2008

Capillary electrochromatography for analysis of proteins and metalloproteinases

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CAPILLARY ELECTROCHROMATOGRAPHY FOR ANALYSIS OF
PROTEINS AND METALLOPROTEINASES

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

Vasudha Salgotra

August 2008

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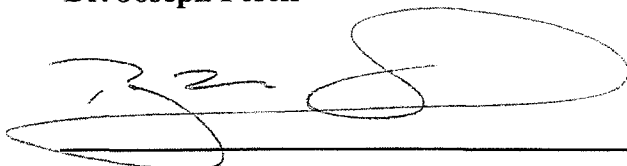789 E. Eisenhower Parkway
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ABSTRACT

CAPILLARY ELECTROCHROMATOGRAPHY FOR ANALYSIS OF
PROTEINS AND METALLOPROTEINASES

by Vasudha Salgotra

Capillary electrochromatography (CEC) is a hybrid technique of high

performance liquid chromatography (HPLC) and capillary electrophoresis (CE),

in which stationary phases used in HPLC are chemically bonded to the inner wall

of the capillary. In this research, open tubular capillaries have been prepared by

etching the inner wall of polyimide coated capillaries. The etching will increase

the overall capacity of the capillary. The stationary phases, C5 and C18, have

been chemically bonded to the etched surface of the capillary utilizing a

silanization/hydrosilation procedure. These capillaries were utilized in qualitative

analysis of metalloproteins. In order to find out the optimal conditions for the

analysis of these proteins, different pH buffers were tested. For comparing and

testing the performance of these capillaries, the proteins were also analyzed on a

bare capillary. It was determined that C5 and C18 capillaries gave highly

efficient and better reproducible results as compared to a bare capillary.

ACKNOWLEDGEMENTS

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1. INTRODUCTION

1.1 Capillary Electrophoresis (CE)

1.1.1 Basics

Capillary electrophoresis (CE) is a separation technique which utilizes narrow bore fused silica capillary, filled with an electrolyte for the separation of compounds of interest. Some common compounds which can be separated using CE are amino acids, polypeptides and proteins. The CE system set up consists of buffer reservoirs filled with an electrolyte solution. The positive anode and negative cathode platinum electrodes are immersed in these buffer vials, which are connected to a high voltage power supply. The fused silica capillary is also immersed in these buffer vials at both the ends of the capillary. The detection window is created on part of the capillary. Figure 1 shows the CE system set up. The usual voltage of 5-30 kV is used for CE experiments which produce a current of 0-100 µA [1].

The separation of species in CE is based on simple theory of electrophoresis, in which charged species get separated depending on their size and charge which together determine their electrophoretic migration velocity [1]. The electrophoretic migration velocity (V_{em}) is calculated as:

$$V_{em} = \mu_{em}. E$$

where μ_{em} is the electrophoretic mobility of the analyte species and E is the electric field applied. The electrophoretic mobility (μ_{em}) is calculated as:

$$\mu_{em} = \frac{q}{6\,\pi\acute{\eta}\,r}$$

where q is the charge on the ions, ή is the viscosity of the medium and r is the radius of

the ions. In the above equation electric field 'E' is defined as:

$$E = \frac{V}{L}$$

where V is the voltage in kV and L is the length of the capillary column.
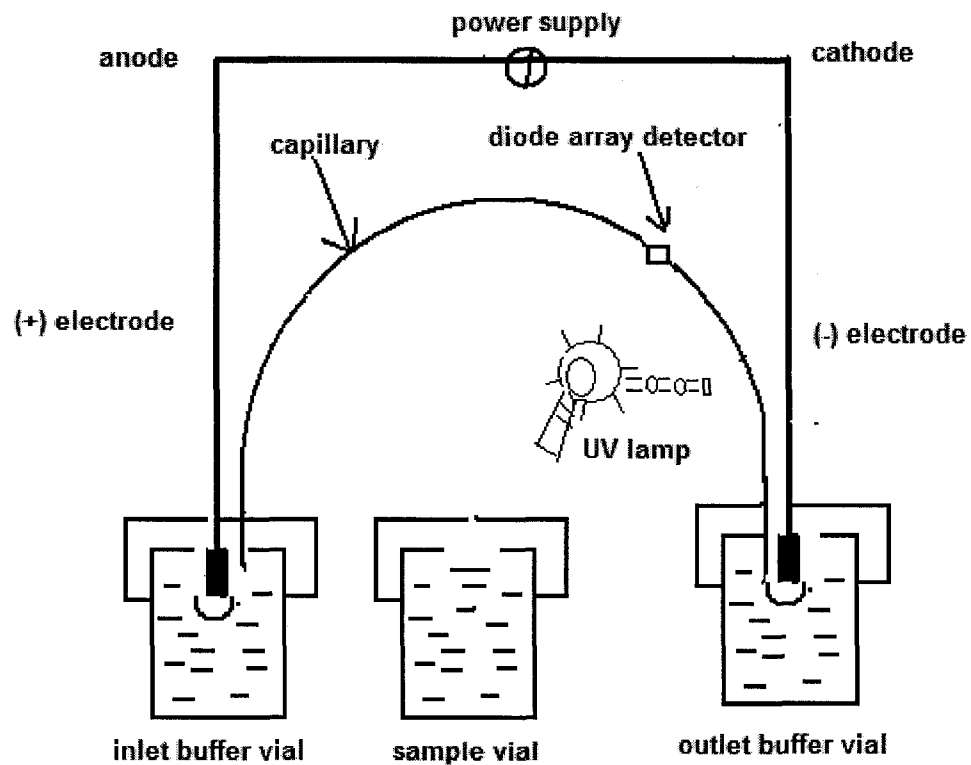
Figure 1: Capillary Electrophoresis system set up.

The buffer solution filled in reservoirs creates an electroosmotic flow (EOF)

when voltage is applied across the vials. In general, the inner surface of the fused silica

capillaries consists of silanol groups, which are weakly acidic and ionize above pH 3.0.

Ionization of these silanols creates charged sites inside the capillaries. These ionized

silanols releases hydrated cations and form an electrical double layer as seen in Figure 2.

This layer consists of a fixed or Stern layer near the capillary surface, and a mobile outer

layer, also called the Gouy-Chapman layer. When voltage is applied to the capillary, the

hydrated outer layer moves towards the cathode and forms the net flow of bulk liquid

inside the capillary [1].

The velocity of electroosmotic flow (V_{eof}) in CE is given by the following

equation:

$$V_{eof} = \mu_{eof}.\ E$$

μ_{eof} is the electroosmotic mobility and E is the electric field applied. In this equation, μ_{eof}

is defined by the next equation:

$$\mu_{eof} = \frac{\varepsilon\ r\ \zeta}{\acute{\eta}}$$

where ε is dielectric constant, r is radii of ion, ζ is zeta potential of the capillary wall and

$\acute{\eta}$ is viscosity of the medium.

The overall velocity of analyte (V) in an electroosmotic flow is a combination of

both electrophoretic migration velocity (V_{em}) and electroosmotic flow velocity (V_{eof}).

This equation is written as:

$$V = V_{em} + V_{eof}$$

1.1.2 Injection systems in CE

There are three types of injection systems in CE: hydrodynamic, siphoning, and

electrokinetic systems as shown in Figure 3. In a hydrodynamic injection system, pressure is applied to the sample vial and this forces the sample into the capillary. This type of injection system gives generally reproducible results. In a siphoning injection system, the level of the sample and buffer vials are changed and this introduces the sample into the capillary. In an electrokinetic injection system, voltage is applied to the sample and the buffer vial and this forces the sample into the capillary [1].
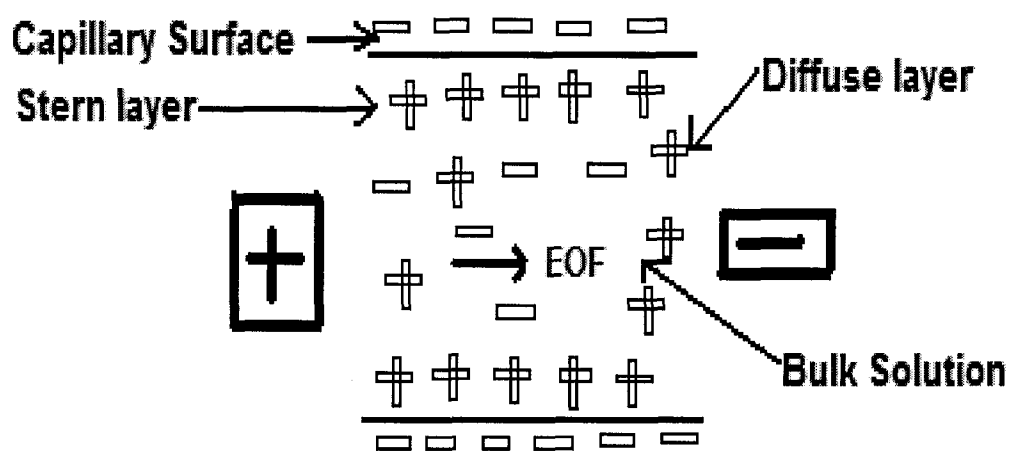
Figure 2: Electrical double layer in CE.

1.2 High Performance Liquid Chromatography (HPLC)

1.2.1 Basics

HPLC is a well known separation technique used for the analysis of many biological, industrial, and pharmaceutical compounds. The basic system set up in HPLC consists of mobile phase reservoirs, a mixer, pumps, a column and a detector. In HPLC,

two phases are involved in the separation of compounds: a mobile and a stationary phase. The stationary phase can be classified as normal or reverse phase, depending upon the type of analyte being analyzed. The partitioning of components between the mobile and stationary phases is the main principle of separation of compounds in HPLC [1].
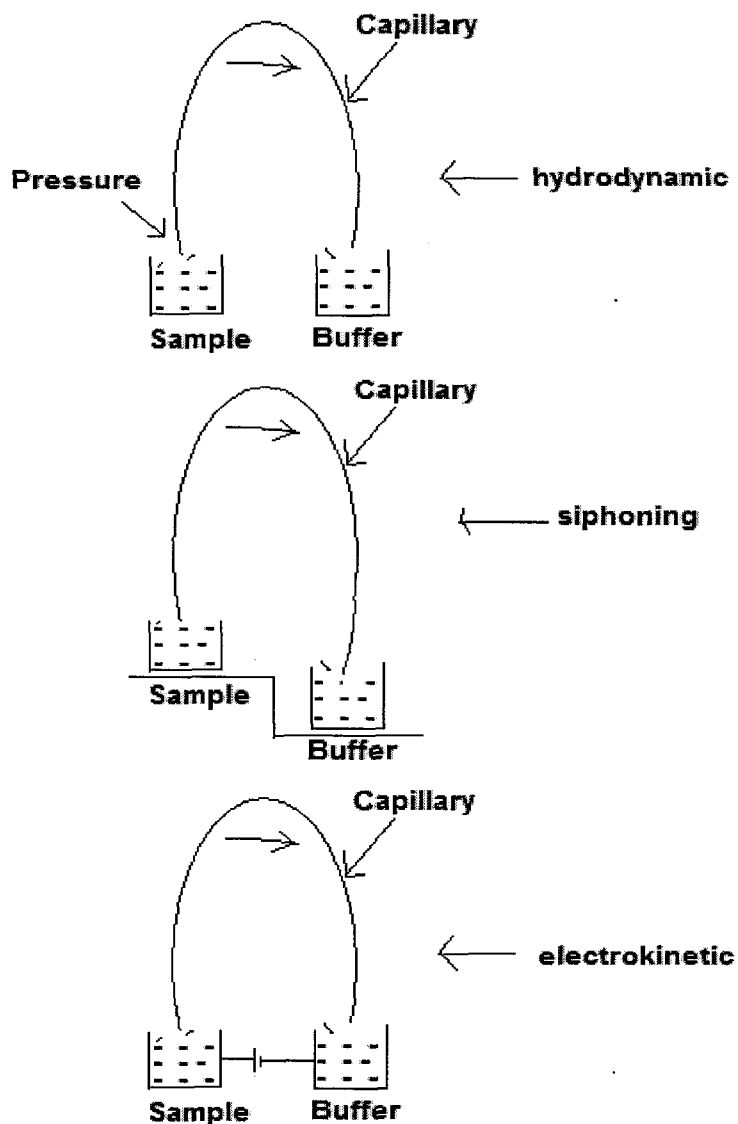
Figure 3: Three types of injection systems in CE.

1.3 Advantages of CE over HPLC

CE exhibits much higher efficiency in comparison to HPLC. This is due to the electrically driven flow in CE, which creates a flat flow profile in CE and reduces mass transfer effects. These reduced mass transfer effects are responsible for giving narrow peaks and increased efficiency in CE. The pressure driven system in HPLC creates a laminar flow profile, which usually increases mass transfer effects. This gives broad peaks in HPLC and lowers its efficiency. Figure 4 shows the flow profile for HPLC and CE. CE does not have any back pressure problem, which is usually a problem in HPLC. CE has greater resolution as compared to HPLC. The consumption of solvents in HPLC is greater as compared to CE. HPLC requires microliters of sample during injection and CE requires nanoliters of sample [2].
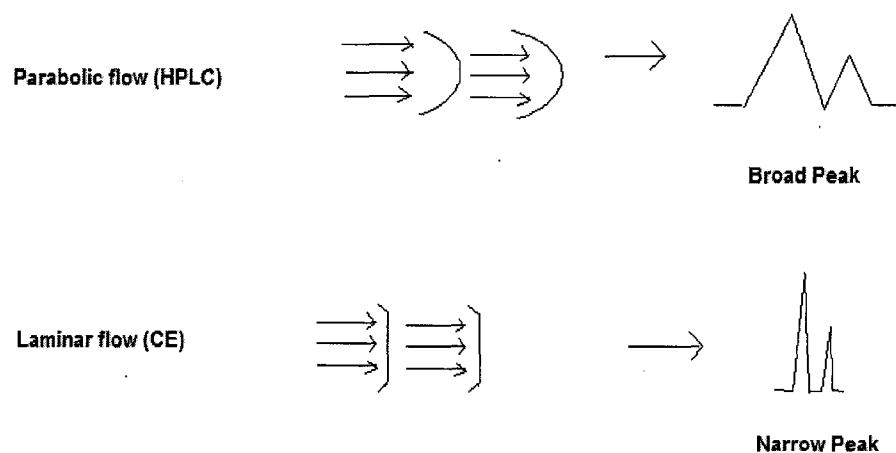
Figure 4: Flow profile for HPLC and CE.

1.4 Capillary Electrochromatography (CEC)

1.4.1 Basics

Capillary electrochromatography (CEC) is a new separation technique which combines both capillary electrophoresis (CE) and high performance liquid chromatography (HPLC). It utilizes narrow bore fused silica capillaries similar to those used in CE, and stationary phases similar to ones used in HPLC. In CEC, the inner surface of the fused silica capillary is modified with stationary phases like HPLC. Because of this hybrid combination, CEC offers both chromatographic and electrophoretic effects for the separation of compounds. Thus, it is used to separate both charged as well as neutral species [3].

The stationary phases in CEC can be attached in three different formats: packed, monolith and open tubular (OT). Figures 5a-c show three different formats of stationary phases in CEC. In the packed format, stationary phase particles of small diameter are introduced into the capillary, and each stationary phase particle forms an electrical double layer around it. In this format, frits are required to be made inside the capillary to hold the packed particles. In the monolith format, stationary phase solution is introduced into the capillary and gets polymerized inside the capillary. This forms a solid rod like stationary phase column in the capillary. In the open tubular format, a stationary phase is chemically bonded or chemically attached to the inner wall of capillary. From these three formats, the open tubular format is simple to make, and has fewer problems of capillary blockage, which is usually seen with the packed and monolith formats [3].

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Polyimide coating capillary wall   Open Segment

Packed bed

Detection Window

Frits

(a)

Polyimide coating capillary wall

Open tubular coating   Detection window

(b)

Polyimide coating capillary wall   open segment

monolith   detection window

(c)
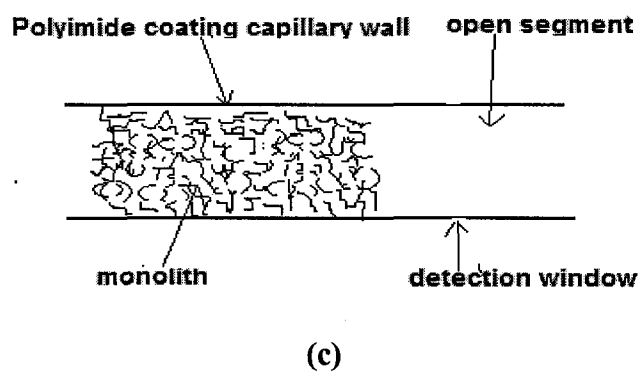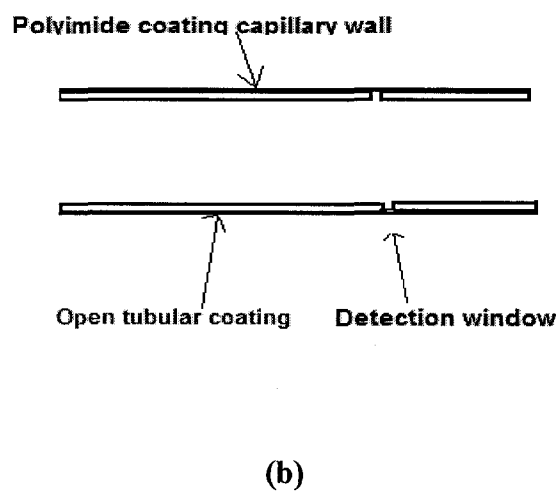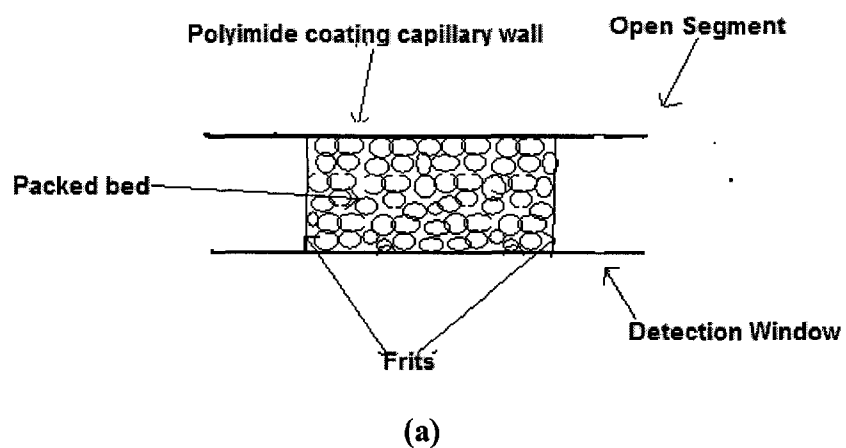
Figure 5: Three different formats of stationary phases in CEC: (a) packed format, (b) open tubular format, and (c) monolith format.

1.4.2 Two common methods for stationary phase bonding

i) Organosilanization method

The organosilanization reaction is the commercial method to attach a stationary phase into the capillary. In this method, a hydroxylated silica surface is reacted with organic moiety to form a siloxane linkage. The main problem with this method is that these capillaries are susceptible to hydrolysis at pH extremes [4]. The general reaction for organosilanization is given as:

$$\overset{\displaystyle |}{\underset{\displaystyle |}{\overset{O}{\underset{O}{Si}}}}\text{—OH} + Cl_3SiCH_2CH_2R \xrightarrow[\text{Toluene}]{} \overset{\displaystyle |}{\underset{\displaystyle |}{\overset{O}{\underset{O}{Si}}}}\text{—O}\overset{OY}{\underset{OY}{Si}}CH_2CH_2R + 3HCl$$

$$\overset{\displaystyle |}{\underset{\displaystyle |}{\overset{O}{\underset{O}{Si}}}}\text{—OH} + Cl\overset{CH_3}{\underset{CH_3}{Si}}CH_2CH_2R \xrightarrow[\text{Toluene}]{} \overset{\displaystyle |}{\underset{\displaystyle |}{\overset{O}{\underset{O}{Si}}}}\text{—O}\overset{CH_3}{\underset{CH_3}{Si}}CH_2CH_2R + HCl$$

Y = -Si-CH$_2$CH$_2$R
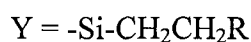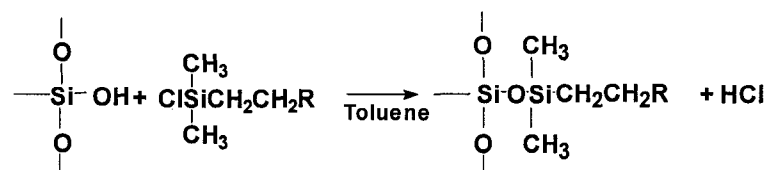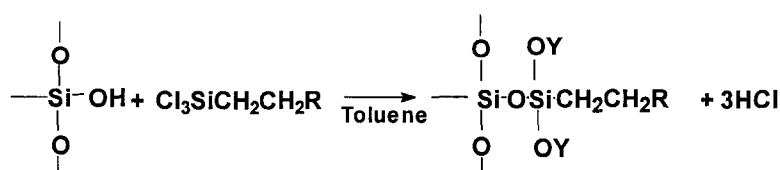
ii) Silanization/Hydrosilation method

This approach is commonly used for the preparation of stationary phase inside capillaries. In the silanization step, silanols are converted to hydride which ultimately lowers the EOF in the capillary. In the hydrosilation step, the hydride capillary is reacted with an organic moiety to attach the stationary phase. This step leads to a direct Si-C bond and enhances capillary stability. The general reaction for silanization and hydrosilation is given as:

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Silanization:

$$\equiv Si\text{-}OH + (OEt)_3Si\text{-}H \xrightarrow{\;\;H^+\;\;} \equiv Si\text{-}O\text{-}\underset{\underset{OY}{|}}{\overset{\overset{OY}{|}}{Si}}\text{-}H + nEtOH$$

Hydrosilation:

$$\equiv Si\text{-}H + R\text{-}CH{=}CH_2 \xrightarrow{\;\;cat.\;\;} \equiv Si\text{-}CH_2\text{-}CH_2\text{-}R$$
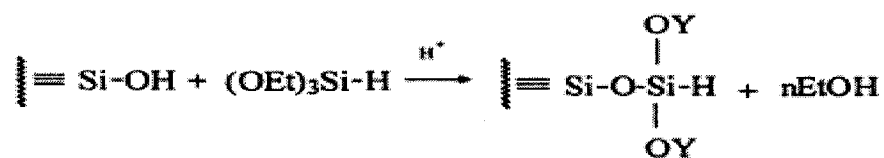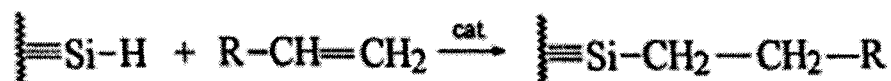
Typically used catalyst is hexachloroplatinic acid

2. THE GOAL OF THE RESEARCH

Open tubular capillary electrochromatography (OTCEC) was used in this study for the analysis of some complex proteins and metalloproteinases. In this research, polyimide coated fused silica capillaries have been etched before attachment of a stationary phase to the capillaries. The etching has been done in order to increase the surface area inside the capillaries, so that more stationary phase can be attached to the inner wall. The silanization /hydrosilation method was used to chemically bond stationary phase into the etched capillary. C18 and C5 capillaries have been prepared for study in this research. A high performance capillary electrophoresis (HPCE) instrument with diode array detector (DAD) was utilized for analysis of these proteins. Different pH buffers and wavelengths have been tested in order to find the optimum conditions for the analysis of these compounds. For further comparison and to test the performance of the C5 and C18 capillaries, these compounds were also analyzed on a bare capillary.

