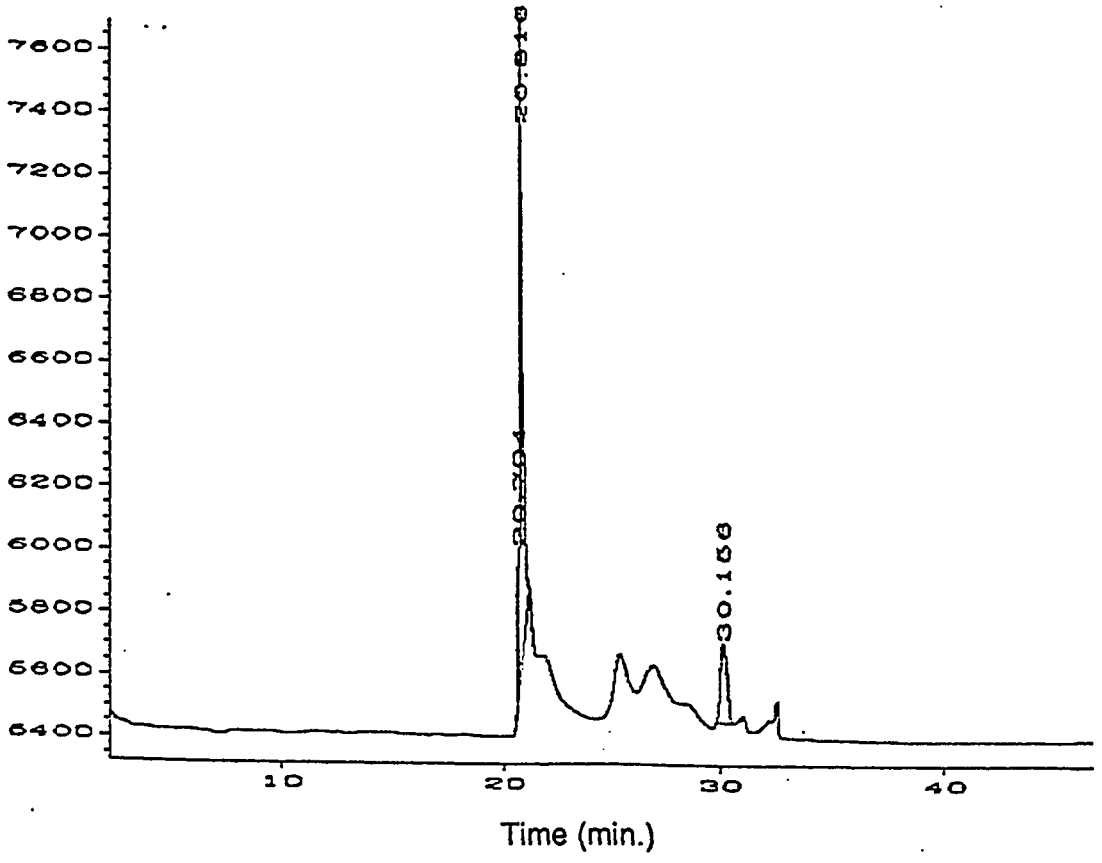


Figure 3.27: Electropherogram of α-Lactalbumin at 110 V/cm, injection 1 (Proposed method).

