2003

OTCEC analyses of ipratropium bromide and PEGylated proteins

Vidhyalakshmi Krishnamoorthi
San Jose State University

NOTE TO USERS

OTCEC ANALYSES OF IPRATROPIUM BROMIDE AND PEGYLATED PROTEINS

A Thesis

Presented to

The Faculty of the Department of Chemistry

San Jose State University

In Partial Fulfillment

Of the requirements for the Degree

Master of Science

by

Vidhyalakshmi Krishnamoorthi

December 2003

APPROVED FOR THE DEPARTMENT OF CHEMISTRY

Dr. Joseph Pesek

Dr. Pam Stacks

Dr. Roger Terrill

APPROVED FOR THE UNIVERSITY

ABSTRACT

OTCEC ANALYSES OF IPRATROPIUM BROMIDE AND PEGYLATED PROTEINS

by Vidhyalakshmi Krishnamoorthi

Ipratropium bromide is a quaternary ammonium compound chemically related to atropine. It blocks the parasympathetic nervous system and induces bronchodilation. The simultaneous determination of ipratropium bromide and related compounds were investigated using open tubular capillary Electrochromatography (OTCEC). It is necessary to develop a technique and also find optimum conditions which can detect the various impurities associated with ipratropium Bromide. These impurities can prove to be fatal if present in high concentrations.

PEGylated proteins are becoming more common as therapeutic agents because they remain longer in the body thereby providing a constant drug level. PEGylation is defined as the modification of a protein by covalent attachment of polyethylene glycol. The crux of this project is to find optimum conditions for the analysis of the PEGylated proteins. OTCEC appears to be a viable method for the analysis of PEGylated proteins.

ACKNOWLEDGEMENT

I would like to thank first and foremost, my advisors, Dr. J. J. Pesek and Dr. Maria Matyska-Pesek, for giving me the opportunity to work on such interesting projects. I would like to express my deepest gratitude to Dr. Maria Matyska-Pesek, from whom I have learnt a lot of qualities for being a good analytical chemist. She has been my mentor and my role model for discipline and professionalism.

I would like to acknowledge the support of the faculty and staff of the Chemistry Department at San Jose State University. I would like to thank, especially, Dr. Pam Stacks and Dr. Roger Terrill (my committee members) for providing valuable insights, suggestions, and feedback.

My appreciation goes out to all the members of my research laboratory (specifically Dr. Brent Dawson) and friends, for providing a congenial atmosphere for me to work in and also for their assistance and inspiration.

Last but not the least, I would like to thank my family (parents, siblings, and my husband) for their support and understanding during the duration of my graduate studies; without their support I would not have been able to complete the thesis in a timely manner with such satisfaction.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. Introduction

1.1 Background

Capillary Electrophoresis (CE) is an important analytical technique that utilizes narrow bore (20-200 μm i.d.) capillaries for the separation of large and small molecules. Pretorius invented it in 1974 and Jorgenson and Lukacs achieved the first effective separation technique in 1981 (1). CE is an important tool for the separation of simple monomers, oligomers, and complex polymeric molecules and has recently been used in the human genome project, in which it plays a crucial role. Previously such separations were done using high performance liquid chromatography (HPLC) in which the molecular components in a mixture are separated due to the affinities/partitioning between a fixed (stationary) phase and a moving (mobile) phase. The output of HPLC is a chromatogram that is based on the retention time while the output for CE is an electropherogram based on the migration time. There can be more than one mode of detection such as UV/Vis, fluorescence, laser-induced fluorescence, mass spectrometry, and electrochemical (2-3).

The advantages of CE are: 1) the flow profile is flat as compared to parabolic for HPLC, 2) the volume requirement of the sample and the mobile phase is much smaller in comparison to HPLC, and 3) gradient flow in HPLC is replaced by isocratic operation in CE.

1.2 Principle

Electrophoresis is the migration of charged species when dissolved, or suspended,

in an electrolyte when a voltage is applied. A schematic diagram of the instrument is shown in Figure 1 (4). The two ends of a capillary are immersed in buffer reservoirs (electrolytes) containing electrodes (platinum) connected to a high voltage power supply. A sample of analyte is injected at one end of its capillary, either by voltage or by pressure, and then an electric field is applied across the capillary.

1.2.1 Electroosmotic flow (EOF)

Electroosmotic flow is the bulk movement of the buffer in the capillary when a potential is applied. Initially a capillary is filled with the buffer solution. The silanol groups, which are negatively charged at the surface of the capillary, attract cations forming a double layer. The inner layer called the Stern layer is held tightly by the Si-O- groups and the outer layer called the Helmholtz layer is mobile as it is farther away from the silanoate group (5). The sample is injected and a voltage is applied across the electrodes wherein the cations move towards the cathode and the anions move towards anode. The positively charged solvated buffer ion move towards the cathode thereby creating a bulk flow that in turn sweeps all the solute species, cations, neutral and anions towards the detector. This flow is called the electroosmotic flow (EOF). This phenomenon is illustrated in Figure 2. The velocity of the electroosmotic flow, v_{EOF}, is given by

$$v_{EOF} = \varepsilon\zeta E / 4\pi\eta$$

ε = dielectric constant of the buffer

ζ = zeta Potential

E = applied electric field (V/cm)

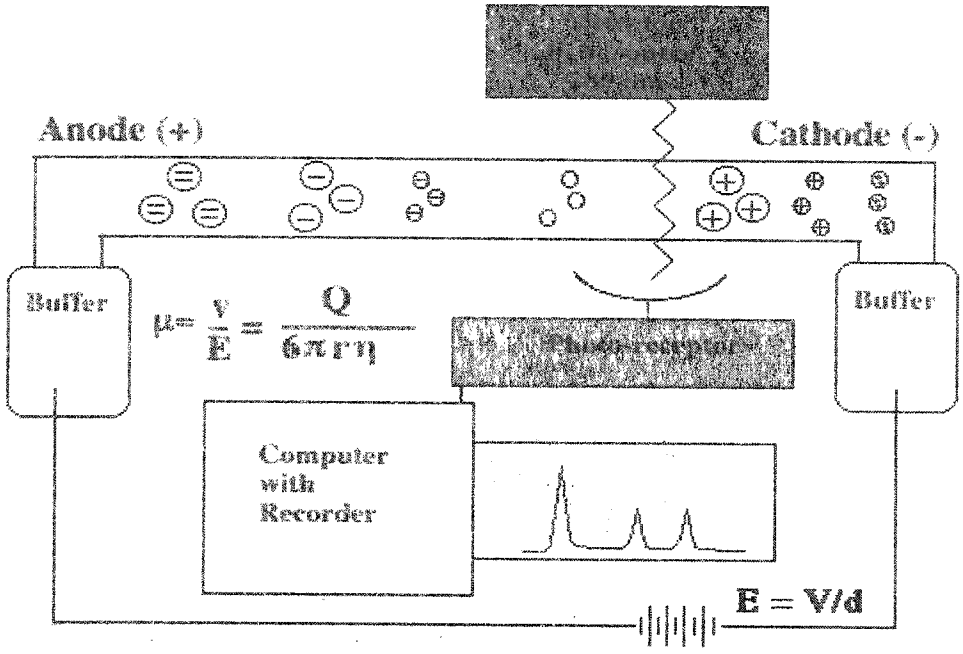


Figure 1: Schematic representation of CE/CEC system
*Figure taken from ref (4)

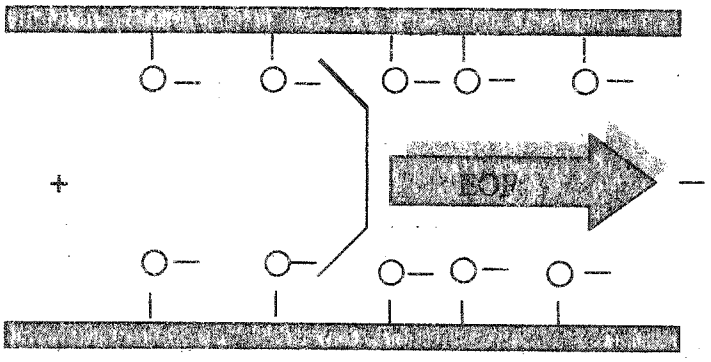


Figure 2: Schematic representation of Electroosmotic flow

η = viscosity of buffer

The electroosmotic mobility, μ_{EOF} of the buffer is given by the: $\mu_{EOF} = \varepsilon\zeta / 4\pi\eta$

1.2.2 Electrophoretic mobility (E_p)

Electrophoretic mobility is movement due to an electric field applied to an electrically charged species. The electrophoretic mobility depends on the charge of the ion. If the species is doubly charged it will reach the cathode much faster than a single positively charge ion. Electrophoretic mobility is given by

$\mu_{EP} = q / 6\pi\eta r$

q = Charge of the ionized species

r = solute radius

η = viscosity of buffer

The mobility depends on the charge as well as the size of the solute and the buffer viscosity.

Net flow: (Net mobility) $\mu = \mu_{EOF} + \mu_{EP}$

1.2.3 Modes of CE

There are different modes of CE:

- Electrophoresis or non-partitioning modes
    - Capillary zone electrophoresis: Separation depends on the charge and size of the analyte, with smaller (more highly charged) species eluted before the larger (less charged) ones (6).

- Capillary isoelectric focusing: Separation of zwitterionic species within a pH gradient.
- Capillary gel electrophoresis: Separation based on molecular size due to sieving of analytes through a network of gel.
- Capillary isotachophoresis: A sample is inserted between a leading electrolyte and a trailing electrolyte without electroosmotic flow. The leading electrolyte has a higher mobility and the trailing electrolyte a lower mobility than ions in the sample zone. Thereby separation relies in the difference in the velocities of the analyte ions.

- Chromatographic or partitioning modes
  - Micellar electrokinetic capillary chromatography: Separation of neutral compounds using surfactant micelles like sodium dodecyl sulfate.
  - Chiral capillary chromatography: Separation involves addition of chiral selectors to the background electrolyte, bound on the capillary or to the gel matrix.
  - Capillary Electrochromatography (CEC): Separation of solutes based on partitioning between a stationary and a mobile phase.

1.3 Capillary Electrochromatography (CEC)

CEC is a hybrid technique with features of HPLC and CE. In CEC, solutes partition between a stationary and a mobile phase. Unlike HPLC, CEC uses an electric field and not pressure to drive the eluent through the column (6). CEC has two modes:

1. the packed capillary configuration that utilizes stationary phase similar to HPLC, and 2. the open tubular CEC (OTCEC) format where the stationary phase is immobilized onto the capillary wall. The advantages of using OTCEC are a low sample requirement (~1.0 picomole), high reproducibility due to silanol groups covered, fast analysis, no packing or frits required, and efficient separation (but not like CE) with the plate count of CEC between CE and HPLC. Figure 3 shows a schematic representation of packed and open tubular columns.

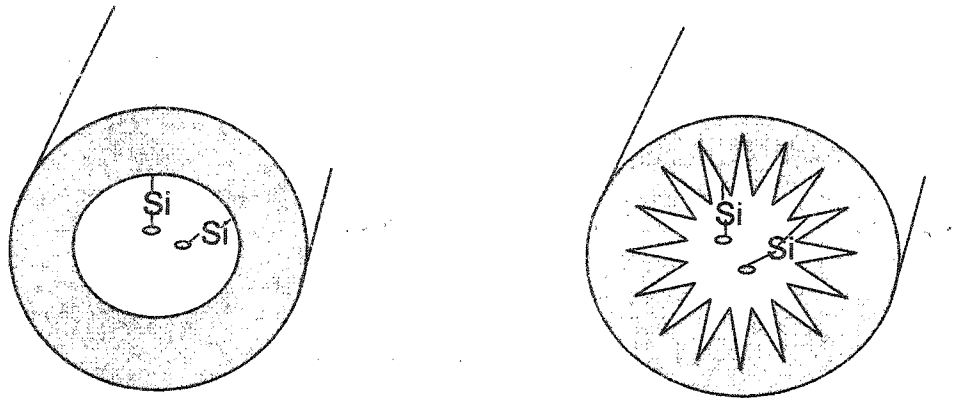
1.3.1 Capillary

For the current research an etched capillary is used. A bare capillary has some drawbacks such as: 1) low capacity of the capillary (small surface area for bonding a stationary phase), and 2) relatively long axial distances (molecules have to migrate to interact with the bonded moiety). Figure 4 shows a schematic representation of a section bare capillary and etched capillary.

The positive side of etching includes: 1) increased overall surface area of the inner bore of the capillary (1000-fold) (7). 2) dissolution and redeposition of silica material during the etching process create radial extensions (8). The analyte therefore has to travel less distance as compared to bare capillary to interact with the stationary phase. Some analytes like proteins tend to adsorb electrostatically due to the negative charge on the capillary. To get a constant EOF for reproducible separations it sometimes becomes necessary to reduce the EOF due the adsorption of proteins on the capillary. The EOF is

Packed column Open tubular column

Figure 3: Schematic representation of packed and open tubular columns



Section of Bare capillary Etched capillary

Figure 4: Schematic representation of bare capillary and etched capillary

eliminated or reduced by either coating the capillary or using certain additives in the buffer. In this research the capillaries were chemically modified with different organic moieties.

1.3.2 Etching and modification of capillary

1.3.2a Etching

To achieve a high surface area the inner walls of the capillaries are etched. The capillaries are filled with conc. HCl by applying a pressure of 60-80 psi and then heated overnight at 80°C in a GC (gas chromatography) oven (9). HCl is removed by passing deionized (DI) water. The capillaries are then filled with 0.1M NaOH and again washed with DI water to remove the NaOH and dried with nitrogen. A 5% w/v solution of ammonium bifluoride in methanol is passed through the capillaries using low pressure and allowed to lie in a horizontal position for an hour. Methanol is completely removed from the capillaries by passing nitrogen at a very low pressure (1-3 psi). The capillaries are heated at 300°C for 2 hours and flushed with nitrogen at a very low pressure and later heated at 400°C (at a nitrogen pressure of 20 psi) for 1 hour. After cooling, methanol is passed through the capillaries and dried with nitrogen.

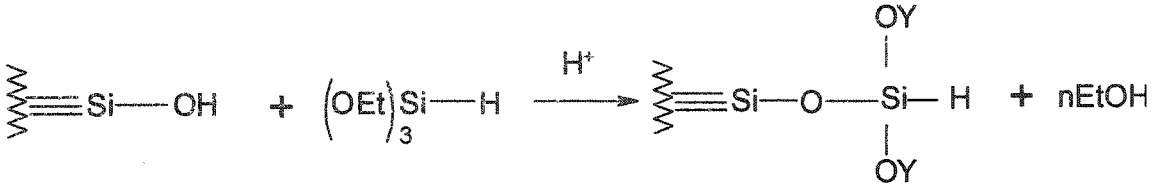
1.3.2b Capillary preconditioning

The capillaries are filled with NaOH and kept horizontal overnight. NaOH is removed using DI water and HCl is passed through it and removed using DI water and then dried with nitrogen and acetone. The capillaries are dried completely by heating at

100°C for 2 days and simultaneously flushing the capillaries with nitrogen at a low pressure.

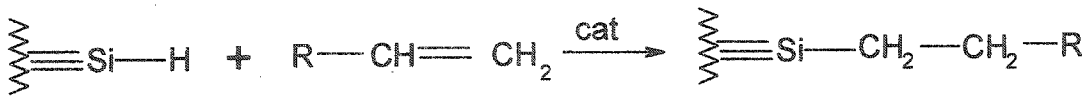
1.3.2c Silanization

The next step is silanization wherein the silanol groups (-SiOH) are converted to Si-H moieties (9-11). The capillary is flushed with a mixture of dioxane / triethoxysilane and 2.3 M HCl, then heated at 90° C for 90 mins and finally washed with toluene.



1.3.2d Hydrosilation

The H of the Si-H can be replaced by a variety of groups such as C18 (octadecyl), cholesteryl or a diol. For the modification with a diol, a mixture of 2ml 7-octene-1,2-diol +2ml toluene + 70µL Pt catalyst (Speier's catalyst) is heated at 70°C for 1 hr and then passed through the capillaries by applying pressure. The capillaries are heated at 100°C for 5 days and the mixture is passed through the capillary every day. Finally the capillaries are washed with toluene for 2 hours at a low pressure and then with methanol for an hour.



catalyst typically hexachloroplatinic acid (Speier's catalyst)

2. Project 1:

Separation of ipratropium bromide, related compounds and impurities in pharmaceutical formulations using OTCEC

2.1 What is ipratropium bromide?

Ipratropium bromide is an anticholinergic bronchodilator. It is a quaternary ammonium compound chemically related to atropine. It is used to prevent the breathing problems associated with asthma, wheezing, chronic bronchitis, and other lung diseases. A bronchodilator relaxes and opens the air passages to the lungs thus making breathing easier. Ipratropium is administered as an aerosol to inhale by mouth. It functions as a drug, by blocking the parasympathetic nervous system and inducing bronchodilation. The parasympathetic nervous system induces a decrease in heart rate and a decrease in blood circulation. Ipratropium bromide is a long-term bronchodilator but not a "rescue medication" (12).

The nervous system is divided into the central nervous system that controls the voluntary organs and the autonomic nervous system. The autonomic nervous system is further is divided into sympathetic and parasympathetic components. The sympathetic nervous system increases the heat rate, and slows peristalsis. The parasympathetic system conserves and restores energy thereby reducing the heart rate and blood pressure. Ipratropium's function is to basically block the parasympathetic nervous system. Figure 5 is a diagram outlining the division of the nervous system. Ipratropium bromide has several advantages over atropine. Atropine inhibits the mucociliary clearance allowing nasal obstruction whereas ipratropium bromide does not. Also ipratropium has no

significant effect on the central nervous system, and is more effective than the β-adrenergic agonists which stimulate through β- adrenergic receptor catalyzing ATP to cAMP thereby relaxing the bronchial smooth muscle .