3. EXPERIMENTAL

3.1 Capillaries and CE instrument

For this research, a fused silica polyimide coated capillaries from Polymicro Technology (Phoenix, AZ) were used. These were bare capillaries with internal diameter of 50 μm and outer diameter of 325 μm. These bare capillaries were modified chemically and used for analysis of proteins and metalloproteinases. The initial length of these capillaries was 90 cm but it changed during the course of modification of capillaries. After modification, the capillaries used during analysis have a length ranging from 40-80cm.

The CE instrument used in this research was 3D Hewlett Packard (HP) with serial number 3423G00376 (Germany). This was an automated instrument equipped with a circular tray consisting of 48 vials positions for holding reagents and samples, a capillary cassette for holding a capillary, a detection cell with diode array detector (DAD) and, a HP chemstation software for controlling all the experimental parameters. The experimental parameters like voltage, pressure, temperature, run time, injection time and, detection wavelength can be controlled by this chemstation software. The diode array detector is a multi-wavelength detector which can measure compounds at different wavelengths simultaneously.

The list of analytical chemicals used in this research work are shown in Table 1 and the list of proteins and metalloproteinases analyzed in this research work are shown in Table 2.

Table 1: List of chemicals used in this research work

Name	Company Names	CAS Registry Number
Ammonium hydrogen difluoride	Aldrich Chemicals	1341-49-7
Sodium hydroxide	Fisher Scientific	1370-73-2
Boric Acid	Bakers Chemical Company	10043-35-3
Triethoxysilane (TES)	Aldrich Chemicals	998-30-1
Dioxane	GFS Chemicals	1764-74-4
1- Octadecene	GFS Chemicals	112-88-9
Pentyne	GFS Chemicals	627-19-0
Hexachloroplatinic acid	Sigma –Aldrich Chemicals	16941-12-1
Toulene	Fisher Scientific	108-88-33
Phosphoric acid	Fischer Scientific	7664-38-2
Tris (hydroxymethyl) aminomethane	Sigma	77-86-1
Citric acid	Bakers Chemical Company	1-0-110
Beta-alanine	Sigma	107-95-9
Lactic acid	J.T. Baker Chemicals	50-21-5
Acetic acid	EM Science	64-19-7
Gama-amino butyric acid	Sigma	56-12-2
(N-morpholino) ethane sulfonic acid	Sigma	4432-31-9
Histidine	Sigma	4998-57-6
2-(N-morpholino) propane sulfonic acid	Sigma	68399-77-9
Imidazole	Eastman Organic Chemicals	288-32-4

3.2 Steps in modification of capillaries:

The steps for modification of the capillaries were provided by Dr. Maria Matyska Pesek. For each step, a modified GC oven assembly was used to fill the capillaries This assembly consists of capillary holder, N_2 supply, and solution vial as shown in Figure 6.

3.2.1 Conditioning of the capillaries

The conditioning of the capillaries was done using conc. HCL. The conc. HCL is filled into the capillaries using N_2 pressure of 30-50 psi. These capillaries were then kept overnight for proper conditioning and dried the next day using N_2 gas. This step was done to clean the capillaries for further modification steps.

3.2.2 Etching of capillaries

The etching of capillaries was done to increase the overall capacity of the capillaries. For etching, a 5% w/v solution of ammonium hydrogen difluoride was prepared in methanol. This etching reagent was filled into the capillaries by applying a N_2 pressure of 30-50 psi. The capillaries were then kept flat for 1 hr. After 1 hr, this solution was removed by applying N_2 pressure. The dried capillaries were then sealed at both ends using a torch and wrapped in an aluminum foil. These wrapped capillaries were then placed in a GC oven at a temperature of $300^{\circ}C$ for 3 hrs with a continuous supply of N_2 gas into the GC oven. After 3 hrs, the capillaries were cut at both ends and flushed with methanol, and then kept flat for 1 hr at room temperature. These capillaries were then dried and emptied using N_2. This procedure will etch the inner walls and

increase the surface area of the capillaries for attachment of a stationary phase. The

characterization of capillaries can be done using scanning electron microscopy (SEM).

The SEM images of bare and etched capillaries are shown in Figure 7 [6].
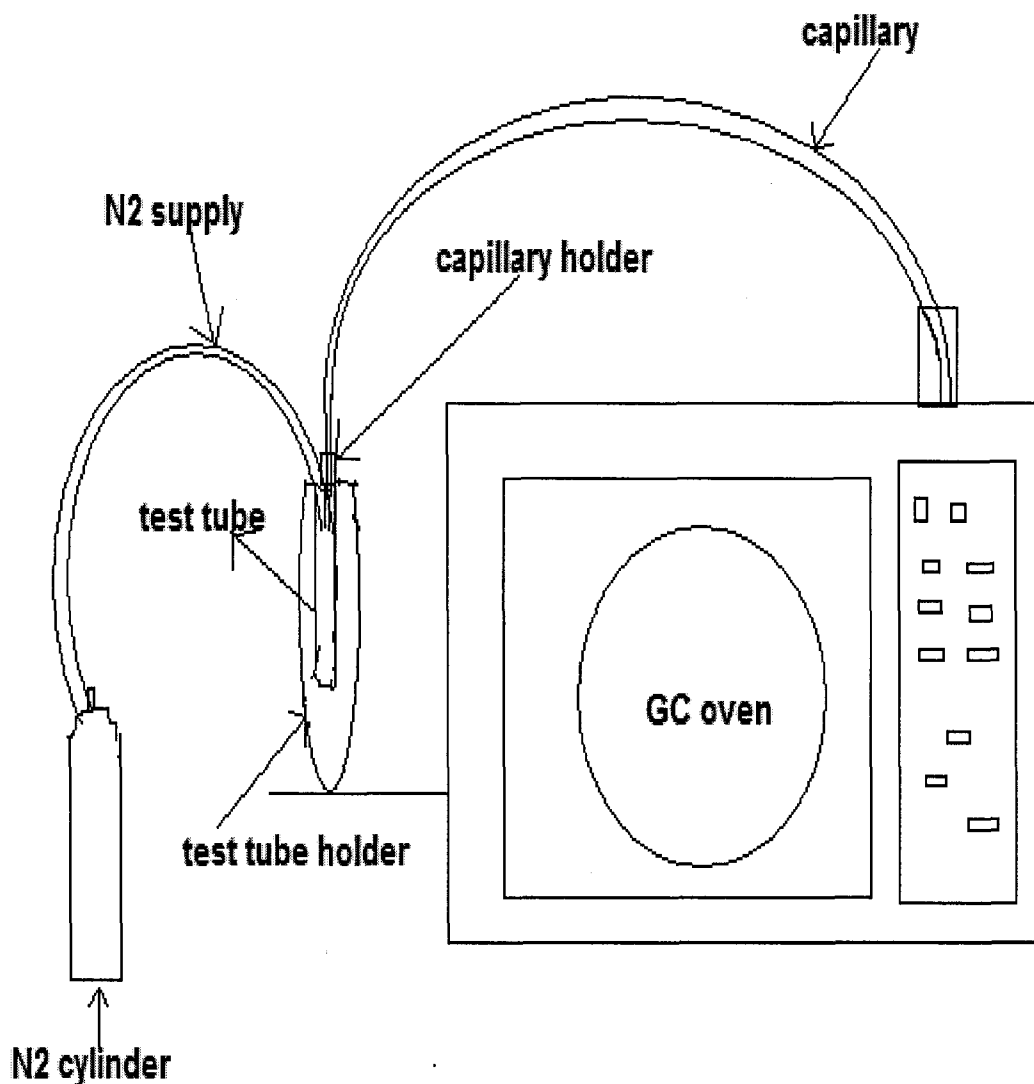
Figure 6: Modified GC oven assembly.

(a)

(b)

Figure 7: SEM images of: (a) bare capillary and (b) etched capillary (at 300°C for 3 hrs)
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3.2.3 Formation of hydride layer

For preparation of the hydride layer, dried capillaries were washed with DI water using N_2 pressure. These capillaries were then filled with freshly prepared 0.1 M NaOH solution and kept overnight at room temperature. The next day, the capillaries were washed with DI water and filled with 0.1 M HCL using N_2 pressure until the effluent became acidic. The acidity of the effluent was tested using indicator paper. The acid filled capillaries were then kept flat for 4 hrs and then washed with DI water. Then the capillaries were dried again using N_2. For complete drying, the capillaries were heated in a GC oven overnight at 100°C. Complete drying of the capillaries is necessary to proceed with the silanization step, otherwise the capillaries may become blocked.

3.2.4 Silanization

In this step, silanol groups (-SiOH) in the capillary are converted to Si-H moieties in an acidic medium. Triethoxysilane (TES) reagent was used to form the hydride layer inside the capillaries. This reagent was prepared by mixing 0.7 ml of TES with 3 ml of dioxane and 50 μl of 2.3 M HCL. After filling the capillaries with TES reagent, capillaries were kept in an oven at 90°C for 90 minutes. These capillaries were then washed with dioxane and toluene and kept flat for 2 hrs. After 2 hrs, these capillaries were dried using N_2. This step is called silanization and will prepare the capillaries for the next hydrosilation step.

3.2.5 Hydrosilation

In the hydrosilation step, a hydride capillary is reacted with an organic moiety to attach a stationary phase. The stationary phase solution was prepared by heating an alkene or alkyne and catalyst solution for 1 hr at 70°C. This solution was then filled into the capillaries using N_2 gas. In this research, C5 and C18 capillaries were prepared and bonded phases are shown in Figures 8a and b. For the C5 capillary, 5 ml of toluene, 1 ml of pentyne and 60 µl of 10 mM platinum catalyst were heated in a reflux set up at 70°C for 1 hr. This solution was then filled into the hydride capillaries using N_2. These capillaries were wrapped in an aluminum foil and placed in a GC oven at 100°C for 5 days. The C5 stationary phase solution was changed every day with fresh solution. Similarly for the C18 capillary, 5 ml of 1- octadecene and 70 µl of platinum catalyst solution were heated in a reflux set up at 70°C for 1 hr. Then this C18 stationary phase solution was filled into capillaries using the same N_2 technique and the capillaries were placed in GC oven at 100°C for 5 days. These capillaries were washed with methanol before analysis.

3.3 Buffers used in the research work

pH 2.14 = 1:10 dilution of tris (0.19 M) and H_3PO_4 (0.3 M).

pH 3.00 = 1:10 dilution of citric acid (0.3 M) and β-alanine (0.25 M).

pH 3.72 = 1:10 dilution of lactic acid (0.3 M) and β-alanine (1.36 M).

pH 4.38 = 1:10 dilution of acetic acid (0.3 M) and gama-amino butyric acid (GABA) (0.375 M).

pH 6.00 = 1:10 dilution of 2-(morpholino) ethane sulphonic acid (MES)

(0.3 M) and histidine (0.21 M).

pH 7.08 = 1:10 dilution of 3-(N-morpholino) propane sulphonic acid

(MOPS) (0.3 M) and imidazole (0.215 M).

pH 8.10 = 1:10 dilution of tris (0.1 M) and boric acid (0.15 M).
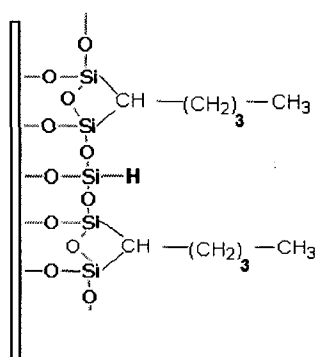
(a)
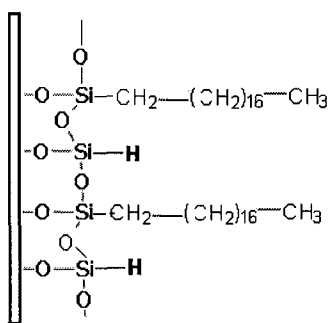
(b)

Figure 8: Stationary phase attachments inside capillary: (a) C5 attachment and (b) C18

attachment.

Table 2: List of compounds studied in this research work

Compounds	Company	Conc. in milliQ H_2O
Transferrin	Sigma	1.4 mg/1.4 ml
Human IgG	Sigma	1.3 mg/1.3 ml
Carbonic anhydrase	Sigma	1.5 mg/1.5 ml
Myoglobin	Sigma	1.2 mg/1.2 ml
Human serum albumin	Sigma	1.3 mg/1.3ml
MMP-1	Biomol International LP	2 μg/40 μl
MMP-3	Biomol International LP	2 μg/40 μl
MMP-7	Biomol International LP	2 μg/40 μl
MMP-8	Biomol International LP	2 μg/40 μl
MMP-9	Biomol International LP	2 μg/40 μl
MMP-10	Biomol International LP	2 μg/40 μl
MMP-11	Biomol International LP	2 μg/40 μl
MMP-12	Biomol International LP	2 μg/40 μl
MMP-13	Biomol International LP	2 μg/40 μl
TIMP-2/MMP-2	R and D systems	2.3 mg/300 μl
TIMP-1/MMP-3	R and D systems	1.6 mg/200 μl
TIMP-2/MMP-3	R and D systems	2.3 mg/300 μl

4. RESULTS AND DISCUSSION

4.1 Proteins on C5, C18 and bare capillaries

Generally, the human body consists of many protein molecules that perform a variety of functions inside the body. For example, proteins like transferrin, myoglobin, and hemoglobin are essential for oxygen transport to muscle tissues in the body. Some proteins act as markers for diseases. Chemically, proteins shift between a number of related structures while performing their biological functions. So, in order to understand the nature, function and their requirement in the human body, it is necessary to purify proteins for in-vitro analysis. Many methods have been utilized for the purification of proteins based on their molecular weight, net charge, and binding affinity. For example, gel electrophoresis is used when protein molecular weight and isoelectric point are known, enzyme assays are used when the protein has some enzymatic activity and electrofocusing is used when proteins bears a charge on it. In addition to all of these techniques, new and more efficient techniques have been developed for accurate identification, characterization, and purification of proteins [7].

This section involves the results and discussion on some of the proteins analyzed in this research work using C5, C18 and bare capillaries. These proteins are transferrin, human serum albumin, human IgG, carbonic anhydrase and myoglobin.

i) Transferrin: Transferrin is an iron binding protein found in blood plasma. It is a glycoprotein which binds less than 0.1% of total body iron. It is mainly produced by the liver in the body. When the liver decreases transferrin production in the body, it impairs

hemoglobin production. Iron deficiency anemia, infection and malignancies decreases

transferrin level in body. The molecular weight of transferrin is around 80 kDa. The

primary protein structure of transferrin consists of 700 amino acids. The blend of alpha

and beta sheets forms the N and C terminal lobes of transferrin. These two lobes are

connected to each other by a short peptide chain and form the hydrophobic site in

transferrin. It has two specific high affinity iron binding sites. This iron is present in the

ferric (Fe^{+3}) state which is surrounded by positively and negatively charged amino acids.

Tyrosine, aspartic acid, histidine and arginine are the most important amino acids which

balance the charge on this protein [8]. The migration times for transferrin on three

different capillaries is shown in Table 3.

Table 3: Migration times for transferrin on bare, C5 and C18 capillaries

		Capillary		
		Bare	C5	C18
pH	2.14	4.73, 5.18	3.86	7.41
	3.0	Multiple peaks	2.95	4.61, 9.68
	3.72	Multiple peaks	3.93, 11.78	4.34, 8.43
	4.38	2.12, 3.14	3.12	3.91, 6.46
	6.0	No peak	No peak	-
	7.08	No peak	No peak	No peak
	8.10	1.51, 1.91	10.93	9.41, 5.03

Table 3 represents the migration times for transferrin with seven different pH

buffers on three different capillaries. According to the above table, transferrin mostly

showed two or more peaks on the C18 and bare capillaries. One peak was observed for transferrin on the C5 capillary at pH 2.14, 3.0, 4.38, and 8.10. These experiments were run three times under the same experimental conditions and found to be reproducible. These peaks for transferrin were also observed at the wavelengths 214, 223, 254, and 270 nm, but the intensity of the peaks decreased as the wavelength increased.

Figures 9, 10, and 11 represent the chromatograms of transferrin at pH 4.38 on the C5, C18, and bare capillaries. At pH 4.38, the C5 capillary gave one peak for transferrin at negative voltage, but the C18 and bare capillaries gave two peaks for transferrin at positive voltage. Due to the complex nature of proteins, it is believed that the two compounds seen on the C18 and bare capillaries could be some structural isomers of the parent compound. Moreover, the migration times for transferrin on the C5 and bare capillaries are shorter when compared to the C18 capillary. This longer migration time on the C18 capillary could be due to the longer C18 carbon chain, which, in turn retains the molecule for a longer period of time on the column, and results in a longer migration time for the compound.

Figures 12, 13, and 14 represent transferrin chromatograms at pH 8.10 on the C5, C18, and bare capillaries. It was clear from these chromatograms that transferrin on the C5 and C18 capillaries had longer migration times than on the bare capillary. At 8.10 pH, one peak was observed for transferrin on the C5 capillary; four peaks were observed on the C18 capillary; and three peaks were obtained on the bare capillary, with a major peak having a large shoulder on it. The tailing and broad peaks observed on the C18 capillary could be due to longer stationary phase interactions of compound on this

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column. The minor peaks on this column could be some chemical impurity or it could be some other isoform of transferrin, which has been resolved on this column. A chemical impurity in the sample could be the result of preparation procedure used for the parent compound. Another isoform of transferrin could be the result of the general heterogeneous nature of the parent compound. The differences in the migration times and peak shapes on the three columns suggests that the three capillary surfaces are fundamentally different and are responsible for variable separation capabilities.

