

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE
ENGINEERING AND TECHNOLOGY

**MELITTIN, A MAJOR COMPONENT OF BEE VENOM: DETERMINATION
BY CAPILLARY ELECTROPHORETIC METHODS**



M.Sc. THESIS

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Department of Chemistry

Chemistry M.Sc. Programme

MAY, 2018

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

**KAPİLER ELEKTROFORETİK YÖNTEMLERLE ARI ZEHRİNDEKİ MAJOR
BİLEŞEN MELİTTİNİN ANALİZİ**

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To my family,



FOREWORD

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ABBREVIATIONS

pH	: Hydrogen Ion Concentration
BGE	: Back Ground Electrolyte
CE	: Capillary Electrophoresis
CZE	: Capillary Zone Electrophoresis
EKC	: Electrokinetic Chromatography
EOF	: Electroosmotic Flow
HPLC	: High Performance Liquid Chromatography
LOD	: Limit of Detection
LOQ	: Limit of Quantification
MCD	: Mast Cell Degranulation
MEKC	: Micellar Electrokinetic Chromatography
MELT	: Melittin
MS	: Multiple Sclerosis
PLA2	: Phospholipase A2
SDS	: Sodium Dodecyl Sulfate
TTAB	: Tetradecyltrimethylammonium Bromide
UV-VIS	: Ultraviolet-Visible Spectral Region



SYMBOLS

E	: Applied Electric Field
F_E	: The Electrical Force
F_F	: The Fractional Force
r	: Ion Radius
V	: The Ion Velocity
q	: Ion Charge
ε	: Dielectric Constant
π	: Pious
η	: Solution Viscosity
μ_{ep}	: Electrophoretic Mobility
μ_{eo}	: Electroosmotic Flow
ζ	: Zeta Potential



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MELITTIN, A MAJOR COMPONENT OF BEE VENOM: DETERMINATION BY CAPILLARY ELECTROPHORETIC METHODS

SUMMARY

In recent times, bee venom from honey bees (*Apis Mellifera*) have become subject of excessive pharmacological and biological investigation because of its form of alternative and preventive medication for treatment of variety of ailments. Numerous studies have been showed its capability in treating arthritis, rheumatism, pain, angiocardopathy, cancerous tumors, sclerosis and skin disease. Bee venom has been used since pheristoric times and in the ancient civilization of China, India, Egypt, Babylon, and Greece for apitherapy, but its composition was established few years ago. Bee venom is natural toxin that produced by the venom gland located in the abdominal cavity of a bee and contains considerable biologically active peptides, including melittin, apamin, adolapin, mast cell degranulation peptide, and enzymes such as phospholipase A2 and hyaluronidase, and also nonpeptides components such as histamine, dopamine and epinephrine. Melittin is the main component and the major pain producing substance of bee venom. It was first identified as a haemolytic factor by Habermann in 1954. Melittin comprises approximately 50% of dry weight of bee venom. It is a small linear peptide composed of 26 amino acids residues. The first twenty amino acids of this peptide are mostly apolar whereas the C-terminal hexapeptide is polar and highly basic.

Capillary electrophoresis (CE) is a modern analytical method that offers advantages of short analysis time and minimum consumption of both reagents and samples. It is well suited for the separation of aminoacids, peptides, proteins and has been used for this purpose. In CE, peptides function differently in terms of separation efficiency than small molecules. They differ in electric charge, relative molecular mass, conformation, hydrophobicity, and specific binding. Attributed to these properties, electrophoretic separation can be achived by differences in electrophoretic mobility, size, charge, hydrophobicity, specific interactions with other biomolecules.

The aim of this study is determination of mellitin content in bee venom via CE. It was observed that separation of basic peptide melittin has loss of efficiency because of adsorption on the internall surface on the capillary wall. The adsorption occurred due to the electrostatic interactions between positively charged residues of the basic peptide and negatively charged fused silica surface. Two strategies have been tried to prevent wall adsorption. The first is to supresss the negative surface charge of the silica capillary by lowering the pH of the separation buffer, and the other is coating the inner wall of capillary with a positively charged polymer to eliminate the wall negative charge.

This study deals with the determination of predominant melittin component using bare fused silica capillary and polymeric coated capillary electrophoresis, and critically compares these two approaches.



KAPİLER ELEKTROFORETİK YÖNTEMLERLE ARI ZEHRİNDEKİ MAJOR BİLEŞEN MELİTTİNİN ANALİZİ

ÖZET

Son zamanlarda, bal arıları (*Apis Mellifera*) tarafından üretilen arı zehri çeşitli hastalıkları alternatif tedavi edici ve önleyici olarak farmakolojik ve biyolojik alanlarda yoğun bir şekilde araştırılmaktadır.

Arı zehri de apiterapideki diğer arı ürünleri olan bal, polen, arı sütü, propolis ve balmumu gibi hastalıkların önleminde ve iyileştirilmesinde kullanılan destek ya da tedavi yöntemlerinden biridir.

Yapılan sayısız çalışmalar ve araştırmalar arı zehrinin eklem ağrısı, romatizma, ağrı, kalp damar sistemi hastalıkları, kanserli tümörler, multipl skleroz, gut, zona, ülser, tendonit, enfeksiyonlar ve deri hastalıklarındaki etkileri kanıtlanmıştır.

Eski çağlardan beri Çin’de, Hindistan’da, Mısır’da, Babil’de ve Yunanistan’da arı zehri kullanılmasına ve geliştirilmesine rağmen, zehrin içeriğini oluşturan bileşenler yakın zamanda çalışılmış ve aydınlatılmıştır.

Doğal bir toksin olan arı zehri, arının karın boşluğunda bulunan zehir salgı bezinden üretilir. Zehir üretimi işçi arının yetişkinliğe gelişimi boyunca artar, kovan savunması ve besin arama işleri ile ilgilenmeye başladığında en üst seviyeye ulaşır. Kraliçe arının zehir üretimi işçi arılara oranla en yüksek seviyede olur.

Arıların zehir keselerinde ortalama 0.15-0.30 mg zehir bulunur. Bu zehir açık renkli olup, kokusuz, keskin ve acı bir tada sahiptir. İçeriğinde birçok kimyasal yapı bulunmaktadır. Bunlar proteinler, enzimler, peptitler, aktif aminler, mineraller ve karbonhidratlardır.

Arı zehri 18 farmakolojik aktiviteye sahip bileşikten oluşmaktadır. Bunlardan başlıcaları, histamin, melittin, apamin, epinefrin, mast hücre degranülasyonu peptidi, fosfolipaz A2, dopamin, hyaluronidaz enzimleridir.

Melittin zehrin major kısmını oluşturan ve asıl acıya sebep olan peptittir. İlk defa 1954 yılında tanımlanmıştır. Melittin kuru arı zehrinin %50’ye yakını oluşturur, farmakolojik ve fizyolojik olarak antibakteriyel, antifungal, sinir sistemi düzenleyici, radyasyondan koruyucu etkilere sahip, suda çözülebilir özelliktedir.

Melittin 26 amino asitten oluşan doğrusal bir yapıya sahip küçük bir peptittir. İlk 20 amino asit kısmı çoğunlukla apolarken, C terminalli kısımdaki 5’li aminoasit bölümü polar ve yüksek derecede baziktir. Çok güçlü antiinflamatuvar özelliğe sahiptir. Kanser, bakteri, mantar, virüs ve enfeksiyonlara karşı engelleyicidir. Membran yüzey gerilimini ve kanın pıhtılaşmasını azaltır. Melittinin daha birçok biyolojik etkileri bilimsel çalışmalarla araştırılmaktadır.

Kapiler elektroforez, kısa analiz süresi, az miktarda çözücü sarfiyatı, küçük numune hacminde örnek enjeksiyonu, kullanım kolaylığı ve yüksek ayırım gücü gibi birçok avantaj sağlayan modern analitik bir metottür.

Kapiler elektroforez metodunda, yüklü tanecikler sıvı ya da katı bir ortamda elektriksel potansiyel uygulanarak, etkin yüzeylerinin büyüklüklerine oranla belirli bir pH da göç etmesi ve ayrılma prensibine dayanmaktadır. Her bir molekül kendisine ait olan elektroforetik hareketliliğe ve sistem içerisinde kullanılan tampon çözeltinin elektrik akım altında sağlamış olduğu elektroosmatik akışa sahiptir. Elektrik akım altında kapiler bir kolonun içerisinde ayırım gerçekleşir. Kapiler elektroforezde proteinlerin, peptitlerin, amino asit ve bir çok benzeri moleküllerin ayırımı gerçekleştirilebilmektedir.