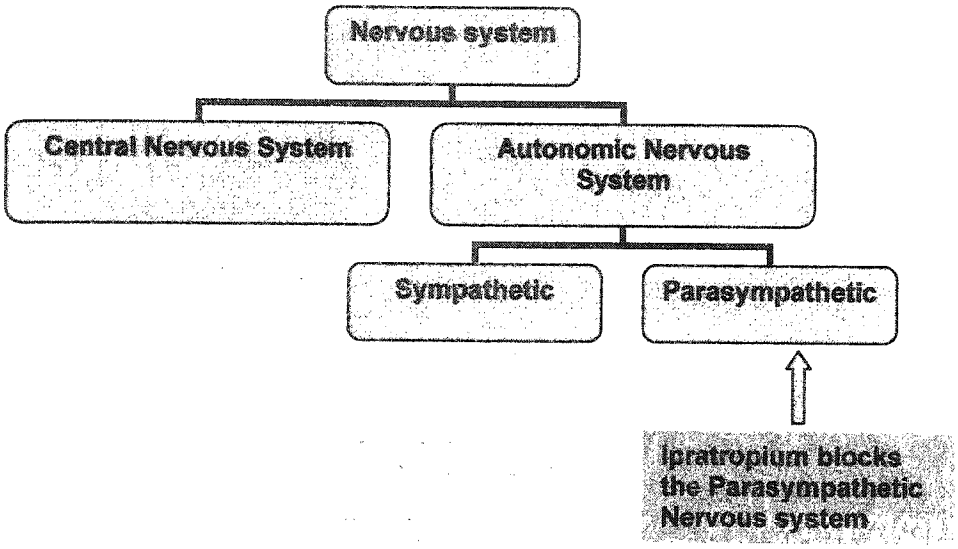


Figure 5: Diagram of the division of the nervous system

During the manufacture of ipratropium bromide (8-isopropyl-nor-atropine-methylbromide) some process impurities are found (13). Some of these impurities are listed below:

- Ipratropium bromide
- Tropic acid (3-hydroxy-2-phenylpropanoic acid)
- Atropic acid (2-phenylpropanoic acid)
- Ipratropium alcohol
- N-isopropyl noratropine (NINA)
- Apo-ipratropium bromide
- 8S-ipratropium bromide

Figure 6 shows the structures of the ipratropium bromide and impurities used in the present research. Ipratropium bromide and related compounds have been analyzed previously using HPLC (13).

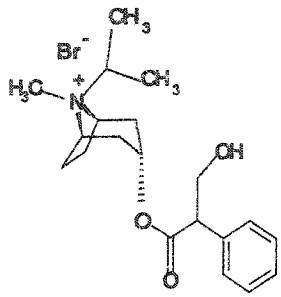
2.2 Goal of Project 1

In view of the above discussion it would be desirable to have a technique, which can detect ipratropium bromide and the various process impurities. These impurities can prove to be fatal if present in high concentrations. Ipratropium alcohol in high doses can prove toxic and has never been separated before (13). This research focuses on finding a technique which is suitable for pharmaceutical analyses in a limited amount of time at low cost. For this to be done various capillaries with varying internal diameters (bare capillary, 50μm; C18, 50μm; cholesterol, 50μm and 20μm; diol, 50μm; 4-cyano-4'-n-pentoxybiphenyl, 50μm; and butyl phenyl, 50μm) were tested to see which would give the best results. Simultaneously experimental conditions were also varied such as pH (2.14, 3.00, 4.41, 6.00, 7.00, and 8.00), voltages (30 kV, 25 kV, 20 kV, 15 kV, and 10 kV), and different modes of injection (hydrodynamic and electrokinetic). OTCEC should be a good analytical technique as the amount of sample required can be micrograms or even less. The primary goal of this project is to find suitable parameters for the detection and separation of these compounds.

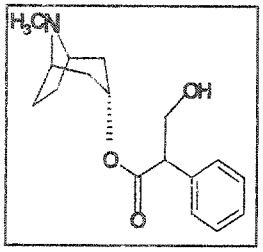
2.3 Experimental

Reagents and material:

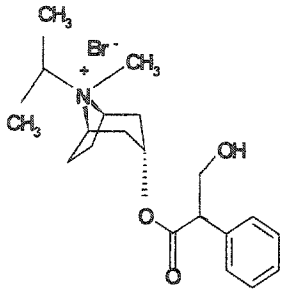
Capillary:



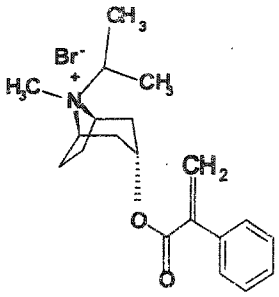
Ipratropium bromide



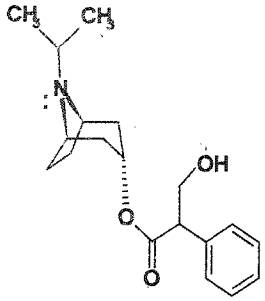
Atropine



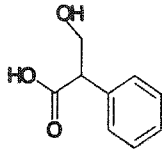
8S-Ipratropium bromide



Apo-ipratropium bromide



N-isopropyl noratropine



Tropic Acid

Figure 6: Ipratropium bromide and process degradation products (13)

The capillary i.d. used was 50μM internal diameter (i.d.) from Polymicro Technologies, Phoenix, Az, USA. Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Ammonium bifluoride, the etching agent, was bought from Aldrich (Milwaukee, WI, USA), triethoxy silane (Huls America, PA, USA), 1-octadecene (Aldrich Co Inc), and hexachloroplatinic acid (Aldrich Co. Inc) were used for the modification of the inner walls of the capillary. A 50μm i.d. capillary (Italy) coated with BDMA (DMA-N+$(CH_3)_2$ 10% functional group) without etching was also used in the research.

Buffers:

The buffers used were as follows: pH 2.14, 3.00, 4.41, 6.00, 7.06, and 8.14. The composition of these buffers is as follows:

pH 2.14, 0.3 M H_3PO_4 and 0.19 M TRIS [tris [hydroxymethyl] amino methane]

pH 3.00, 0.3M citric acid and 0.25 M β-alanine

pH 4.41, 0.3M acetic acid and 0.375 M γ-amino butyric acid

pH 6.00, 0.3M MES [(2-[N-morpholino] ethane sulfonic acid)] and 0.21 M l-histidine [l-α-amino-β-imidazole propionic acid]

pH 7.06, 0.3M MOPS [(2-[N-morpholino] propane- sulfonic acid)] and 0.215 M imidazole

pH 8.14 0.1M TRIS and 0.15 M boric acid

TRIS, MES, MOPS, L-histidine, GABA, and citric acid were purchased from Sigma Chemical Co (St. Louis, MO); boric acid and glacial acetic acid were purchased from

American Scientific Products (CA), and imidazole from Calbiochem (San Diego, CA). Table 1 lists the compounds (ipratropium bromide and impurities) used.

Table 1: Ipratropium bromide and impurities used in the research

Name of compound	*Manufacturers name*
Tropic acid	Sigma (St, Louis MO)
Atropic acid	Sigma (St, Louis MO)
N-isopropyl noratropine (NINA)	Sifavator (Milan, Italy)
Apo-ipratropium bromide	Sifavator (Milan, Italy)
8S-ipratropium bromide	Sifavator (Milan, Italy)
Ipratropium bromide	Sifavator (Milan, Italy)
Ipratropium alcohol	Dey Labs (Napa, CA)

2.3.1 Instruments

Two high performance capillary electrochromatography (HPCE) instruments were used for the research project. One was an Applied Biosystems Model 270-A-HT capillary electrophoresis system (Foster City, CA, USA) having a UV detector. The other HPCE instrument used was an Agilent (Braunschweig, Germany) 3D Capillary

electrophoresis instrument having a UV detector. The latter was automated whereas the former is a manual one.

The oven used for etching of capillaries previously described was part of a Hewlett-Packard Model 5890 gas chromatograph. The GC oven was used for the control of the temperature and also modified such that multiple capillaries could be accommodated.

2.3.2 Sample preparation

The stock sample solution was prepared by dissolving 0.5 mg sample in 0.5 ml pH 2.14 (1:100) buffer. The individual sample is prepared by adding 20 μL of stock solution to 120 μL of buffer. The buffer is prepared by diluting pH 2.14 buffer by 100 times.

2.4 Results and Discussion

2.4.1 Bare capillary

Analysis of ipratropium bromide has been done previously using HPLC. Every technique has its advantages and disadvantages. OTCEC combines HPLC and CE as the separation process is based on partitioning between phases like HPLC and it is also based on electroosmosis (EOF) as a main driving force with a characteristic flat flow pattern as in CE, thus giving a better result (14). The capillaries are also etched to give a higher surface area. The result from a bare capillary without any chemical modification is shown in Figure 7. Only three peaks are seen, indicating that not all the components are

resolved and separated. The peaks were the combination of more than one compound and were difficult to assign. In Figure 8, it is seen that changing the pH of the buffer doesn't give any better results [7]. In Figure 9, the inner wall of the bare capillary is chemically modified with β-dimethyl aryl amide group (BDMA). But it is seen that there is not much difference. Only one peak is seen, indicating that the components are not separated.

2.4.2 Etched capillaries

The etching process increases the surface of the inner walls of the capillary by about 1000 times. This in turn increases the amount of stationary phase bonded to the walls and thus the sample capacity. The silica is redeposited on the walls during etching thereby creating radial extensions that will decrease the axial distances. The etched capillary is chemically modified with different groups. By chemically modifying the capillaries, both the electroosmotic flow and the adsorptive properties are affected. The interaction of ipratropium bromide and similar compounds with the stationary phase can be seen in Figure 10. The capillary being used was etched modified with 4-phenyl – 1-butene (50 μm i.d.) using a pH 3.00 (1:10 dilution) buffer. Some peaks are observed indicating partial separation. Different voltages and different pHs did not give any better results.

In Figure 11, it can be seen that an etched capillary modified with C_{18} gave better results. The resolution is improved and some partial separation is seen. Tropic acid and ipratropium alcohol both have the same migration time as well as apo-ipratropium

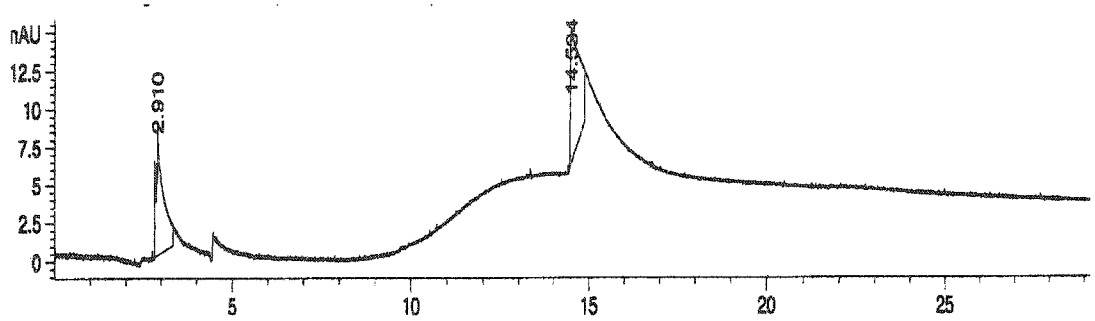


Figure 7: Electropherogram of ipratropium mixture using a bare capillary, 50 µm i.d., 55.5/47 cm, pH 2.14 buffer (1:10), detection at 220nm, 30 kV, injection 5s@50mbar, and sample prepared in pH 2.14 buffer (1:100)

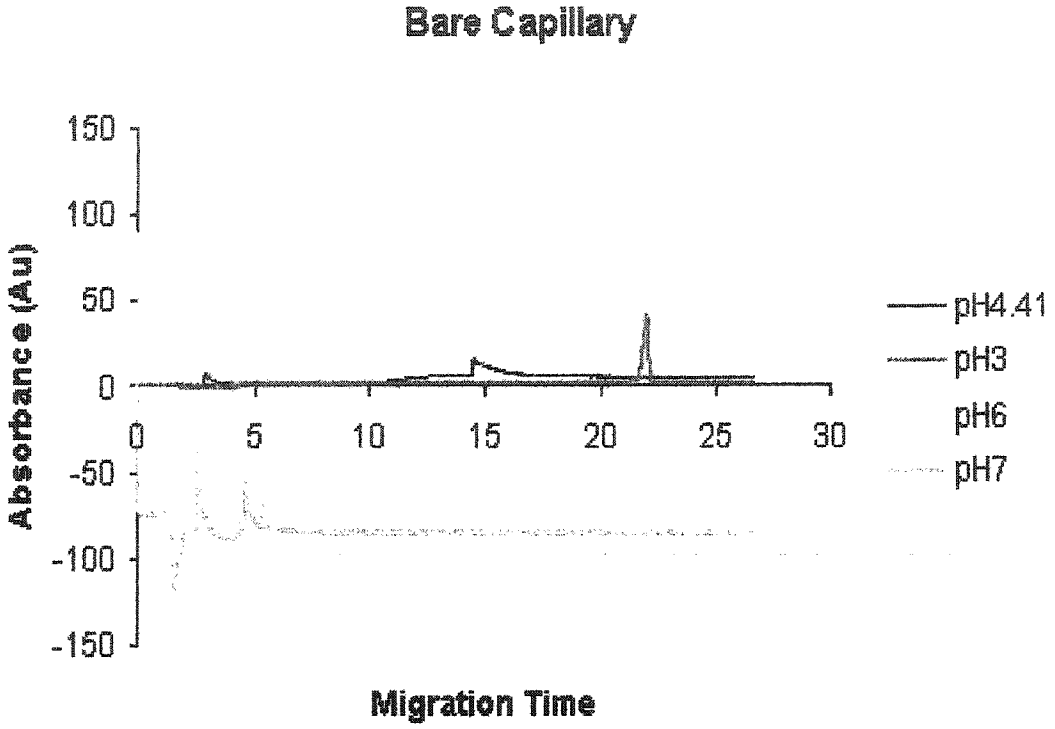


Figure 8: Separation of ipratropium mixture on a bare capillary, 50 µm i.d., at pH 3.00, 4.41,6.00 and 7.06, 55.5/47 cm, buffer pH 2.14(1:10), 30 kV, injection 5s@50mbar, sample prepared in pH 2.14 buffer (1:100),and detection at 220 nm

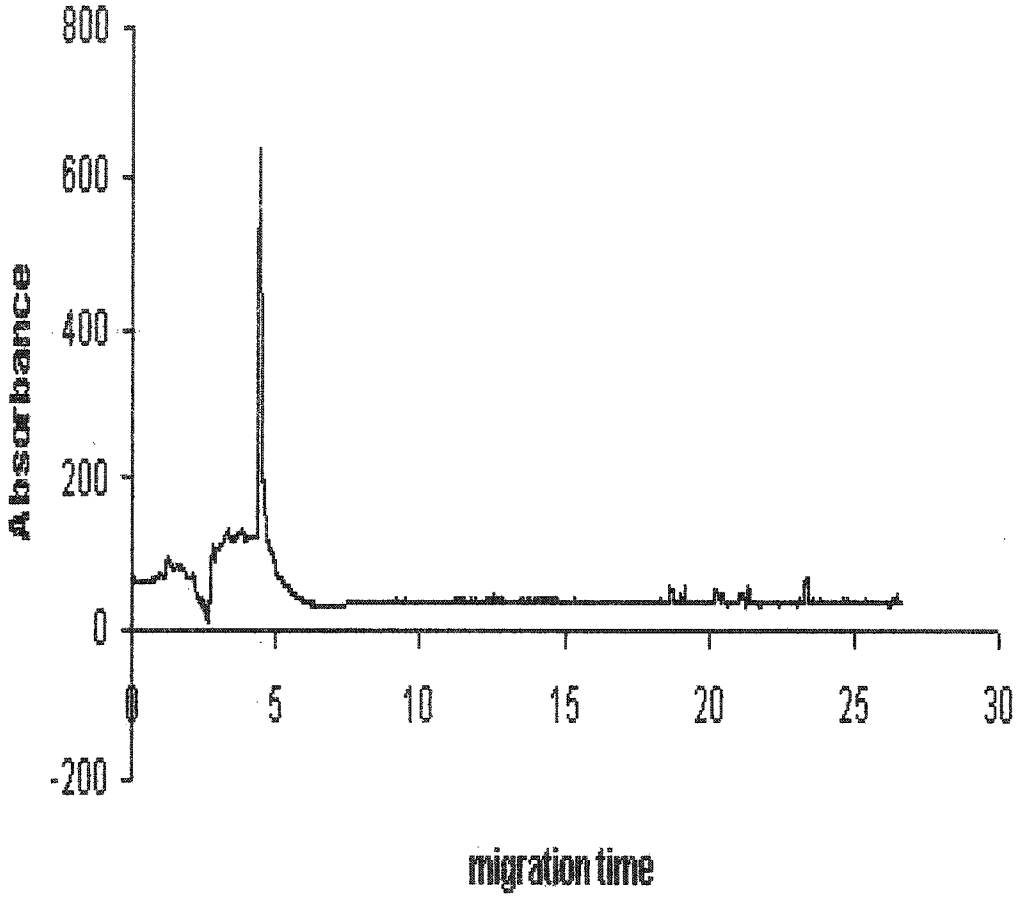
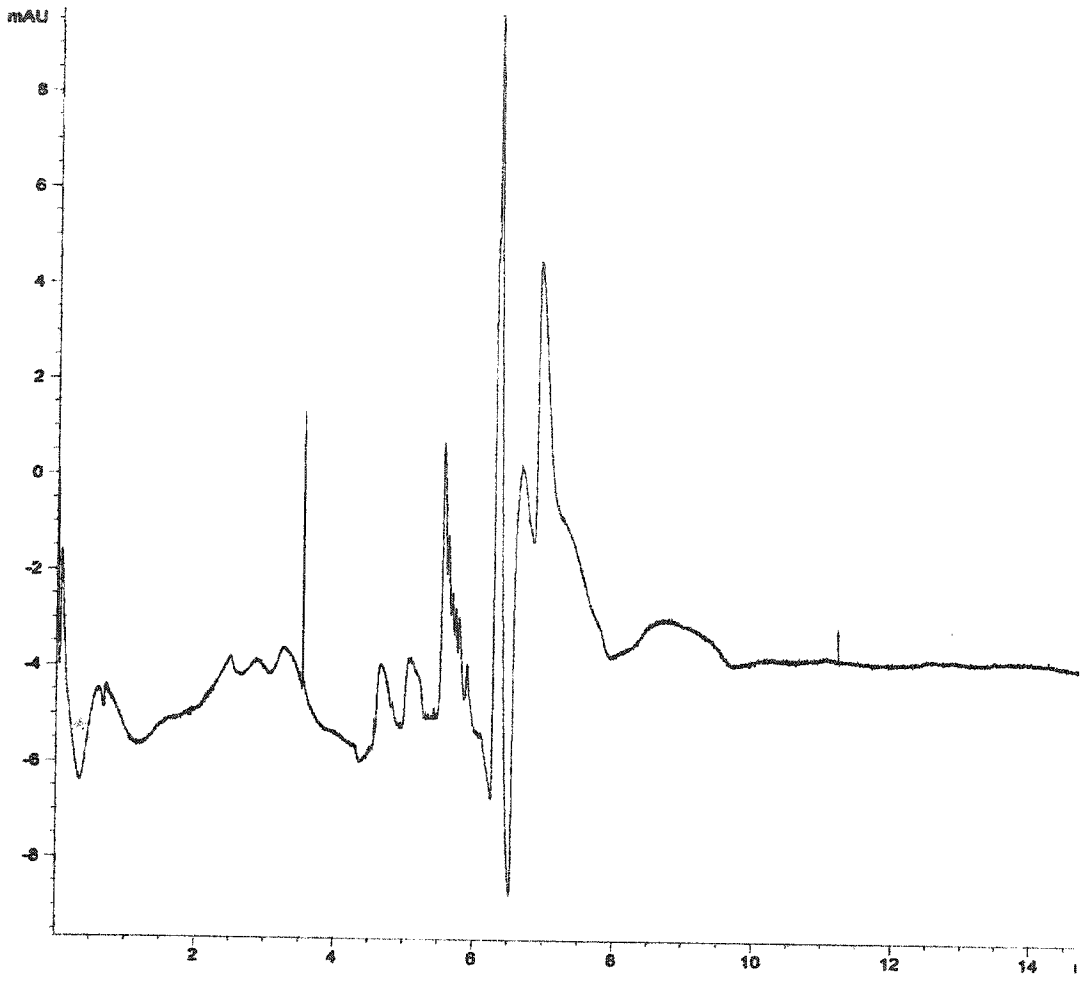