Figure 9: Electrochromatogram of transferrin on the C5 capillary, 50 μm i.d, l=45 cm, l_{eff} = 36.5 cm, pH 4.38 (1:10), applied voltage = -25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.
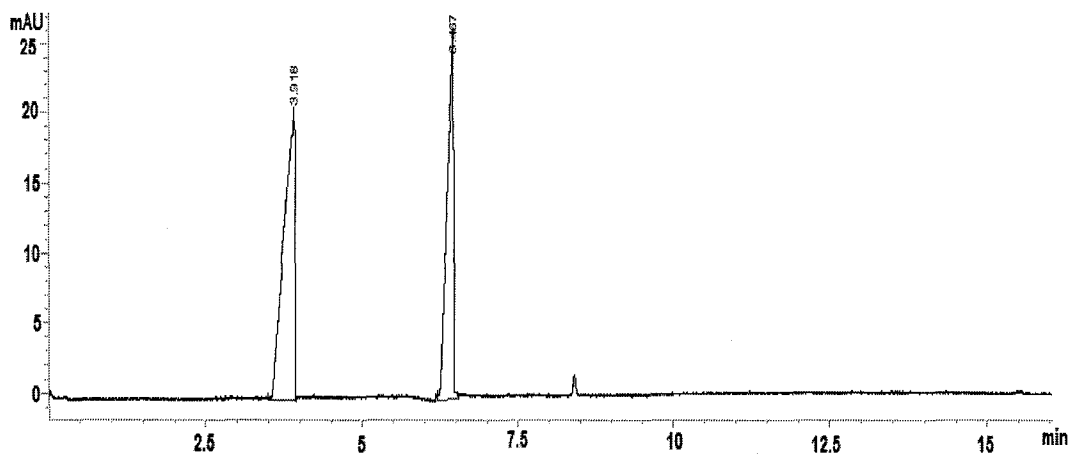
Figure 10: Electrochromatogram of transferrin on the C18 capillary, 50 μm i.d, l = 58.5 cm, l_{eff} = 50.0 cm, pH 4.38 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.
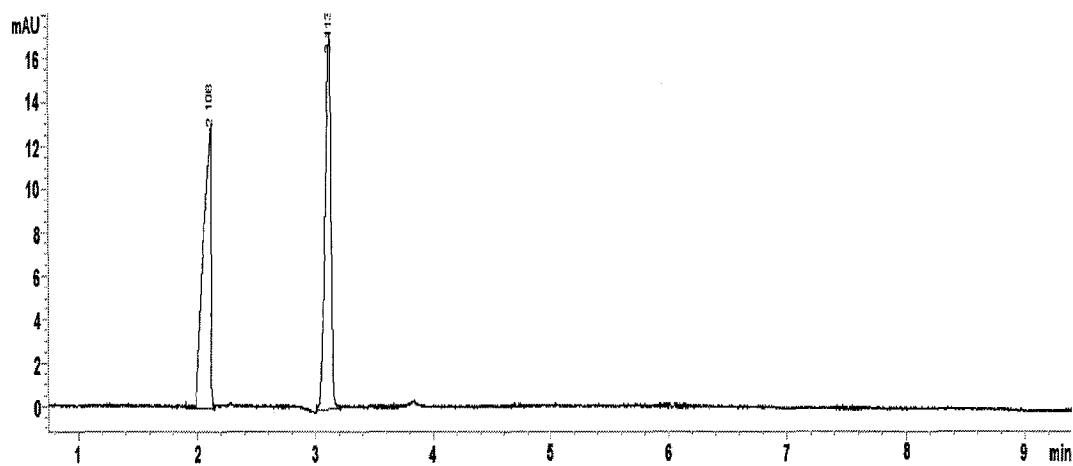
Figure 11: Electrochromatogram of transferrin on the bare capillary, 50 μm i.d, l = 51.4 cm, l_{eff} = 42.9 cm, pH 4.38 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s, and detection at 210 nm.
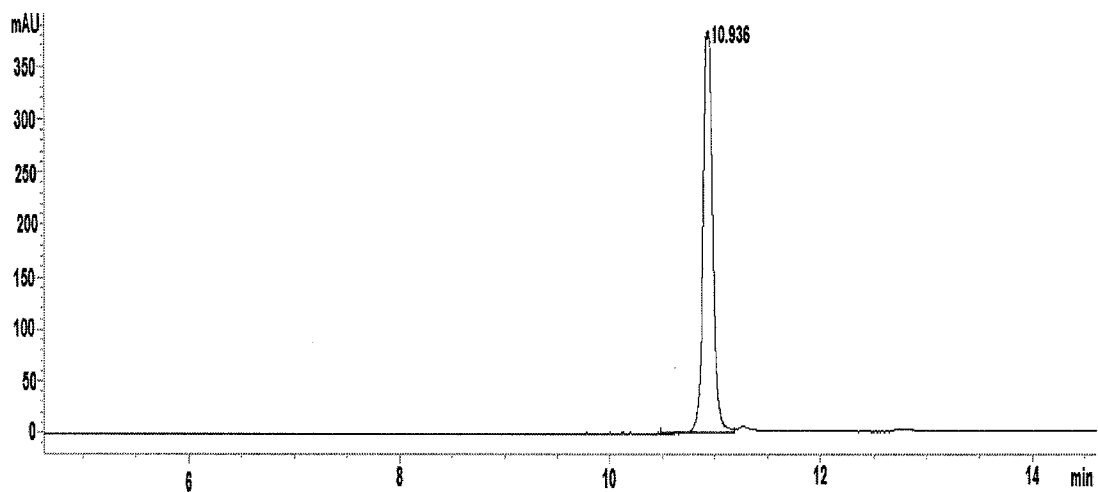
Figure 12: Electrochromatogram of transferrin on the C5 capillary, 50 μm i.d, l=45 cm, l_{eff} = 36.5 cm, pH 8.10 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.
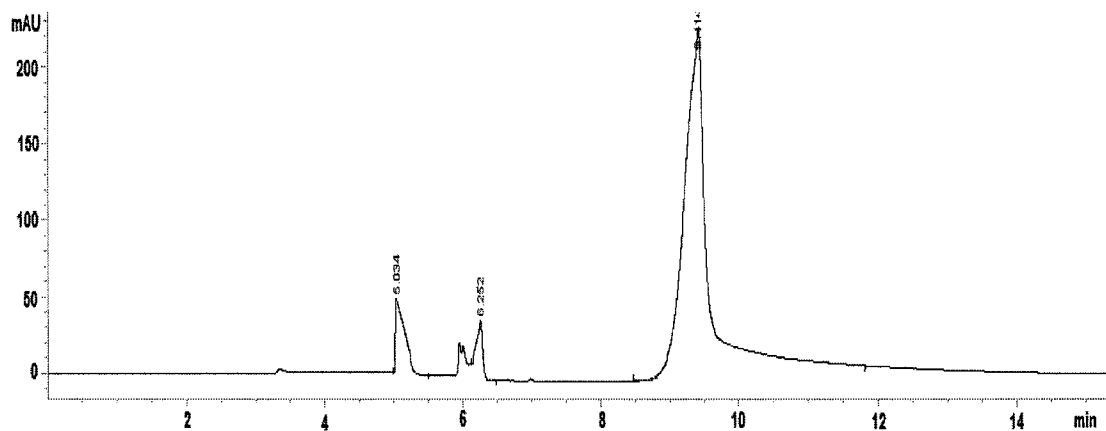
Figure 13: Electrochromatogram of transferrin on the C18 capillary, 50 μm i.d, l = 58.5 cm, l_{eff} = 50.0 cm, pH 8.10 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.
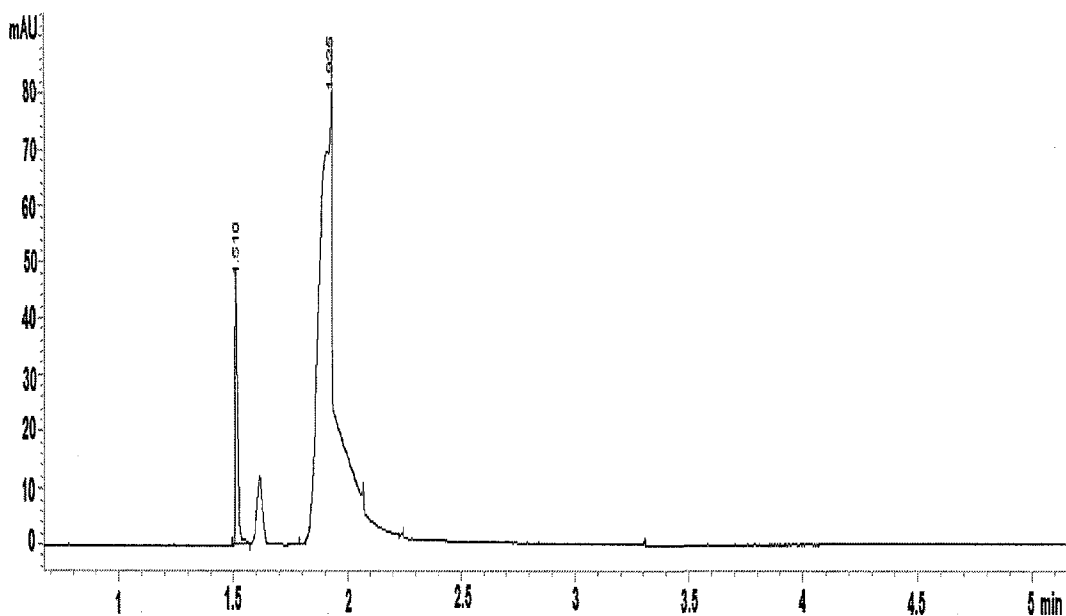
Figure 14: Electrochromatogram of transferrin on the bare capillary, 50 μm i.d, l = 51.4 cm, l_{eff} = 42.9 cm, pH 8.10 (1:10) buffer, applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.

ii) Human IgG: IgG is a glycoprotein found in human plasma. This protein serves as the major effector molecule in humoral response. IgG is the major immunoglobin secreted at the end of primary immune and memory responses. This protein diffuses from blood into other extracellular fluids, particularly in inflamed microvasculature, and also crosses the fetal circulation. IgG accounts for approximately 70% of adult serum immunoglobin. IgG helps in removal of infectious agents like bacteria, viruses, and toxins from the body [9]. Table 4 shows the migration times for human IgG on the C5, C18, and bare capillaries at seven different pH buffers.

Table 4: Migration times for human IgG on bare, C5, and C18 capillaries

		Capillary		
		Bare	C5	C18
pH	2.14	4.68, 5.59	No peak	8.45
	3.0	2.83, 4.67	No peak	4.88, 10.03
	3.72	2.85, 4.59	8.27	4.38, 8.39
	4.38	1.92, 2.54	8.36	4.32, 7.34
	6.0	No peak	No peak	-
	7.08	No peak	No peak	No peak
	8.10	1.52	3.06, 9.59	5.03, 6.22

In the above table, a single peak for Human IgG was observed at pH values of

3.72 and 4.38 on the C5 capillary, at pH 2.14 on the C18 capillary, and at pH 8.10 on the

bare capillary. Under other pH conditions, human IgG gave either two or no peaks on the

three columns. These peaks for IgG were also observed at other wavelengths such as

214, 223, 254, and 270 nm, but the intensity of the peaks decreased as the wavelength

increased. These experiments were run three times under the same experimental

conditions and found to be reproducible.

Figures 15, 16, and 17 show the chromatograms of Human IgG at pH 3.72 on the

C5, C18, and bare capillaries. From the chromatograms it is clear that at pH 3.72, the C5

capillary gave a single and symmetric peak, whereas the C18 and bare capillaries gave

two peaks. The reason for two peaks on the C18 and bare columns could be some

isoform of the parent compound or it could be some chemical impurity. The first peak on

the C18 and bare columns was asymmetric. This type of peak could be due to difference

in conductivities of the sample and the buffer solution. The second peak on the C18 and

bare columns was more symmetric than the first peak, but it still showed some tailing.

Figures 18, 19, and 20 represent the chromatograms for human IgG at pH 4.38 on

the C5, C18, and bare capillaries. On comparing the chromatograms of the C5, C18, and

bare capillaries, it is observed that the C5 capillary gave better results for human IgG at

this pH. The C18 and bare capillaries gave two peaks, in which the first peak was

asymmetric and broad compared to the second peak. It can also be seen that the first

peak for human IgG on the bare column also had shoulder on the left side of peak, which

could be a third component, not well separated on this column. But it is clear from these

chromatograms, that the three surfaces behave differently from each other under the same

experimental conditions and for the same compound.

Figure 15: Electrochromatograms of human IgG on the C5 capillary, 50 μm i.d, l=45 cm,

l_{eff} =36.5 cm, pH 3.72 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50

mbar pressure for 8 s and detection at 210, 214, 223, and 254 nm.

Figure 16: Electrochromatograms of human IgG on the C18 capillary, 50 μm i.d, l = 58.5 cm, l_{eff} = 50.0 cm, pH 3.72 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210, 214, 223, 254, and 270 nm.

Figure 17: Electrochromatograms of human IgG on the bare capillary, 50 μm i.d, l = 51.4 cm, l_{eff} = 42.9 cm, pH 3.72 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210, 214, 223, 254, and 270 nm.
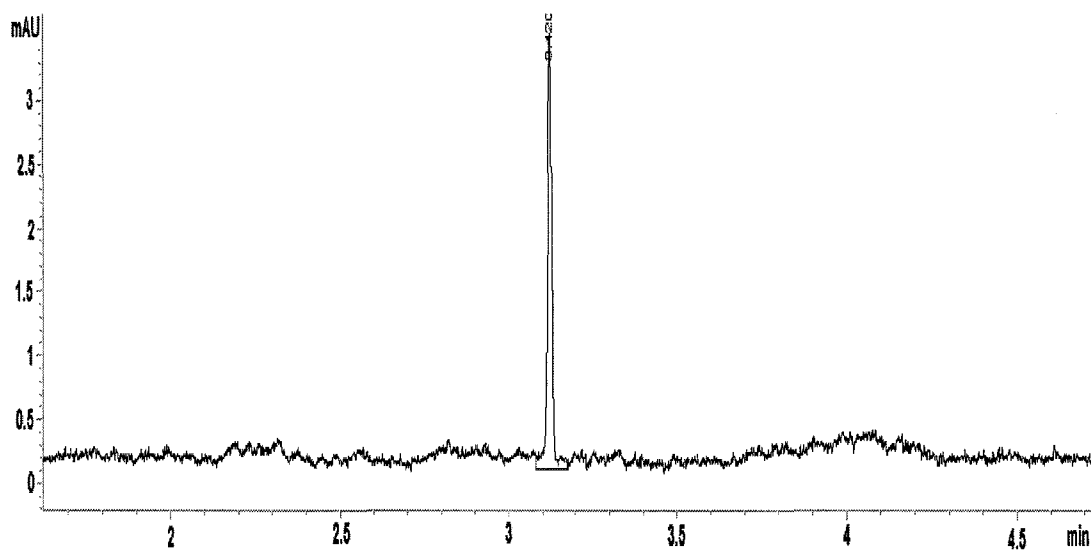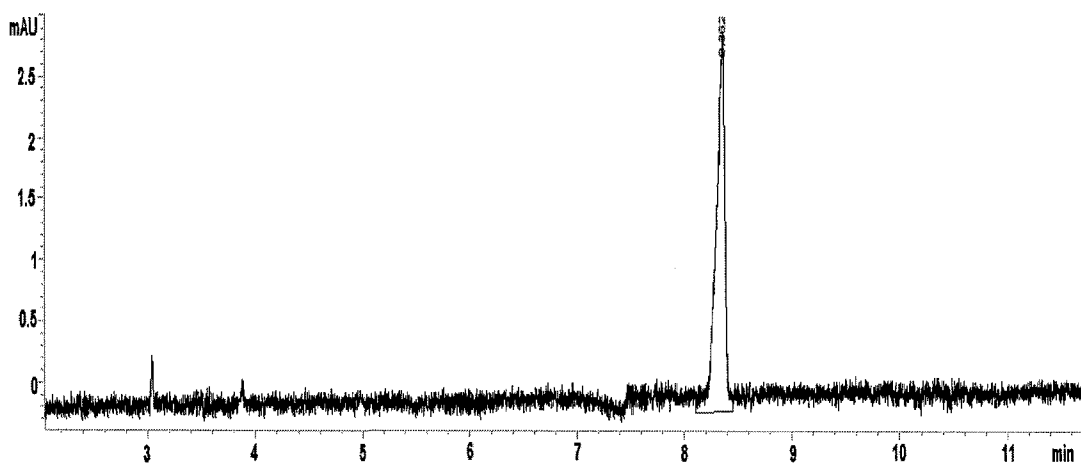
Figure 18: Electrochromatogram of human IgG on the C5 capillary, 50 μm i.d, l=45 cm, l_{eff} = 36.5 cm, pH 4.38 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.
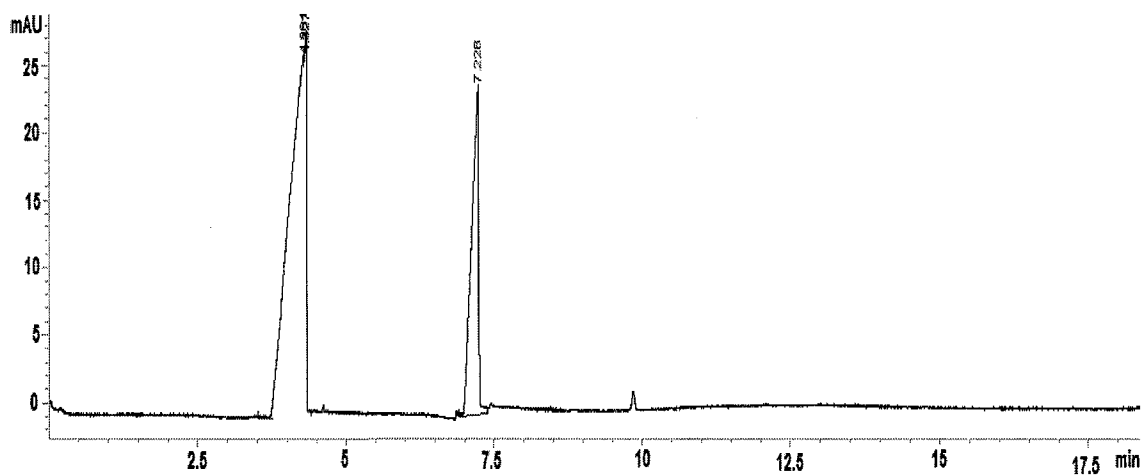
Figure 19: Electrochromatogram of human IgG on the C18 capillary, 50 μm i.d, l = 58.5 cm, l_{eff} = 50.0 cm, pH 4.38 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.
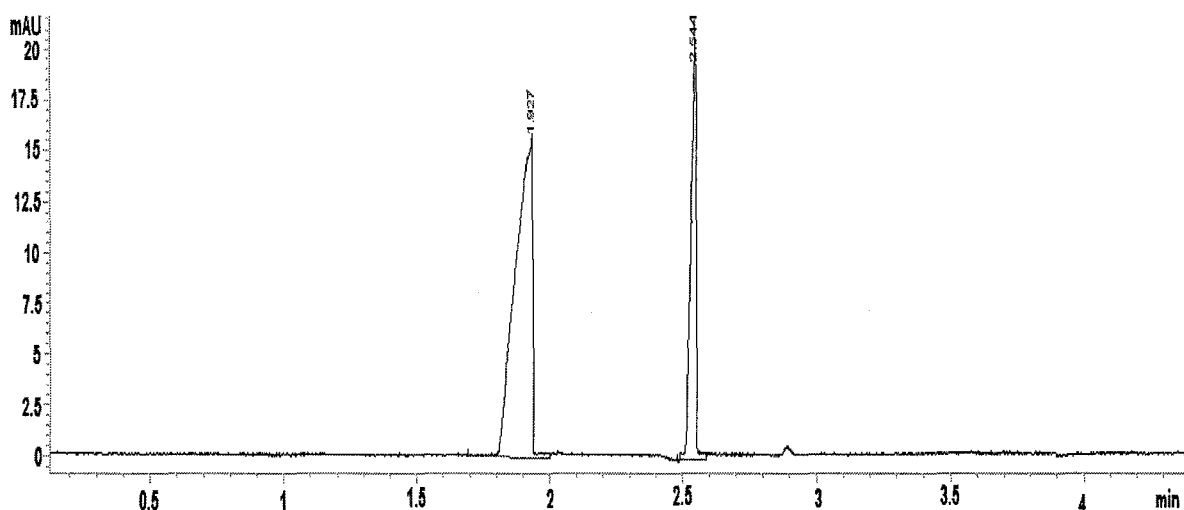
Figure 20: Electrochromatogram of human IgG on the bare capillary, 50 μm i.d, l = 51.4 cm, l_{eff} = 42.9 cm, pH 4.38 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.

iii) Carbonic Anhydrase: Carbonic anhydrase is a metalloenzyme that catalyzes the conversion of carbon dioxide into bicarbonate ions and protons. The active zinc ion of this enzyme is coordinated by three histidine side chains and the fourth position is taken by a water molecule, which polarizes the -OH bond and makes it more negative. Different forms of this enzyme are found in nature. For example, the alpha form of carbonic anhydrase that is found in animals converts bicarbonate to carbon dioxide which maintain acid-base balance in blood and helps in transportation of carbon dioxide out of tissues. The beta form of carbonic anhydrase that is found in plants helps in photosynthesis by converting carbon dioxide to organic carbon sugars [10]. Table 5

represents the migration time for carbonic anhydrase in seven pH buffers and on three different capillaries.

Table 5: Migration times for carbonic anhydrase on bare, C5 and C18 capillaries.

pH	Capillary		
	Bare	C5	C18
2.14	Multiple Peaks	No peak	6.95
3.0	Multiple Peaks	10.68	4.63, 9.41
3.72	Multiple Peaks	No peak	4.23, 7.93
4.38	2.06, 2.99	No peak	No peak
6.0	Multiple Peaks	5.34	-
7.08	2.90	No peak	10.91
8.10	Multiple Peaks	13.40	No peak

In the above table, one peak for carbonic anhydrase was observed at pH 3.0, 6.0, and 8.10 on the C5 capillary; at pH 2.14 and 7.08 on the C18 capillary; and at pH 7.08 on the bare capillary. In other pH buffers, either multiple or no peaks were observed on the three columns. These peaks for carbonic anhydrase were also observed at other wavelengths such as 214, 223, 254, and 270 nm, but the intensity of the peaks decreased as the wavelength increased.

Figures 21, 22, and 23 show the chromatograms for carbonic anhydrase on the C5, C18, and bare capillaries at pH 3.0. From these chromatograms it is clear that at pH 3.0, the C5 capillary gave single, narrow, and symmetric peak; the C18 capillary gave two peaks, in which the first peak was of lower intensity with small shoulder on the left side, and the second peak was asymmetric and of higher intensity; and the bare capillary

gave five peaks in which one peak was major and all other peaks were minor. The minor peaks in the chromatograms could be due to impurities. Regarding the migration time on the three columns, the compound was eluted faster on the bare column than the C5 and C18 columns.

Figures 24, 25, and 26 represents the chromatograms of carbonic anhydrase at pH 7.08 on the C5, C18, and bare capillaries. In Figure 24, the C5 capillary gave no peak at pH 7.08 but an inverted dip was noticed which could be due to sample solvent used in the analysis. In Figure 25, one major peak and two minor broad peaks were observed on the C18 capillary. These minor peaks could be some impurity in the parent compound. In Figure 26, one major narrow peak can be seen on the bare capillary but this major peak also had minor peak not well separated from it. Thus the bare and C5 capillaries are not the best columns for the analysis of this protein
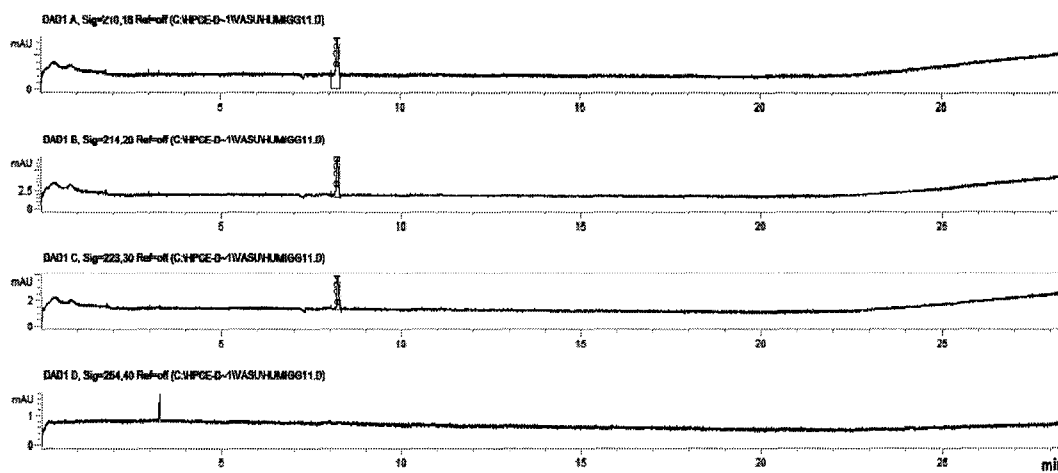

Figure 21: Electrochromatogram of carbonic anhydrase on the C5 capillary, 50 μm i.d, l=45 cm, l_{eff} = 36.5 cm, pH 3.0 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.

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Figure 22: Electrochromatogram of carbonic anhydrase on the C18 capillary, 50 μm i.d, l = 58.5 cm, l_{eff} = 50.0 cm, pH 3.0 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.

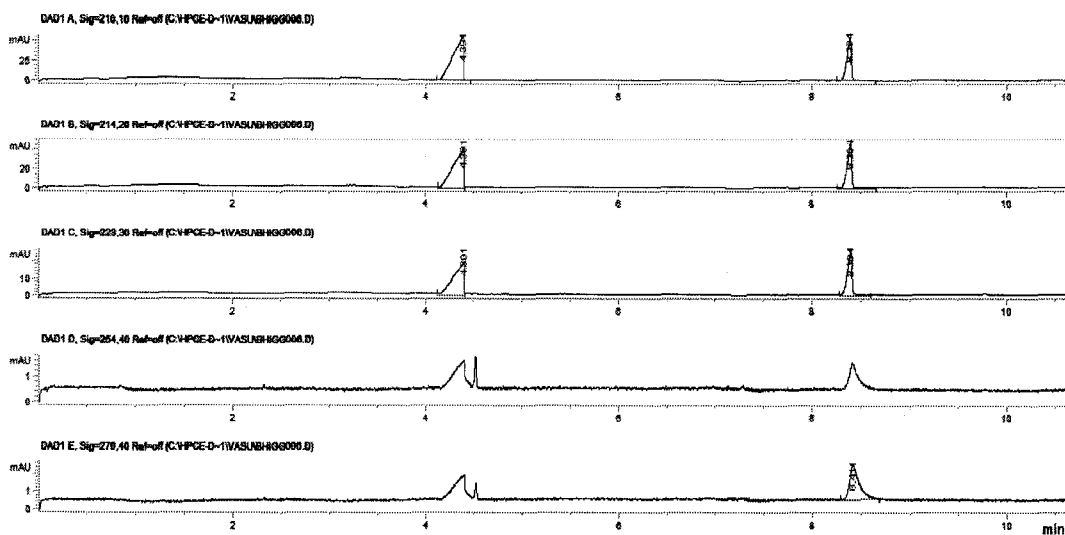Figure 23: Electrochromatogram of carbonic anhydrase on the bare capillary, 50 μm i.d, l = 51.4 cm, l_{eff} = 42.9 cm, pH 3.0 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.

