การพัฒนาวิธีตรวจวัดเมทิลพาราเบน เอทิลพาราเบนและโพรพิลพาราเบนโดยใช้อัลตราเพอร์ฟอร์มานซ์ลิควิดโครมาโทกราฟีร่วมกับการตรวจวัดทางเคมีไฟฟ้า

นางสาวมณีนุช ชูโต

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตร์มหาบัณฑิต
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METHOD DEVELOPMENT FOR DETERMINATION OF METHYLPARABEN, ETHYLPARABEN AND PROPYLPARABEN BY ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED WITH ELECTROCHEMICAL DETECTION

Miss Maneenuch Chuto

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Chemistry
Department of Chemistry
Faculty of Science
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Thesis Title	METHOD DEVELOPMENT FOR DETERMINATION OF METHYLPARABEN, ETHYLPARABEN AND PROPYLPARABEN BY ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED WITH ELECTROCHEMICAL DETECTION
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มณีนุช ชูโต : การพัฒนาวิธีตรวจวัดเมทิลพาราเบน เอทิลพาราเบนและโพรพิลพาราเบนโดยใช้อัลตราเพอร์ฟอร์มานซ์ลิควิดโครมาโทกราฟีร่วมกับการตรวจวัดทางเคมีไฟฟ้า (METHOD DEVELOPMENT FOR DETERMINATION OF METHYLPARABEN, ETHYLPARABEN AND PROPYLPARABEN BY ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED WITH ELECTROCHEMICAL DETECTION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร. อรวรรณ ชัยลภากุล 96 หน้า

ในงานวิจัยนี้ได้พัฒนาวิธีตรวจวัดสารกันบูดในกลุ่มพาราเบน ได้แก่ เมทิลพาราเบน เอทิลพาราเบน และโพรพิลพาราเบน โดยใช้เทคนิคอัลตราเพอร์ฟอร์มานซ์ลิควิดโครมาโทกราฟีร่วมกับการตรวจวัดทางเคมีไฟฟ้าโดยใช้ขั้วไฟฟ้าเพชรที่โดปด้วยโบรอน โดยมีจุดประสงค์หลักคือ การวิเคราะห์ให้ได้ผลเร็ว ทั้งนี้จึงได้เลือกใช้คอลัมน์ชนิดโมโนลิทขนาดเล็ก (25 มิลลิเมตร x 4.6 มิลลิเมตร) ร่วมกับเทคนิคอัลตราเพอร์ฟอร์มานซ์ลิควิดโครมาโทกราฟี ข้อดีของคอลัมน์ชนิดนี้คือให้แรงดันกลับต่ำ แม้เพิ่มอัตราการไหลของเฟสเคลื่อนที่ ภาวะการทดลองที่เหมาะสมในการแยกคือ เฟสเคลื่อนที่ประกอบด้วย อะซิโตไนไทรล์และฟอสเฟตบัฟเฟอร์ พีเอช 5 ความเข้มข้น 0.05 โมลาร์ ในอัตราส่วน 25:75 (v/v) โดยมีอัตราการไหลของเฟสเคลื่อนที่ผ่านคอลัมน์ที่ 2.5 มิลลิลิตรต่อนาที สำหรับการตรวจวัดทางเคมีไฟฟ้าโดยใช้ขั้วไฟฟ้าเพชรที่โดปด้วยโบรอนเป็นขั้วไฟฟ้าใช้งานนั้น ได้ทำการหาภาวะที่ เหมาะสมสำหรับการตรวจวัดด้วยเทคนิคแอมเพอโรเมตรี พบว่า ค่าศักย์ไฟฟ้าที่เหมาะสมสำหรับการตรวจวัดสารในกลุ่มพาราเบนนี้คือ 1.5 โวลท์ (เมื่อเทียบกับขั้วไฟฟ้าอ้างอิงซิลเวอร์ในซิลเวอร์คลอไรด์) จากภาวะที่เหมาะสมข้างต้น พบว่าสามารถวิเคราะห์พาราเบนทั้งสามชนิดได้อย่างรวดเร็วภายใน 2 นาที มีช่วงของความเป็นเส้นตรงที่ 0.1-50 มิลลิกรัมต่อลิตรและมีขีดจำกัดการตรวจวัดที่ 0.03 มิลลิกรัมต่อลิตร วิธีที่พัฒนาขึ้นนี้สามารถนำไปประยุกต์กับตัวอย่างจริง โดยตัวอย่างจริงทั้ง 6 ชนิดนี้ประกอบด้วย ตัวอย่างน้ำอัดลม วุ้น ยาสีฟัน น้ำยาบ้วนปาก โลชั่นและแป้ง จากการทดลองวิเคราะห์ในตัวอย่างจริงที่ความเข้มข้น 3 ค่า คือ 12 22 และ 32 มิลลิกรัมต่อลิตรพบว่าให้ค่าร้อยละของการคืนกลับที่ 80.3-98.8 ดังนั้นวิธีดังกล่าวสามารถวิเคราะห์สารกันบูดในกลุ่มพาราเบนในตัวอย่างหลายชนิดได้อย่างมีประสิทธภาพและรวดเร็ว

ภาควิชา.....เคมี.....ลายมือชื่อนิสิต.....
สาขาวิชา.....เคมี.....ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....
ปีการศึกษา.....2554.....

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KEYWORDS : PARABENS / ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHY / MONOLITHIC COLUMN / BORON-DOPED DIAMOND ELECTRODE / AMPEROMETRY

MANEENUCH CHUTO : METHOD DEVELOPMENT FOR DETERMINATION OF METHYLPARABEN, ETHYLPARABEN AND PROPYLPARABEN BY ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED WITH ELECTROCHEMICAL DETECTION. ADVISOR: ASSOC. PROF. ORAWON CHAILAPAKUL, Ph. D., 96 pp.

In this study, a new technique was developed using rapid ultra-performance liquid chromatography (UPLC)-based separation coupled with electrochemical detection by a boron-doped diamond (BDD) electrode for the detection and quantification of three commonly used parabens (methylparaben (MP), ethylparaben (EP) and propylparaben (PP)). The ultimate goal is aimed to reduce the analysis time by using UPLC coupled with a short reverse phase C18 monolithic column (25 mm x 4.6 mm). Operating the monolithic column at low back-pressure resulted in high flow rates. A mobile phase consisting of a 25:75 (v/v) ratio of acetonitrile: 0.05 M phosphate buffer (pH 5) at a flow rate of 2.5 mL min^{-1} was used to perform the separation. The amperometric detection with the BDD electrode was found to be optimal and reliably reproducible at a detection potential of 1.5 V vs. Ag/AgCl. Under these conditions, the separation of the three target analytes (MP, EP and PP) was achieved in 2 min and was linear within a sample concentration range of 0.1 to 50.0 mg L^{-1} ($r^2 > 0.997$). This method was successfully applied to determine the concentrations of each paraben in six real samples with the recoveries ranging from of 80.3 - 98.9% for all three parabens from samples spiked at 12, 22 and 32 mg L^{-1}. Therefore, the proposed method can be used as an alternative rapid and selective method for the determination of paraben levels in real samples.

Department : Chemistry Student's Signature .....
Field of Study : Chemistry Advisor's Signature .....
Academic Year : 2011

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LIST OF ABBREVIATIONS

Ag/AgCl	Silver/silver chloride
AOAC	Association of Analytical Communities
BDD	Boron-doped diamond
CE	Capillary electrophoresis
CV	Cyclic voltammetry
CVD	Chemical vapor deposition
^{o}C	Degree Celsius
E	Potential
E^{0}	Formal reduction potential
EB	Ethylparaben
EEC	European Economic Community
E_{pa}	Anodic peak potential
E_{pc}	Cathodic peak potential
GC	Gas chromatography
HPLC	High performance liquid chromatography
IUPAC	International Union for Pure and Applied Chemistry
$i_{p,a}$	Anodic peak current
$i_{p,c}$	Cathodic peak current
L	Liter
M	Molar
MB	Methylparaben
mL	Milliter

MS	Mass spectroscopy
min	Minute
N	Noise
n	Number of electron
nm	Nanometer
PB	Propylparaben
RE	Reference electrode
S	Signal
SD	Standard deviation
SEM	Scanning electron microscopy
UPLC	Ultra-performance liquid chromatography
RSD	Relative standard deviation
WE	Working electrode
μA	Microampere

CHAPTER I

INTRODUCTION

1.1 Introduction

Parabens are an ester of *para*-hydroxybenzoic acid. They are effective chemical preservatives that have been used in several kinds of products such as food, cosmetics and pharmaceutical products since more than 80 years ago because they are broad spectrum, low toxicity, have a good stability and are non-volatile [1]. The actions of pareben preservatives are that they have an inhibitory effect on membrane transport and processes of mitochondrial functions [2]. The antimicrobial activities of the parabens appear to increase with the increasing chain length but they also have lower solubility in water [3]. Parabens are synthesized from esterfication of *para*-hydroxybenzoic acid with alcohol (methyl-, ethyl-, propyl- and etc.). In this research, the common parabens such as methylparaben (MB), etylparaben (EB) and propylparaben (PB) were investigated.

Normally, the toxicology of parabens is low because their quick hydrolysis. However, there are many researches which show an endocrine disrupting [4,5], weak estrogenic of parabens [6,7] and parabens found in breast cancer [8]. The European Economic Community (EEC) directly permits the use of parabens in cosmetics with a maximum concentration for total parabens of 0.8% (w/w) and for single parabens of 0.4% (w/w) [9]. The maximum concentration of parabens in foods is 0.1% (w/w) and in pharmaceuticals is 1% (w/w) [10,11].

Furthermore, the parabens are the cause of contact dermatitis on the skin called paraben paradox. Paraben paradox was first referred to in 1973 by Fisher [12]. Therefore, the given content of parabens in products should be considered. The separation methods for parabens have been reported: high-performance liquid

chromatography (HPLC) [13-16], gas chromatography (GC) [17-20], capillary electrophoresis (CE) [1,21]. The common detection of parabens analysis are ultraviolet (UV) detection [13-16], chemiluminescent (CL) detection [22,23] and mass spectrometric (MS) detection [13, 15, 20]. However, these separation methods are time-consuming. For the detection method such as the ultraviolet detection there is a high detection limit and although chemiluminescent detection and mass spectrometric detection are low detection limits but it is difficult to select a chemilumenescent generator. For mass spectrometric detection, there is high cost for the equipment and an specialized labor. A few years ago, the new technique was developed from a conventional high-performance liquid chromatography (HPLC) called ultra-performance liquid chromatography or UPLC. This technique achieved good resolution, high speed and good sensitivity [24]. There are many benefits about UPLC. For example, it can operate at 1000 bar (15,000 psi) which is a much higher pressure than HPLC and can use higher flow rates for fast analysis.

Furthermore, the short monolithic column has been investigated [25,26]. The monolithic columns were developed based on a new sol–gel technology. They provide excellent separation in a fraction of time owing to their highly porous monolithic rods of silica. The pore structure provides a combination of macropores and mesopores that have a much higher porosity (about 15%) than conventional particulate columns [27]. Regarding electrochemical detection, the electrochemical detection is an alternative method for determination of parabens in several samples such as food, personal care and cosmetic products. A few electrochemical detections have been reported [28-30]. Compared to spectrophotometric techniques, electrochemical detection requires a low cost instrumentation. In addition, this technique offers fast analysis and highly sensitive.

1.2 Objectives and scopes of the thesis

In this research, a rapid determination of three parabens including methylparaben, ethylparaben and propylparaben by ultra-performance liquid chromatography coupled with electrochemical detection were developed. Then the

proposed method was applied to detect three parabens in real samples such as softdrink, jelly, toothpaste, mouthwash, lotion and BB powder sample.

To achieve this objective, there are two parts of work consisting of an electrochemical detection and separation part. The electrochemical detection part was investigated to improve the sensitivity of the parabens detection at low concentration levels ($\mu g\ L^{-1}$) at boron-doped diamond electrode. The supporting electrolytes, pH of buffer, concentration and working potential were reported. The separation part was optimized for fast analysis and good resolution. A ratio of the mobile phase and flow rate were investigated. In addition, the analytical performance of the proposed method such as linearity range, limit of detection, and limit of quantitation were studied. Finally, the developed method was applied to analyze three paraben preservatives in various kinds of samples.

CHAPTER II

THEORY AND LITERATURE SURVEY

2.1 Parabens

The parabens or *p*-hydroxybenzoic acid are synthetic preservatives such as antimicrobial substances which inhibits the growth of bacteria, yeasts, fungi and other micro-organisms. They have a six-member carbon ring with a hydroxyl group of the ring and a side chain called an alkyl ester on the opposite side of the ring. They are commonly used in food, cosmetics, and pharmaceutical products because they are broad spectrum antimicrobial and antifungal agents especially effective against molds and yeasts with a low toxicity to humans, have a good stability, are effective over a wide pH range and non-volatile. The actions of parabens are inhibition of membrane transport and mitochondrial function processes. The activities of the parabens appear to increase with the increasing chain length but they also have lower solubility in water. With the combination of two or more parabens, their antimicrobial performance is enhanced because it is a synergistic effect. In this work, the separation of three commonly used parabens, methylparaben (MP), ethylparaben (EP) and propylparaben (PP) (**Figure 2.1**) was reported.

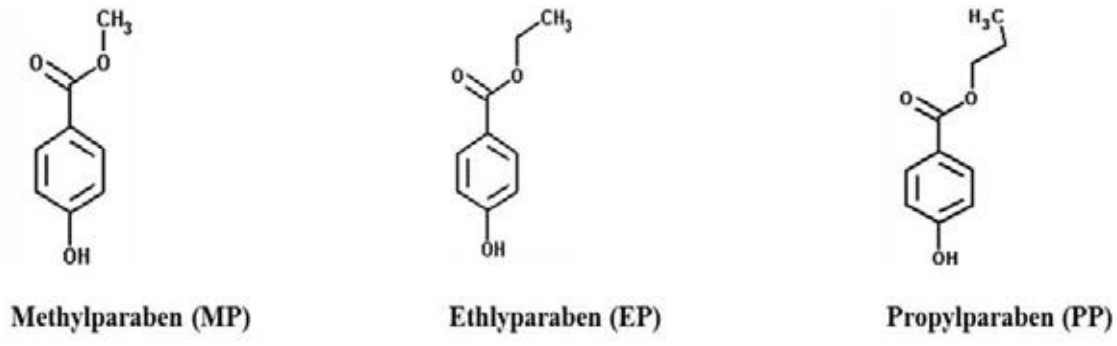


Figure 2.1 The chemical structure of three parabens

2.2 Liquid chromatography [31-32]

The chromatography is a separate method which is based on a distribution between two phases called the stationary phase and the mobile phase. From the official definitions of chromatography by IUPAC, "the mobile phase is a fluid which percolates through or along the stationary bed, in a definite direction. It may be a liquid or a gas or a supercritical fluid" and the stationary phase is one of the two phases forming a chromatographic system. It may be a solid, a gel or a liquid. If it is a liquid, it may be distributed on a solid. This solid may or may not contribute to the separation process. The liquid may also be chemically bonded to the solid (Bonded Phase) or immobilized onto it (Immobilized Phase). Therefore, the characteristics of the mobile phase can be divided into three basic types of chromatography. They include liquid chromatography (LC), gas chromatography (GC) and supercritical-fluid chromatography (SFC). The classification of chromatographic systems is shown in Figure 2.2.