Figure 9: Separation of ipratropium mixture on a 50 µm i.d. bare capillary modified with BDMA (DMA-N +$(CH_3)_2$ 10% functional groups), pH 3.00 (1:10) buffer, 30 kV, injection 5s@50mbar, and sample prepared in pH 2.14 buffer (1:100), and detection at 220 nm



Migration time

Figure 10: Separation of ipratropium mixture on an etched capillary modified with 4-phenyl -1- butane, 50 µm i.d., pH 3.00 (1:10) buffer, 30 kV, injection 5s@50mbar, sample prepared in pH 2.14 buffer (1:100), and detection at 220 nm

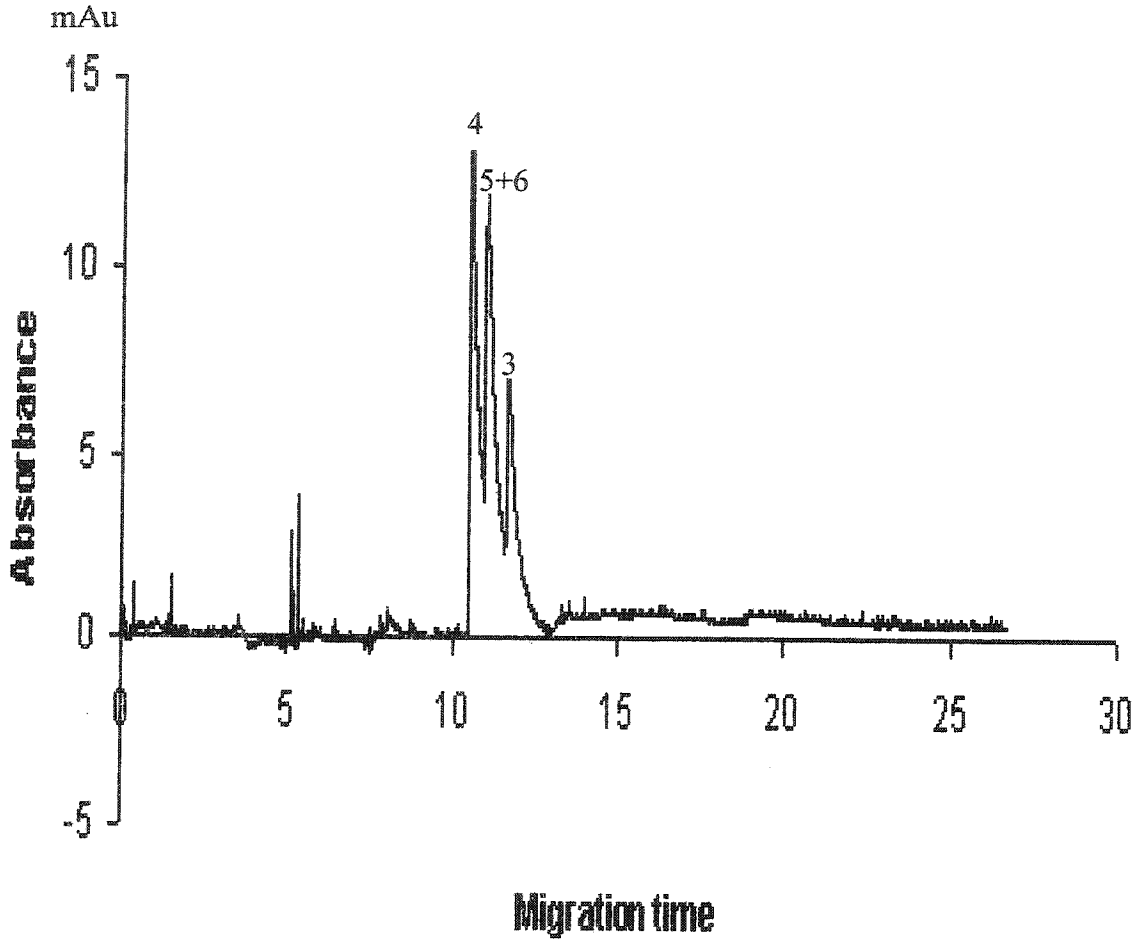


Figure 11: Separation of ipratropium mixture on an etched capillary modified with C18, 50 µm i.d., 59.6/51.3cm, pH 3.00 (1:10) buffer, 30 kV, injection 5s@50mbar, sample prepared in pH 2.14 buffer (1:100), and detection at 220 nm

bromide and 8S-ipratropium bromide by referring to the individual electropherograms. NINA and apo-ipratropium bromide have different migration times. In the next electropherogram, Figure 12, the same capillary and the same conditions are used except the buffer is pH 2.14 (1:10). It is seen that the components are better resolved. The peak shape is also better than the previous electropherogram. Ipratropium alcohol is resolved in the mixture that was not achieved before using HPLC or CE. The electropherograms of the various compounds are also seen with the mixture to show the individual migration times. The temperature used was room temperature.

2.4.3 Effect of voltage

An increase in voltage causes an increase in electrophoretic mobility, resulting in a faster migration time. In Figures 13a and 14, it is seen that at 10kV the migration time is longer than at 15kV. But with a further increase in voltage, the migration time does not change much. This observation is the same even after changing the pH to 3.00. It was assumed that this could have been an instrumental error with some fluctuations in the current. To prove this assumption a graph of current vs. voltage was plotted as shown in Figure 13b, but it showed a straight line indicating no problem with the instrument. This behavior cannot be explained at this point. More experiments need to be done.

2.2.4 Capillaries

An etched capillary modified with 4-cyano-4-pentoxy biphenyl capillaries gave in

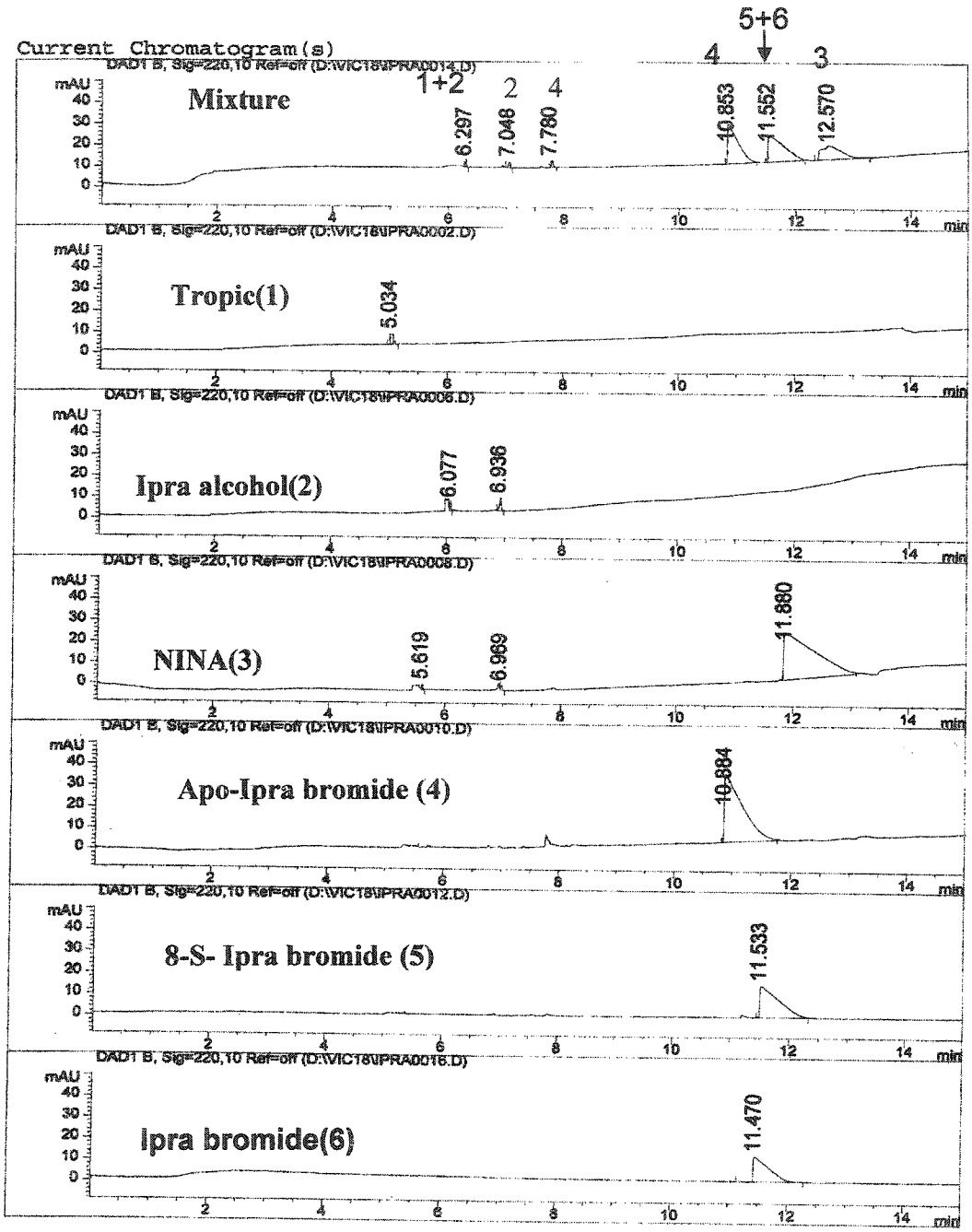


Figure 12: Electropherograms of ipratropium mixture and individual impurities on an etched capillary modified with C18, 50 µm i.d., , 59.6/51.3 cm, buffer pH 2.14 (1:10), 30 kV, injection 5s@50mbar, sample prepared in pH 2.14 buffer (1:100), and detection at 220 nm.

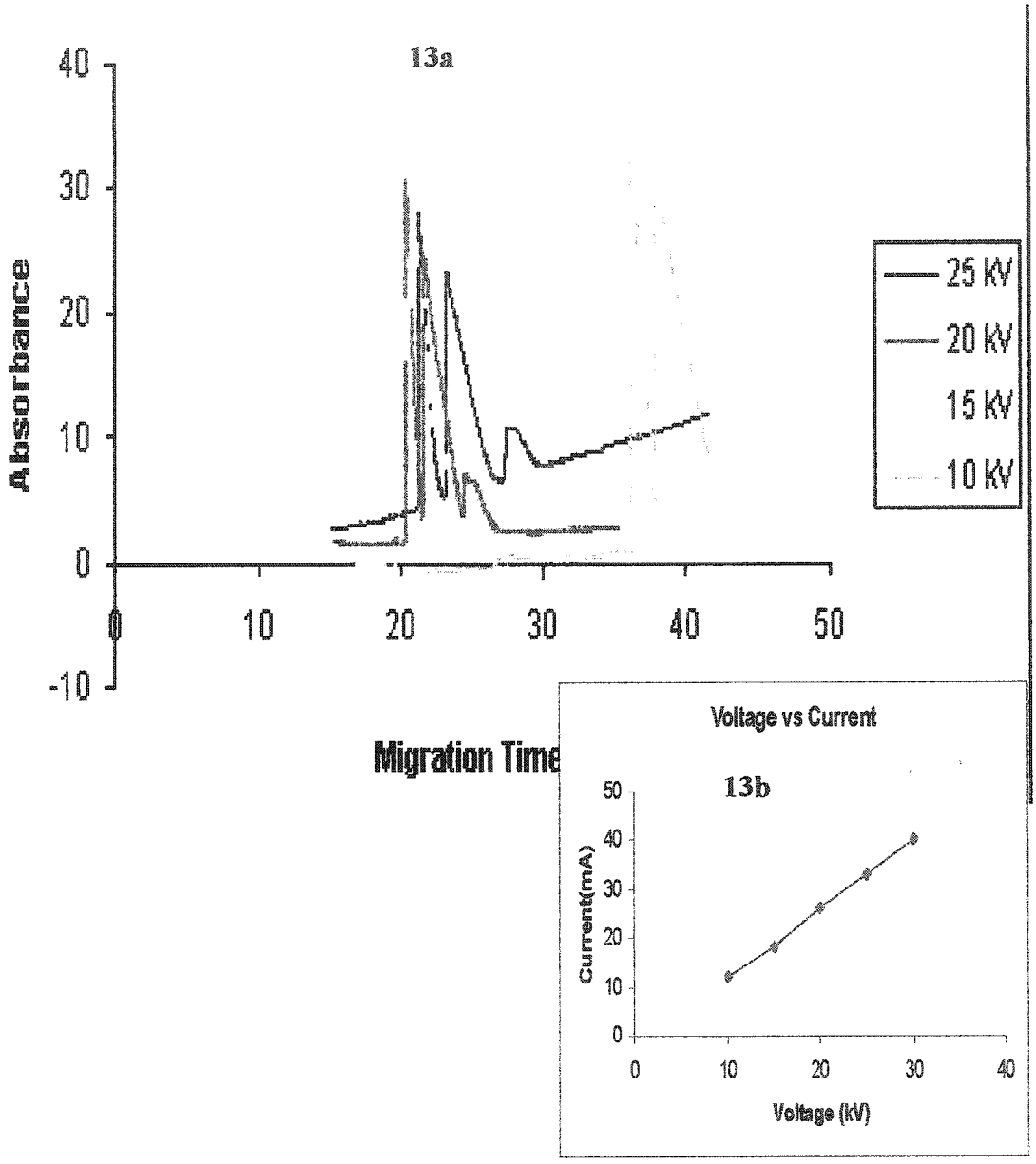


Figure 13: (a) Separation of ipratropium mixture on an etched capillary modified with C18, 50 µm i.d., , 59.6/51.3 cm, pH2.14 (1:10) buffer, at different voltages 25, 20, 15 and 10 kV, injection 5s@50mbar, sample prepared in pH 2.14 buffer (1:100), detection at 220 nm (b) Voltage vs Current