Figure 24: Electrochromatogram of carbonic anhydrase on the C5 capillary, 50 μm i.d,

l=45 cm, l_eff = 36.5 cm, pH 7.08 (1:10), applied voltage = 25 kV, hydrodynamic injection
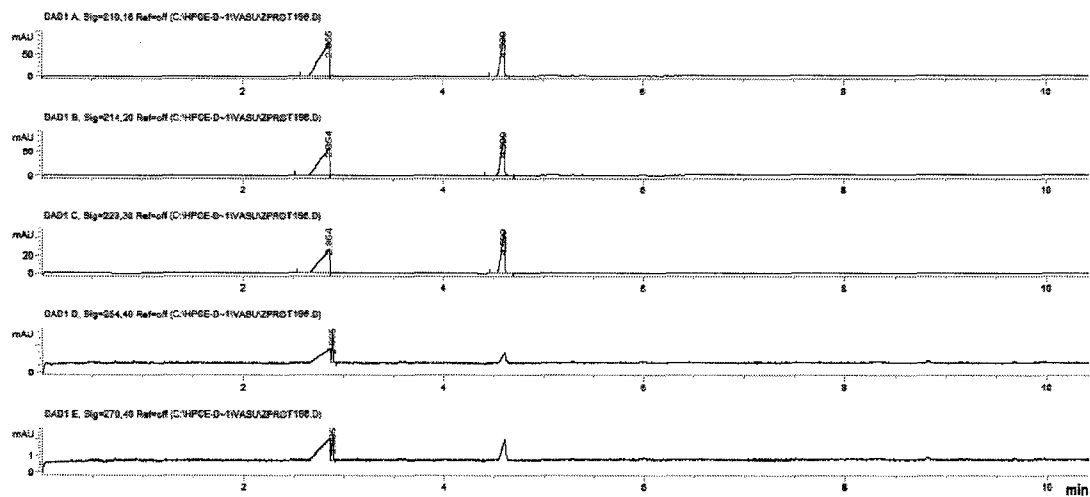
at 50 mbar pressure for 8 s and detection at 210 nm.

Figure 25: Electrochromatogram of carbonic anhydrase on the C18 capillary, 50 μm i.d, l

= 58.5 cm, l_eff = 50.0 cm, pH 7.08 (1:10), applied voltage = 25 kV, hydrodynamic

injection at 50 mbar pressure for 8 s and detection at 210 nm.

Figure 26: Electrochromatogram of carbonic anhydrase on the bare capillary, 50 μm i.d, l = 51.4 cm, l_{eff} = 42.9 cm, pH 7.08 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.

iv) Myoglobin: Myoglobin is a single chain globular protein containing heme as the prosthetic group in the center surrounded by apoprotein. This protein consists of a porphyrin ring with an iron center, in which a proximal histidine group is attached directly to the iron center and a distal histidine group opposite to it, not bonded to iron. This protein consists of 153 amino acids and has a molecular weight of 167 kDa. It is the primary oxygen carrying species of muscle cells in the human body. Its high concentration in muscle cells helps in holding breath for a long period of time. Myoglobin also serves as a storage site for oxygen [11,12]. Table 6 shows the migration times for myoglobin in seven pH buffers and on three different capillaries.

Table 6: Migration times for myoglobin on bare, C5 and C18 capillaries

		Capillary		
		Bare	C5	C18
pH	2.14	4.49	No peak	No peak
	3.0	5.04, 2.68	10.54	12.54
	3.72	2.81, 5.26	11.97	4.32
	4.38	2.16	No peak	No peak
	6.0	Multiple peaks	No peak	-
	7.08	No peak	No peak	No peak
	8.10	1.63	No peak	7.54

According to the above table, one peak for myoglobin was observed at pH 2.14, 4.38, and 8.10 on the bare capillary; at pH 3.0 and 3.72 on the C5 capillary; and at pH 3.0, 3.72, and 8.10 on the C18 capillary. In other pH buffers, either no peak or multiple peaks were observed on these columns. Similar peaks were also seen at higher wavelengths of 210, 214, and 223 nm, and were found to be reproducible on three consecutive injections of this compound.

Figures 27, 28, and 29 show the chromatograms of myoglobin on the C5, C18, and bare capillaries at pH 3.0. At pH 3.0, the C5 column gave one narrow peak; the C18 column gave one broad tailing peak; and the bare column gave two peaks, one major broad and tailing and the other minor, sharp and narrow. The tailing peak on the C18 column could be due to stronger interaction of the compound with the stationary phase.

The minor peak on the bare column could be an impurity or it could be some other isoform of myoglobin.

Figures 30 and 31 show the chromatograms for myoglobin on the bare capillary at pH 2.14 and 4.38. In these chromatograms, one major peak and two minor peaks were observed for myoglobin at pH 2.14, but the minor peaks were not well separated from the major one. At pH 4.38, one narrow, sharp peak was observed. At pH 4.38 a shorter migration time was observed as compared to pH 2.14. It is likely that the shorter migration time at pH 4.38 is due to the deprotonation of silanols at higher pH, which increases the EOF in the capillary and in turn reduces the migration time of the compound in the column.
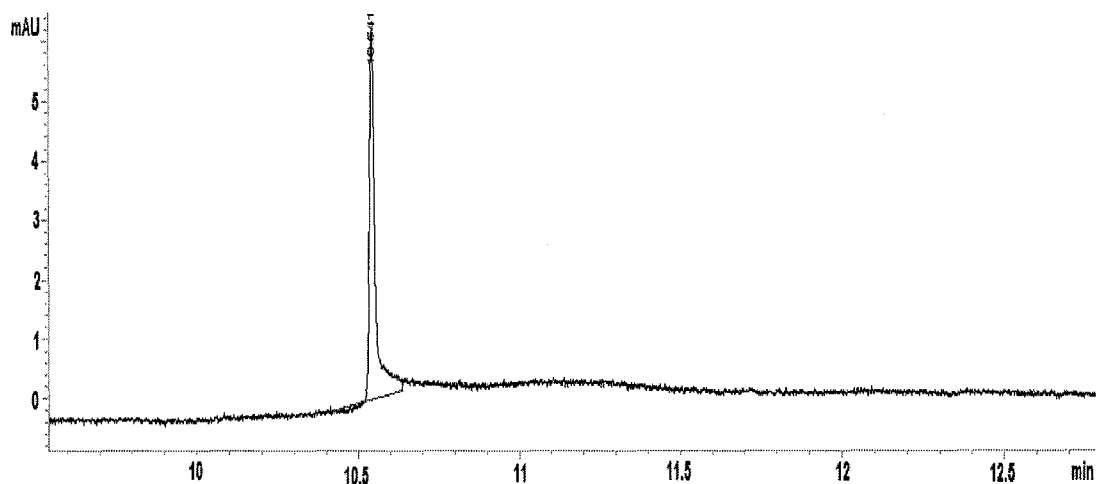
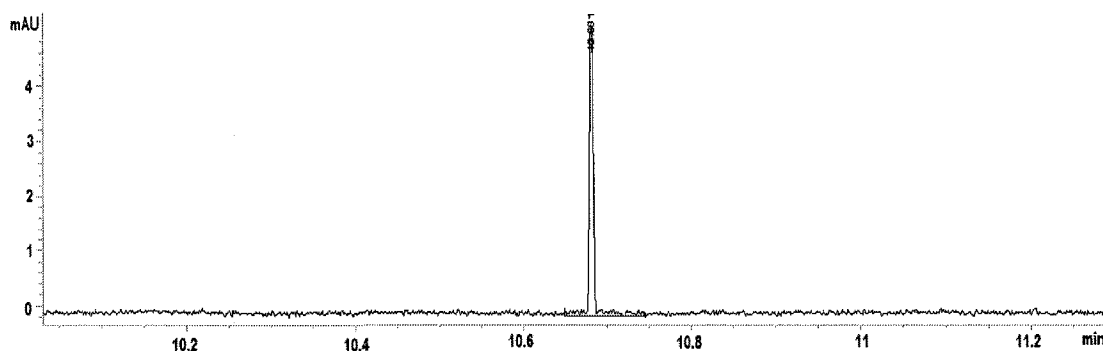Figure 27: Electrochromatogram of myoglobin on the C5 capillary, 50 μm i.d, l=45 cm, l_{eff} = 36.5 cm, pH 3.0 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210nm.

Figure 28: Electrochromatogram of myoglobin on the C18 capillary, 50 μm i.d, l = 58.5 cm, l_{eff} = 50.0 cm, pH 3.0 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.
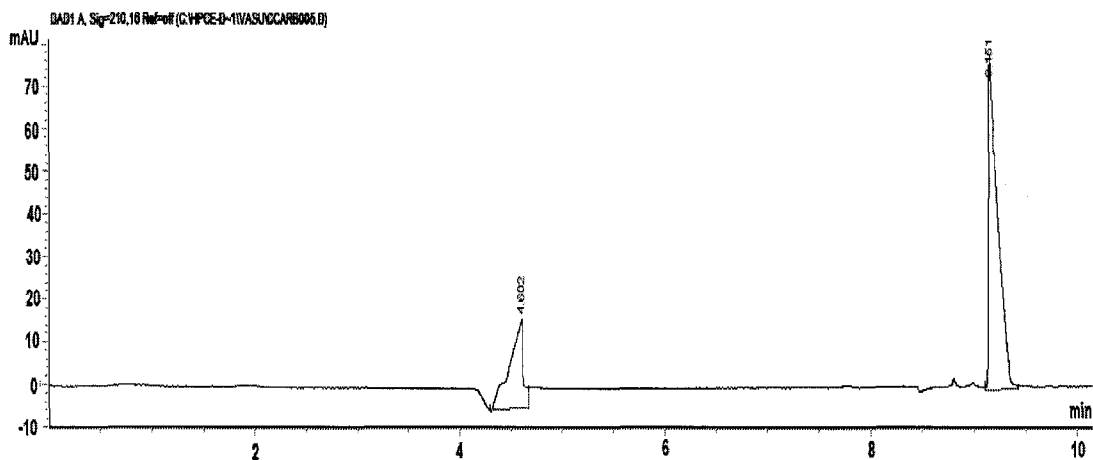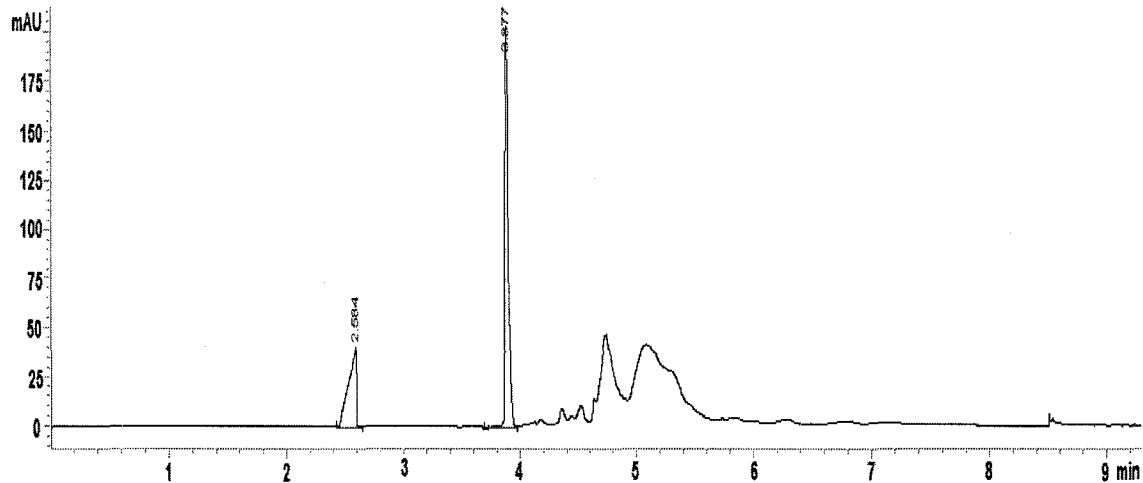

Figure 29: Electrochromatogram of myoglobin on the bare capillary, 50 μm i.d, l = 51.4 cm, l_{eff} = 42.9 cm, pH 3.0 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.

Figure 30: Electrochromatogram of myoglobin on the bare capillary, 50 μm i.d, l = 51.4 cm, l_{eff} = 42.9 cm, pH 2.14 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.
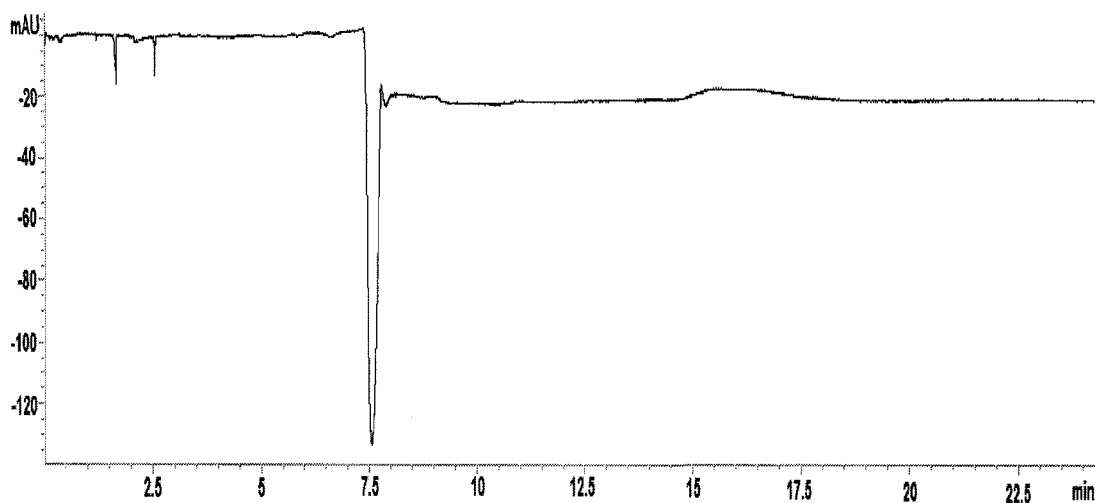

Figure 31: Electrochromatogram of myoglobin on the bare capillary, 50 μm i.d, l = 51.4 cm, l_{eff} = 42.9 cm, pH 4.38 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.

v) Human Serum Albumin: Human serum albumin is the most abundant protein found in blood plasma and mainly formed by the liver. The main function of this protein is to maintain the osmotic pressure balance in the blood compartment. It also serves as the carrier for molecules of low water solubility such as bile salts, bilirubin, free fatty acids, calcium, iron, and some drugs. The decrease and increase in the level of albumin in the body leads to hypoalbumineamia and hyperalbumineamia. Hypoalbumineamia can be caused by liver disease, nephrotic syndrome, burns, malabsorption, malnutrition, late pregnancy and malignancy. Hyperalbumineamia can be caused by severe dehydration [9]. Table 7 shows the migration times for human serum albumin in seven different pH buffers and on three different capillaries.

Table 7: Migration times for human serum albumin on bare, C5 and C18 capillaries

		Capillary		
		Bare	C5	C18
pH	2.14	22.50, 4.43	No peak	5.85
	3.0	Multiple peaks	No peak	4.75, 10.30
	3.72	Multiple peaks	No peak	4.25, 8.45
	4.38	1.99, 2.88	No peak	4.27, 7.46
	6.0	Multiple peaks	No peak	-
	7.08	No peak	No peak	No peak
	8.10	1.46, 1.95	No peak	5.47, 6.73

According to the above table, the C5 capillary gave no peak for albumin in any of the buffers. On the C18 capillary, a single peak was observed at pH 2.14 and two peaks

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were obtained at pH 3.0, 3.72, 4.38, and 8.10. And on the bare capillary usually two or multiple peaks were observed. These peaks for human serum albumin were also observed at higher wavelengths, 214, 223, 254, and 270 nm, but the intensity of the peaks decreased as the wavelength increased. These results were found to be reproducible on three consecutive injections of the parent compound.

Figures 32 and 33 show the chromatograms for human serum albumin at pH 2.14 on the bare and C18 capillaries. In these chromatograms, the single peak obtained on the C18 capillary was broad and asymmetric. This shape of peak could be due to difference in conductivities of the sample and the buffer or it could be two or more peaks not well separated from each other. On the bare capillary two peaks were observed, one major and one minor. The major peak was only slightly asymmetric but the minor peak was quite asymmetric. The minor peak on the bare capillary could be an impurity or some structural isomer of albumin.

Figures 34 and 35 show the chromatograms for human serum albumin on the bare and C18 capillaries at pH 3.0. In these chromatograms, one major broad peak, two minor narrow peaks and two or three small shoulders were observed on the bare capillary. The major peak was broad and tailed. The shoulders and minor peaks could be some impurities in the parent compound. On the C18 capillary, two major peaks were observed with a negative dip before the first peak. The major peaks could be structural isomers of albumin and the negative dip could be due to the sample solvent used in the analysis. But it is clear from these chromatograms that the results of the three surfaces are fundamentally different from each other and have variable analytical capabilities.

Figure 32: Electrochromatogram of human serum albumin on the C18 capillary, 50 μm i.d, l = 58.5 cm, l_{eff} = 50.0 cm, pH 2.14 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.
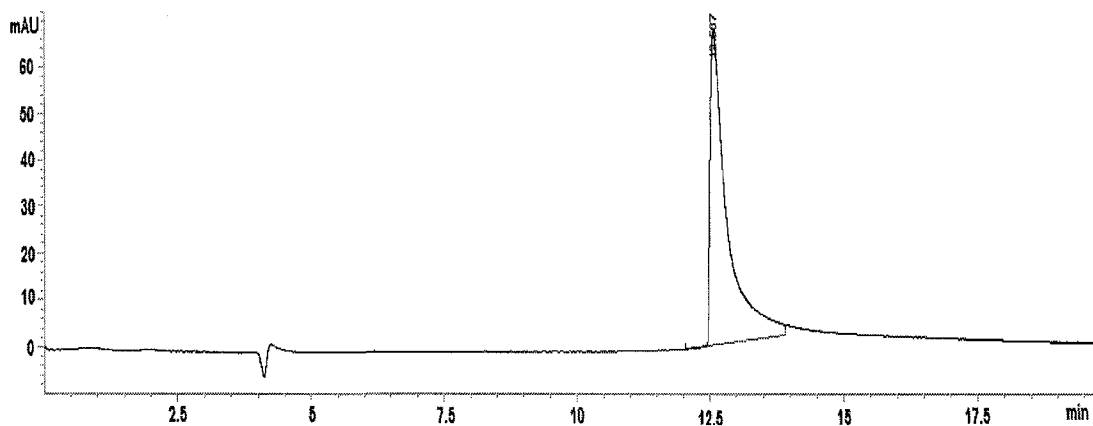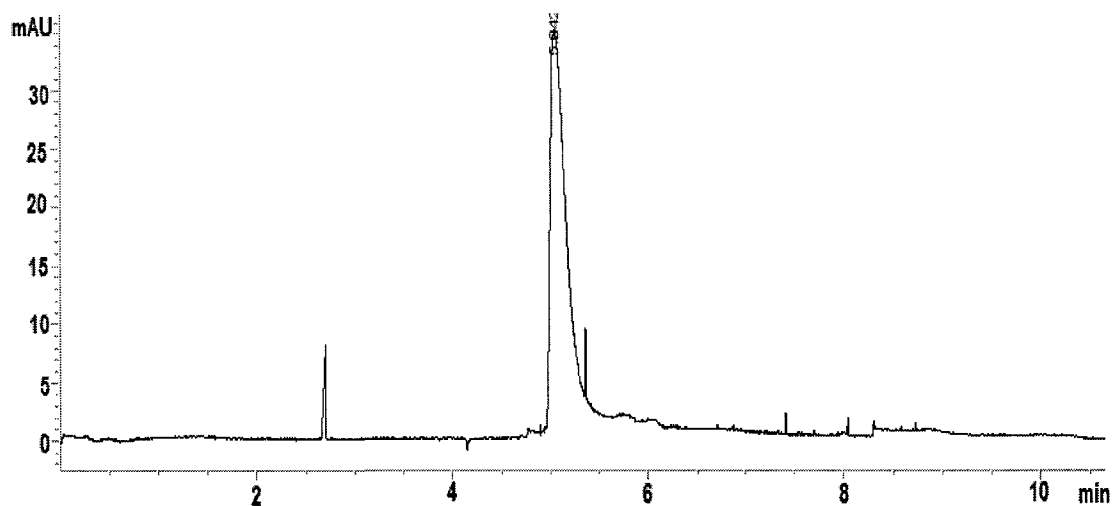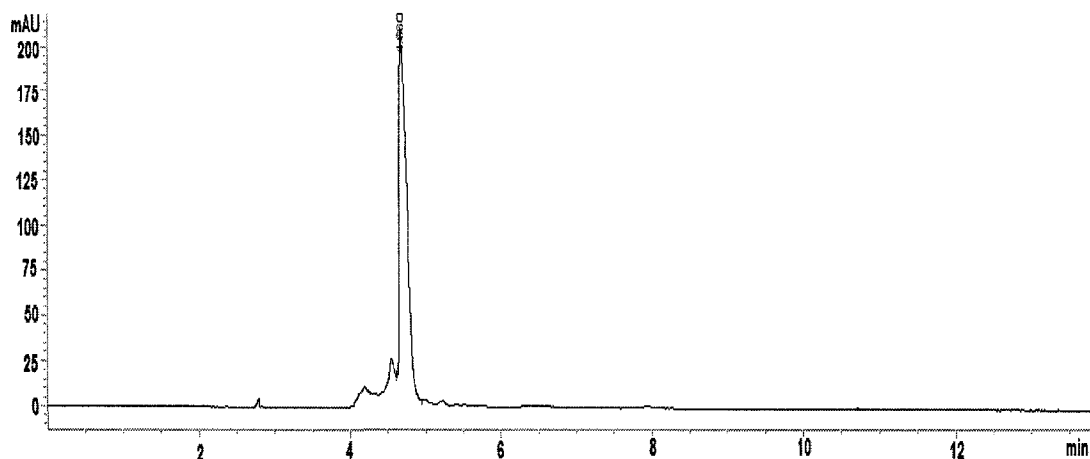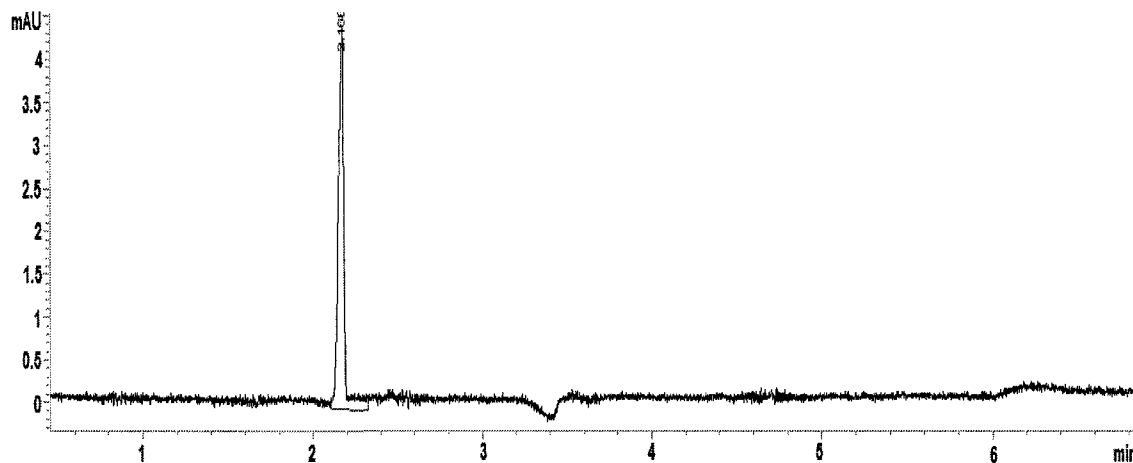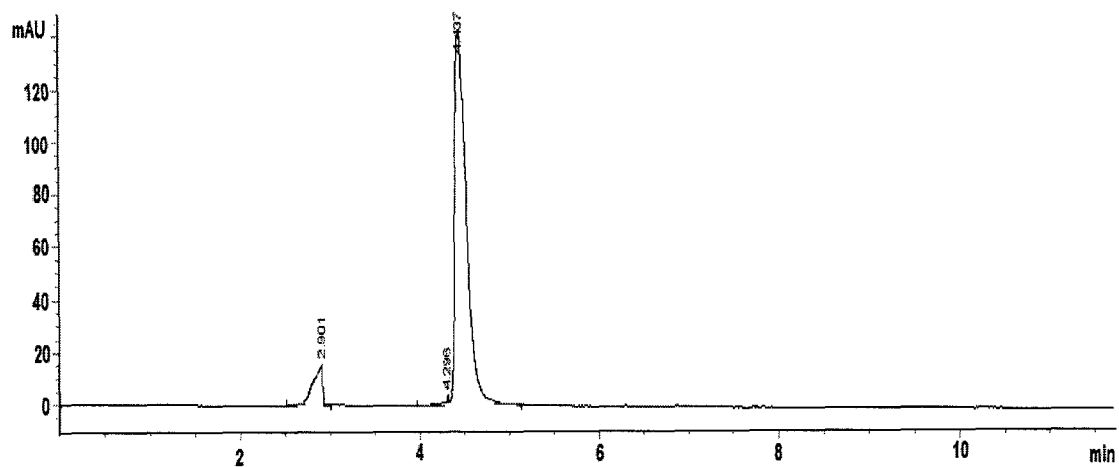