Kapiler kolonun iç yüzeyi silanol grupları ile kaplıdır, pH'ın 2 değerinden büyük durumlarda silanol gruplar deiyonize olarak, tampon çözeltiden gelen pozitif yüklerle çift katlı yük katmanını oluşturur ve elektroosmatik akış sağlanmış olur. Bileşiklerin ayrılmasında hem elektroosmatik akış hem de moleküllerin elektroforetik hareketliliği ile sağlanır.

Sağladığı yüksek verim ve seçicilik sayesinde kapiler elektroforez protein, peptit ve aminoasit araştırmalarında tercih edilen bir ayırıştırma yöntemidir. Peptit ayırımı küçük moleküllere benzese de peptidi oluşturan aminoasitlerden ötürü farklıdır. Peptitlerin farklı elektroforetik hareketliliği, moleküler ağırlık, konformasyon, çözünürlük, yük, boyut, hidrofobisite ve diğer biyomoleküllerle etkileşimi özelliklerinden dolayı sağlanır.

Analizler rutin bir şekilde gerçekleştirilmiştir. Yapılan çalışmalarda çözeltilerdeki melittin peptit konsantrasyonları Agilent 1600 kapiler elektroforez cihazı ile belirlenmiştir. Melittin peptitinin etkin ayırımı kapiler duvarın iç yüzeyine yapışma özelliği gösterdiği için düşük seviyelerde gözlenmiştir. Bu durum negatif yüklü silanol gruplarıyla melittin peptitinin pozitif yüklü kısımları arasındaki elektrostatik etkileşiminden kaynaklanmaktadır. Pozitif yük ve negatif yük etkileşimini engellemek için iki farklı yaklaşım çalışılmıştır.

Bu çalışmanın ilk bölümünde, silanol gruplarının deiyonize olmasını engellemek ve elektroosmatik akışı yok etmek için çalışma tamponunun pH'ı 2 den daha küçük bir değer olarak seçildi.

Denemeler sonucunda, düşük pH çalışmasında melittinin ayrılmasında en uygun ayırma şartlarının, çalışma tamponu olarak pH: 1,55'de fosforik asit çözeltisi, 25 kV voltaj 1000 mbar basınçla 6 saniye örnek enjeksiyonu olduğu tespit edilmiştir. Kalibrasyon eğrisinin doğrusal aralığı 70-350 µM aralığında, 0,983 korelasyonla tespit edilmiştir Bu optimal şartlar altında yöntemin melittin peptiti için tayin sınırı (LOD) 19,3 µg/mL olarak tespit edilmiştir.

Bu çalışmanın ikinci bölümünde, bir kapiler kaplama işlemi tanımlanmıştır. Kaplama malzemesi olarak polietilenimin (PEI) kullanılmış, böylece melittin peptitinin pozitif yüklü kısımlarının kapiler kolonun negatif silanol grupları içeren iç yüzeyle etkileşimi engellenmeye çalışılmıştır.

Kaplama prosesi kapiler kolondan 1000 mbar basınçta 30 dakika boyunca, 1 M sodyum hidroksit çözeltisi, ardından 1000 mbar basınçta 15 dakika boyunca su, 1000 mbar basınçta 10 dakika boyunca %10'luk PEI çözeltisi geçirip, daha sonra kapilerin içinde PEI çözeltisini 1 saat bekletmek, ardından 1000 mbar basınçta hava ile polimer çözeltisini çıkarmak, en son olarak da 15 dakika boyunca 1000 mbar basınçta deiyonize su ve 15 dakika boyunca 1000 mbar basınçta çalışma tamponu geçirilerek tamamlanmıştır.

PEI molekölü silika kapiler yüzeyindeki negatif yüklü silanol grupları ile güçlü bir etkileşime girer. Kapiler elektroforezde kaplayıcı olarak katyonik bir polimer olan polietileniminin kullanılmasıyla beraber, kapiler içindeki elektroosmotik akış katotdan anoda doğru yönelir. Adsorplanan PEI tabakası geniş bir pH aralığında pozitif yüke sahiptir.

PEI ile kaplanmış kapilerde elektroosmotik akış büyüklüğü ve stabilitesi, farklı tampon çözeltileri, tampon konsantrasyonları ve pH'larda, iyonik şiddet ve organik çözücü eklemeleri gibi şartlar altında incelenmiştir. Geliştirilen kaplama işlemi daha sonra melittin peptidi analizinde uygulanmıştır.

Bu çalışmadaki denemeler sonucunda, kapiler kolonun %10'luk PEI çözeltisi ile kaplanmış, melittin peptidinin ayrılmasında en uygun ayırma şartlarının, çalışma tamponu olarak pH: 5.50'de asetik asit çözeltisi, -25kV voltaj 50 mbar basınçla 6 saniye örnek enjeksiyonu olduğu tespit edilmiştir. Kalibrasyon eğrisinin doğrusal aralığı 35-350 µmol/L aralığında, 0,996 korelasyonla tespit edilmiştir. Bu optimal şartlar altında yöntemin melittin peptidi için tayin sınırı (LOD) 10.0 µg/mL olarak tespit edilmiştir.

İki yöntemin de geliştirilmesindeki en önemli amaç melittin peptidinin pozitif yüklü kısımlarıyla, kapiler kolonun iç yüzeyindeki negatif yüklü silanol gruplarının etkileşimini olabildiğince engellemek ya da yok etmektir. Birinci yöntemde elektroosmotik akış çalışılabilecek en düşük pH değerlerini kullanarak olabildiğince yok edilmeye çalışılmış, böylece duvar negatif yükü bastırılmıştır. Melittinin duvara yapışmadan ayrımı ve analiz tekrarlanırlığı sağlanmıştır. Diğer yöntemde ise, kapiler kolonun iç yüzeyi pozitif yüklü PEI polimeri ile kaplanmış. Böylelikle melittinin pozitif yüklü kısımlarıyla negatif yüklü silanoller arasındaki etkileşim engellenmiştir. Bu çalışmada da melittinin ayrımı ve tekrarlanabilirlik sonuçları sağlanmıştır.

Sonuç olarak her iki yöntemde de arı zehiri içinde melittin tayini hassas ve tekrarlanabilir şekilde tespit edilmiştir. Deney süresinin daha kısa olması nedeniyle gerçek örnekte miktar tayini silika kapilerde geliştirilen yöntemle yapılmış ve Karadeniz Bölgesi arılarından toplanan kuru arı zehri örneğinde melittin 245 mg/g olarak bulunmuştur.



1. INTRODUCTION

1.1 Capillary Electrophoresis

In recent years, capillary electrophoresis (CE) has a very compelling effect on the field of analytical chemistry as a resourceful technique of high speed, sensitivity, low limit of detection, and small amounts of samples and reagents. Rapid, reproducible separations of wide range of species, including inorganic ions, carbohydrates, chiral molecules, DNA, nucleotides, vitamins, peptides, proteins and amino acids have been studied. CE is a well-established separation technique in a great variety of research fields [1].

1.2 Historical Background and Developments

Electrophoresis is a general term that explains separation of charged particles under the influence of an electrical field. The history of capillary electrophoresis started back at 19th century, when Lodge described in his thesis ‘‘hydrogen ion movements in a tube of phenolphthalein jelly’’ in 1886 [2,3]. Later that, Smirnow, Hardy, and Field had taken the development of electrophoresis systems. Their electrophoresis system studies were performed in U shaped glass tubes with electrodes attached to each of tubes’s arms [4-7]. In 1937, Swedish Biochemist Arne Tiselius utilized the electrical charged carried by macromolecules to achieve separation of blood plasma and used this study in his thesis with the title of ‘‘The Moving Boundary Method to Study the Electrophoresis of Proteins’’[8] Tiselius was the first performed electrophoresis experiments on proteins by placing protein mixtures between conducting media solutions in a U shaped tube and applying the electric field, sample species migrated in a direction by their charge and mobility ratio [8]. He was awarded with Nobel Prize in 1948 for his pioneering work on the electrophoretic mobility. Professor Tiselius continued his explorations in the field of electrophoresis and a number of more results were published. He also resolved band boardening problem due to electrical heating by introducing effecient cooling technique of the

electrophoresis cell by fast circulation of water at 4°C. Afterwards, Virtanen and then Mikkers developed electrophoresis method using smaller diameter columns, anyhow studies were not able to display high efficiencies because of inability to produce durable capillary column [9]. In the beginning of 1980s, Jorgenson and Lukacs advanced the technique by executing electrophoresis in a very thin capillary with inner diameter less than 100 μm leading to capillary electrophoresis [11-12]. Meanwhile, Japanese Chemist Watanabe Terabe described a new capillary electrophoresis method. Terabe developed micellar electrokinetic chromatography (MEKC) as a practical separation of neutral molecules and published first article about MEKC in 1984 [13]. To date, capillary electrophoresis has become a powerful analytical technique which successfully used for separation of small ions, neutral molecules, and large molecules [14]. It is being utilized in widely different fields, such as analytical chemistry, forensic chemistry, clinical chemistry, organic chemistry, natural products, pharmaceutical industry, chiral separations, molecular biology, and others [15-16]. Over the past three decades, have been a period of rapid growth for CE, which is proven by rising number of publications, scientific meetings, commercial instruments and separation methodologies related to this technique. Undoubtedly, there will further developments in CE. And it is not surprising to understand that the growth of capillary electrophoresis can be attributed to previous developments and inventions in electrophoresis [17].