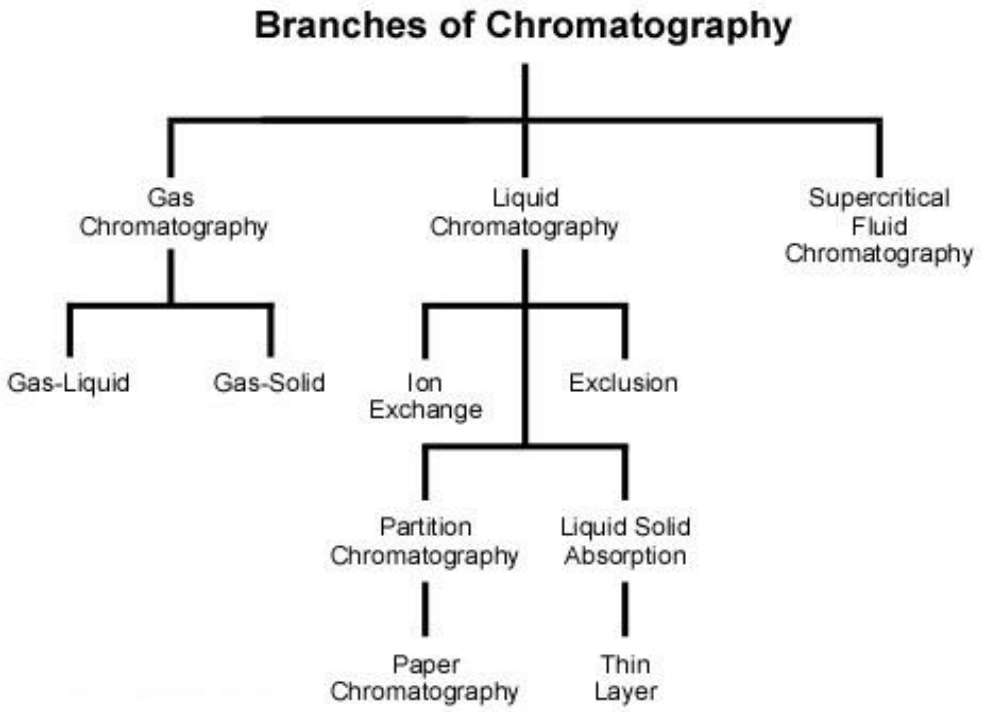


Figure 2.2 Classification of chromatographic systems

The interaction of chemical structure between analytes and the stationary phase cause the separation of each of the analytes. For the normal phase system, this mode is carried out with a polar stationary phase and a nonpolar mobile phase and the other one is a reverse phase system which consists of a nonpolar stationary phase and a polar mobile phase.

2.2.1 High-performance liquid chromatography [33-34]

High performance liquid chromatography (HPLC) is a part of liquid chromatography. It is widely used for the separation of compounds. For the stationary phase, it can be a solid or liquid supported on a solid and the mobile phase is a liquid. There are four types of high-performance liquid chromatography, including (1) adsorption, (2) partition, (3) ion-exchange and (4) exclusion. These modes of separation are based on interactions between the sample molecules or ions and the stationary and mobile phase. The schematics for the four modes are shown in Figure 2.3.

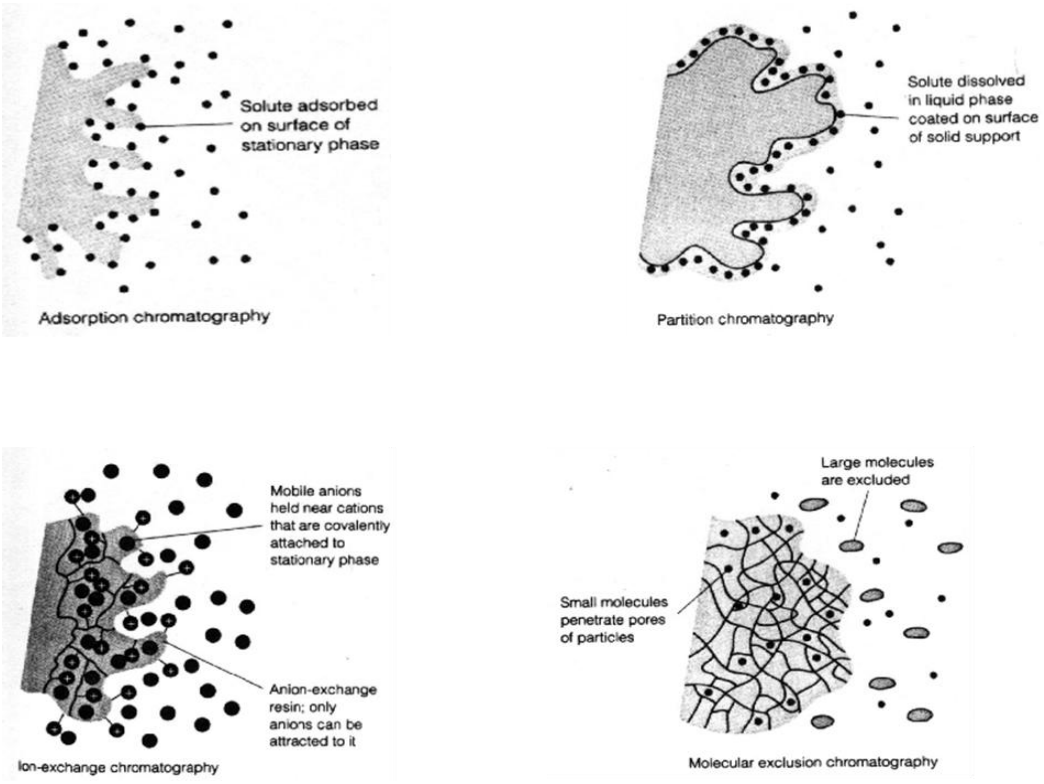


Figure 2.3 Schematic of the four types of liquid chromatography.

2.2.2 Ultra-performance liquid chromatography [35-36]

For many years ago, HPLC has been widely used in the laboratories because they are an efficient method for separating the compounds in a mixture. In recent years, the researchers have become interested in a rapid separation for high-throughput analysis. The ultra-performance liquid chromatography called UPLC is a new technology which improves speed, resolution and sensitivity of analysis. UPLC is a technology which has been developed from HPLC and uses the same principles as HPLC. The packing materials of the column are an important factor for development of separation. The Van Deemter plot was presented in Figure 2.4, which describe a relationship between the linear velocity or flow rate and plate height (HETP) which can be investigated using a chromatographic performance. According to the Van Deemter equation, the particle sizes decrease to less than 2.5 µM which can increase the efficiency separation at increased flow rates. UPLC was developed from HPLC which can use a smaller particle column with a higher flow rate to produce fast analysis while maintaining the low HETP and high resolution.

$$H = A + B/u + (CS + CM)u$$

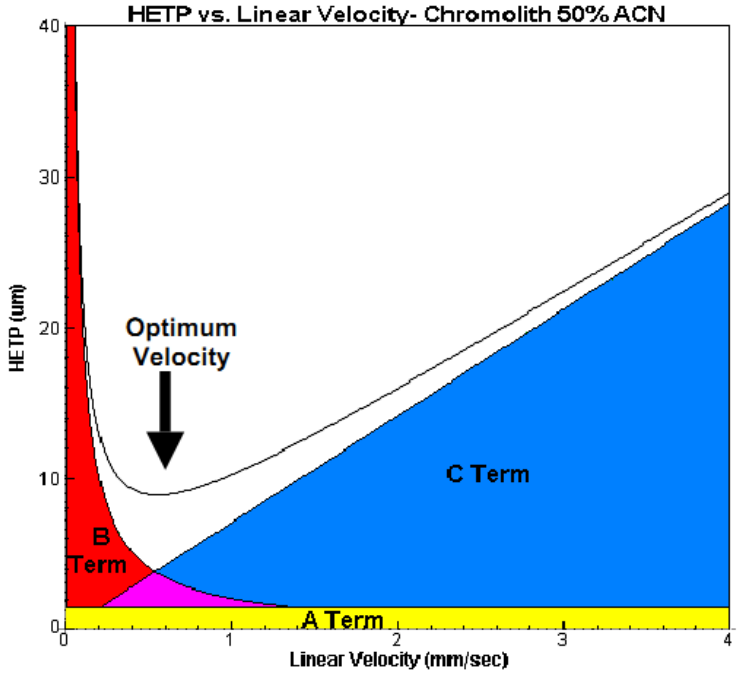


Figure 2.4 Van Deemter equation and Van Deemter plot

UPLC has several advantages for rapid separation, such as using a smaller particle column and/or higher flow rates for increased speed. UPLC can operate at a much higher pressure; 1000 bar (15,000 psi), which reduces the analysis time and so allows a higher sample throughput. The UPLC feature is shown in Figure 2.5.

Figure 2.5 The Ultra-performance liquid chromatography (Shimadzu LC-20ADXR UFLC)

2.3 Instrument of UPLC system [37-38]

2.3.1 Pump

The pump of ultra-performance liquid chromatography is a similar function of the pump in HPLC but it has been developed for high pressure. The UPLC can be operated at higher pressure than the conventional HPLC (15,000 psi). The solvent delivery method is a parallel-type double plunger with two inlet check valves. An operation temperature works in a range at 4°C to 35°C.

2.3.2 Autosampler

In UPLC, an autosampler must offer precise, accurate and speedy measurements to achieve the efficiency of analysis results. Conventional injection valves, either automated or manual cannot be used at extreme pressures. The analysis

time can be reduced owing to the high-speed vertical movement of the needle which enables ultra-high-speed sample processing and minimized carryover. An analysis cycle of UPLC and a comparison of analysis time between HPLC and UPLC injection were presented in Figure 2.6 and 2.7. The injection modes are total-volume sample injection and variable injection volumes that can be set in a range at 0.1 – 50 µL. The operation temperature and pH are working in a range between 4°C to 35°C and pH 1 to pH 14.

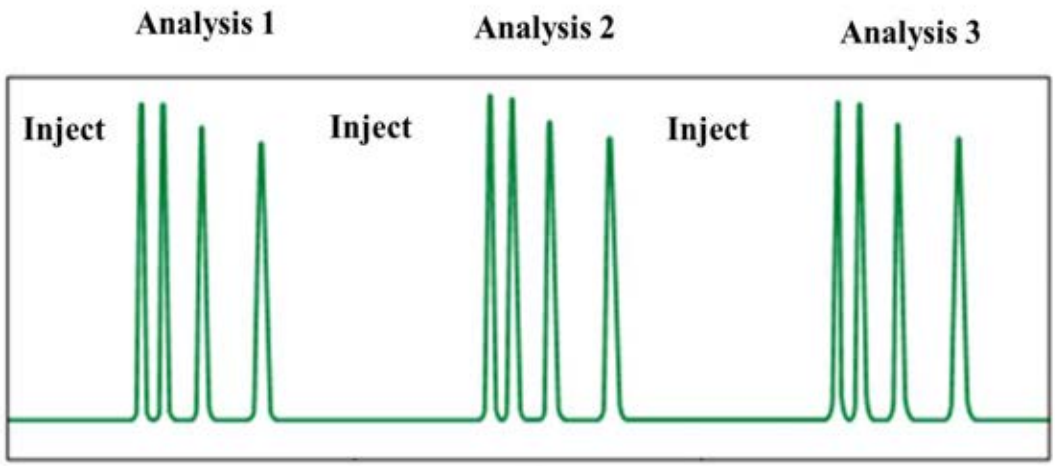


Figure 2.6 An analysis cycle of UPLC that reduced the total analysis time.

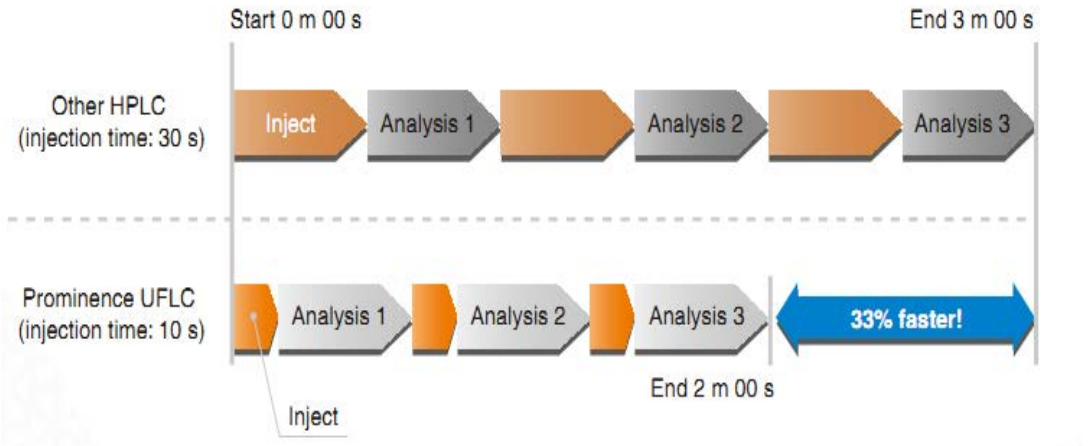


Figure 2.7 The comparison of the analysis time between HPLC and UPLC by using a 30 sec analysis time for each analysis.

2.3.3 Column manager

Column oven supports stable analysis that is not influenced by the ambient temperature. It can be operated in a wide range of temperatures from 10°C to 85°C.

2.3.4 Column [39]

The columns are an important key for fast analysis in UPLC. The small particle columns can obtain better resolutions, faster separation and increased sensitivity. For fast separation, due to Van Deemter theory, an ability to work at increased flow rate without any loss of efficiency. However, the flow rate is proportional to back pressure. Therefore, the smaller particle sizes require much higher operating pressures as which are possible with UPLC technology. Thus, the column can be shortened for fast analysis without any loss of resolution. The conventional columns are sub-2 μM packing column, the most widely used type of particle-packed column which has bonded-phase packing where liquids are coated on a packing material. The packing columns have a very high back pressure and even though UPLC can work at high pressures, it limits the increase in the flow rate. Recently, monolithic columns were discovered and used as an alternative material to conventional columns. The advantages of monolithic columns are high porosity, low back pressure and better mass transfer properties at a high linear flow rate.

2.3.4.1 Monolithic column [27,35,40-41]

The monolithic columns are a new type of stationary phase. Monolith columns were developed based on a new sol–gel technology. A column consists of one piece of solid that possesses interconnected skeletons and interconnected flow paths. They can be constructed by a synthesis of organic materials such as acrylate resins, natural polymers such as cellulose and inorganic materials such as silica. In this research, the use of silica-based monolithic columns are studied. The skeleton of the monolith column is shown in Figure 2.8. The silica-based monolith columns were obtained by sol-gel technology which includes the hydrolysis and polycondensation of alkoxysilanes (e.g. tetramethoxysilane or

tetraethoxysilane) in the presence of water-soluble polymers such as poly (ethyleneoxide) or polyethyleneglycol.