Figure 33: Electrochromatogram of human serum albumin on the bare capillary, 50 μm i.d, l = 51.4 cm, l_{eff} = 42.9 cm, pH 2.14 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm
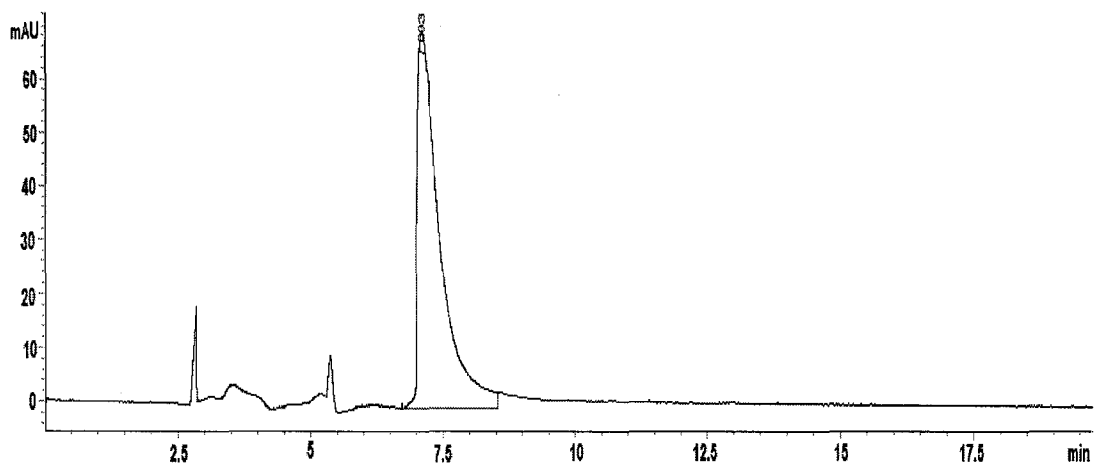
Figure 34: Electrochromatogram of human serum albumin on the bare capillary, 50 μm

i.d, l = 51.4 cm, l_{eff} = 42.9 cm, pH 3.0 (1:10), applied voltage = 25 kV, hydrodynamic
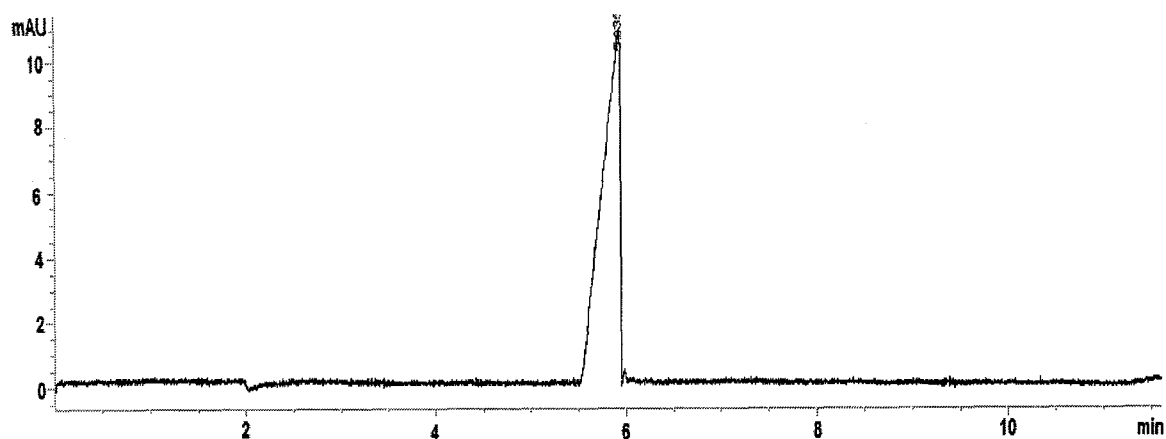
injection at 50 mbar pressure for 8 s and detection at 210 nm.
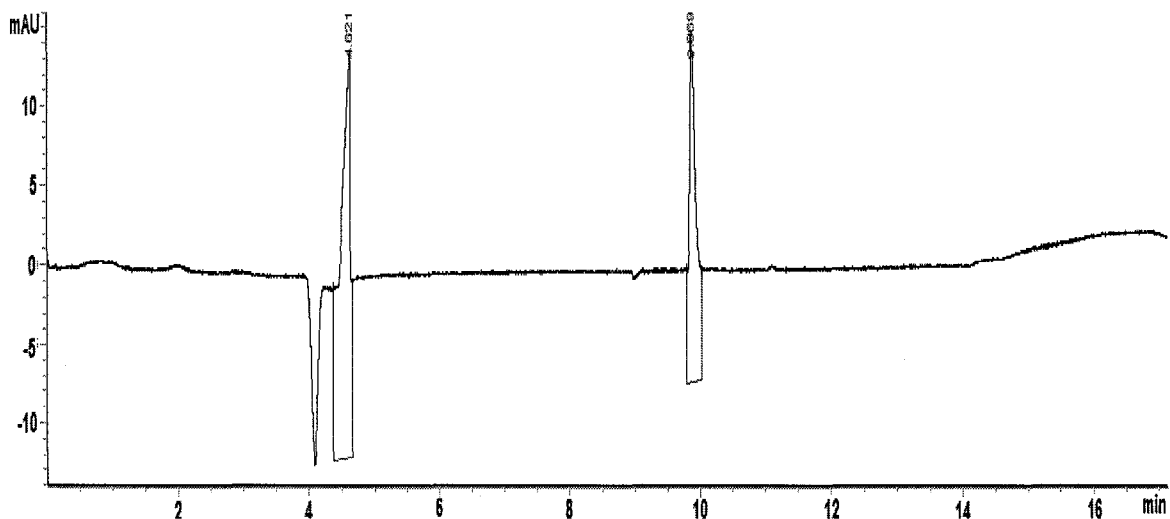
Figure 35: Electrochromatogram of human serum albumin on the C18 capillary, 50 μm

i.d, l = 58.5 cm, l_{eff} = 50.0 cm, pH 3.0 (1:10), applied voltage = 25 kV, hydrodynamic

injection at 50 mbar pressure for 8 s and detection at 210 nm.

4.2 Metalloproteinases on C5, C18 and bare capillaries

Metalloproteinases are family of enzymes which can be classified by the most important functional group in their active site. Matrix Metalloproteinases (MMPs) are extra cellular proteases that cleave a wide variety of substrates such as basement membrane, extra cellular matrix components, death factors, cytokines, cell and matrix adhesion molecules. These MMPs are expressed as diagnostic markers for diseases like cancer, angiogenesis, arthritis, inflammation, periodontal disease, emphysema, multiple necrosis and chronic wounds. The common structure of MMPs consist of a predomain, a prodomain, a catalytic domain and a C-terminal hemopexin domain. The catalytic domain is the only active site of the MMPs. The zinc ion is bonded coordinately to the catalytic domain and is activated by breaking one of the coordinate bonds via elimination of the prodomain by a proteolytic enzymes. This section involves the results and discussion on some of the MMPs analyzed in this research work.

(i) MMP multipack 1: Multipack 1 is a pack of 2μg each of active recombinant catalytic domains of MMP-1, MMP-8, MMP-13, and MMP-9 formed by E. coli bacteria. MMP 1, 8, and13 are collagenases and MMP-9 is a gelatinase enzyme. These MMPs are involved in the study of enzyme kinetics, comparative studies of substrate and inhibitor specificities, and cleavage of target proteins. These enzymes lack a C-terminal hemopexin domain so they cannot cleave collagen molecules but are able to cleave gelatin, casein, and other peptide substrates [13]. Multipack 1 is not a mixture of the above mentioned MMPs but involves separate MMP compounds. Table 8 shows the list of MMP multipack 1 and their molecular weights.

Collagenases in general are enzymes that break a peptide bond in collagen. Collagenase production is increased during an immune response by cytokines, which encourage cells such as fibroblasts and osteoblasts, and cause indirect tissue damage. Gelatinases are proteolytic enzymes that hydrolyse gelatin into subcompounds like polypeptides, peptides, and amino acids that can cross the cell membrane and be used by the organism [14].

Table 8: List of MMP multipack 1 and their molecular weights

MMP	Group Name	Molecular weight
MMP-1	Collagenase	19.9 kDa.
MMP-8	Collagenase	20.3 kDa.
MMP-9	Gelatinase	39.0 kDa.
MMP-13	Collagenase	20.4 kDa.

(ii) MMP multipack 2: Multipack 2 is a pack of 2μg each of the active recombinant catalytic domain of MMP-7, MMP-12, MMP-3, MMP-10 and MMP-11 formed by E. coli bacteria. MMP-7 is classified as a matrilysin enzyme; MMP-12 is a metalloelastase; and MMP 3, 10, and 11 are stromelysin enzymes. These enzymes are used for the study of enzyme kinetics, to cleave target substrates and for screening of inhibitors. Multipack 2 also involves separate MMP compounds. Table 9 shows the list of MMP multipack 2 and their molecular weights.

Matrilysin is expressed in a variety of tumors ranging from adenomas to carcinomas and adenocarcinomas of the breast, colon, stomach, lungs, and skin; where it may be involved in tumor formation as well as tumor degradation. Metalloelastase is implicated in elastin degradation and macrophage migration in many pathological conditions. It generates angiostatin and prevents tumor angiogenesis. Transformed epithelial cells express MMP-12 in skin cancer. Stromelysins degrade proteoglycan core proteins, fibronectin, elastin, gelatin and several collagen types which participates in wound healing, tumor invasion and inflammation [14].

Table 9: List of MMP multipack 2 and their molecular weights

MMP	Group Name	Molecular Weight
MMP-7	Matrilysin	20.4 kDa.
MMP-3	Stromelysin	19.5 kDa.
MMP-10	Stromelysin	19.4 kDa.
MMP-11	Stromelysin	19.3 kDa.
MMP-12	Metalloelastase	20.3 kDa

Figures 36, 37, and 38 show the graph of migration time vs pH for multipack 1 on the C5, C18 and bare capillaries. For MMP multipack 1 on the C5 column, the migration time for the MMPs decreased from the lowest pH value measured up to 3.72. Above pH 3.72, the migration time increased up to pH 7.08 and then again it decreased as the pH was raised to 8.10. The largest difference in migration times between individual MMPs was observed at pH 6.0 and 7.08. This difference could be due to difference in EOF

behavior at pH 6.0 and 7.08 or it could be due to stationary phase interactions of individual MMPs in these two buffers.

The graph for MMP multipack -1 on the C18 column decreased from the lowest value to pH 4.38 and then increased up to pH 7.08 and then again decreased to a pH of 8.10. It should be noted that the migration time for individual MMPs on the C18 column was greater than on the C5 column. This difference could be due to greater stationary phase interactions on the C18 column, which retains the compound for a longer period of time and leads to increased migration time.

The migration time vs pH graph for multipack 1 on the bare capillary column was different than the C5 and C18 capillaries. On the bare column, the migration time for MMP multipack 1 decreased from pH 2.10 to 4.38, and then it increased up to pH 6.0. But after pH 6.0, it decreased as the pH was raised to 8.10. This pattern on the bare capillary could be due to different EOF behavior on this column, since there are no stationary phase interactions present.

Based on the previous studies, the usual behavior on a bare column was a decrease in migration time with an increase in pH. The reason for this pattern on a bare column is due to deprotonation of silanols at higher pH, which increases the EOF and leads to a shorter migration time. But in this study, it was not clear that why the MMPs behave differently from the usual behavior on a bare column. It could be due to either changes in ionization or conformation of the protein. This observation should be investigated in future studies.

Figure 36: Migration time vs pH graph for MMP multipack 1 on the C5 capillary.

Figure 37: Migration time vs pH graph for MMP multipack 1 on the C18 capillary.

Figure 38: Migration time vs pH graph for MMP multipack 1 on the bare capillary.
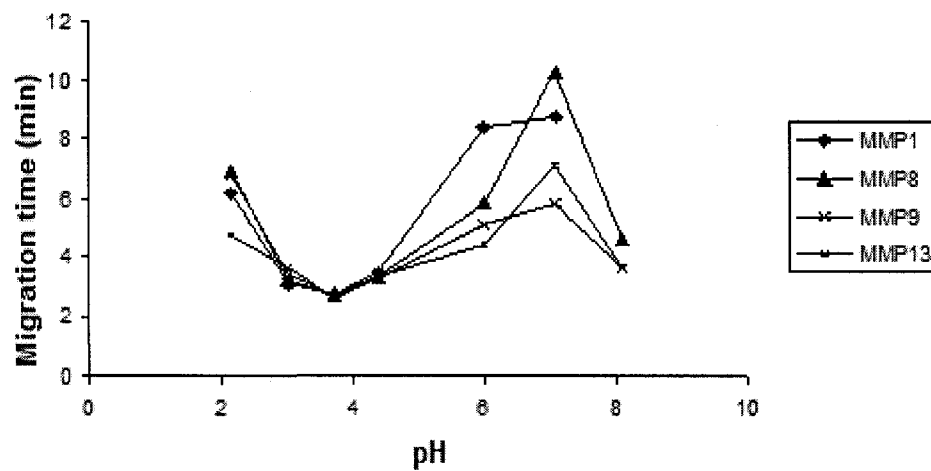

Figure 39: Migration time vs pH graph for MMP multipack 2 on the C5 capillary.
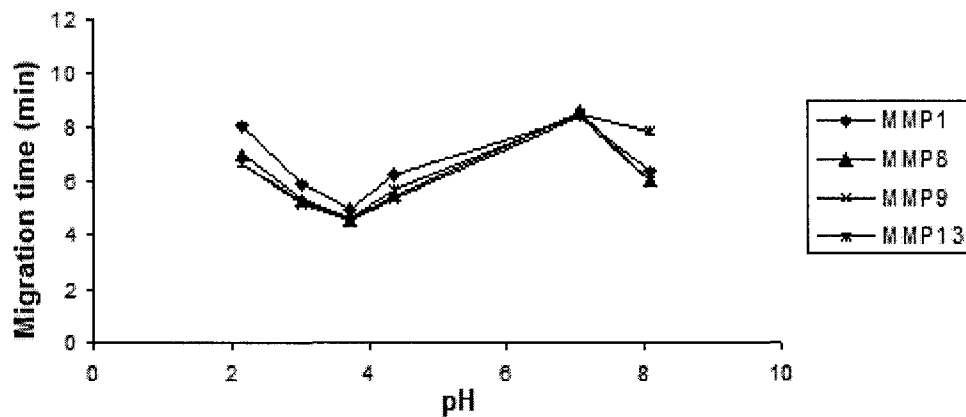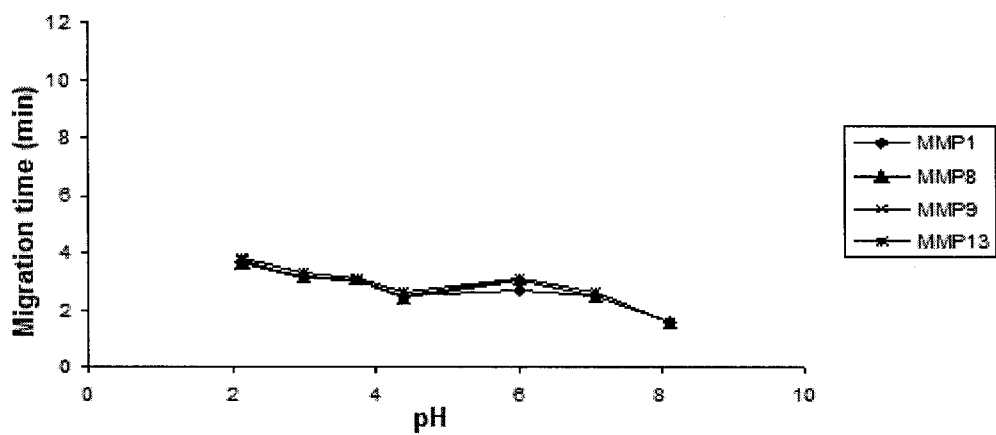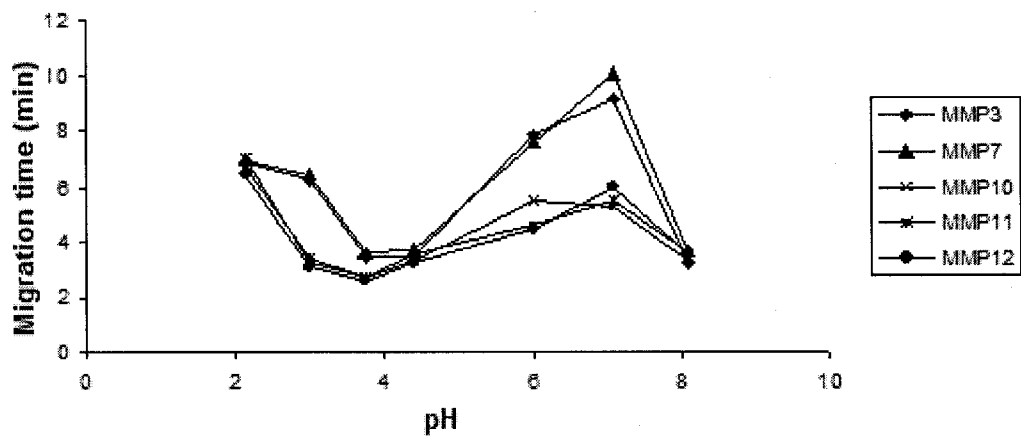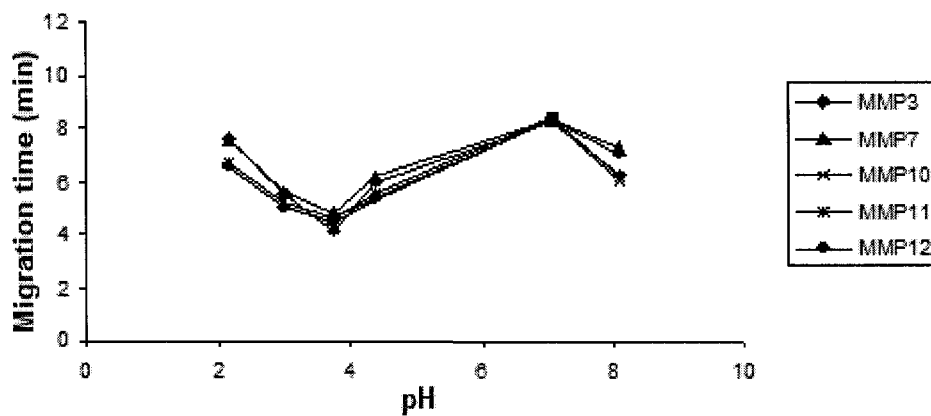
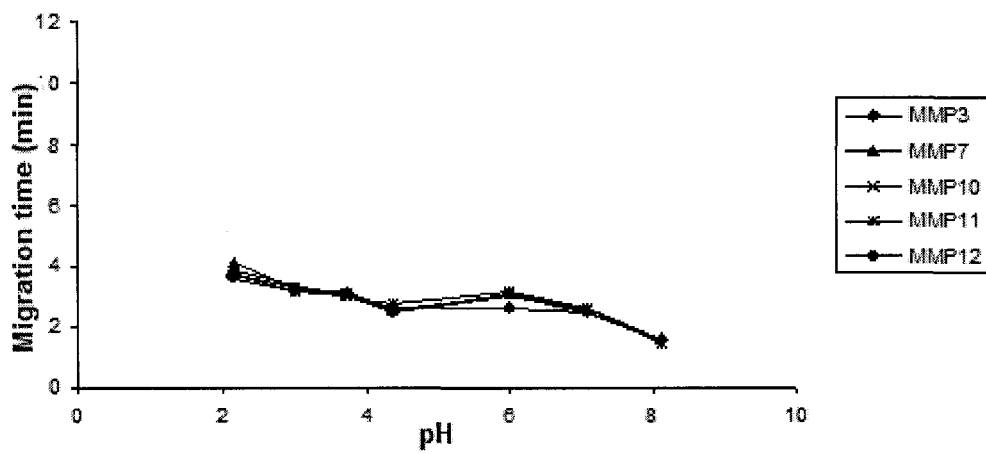Figure 40: Migration time vs pH graph for MMP multipack 2 on the C18 capillary.