1.3 Capillary Electrophoresis Theory

Electrophoresis is simply defined as the dissimilarity in the electrophoretic and electroosmotic mobilities of molecules. Electroretic separation is based on these molecules dissimilar velocities in an electrical field[18]. The velocity of an ion is defined in equation 1.1[17]

$$v = \mu_e \cdot E \quad (1.1)$$

Where μ_e is mobility and E is electrical field.

The mobility is determined by electric force that the molecules experiences, balanced by its frictional force. The electrical force can be given in equation 1.2[18]

$$F_E = q \cdot E \quad (1.2)$$

Where q is the charge of particle.

The fractional force can be given in equation 1.3 [18]

$$F_F = -6. \eta. \pi. r. v \quad (1.3)$$

Where η is viscosity of separation medium, r is radius of particle.

At this point, the forces are equal but opposite, calculation shown in equation 1.4 [18]

$$6. \eta. \pi. r. v = q. E \quad (1.4)$$

Solving the equation, electrophoretic mobility in terms of physical parameters given by in equation 1.5 [18]

$$\mu_e = q / 6. \eta. \pi. r \quad (1.5)$$

From this equation it is showed that small, highly charged species have high mobilities whereas large, minimally charged species have low mobilities[19].

1.4 Basic Principle of Electrophoretic Separation

Electrophoresis is definition for dissimilar migration of charged species in an electric field. Separation observed under an electric field, inside a thin fused silica capillary column with a diameter between 10 μ m and 100 μ m. The separations are performed on the basic instrumental set-up of a CE system, which is illustrated in Figure 1.1, consist of a high voltage power supply, a fused silica capillary, two buffer reservoir, two electrodes, a computer control, and an on-column detector. Sample injection is done by temporarily replacing one of the buffer reservoir with a sample vial.

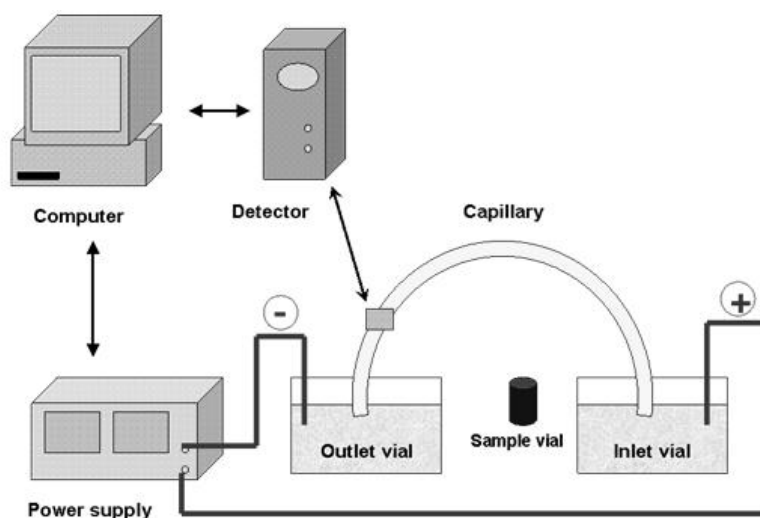


Figure 1.1 : Basic instrumental set-up of a capillary electrophoresis system[20]

Under applied electric field, positively charged species move to the cathode, and negatively charged species move to the anode in conducting medium. Separation depends on differences in charge to size ratios.

1.4.1 Electrophoretic Mobility

Electrophoretic mobility is the fundamental parameter which determines the efficiency of separation based on charge to size ratio. The symbol for electrophoretic mobility is μ_{ep} . In simply, ion placed in a electrical field experiences force opposes the forward movement and backward movement. Velocity of species in constant electrical field depends on the balance between the two forces. Electrophoretic mobilities of species are shown in Figure 1.2. Positively charged species move towards to cathodic side, and negatively charged species move toward opposite electrode due to the coulombic force.

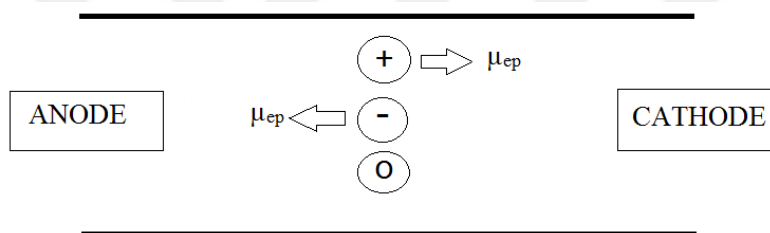


Figure 1.2 : Electrophoretic mobilities of species[21]

Neutral species have no electrophoretic mobility, so only conducting medium is effectly adjust their movement towards to cathodic side.

1.4.2 Electroosmotic Flow

Another important parameter in CE is electroosmotic flow (EOF), which refers to the flow of solvent in capillary columns. The inner surface of a fused silica capillary is covered with silanol groups, they are ionized at pH greater than two. The negatively charged surface is counterbalanced by positive ions from the conducting medium forming the electrical double layer. When a voltage applied across the capillary, electrical double layer's cations migrate in the direction of the cathode, carrying water with them which creates electroosmotic flow[21]. The electroosmotic flow direction is shown in Figure 1.3.

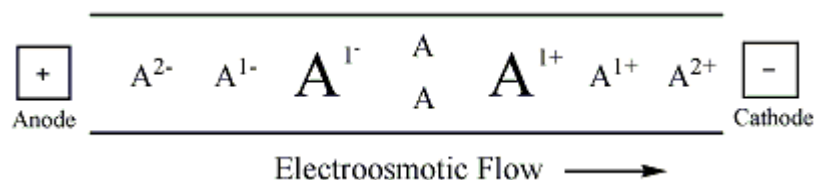


Figure 1.3 : Electroosmotic flow direction[22]

Velocity of the electroosmotic flow through a capillary is defined by the Smoluchowsky equation in equation 1.6 [23]

$$V = \frac{\epsilon \cdot \zeta \cdot E}{4 \cdot \pi \cdot \eta} \quad (1.6)$$

The zeta potential is related to surface charge of the capillary surface, and to ionic nature of conducting medium. Since the charge per unit surface area is strongly pH dependent, the magnitude of the electroosmotic flow varies with pH. This is an inverse relationship, increasing the concentration of the electrolyte decreases the electroosmotic flow[24].

For cations, which move in the same direction as the electroosmotic flow to the cathode, and anions is in the opposite direction of electroosmosis, so for at moderate pH values, electroosmotic flow is generally higher than electrophoretic mobility causing anions to migrate towards the cathode, which is where the detector is typically located. At lower pH, electroosmosis is weak and anions may never reach the detector unless the polarity of the instrument is reversed in order to change the location of the detector from the cathode end to the anode end of the capillary. Electroosmotic flow can be diminished by coating the capillary with a coating reagent that restrain ionization of the silanol groups, such as polyacrylamide or polyethylenimine. A zwitterion such as a peptide can be separated under each of the two conditions. At high pH values, EOF is large and the peptide is negatively charged. Despite the peptide's electrophoretic mobility towards the positive electrode, the EOF is overpowering, and the peptide moves towards the cathodic side. At low pH values, the peptide is positively charged and EOF is very small. Thus, peptide electrophoretic mobility and EOF are towards the cathodic side. In special cases, separation of proteins and peptides in bare fused silica capillaries could be prevented by the adsorption of species at the capillary inner wall. The main cause of protein and peptide adsorption is the electrostatic interaction between the positively charged residues of the protein and the deionized negatively charged silanols of the fused silica surface. Adsorption of the analytes is unwanted in capillary electrophoresis

because it causes peak dispersion, asymmetry, poor reproducibility of migration time and low protein recovery. Various methods have been attempted to prevent interaction between positive charged species and the fused silica surface. The most successful methods are the ones whereby the silanol groups are dynamically coated by a immobilized polymer groups such as polyvinylalcohol, polyethyleneimine, polyacrylamide, polyoxyethylene and polysaccharides. The use of such polymers as coating reagents has led to high efficiencies and reproducible separations[24-30].