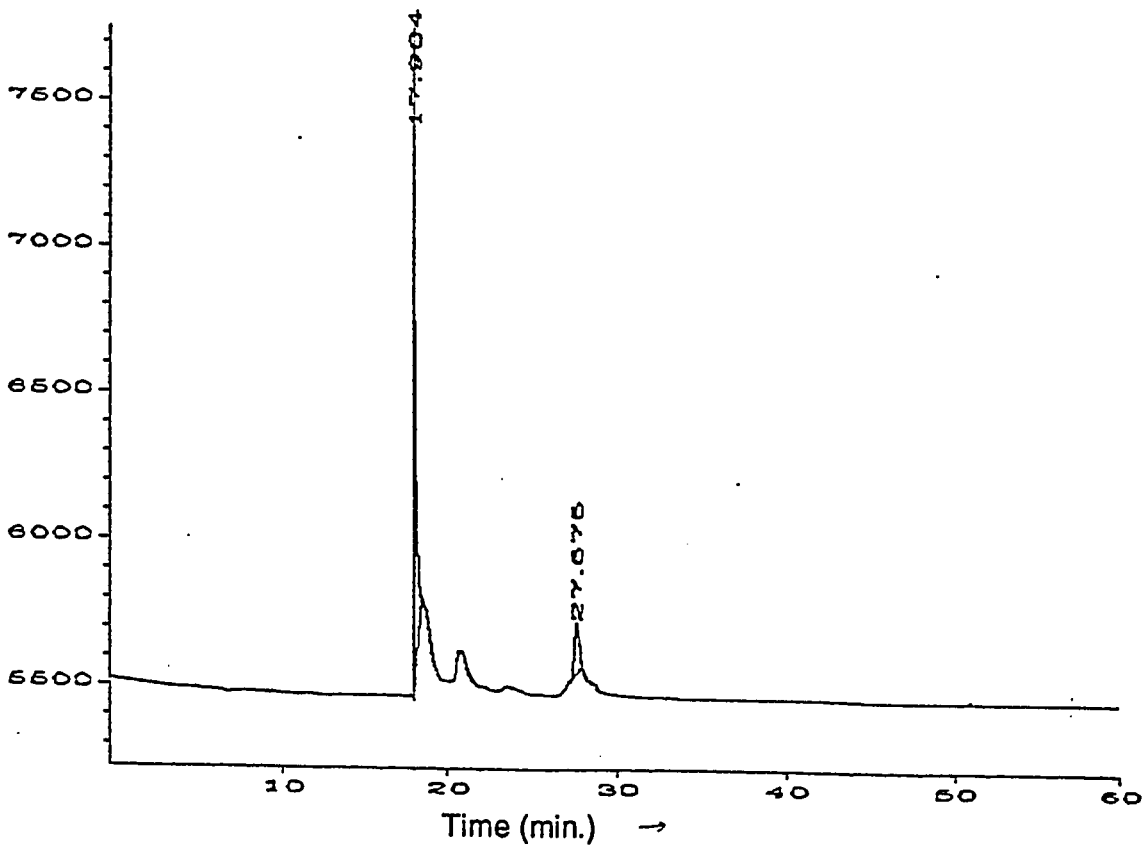


Figure 3.28: Electropherogram of α-Lactalbumin at 110 V/cm; injection 2 (Proposed method).