Figure 41: Migration time vs pH graph for MMP multipack 2 on the bare capillary.

Figures 39, 40, and 41 show the graph of migration time vs pH for MMP multipack 2 on the C5, C18, and bare capillaries. The behavior of MMP multipack 2 was similar to that obtained for MMP multipack1 on the C5, C18, and bare capillaries. The reasons for this type of behavior for MMP multipack 2 are also due to different stationary phase interactions and electroosmotic flow behavior in three column surfaces. The migration time for both MMP multipack 1 and MMP multipack 2 decreases from C18 column to C5 column and then C5column to bare column. That means due to greater stationary phase interactions, C18 column retain the compounds for longer period of time than C5 and bare column, and C5 column retain the compounds for longer period of time than bare column. This in turn will effect the migration time for the compounds on three columns. The following are some of the chromatograms obtained for multipack 1 and multipack 2 on three different capillaries and some different pH buffers. The shape and migration time for these MMPs differs with different column surfaces and different pH buffers.

Figures 42, 43, and 44 show the chromatograms for MMP1 on the C5, C18, and bare capillaries at pH 2.14. The major peaks obtained on the C5 and C18 capillaries were sharp and symmetrical but the peak obtained on the bare capillary was slightly asymmetric. The peak obtained on the C18 column had a longer migration time compared to the C5 and bare columns. The C5 column had a longer migration time than the bare column. So it was clear here that a longer carbon chain resulted in a longer migration time. This observation means that the more hydrophobic C18 column resulted in stronger stationary phase interactions which retained the molecule for a longer time.

Figure 42: Electrochromatogram of MMP-1 on the C5 capillary, 50 µm i.d, l = 49.5 cm, l_{eff} =41.0 cm, pH 2.14 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 3 s and detection at 210 nm.

Figure 43: Electrochromatogram of MMP-1 on the C18 capillary, 50 µm i.d, l = 58.5 cm, l_{eff} = 50.0 cm, pH 2.14 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.

Figure 44: Electrochromatogram of MMP-1 on the bare capillary, 50 μm i.d, l = 51.4 cm, l_{eff} = 42.9 cm, pH 2.14 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.

Figures 45, 46, and 47 show the chromatograms for MMP1 on the C5, C18, and bare capillaries at pH 3.0. Under these conditions, single peaks were observed for MMP-1 on the C5 and C18 columns. The bare column gave two peaks, one major and one minor. The minor peak could be some impurity in the parent compound.

Figures 48, 49, and 50 show the chromatograms of MMP-3 on the C5, C18, and bare capillaries at pH 2.14. Single sharp peaks were obtained for MMP-3 on the C5and C18 columns. A small shoulder was observed on the major peak for the bare column. This shoulder was not well separated from the major peak and could be considered second peak on the bare column.

DAD1 A, Sig=210,18 Ref=off (C:\HPCE-D~1\VASU\HMP10001.D)
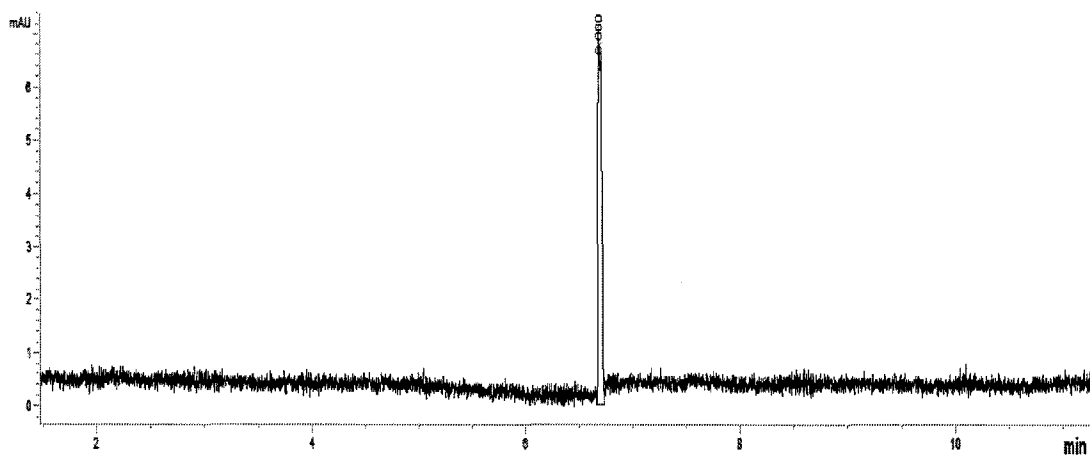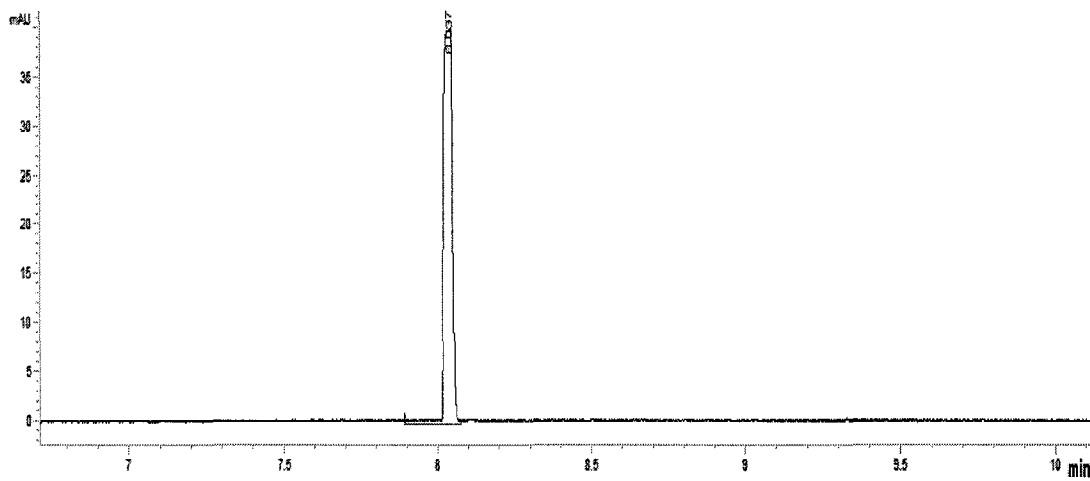
Figure 45: Electrochromatogram of MMP-1 on the C5 capillary, 50 μm i.d, l = 49.5 cm, l_{eff} =41.0 cm, pH 3.0 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 3 s and detection at 210 nm.

DAD1 A, Sig=210,18 Ref=off (C:\HPCE-D~1\VASU\FMMP1003.D)

Figure 46: Electrochromatogram of MMP-1 on the C18 capillary, 50 μm i.d, l = 58.5 cm, l_{eff} = 50.0 cm, pH 3.0 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.
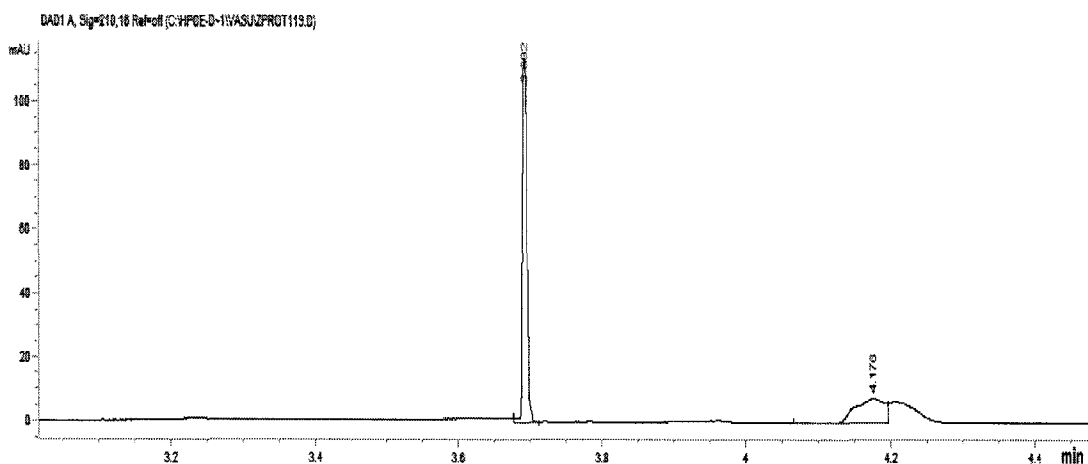
Figure 47: Electrochromatogram of MMP-1 on the bare capillary, 50 μm i.d, l = 51.4 cm, l$_{eff}$ = 42.9 cm, pH 3.0 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.
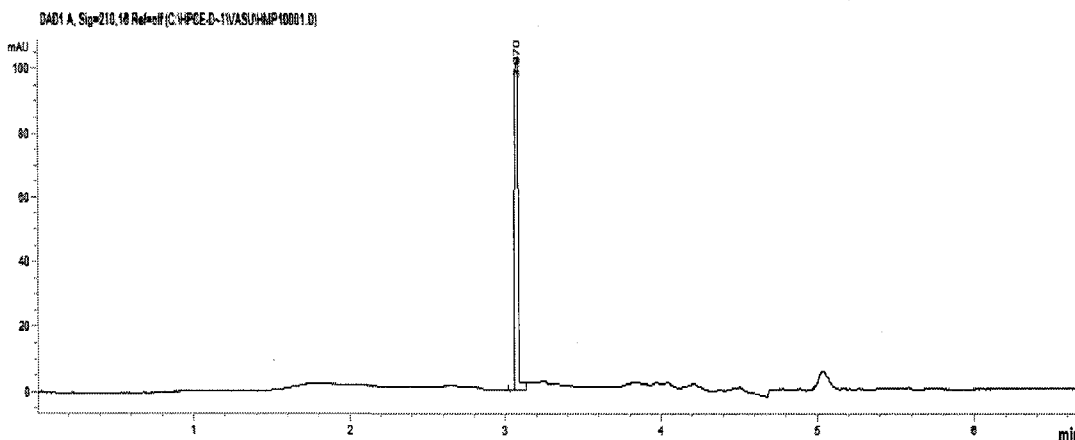

Figure 48: Electrochromatogram of MMP-3 on the C5 capillary, 50 μm i.d, l = 49.5 cm, l$_{eff}$ =41.0 cm, pH 2.14 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 3 s and detection at 210 nm
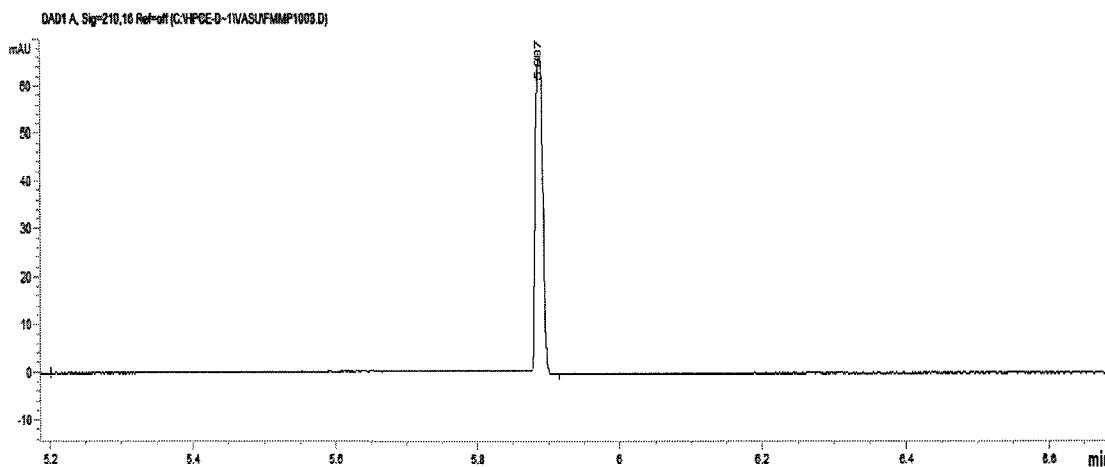
Figure 49: Electrochromatogram of MMP-3 on the C18 capillary, 50 μm i.d, l = 58.5 cm, l_{eff} = 50.0 cm, pH 2.14 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.
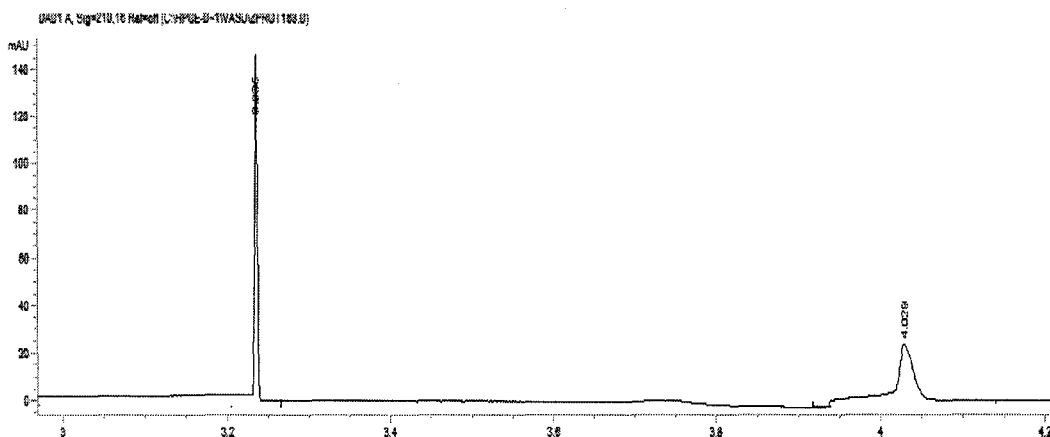

Figure 50: Electrochromatogram of MMP-3 on the bare capillary, 50 μm i.d, l = 51.4 cm, l_{eff} = 42.9 cm, pH 2.14 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.

Figures 51, 52, and 53 are the chromatograms for MMP-3 on the C5, C18, and bare capillaries at pH 8.10. The peaks obtained for MMP-3 at pH 8.10 are asymmetric in shape. The peaks are somewhat broad with a negative dip on the right side, which is not considered very useful for quantitative determinations.

Overall the differences in the migration times and peak shape suggest that the three columns have different separation capabilities. These results were also found to be reproducible on three consecutive injections of this compound.
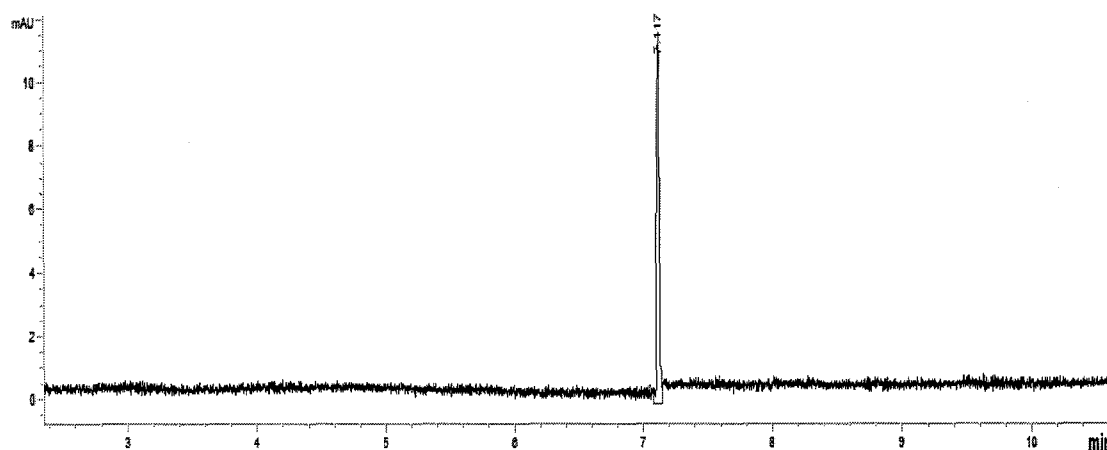

Figure 51: Electrochromatograms of MMP-3 on the C5 capillary, 50 μm i.d, l = 49.5 cm, l_{eff} =41.0 cm, pH 8.10 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210, 214, and 223 nm.
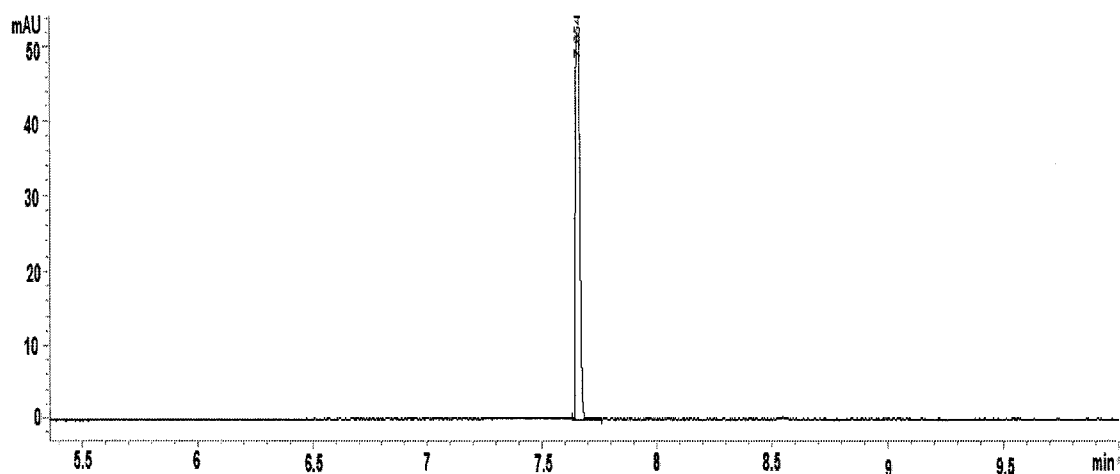
Figure 52: Electrochromatograms of MMP-3 on the C18 capillary, 50 μm i.d, l = 58.5 cm, l_{eff} = 50.0 cm, pH 8.10 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210, 214, and 223 nm.
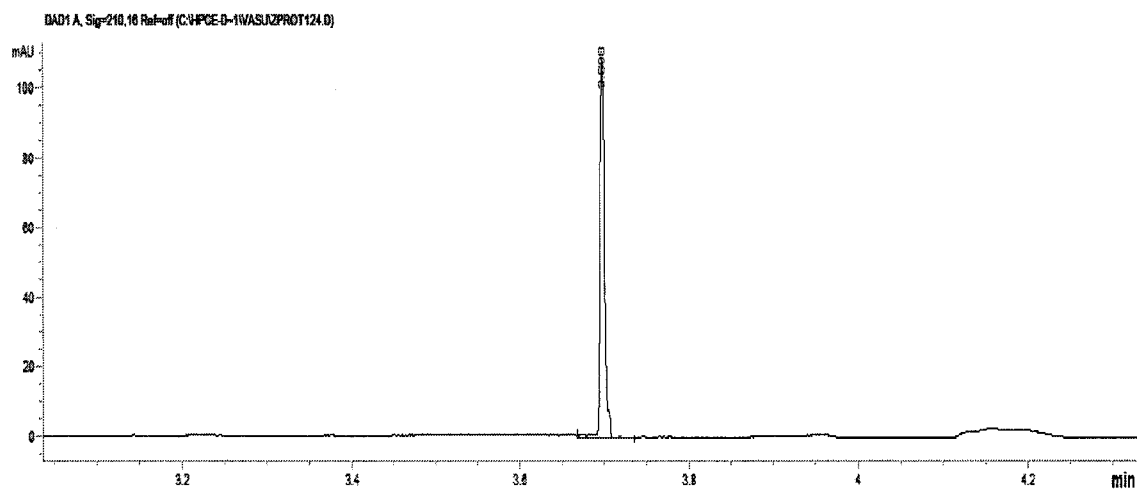

Figure 53: Electrochromatograms of MMP-3 on the bare capillary, 50 μm i.d, l = 51.4 cm, l_{eff} = 42.9 cm, pH 8.10 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210, 214, and 223 nm.

