IMPROVEMENTS OF DETECTION SENSITIVITY IN CAPILLARY ELECTROPHORESIS (CE) FOR PEPTIDES

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THIS DISSERTATION IS SUBMITTED AS PARTIAL FULFILLMENT FOR BACHELOR OF SCIENCE WITH HONOURS

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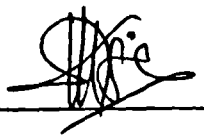

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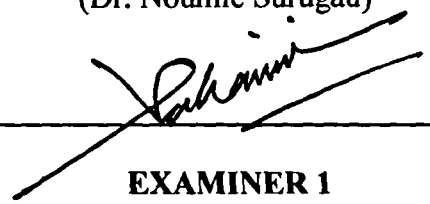

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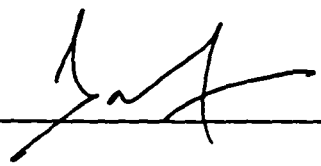

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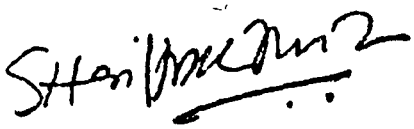

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ABSTRACT

Application of CE to peptide samples is limited due to poor concentration limit of detection (CLOD). At very low concentration, peptide is undetectable by CE with UV absorption detection. The lack of detection sensitivity of CE occurs as a result of small sample volume mean limiting loading capacity and short optical path length for detection. The main objective of this project is to analyze two preconcentration techniques that is sample stacking and field-enhanced sample injection in improving detection sensitivity of CE for peptide analysis. The second objective is to compare these two techniques and determine which technique is more effective in improving the detection sensitivity of CE. In sample stacking, peptides were prepared in 100% (v/v), 50% (v/v), 40% (v/v), 30% (v/v), 20% (v/v) and 10% (v/v) of phosphate buffer while BGE is always 100% (v/v) of phosphate buffer. The result shows significant increase in value of peak height gradually from 100% (v/v) to 10% (v/v) phosphate buffer. The best result for sample stacking was obtained when peptides were prepared in 10% (v/v) phosphate buffer. FESI use the same concept as sample stacking but differ in term of the injection procedure and focusing process. The best result for FESI was obtained when peptides were prepared in 20% (v/v) phosphate buffer. In 10% (v/v) phosphate buffer, only 8 peptides were detected instead of 9. The lost peptide was identified as methionine enkephalin. Injection of short plug of dilute buffer before injecting peptides in FESI ensures amplified field thus produce higher peaks. For comparison, FESI is more effective in improving detection sensitivity of CE in peptide analysis based on high significant peak that can be observed from electropherogram compared to sample stacking. As conclusion, the aim of this project has successfully been achieved.

ABSTRAK

Aplikasi CE kepada sesetengah sampel biologi adalah terhad disebabkan had pengesanan kepekatan (CLOD) yang rendah. Pada kepekatan yang amat rendah peptida tidak dapat dikesan dengan menggunakan CE bersama dengan pengesan serapan ultralembayung. Kekurangan sensitiviti pengesanan pada CE berlaku disebabkan isipadu sampel yang kecil yang bermaksud kapasiti muatan yang terhad dan juga panjang optikal yang pendek untuk pengesan. Objektif utama projek ini ialah untuk menganalisis dua teknik prakepekatan iaitu longgokan sampel dan suntikan sampel medan peningkatan untuk analisis peptida. Objektif kedua ialah untuk membandingkan kedua-dua teknik ini dan menentukan teknik mana yang lebih efektif dalam meningkatkan sensitiviti pengesanan pada CE. Dalam longgokan sampel, peptida disediakan dalam larutan fosfat berkepekatan 100% (v/v), 50% (v/v), 40% (v/v), 30% (v/v), 20% (v/v) dan 10% (v/v) manakala kepekatan BGE adalah sentiasa 100% (v/v). Keputusan menunjukkan peningkatan yang signifikan dalam nilai tinggi puncak dari 100% (v/v) ke 10% (v/v). Keputusan terbaik diperolehi apabila peptida disediakan dalam larutan fosfat berkepekatan 10% (v/v). FESI menggunakan konsep yang sama seperti longgokan sampel tetapi berbeza dari segi prosedur suntikan dan proses pemfokusan. Keputusan terbaik untuk FESI diperolehi apabila peptida disediakan dalam larutan fosfat berkepekatan 20% (v/v). Dalam larutan fosfat berkepekatan 10% (v/v), hanya 8 peptida dapat dikesan dan bukannya 9. Peptida yang hilang dikenalpasti sebagai methionine enkephalin. Suntikan sedikit larutan penimbal sebelum suntikan sampel memastikan medan amplifikasi seterusnya menghasilkan puncak-puncak yang lebih tinggi. Untuk perbandingan, FESI didapati lebih efektif dalam miningkatkan had pengesanan kapilari elektroforesis dalam analisis peptida berdasarkan kepada ketinggian puncak yang signifikan yang boleh diperhatikan daripada elektroferogram berbanding longgokan sampel. Kesimpulannya, objektif projek ini berjaya dicapai.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF UNITS, SYMBOLS AND ABBREVIATIONS