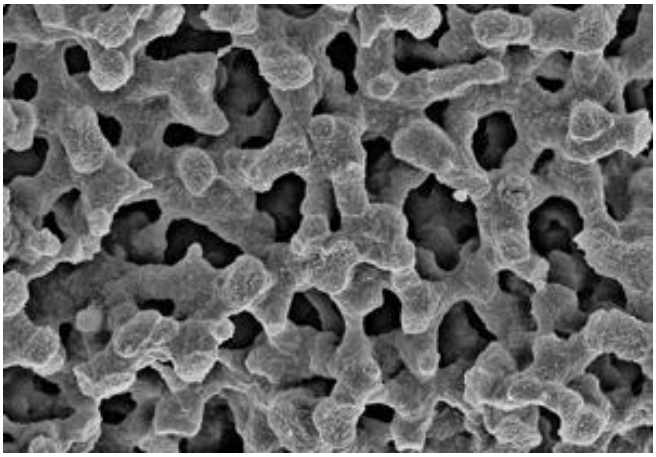


Figure 2.8 A skeleton of monolith column

The monolith in this research is a Chromolith Flash and marketed by Merck (Darmstadt, Germany). The monolith particles consist of double pore sizes such as macropores and mesopores. The double pore structure of a Chromolith column provides a combination of macropores of 2 µm in diameter and mesopores with an average pore diameter of approximately 13 mm. The macropores allow rapid flow of a mobile phase at low pressure and the mesopores from the porous structure provide a very large active surface area for high efficiency separations. The bimodal pore structure of the Chromolith column is shown in Figure 2.9.

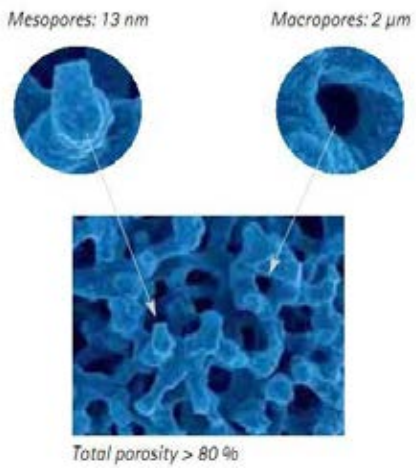


Figure 2.9 A bimodal pore structure of Chromalith column

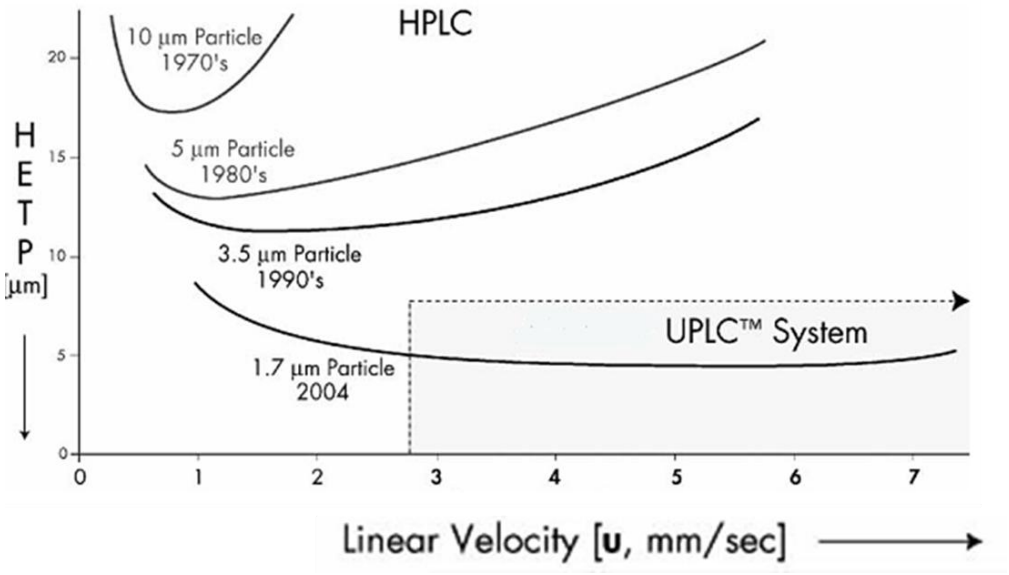
The Chromolith column provides excellent separations in a fraction of time. It can be operated at higher flow rate at low-back pressure and separations can be performed in a shorter analysis time due to minimizing the length of the column.

2.4 The Advantage of small particles [42-46]

The UPLC technology gives advantages such as increased speed, resolution and sensitivity afforded by smaller particles. The UPLC was developed from the HPLC method. Therefore, UPLC can be explained by the HPLC theory.

2.4.1 Speed without loss of efficiency

According to the Van Deemter plot, the smaller particles in the column provide an ability to work at a higher flow rate without a loss of efficiency. The Van Deemter equation and Van Deemter plot are shown in Figure 2.10.



$$H = 2\gamma d_p + \frac{2\lambda D_m}{\mu} + \left(\frac{1}{96}\frac{d_p^2}{D_m}\frac{11k^2+6k+1}{(k+1)^2} + \frac{2}{3}\frac{d_s^2}{D_s}\frac{k}{(k+1)^2} \right)\mu$$

A term $\frac{B}{\mu}$ term; Cm; Cs; C term

H = Height equivalent to a theoretical plate

A = Eddy diffusion and mobile phase mass transfer

B = Longitudinal molecular diffusion

μ = Linear velocity

C_m = Mass transfer coefficients for the stationary phase

C_s = Mass transfer coefficients for the mobile phase

Figure 2.10 The Van Deemter plot, the evolution of particles sizes over the last three decades.

A-term: Eddy diffusion [47-49]

Eddy diffusion in the Van Deemter equation is independent of the flow velocity of the mobile phase whereby the mobile phase move flow passes through a packed column. Solute molecules will take different paths through the stationary phase at random. Eddy diffusion is important for columns with internal diameters of 2 - 5 μm. The A value depends on the particle size and geometrics of particles. Therefore, when the pariticles sizes were reduced, the contribution from the eddy difffusion in the Van Deemter equation will be reduced. It produces the efficiency of column. (Figure 2.11).

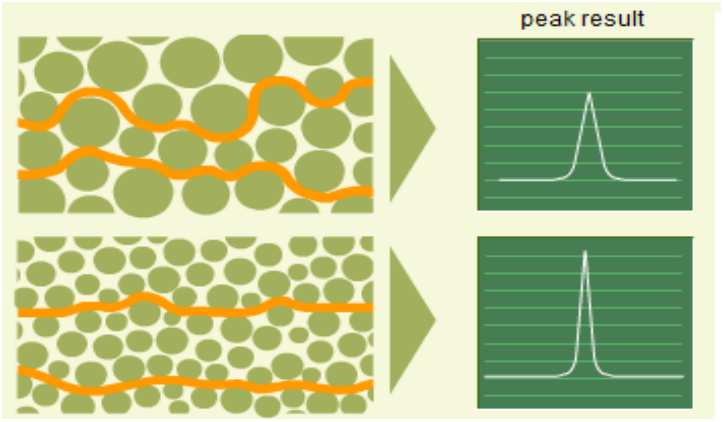


Figure 2.11 The effect of particles size in A-term

B-term: Longitudinal diffusion

B-term in the Van Deemter equation refers to longitudinal diffusion. It depends on a diffusion of individual analyte molecules in the mobile phase along the longitudinal direction of a column. Molecular diffusion takes place independently of the longitudinal flow direction. The higher flow rates reduce analysis time, and shorter time of the analyte molecules in the column reduces the effects of longitudinal diffusion (peak broaden (Figure 2.12). This reduction can improve the separation efficiency.

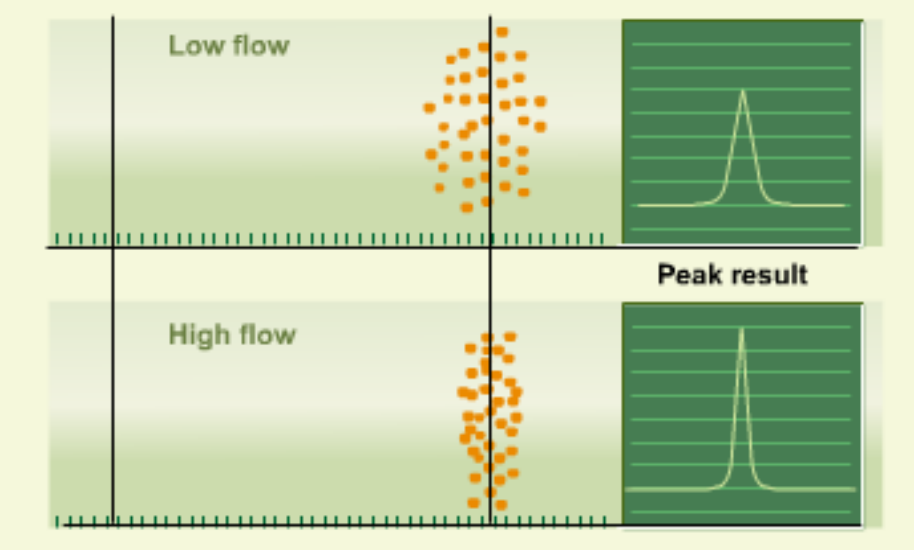


Figure 2.12 The effect of the flow rate in B-term

Longitudinal diffusion is proportional to the time that sample spends in the column and the diffusion coefficient of the analytes in the mobile phase. For molecular diffusion the coefficient depends on the viscosity, temperature and molecular size.

C-term: Mass transfer

C-term is an analyte mass transfer coefficient. It is the most important factor in liquid chromatography, especially at commonly used mobile phase velocities. It relates to the mass transfer of sample components in both the mobile phase and stationary phase during separation. The C-term is divided into two separate mass

transfer terms which are C_m and C_s. C_m-term describes the resistance to mass transfer in the mobile phase. It depends on the diffusion coefficient of the analytes in the mobile phase; the diffusion coefficient in a liquid chromatography is directly proportional to temperature, molecular mass of the mobile phase and mobile phase viscosity. C_s-term describes the resistance to mass transfer in the stationary phase. In the solid stationary phase, it can be ignored because of the rapid transfer of analytes on the surface of adsorbent. For the liquid stationary phase, it depends on the thickness of the film, the diffusion coefficient of analytes in the stationary phase and the geometry of particles.. The contribution of the two C-terms to the overall peak broadening appears to increase with the linear velocity of the mobile phase.

As with the above, the smaller particles of the stationary phase reduce A and C-term where the A-term contributes less to H and the B-term becomes less significant to the value of H when increasing the flow rate. As a result, the speed separation can be obtained with the same resolution. Moreover, the length of the column is an important key to rapid separation. In this research, the shorter monolith column has been developed for fast analysis.

2.4.2 Resolution

In chromatography, the resolution is determined by the distance between the two peaks. A better measure of separation is provided by resolution (R_s). It takes into account both retention difference, column efficiency and symmetrical peaks of Gaussian shape as shown by Figure 2.13.

$$Rs = \underbrace{\frac{\sqrt{N}}{4}}_{\text{Physical}} \left(\frac{\alpha - 1}{\alpha}\right) \underbrace{\left(\frac{k}{k+1}\right)}_{\text{Chemical}}$$

Figure 2.13 The resolution equation shows the relationship between the resolution and the efficiency value

This equation presents a relationship between resolution (R_s), retention (k), sensitivity (α) and efficiency (N). The k and α parameters describe the interaction of

analyte molecules, the stationary phase and the mobile phase, and the N value describes the physical process of band-broadening during the separation. The resolution is proportional to the square root of N. Therefore N is inversely proportional to the particle size (dp).

$$N \alpha \frac{L}{dp}$$

In this relationship, the reduction of the particle size can be improved a column efficiency that relates to a resolution parameter. The particle size of the packing column was reduced to 1.7 µM (UPLC-system) which offers a better resolution value at 1.7 fold when compared to a conventional HPLC column (5 µM). Moreover, the optimum flow; F_{opt} is inversely proportional to the particle size (dp). The relationship between the flow rate and the efficiency value is shown in Figure 2.14. As the result, a higher flow rate can be used for faster separation.

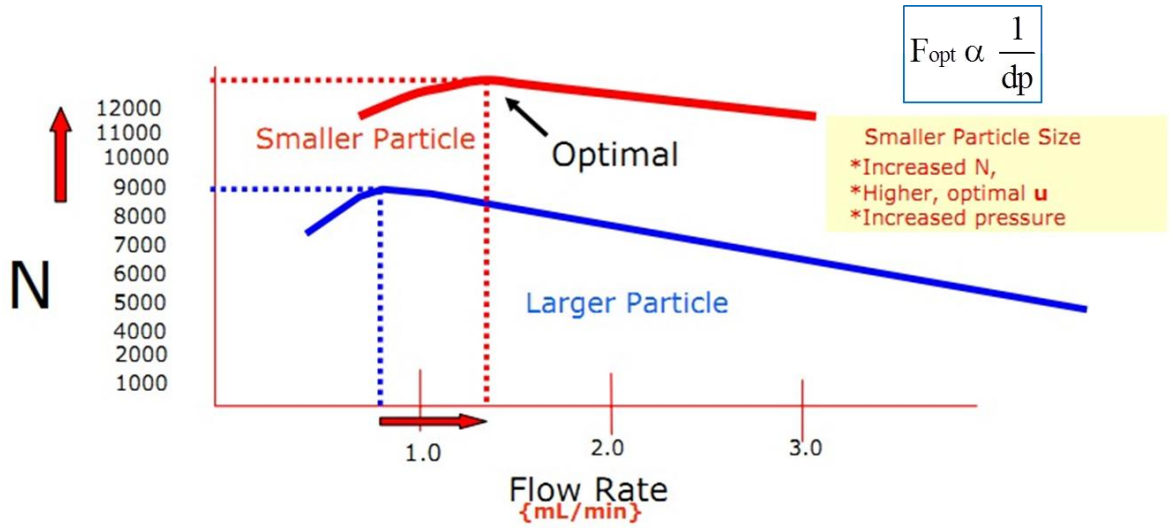


Figure 2.14 A relationship between the flow rate and the efficiency (N value) of the large particles and small particles.

2.4.3 Sensitivity

An increase of sensitivity is discussed on the relationship between an efficiency and peak width that N is inversely proportional to the square of the peak width (w). Also the peak height is inversely proportional to the peak width.

$$N \alpha \frac{1}{w^2}$$

$$\text{Peak height } \alpha \frac{1}{w}$$

So the particle size decreases to increase N and subsequently R_s. The increasing of the resolution is defined by a narrower peak. Since narrower peaks are taller peaks, sensitivity is therefore also increased.

2.5 Fundamentals of Electrochemistry [50]

Electrochemistry is a branch of chemistry which involves the relationship of electricals such as current, potential or charge and chemical reaction. The main part of the field is the study of chemical changes caused by the passage of an electric current and the production of electrical energy by chemical reactions. The electrochemical cells include a galvanic and electrolytic cell. A galvanic cell is one in which redox reactions occur spontaneously at the electrode. They are connected externally by a conductor (Figure 2.15a). These cells convert chemical energy into electrical energy. Commonly, a galvanic cell is used in batteries. An anode (oxidation) is a negative and a cathode (reduction) is a positive electrode. An electrolytic cell non-spontaneously reacts with energy from a battery or an external source which must be supplied to induce the electrolysis reaction (Figure 2.15b). These cells convert electrical energy into chemical energy. An anode (oxidation) is a positive and cathode (reduction) is a negative electrode and an external battery supplies the electrons through the cathode and they come out through the anode.