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Figures 54, 55, and 56 are electrochromatograms for MMP-8 on the C5, C18 and bare capillaries at pH 2.14. At this pH, the C5 and C18 columns gave a single, narrow sharp peak; and the bare capillary gave one major peak and one minor peak. This minor peak observed on the bare column was not well separated from the major peak. The migration time was observed to be less on the bare capillary as compared to the C5 and C18 capillaries. It is believed that this is due to greater EOF and negligible stationary phase interactions on the bare column.
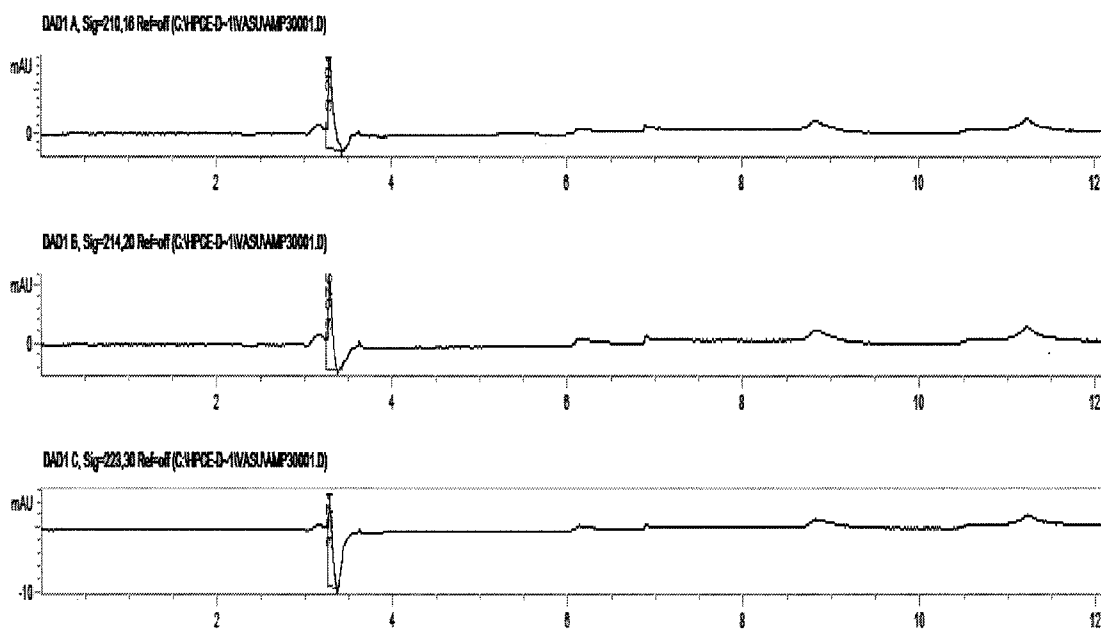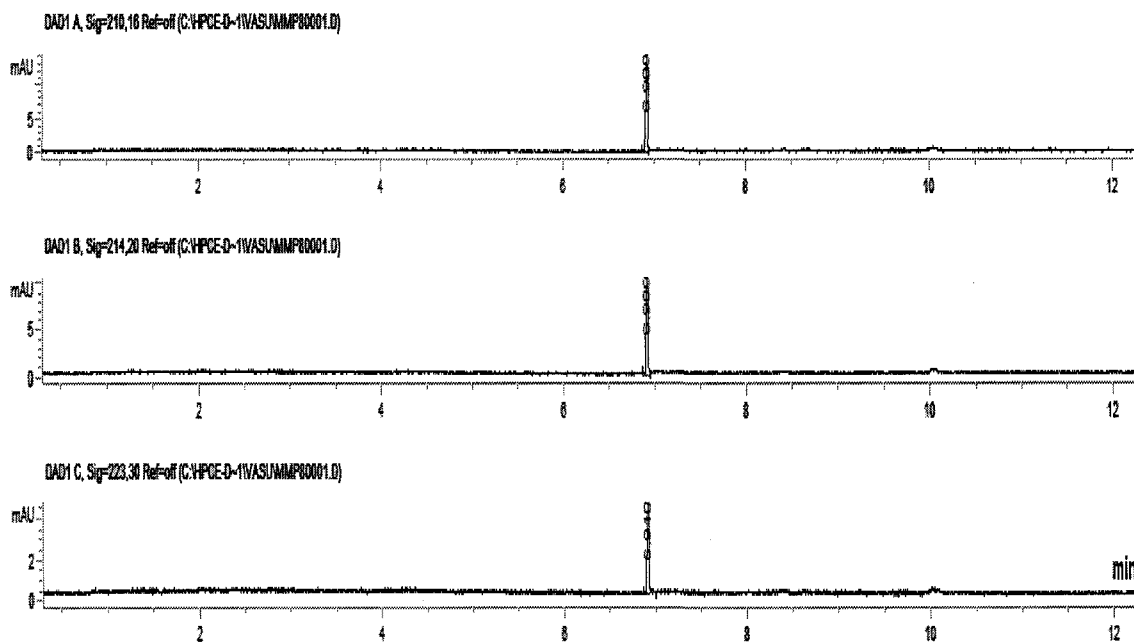

Figure 54: Electrochromatograms of MMP-8 on the C5 capillary, 50 μm i.d, l = 49.5 cm, l_{eff} = 41.0 cm, pH 2.14 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210, 214, and 223 nm.
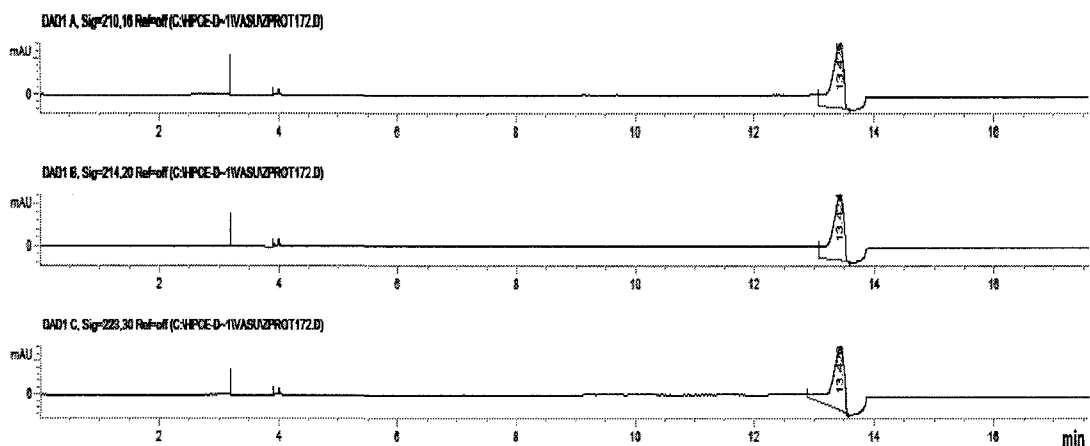
60
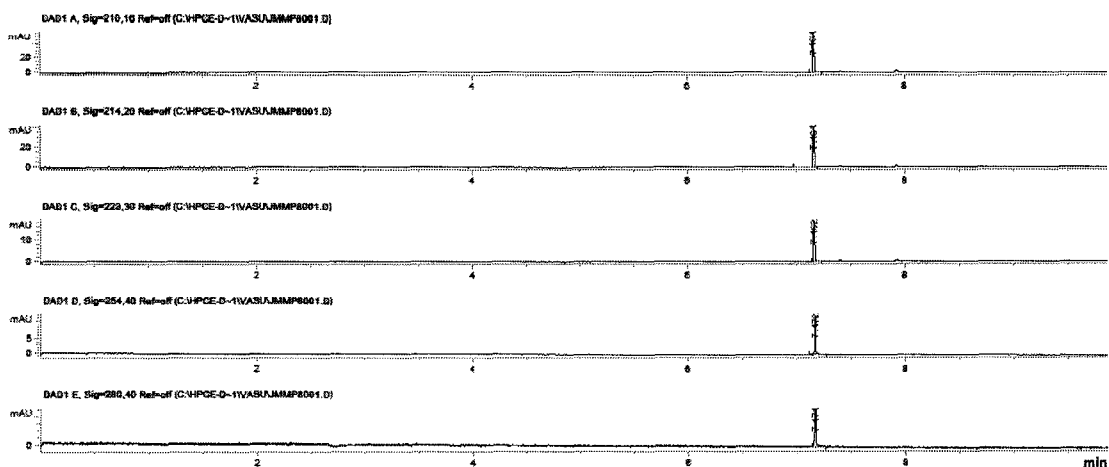
Figure 55: Electrochromatograms of MMP-8 on the C18 capillary, 50 μm i.d, l = 58.5 cm, l_eff = 50.0 cm, pH 2.14 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210, 214, 223, 254, and 280 nm.
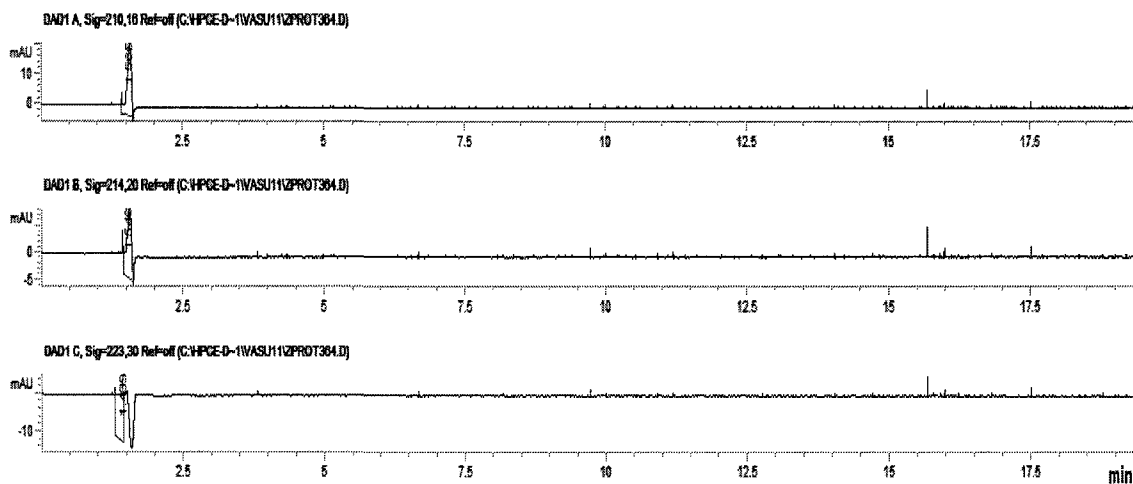

Figure 56: Electrochromatograms of MMP-8 on the bare capillary, 50 μm i.d, l = 51.4 cm, l_eff = 42.9 cm, pH 2.14 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210, 214 and 223, 254, and 270 nm.

Figures 57, 58, and 59 are the electrochromatograms for MMP-8 on the C5, C18

and bare columns at pH 7.08. Here, the peaks obtained on C5 and C18 columns were

broader than the peak obtained on the bare column. Broad peaks on the C5 and C18

capillaries could be due to stationary phase interactions inside the capillary, which

retained the compound for longer period of time. The uneven baseline observed in these

chromatograms could be due to solvent effects or could be some small variations in

voltage or current during analysis. The results obtained for MMP-8 at this pH were found

to be reproducible on three consecutive injections of this compound.
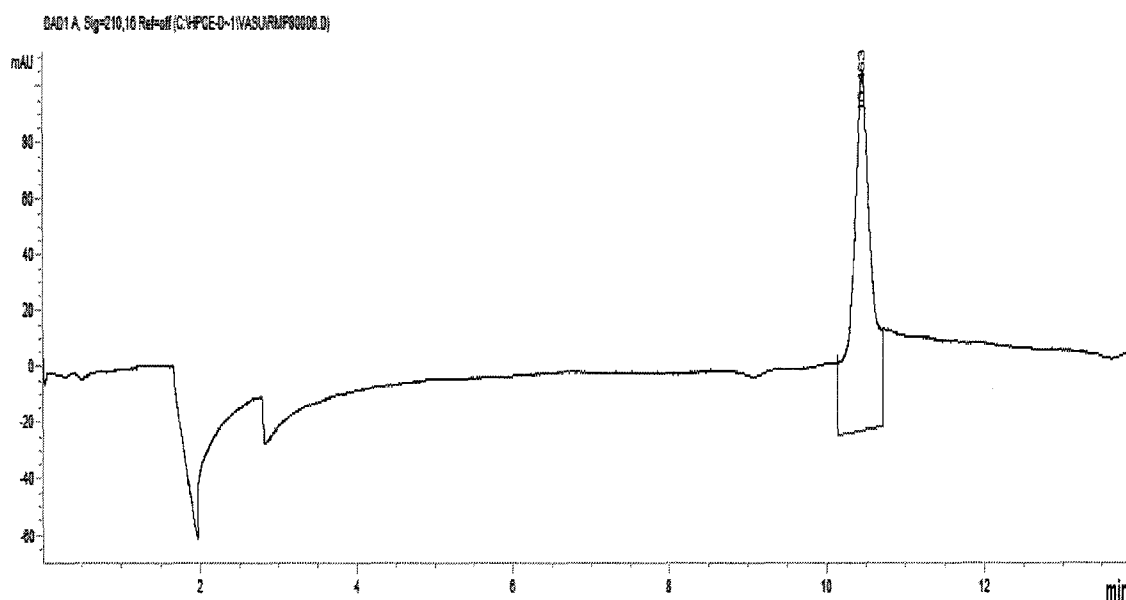
Figure 57: Electrochromatogram of MMP-8 on the C5 capillary, 50 μm i.d, 1 = 49.5 cm,

l_{eff} =41.0 cm, pH 7.08 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50

mbar pressure for 8 s, and detection at 210 nm.

Figure 58: Electrochromatogram of MMP-8 on the C18 capillary, 50 μm i.d, l = 58.5 cm, l_{eff} = 50.0 cm, pH 7.08 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.
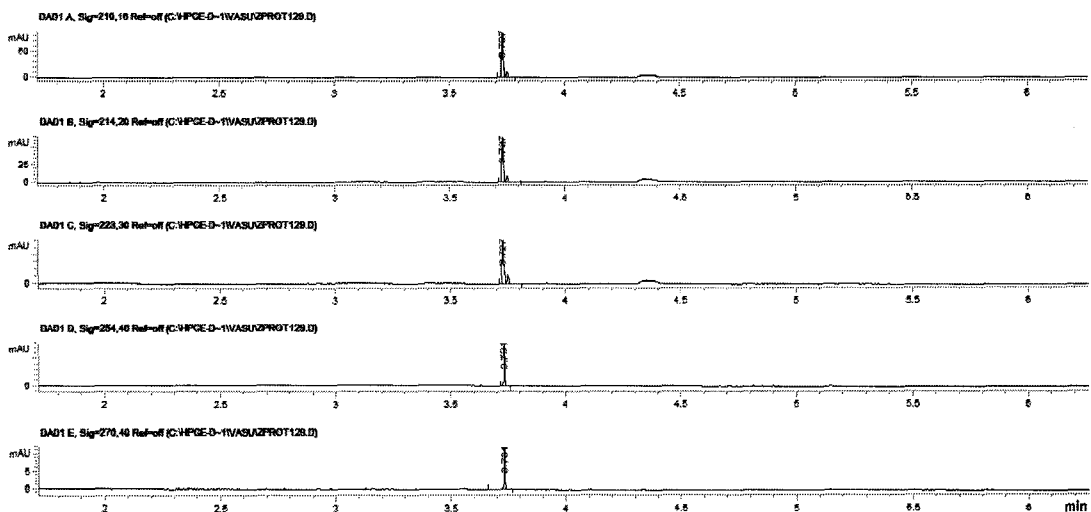

Figure 59: Electrochromatogram of MMP-8 on the bare capillary, 50 μm i.d, l = 51.4 cm, l_{eff} = 42.9 cm, pH 7.08 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.

Figures 60, 61, 62, 63, 64, and 65 are the chromatograms for MMP-10 on C5,

C18, and bare capillaries at pH 3.72 and 3.0. As shown in Figures 60, 61 and 62, two

peaks were observed; one major and one minor at 210, 214, and 223 nm. The main point

is that the minor peak had relatively low intensity above 223 nm. The minor peak for

MMP-10 cannot be easily detected at higher wavelengths. Regarding the migration time

on the three columns, the bare column eluted the compound faster than C5 and C18

columns.

Figure 60: Electrochromatograms of MMP-10 on the C5 capillary, 50 μm i.d, l = 49.5

cm, l_{eff} =41.0 cm, pH 3.72 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50
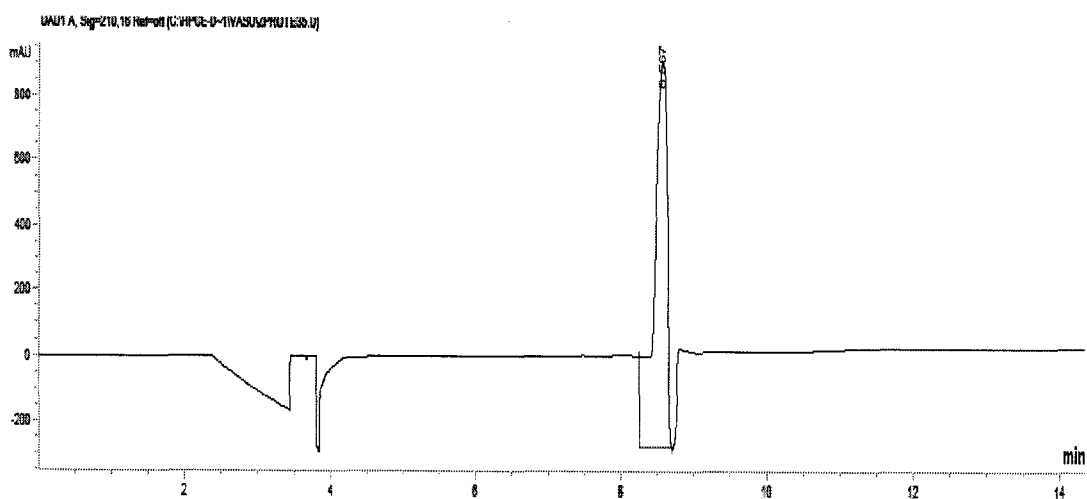
mbar pressure for 8 s and detection at 210, 214, 223, 254, and 270 nm.

Figure 61: Electrochromatograms of MMP-10 on the C18 capillary, 50 μm i.d, l = 58.5 cm, l_{eff} = 50.0 cm, pH 3.72 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210, 214, 223, 254, and 270 nm.
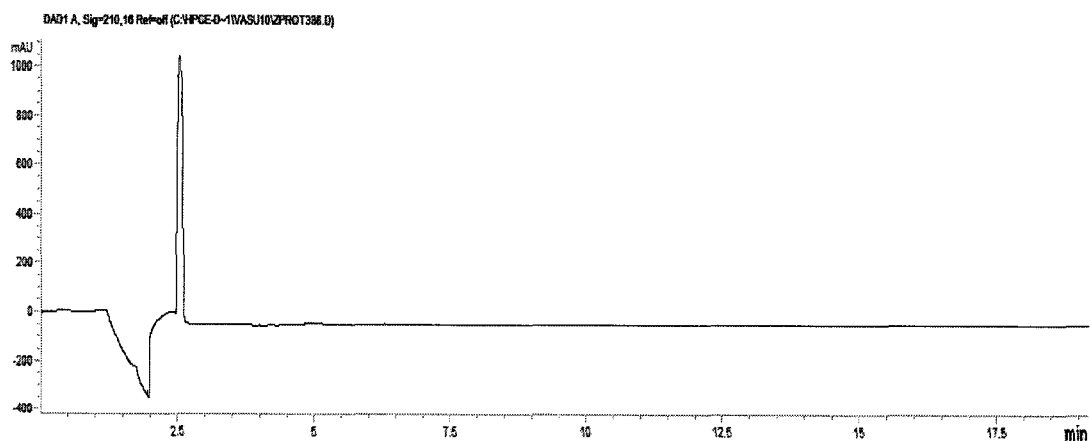

Figure 62: Electrochromatograms of MMP-10 on the bare capillary, 50 μm i.d, l = 51.4 cm, l_{eff} = 42.9 cm, pH 3.72 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210, 214, 223, 254, and 270 nm.

In the electrochromatograms shown in Figures 63, 64, and 65, two peaks were obtained for MMP-10 at pH 3.0 on the three columns: one major and one minor. The major peak was narrow and the minor peak was slightly broader than the major peak. The minor peak could be some impurity in the sample, which can be detected at this wavelength. The migration time for MMP-10 decreased when comparing the C18 to the C5 to the bare capillaries. This was again due to greater stationary phase interactions on C18 column and negligible interactions in the bare capillary column. Thus the variable migration times and peak shapes obtained for MMP-10 were due to three different surfaces each having its own separation capabilities.
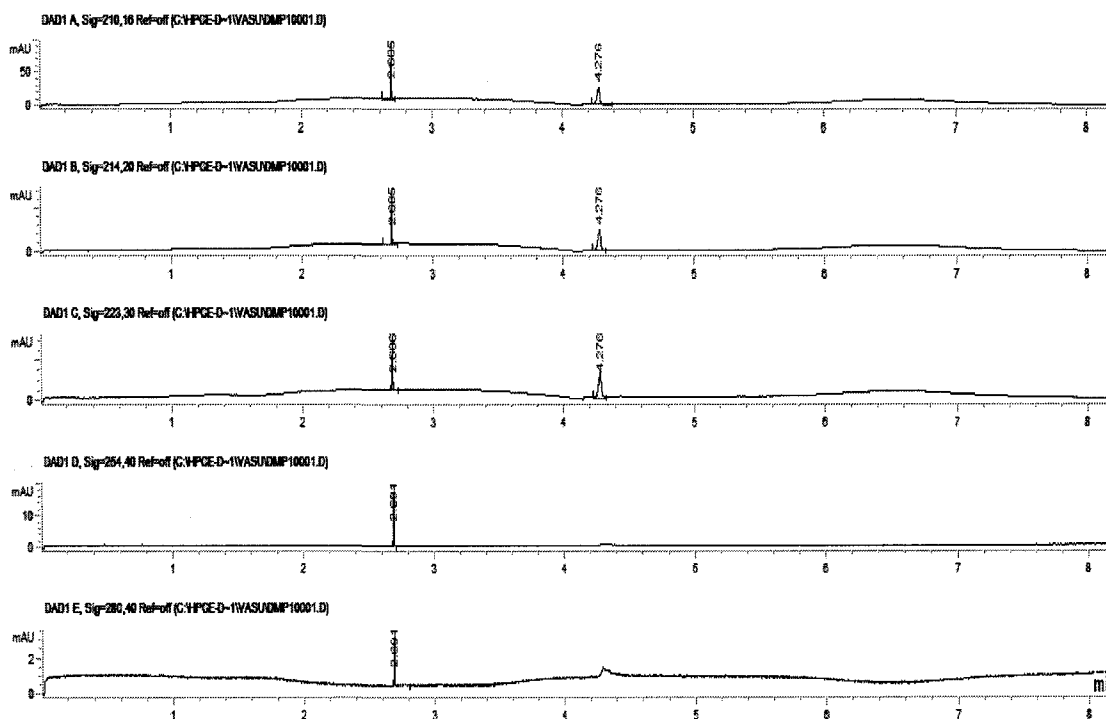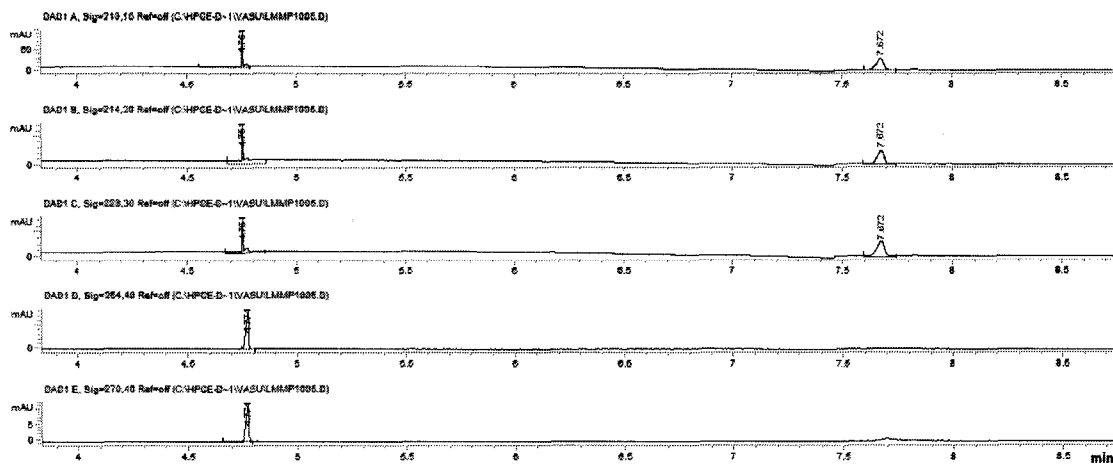

Figure 63: Electrochromatogram of MMP-10 on the C5 capillary, 50 μm i.d, $l = 49.5$ cm, $l_{eff} = 41.0$ cm, pH 3.0 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.