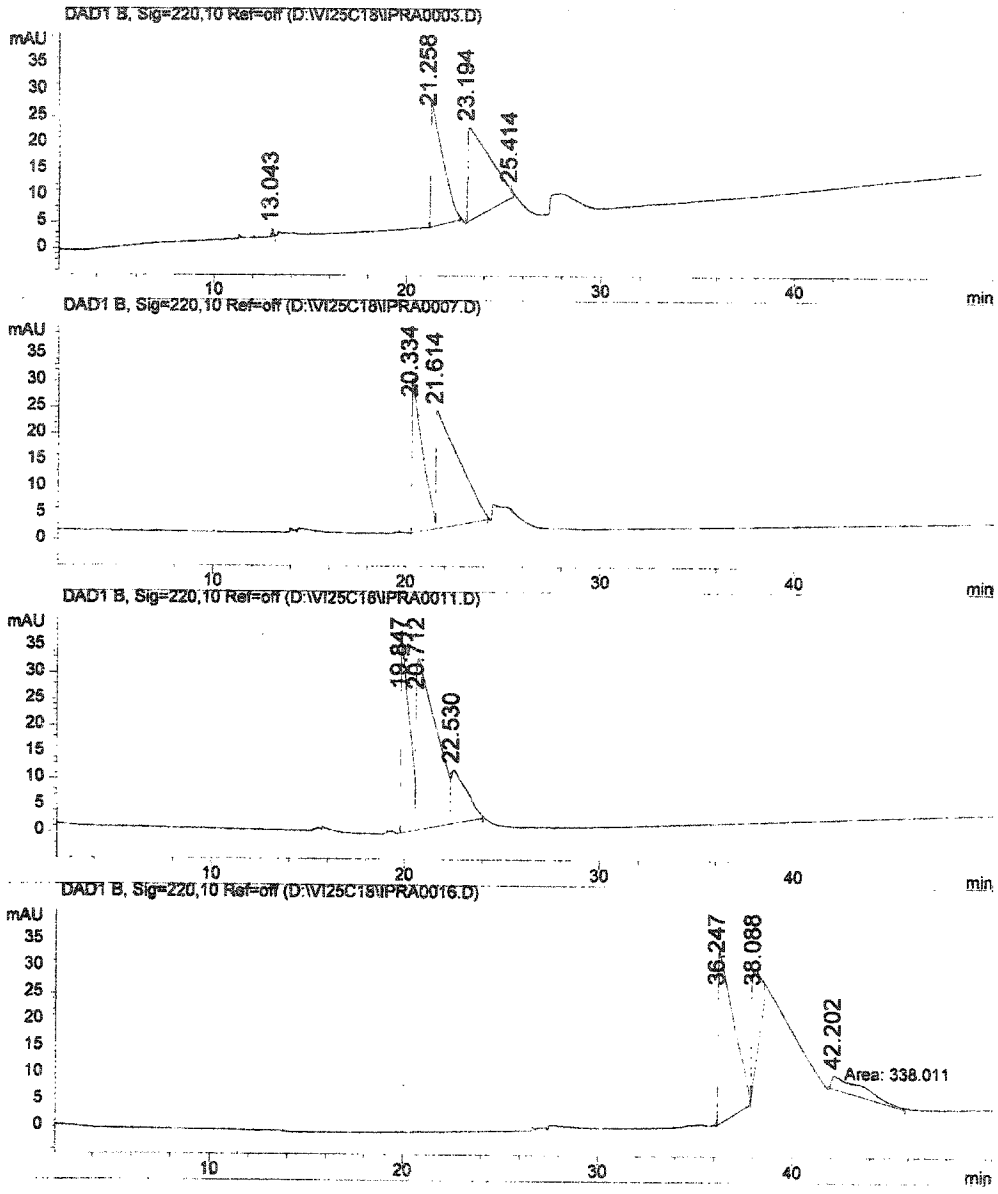


Figure 14: Separation of ipratropium mixture on an etched capillary modified with C18, 50 µm i.d., 59.6/51.3 cm, pH 3.00 (1:10) buffer, at different voltages 25, 20, 15, and 10 kV, injection 5s@50mbar, and sample prepared in pH 2.14 buffer (1:100), and detection at 220 nm

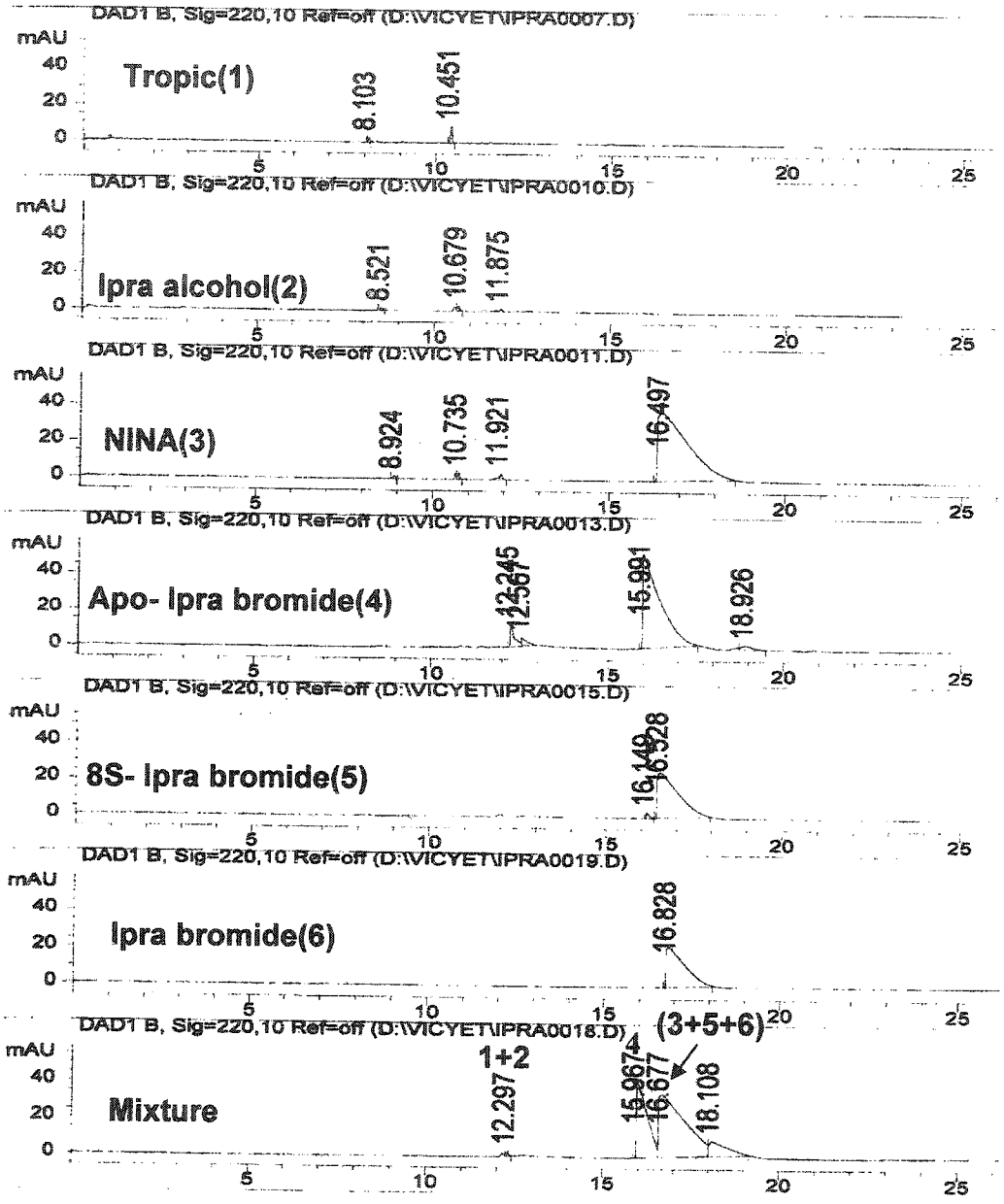


Figure 15: Electropherograms of ipratropium mixture and individual impurities on an etched capillary modified with 4-Cyano-4'-pentoxy biphenyl, 50 µm i.d., 61.7/70.0cm, buffer pH2.14 (1:10), 25 kV, injection 5s@50mbar, sample prepared in pH 2.14 buffer (1:100), and detection at 220 nm

general good results. Using 25 kV and a pH 2.14 (1:10) buffer, better results than bare capillary are obtained as seen in Figure 15. When an etched column modified with cholesterol was used the resolution improved considerably and peak shape improved too. The injection done in this case was electrokinetic and the internal diameter used was 20 μm. The electropherogram is seen in Figure 16. Decreasing the internal diameter provides more interaction with the stationary phase giving better resolution.

2.4.5 Addition of methanol

In Figure 17, different concentrations of methanol (MeOH) are used with the pH 2.14(1:10) buffer. The solute mobility changes with the addition of MeOH. Due to the organic solvent, the dielectric of the background electrolyte decreases and thereby the migration time is longer. Addition of MeOH decreases the EOF due to the decreased zeta potential resulting in a lower current and less Joule heating. It is seen in the figure that at 20% MeOH added to the buffer the electropherogram gives better resolution and symmetrical peaks are observed.

2.5 Conclusions

The bare and etched capillaries were compared to determine which gave the best separation of ipratropium mixture. The etched capillary gave more resolved peaks than the bare capillary. The etched capillary modified with different moieties such as C_{18}, cholesteryl, diol and others was tried, of which C_{18} gave the best results. The goal of the project being the determination of optimum conditions for separation of ipratropium

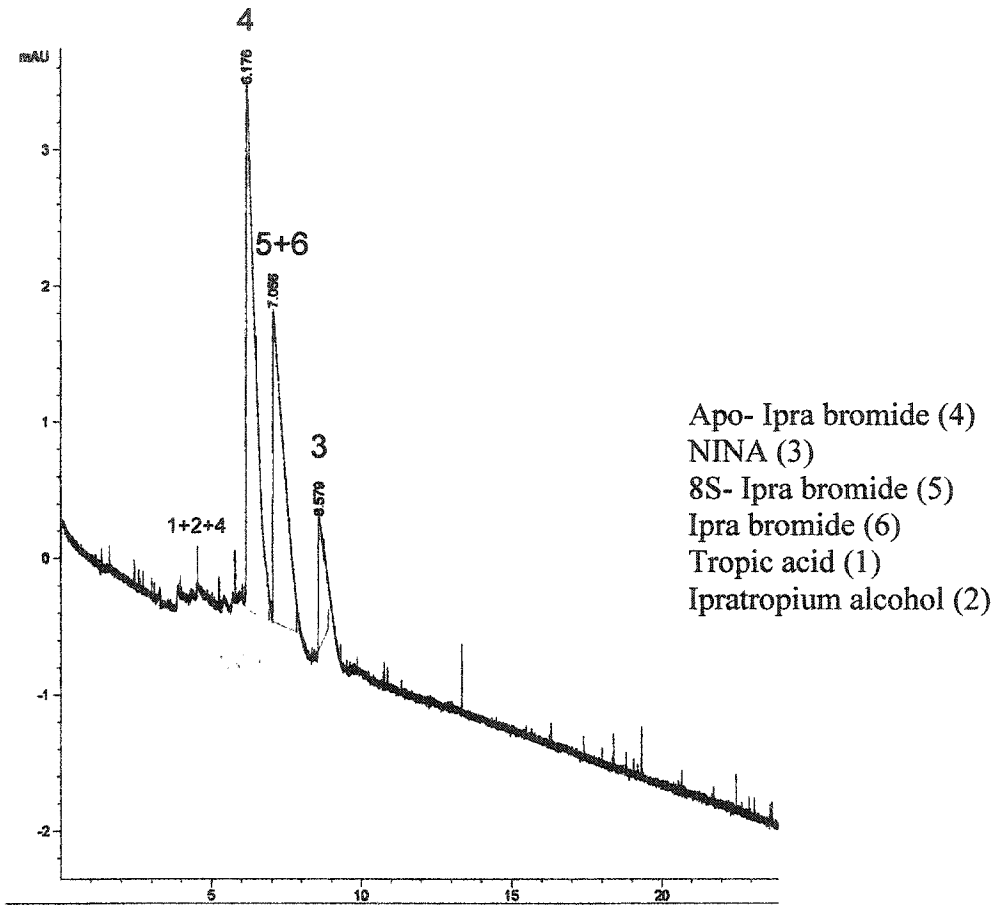
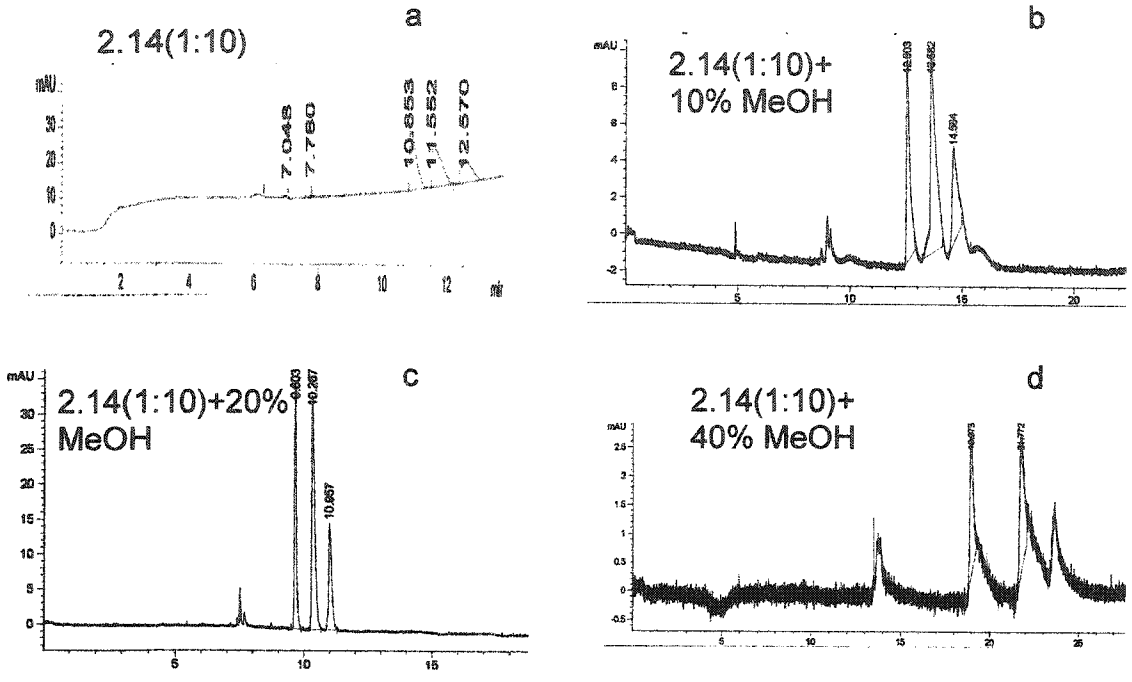


Figure 16: Electropherograms of ipratropium mixture and individual impurities on an etched capillary modified with cholesterol, 20 µm i.d., 33.4/25 cm, buffer pH 2.14 (1:10), 30 kV, injection 5s@50mbar, sample prepared in pH 2.14 buffer (1:100), and detection at 220 nm.



Apo- Ipra bromide(4) , NINA(3), 8S- Ipra bromide(5),Ipra bromide(6)

Figure 17: Separation of ipratropium mixture on an etched capillary modified with C18, 50 µm i.d., 59.6/51.3 cm, at pH 2.14 (1:10) buffer, at different concentrations of methanol a: without methanol, b: 10%, c: 20% and d: 50% added to the pH 2.14 (1:10) buffer, injection 5s@50mbar, sample prepared in pH 2.14 buffer (1:100), and detection at 220 nm.

bromide and its impurities, a variety of conditions was tested. The pH 2.14 (1:10) buffer was the best among those tried (3.00, 4.41, 6.00 and 7.06 all at 1:10 dilution). An applied voltage of 30 kV was best able to resolve the peaks. The addition of methanol showed some interesting results. Adding 20% MeOH to the buffer gave the best resolution and very symmetrical peaks were obtained as compared to just the pH 2.14 (1:10) buffer. When a cholesteryl modified etched capillary with an internal diameter of 20 μm i.d. was used, better results were obtained compared to a 50 μm i.d. capillary with the same surface modification.

The main toxic impurity of ipratropium bromide, being the ipratropium alcohol, has not been successfully analyzed to date. In the present research, ipratropium alcohol was resolved in the mixture with a pH 2.14 (1:10) buffer on an etched capillary modified with C18, 50 μm i.d., 59.6/51.3 cm, 30 kV, injection 5s@50mbar, sample prepared in pH 2.14 buffer (1:100), and detection at 220 nm. The results indicate that the etched modified capillaries are able to resolve the various impurities. The electropherograms obtained were also very reproducible suggesting the use of OTCEC is a technique that can be validated for industrial use.

2.6 Future studies

It is also necessary to find a method to separate R and S ipratropium bromide. The R form being ipratropium bromide and the S form is the 8S- ipratropium bromide. Both the R and S have the same migration time under the conditions used in this study.

Ipratropium alcohol is a toxic impurity and has never been analyzed successfully before. It is necessary to study the structure of ipratropium alcohol. The structure of ipratropium alcohol is similar to ipratropium bromide but due to the company's proprietary policy for the structure of ipratropium alcohol was not known during the time of research.

3. Project 2

Separation of proteins that are modified by attachment of polyethylene glycol (PEG)

3.1 Introduction

Over the last decade there has been an increased interest in using proteins and peptides as therapeutic agents for various diseases including cancer. The problem with using a protein as a drug is its solubility in body fluids and the need for frequent dosage requirement as it is removed out of the body rapidly. In particular, the smaller proteins are most affected which can limit the therapeutic efficacy. There have been various methods devised to circumvent these problems, but not many have been able to reach the clinical trial stage. Polyethylene glycols and dextrans are used to modify proteins to address some of the problems mentioned above. The additive does retain the pharmacological activity of the protein, though to a lesser extent compared to the native compound (15). There is an increased plasma life of the protein which compensates for the reduction in the pharmacological effects of the polymer-protein conjugation; thereby there is a net increase in the pharmacological activity of the protein.

Poly (ethylene glycol) "PEG" [$HO\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2OH$] has varying molecular weights (MW ~5000 kD or more) and contains terminal hydroxyl groups and a carbinol moiety. The polymer is usually linear at MW < 10 kD, however higher MW compounds have some degree of branching. PEG is soluble in water, toluene, methylene chloride, and many organic solvents but is not soluble in ethyl ether, hexane, and ethylene glycol. PEG is one of the most universal biocompatible polymers and is non toxic for internal consumption (FDA approved). PEGylation is defined as the

modification of protein by covalent attachment of polyethylene glycol. PEGylation was first developed in the 1970's by Abuchowski and colleagues (16). The modification by attaching PEG improves solubility of biomaterials like proteins (Pegylated protein having a large surface area) with body fluids. There is improved stability as well as solubility (even "brick dust" and "sand" can be rendered water soluble), reduced immunogenicity and proteolysis, and extended time for removal from the body.