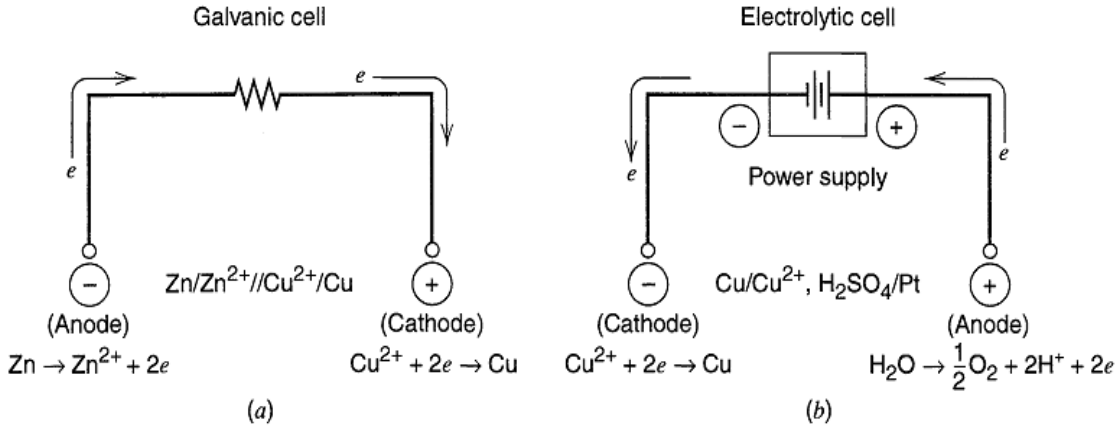


Figure 2.15 (a) Galvanic cell (b) Electrolytic cell

2.5.1 Mass transport [51-53]

One of fundamentals of electrochemistry is Faraday's law. The faradaic current that flows at any time is directly proportional to the rate of electrochemical detection at the electrode. In addition, the current depends on two processes. 1) the rate from bulk of solution to the electrode called mass transfer 2) the rate that electrons can transfer across the interface, or charge transfer. In mass transport there are three mechanisms to describe the transport of reactants or charges to the electrode.

2.5.1.1 Diffusion

The movement of the molecules is performed by the concentration gradient. The diffusion depends on the difference in the concentration between the two points. It transfers from a higher concentration to a lower concentration (Figure 2.16). This rate is directly proportional to the concentration difference.

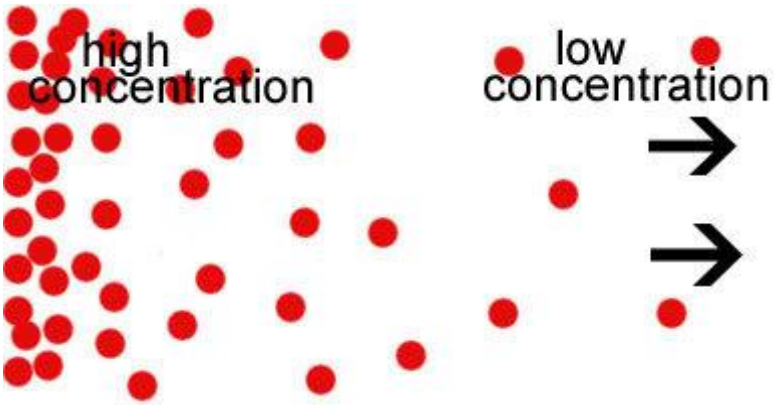


Figure 2.16 Diffusion by a concentration gradient

2.5.1.2 Migration

In the bulk solution, the total current is carried mainly by migration. The migration processes are a movement of ions which respond to an electric field (Figure 2.17). It is proportional to 1) the charge of the ion, 2) the concentration of ions, 3) the diffusion coefficient and 4) the magnitude of the electric field gradient. This process causes anions of analyte to be attracted to the positive electrode and cations of analyte to the negative electrode. They are an undesired process. Commonly, the analyte species can migrate less by using a supporting electrolyte. A supporting electrolyte is a fully dissociated electrolyte that acts as a charge carrier.

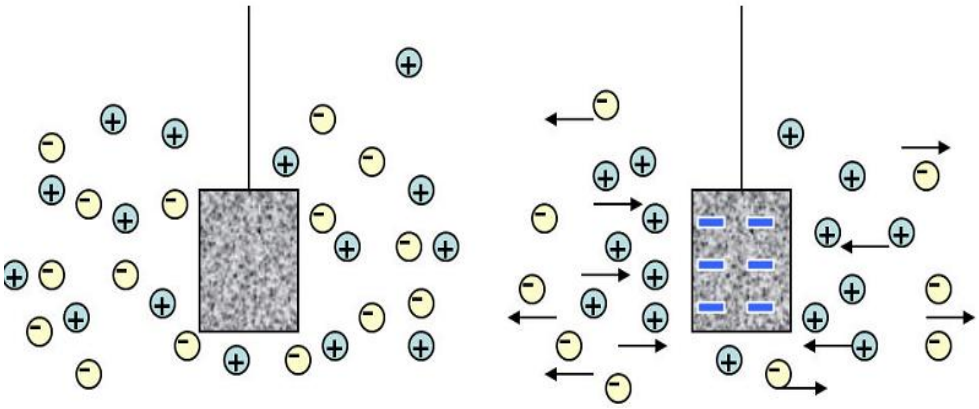


Figure 2.17 Migration by electric field of elecltrode

2.5.1.3 Convection

The movement of species is forced by a mechanical method such as vibration or stirring (Figure 2.18). The convection causes the thickness of the diffusion layer at the surface of an electrode to decrease and leads to a decrease in the concentration polarization. It can be eliminated by using an unstirred solution.

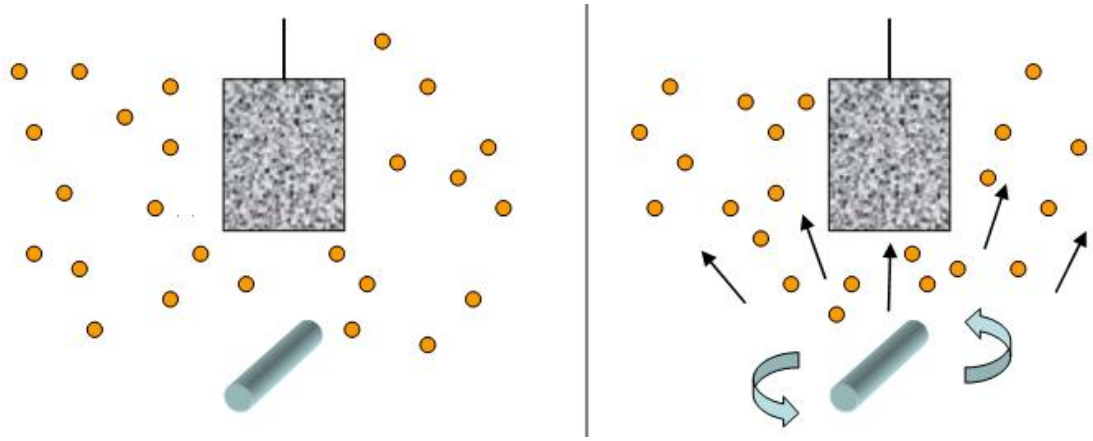


Figure 2.18 Convection by vibration or stirring

2.5.2 Voltammetry [33,50]

Votammetry is an electrochemical technique which applies a potential to an electrochemical cell and measure the current as a function of applied potential. There are many typical excitation signals to the working electrode that are shown in Figure 2.19. The resulting current is proportional to the concentration of electrochemical species and the current measured is the anodic current (oxidation reaction) and cathodic current (reduction reaction) at the surface of an electrode. The resulting current plot of current versus applied potential is called the voltammogram. The classical voltammetric excitation signal is a linear scan as shown in Figure 2.19(a). The pulse excitation signals are shown in Figure 2.19(b) and Figure 2.19(c). In this research, cyclic voltammetry and amperometry were used for studying the electrochemical reaction of target analytes.

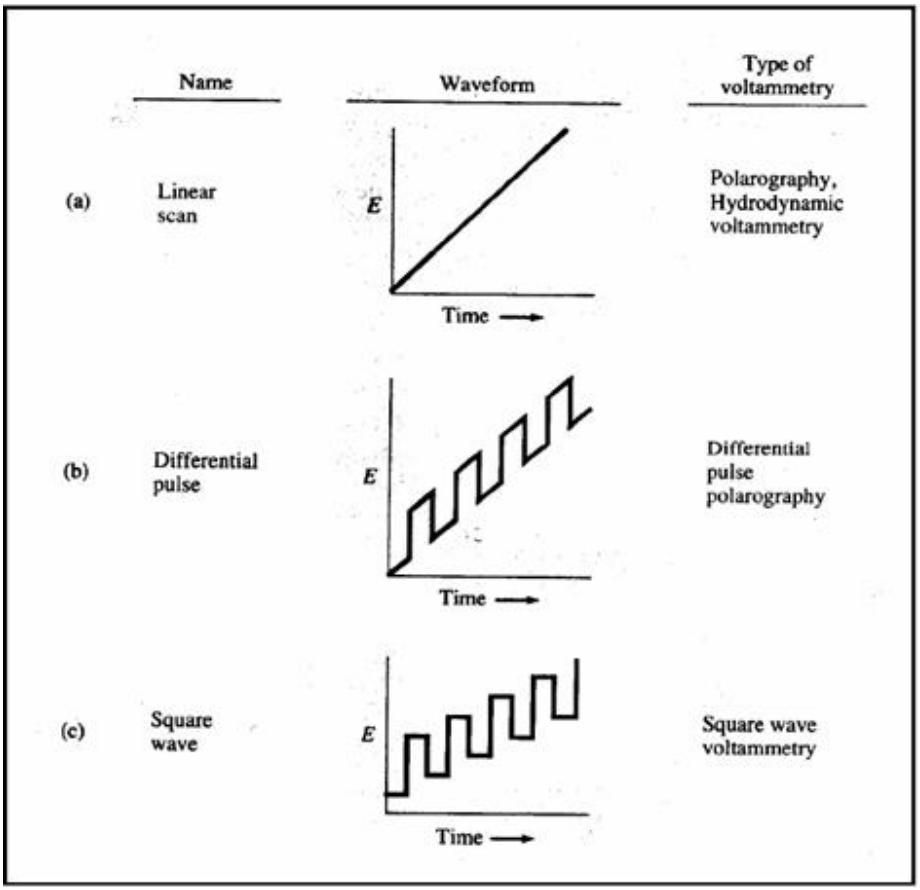


Figure 2.19 The characteristic of excitation signal of voltammetry

2.5.2.1 Cyclic voltammetry [53-55]

In this section, which is called cyclic voltammetry or CV; it has become a very popular technique for initial electrochemical studies. Cyclic voltammetry consists of applied potential at a working electrode in both the forward and reverse directions, using a triangular potential waveform (Figure 2.20a). This is the rapid-voltage scan technique in which the sweep rates are fast at 20-400 mV s^{-1}. During the potential sweep, the potentiostat measures the current resulting from the applied potential. The resulting current-potential plot is called a cyclic voltammogram (Figure 2.20b).

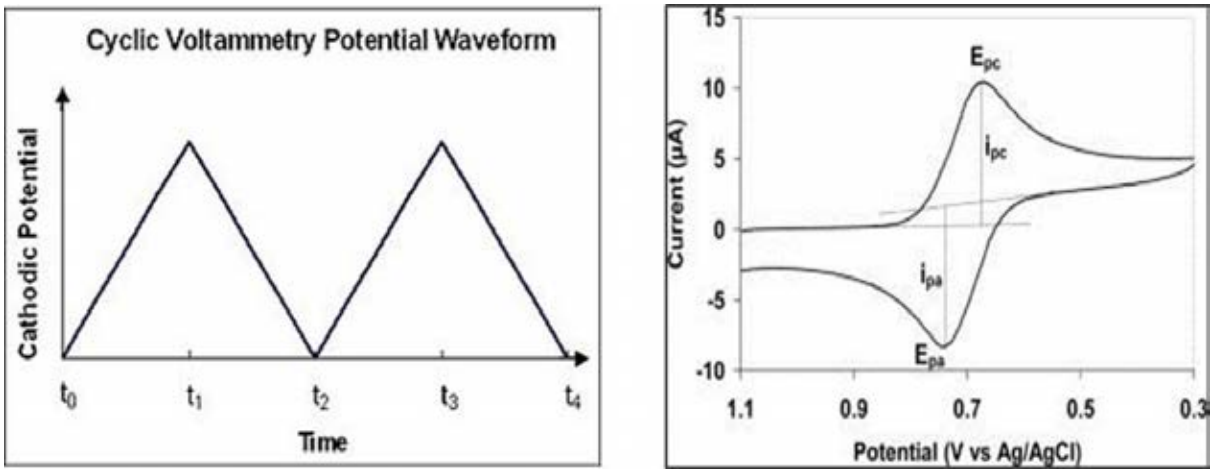


Figure 2.20 (a) Cyclic voltammetry waveform (b) Cyclic voltammogram

As cyclic voltammogram in Figure 2.20b, the applied potential approaches the characteristic E^0 for the redox reaction. At the beginning which is zero, a cathodic current is obtained whereby a reduction process occurs.

$$O + e^- \rightarrow R$$

When the direction of potential sweep is reversed (reverse scan). R molecules are reoxidized back to O and an anodic current occurs.

$$R \rightarrow O + e-$$

The response of a reversible system is shown in Figure 2.20b. In a cyclic voltammogram, the parameters can be measured such as an anodic and cathodic peak potential ($E_{p,c}$ and $E_{p,a}$), an anodic and cathodic current ($I_{p,c}$ and $I_{p,a}$). All of these can be diagnose a reaction of the compound.

For a reversible process, the peak current (at 25°C) is given by the Randles-Sevcik equation as follows

$$I_p = (2.69\times10^5)n^{3/2}ACD^{1/2}\nu^{1/2}$$

Where

n = number of electron

A = electrode area (cm^2)

C = concentration (mol cm^{-3})

D = diffusion coefficient ($cm^2\ s^{-1}$)

ν = potential scan rate ($V\ s^{-1}$)

According to this equation, the resulting currents are directly proportional to the concentration and square root of the scan rate. For a simple reversible system, a cathodic peak current is equal to an anodic peak current ($I_{p,\ a} = I_{p,\ c}$,) and the differential between two peak potential is given by

$$\Delta E_p = E_{p,\ a} - E_{p,c} = 0.059/n \ \text{V}$$

Therefore, this equation can be determined by the number of electrons transferred, a fast one-electron process shows ΔE_p at 0.059V. And the peak potential is related to a formal potential (E^0) as follows

$$E^0 = \frac{E_{p,\ c} + E_{p,\ a}}{2}$$

For an irreversible process, the cathodic and anodic peak are reduced in size and separated. Commonly, the irreversible processes are presented to a shift of the peak potential with a scan rate. The separation between the peak potential and the half-peak potential is $48/\alpha n$ mV. Furthermore, the peak current is proportional to the bulk concentration.

For a quasi-reversible process, the charge transfer and mass transfer will control the current. The characteristic of cyclic voltammogram depends on a function of $k^0 / (\pi aD)^{1/2}$, where $a = nFv/RT$. When $k^0 / (\pi aD)^{1/2}$ increases, the cyclic

voltammogram approaches the reversible process, k^0 / $(\pi aD)^{1/2}$ decreases, and the process exhibits an irreversible system.

2.5.2.2 Amperometry [56]

Amperometry is a class of electrochemistry in which the applied potential is held constant at the working electrode and measures the resulting current that directly proportional to the concentration of analytes. The applied potential is high enough to initiate the oxidation or reduction process. It has been used as a detection method in the flow system. Therefore, the amperometric detection has been developed for chromatographic detection. It is an alternative method for the determination of electroactive analytes. Commonly, the measurement by amperometric detection in the flow system as chromatography will decrease the thickness of diffusion layer at the working electrode surface. As a result, the measured current is higher. The potential waveforms normally used in amperometric detection are presented in Figure 2.21.