CE	Capillary Electrophoresis
CZE	Capillary Zone Electrophoresis
CGE	Capillary Gel Electrophoresis
MECC	Micellar Electrokinetic Capillary Electrochromatography
CITP	Capillary Isothachorphoresis
DNA	Deoxyribonucleic Acid
R	Side Chains of Amino Acids
υ_{eof}	Velocity of Electroosmotic Flow
$\mu_{eof,}$	Electroosmotic mobility
ε	Dielectric constant of the buffer
ζ	Zeta Potential
E	Applied electric field
η	Viscosity of the buffer
UV	Ultraviolet
kV	Kilovolt
µA	Microampere
W	Watt
nm	Nanometer
A	Absorbance
a	Absorptivity
b	Path length
C	Solute Concentration
CLOD	Concentration Limit of Detection
BGS	Background Solution
FESI	Field-Enhanced Sample Injection
FASS	Field-Amplified Sample Stacking
BGE	Background Electrolyte
ITP	Isotachorphoresis
tITP	Transient Isotachorphoresis

HPLC	High Performance Liquid Chromatography
mPC-CE	Membrane Preconcentration Capillary Electrophoresis
EOF	Electroosmotic Flow
HF	Hydrogen Fluoride
%	Percentage
cm	Centimeter
mL	milliliter
NH_3	Ammonia
H_3PO_4	Phosphoric Acid
mM	milimolar
v/v	volume/volume
µm	micrometer
K	Kelvin
psi	Pound per square inch
s	seconds
NaOH	Sodium Hydroxide
μgml^{-1}	microgram/mililiter

LIST OF APPENDIX

CHAPTER 1

INTRODUCTION

1.1 Capillary electrophoresis (CE)

Capillary electrophoresis (CE) has a very good sensitivity based on mass detection. A feature, which is important when the sample size is very limited as in analyzing a single cell, thus a minute amount of sample is sufficient (Shihabi, 2000). In the last two decades, various CE modes have been developed such as capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), micellar electrokinetic capillary electrochromatography (MECC), and capillary isothachorphoresis (CITP) (Wu, 2003). Since it operates using distinct separation mechanism (based on differences in the analytes' charge to size ratio), it can serve as an alternative method for samples that are not easily deal with chromatography-based techniques (Monton & Terabe, 2006).

CE is quite useful in the field of peptide analysis, especially for small polar peptide separation, which is difficult to perform using other techniques (Messana *et al.*, 1997). CE also offers several advantages over other separation mechanisms, including short analysis times, low sample volumes, and high-resolution separations (Deterding *et al.*,2003). Furthermore, samples losses also are minimized due to limited surface area and volume of the capillary. However, limited surface area and volume mean limited loading capacity, which results in poor concentration limit of detection (CLOD). This problem has proven to be a major limitation of CE in the analysis of many biologically derived analyte mixtures (Yang *et al.*, 1999).

Thus, CE has less than desirable sensitivity based on concentration especially when compared to high-performance liquid chromatography. Because of the need for better sensitivity of detection in CE, sample concentration becomes crucial for the widespread use of this technique in the practical analysis (Shihabi, 2000).

Other problems in applying CE to some biological samples such serum, peptide and plasma is high concentration of inorganic constituents in the samples may reduce the efficiency of CE separation. Some components also can bind to capillary wall and affect the migration time, thus result in interfering peaks in electropherograms (Wu, 2003).

1.2 The importance of peptides

Peptides represent a large group of biomolecules playing vitally important role in living organisms. Natural peptides are composed of approximately 20 amino acids.

Peptide molecules differ in terms of their electric charge, size, shape, hydrophobicity and specific binding capacity. These features make them can be separated by electromigration methods. More recently, peptides and proteins belong to the largest classes of compounds separated by capillary electrophoresis (Kašička, 1999).