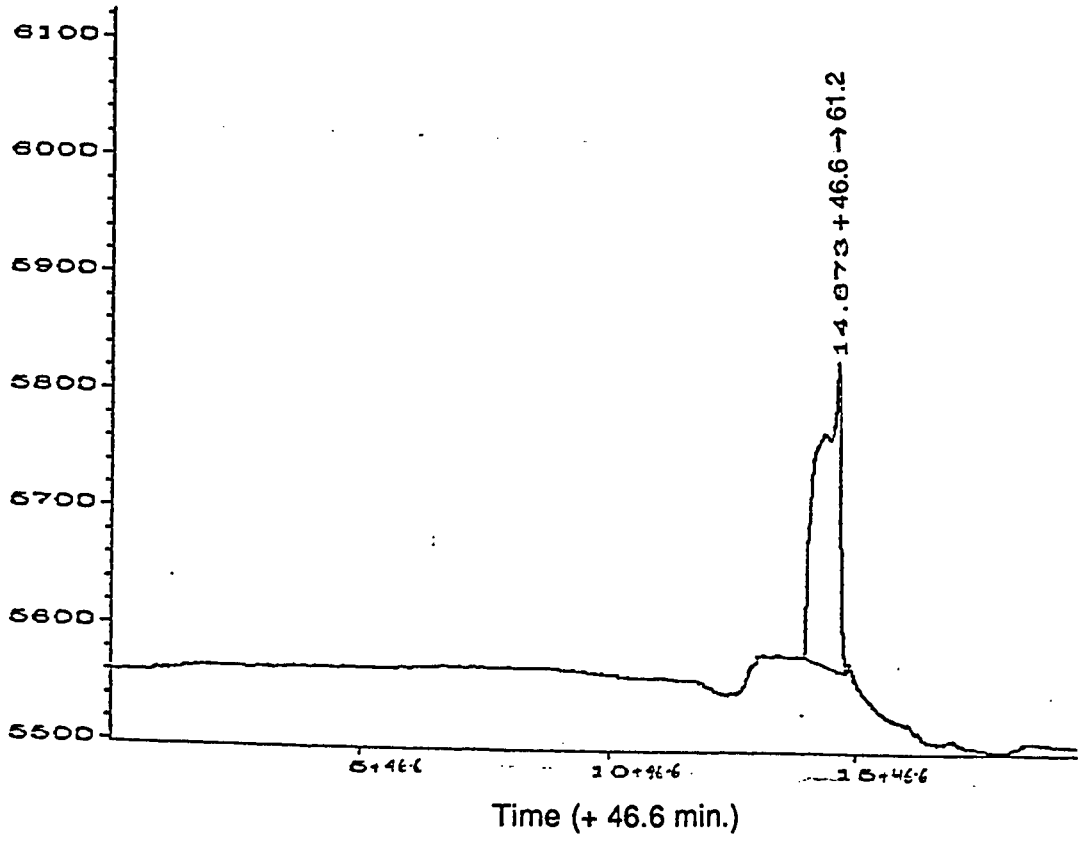


Figure 3.29: Electropherogram of β-Lactoglobulin at 110 V/cm, injection 1 (Proposed method).

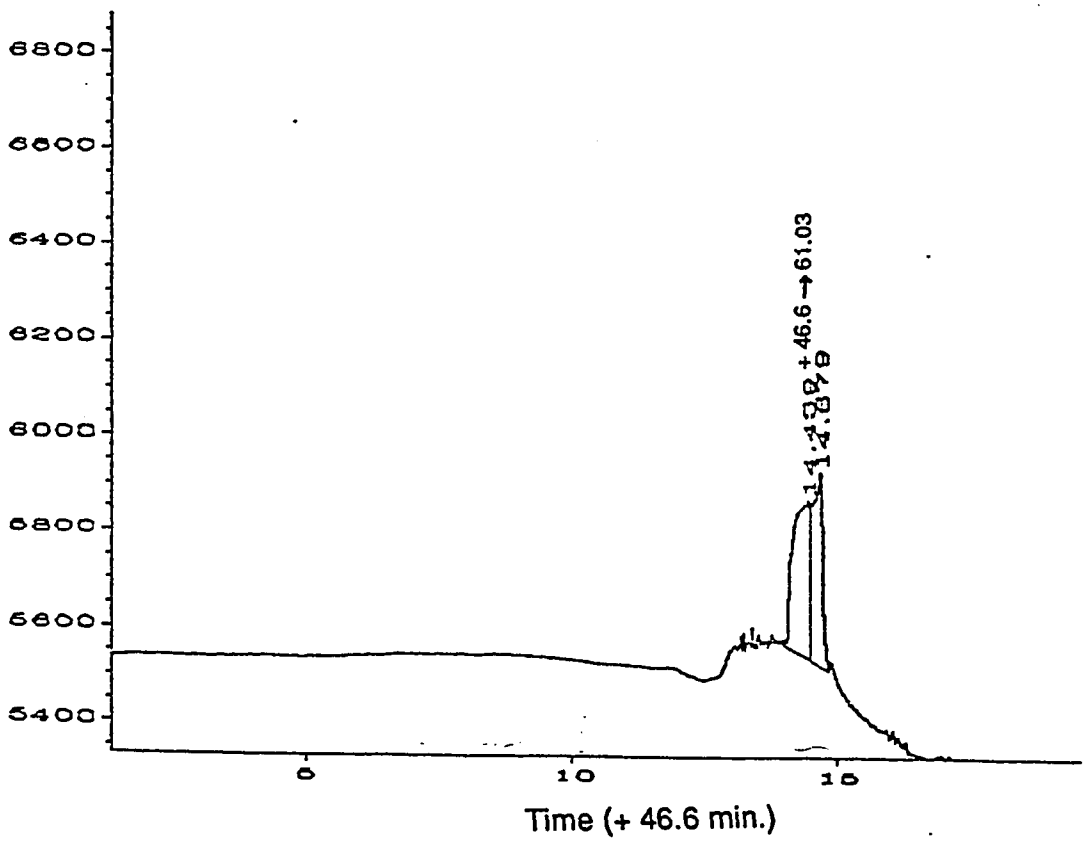


Figure 3.30: Electropherogram of β-Lactoglobulin at 110 V/cm, injection 2 (Proposed method).