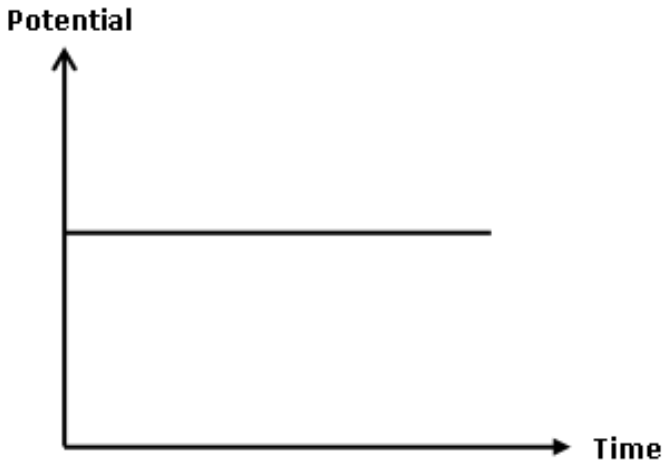


Figure 2.21 The potential waveform of amperometry

2.5.3 Working electrode

In an electrochemical cell, the reaction occurs at only the electrode's surface. The working electrode is the electrode at which the reaction of interest occurs. The materials for making the working electrode strongly influence the efficiency of the electro analysis technique. It should provide high signal-to-noise ratios. The potential electric conductivity, geometry, mechanical properties, cost, availability and surface reproducibility must be considered. The selection depends on two factors, the redox behavior of the target and the background current. The most popular materials are mercury, carbon, and noble metals. In this research, the electrodes structured made from carbon is interested.

In electrochemistry, carbon electrodes such as carbon fiber, glassy carbon, carbon nanotubes, diamond and various forms of carbon are commonly used because they have low cost, simple preparation methods, large surface area and a relatively wide potential window of water stability. The various carbon conductive forms and kinetics of electron which transfer on it depend on the surface structure.

2.5.3.1 Boron-doped diamond electrode [56-59]

A diamond is an allotrope of carbon which is an extremely hard crystalline. Boron-doped diamond (BDD) is an alternative to the original carbon electrode. In the original carbon electrodes, there are many drawbacks as electrode fouling limits their long term stability cause to polishing of the electrode and limited potential window. BDD is a novel carbon material which has many advantages such as (1) wide potential window in aqueous media; the characteristics of the BDD electrode are hydrophobic and not attractive for adsorption, it is an extremely low catalytic activity for hydrogen and oxygen generation. (2) low and stable background current; the low electrostatic capacity minimizes the background current. (3) high reproducibility and stability; it is impassive to the presence of oxygen dissolved in aqueous solution and has a high resistance to deactivation due to low adsorption of polar molecules. (4) resistance to fouling. An SEM image of a boron-doped crystalline diamond is shown in Figure 2.22 and a comparison of electrochemical

properties of boron-doped diamond thin film, glassy carbon, platinum and gold electrode are presented in Figure 2.23.

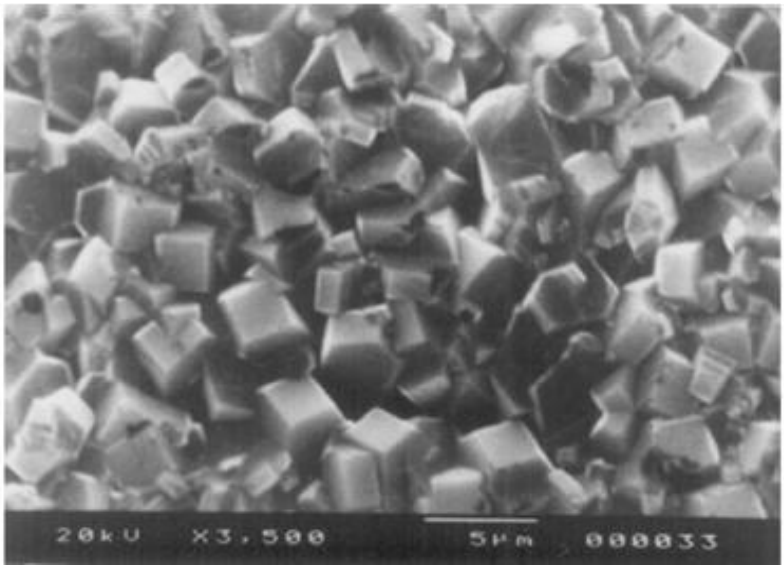


Figure 2.22 Scanning electrode microscopy (SEM) of boron-doped crystalline diamond

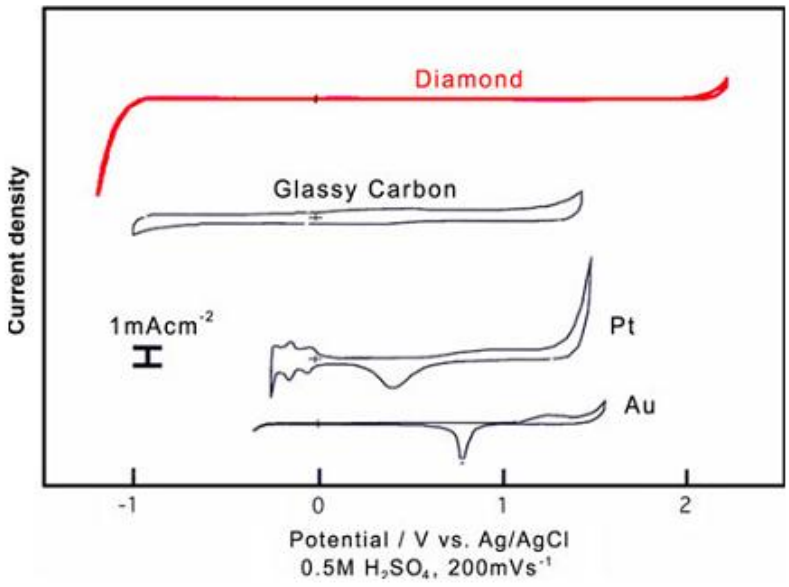


Figure 2.23 Cyclic voltammogram of various electrodes in 0.5 M H_2SO_4

The wide potential window on the anodic potential, compared to glassy carbon has enabled the detection of a wide range of compounds. Moreover, compared to mercury, a wide potential in cathodic potential has enabled us to detect the trace metals. Recently, BDD electrodes are a major innovation to new application not only in the electrochemistry field such as material science, analytical chemistry, environmental science and biological science. Boron is widely used as a dopant on diamond electrodes because boron has a low charge carrier activation energy and causes a p-type semiconductor behavior in diamonds. All of these, an innovation of electrode material with several good properties of diamond as hardness, conductivity, optical transparency and chemical inertness have been developed. The diamond electrode was doped with boron using a chemical vapor deposition (CVD) of diborane, trimethyl borane, organic borate in a source gas. At a low concentration of boron, it promoted p-type semiconductivity in the diamond, in which the acceptor level is 0.37 eV above the valence band. At a high concentration of above 10^{17} to 10^{18} cm^{-3}, an impurity band forms and the acceptor gap is reduced. At a boron concentration from 10^{20} to 10^{21} cm^{-3}, it is a semimetal.

CVD techniques were classified into three types, plasma -assisted CVD, hot filament-assisted and combustion flames. In this research, a microwave plasma-assisted method is described because this is a most common method. In a plasma-assisted technique, the character of the plasma is to generate atomic hydrogen and to produce the carbon for the growth of the diamond. A deposition process is a combination of two steps which are a homogeneous process in a plasma bulk and a heterogeneous process on a plasma surface area. The first procedure is the dissociation of the hydrogen source into atomic hydrogen and the formation of the intermediate species. Generally, the excitation frequency is 2.45 GHz for a microwave plasma CVD. A schematic of a microwave plasma system is shown in Figure 2.24.

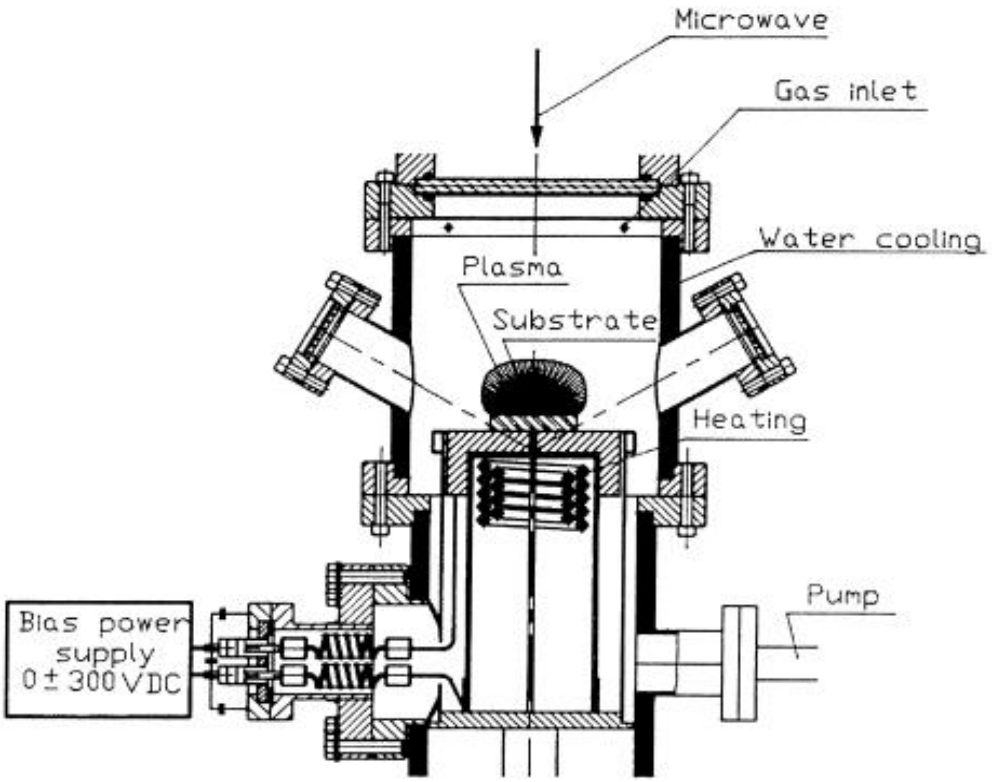


Figure 2.24 Schematic of microwave plasma-enhanced CVD reactor

2.6 Literature surveys

2.6.1 Conventional method for Paraben preservatives detection

Several analytical methods for the determination of the presence and quantity of parabens have already been developed. Chromatographic methods are widely used, especially high-performance liquid chromatography (HPLC) or gas chromatography (GC), although capillary electrophoresis and the flow injection have also been reported. These are coupled with standard detection methods, most often ultraviolet (UV) detection, but also chemiluminescent (CL) and mass spectrometric (MS) detection have been reported.

In 1998, Sottofattori et al. presented a HPLC method for the determination of multiple components in a commercial cosmetic cream [60]. In this work, HPLC was used for the simultaneous determination of magnesium ascorbyl phosphate, imidazolidinylurea, a mixture of methyl-, ethyl-, propyl-, butyl- parabens, and ascorbyl palmitate (VII). The method was developed by using a cyano-propyl stationary phase and a phosphate buffer and methanol as a mobile phase in gradient elution system with a flow rate of 1.0 mL min^{-1}. A good separation was obtained in 12 min. In order to overcome some problems from the difference in the composition of the compound, the UV multi-wavelength detector was selected at 220 and 240 nm. Finally, the developed method can be applied in cosmetic cream.

In the same year, Wang et al. reported the developed method for parabens detection in cosmetic products by a supercritical fluid extraction and capillary zone electrophoresis [61]. This paper developed a method for the determination of four parabens such as methyl-, ethyl-, propyl- and butylparaben in eleven cosmetic products by the improvement of supercritical fluid extraction (SFE) with CO_2 and capillary zone electrophoresis (CZE). The multi-wavelength diode array detector was set at the 230 and 255 nm. The sample preparation by the supercritical fluid CO_2 has good results with 12 100 kPa SFE-CO_2 with 0.05% acetonitrile at 40^0C. For a separation by CZE, the optimum condition was 30 mM sodium tetraborate buffer (9.18) in 5% acetonitrile, 96 cm of fused-silica capillary tube and appile

voltage at 25 kV. This method has a good recovery (97.3-99.9%), resolution (R=1.70) and detection limits (LOD) ranging from 0.97 to 1.33 ng for the parabens determination.

In 2005, Saad et al. described the simultaneous determination of benzoic acid, sorbic acid, methylparaben and propylparaben in foodstuffs by high-performance liquid chromatography [3]. In this paper, the HPLC method for determination of four types of preservative were studied. The separation was carried out with a gradient elution using a mixture of acetate buffer pH 4.4 and methanol. A Supelco 516 C18 column (15 cm×4.6mm, 5 μm) was selected. For the detector, the UV detector was set at 254 nm. Under these conditions, the determination of parabens was achieved in 23 min. The limits of detection (LOD) were 0.5, 0.1, 0.3 and 0.1 mg L^{-1} for benzoic acid, sorbic acid, methylparaben and propylparaben, respectively. The developed method was applied in sixty seven food samples.

In the same year, Zhang et al. developed a high performance liquid chromatography method coupled with chemiluminescence detection for determination of parabens in wash-off cosmetic products and foods samples [62]. The four common paraben preservatives which were methylparaben, ethylparaben, propylparaben and butylparaben were studied. The good separation was achieved in 8.5 min using an isocratic elution with methanol and water (60:40 v/v) at a flow rate of 1.0 mL min^{-1}. The reverse phase XDB-C8 column was used. For chemiluminescence procedure, the reaction between parabens with cerium (IV) and rhodamine 6G in the strong sulfuric acid were investigated. The detection limits (LOD) were 1.9×10^{-3} to 5.3×10^{-3} mg L^{-1} for four parabens detection. The proposed method was applied in wash-off cosmetics.

In 2007, Shabir developed a determination and validation method for detected preservatives by HPLC [63]. The preservatives such as benzyl alcohol, ethylene glycol monophyl ether, methyl hydroxybenzoate, ethyl hydroxybenzoate, propyl hydroxybenzoate, and butyl hydroxybenzoate were studied. The preservatives were separated using a reversed-phase C18 Lichrospher column (250 mm x 4.0 mm, 5 μm) with a gradient elution of a water and acetonitrile at a flow rate of 1.0 mL min^{-1}. The temperature was set at 30 ± 0.5 ^{0}C and detected at 258 nm. In this condition, the

five compounds were separated into 12 min. The limits of detection (LOD) were 2, 1 and 0.024 mg L^{-1} for methylparaben, ethylparaben, and propylparabens, respectively.

In 2010, Shanmugram et al. repored a method for paraben preservative detection in human breast cancer [64]. The preservatives such as methylparaben, ethylparaben, propylparaben and butylparaben were detected by gas chromatography and mass spectrometry (GC-MS). The parabens were separated by DB-1 fused silica capillary column (30 m×0.32 mm i.d., 0.25 μm film thickness) and helium was used as a carrier gas at a flow rate of 2.25 mL min^{-1}. The separation was achieved within 7 min. The limits of detection (LOD) were 2.02, 1.05, 1.71 and 3.75 ng g^{-1} for methylparaben, ethylparaben, propylparaben and butylparaben.