Separation and identification of peptides derived from an individual protein or from protein mixtures is becoming increasingly important in the burgeoning field of proteomics (Sheng *et al.*, 2000). Besides, the availability on the market of new peptide and proteins drugs with therapeutically activity in low concentrations increase rapidly. Peptide and protein class demands sensitive bioanalytical methods compared to most of the conventional drugs for determination in biological matrices. Increasing challenge for many researchers to establish peptide profiles of healthy biosystems and to study modifications of these profiles in the case of disease also a reason for the increasing interest in the development of sensitive method for study of peptide (Stroink *et al.*, 2001).

Analytical chemists have attempted to develop sample fractionation, separation, concentration, and detection methods that possess sufficient resolution to separate large numbers of peptides, as well as be sensitive enough and with the dynamic range to detect those peptides in low abundance (Issaq *et al.*, 2002)

1.3 Objectives of the study

The objectives of this study are:

1) To analyze two preconcentration techniques, namely sample stacking and field-enhanced sample injection in improving the detection sensitivity of CE for peptide analysis.
2) To compare these two techniques and determine which technique is more effective in improving the detection sensitivity of CE.

1.4 Scope of the study

In this study, model peptides that will be used are a mixture of nine hormones peptide (P2693) obtained from Sigma-Aldrich in the highest grade available. Capillary electrophoresis that is used is Beckman P/ACE MDQ (Beckman Coulter, Inc., Fullerton, CA). After separation, detection process will use UV absorbance detection at 190-200 nm.

CHAPTER 2

LITERATURE REVIEW

2.1 An Overview of Electrophoresis

Electrophoresis is the movement of electrically charged particles or molecules in a conductive liquid medium, usually aqueous, under the influence of an electric field. The ends of a glass tube that is filled with an aqueous buffer, called the electrolyte or run buffer, are connected to containers filled with the same buffer. Also in these containers are electrodes that are connected to power supply. The rates and direction of migration of analytes depend on the sizes of the ions and the magnitudes and signs of their charges (Baker, 1995).

A particular strength of electrophoresis is its unique ability to separate charged macromolecules of interest in the biotechnology industry and in biochemical and biological research. Electrophoresis has been the powerhouse method of separations of proteins and nucleic acids for many years. For example, in order to sequence DNA it is necessary to distinguish between long-chain polynucleotides and differing by only

a single nucleotide. Only electrophoresis has sufficient resolving power to handle this problem (Skoog *et al.*, 1998).

A smaller ion will move faster than a larger ion of the same charge. An ion with a higher charge will migrate faster than one with a lower charge, if they are the same size. Neutral molecules will not be influenced by the electric field.

Under the influence of electric field, the buffer and the neutral molecules also move through the tube due to eletroosmosis. This electroosmotic flow of the buffer moves toward the negative electrode and carries the solutes with it. The separated molecules can be analyzed by placing some type of detector near the end of the tube that senses the molecules as they move through the tube. The output of the detector is a plot of detector response versus time, and is called an electropherogram (Baker, 1995).

2.2 Peptides

Peptide are composed of approximately 20 amino acid species linked by peptide bonds and sometimes also cross-linked by the disulfide bridges (Kašička, 1999). Amino acids are organic molecules possessing both carboxyl and amino groups. At the centre of amino acid is an asymmetric carbon atom called the alpha (α) carbon. Its four different partners are an amino group, a carboxyl group, a hydrogen atom, and a variable group symbolized by *R*. Figure 2.1 shows general formula of an amino acid.

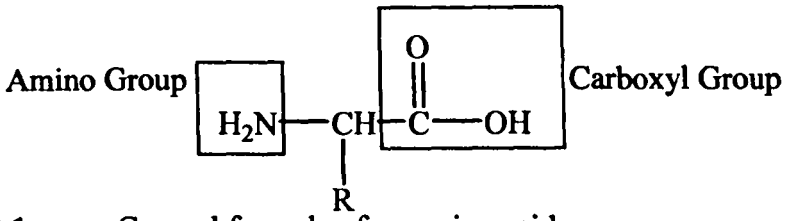

Figure 2.1 General formula of an amino acid

Amino acids are grouped according to the properties of their side chains (*R* groups). Figure 2.2 shows chemical structure of nonpolar, aliphatic *R* groups of amino acids that build peptide and then proteins. Figure 2.3 shows polar, uncharged *R* groups of amino acids. Figure 2.4 shows aromatic *R* groups of amino acids. Figure 2.5 shows positively *R* groups of amino acids. Figure 2.6 shows negatively *R* groups of amino acid. In these 5 figures, the area in the box represents *R* group, the side chain that divide by acidic and basic. The *R* group differs with the amino acid. The physical and chemical properties of the side chain determine the unique characteristics of a particular amino acid (Campbell & Reece, 2002).