Renal filtration is slowed down by increased molecular size. Studies have shown that each ethylene oxide unit is associated tightly with 2 – 3 water molecules thus giving the PEG species its solubility properties of 5 to 10 times as large as the solubility of a protein of similar MW. The PEG molecule is heavily hydrated in an aqueous medium and has a very rapid motion. PEG sweeps out a large volume, thereby preventing interference of other molecules which might try to conjugate with the protein. Thus when attached to a drug, PEG chains protect the drug molecules from immune response and other disruptive mechanisms (17). PEGylated proteins are introduced by injections. Drugs with a MW below 20 kD are cleared via the kidney and excreted through the urine, whereas drugs with a MW above 20 kD, are cleared more slowly through kidney due to the increased size, with removal by the liver increasing with MW (18).

3.2 Chemistry of PEGylation

PEGylation chemistry can be divided into first- and second-generation approaches (19). The synthesis of PEG involves anionic ring opening polymerization of ethylene oxide which is initiated by the nucleophilic attack of hydroxide ion on the epoxide ring.

The first generation chemistry is restricted to low MW monofunctional PEG (MPEG) CH_3O -$(CH_2CH_2O)_n$-CH_2CH_2OH.

Coupling of PEG to a protein requires the activation of the PEG by derivatizing one or both end termini to have a reactive functional group. The typical amino acids that are reactive towards PEGylation are lysine, cysteine, arginine, histidine, N-terminal amino acids, and the C-terminal carboxylic acid. But the most common method utilized is using functional groups (on PEG) that are reactive towards the lysine and the N-terminal amino acid groups in the proteins. In the case of polypeptides addition involves the lysine amino acids. Listed below are the PEGylated proteins used in this research with their sequence (20). The bold "**k**" indicates the amino acid lysine.

Catalase

adnrdpasdq m**k**hwkeqraa q**k**pdvlttgg gnpvgd**k**lns ltvgprgpll vqdvvftdem ahfdreripe rvvha**k**gaga fgyfevthdi trys**k**a**k**vfe hig**k**rtpiav rfstvagesg sadtvrdprg fav**k**fytedg nwdlvgnntp iffirdallf psfihsq**k**rn pqthl**k**dpdm vwdfwslrpe slhqvsflfs drgipdghrh mdgygshtf**k** lvnadgeavy c**k**fhy**k**tdqg i**k**nlsvedaa rlahedpdyg lrdlfnaiat gnypswtlyi qvmtfseaei fpfnpfdlt**k** vwphgdypli pvg**k**lvlnrn pvnyfaeveq lafdpsnmpp giepspd**k**ml qgrlfaypdt hrhrlgpnyl qipvncpyra rvanyqrdgp mcmmdnqgga pnyypnsfsa pehqpsaleh rthfsgdvqr fnsanddnvt qvrtfyl**k**vl neeqr**k**rlce niaghl**k**daq lfiq**kk**av**k**nfsdvhpeygs riqalld**k**yn ee**k**p**k**n

Asparaginase

mkkilllatg gtiasvegne glapglsaee llnyfsrssr nleidckilm nidstnmqpe hwkeianavf nhyddydgfv ithgtdtlay tssalsymlq glrkpvvltg sqvpisfkkt dakknvadal rfacedvggv fivfdsrvii gtravkmrtk sydafesvny pyvaevned evkyhwkptss hnqlsintnl ctdvflmkly pgtkpeifdc lkdlykgiii esfgnggipf egrnllskiq eltemgvavv ittqcleege dillyevgrk vaqhqvilsg dmnteaiiak lmwalgktnk leeikkiiee plsydltiks dkdw

protease

mmrkksfwlg mltafmlvft mafsdsasaa qpaknvekdy ivgfksgvkt asvkkdiikesggkvdkqfr iinaakakld kealkevknd pdvayveedh vahalaqtvp ygiplikadkvqaqgfkgan vkvavldtgi qashpdlnvv ggasfvagea yntdgnghgt hvagtvaald nttgvlgvap svslyavkvl nssgsgtysg ivsgiewatt ngmdvinmsl ggpsgstamk qavdnayarg vvvvaaagns gssgntntig ypakydsvia vgavdsnsnr asfssvgaele vmapgagvy styptstyat lngtsmasph vagaaalils khpnlsasqv rnrlsstatylgssfyygkg linveaaaq

chymotrypsin

cgvpaiqpvl sglsrivnge eavpgswpwq vslqdktgfh fcggslinen wvvtaahcgvttsdvvvage fdqgssseki qklkiakvfk nskynsltin nditllklst aasfsqtvsa vclpsasddf aagttcvttg wgltrytnan tpdrlqqasl pllsntnckk ywgtkikdam icagasgvss cmgdsggplv ckkngawtlv givswgsstc ststpgvyar vtalvnwvqqtlaan

Superoxide dismutase

atkavcvlkg dgpvqgtihf eakgdtvvvt gsitgltegd hgfhvhqfgd ntqgctsagphfnplskkhg

gpkdderhvg dlgnvtadkn gvaivdivdp lislsgeysi igrtmvvhek pddlgrggne estktgnags rlacgvigia k

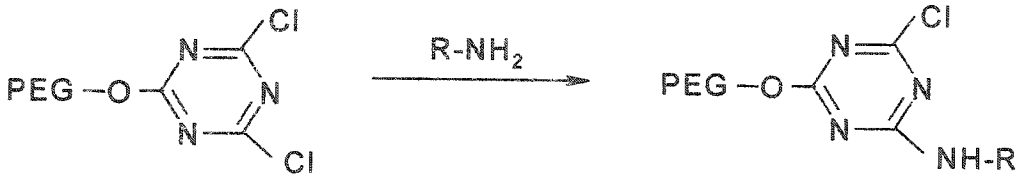
Lipase

mklalalsli asvaaaptak langdtitgl naiineaflg ipfaeppvgn lrfkdpvpys gslngqkfts ygpscmqqnp egtfeenlgk taldlvmqsk vfqavlpqse dcltinvvrp pgtkaganlp vmlwifgggf eigsptifpp aqmvtksvlm gkpiihvavn yrvaswgflag ddikaegsg naglkdqrlg mqwvadniag fggdpskvti fgesagsmsv lchliwndgd ntykgkplfr agimqsgamv psdpvdgtyg neiydlfvss agcgsasdkl aclrsassdt lldatnntpg flaysslrls ylprpdgkni tddmyklvrd gkyasvpvii gdqndegtif glsslnvttn aqarayfkqs fihasdaeid tlmaaypqdi tqgspfdtgi fnaitpqfkr isavlgdlaf iharryflnh fqggtkysfl skqlsglpim gtfhandivw qdyllgsgsv iynnafiafa tdldpntagl lvnwpkytss sqsgnnlmmi nalglytgkd nfrtagydal mtnpssffv

The first generation methoxypolyethylene (MPEG) glycol used in this research were MPEG activated with cyanuric chloride (Avg. Mol Wt 5000), MPEG tresylate (Avg. Mol Wt 5000), MPEG succinate N-hydroxysuccinimide ester (Avg. Mol Wt 5000), and MPEG p-nitrophenyl carbonate (Avg. Mol Wt 5000). This information was provided by Sigma-Aldrich. The reactions below show (1) MPEG- tresylate with alkaline phosphatase (R-NH_2), (2) MPEG dichlorotriazine with a protein, and (3) other MPEG derivatives such as succinimidyl carbonate and p-nitrophenyl carbonate (19, 21, and 22).

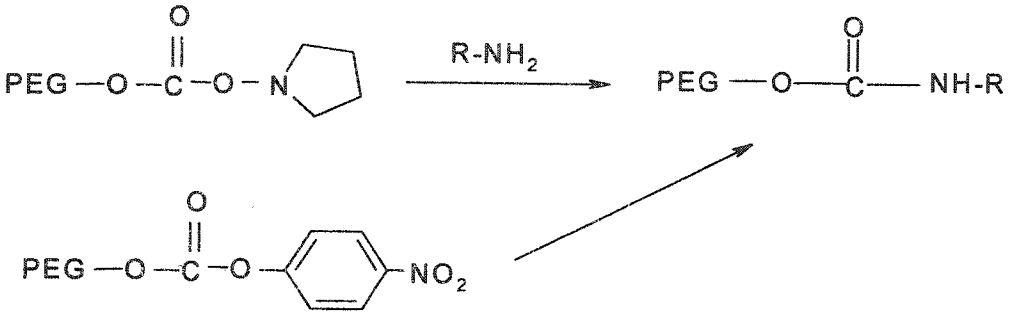
MPEG tresylate: $PEG\text{-}O\text{-}SO_2\text{-}CH_2CF_3 \xrightarrow{R\text{-}NH_2} PEG\text{-}NH\text{-}R$ (1)

MPEG dicholortriazine:



(2)

Succinimidyl carbonate



(3)

P-Nitrophenyl carbonate

The PEGylated proteins and their roles are listed below.

Table 2: PEGylated proteins used in the research

PEGylated Protein	***Protein origin***	***Function of the PEGylated protein***
Catalase – PEG	Bovine liver	Anti oxidant enzyme
Asparaginase – PEG	-	leukemia and lymphoma treatment
A- chymotrypsin – PEG	Bovine pancreas	Acts as a catalyst for peptide synthesis
Lipase – PEG	Candida rugosa	Anti cancer agent
Superoxide dismutase – PEG	Bovine erythrocytes	inhibits lipid peroxidation (Anti Oxidant)
protease – PEG	Bacillus licheniformis	formation of acid-amide bonds

The major problem involved in the 1st generation PEG's is that MPEG-OH is often contaminated with the PEG diol (HO-PEG-OH) resulting in weak (unstable) linkages during preparation. The diol impurities may range from 10 to 15%. Diol impurities are found due to presence of water during the base catalyzed polymerization of ethylene oxide to form MPEG and increases with increasing MW of MPEG. Diol activation tends to produce cross linking and aggregation. Also polydispersity for first generation compounds is less for higher MW PEG's (1.01 as compared to 1.1 respectively) (19). Polydispersity (MW / Mn) indicates if the peak is homogeneous with respect to molar mass (23). A homogeneous sample contains only one type of molecule of the defined MW, the average mass is independent of the method of averaging, and the polydispersity will be equal to one. For a sample made up of mixture of molecules having different molar masses the polydispersity will be greater than one.

3.3 Goal of Project 2

OTCEC shows immense potential in the analysis of proteins. This research involves the study of PEGylated proteins. Similar to the ipratropium bromide project different capillaries with different conditions are used to study these proteins. Due to different point of attachments of the PEG (different amine sites) there can be a number of positional isomers, which have an influence on the activity of the PEGylated protein and thus on the effective treatment. Positional isomers are calculated by (19):

$$P = \frac{N!}{(N-k)! * k!}$$

Where N=Number of positional isomers

K= Number of sites modified by PEG

It is also important in the case of proteins to show the peaks are reproducible in order to prove that the peaks are not mere background noise. Peaks observed for PEGylated proteins were found to be broad with other techniques indicating poor resolution of the isomers.

3.4 Experimental

All the instruments used in this section are the same as those of Project 1. The buffers used were the same as prepared earlier for Project 1.

The PEGylated proteins were bought from Sigma-Aldrich. The number of PEGs per mol of protein and the approximate pI of the unmodified protein (without PEG) are listed in the table below.

Table 3: List showing PEGylated proteins, the number of moles of PEG attached and the pI of the native protein without the PEG

Proteins-PEG	PEG per mole protein	~pI of unmodified protein
Catalase	40	5.4
Asparaginase	40	4.8
α- chymotrypsin	9	8.5
Lipase	16	6.1
Superoxide dismutase	12	5.6
protease	6	8.8

The PEGylated protein sample was prepared by using 1mg of protein in 1ml pH 2.14 (1:100) buffer.

3.5 Results and Discussion

PEGylated proteins have been analyzed previously using various techniques such as size exclusion chromatography. Electropherograms of PEGylated proteins have always been a subject of interest since people have not been able to successfully achieve good resolved peaks with reproducible results. The basic idea of this research is to be able to determine the conditions that maximize the protein-stationary phase interactions with good reproducibility, good resolution and good peak shape.

3.5.1 Bare capillary

Figure 18 shows electropherograms of PEGylated proteins on a bare capillary without any chemical modification. Not many peaks or in some cases none are seen. Analytes adsorb on the capillary wall and/or not resolved. Also it was observed that these peaks were not very reproducible between runs.

3.5.2 Etched capillary modified with cholesterol

It has already been discussed in Project 1 that an etched capillary gives better results than a bare capillary. In Figure 19, it is seen that each of the PEGylated proteins shows a different number of peaks under the same conditions. It depends on their positional isomers and interactions with the stationary phase which in this case is cholesterol. The number of peaks depends on the number of the PEG species bonded to the proteins and also the location at which it is bonded. So different PEGylated proteins show different number of peaks, but it needs to be observed which condition

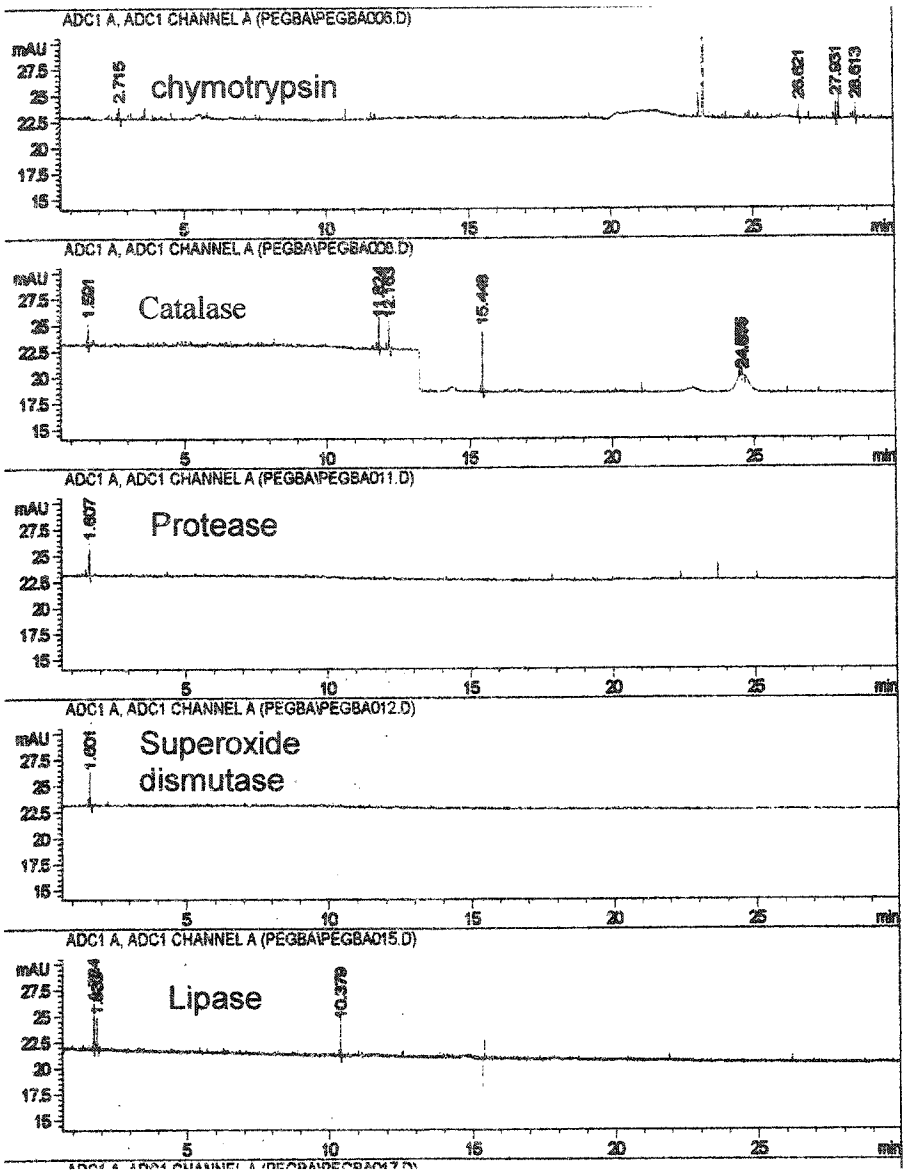


Figure 18: Electropherograms of individual PEGylated proteins using a bare capillary, 50 µm i.d., 51.0/25 cm, pH 2.14 (1:10) buffer, detection at 220nm, 30 kV, injection 5s@50mbar, and sample prepared in pH 2.14 buffer (1:100).