Another aspect of EOF is flow profile. The flow profile of the capillary the velocity of liquid is nearly uniform, resulting electroosmotic flow. Compared to laminar flow profile, seen in high performance liquid chromatography (HPLC) system that are pumped by a pressure differential, electroosmotic flow profile gives much less dispersion and high separation efficiency. A comparison of laminar flow and electroosmotic flow is shown in Figure 1.4.

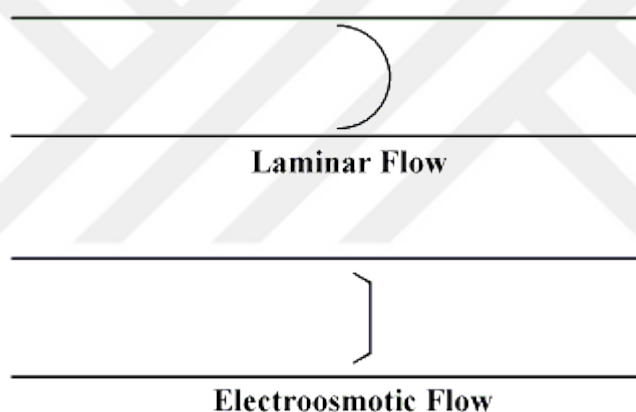


Figure 1.4 : Flow profiles of electroosmotic flow and laminar flow[31]

1.4.3 Electroosmotic flow control

Electroosmosis can be changed by a variety of methods. Fundamentally, control of EOF requires adjustment of the capillary surface charge or conducting media viscosity. There are several methods to control EOF, as detailed in Table 1.1

Table 1.1 : Methods to control electroosmotic flow[32]

Variable	Result	Comment
Electric field	Proportional change in EOF	<ul style="list-style-type: none"> • Efficiency and resolution decrease when lowered • Joule heating may result when increased
Buffer pH	EOF decreased at low pH and increased at high Ph	<ul style="list-style-type: none"> • Most convenient and useful method to change EOF • May change charge or structure of solute • High ionic strength generates increased high current and possible Joule heating • Low ionic strength problematic for sample adsorption • May distort peak shape if conductivity different from sample conductivity • Limits sample stacking if reduced
Ionic strength or buffer concentration	Decreases zeta potential and EOF when increased	<ul style="list-style-type: none"> • Often useful since temperature is controlled instrumentally
Temperature	Changes viscosity 2 – 3% per°C	<ul style="list-style-type: none"> • Complex changes, effect experimentally • May alter selectivity
Organic modifier	Changes zeta potential and viscosity	<ul style="list-style-type: none"> • Anionic surfactants can increase EOF Cationic surfactant can
Surfactant	Adsorbs to capillary wall via hydrophobic and/ or ionic interactions	<ul style="list-style-type: none"> • Decreases EOF by shielding surface charge and increasing viscosity
Neutral hydrophilic polymer	Adsorbs to capillary wall via hydrophobic interactions	<ul style="list-style-type: none"> • Many modifications possible (hydrophilicity or charge) • Stability often problematic
Covalent coating	Chemical bonding to capillary wall	

1.5 Capillary Electrophoresis Instrumentation

CE can be performed with comparatively simple instrumentation as shown in Figure 1.1. A capillary containing a suitable separation medium spans two buffer reservoirs, to which the high voltage power supply is connected via the two platinum

electrodes. Following the introduction of sample at the capillary inlet, a high voltage is applied, thus driving the species to move inside the capillary with different velocities. Somewhere close to the capillary outlet, an on-line detector is installed to monitor the separation process. The resulting signals are delivered to the data acquisition device, and finally the result is presented in the form of an electropherogram[33].

1.5.1 High Voltage Supply

In CE a DC power supply is used to apply up to about 30 kV and current levels of 200 to 300 mA. Steady regulation of the voltage is needed to maintain high migration time reproducibility. The power supply should have the capability to switch polarity. Under normal conditions the EOF is in the direction of the cathodic side. On these terms, injection is made at the anodic side[34]. In switched polarity mode, EOF is in the direction of the anodic side. Injection is done at the cathodic side using a fused silica capillary modified by adsorption of polymer group as coating reagent on its inner wall.

1.5.2 Background Electrolyte

The background electrolyte (BGE) should principally provide a suitable migration of the species, along the detector in a reasonable time. Moreover, minor peak broadening and other migration interferences should not be present. When the principal requirements are accomplished, and, a suitable BGE is selected which provides a suitable migration and detection[35]. Convenient BGEs should contain enough ions to conduct the electric current. The concentration of species should not be too high, in order to avoid excessive Joule heating. The voltage needed for a specific time of analysis can be calculated from the mobilities of the species. At least one component of the BGE must have significant buffering capacity at the selected pH. A ion of the BGE should be selected, in this way, its mobility is close to those of sample ions; then the electrodispersion of sample peaks is minimum and peaks are practically symmetric and sharp. Samples with UV-transparent components may be detected by using the indirect detection mode with a BGE where a suitable UV-absorbing ion or counterion is used. Generally, EOF is present in fused-silica capillaries migrating to the cathode. Thus, cations and anions can be detected by using cathodic mode. Anions with mobilities lower than that of the EOF can be

detected. Anions with higher mobilities should be analyzed in the anodic mode. The EOF can be suppressed or even reversed by adding suitable surfactants, such as cetyltrimethyl ammonium bromide (CTAB); however, the risk that it brings some more ions into the BGE should always be considered. The background electrolyte composition is the key parameter for clear separation.

1.5.3 Autosampler

In CE an autosampler has two functions. It should transport both the sample vials and buffer vials to the capillary ends. For automated method development, usually a single sample vial and numerous buffer vials are used. Conversely, for routine analysis, numerous samples but only a single buffer is used. Thus it is important to have a high vial capacity and the capability of random access to the vials. In either case, it is important that the anodic and cathodic reservoirs contain the same buffer composition. Differences in the reservoir contents can result in absorbance fluctuations, current changes, and variations in EOF. Cooling of the autosampler is required if thermally sensitive samples are used. Thermostating is also beneficial in reducing sample evaporation which can increase sample concentration and limit quantitative analysis. Similarly, tightly capped sample vials also limit evaporation [36].

1.5.4 Sample Introduction

To achieve high efficiency and good quantitative results, sample injection must be done in a routine and reproducible manner. Since the injection volume in CE is in the nanoliter range, which deters the use of regular injection methods, alternative approaches have to be sought. Hydrodynamic injection and electrokinetic injection are the mostly used sample injection techniques.

1.5.4.1 Hydrodynamic injection

Hydrodynamic injection introduces a sample based on a pressure difference in the two sides of the capillary. For a laboratory-built instrument, this is done by simply lifting up the sample vial together with the capillary inlet for a certain period of time. The hydrodynamic force will do pump affect to sample solution into the capillary. For commercial instruments, the pressure drop is created by either applying pressure at the inlet side, or commanding a vacuum at the outlet vial [36].

1.5.4.2 Electrokinetic injection

Electrokinetic injection is based on the transportation of sample ions by electrophoretic movement and EOF. Normally a lower voltage than separation purpose is applied for a certain amount of time to allow species to move inside the fused silica capillary[36].

1.5.5 Capillary system

Ideal properties of the capillary material include being chemically and electrically inert, UV-Visible transparent flexible and robust, and inexpensive. Fused silica capillaries are used widely in CE, largely due to their strength, flexibility and most predominantly their UV transparency. Generally the fused silica capillary is coated with a layer of polyimide to strengthen its durability. For on-column optical detection a small segment of this coating needs to be removed to provide the detection window. Layer of polyimide could easily removed by applying heat by a heat source such as lighter or scrubbing the glass stream on desired area for detection window. The most commonly used CE capillaries have inner diameter vary between 20 and 75 μm , outer diameter 100-400 μm and are about 30-100 cm in length [36]. Fused silica capillaries with internal diameters ranging from 10 to 200 μm with a range of outer diameters are available, however, 25 to 75 μm id and 350 to 400 μm od are typical. From an analysis time perspective, capillaries as short as possible should be used.