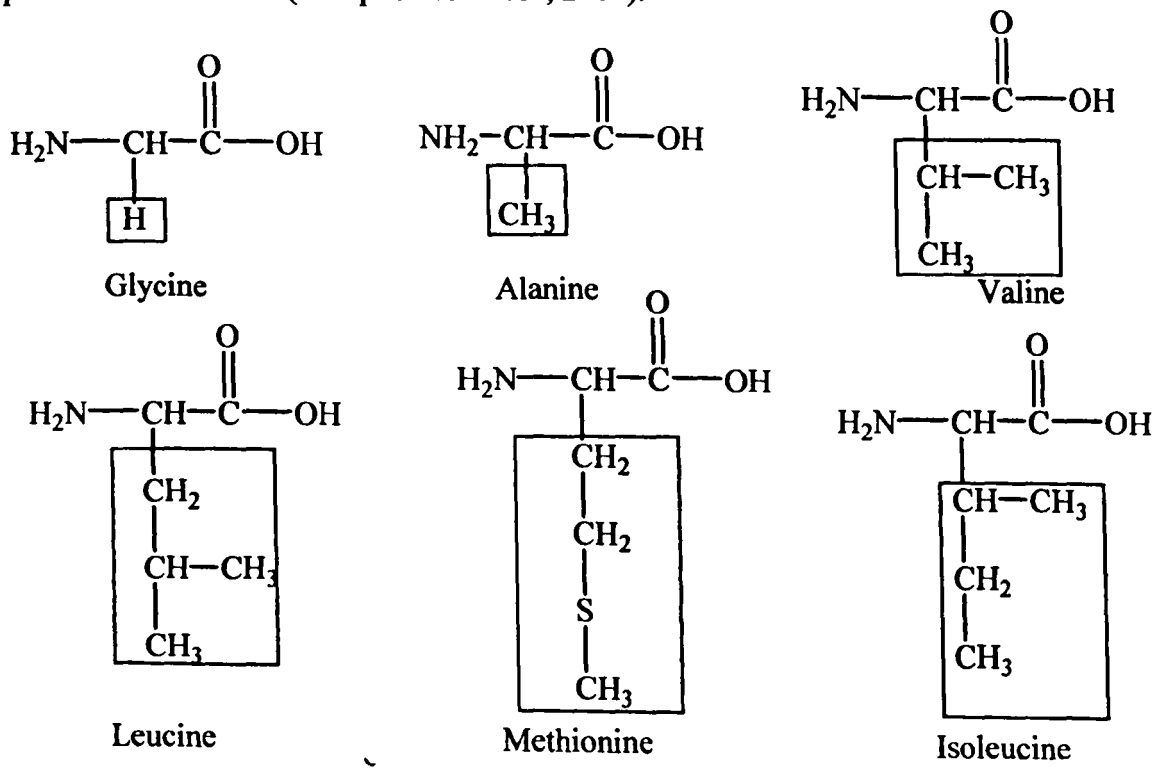

Figure 2.2 A *R* groups of nonpolar and aliphatic amino acids

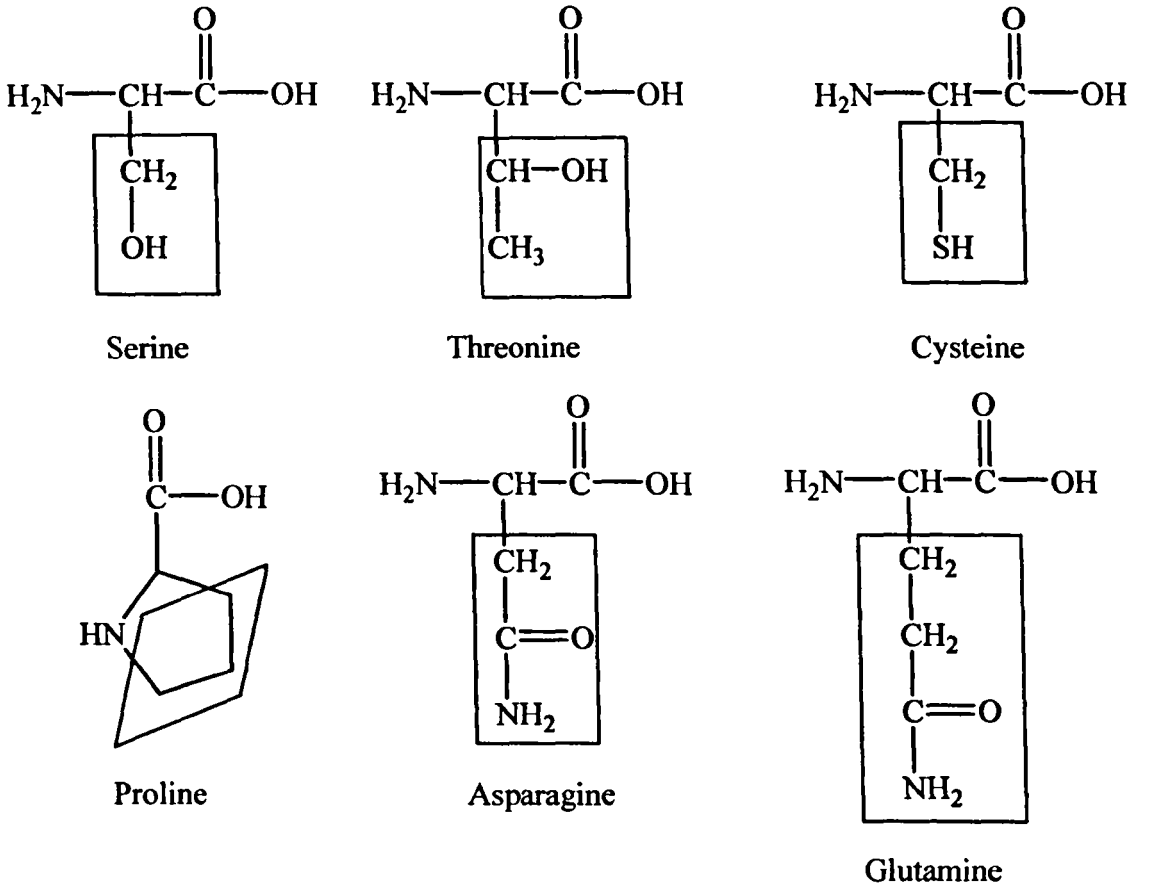

Figure 2.3 A *R* groups of polar