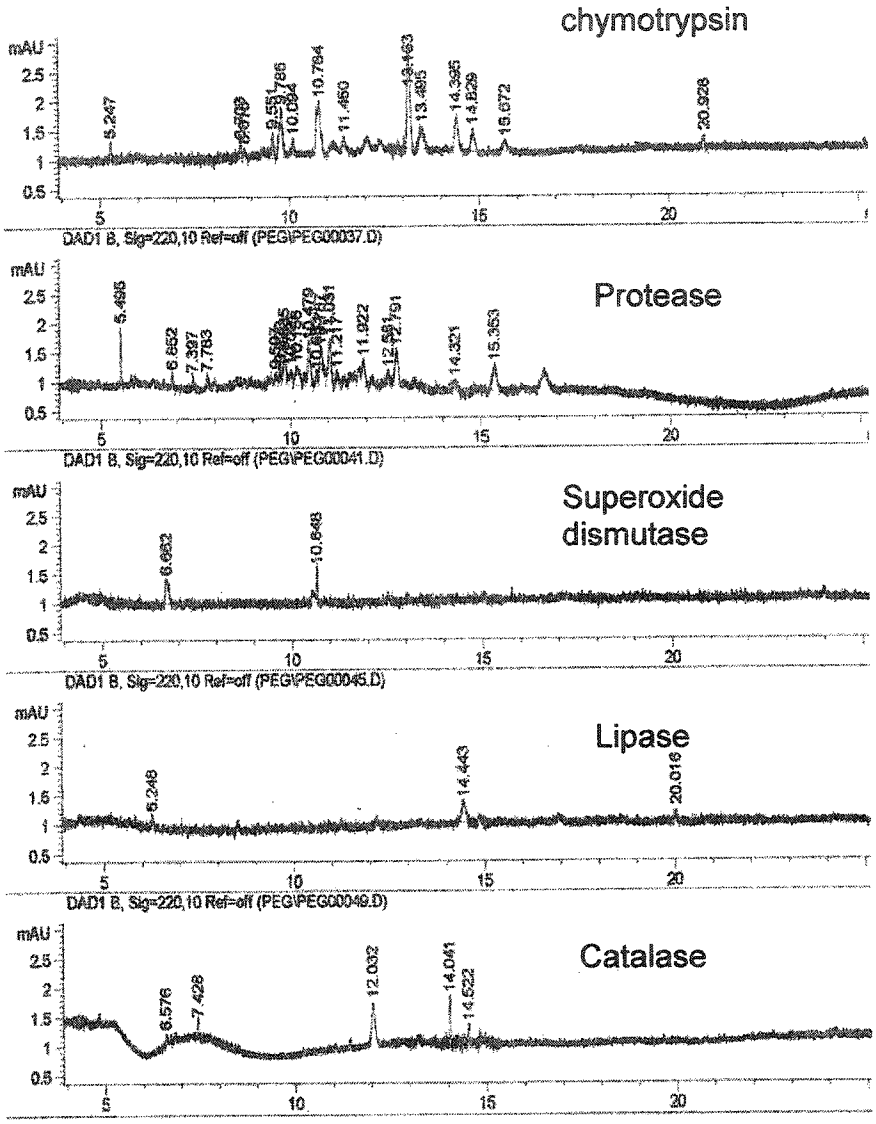


Figure 19: Electropherograms of individual PEGylated proteins using an etched capillary modified with cholesteral, 50 µm i.d., 58/49.5 cm, pH 2.14 (1:10) buffer, detection at 220nm, 30 kV, injection 5s@5", and sample prepared in pH 2.14 buffer (1:100).

successfully achieves the peaks corresponding to the different positional isomers of that protein.

3.5.3 Different voltages with cholesteryl capillary

As seen in Figure 20 the electropherogram of chymotrypsin at different voltages for a pH 2.14 (1:10) buffer solution shows the peaks decreasing with decreasing voltage. This maybe due to the fact that increasing the voltage, decreases the migration time. So the window just shows a 30 minute run, thus explaining the number of peaks be less as they may appear after 30 minutes. Thus in case of chymotrypsin at pH 2.14 (1:10) buffer the 30 kV is best voltage for a good number of peaks are observed. When a negative voltage was applied and compared to the positive voltage for all the PEGylated proteins there are no peaks seen in the -20 kV electropherogram. This can be seen in Figure 21a and 21b for chymotrypsin as well as protease respectively. This indicates that no negative species of PEGylated proteins are present thereby no peaks at negative voltage where the charge on electrodes are reversed. In Figure 22, catalase shows a larger number of peaks at 20 kV than at 30 kV which is seen in Figure 19.

3.5.4 Different pHs with cholesteryl capillary

Figure 23 shows the electropherograms of chymotrypsin at pH values of 2.14, 3.00, 4.41, 6.00, and 7.06, all at 1:10 buffer dilution. The number of peaks observed decreased with increasing pH. This is seen in all the PEGylated proteins studied except lipase where the greatest number of peaks seen is at pH 4.41 (1:10) and 30 kV. The

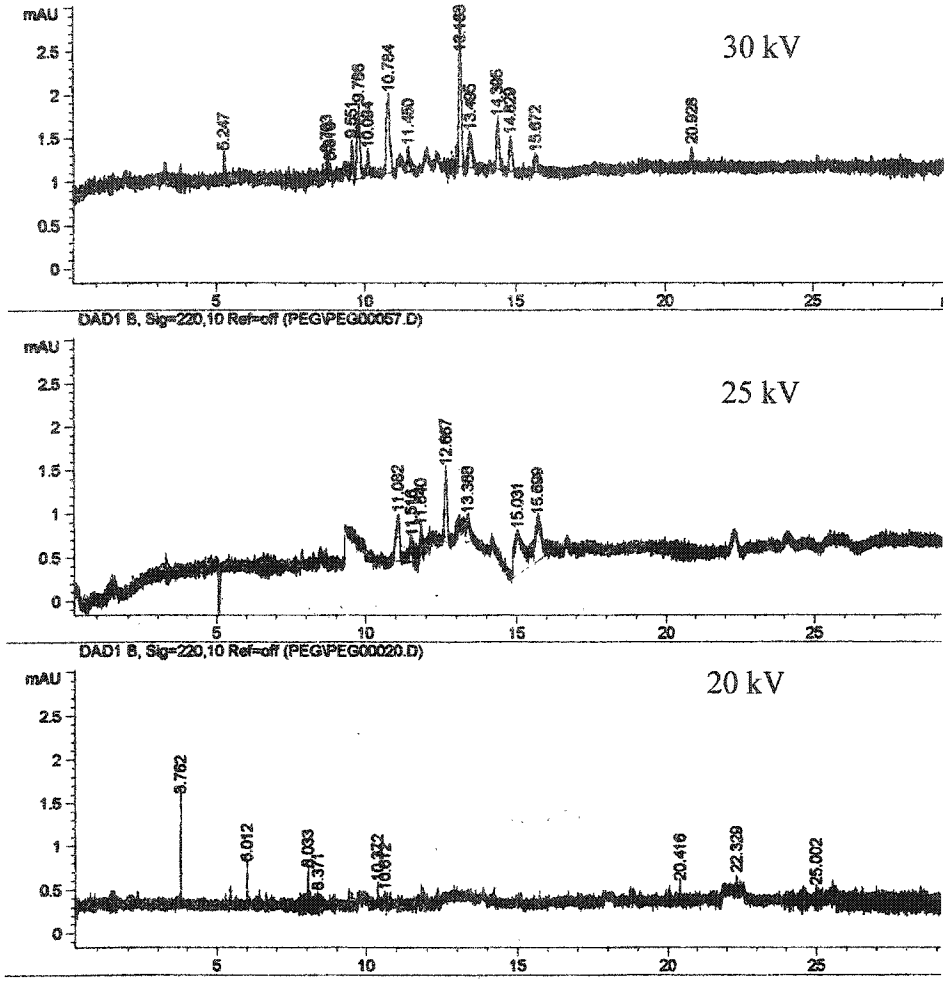


Figure 20: Electropherograms of PEGylated chymotrypsin using an etched capillary modified with cholesterat at voltages 30, 25 and 20 kV, 50 µm i.d., 58/49.5 cm, pH 2.14 (1:10) buffer, detection at 220nm, injection 5s@5", and sample prepared in pH 2.14 buffer (1:100).

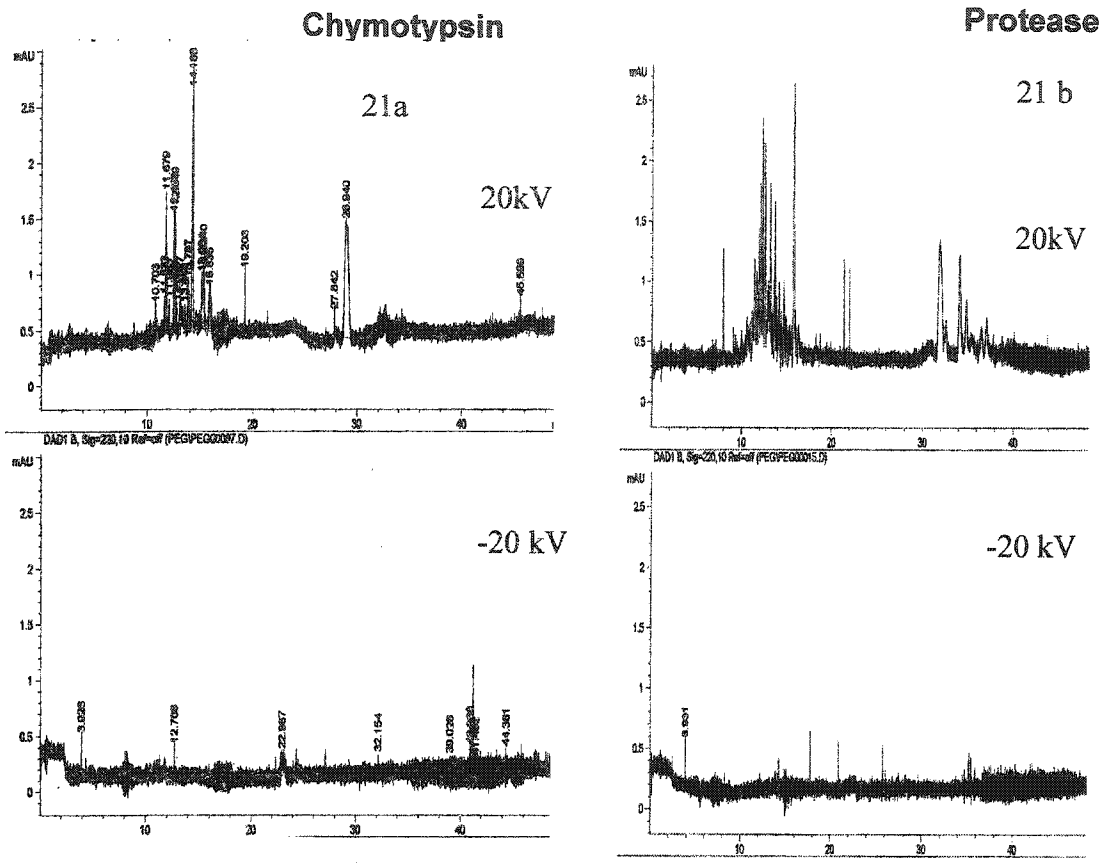


Figure 21: a: Electropherograms of PEGylated chymotrypsin using an etched capillary modified with cholesterol at voltages 20 kV and -20 kV; b: Electropherograms of PEGylated protease using an etched capillary modified with cholesteryl at voltages 20 kV and -20 kV, 50 μm i.d., 58/49.5 cm, pH 2.14 (1:10) buffer, detection at 220nm, injection 5s@5", and sample prepared in pH 2.14 buffer (1:100).

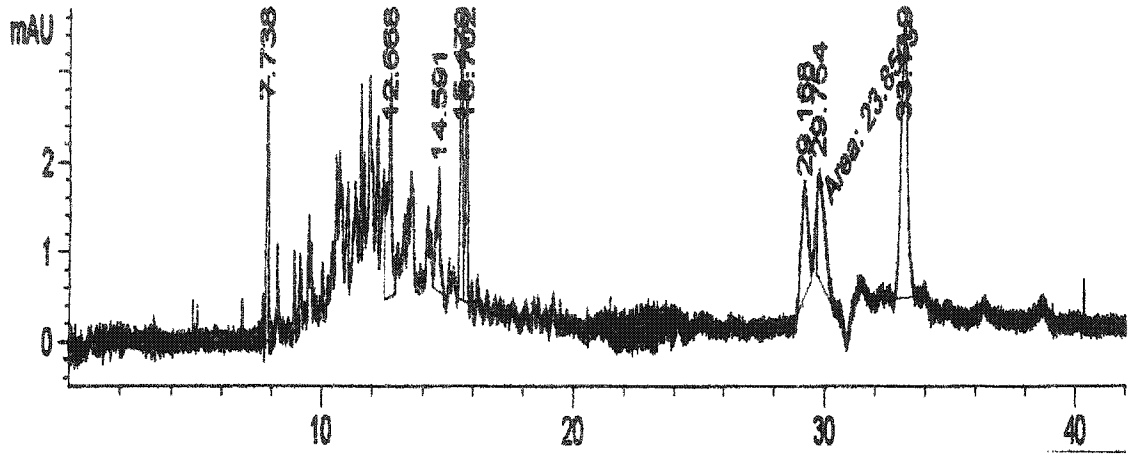


Figure 22: Electropherogram of PEGylated catalase using an etched capillary modified with cholesterol at 20 kV, 50 µm i.d., 58/49.5 cm, pH 2.14 (1:10) buffer, detection at 220nm, injection 5s@5", and sample prepared in pH 2.14 buffer (1:100).

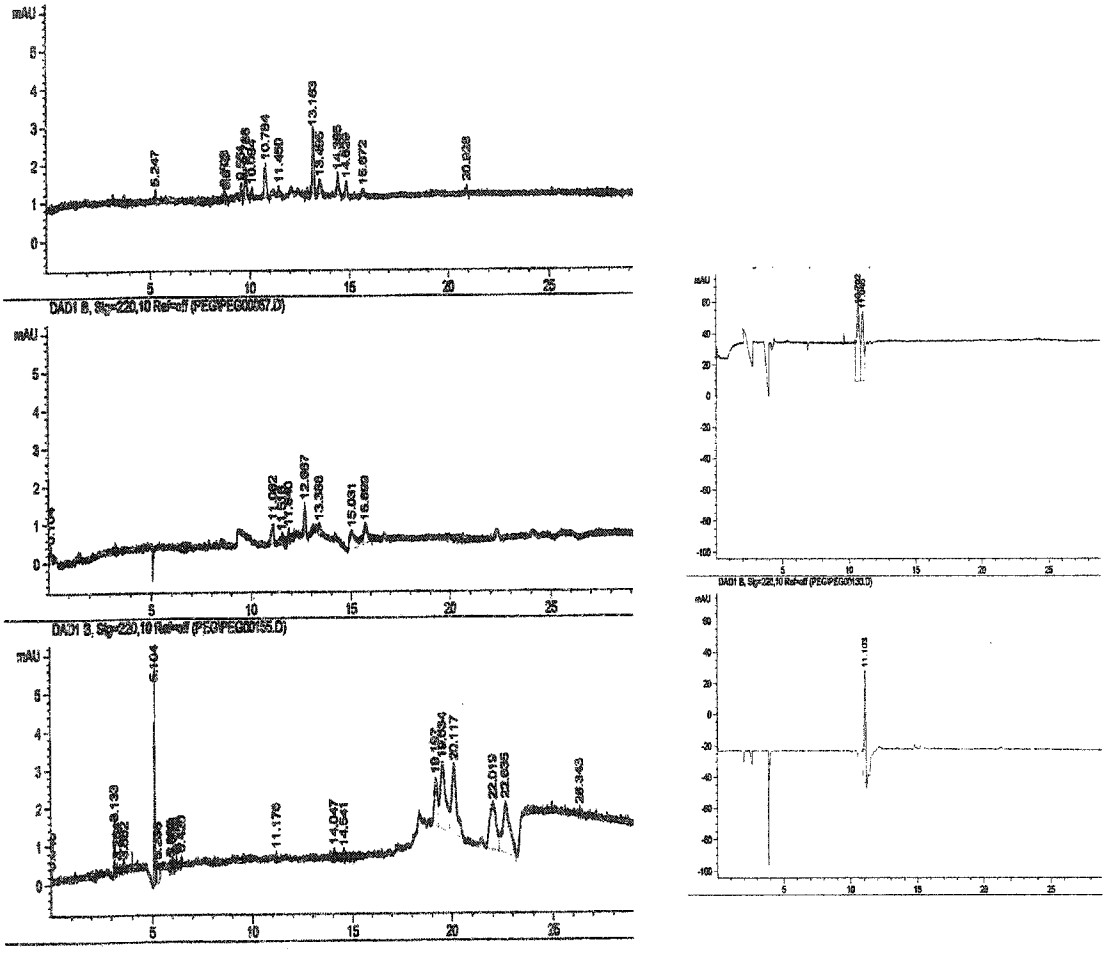


Figure 23: Electropherograms of PEGylated chymotrypsin using an etched capillary modified with cholesterol at 30 kV, 50 µm i.d., 58/49.5 cm, at pH 2.14, 3.00, 4.41, 6.00, and 7.06 all at 1:10 dilution, detection at 220nm, injection 5s@5", and sample prepared in 2.14 buffer (1:100)

observation that the number of peaks decreased with increasing pH is due to the fact that the proteins get deprotonated. So it's difficult for the proteins to go towards the cathode and therefore fewer peaks are observed.

3.5.5 Different percentage of methanol in the buffer with cholesteryl capillary

As in the case of Project 1, methanol was added to the buffer in different concentrations ranging from 10 to 90 %. In the case of pH 2.14 buffer (1:10) the electropherogram for chymotrypsin at 30 kV doesn't show any improved separation. Addition of methanol increases the migration time of the analyte species. So probably this explains why the protein peaks are not seen in the 30 minute window run. The electropherograms with different % MeOH in pH 2.14 (1:10) buffer of chymotrypsin and protease can be seen in Figure 24 and Figure 25 respectively

3.5.6 Etched capillary modified with C_{18}

As compared to cholesteryl modified capillaries, C_{18} with pH 2.14 (1:10) buffer and 30 kV does not look promising. As shown in Figure 26, there are hardly any peaks seen for the all the PEGylated proteins studied. It might be due to the fact that there are hardly any interactions of these proteins at this particular condition or maybe due to very strong interactions they are adsorbed on to the stationary phase.