2.6.2 Electrochemical detection for parabens

In 1997, Kang et al. reported the HPLC-electrochemical detection for methylparaben, propylparaben and thimerosal detection in pharmaceutical products [28]. In this paper, the chromatographic coupled with the electrochemical detection for preservatives detection has been developed. The three compounds were separated using a C_{18} silica column with a methanol and phosphoric acid as a mobile phase in a ratio of 59:41v/v, a flow rate at 1.0 mL min^{-1}. The elution time was less than 20 min. For amperometric detection, the potential of 1.25 V versus Ag/AgCl was applied to a glassy carbon which is the working electrode. The limits of detection (LOD) were 50, 100 and 250 μg L^{-1} for methylparaben, propylparaben and thimerosal, respectively.

In 2010, Chu et al. developed a method for the determination of parabens by capillary zone electrophoresis coupled with electrochemical detection in soy sauces [1]. The four common parabens such as methylparaben, ethylparaben, propylparaben and butylparaben were well separated using a fused-silica capillary (70 cm length of 25 μm i.d.) with a borax buffer pH 9.94 and a separation voltage was selected at 16 kV. Under these conditions, the separation was obtained within 16 min. For the electrochemical detection, a carbon-disk working electrode was selected with applied potential at 0.95 V versus SCE. In this work, the limits of detection were 44 to 57 μg L^{-1} for four parabens.

In 2011, Martins et al. reported a determination of parabens using high-performance liquid chromatography coupled with electrochemical detection using boron-doped diamond electrode [29]. The three parabens such as methylparaben, ethylparaben and propylparaben were studied. The separation of parabens were carried out using a Waters X Terra^{TM} C8 analytical column (250 mm×4.6 mm, 5 μm) with a mixture of acetonitrile and disodium phosphate pH 7.0 (40:60 v/v) at a flow rate of 1.0 mL min^{-1}. The elution time was less than 10 min. For amperometric detection, in three pulse modes were (1) initial potential at 1.2 V (2) medium potential at -0.5 V (3) end potential at -2.0 V. The limit of detection (LOD) was 0.4 μg mL^{-1} for all parabens.

CHAPTER III

EXPERIMENTAL

3.1 Chemicals and reagents

Chemicals and their suppliers are summarized in Table 3.1

Table 3.1 List of chemicals and reagents and their suppliers

Chemicals/ Reagents	**Suppliers**
Methylparaben	Sigma-Aldrich (USA)
Ethylparaben	Sigma-Aldrich (USA)
Propylparaben	Sigma-Aldrich (USA)
Potassium dihydrogen orthophosphate	Merck (Germany)
disodium hydrogen phosphate dihydrate	Merck (Germany)
Sodium acetate	Fluka (Switzerland)
Acetic acid	Merck (Germany)
ortho-phosphoric acid 85%	Merck (Germany)
Sodium hydroxide	Merck (Germany)
Methanol (HPLC grade)	Merck (Germany)
Acetonitrile (HPLC grade)	Merck (Germany)
2-propanol	Merck (Germany)

3.2 Instruments and equipment

Table 3.2 List of instruments and their suppliers

Instruments and equipment	Suppliers
UPLC LC-20AD XR pump	Shimadzu (Japan)
UPLC SIL-20A XR autosample	Shimadzu (Japan)
UPLC CTO-20AC column oven	Shimadzu (Japan)
UPLC SPD-M20A diode array detector	Shimadzu (Japan)
CHI1232A	CH Instrument (USA)
Monolith column Chromolith® Flash RP-18 endcapped column (25 mm x 4.6 mm)	Merck (Germany)
Electrochemical flow cell	Bioanalytical System (USA)
Teflon cell gasket	Bioanalytical System (USA)
PEEK tubing (0.25 mm. i.d.)	Upchurch (USA)
Teflon tubing (1/10 inch i.d.)	Upchurch (USA)
Silver/silver chloride electrode (Ag/AgCl)	Bioanalytical System (Japan)
Boron-doped diamond electrode (BDD)	Toyo Kohan Co., Ltd. (Japan)
Platinum wire	Bioanalytical System (USA)
Stainless-steel tube	Bioanalytical System (USA)
pH meter	Metrohm
Milli Q water system $R \geq 18.2$ MΩcm	Millipore (USA)

Table 3.2 (Continued)

Instruments and equipment	Suppliers
Centrifuge	Cole parmer (USA)
Analytical balance	Mettler Toledo (Switzerland)
Vortex mixer VTX-3000L	Mixer Uzusio LMS (Japan)
Autopipette	Eppendorf (Germany)
Filter membrane (0.20 μm)	National scientific
Nylon syringe filters (0.20 μm)	ChroMex
Ultrasonic bath	ULTRAsonik 28H, ESP Chemicals, Inc., (USA)
Vacuum pump	GAST (USA)

3.3 Preparation of the chemical solution

3.3.1 Cyclic voltammetry

3.3.1.1 Supporting electrolyte

3.3.1.1.1 Type of supporting electrolyte

The type of supporting electrolytes consisting of the 0.1 M acetate buffer pH 4.0 and 0.1 M phosphate buffer pH 4.0 have been investigated. The 0.1 M acetate buffer pH 4.0 was prepared by mixing the sodium acetate 0.2259 g and 83.4 mL of acetic acid and then adjusting the volume to be 100 mL in a volumetric flask by adding Milli Q water. In addition, 0.1 M phosphate buffer pH 4.0 was prepared by weighing 1.3500 g of potassium dihydrogen orthophosphate and 0.0142 g of disodium hydrogen phosphate dihydrate, the mixture was dissolved with Milli Q

water and then adjusting the volume into 100 mL in a volumetric flask and then adjusting to pH 4.0 with phosphoric acid.

3.3.1.1.2 pH of supporting electrolyte

The pH of the phosphate buffer solution was studied. Each pH of 0.05 M of phosphate buffer solution was prepared by weighing potassium dihydrogen orthophosphate and disodium hydrogen phosphate dihydrate (Table 3.3). The mixture was transferred into a 100 mL volumetric flask and adjusting the volume with milli Q water. The pH ranges at 4-8 were adjusted with phosphoric acid to acidity.

Table 3.3 The preparation of each pH of phosphate buffer solution

pH	Potassium dihydrogen orthophosphate (g)	Di-sodium hydrogen phosphate dehydrate (g)
4*	0.6750	0.0071
5	0.6750	0.0071
6	0.6049	0.0988
7	0.2810	0.5224
8	0.0252	0.8570

* Firstly, the phosphate buffer was prepared at pH 5.0 and adjusted to pH 4.0 with phosphoric acid.

3.3.1.1.3 Concentration of supporting electrolyte

The concentration of the supporting electrolyte has been investigated. The preparation of each concentration ranges at 0.05-0.3 M of phosphate buffer pH 5.0 are presented in Table 3.4.

Table 3.4 The preparation of each concentration of phosphate buffer solution

Concentration (M)	Potassium dihydrogen orthophosphate (g)	Disodium hydrogen phosphate dihydrate (g)
0.05	0.6750	0.0070
0.10	1.3500	0.0142
0.20	2.7000	0.0285
0.30	4.0500	0.0427

3.3.1.2 Standard stock solutions

Each standard stock solution of three parabens (1000 µg mL^{-1}) was prepared by dissolving 10 mg of each paraben in 10 mL of an acetonitrile: Milli-Q water (50:50; v/v) solution in a volumetric flask and stored at 4^{o} C in an amber bottle. The working solutions were prepared by suitable dilution of the stock standard solutions with an acetonitrile: Milli-Q water (50:50; v/v) solution.

3.3.1.3 Working standard solutions

For cyclic voltammetric experiment, the volume of solution in the electrochemical cell was 3 mL. 50 µg mL^{-1} of each paraben solution was prepared by pipetting 0.15 mL of each standard stock solution 1000 µg mL^{-1} into the electrochemical cell and adjusting to 3 mL with 2.85 mL of supporting electrolyte.

3.3.2 Ultra-performance liquid chromatography coupled with electrochemical detection

3.3.2.1 Mobile phase

The mobile phase for UPLC consisted of the 0.05 M phosphate buffer solution pH 5.0 and acetonitrile in the ratio of 75:25 (v/v). The refreshed 0.05 M

phosphate buffer solution (pH 5) was prepared daily by dissolving 6.75 g of potassium dihydrogen phosphate and 0.07 g of disodium hydrogen phosphate dihydrate in a 1,000 mL volumetric flask with Milli Q water and adjusting the pH by using phosphoric acid. All solutions and solvents were filtered through 0.22 µm Nylon membranes and degassed by an ultrasonic bath.

3.3.2.2 The standard stock solution

A standard stock solution (1000 µg mL^{-1}) of three mixed parabens solution was prepared by dissolving 10 mg of of methylparaben, ethylparaben and propylparaben with acetonitrile: Milli Q water (50: 50; v/v) in a 10 mL volumetric flask and stored at 4 ºC in the amber bottle.

3.3.2.3 The working standard solution

A working standard solution (10 µg mL^{-1}) of three mixed parabens solution was prepared by pipetting 0.1 mL of the standard stock solution (1000 µg mL^{-1}) and diluting it with acetonitrile: Milli Q water (50: 50; v/v) in a 10.00 mL volumetric flask.

3.3.3 Sample preparation

All the real samples are presented in Table 3.5. One milliliter of samples A, D and F were extracted with 2.5 mL of methanol. The solutions were sonicated for 15 minutes in an ultrasonic bath. For samples B, C and F, fifty milligrams of sample F and three hundred milligrams of samples B and C were extracted with 1.5 mL of methanol and then vortexed for 3 minutes in a vortex mixer. The mixtures were centrifuged for 10 minutes at 6000 rpm and then appropriately diluted so that the concentration of parabens in the final test solution was within the linear dynamic range (0.1–50 µg mL^{-1}). The collected supernatants were filtered through 0.22 µm nylon membrane filter before being injected into the UPLC-EC system.

Table 3.5 The real product samples used in this research

Type	Samples	Remarks
Food	Soft drink	A
	Jelly	B
Personal care	Toothpaste	C
	Mouthwash	D
Cosmetic	Lotion	E
	BB powder	F

3.4 Procedure

3.4.1 Cyclic voltammetry

Electrochemical measurements were performed using CH Instrument potentiostat with a standard three electrode configuration, including a boron-doped diamond (BDD) working electrode, an Ag/AgCl reference electrode and a platinum counter electrode in 0.05 M phosphate buffer pH 5.0 with a volume of 3 mL. Before use, the BDD working electrode was sonicated with Milli Q water. After use, it was cleaned with 2-propanol and Milli Q water.

3.4.1.1 The type of supporting electrolyte

The electrochemical behavior of 50 µg mL^{-1} methylparaben, ethylparaben and propylparaben in various supporting electrolytes, including a phosphate buffer solution and acetate buffer solution were studied by cyclic voltammetry on a BDD electrode. The potential was scanned from 1.0 to1.8 V with a scan rate of 50 mV s^{-1}.

3.4.1.2 The pH of the supporting electrolyte

The electrochemical behavior of 50 μg mL^{-1} of each paraben in several pH of phosphate buffer solution was investigated by cyclic voltammetry using a BDD working electrode. The pH range of the phosphate buffer solution was 4 to 8 and the peak current was plotted as a function of pH. The potential was scanned from 1.0 to1.8 V with a scan rate of 50 mV s^{-1}.

3.4.1.3 The concentration of the supporting electrolyte

The electrochemical behavior of 50 μg mL^{-1} of each paraben in variation of phosphate buffer solution concentration was investigated by cyclic voltammetry. The concentrations of 0.05, 0.1, 0.2 and 0.3 M were studied. The potential was scanned from 1.0 to1.8 V at a scan rate of 50 mV s^{-1}.

3.4.1.4 The Scan Rate Dependence Study

The effect of the scan rate on the electrochemical behaviors of parabens was investigated by a variation of the scan rate in cyclic voltammetry. The solutions of 50 μg mL^{-1} methylparaben, ethylparaben and propylparaben in 0.05 mM phosphate buffer solution (pH 5.0) were prepared. The potential was scanned from 1.0 to1.8 V at a scan rate of 50, 100, 200, 300 and 500 mV s^{-1}. The peak currents obtained from cyclic voltammogram at each scan rate were plotted as a function of the square root of the scan rate.

3.4.2 UPLC-EC

The UPLC-EC measurement, Ultra-performance liquid chromatography was performed using a Shimadzu LC-20ADXR UFLC equipped with high-pressure binary pumps. The Chromolith® Flash RP-18 endcapped column (25 mmx4.6 mm) from Merck was used. An electrochemical detector consisted of a CH Instrument potentiostat, BDD electrode as an amperometric detector, and a thin layer flow cell (Figure 3.1). The UPLC-EC system was carried out in the mobile phase, which consisted of a phosphate buffer solution (pH 5) and acetonitrile in the ratio of 75:25 (v/v), with applied potential as 1.5 V vs. Ag/AgCl. The flow rate was used at 2.5 mL

min^{-1}. The experiment was performed at room temperature (25^{o} C). The optimal conditions in UPLC-EC were utilized for separation of methylparaben, ethylparaben and propylparaben as shown in Table 3.6.

The thin-layer flow cell consisted of three electrodes: a BDD working electrode, a Ag/AgCl reference electrode and a stainless steel tube counter electrode. The geometric area of the BDD electrode in the flow cell was estimated to be 0.42 cm^2 using a 1 mm thick silicon rubber gasket as a spacer. An electrochemical analyzer (CHI1232a, CH-instrument, USA) was used for amperometric control and signal processing.

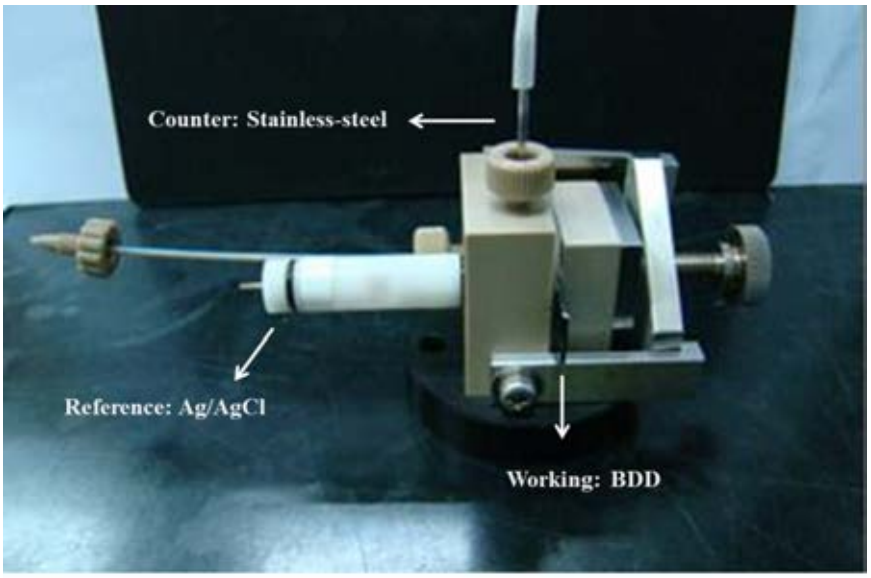


Figure 3.1 A thin-layer flow cell

Table 3.6 The UPLC-EC conditions for the detection of three paraben preservatives

UPLC parameters	**UPLC conditions**
Column	Reverse phase monolith C18 silica-based (25 mm x 4.6 mm i.d.)
Mobile phase	Acetonitrile:0.05 M Phosphate buffer (pH 5) (25:75, v/v)
Flow rate	2.5 mL min^{-1}
Injection volume	20 μL
Temperature	25 ^{0}C
Detector	Amperometric detection at 1.5 V vs. Ag/AgCl

3.4.2.1 Ratio of mobile phase

The separation of three parabens such as methylparaben, ethylparaben and propylparaben in various ratios of the mobile phase were studied. The mobile phase has a direct effect on the performance of separation and sensitivity. The mobile phase was a mixture of acetonitrile and 0.05 M phosphate buffer pH 5.0. The optimum ratio of the mobile phase was investigated from 25:75, 30:70, 35:65 and 40:60 (v/v).