Figure 64: Electrochromatogram of MMP-10 on the C18 capillary, 50 μm i.d, l = 58.5 cm, l_{eff} = 50.0 cm, pH 3.0 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.
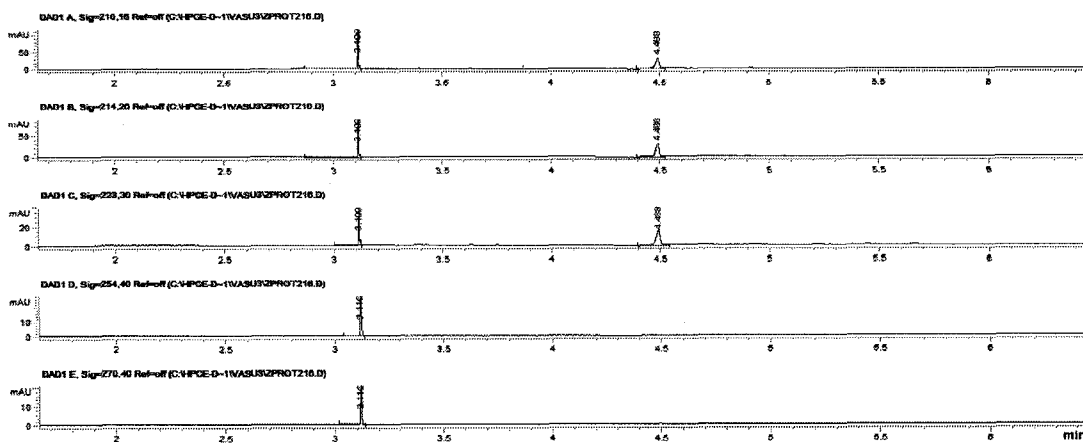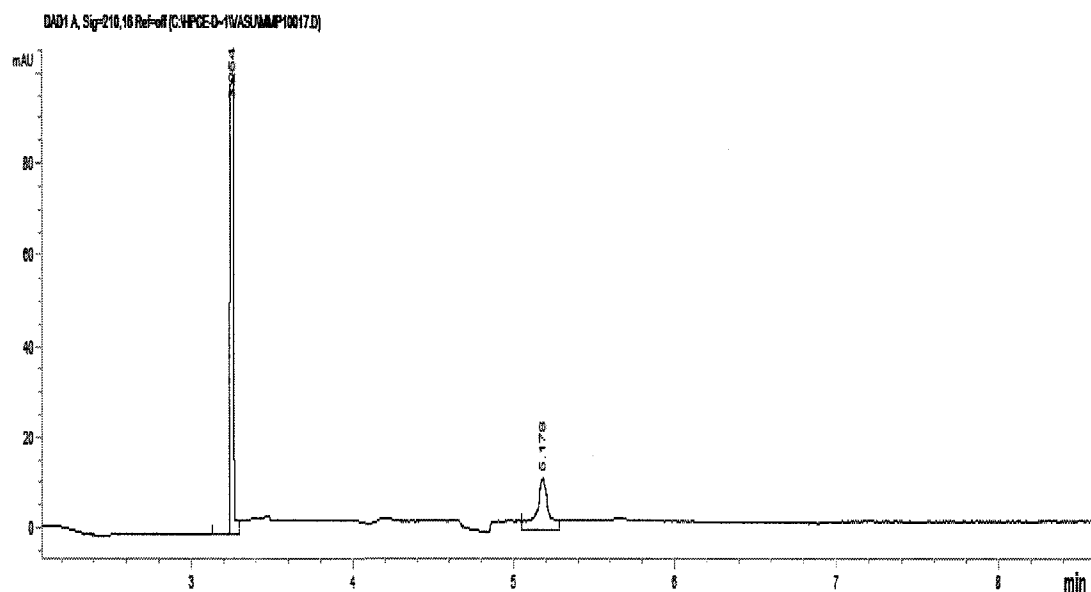

Figure 65: Electrochromatogram of MMP-10 on the bare capillary, 50 μm i.d, l = 51.4 cm, l_{eff} = 42.9 cm, pH 3.0 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.

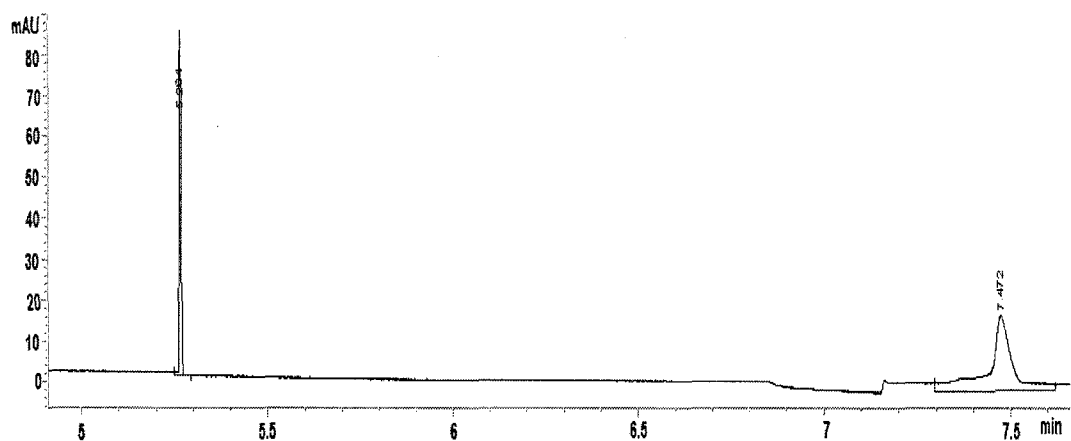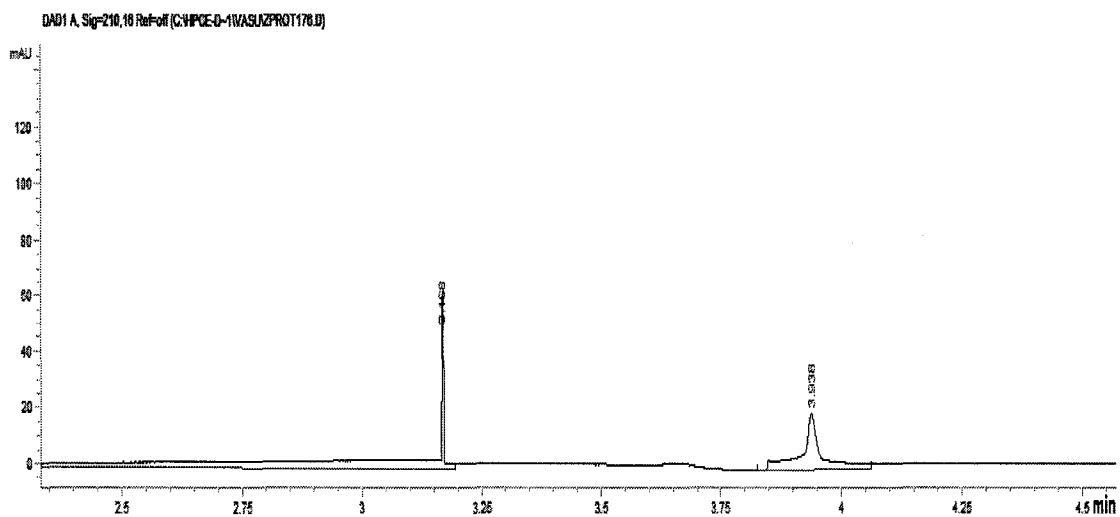**iii) TIMP/MMP standard mixtures:** TIMP is the abbreviation for the tissue inhibitor of metalloproteinases. Standard mixtures of MMP-2/TIMP-2, MMP-3/TIMP-1 and MMP-3/TIMP-2 were also investigated in this research project. TIMP-1 is a tissue inhibitor of many MMPs and has a molecular weight of 28 kDa. It can also inhibit angiogenesis and promote cell growth. TIMP-2 is a tissue inhibitor of metalloproteinase-2 and has a molecular weight of 22 kDa. TIMP-2 inhibits most MMPs but particularly MMP-2. It can also inhibit angiogenesis and promote cell growth [13].

Figures 66 and 67 show the chromatograms for TIMP-2/MMP-2 on the C18 column at pH 2.14 and 3.72. In the chromatograms, the minor peak obtained was assigned to MMP-2 and major broad peak was for TIMP-2. In both chromatograms, the two peaks were well resolved. The broad peak for TIMP-2 could be due to stationary phase interactions of the C18 column, which retained TIMP-2 for longer period of time.

Figures 68 and 69 show the chromatograms for TIMP-2/MMP-3 on the C18 column at pH 2.14 and 3.72. In these chromatograms, the two peaks were well separated but the peak for TIMP-2 was asymmetric as compared to the peak obtained for MMP-3. This is again due to stationary phase interactions which lowers the rate of mass transfer and results in a broader peak.

Figures 70 and 71 show the chromatograms of TIMP-1/MMP-3 on the C18 column at pH 2.14 and 3.72. In these chromatograms, the two peaks were well separated but the peak for TIMP-1 was broader and asymmetric as compared to MMP-3. The C5 and bare columns were not successful in separation of these standard mixtures of TIMP/MMPs. So only C18 column can be used for separation of these mixtures.

Figure 66: Electrochromatogram of TIMP-2/MMP-2 mixture on the C18 capillary, 50 μm i.d, l = 58.5 cm, l$_{eff}$ = 50.0 cm, pH 2.14 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.

Figure 67: Electrochromatogram of TIMP-2/MMP-2 mixture on the C18 capillary, 50 μm i.d, l = 58.5 cm, l$_{eff}$= 50.0 cm, pH 3.72 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.
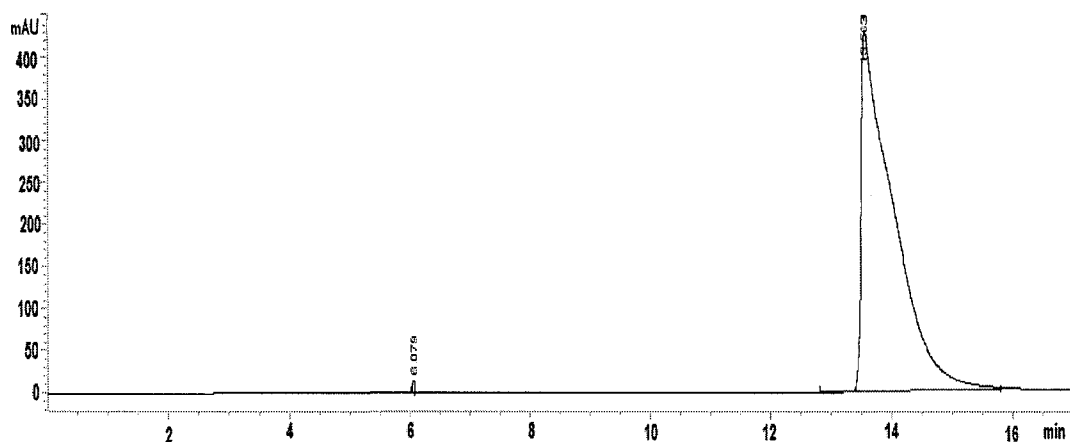
Figure 68: Electrochromatogram of TIMP-2/MMP-3 mixture on the C18 capillary, 50 μm

i.d, l = 58.5 cm, l_{eff} = 50.0 cm, pH 2.14 (1:10), applied voltage = 25 kV, hydrodynamic

injection at 50 mbar pressure for 8 s and detection at 210 nm.
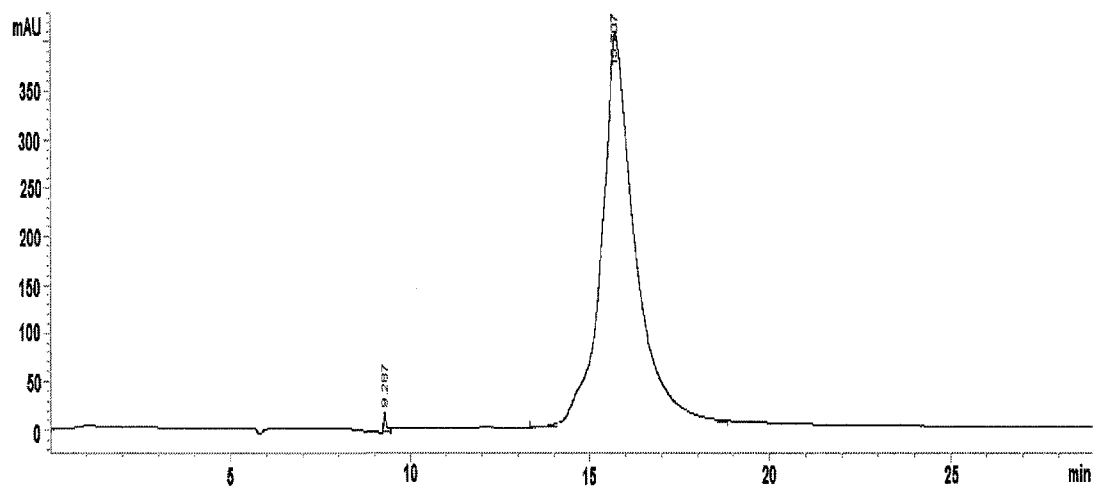
Figure 69: Electrochromatogram of TIMP-2/MMP-3 mixture on the C18 capillary, 50 μm

i.d, l = 58.5 cm, l_{eff}= 50.0 cm, pH 3.72 (1:10), applied voltage = 25 kV, hydrodynamic

injection at 50 mbar pressure for 8 s and detection at 210 nm.

70
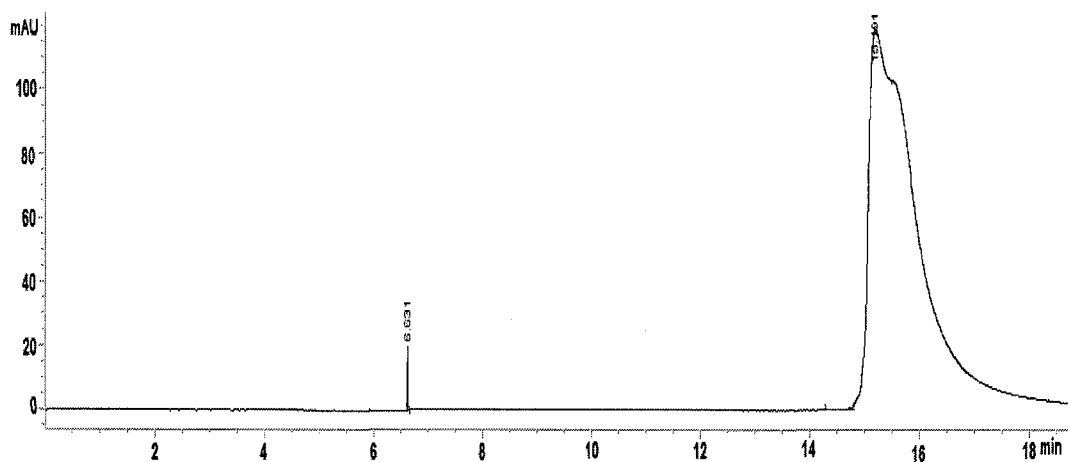
Figure 70: Electrochromatogram of TIMP-1/MMP-3 mixture on the C18 capillary, 50 μm i.d, l = 58.5 cm, l_{eff} = 50.0 cm, pH 2.14 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.
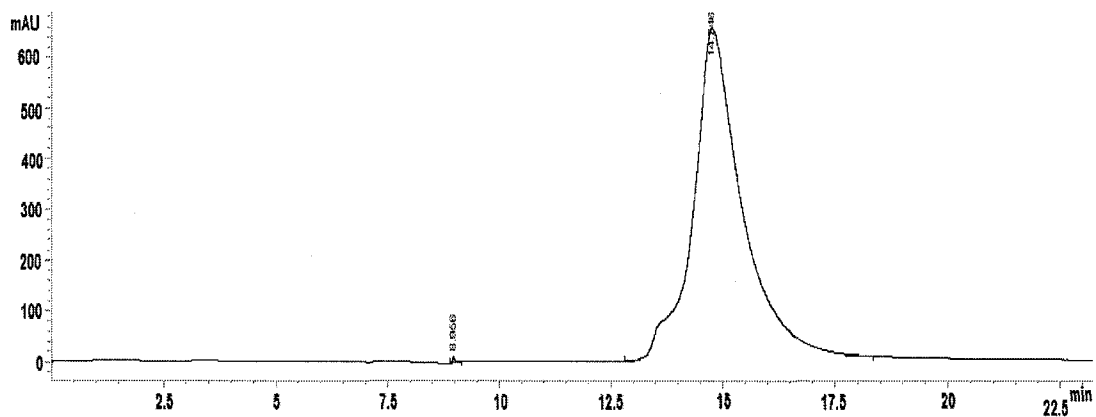

Figure 71: Electrochromatogram of TIMP-1/MMP-3 mixture on the C18 capillary, 50 μm i.d, l = 58.5 cm, l_{eff} = 50.0 cm, pH 3.72 (1:10), applied voltage = 25 kV, hydrodynamic injection at 50 mbar pressure for 8 s and detection at 210 nm.
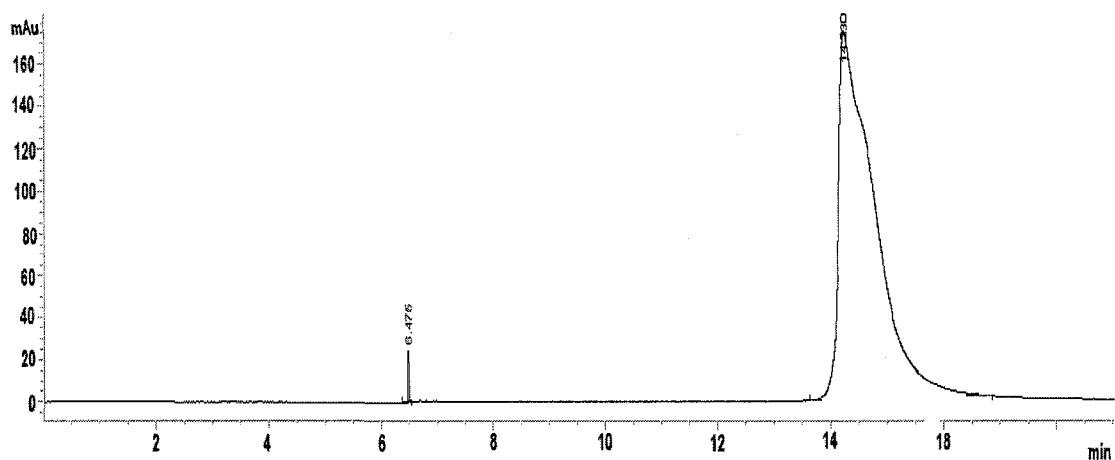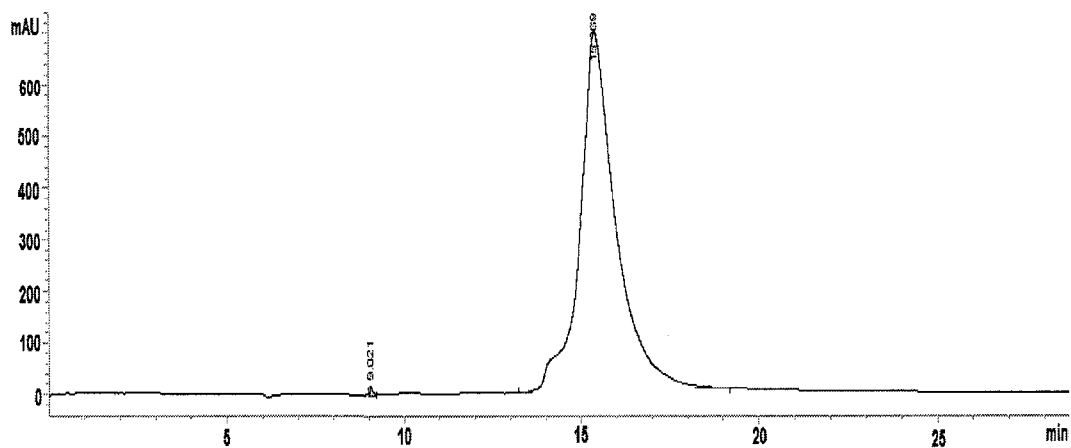
5. CONCLUSION

The modification of capillaries by the silanization/hydrosilation procedure is a useful approach for the development of capillary electrochromatography. The C5 and C18 capillaries made using this technique were successful for analyzing proteins and metalloproteins using appropriate buffers and experimental conditions. In order to obtain a better comparison, proteins were also analyzed on a bare capillary as well. As a result, some proteins gave good results on the C5 capillary; some proteins gave good results on the C18 capillary while some had optimal performance on the bare capillary. Because of the heterogeneous nature of proteins, each protein behaves differently depending on the buffer and capillary column selected. The variable results obtained on the three columns confirms that the three surfaces are different with each having unique separation capabilities.

In summary, transferrin gave good results on the C5 capillary at pH 4.38 and 8.10 in comparison to the C18 and bare capillaries. For human IgG, the C5 capillary can be considered good at pH values of 3.72 and 4.38. Carbonic anhydrase can be analyzed best on the C5 capillary at pH 3.0 and on the C18 column at pH 7.0. Symmetrical sharp peaks can be obtained for myoglobin on the C5 capillary at pH 3.0, on the C18 capillary at pH 8.10 and on the bare capillary at pH 4.38. The C18 and bare capillaries were good for human serum albumin at pH 2.14. The behavior of all MMPs were also different on three capillaries. The migration time vs pH graphs for MMPs show different behavior on three columns. And the chromatograms show different shapes and migration times for these

MMPs on the three columns. For standard TIMP/MMP mixtures, the C5 and bare columns were not considered good for separation of these mixtures at any pH values tested. However, the C18 capillary can be useful for separation of TIMP/MMP standard mixtures. It has also been noticed that pH 6.0 did not give any results on C18 column for these compounds. This investigation was successful in determining the optimum conditions for the analysis of these proteins under appropriate conditions, but further studies are needed to improve understanding of how etched chemically modified capillaries function in open tubular CEC.

6. FUTURE STUDIES

Future studies can be done using different organic solvents during analysis to improve the quality of the peaks obtained and perhaps to resolve additional components. The use of a variety of detectors such as a mass spectrometer and fluorescence can be helpful in studying the unknown peaks obtained during analyses.

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