3.5.7 Variable voltage studies for C_{18} capillary

Figures 27a and b and 28a and b shows the electropherograms of chymotrypsin

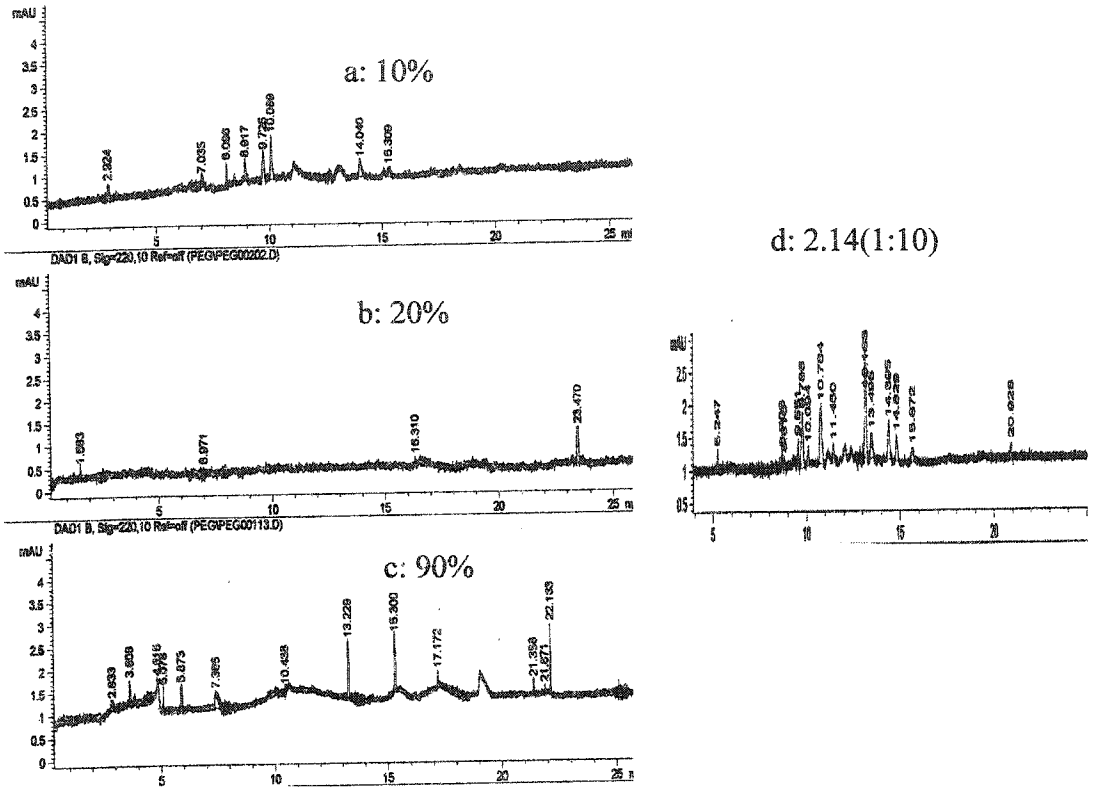


Figure 24: Electropherogram of PEGylated chymotrypsin using an etched capillary modified with cholesterol at 30 kV, 50 µm i.d., 58/49.5 cm, pH 2.14 (1:10) buffer modified with addition of a: 10%, b: 20%, c: 90% methanol, and d: with no Methanol, detection at 220nm, injection 5s@5", and sample prepared in 2.14 buffer (1:100).

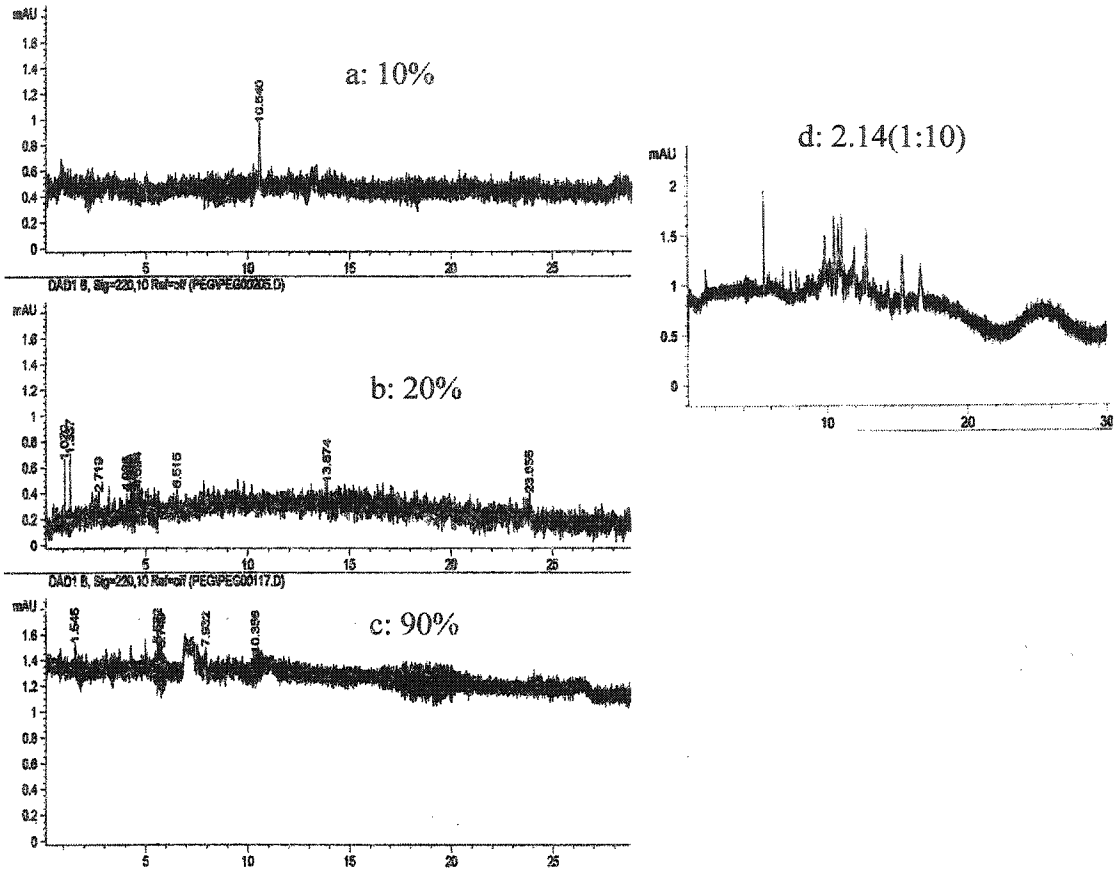


Figure 25: Electropherogram of PEGylated protease using an etched capillary modified with cholesterol at 30 kV, 50 µm i.d., 58/49.5 cm, pH 2.14 (1:10) buffer modified with addition of a: 10%, b: 20%, c: 90% Methanol, and d: with no Methanol, detection at 220nm, injection 5s@5", and sample prepared in 2.14 buffer (1:100).

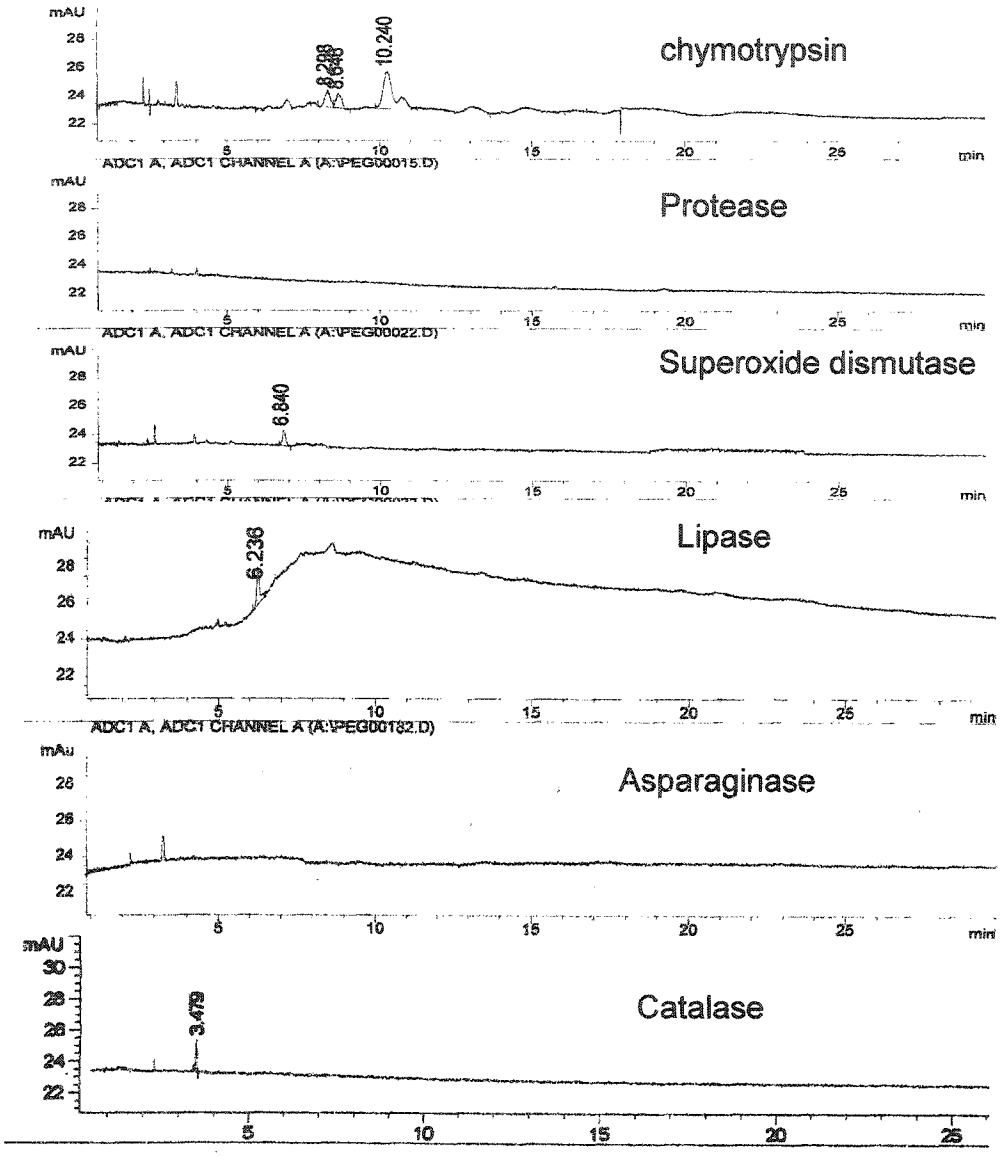


Figure 26: Electropherograms of individual PEGylated proteins using an etched capillary modified with C18, 50 µm i.d., 50.6/25.3 cm, pH 2.14 (1:10) buffer, detection at 220nm, 30 kV, injection 5s@5", and sample prepared in pH 2.14 buffer (1:100).

and protease, respectively, at different voltages for a pH 2.14 (1:10) buffer solution. As is seen in Figure 27, the peak shape and the number of peaks resolved at 25 kV looks the best for both the PEGylated proteins. When a negative voltage of -20 kV is applied and the electropherogram compared with a positive 20 kV it is observed that there are fewer peaks at -20 kV which is seen in Figure 28. And when the injection time is increased from 5 to 10 s, it is seen that the peaks also increase in height suggesting they are not noise. Also the peaks are reproducible as seen in the figure with different electropherograms as seen in Figure 28. The peaks seen at -20 kV must be due to the compounds having some degree of substitution where the E_p goes in the opposite direction and the overall charge is negative. Superoxide dismutase at 20 kV gives a larger number of peaks as shown in Figure 29 than at 30 kV.

3.5.8 Variable pH studies with C_{18} capillary

Figures 30 and 31 shows the electropherograms of PEGylated chymotrypsin and protease, respectively, at pH values 2.14, 3.00, 4.41, 6.00, and 7.06, all at 1:10 dilution. As in the case of the cholesterol modified column, the number of peaks observed keeps decreased with increasing pH due to the fact that the proteins get deprotonated with increasing pH and are less likely to move toward the cathode.

3.5.9 Different percentage of methanol added to the buffer

Methanol when added to the buffer in different concentrations ranging from 10 to

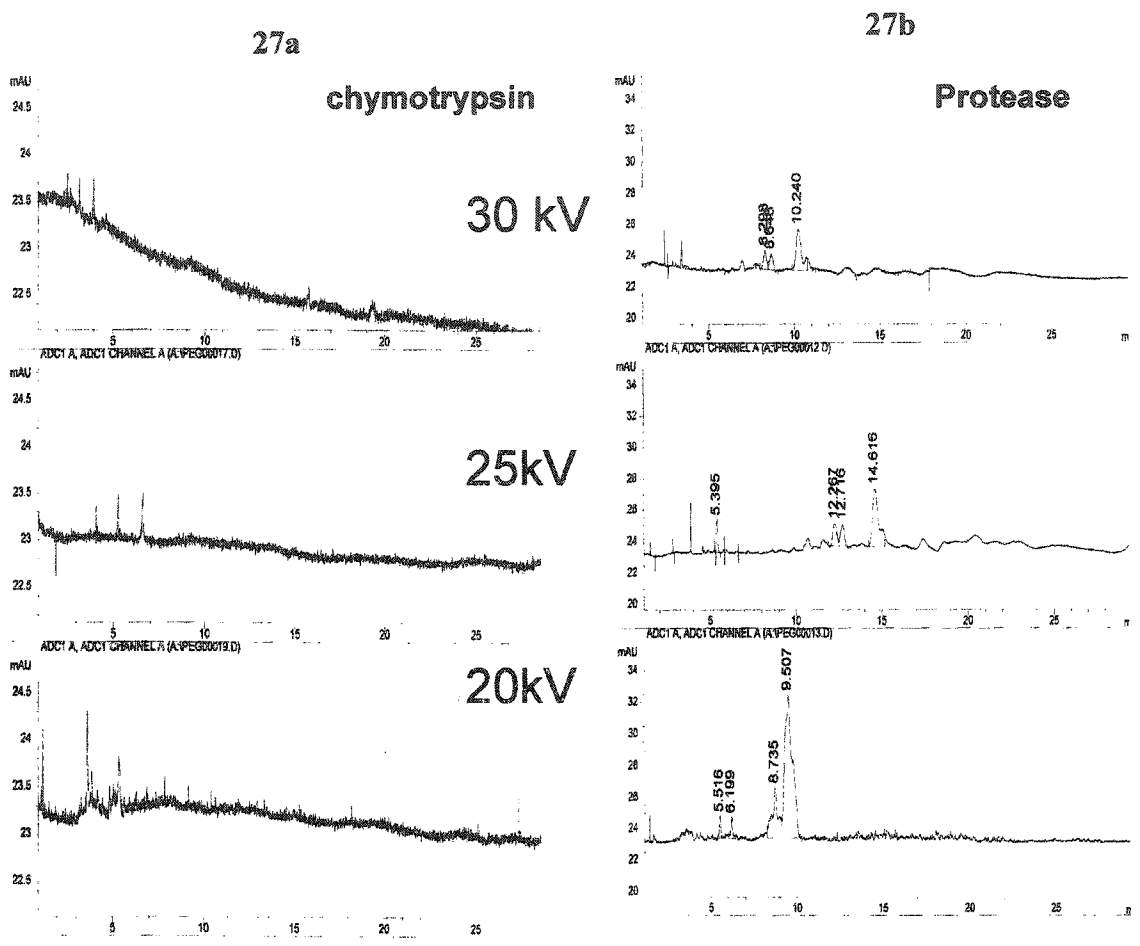


Figure 27: a: Electropherograms of PEGylated chymotrypsin using an etched capillary modified with C18 at voltages 30, 25 and 20 kV, 50 µm i.d.; b: Electropherograms of PEGylated protease at voltages 30, 25 and 20 kV, 50 µm i.d., 50.6/25.3cm, at pH 2.14 (1:10) buffer, detection at 220nm, injection 5s@5", and sample prepared in pH 2.14 buffer (1:100).

28a

28b

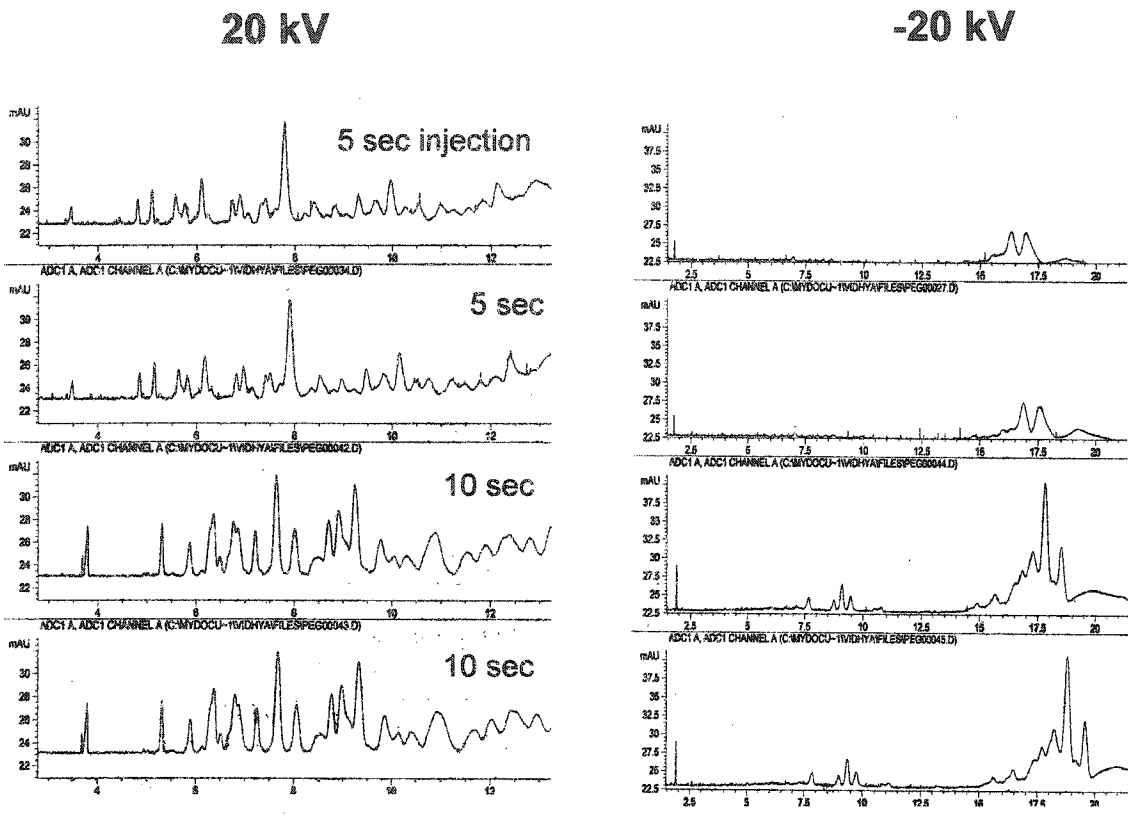


Figure 28: a: Electropherograms of PEGylated chymotrypsin using an etched capillary modified with C18 at voltages 20 kV, and 50 µm i.d. at 5 and 10 sec injection; b: At -20 kV.