3.4.2.2 Flow rate of the mobile phase

The separation of three parabens in several flow rates of the mobile phase were investigated. The flow rate of the mobile phase has an effect on the resolution and the elution time. The optimum flow rate was studied from 1.5, 2.0, 2.5 and 3.0 mL min^{-1}.

3.4.2.3 ECD Potential

The detection of three parabens in various applied potential on a BDD electrode was studied. The applied potential on BDD has an effect on the sensitivity of analytes. The applied potential was studied from 1.3, 1.4, 1.5 and 1.6 V versus Ag/AgCl.

3.4.2.4 UPLC column type

A comparative study on the column performance between a microparticle C18 packed column (Shim-pack XR-ODS II) and two reverse C18 monolithic columns (Chromolith® Performance and Chromolith® Flash) was investigated. The separation of three parabens in different column was obtained using UPLC-ECD method.

3.5 Method validation

The analytical performances of the developed method for determination of methylparaben, ethylparaben and propylparaben, including linearity range, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ) were evaluated.

3.5.1 Linearity

A stock standard solution (1000 μg mL^{-1}) of mixed parabens was diluted to a concentration range from 0.1 to 50.0 μg mL^{-1}. The solution was determined by UPLC-ECD under the optimum condition in three replicates of each concentration. The peak currents obtained from UPLC-ECD were plotted as a function of concentration to obtain the linearity relationship.

3.5.2 Accuracy and precision

The accuracy is evaluated by terms of percent recovery (% recovery), as follows

$$\% \text{ recovery} = \frac{\text{spike}_{\text{matrix}}}{\text{spike}_{\text{blank}}} \times 100$$

For the precision, the repeated analysis of spiked samples is studied in one day (intra-day) and three different days (inter-day). The standard solution was spiked in the samples with different known paraben concentrations (12, 22 and 32 μg mL^{-1} to represent a low, medium and high level, respectively) that each concentration level was repeated in triplicate. The precision is evaluated in terms of the relative standard deviation (%RSD), as follows

$$\%\text{RSD} = \frac{\text{standard deviation}}{\text{Mean}} \times 100$$

3.5.3 Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were obtained from the concentrations that provided a current response three times higher (S/N≥3) and ten times higher (S/N≥10) than the noise. The slope of the linearity was used to calculate the LOD and LOQ.

3.5.4 Comparison of Methods between the UPLC-ECD and UPLC-UV

The results from the proposed method were compared to the standard method. The blank samples were spiked at 12, 22 and 32 μg mL^{-1} and detected by UPLC-UV method. The results using UPLC-ECD and standard UPLC-UV method were compared using a paired-t-test.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Cyclic voltammetry (CV)

The electrochemical behaviors of methylparaben (MB), ethylparaben (EB) and propylparaben (PB) have been investigated using the cyclic voltammetric method. Electrochemical measurements were performed using a CH Instrument potentiostat with a standard three electrode configuration including a BDD working electrode, a platinum counter electrode and an Ag/AgCl reference electrode. In this section, the effect of type of supporting electrolyte, pH and concentration of the supporting electrolyte and scan rate will be discussed.

4.1.1 Type of supporting electrolyte

The electrochemical behaviors of the three parabens (50 mg L^{-1}) in two commonly supporting electrolytes, including the acetate buffer (pH 4.0) and phosphate buffer (pH 4.0) were studied using cyclic voltammetry with a potential range of 1.0 to 1.8 V at a scan rate of 50 mV s^{-1}. The voltammograms of the three parabens in two types of supporting electrolyte were presented in Figure 4.1.

In these results, the oxidation peaks of the three parabens in acetate buffer (pH 4.0) and phosphate buffer (pH 4.0) were obtained at 1.5 V and 1.3 V versus Ag/AgCl, respectively and the voltammogram of three parabens in phosphate buffer (pH 4.0) were well-defined which is important to interpret cyclic voltammetry results. Therefore, phosphate buffer solution was selected as the suitable supporting electrolyte for determination of three parabens.

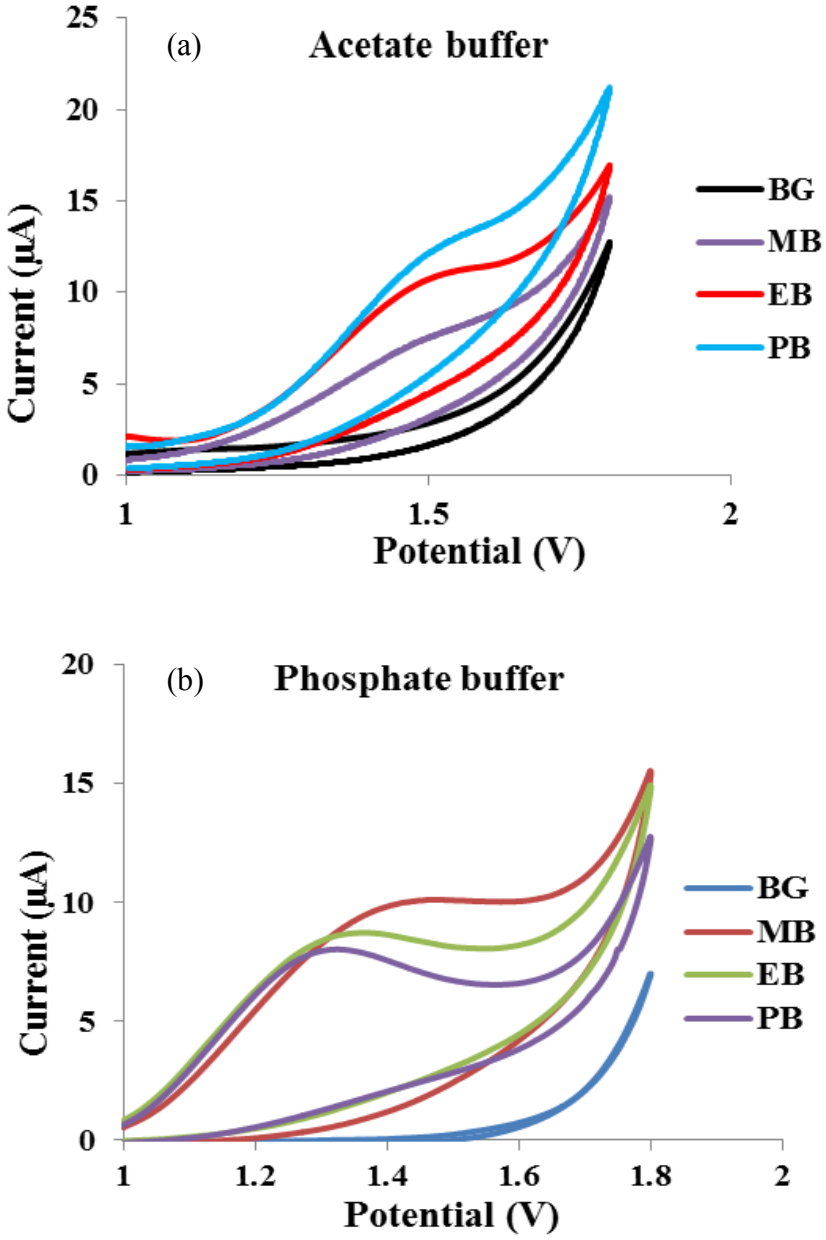


Figure 4.1 The chromatogram of three parabens (each 50 mg L^{-1}) in 0.1 M acetate buffer pH 4.0 (a) and 0.1 M phosphate buffer pH 4.0 (b) on BDD electrode. The scan rate was 50 mV s^{-1}.

4.1.2 Effect of pH of phosphate buffer solution

To study the effect of pH buffer on the electrochemical reaction of 50 mg L^{-1} of MB, EB and PB, the cyclic voltammograms were recorded at pH solutions of 4, 5, 6, 7 and 8. The relationships between current of the three parabens and the buffer pH are shown in Figure 4.2.

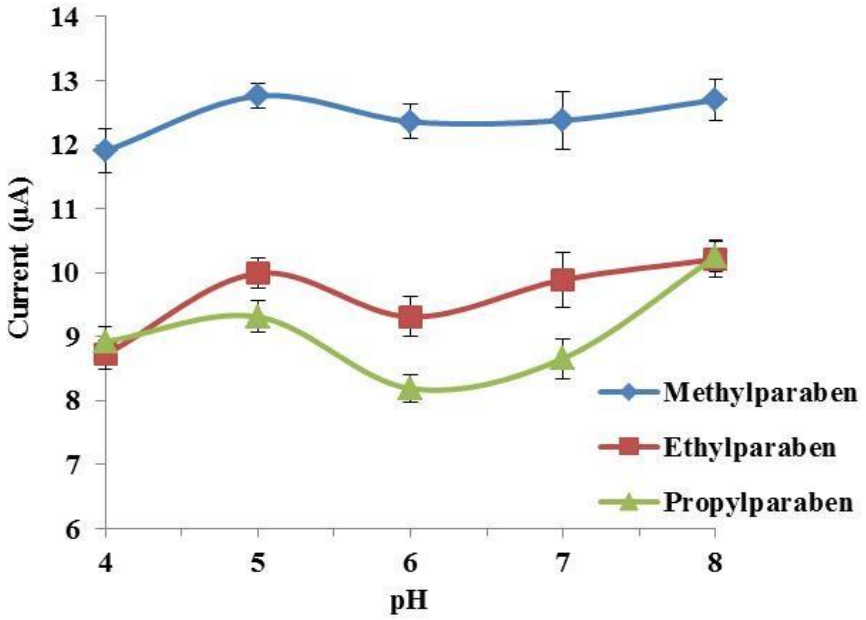


Figure 4.2 Effect of the pH on the obtained peak current of each paraben at 50 mg L^{-1} by cyclic voltammetry on the BDD electrode. The scan rate was 50 mV s^{-1}.

For all three parabens, the best operating current was obtained at pH 5. An equivalent or greater current was observed at pH 8, but high pH values will dissolve the silica and cause an increased void volume in the column. Therefore, 0.05 mol L^{-1} phosphate buffer at pH 5.0 was selected for the determination of parabens.

4.1.3 Concentration of phosphate buffer solution

The electrochemical behavior of 50 µg mL^{-1} of each paraben in variations of phosphate buffer solution concentration was studied because the migration process depends on the concentration of the supporting electrolyte. The concentrations of 0.05, 0.1, 0.2 and 0.3 M were investigated. The potential was scanned from 1.0 to 1.8

V at a scan rate of 50 mV s^{-1}. In these results, the concentrations in a range at 0.05, 0.1, 0.2 and 0.3 M have no effect on the resulting current of all analytes (data not show). Thus, the concentration of phosphate buffer pH 5.0 at 0.05 M was selected for the determination of the three parabens.

4.1.4 Scan rate dependence study

The effect of the scan rate on the electrochemical behaviors of methylparaben, ethylparaben and propylparaben were investigated by variation of the scan rate. The potential was scanned from 1.0 to1.8 V at a scan rate of 0.05, 0.1, 0.2, 0.3 and 0.5 V s^{-1}. The relationship between the resulting current versus the square root of the scan rate ($v^{1/2}$) for the three parabens were presented in Figure 4.3 to 4.5. It is shown to be highly linear at R^2 >0.99. In these results it can be explained that the transportation method of these analytes was controlled by diffusion process.

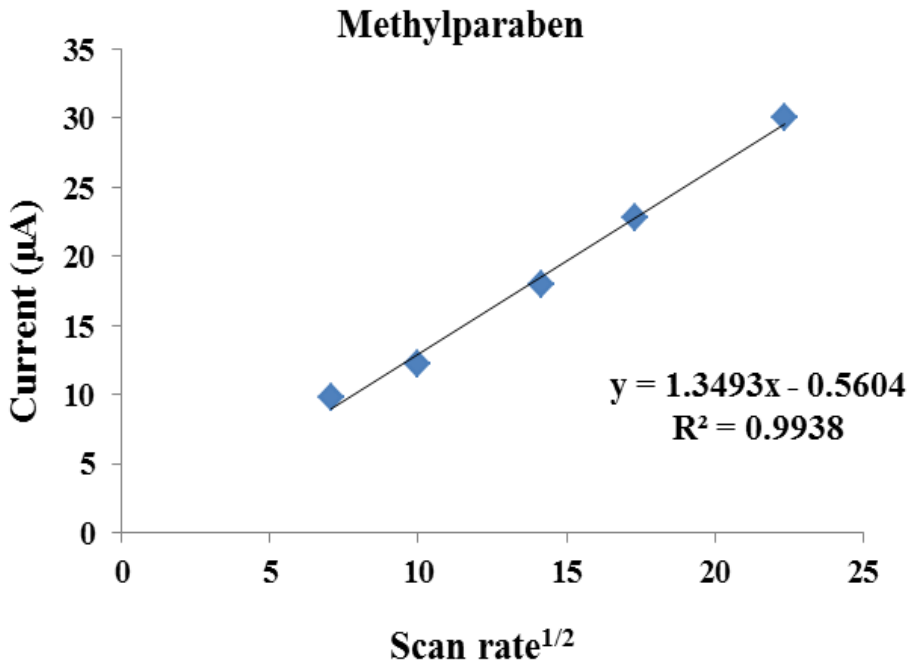


Figure 4.3 The relationship of the current response of methylparaben versus the square root of the scan rate of 50,100, 200, 300 and 500 mV s^{-1}.

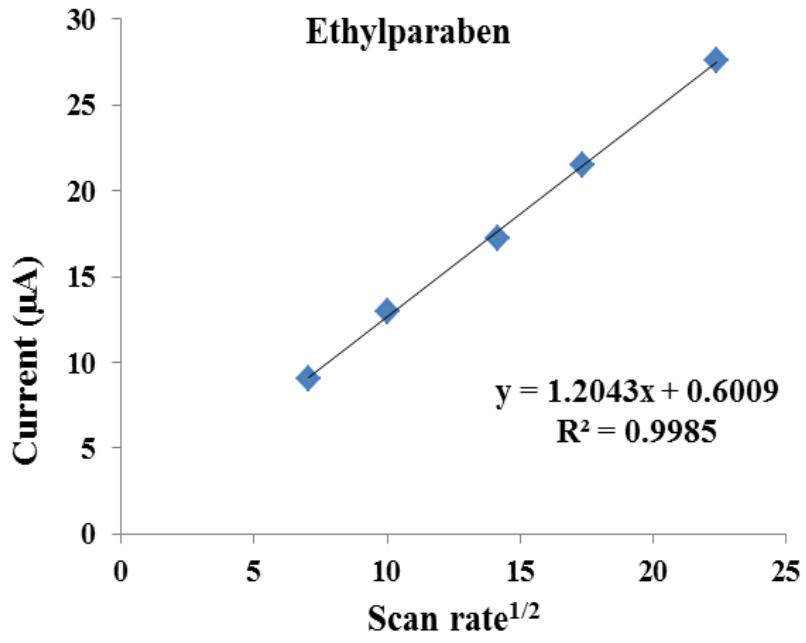


Figure 4.4 The relationship of the current response of ethylparaben versus the square root of the scan rate of 50,100, 200, 300 and 500 mV s^{-1}.