One of the most important factors leading to good reproducibility is capillary conditioning. Above pH:2, inside the capillary wall, all silanol groups deionized their anionic form with the help of conducting medium. Effect of the applied electric field, the negatively charged wall attracts positively charged ions from the conducting medium, form an electrical double layer. Surface of fused silica after hydrolysis is shown in Figure 1.5

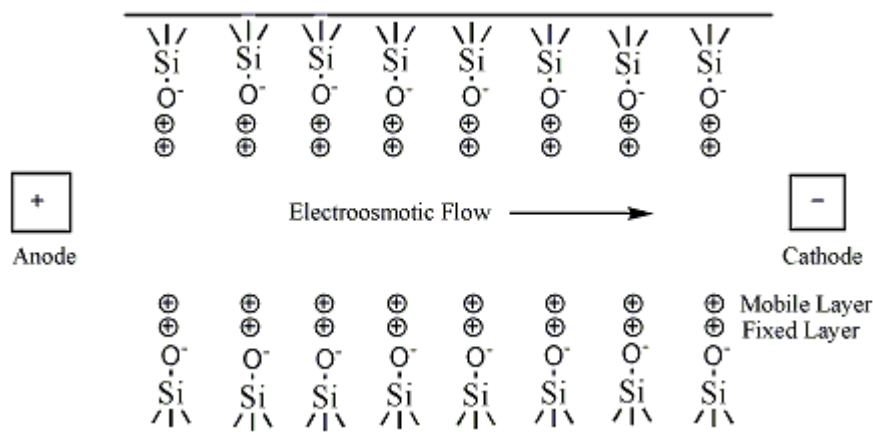


Figure 1.5 : Surface of fused silica after hydrolysis [37]

Within the capillary walls sometimes electrical double layer led loss in efficiency compared to predicted theory. Positively charged species such as proteins adsorption on the internal surface of negatively charged silanol groups can cause fundamental problems. To prevent interaction between strong positively charged species and fused silica surface with silanol groups, inside the capillary column can be internally coated. The use of such polymeric coatings led to high efficiency and reproducible separations[38].

Temperature control is also important to reproducible separations in capillary column. However, temperature regulation is complicated by several factors. Simply, the passage of electrical current through the conducting media filled capillary results in the production of undesired heat. This undesired heating effect is innate in electrophoretic separations and is called Joule heating. A layer of silica and a layer of polyimide separate the small fluid volume inside the capillary from the thermostatted medium. Any heat must be transferred to and from the separation buffer through these layers. Although the temperature inside the capillary cannot easily be measured it can be estimated. Equation in 1.7 is used to estimate the Joule heating [39].

$$Q = E^2 \Lambda c \quad (1.7)$$

Where Q is the heat generated, E is the voltage gradient, Λ is the molar conductivity of conducting medium and c is the concentration of conducting medium.

The temperature gradient within the capillary is parabolic. The difference in temperature between the center of the capillary and the wall will increase with increasing capillary internal diameter. Current will also increase as the diameter

increases. The combination of these two factors can lead to the conclusion that heating will cause band broadening and that the band broadening will increase as the internal capillary diameter increases. Joule heating illustration is shown in Figure 1.6. Joule heating occurs when current passes through the conducting media filled capillary. The temperature can be expected to be highest in the center of the capillary. Each layer through which the heat must pass will have its own thermal conductivity leading to a complex pattern. In systems with moderate temperature control, this thermal band broadening problem does not become a problem unless the capillary diameter is larger than about 100 μm [39].

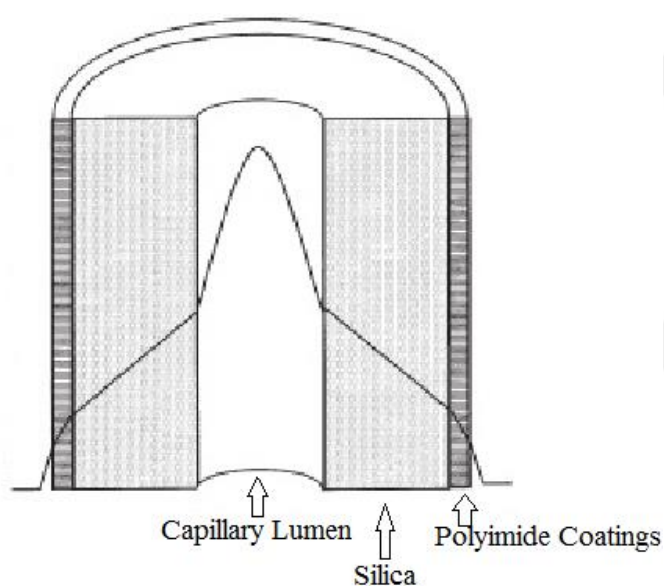


Figure 1.6 : Joule heating illustration[39]

1.5.6 Detector

The internal diameters of capillaries vary between 100 μm down to about 10 μm . Because of these small surface, sample volumes has to be smaller. For these reasons, the choice of detector should be done by the requirement of the application in terms of detection limit, selectivity, miniaturization, also commercial availability, inexpensive, robustness and easy to use. Some features of the main detection methods for CE is detailed in Table 1.2 Main points to be considered when deciding which detection method is most suitable is sensitivity and selectivity requirements.

Table 1.2 : The main detection methods for CE[40].

Method	Features	Detection Requirements
UV-VIS absorption	Readily available commercially	Chromophore group Absorb UV above 190nm
Indirect UV- VIS absorption	Compromise with poorer detection for nonabsorbing species	Derivatized to contain chromophore group
Fluorescence	Good detection limits but most species require derivetization	Fluorophore groups Derivatized to contain fluorophores
Laser induced fluorescence	Elaborate, excellent detection limits	Fluorophore groups Derivatized to contain fluorophores
Conductometry	Good for small ions	Solutes need to be charged
Amperometry	Simple but only possible for electroactive ions	No special requirements
Mass spectrometry	Provides information on peak identity, expensive, interfaces	No special requirements

1.5.6.1 UV-VIS absorption

UV–VIS absorption is the most used detection mode available in CE systems. The main reason due to the fact that most species contain one or more chromophores. Addition to that it is the most advantageous type detector due to the its commercial

availability, simplicity, versatility, and relatively low cost. Fused silica column normally used for CE has a UV cut-off around 170 nm and this is suitable for UV detection. The layer of polyimide coating on the outside can be removed to form a detection window. In on-column detection the path length is defined by the inner diameter of the capillary. This limits the sensitivity of absorbance detection techniques, since sensitivity is proportional to path length. Another consideration is that with small capillaries, ideally only the capillary is illuminated during detection in order to reduce stray light [41]. UV-VIS detector covers 190nm to 700nm absorption range. For compounds that do not exhibit UV absorption, indirect detection can be applied. An absorbing co-ion is added to the background electrolyte, and this is displaced by the species. At the position of the species, a negative peak will appear. The displacement depends on the charges of the probe and the analytes and on their mobilities. Each fluctuation in the probe concentration is detected as noise. In principle, indirect UV detection is universal but optimization is rather complicated. The choice of the monitoring ion and other components of the BGE needs distinctive attention[42].

1.5.7 Data processing and instrument control

Data processing is the final and essential part of the CE equipment, which provides direct information about the analysis. Electropherograms can be obtained at this diverse data processing, which provides the accurate information about the analysis or analytes[43]. Qualitative and quantitative analysis is calculated by using these electropherograms.

Two types of peak detection methods used in data analysis. Slope sensitive algorithm and the moving median filter algorithm. Slope sensitive algorithms is about the slope of the baseline over some interval of time. When the slope exceeds a pre-determined value, a peak is said to have begun. The point at which the slope goes to zero identifies the peak apex, and the point at which the slope returns to the starting value defines the peak end. In this detection method peaks independently of the baseline shape. Moving median filter algorithms has a contrasting approach. Peaks are relatively high frequency events when compared to baseline drift. These algorithms seek to define how the baseline would look in the absence of peaks by filtering out all impulse events.

1.6 Operation Modes in Capillary Electrophoresis

1.6.1 Capillary zone electrophoresis

One of the capillary electrophoresis format is capillary zone electrophoresis and it is the simplest and widespread mode among the various CE modes. The analysis operated by the use of open capillaries and relatively low viscosity electrolyte systems. Species migrate with EOF and separate into the bands according to differences in their electrophoretic mobilities. It is used for a wide range of species [44].