Chapter 4

Conclusions

The purpose of our research project was to prepare wall-bonded, gel-filled capillaries via hydride modified quartz glass surfaces. The chlorination-reduction reaction of native capillary resulted in fairly stable Si-H groups on its surface. The olefin, allylmethacrylate was bonded to the hydrided surface. The IR CH_2 stretching and bending of the propyl chain in the olefin bonded silica and carbon analysis data show reasonable bonding of the olefin on the hydrided surface. The reaction time and the amount of catalyst were found to be important factors during the bonding of an olefin on the silica.

The polyacrylamide gel was filled in the modified capillary. The use of an ultrasonicator and vacuum for degassing the solution was found to be very effective in avoiding the bubbles formed due to dissolved air. The appearance of voids was avoided by filling the capillary under a high pressure (about 6000 psi) for at least an hour. The use of urea in the formation of the gel was quite essential to obtain a clear gel, which has better mechanical strength, instead of a turbid gel. Polyethyleneglycol, often used a stabilizer, formed a gel that was very turbid. The capillary containing this type of gel could stand an electric field of 150 V/cm for only two hours. It was observed that dihydroxyethylene bisacrylamide (DHEBA) acts as a better cross-linker, as compared to bisacrylamide, in terms of stability under a given electric field. The capillary with bisacrylamide could stand an electric field of 180 V/cm for only six hours whereas the one with DHEBA was stable for 13-20 hours under the same field.

Four proteins were tried on the capillary prepared by Karger's method with slight modifications. The separation was completed within 30 minutes for α-lactalbumin, β-lactoglobulin, and trypsinogen under an electric field of 180 V/cm. This time was significantly less as compared to Karger's work where the separation took about 50 minutes under an electric field of 400 V/cm. The migration distance of the capillary used was 23 cm as compared to 20 cm, which again reflects faster analysis. In the hydrided capillary prepared by our proposed method, the α-lactalbumin could be detected in only 20 minutes at 156 V/cm in comparison to 22 minutes at 400 V/cm in Karger's method. The β-lactoglobulin was detected in 62 minutes at 105 V/cm as compared to 25 minutes at 400 V/cm in Karger's method. The migration distance was 24 cm as compared to 20 cm in Karger's data.

A quicker method for chlorination and reduction procedure is required. Further improvements are required to achieve capillaries with high stability. Experimental procedures have to be advanced in order to obtain higher reproducibility of the results. Also, other gels and buffer media must be investigated to improve gel stability.

Bibliography

[1] A.S. Cohen, S. Terabe, J.A. Smith, and B.L. Karger, *Anal Chem, 59*, **1987**, 1021-1027.

[2] M.J. Gordon, X. Huang, S.L. Pentoney, R.N. Zare *Science, 242*, **1988**, 224-228.

[3] A.S. Cohen, A. Paulus, B.L. Karger, *Chromatographia, 24*, **1987**, 15-24.

[4] J.W. Jorgenson and K.D. Lukacs, *Science, 222*, **1983**, 266-272.

[5] A.G. Ewing, R.A. Wallingford, T.M. Olefirowicz, *Anal Chem, 61(4)*, **1989**, 292-303.

[6] A. Chrambach and D. Rodbard, *Science, 172*, **1971**, 440-451.

[7] B.D. Hames, *Gel Electrophoresis*, **1984**.

[8] A.S. Cohen and B.L. Karger, *Journal of Chromatography, 397*, **1987**, 409-417.

[9] B.L. Karger, *U.S. Patent No. 4, 865, 707*, **1989**.

[10] J.E. Sandoval and J.J. Pesek, *Anal Chem, 61*, **1989**, 2067-2075.

[11] A.T. Andrews, *Electrophoresis*, Oxford Science Publications, **1989**.

[12] G.E. Berendsen, K.A. Pikaart, and L.D. Galan *J Liq Chromatography, 3(10)*, **1980**, 1437-1464.