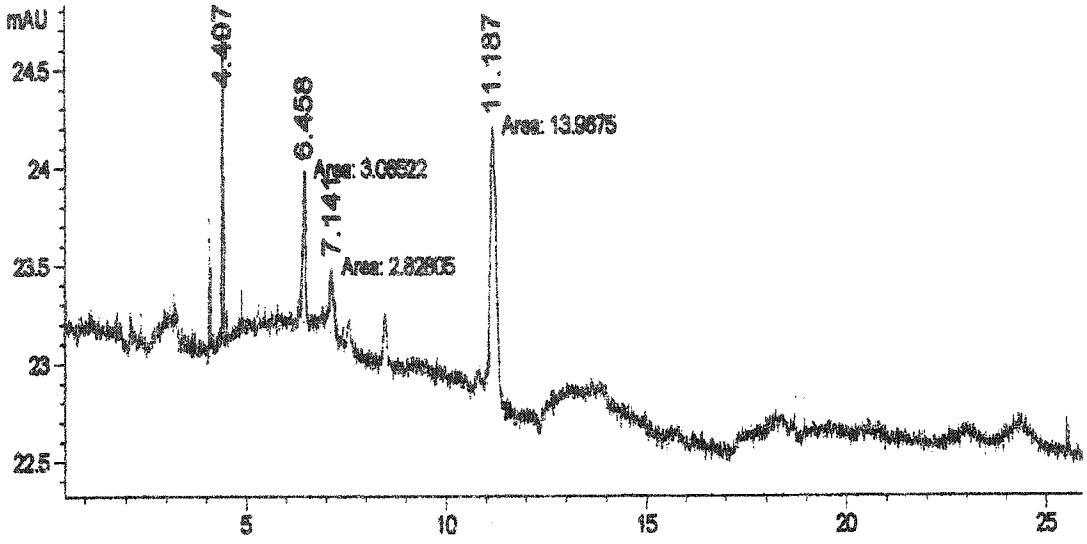


Figure 29: Electropherogram of PEGylated Superoxide dismutase using an etched capillary modified with C18 at voltage 20 kV, 50 µm i.d., 56.7/28.3cm, pH 2.14 (1:10) buffer, detection at 220nm, injection 5s@5", and sample prepared in pH 2.14 buffer (1:100).

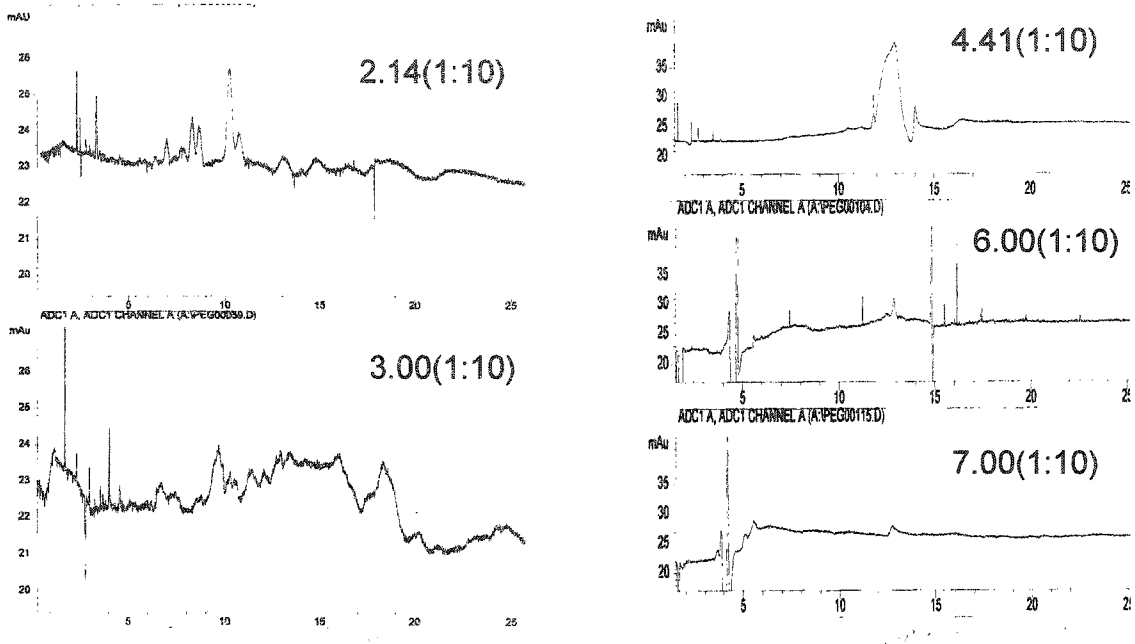


Figure 30: Electropherograms of PEGylated chymotrypsin using an etched capillary modified with C18 at 30 kV, 50 µm i.d., 50.6/25.3cm , in pH 2.14, 3.00, 4.41, 6.00, and 7.06 buffer all at 1:10 dilution, detection at 220nm, injection 5s@5", and sample prepared in pH 2.14 buffer (1:100).

90 % doesn't show any improved separation for any of the PEGylated proteins studied. As discussed, methanol when added increases the migration time of the analyte species. This leads to the disappearance of some peaks from the 30 minute run, as seen in all the electropherograms. Figure 32 and 33 shows an example for PEG-chymotrypsin and PEG-protease.

3.6 Conclusion

PEGylated proteins are becoming more common in drug industry because they remain in the body for a longer time thereby providing a constant drug level. It has been shown that OTCEC could be an important tool in the analysis of PEGylated proteins. The analysis of PEGylated proteins is growing due to its expanding market. Since not much was known about the distribution of species in PEGylated proteins, this research can be used as a stepping-stone for further investigation.

Due to different points of attachments of the PEG (different amine sites) there can be a number of positional isomers, which can have an influence on the activity of the PEGylated protein. The multiplicity of potential sites indicates reaction stoichiometries must be carefully controlled. It is therefore necessary to show the products are reproducible. From this study it is demonstrated that all the peaks in the sample studied are reproducible. In the results it is seen that cholestrol and C18 both show multiple peaks in a pH 2.14 buffer. For some proteins the optimum conditions have not been found yet but for others the conditions could be close to its optimum values. Increasing the pH decreases the number of peaks seen. Addition of MeOH doesn't show improved

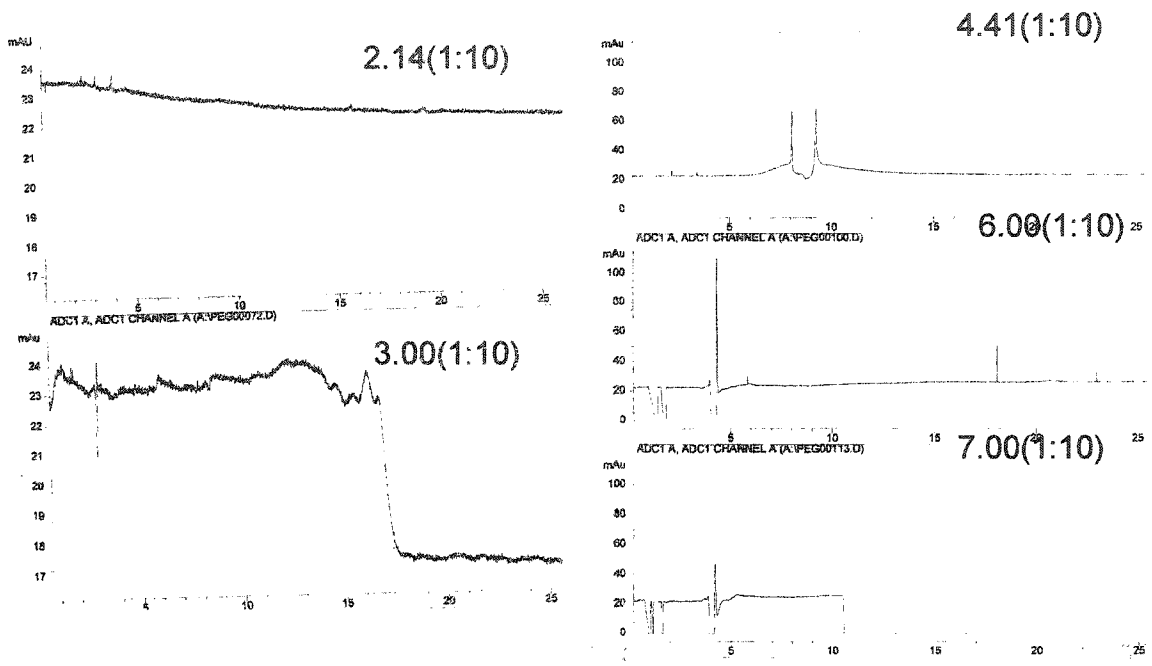


Figure 31: Electropherograms of PEGylated protease using an etched capillary modified with C18 at 30 kV, 50 µm i.d., 50.6/25.3cm , in pH 2.14, 3.00, 4.41, 6.00, and 7.06 buffer all at 1:10 dilution, detection at 220nm, injection 5s@5", sample prepared in pH 2.14 buffer (1:100).

32a 32b

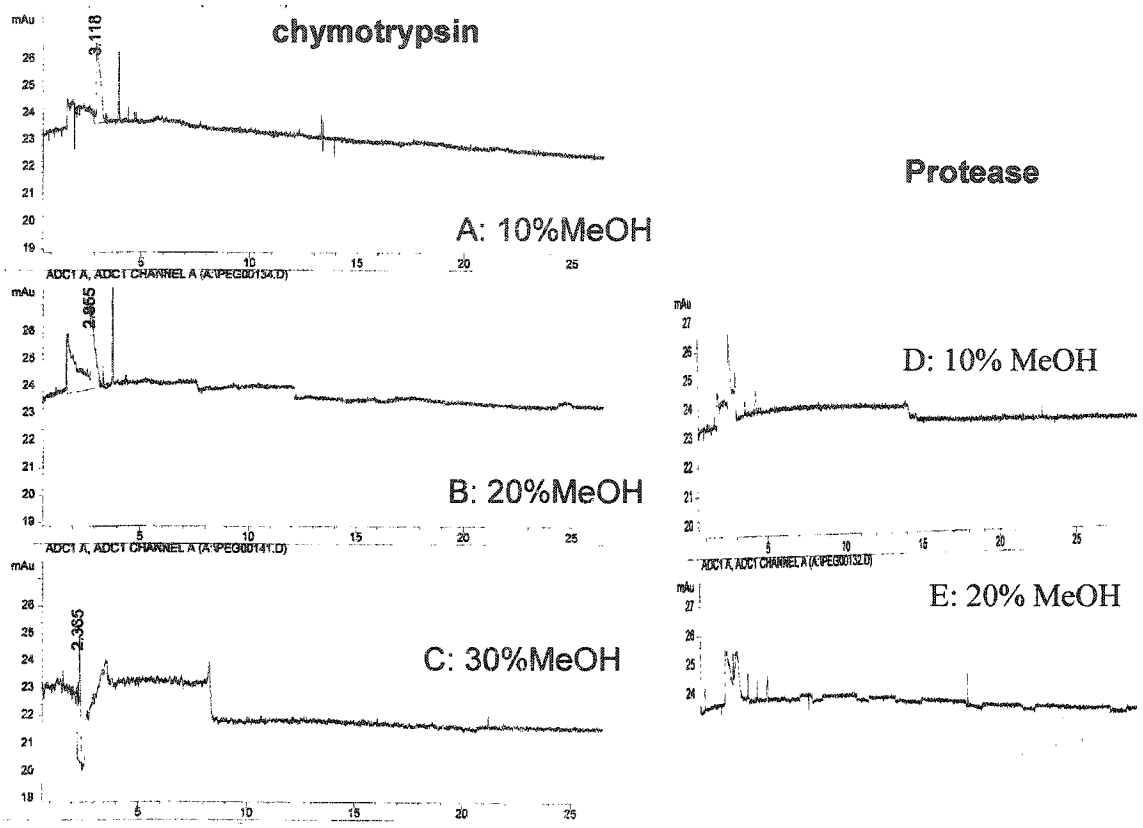


Figure 32: Electropherogram of PEGylated Chymotrypsin (32a) and protease (32b), using an etched capillary modified with cholesterol at 30 kV, 50 µm i.d., 58/49.5 cm, pH 2.14 (1:10) buffer modified with addition of A: 10%, B: 20%, C: 30% methanol for chymotrypsin, and D: 10%, and E: 20% methanol for protease, detection at 220 nm, and injection 5s@5". Sample prepared in 2.14 buffer (1:100).

separation of peaks and actually shows loss of peaks during the thirty-minute run. The table below the best conditions found are listed for each PEGylated protein studied.

Table 4: List if optimum conditions for each PEGylated protein during the time of study

Proteins	**# of peaks**	**Capillary**	**kV andpH**
chymotrypsin	15	cholesterol	30 kV and 2.14(1:10)
protease	25	C18	20 kV 2.14(1:10)
Catalase	25 27	C18 cholesterol	20 kV 2.14(1:10)
Lipase	7	cholesterol	30 kV 4.41(1:10)
Superoxide dismutase	8	C18	20 kV 2.14(1:10)
Asparaginase	9 (10s injection)	cholesterol	30 kV 2.14

3.7 Future studies

Future work will involve finding the correlation between number of peaks observed to the number of positional isomers. Basically the number of positional isomers depends on the number of lysines present, as the PEG gets attached to the proteins mainly at this amino acid. A well-characterized standard will be required to identify positions of the different PEG protein peaks. The position of the PEG's attached to the protein can probably determined by using mass spectrometry in conjunction with the OTCEC and the sequence that we have. This will provide more insight on the positional isomers.

Analysis of PEGylated proteins using capillaries with various other moieties attached to the inner walls can be tested. Different i.d.'s of capillaries can be compared. The transition from first-generation chemistries to second-generation chemistries is taking place rapidly. Analysis of second generation PEGylated proteins opens a new avenue as they are proving to be superior to the first generation. Second generation PEGylation reagents avoid weak linkages and also can be purified to remove diol contaminants, which is seen in first generation proteins and as a consequence, high-MW PEG's can be used. Hence the next step will be to look at these second generation PEGylated proteins and see if the reproducible results achieved with first generation can be achieved with the second generation PEGylated proteins.

References

1. Jorgenson, J. W.; Lukacs, K. D. *Anal. Chem.*, **1981**, 53, 1298.

2. Blanc, T.; Schaufelberger, D. E; Guzman, N. A. In. *Capillary electrophoresis*, Ewing G.W (Ed.), Analytical instrumentation handbook, 2nd ed. New York, Marcel Dekker, **1997**, 1351.

3. Knox, J. H.; Boughtflower, R. *Trends in Anal. Chem.*, **2000**, 19, 643.

4. Natural Toxins Research Center at Texas A&M University – Kingsville Home Page. http://ntri.tamuk.edu/ce/ce.html (accessed Feb 2003).

5. Skoog, D.A; Holler, J. F; West D. M. *Analytical Chemistry*, Saunders publication, 7th Ed, **1991**, 551.

6. Schwartz, H.E; Palmieri, R.H.; Nolan, J. A; Brown, R. *Introduction to Capillary Electrophoresis*, Beckman Instruments Inc, **1993**, 12.

7. Matyska, M. T.; Pesek, J. J.; Boysen, I.; Hearn, T. W. *Electrophoresis*, **2001**, 22, 2620.

8. Pesek, J. J.; Matyska, M. T.; Cho, S. *J. Chromatogr.* A, **1999**, 845, 237.

9. Pesek, J. J.; Matyska, M. T. *J. Chromatogr. A*, **1996**, 736, 255.

10. Matyska, M. T.; *Chem. Anal.* (Warsaw), **1998**, 43, 637.

11. Pesek, J. J.; Matyska, M. T.; Sandoval, J. E.; Williamsen, E. *J. Liq. Chromatogr.*, **1996**, 19, 2843.

12. Aaron, S. D. *J. Asthma.*, **2001**, 38, 521.

13. Simms, P. J.; Towne, R. W.; Gross C. S. *J. Pharm. Biomed. Anal.*, **1998**, 17, 841.

14. Pesek, J. J; Matyska, M. T.; Tran, H. *J. Sep. Sci.*, **2001**, 24, 729.

15. Mehvar, R. *J. Pharm. Pharmaceut. Sci.*, **2000**, 3(1), 125.

16. Abuchowski, A.; Van, E. T.; Palczuk, N. C. *J. Biol. Chem.*, **1977**, 252, 3578.

17. Kozlowski, A.; Charles, S. A.; Harris, J. M. *Bio. Drugs*, **2001**, 15(7), 419.

18. Yamoaka, T.; Tabata, Y.; Ikada, Y. *J. Pharm Sci*, **1994**, 83, 601.

19. Roberts, M. J.; Bentley, M. D.; Harris J. M. *Advanced Drug Delivery Reviews*, **2002**, 54, 459.

20. National Center For Biological Information Home Page. http://www.ncbi.nlm.nih.gov/ (accessed March 2003).

21. Yoshinaga, Y.; Harris, J. M. *J. Bioact. Comp. Poly.*, **1989**, 4, 17.

22. Harris, J. M. *J. Macromol. Sci. Rev. Macromol. Chem. Phy.*, **1985**, C25, 325.

23. American Polymer Standards Corporation Home Page. http://www.ampolymer.com/question.htm (accessed January 2003).