1.6.2 Capillary gel electrophoresis

In capillary gel electrophoresis, separation takes place on viscous drag. The capillary is filled with a gel or viscous solution. That is why it is also called slab gel electrophoresis and has been used in biology research for the size based separation such as biomacro molecules consisting of numerous repeat charge units as DNA fragments. Mostly crosslinked polyacrylamide and agarose gel has been used as polymeric system in this mode. Charged species are resolved according to their physical sizes rather than their electrophoretic mobility[45].

1.6.3 Capillary isoelectric focusing

Capillary isoelectric focusing (CIEF) combines the high resolving power of conventional gel iso electric focusing with the advantages of CE instrumentation. In this method, zwitterions are separated according to their isoelectric points in a pH gradient formed by carrier ampholytes when an electric potential is applied. CIEF is also widely used for examining the distribution of carbohydrate isoforms of glycoproteins. In other techniques, such as CZE, these proteins tend to move as diffuse bands and to generate broad peaks. CIEF can often resolve these bands into peaks that differ by as little as one charged sugar group[46].

1.6.4 Capillary isotachopheresis

Capillary isotachopheresis is a moving boundary electrophoretic format. Separation results from the different mobilities of identically charged ions in a discontinuous electrolyte system with two different solutions, a leading and a terminating electrolyte. The sample is inserted between these two electrolyte solutions. When the

electric field is applied, all negatively charged ions begin to move towards the anode, arranging themselves in order of mobility. Separation of the sample ions takes place between the leading and terminating electrolytes during migration. When the system has reached equilibrium, each individual ionic sample component moves separately as a pure band. Each pure band is stacked between the sample component of next highest and lowest mobility[47].

1.6.5 Micellar electrokinetic electrophoresis

Micellar electrokinetic chromatography, denoted as MEKC and often called micellar electrokinetic capillary chromatography (MEKC), was originally developed by Terabe [48] for separation of neutral compounds which cannot be separated by capillary zone electrophoresis. Applying an ionic surfactant such as sodium dodecylsulfate (SDS) in a concentration higher than the critical micelle concentration and a high pH value makes a separation possible based on the differential partition of the analytes between the running buffer and the surfactant micelles. The micelles form a pseudostationary phase moving with the migration velocity and direction which is different to the background electrolyte. The selectivity of the separation depends sensitively on the differences of distribution constants between the electrolyte solution and the micelles for neutral analytes to be resolved, and in the case of ionized analytes on the differences of distribution constants. The surfactants are the selectivity determining factor. They can be categorized as anionic surfactants like sulfates, sulfonates or carboxylates, bile salts, cationic surfactants containing quaternary ammonium head groups, and nonionic surfactant. Moreover, two different surfactants, ionic and nonionic, can be applied forming mixed micelles which may result in a different selectivity and an improved resolution [49-50].

MEKC is useful for a wide range of small molecules such as drugs, pesticides, and food additives that are not charged and are sufficiently hydrophobic to associate with the micelle. While SDS is probably the most widely used detergent for this purpose, cationic detergents such as tetradecyltrimethylammonium bromide (TTAB) can also be employed. Nonionic detergents by themselves do not provide mobility to uncharged analytes, but in combination with charged detergents they will modify the separation. Some detergents are useful in specific applications [51].

1.6.6 Capillary electrochromatography

Capillary electrochromatography (CEC) is a hybrid technique employing CE and HPLC. This hybrid technique was proposed by Pretorius in 1974 [52]. It is a partitioning method in which molecules distribute between a stationary and moving phase. Separations for CEC are a consequence of both differential partitioning and electrophoretic migration in mobile phases, which typically include a conducting media [44]. This technique is currently used for the separation and analysis of a broad spectrum of compounds, both low and high molecular, organic and inorganic compounds.

The separation happens in narrow bore packed column where the liquid mobile or moving phase is driven not by hydraulic pressure as in HPLC but by electroosmosis. This flow derives from the electrical double layer surrounding both the inner capillary wall and the individual stationary phase particles. A plug-like flow profile is maintained through the packed bed, leading to very high efficiencies limited mainly by the solute diffusion coefficient [53-60].



2. BEE VENOM

Apitherapy or bee therapy is simply the medical use of products made by honey bees. Since ancient times, honey bees and their associated products including honey, pollen, propolis, royal jelly, bee's wax, and bee venom have been used by mankind. In the ancient civilization of Greece, Babylon, Egypt, China, and India bee venom (BV) has been used for apitherapy and nowadays it still gets the most attention. BV is produced by two glands associated with the stings apparatus of worker bees. Production of BV is increased while the bee is getting adult and become highest when needed for hive defence and foraging[61]. The queen bee's production of BV is the highest compared to worker bees. A full venom sac has 0.15-0.30 mg venom. It is clear, odourless, watery liquid and its dried form takes on a light yellow colour. For commercial purpose, BV is extracted from bee glands or sticks, after collected by a electrical shock instrument and commercial BV preparations are light brown due to the oxidation of some of the proteins. A large number of studies have been carried out on the composition of BV [62].

BV is natural toxic and has a complex mixture of enzymes, proteins, aminoacids, peptides, biogenic amines, sugars and minerals. BV contains various peptides including melittin, apamin, adolapamin and mast cell degranulating peptide [63, 64]. Enzymes are composed of phospholipase A2 (PLA2), hyaluronidase, acid phosphomomesterase, α -D-glucosidase and lysophospholipase, as well as non-peptides such as histamine, dopamine and norepinephrine [65]. Bee venom therapy is a treatment method that may be thousands of years old and involves the application of live bee stings to the patient's skin or in recent years, the injection of bee venom into the skin with a hypodermic needle [66]. Many experiments on the biological and pharmacological activities of bee venom have been studied [67]. BV components have different effects on the central and the peripheral nervous systems. Reports have been published on the use of BV to treat patients with different degenerative diseases of the nervous system, such as multiple sclerosis, Alzheimer's disease and

Parkinson's disease [64-66]. BV has also been reported to act against different types of cancers in cell and animal experiments [67, 68]. Thus, melittin, a powerful anti-cancer peptide, might be a better choice than whole BV for treating various diseases. BV acupuncture and melittin have been used to control neuropathy caused by cancer chemotherapy [69-70]. Because, most human deaths resulting from one or a few bee stings are due to allergic reactions, heart failure, or suffocation from swelling around the neck or the mouth, as compared with other human diseases, accidents and other unusual cases, deaths due to bee stings are rare, indicating that BV is very safe for treating diseases in humans [71]. Majority of these studies have proven the effectiveness of bee venom in treating pathological conditions such as arthritis [68], pain [69, 70] and cancerous tumors [71, 72] among others.

2.1 Melittin

Melittin (MELT) is the main component of bee (*Apis Mellifera*) venom, accounting approximately 50% by weight of dry venom. It was first identified as hemolytic polypeptide by Haberman and Neumann in 1957 [64]. This small peptide has a linear structure consist of twenty six aminoacids (NH₂-Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Th-Gly-Leu-Pro¹-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln-CONH₂) [74] with no disulfide bridge in which N- terminal part of the molecule is mostly hydrophobic while C- terminal part is hydrophilic and it is strongly basic. Although MELT is a monomer with a molecular weight of 2.8 kDa, MELT solutions have molecular weights of 11.8-13.8 kDa which suggests aggregations[75]. The structure of MELT is shown in Figure 2.1

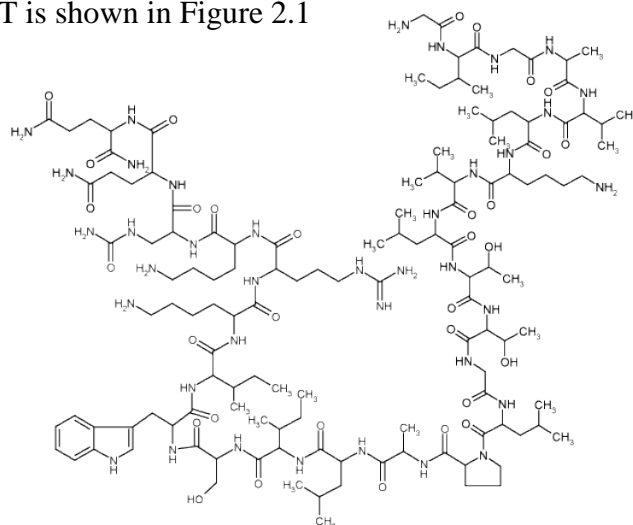


Figure 2.1: Structure of melittin [76]

It is water-soluble as a tetramer, but it spontaneously integrates into lipid bilayers and is thought to work as a hemolytic agent. Each melittin chain is composed of two α -helical segments and its overall shape is that of a bent rod. 20 amino acid residues after the NH_2 -terminal are arranged asymmetrically about the bent rod according to their polarity. Ten large apolar residues in each chain are on one side of the bent rod, and the opposite face contains four polar side chains, four small apolar side chains, and the prolyl residue. The C-terminal 6 residues of the chain are entirely polar. The four chains in the tetramer are packed together with nearly perfect 222 symmetry in such a shape that the interior is composed almost entirely of apolar side chains and the surface is coated with polar residues.