and uncharged groups of amino acids

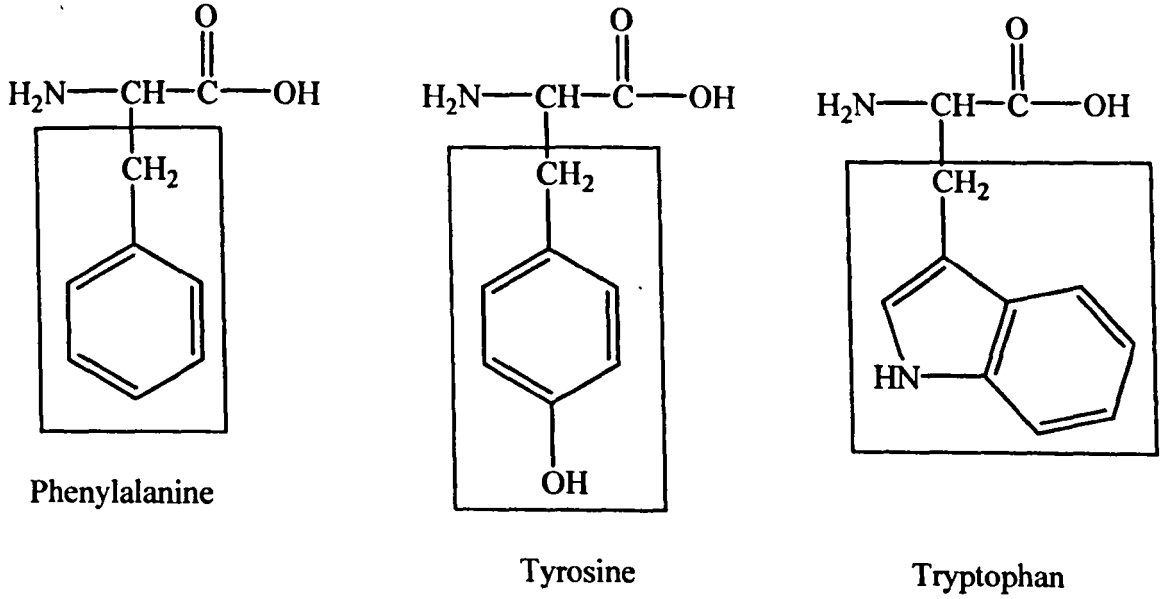

Figure 2.4 Aromatic *R* groups of amino acids

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