The contacts between melittin subunits in the tetramer forms are nearly all hydrophobic and have been analyzed in terms of helix-helix crossings. The balance between this hydrophobic adhesion of the monomers and the electrostatic repulsion of the 24 positive charges in the tetramer is such that melittin is tetrameric at concentrations prevailing in the venom sac of the bee and monomeric at the minimum concentrations required for cell lysis. The 3D structure of melittin peptide has been shown in Figure 2.2

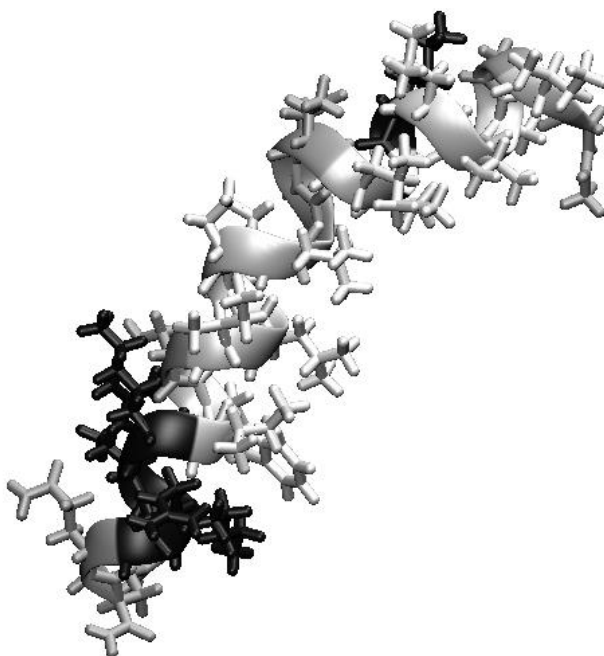


Figure 2.2: 3D structure of melittin peptide [77]

The investigated biological functions are summarized in Table 2.1.

Table 2.1: The investigated biological functions of melittin [78].

Major	Others	Adverse effects	Medical application
Hemolytic activity	Inhibits well-known transport pumps (such as Na ⁺ -K ⁺ -ATPase, H ⁺ -K ⁺ -ATPase)	Initiates various allergic reactions	Arthritis
Anti-inflammatory activity	Activates phospholipase A2	Lyses erythrocytes	Cancer
Anticancer, antibacterial, antifungal, antiviral activities	Diminishes membrane surface tension	Creates cytotoxicity in human peripheral blood lymphocytes	Diseases of central and peripheral nervous systems
	Stimulates smooth muscle	Modulates gene expression related to apoptosis, DNA damage response, and oxidative stress	Skin diseases
	Lowers blood coagulation		Heart and blood system related diseases
	Influences central nervous system (CNS)		Frozen shoulder
	Increases capillary permeability		Asthma, bronchitis

In the past few years, a number of studies regarding the beneficial role of MELT published stating that it possesses radioprotective, antimutagenic, anti-inflammatory, antinociceptive, and anticancer activities[79].

2.2 Literature Review

Qi, Zhu, Zhao, Fang and Sun (1994) conducted studies to purity control of different BV MELT preparations by CZE [80]. They developed a rapid CZE method for the determination of MELT during its purification from BV by column chromatography. The group used low-pH buffer to decrease adsorption of MELT on capillary column. Relative standard deviations of the migration time of melittin and of the peak are were 1.2 and 2.3% , respectively.

Pacakova et al. (1995) studied the HPLC and CE methods for the determination of PLA2 and MELT in BV [81]. They found that the results obtained indicate that both HPLC and CE can be readily used to differentiate BV. They also compared HPLC method with CE. In their prior studies have identified CE analysis is faster, efficiencies are better and costs are much lesser.

In other study and related research, Pacakova and Stulik (2000) examined the validation of a method for determination of PLA2 and MELT in BV [82]. They found that the limits of detection and quantitation were 4.5 and 15 $\mu\text{g/mL}$, respectively, for phospholipase A2 1.6 and 6 $\mu\text{g/mL}$, respectively, for melittin. The reproducibility of the measurements with 2 different CE instruments was satisfactory; the mean concentration and relative standard deviation (RSD) values for phospholipase A2 and melittin were 14.4% (RSD, 1.3%) and 51.4% (RSD, 1.1%), respectively.

Kokot and Matysiak (2009) conducted a new CZE-DAD method for BV analysis and standardization of the product [83]. The aim of this study was to develop a new method for BV analysis using cytochrome c as an internal standard. Their developed method was applied for quantitative analysis of 38 BV samples. The content of analysed in MELT peptide were 24.50% and 60.27% (mean 45.91 ± 9.78) respectively.

In another related research, Hagi et al., (2013) developed a reverse phase HPLC method and validated for the analysis of BV samples and drug products [84]. Limits of detection and quantification for melittin were 1.1 and 3.2 µg/ml respectively.



3. EXPERIMENTAL

3.1 Materials and Solutions

Standart substance of melittin was from Sigma-Aldrich (Steinheim, Germany) which Doç.Dr. Sibel Döşler kindly provided us. Phosphoric acid, acetic acid, hydrochloric acid, and sodium hydroxide were purchased from Merck (Darmstadt, Germany). Polyethyleneimine (PEI) was purchased from Fluka (Steinheim, Germany). All solutions were prepared with water purified by an Elga Purelab Option-7-15 model system (Elga, UK).

3.2 Preparation of Standard Solutions

Melittin stock solution was prepared in deionized water and stored at deep freeze.

3.3 Preparation of Bee Venom Samples

One mg of crude bee venom sample was accurately weighed. Melittin was extracted with deionized water. The mixture was vortexed for 5 min at 2500 rpm and sonicated for 30 min. The supernatant was filtered from 0.45 µm microfilter and directly injected to CE.

3.4 Instrumentation and Analysis Conditions

Separations were performed with an Agilent 1600 capillary electrophoresis system (Waldbronn, Germany) equipped with a UV. Separations were carried out in bare fused silica and PEI coated fused silica capillaries with 50 µm i.d. (Polymicro Technology, Phoenix, AZ, USA). The total length of the capillary was 65 cm and the length to the detector was 57 cm. In bare fused silica column, the separation was performed at 25 kV. In PEI coated column, the separation was performed at -25 kV. The temperature was set at 25 °C. Injections were made at 50 mbar for 6 s. The new

fused silica capillary was conditioned prior to use by rinsing with 1 M NaOH for 30 min and with deionized water for 10 min. For bare fused silica column, capillary was flushed successively by 1 M HCl for 15 min, deionized water for 10 min, and working buffer for 10 min at the beginning of every working day, 1 M HCl for 5 min, water for 2 min, and 5 min buffer between runs. For PEI coated column studies, running buffer for 15 min at the beginning of every working day, and running buffer for 2 min between runs. The data processing was carried out with the Agilent ChemStation software. For PEI coated column studies, capillary was flushed with working buffer for 15 min at the beginning of every working day, and running buffer for 2 min between runs.

3.5 Dynamic Coating Process for Capillary

Coating process was performed as described in the literature [38]. PEI polymer used as coating reagent. The fused silica capillary first etched by flushing the capillary with solution of 1 M sodium hydroxide for 30 min at 1000 mbar and water at 1500 mbar for 15 min then PEI solution in water at 1500 mbar for 10 min and a solution of PEI left in the capillary for one hour. Next the PEI polymer solution was pressed out of the capillary with air 1500 mbar. Finally the capillary was rinsed with water for 15 min and running buffer for 15 min. Running buffer for 15 min at the beginning of every working day, and running buffer for 2 min between runs.

4. RESULTS AND DISCUSSIONS

4.1 CE Method Optimization

In this study, phosphoric acid as working buffer was selected for bare fused silica capillary and acetic acid working buffer for PEI coated fused silica capillary. Different molarities were tested to obtain the desired resolution in real samples and stock solutions.

4.1.1 Low-pH buffer studies

Separation and identification of bee venom component melittin in low-pH studies were obtained in 100 mM phosphoric acid pH 1.55. Above pH:2, EOF was small as possible as with unionized silanol groups in the the capillary column, resolutions and efficiencies were high because of the positively charged MELT peptide in bare fused silica capillaries were not adsorped at the capillary inner wall. Electropherogram representing melittin in low buffer pH was given in Fig.4.1 A

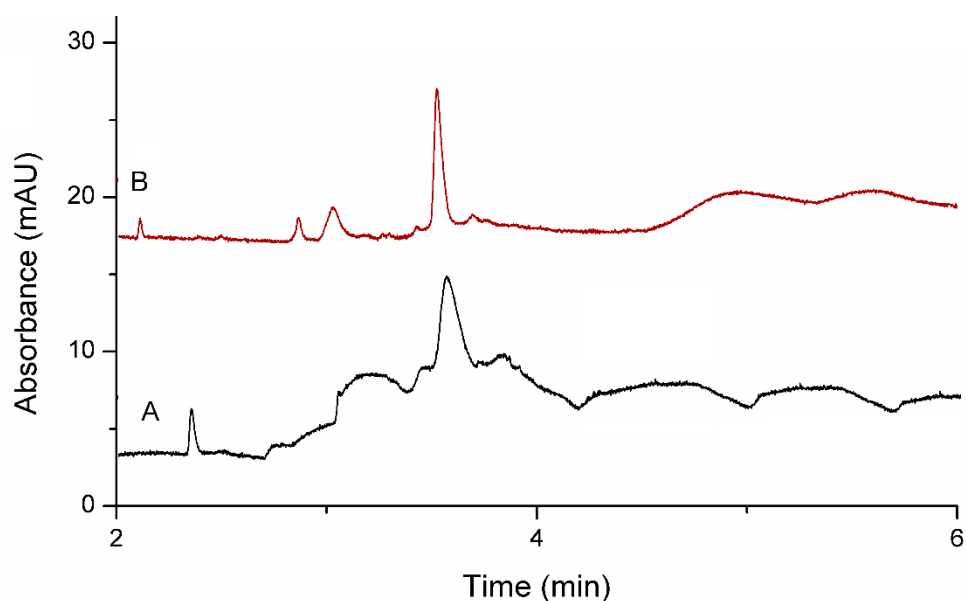


Figure 4.1 : Electropherogram of (A) 175µg/mL standard melittin solution (B) bee venom sample extract. Analysis conditions: Bare fused silica column (50 µm x 57 cm), Buffer: 100 mM phosphoric acid at pH 1.55; Voltage: 25 kV; Temperature: 25 °C; Injection: 50 mbar, 6 s; Detection: UV-DAD detector, λ: 200 nm.

4.1.2 Dynamic coated capillary studies

PEI coated fused silica capillary was studied to enhance the separation of MELT to diminish peptide adsorption to the negatively charged silanols of the fused silica surface. PEI coated silica capillary has changed the normal condition to reversed condition, injections made at cathodic side of CE and MELT migrate with EOF to anode. High efficiencies and resolutions were obtained in 50mM acetic acid pH 5.50.

Electropherogram of melittin in dynamic coated capillary was given in Fig.4.2.

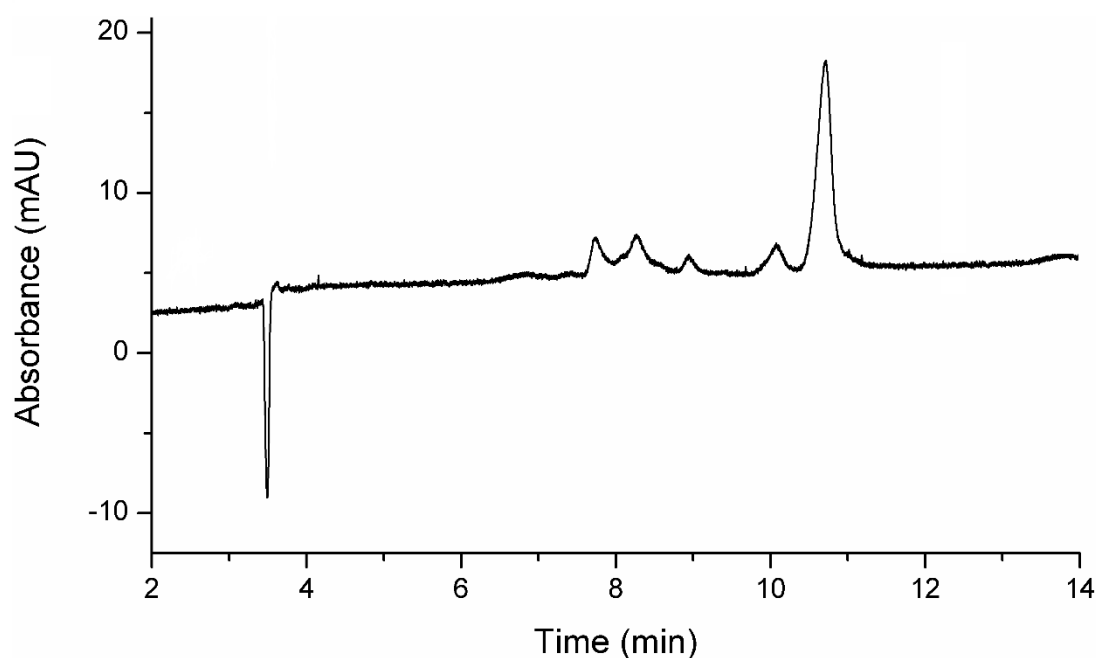


Figure 4.2: Electropherogram of 175 $\mu\text{g/mL}$ standard melittin solution. Analysis conditions: PEI-coated silica column (50 μm x 57 cm), Buffer: 50 mM acetic acid pH 5.50; Voltage: -25 kV; Temperature: 25 $^{\circ}\text{C}$; Injection: 50 mbar, 6 s; Detection: UV-DAD detector, λ : 200 nm.

4.2. Method Validations for Low-pH buffer studies and dynamic coated capillary studies

Method validation data for two experimental designs were given in Table 4.1. Calibration curves were constructed by plotting corrected peak areas versus analyte concentrations. For the intra-day precision of the methods, MELT standard was injected 5 times in one day and for the inter-day precision, MELT standard was injected 15 times in three days. The LOD values were calculated as three times the average noise taken for three different baseline areas. LOQ values were given as ten times the average noise.

Table 4.1. Method validation data for two experimental designs for melittin

Analytical Parameter	Low-pH buffer	PEI-coated capillary
Intra-day precision (n=5)		
Corrected peak area (RSD, %)	2.54	1.25
Migration time (RSD, %)	3.12	2.13
Inter-day precision (n=15)		
Corrected peak area (RSD, %)	3.42	2.57
Migration time (RSD, %)	4.84	3.16
Linearity		
Linear range ($\mu\text{mol/L}$)	70-350	35 - 350
Regression equation	$y=0.0004x-0.0158$	$y=0.0004x-0.0052$
Correlation coefficient	0.983	0.996
LOD, $\mu\text{mol/L}$	19.3	10.0
LOQ, $\mu\text{mol/L}$	64.9	33.3

As seen from Table 4.1, precisions and sensitivities of both methods are satisfactory for melittin analysis. Repeatabilities in PEI-coated capillary seem a little better than the repeatabilities in low pH buffer. Also LOD and LOQ values of analyte are lower in coated capillaries. On the other hand, in the method applied in low pH buffer, the migration time of melittin is considerably shorter. Both methods are suitable for analyzing melittin in bee venom and competing with each other.

4.3.Determination of MELT concentration in Bee Venom

In this study, due to the advantage of short analysis period, the method using low pH buffer was preferred for the analysis of real bee venom sample. Sample solutions were prepared as described in the experimental part. Each sample was analysed three times. Melittin concentration of the bee venom sample is given in Table 4.2.

Table 4.2 : Melittin in bee venom sample

	Melittin ($\text{mg g}^{-1} \pm \text{SD}$)*
Bee venom	245 ± 34

*n=3



5. CONCLUSION

The major difficulty in application of CE to basic peptides such as MELT is their basic tendency for adsorption on capillary wall due to electrostatic attraction between positively charged species and negatively charged silanol groups of capillary wall. To disqualify the adsorption, two different methods are studied. The first approach is using pH lower than 2 in order to suppress negative wall charge greatly. The second approach is coating of the fused silica capillary surface with a positively charged polymer, PEI. This approach eliminates surface charge of the capillary wall. Precisions and sensitivities of both methods are satisfactory for melittin analysis. Thereby, both methods are suitable for analyzing melittin in bee venom and competing with each other.



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