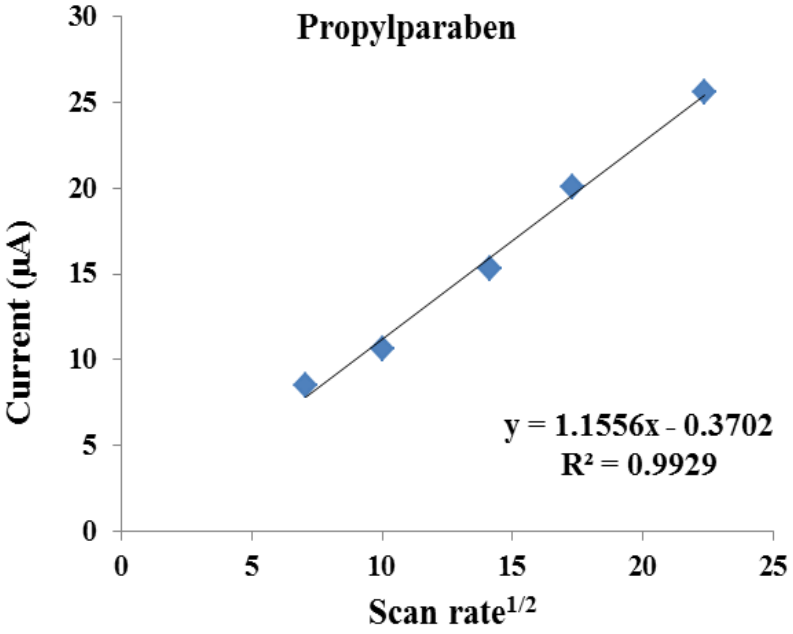


Figure 4.5 The relationship of the current response of propylparaben versus the square root of the scan rate of 50,100, 200, 300 and 500 mV s^{-1}.

The resulting currents were obtained by cyclic voltammetry on a BDD electrode, potential scan at 1.0 to 1.8 V versus Ag/AgCl and the scan rate was 0.05 V s^{-1}. The oxidation peak potential of three parabens in 0.05 M phosphate buffer solution (pH 5.0) at a BDD electrode were shown in **Table 4.1.**

Table 4.1 The oxidation peak potential of each 50 mg L^{-1} of three standard parabens in 0.05 M phosphate buffer solution (pH 5.0) at a BDD electrode, scan rate 0.05 V s^{-1}

Analytes	**Oxidation potential (V versus Ag/AgCl)**
Methylparaben	1.24
Ethylparaben	1.26
Propylparaben	1.23

4.2 Optimal conditions of UPLC-EC

4.2.1 Ratio of the mobile phase

The separation of mixed MP, EP and PP (each at 10 mg L^{-1}) was performed using the C18 short monolithic column in the isocratic mode. The acetonitrile: 0.05 mol L^{-1} phosphate buffer (pH 5) was investigated between a 25:75 and 40:60 (v/v) ratio at a flow rate of 1.5 mL min^{-1} (Figure 4.6). The optimal (v/v) ratio of acetonitrile: 0.05 mol L^{-1} phosphate buffer pH 5 was found to be 25:75 (v/v) owing to the best resolution. As the acetonitrile concentration decreased, the resolution of the three parabens increased.

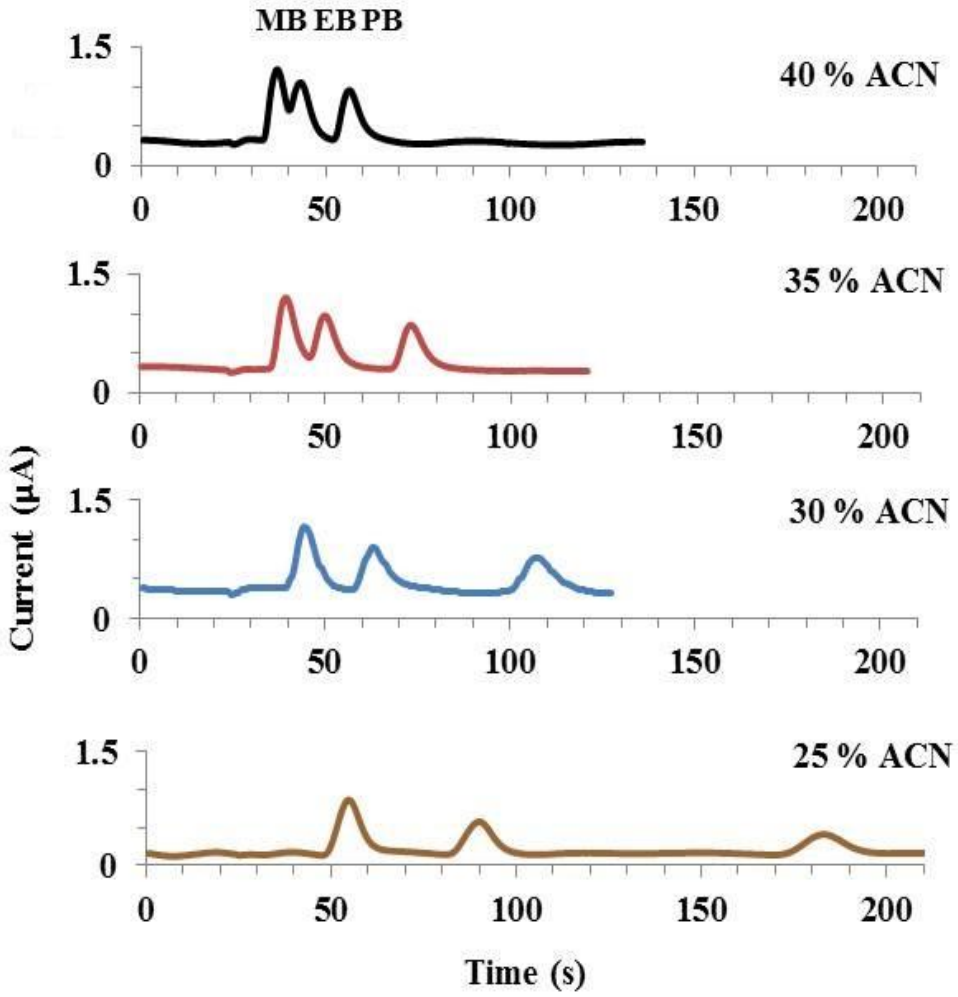


Figure 4.6 UPLC-ECD chromatogram of the three parabens at 10 ppm separated on a C18 monolithic column at a flow rate of 1.5 mL min^{-1} with different (v/v) ratios of acetonitrile (ACN): 0.05 M phosphate buffer (pH 5.0) as the mobile phase. The detection potential was 1.5 V versus Ag/AgCl using a BDD electrode. Peaks labeled MP, EP and PP are methylparaben, ethylparaben and propylparaben, respectively.

4.2.2 Effect of the flow rate

The flow rate of the mobile phase (acetonitrile and 0.05 M phosphate buffer (pH 5.0)) in the ratio at 25:75 (v/v) was investigated. The optimum flow rate was studied from 1.5, 2.0, 2.5 and 3.0 mL min^{-1}. As expected, the elution time decreased after increasing the flow rate, whereby an increased flow rate of 2.5 mL min^{-1} was evaluated. It yielded a good separation of the three parabens (10 mg L^{-1}) within a much shorter elution time of 2 min (Figure 4.7). The retention time of the three parabens was 0.6, 0.9 and 1.8 min for MB, EB and PB, respectively. In the monolith column it showed a total porosity greater than 80%, so a higher flow rate could be achieved without a loss of resolution. In this research, the flow rate of 2.5 mL min^{-1} was selected for rapid separation of the three parabens.

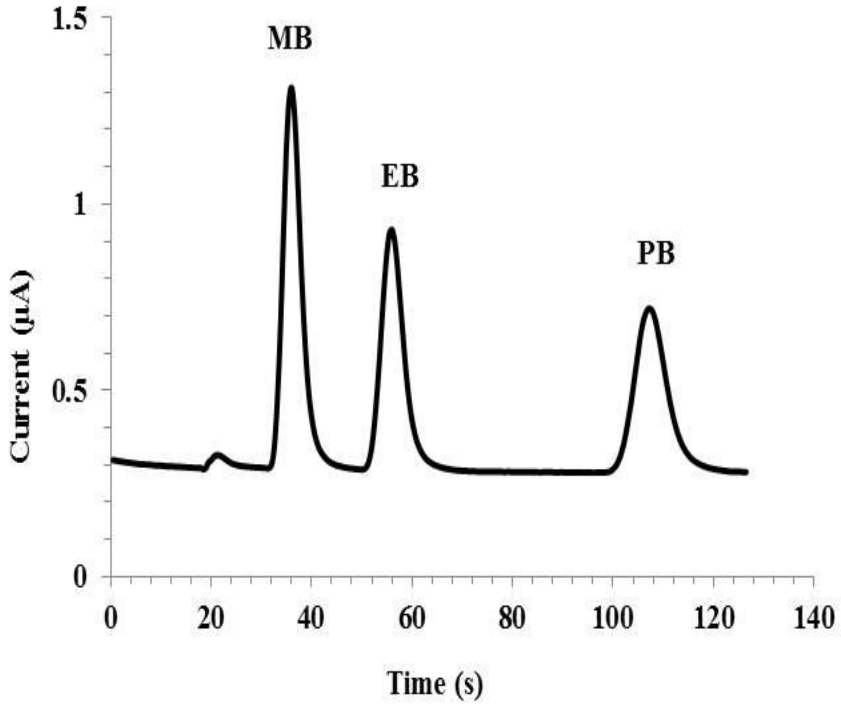


Figure 4.7 UPLC-EC chromatogram of the three parabens at 10 mg L^{-1} separated on a C18 monolithic column (**A**) at a flow rate of 2.5 mL min^{-1} with 25:75 ratios of acetonitrile (ACN): 0.05 M phosphate buffer (pH 5.0) as the mobile phase. The detection potential was 1.5 V versus Ag/AgCl using a BDD electrode.

4.2.3 Optimum potential of amperometric detection

The detection of three parabens in various applied potential on a BDD electrode was studied. The applied potential on BDD has an effect on the sensitivity of analytes. The applied potential was investigated at 1.3, 1.4, 1.5 and 1.6 V versus Ag/AgCl. The results were obtained by recording the peak area of mixed parabens (10 mg L^{-1}) at each applied potential (Figure 4.8). The signals increased when the potential increased up to 1.5 V versus Ag/AgCl. Therefore, a detection potential at 1.5 V versus Ag/AgCl was selected as the optimal potential for the determination of methylparaben, ethylparaben and propylparaben.

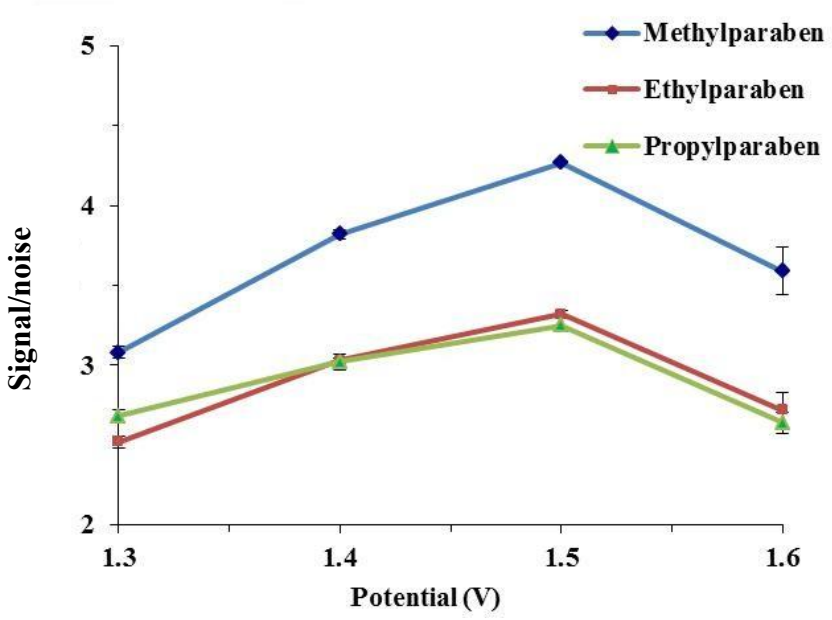


Figure 4.8 The effect of the detection potential on the peak current obtained from the BDD electrode with 10 mg L^{-1} of paraben in acetonitrile (ACN): 0.05 M phosphate buffer (pH 5.0) as the mobile phase at a flow rate of 2.5 mL min^{-1}.

4.2.4 Optimal conditions of UPLC-EC

The optimal conditions in UPLC-EC (**Table 4.2**) were utilized for the separation of methylparaben, ethylparaben and propylparaben using the short monolith column. In these conditions, the separation of the three parabens was achieved into 2 min. The chromatogram of the three parabens (10 mg L^{-1}) is presented in **Figure 4.7**.

Table 4.2 The UPLC-EC conditions for the detection of the three parabens.

UPLC parameters	**UPLC conditions**
Column	Chromolith® Flash RP-18e silica-based (25 mm x 4.6 mm i.d.)
Mobile phase	Acetonitrile:0.05 M Phosphate buffer (pH 5) (25:75, v/v)
Flow rate	2.5 mL/min
Injection volume	20 μL
Temperature	25 ^{0}C
Detector	Amperometric detection at 1.5 V vs. Ag/AgCl

4.2.5 Comparison of the UPLC column

A comparative study on the column performance between a microparticle C18 packed column (Shim-pack XR-ODS II) and two reverse C18 monolithic columns (Chromolith® Performance and Chromolith® Flash) was performed. The retention times for the three parabens by UPLC-ECD varied significantly between the three different columns **(Table 4.3)**, with the C18 monolithic column under these conditions having by far the fastest analytical speed. The shim-pack XR-ODS II C18 packing column, which generated the highest back-pressure, had a longer elution time than the short C18 monolithic column (Chromolith® Flash RP18), whereas the extended monolith column had a longer time than the short column. Thus, the Chromolith® Flash RP-18 column, which is very short and perfect for ultra-fast analysis, was selected as the most suitable column when the speed of the analysis is an important factor.

Table 4.3 The elution times for the three parabens from three different columns used in the UPLC-ECD

Analytical column	**Flow rate**	**Elution time (min)**		
	(mL min^{-1})	**MP**	**EP**	**PP**
Shim-pack XR-ODS II (100 mm × 2.0 mm i.d.)	0.6*	2.2	4.3	9.6
Chromolith® Performance (100 mm × 4.6 mm i.d.)	2.5	1.7	3.0	6.6
Chromolith® Flash (25 mm × 4.6 mm i.d.)	2.5	0.6	0.9	1.8

* At the maximum pressure of the column

4.3 Method validation

4.3.1 Linear range and Calibration curve

After the optimized conditions were obtained, the linearity and calibration curve of the developed method for simultaneous determination of methylparaben, ethylparaben and propylparaben were investigated under these conditions**. Figure 4.9** shows the chromatogram of the three parabens (10 mg L^{-1}) at several concentrations ranged from 0.1 to 50.0 mg L^{-1}. In addition, the calibration of the peak areas against the respective paraben concentrations was plotted. These calibration curves of the three parabens are shown in **Figure 4.10**. The linear relationship was determined for all of the three parabens within the range of 0.1 to 50 mg L^{-1}, with coefficients of determination (r^2) ≥ 0.997 for the three parabens.

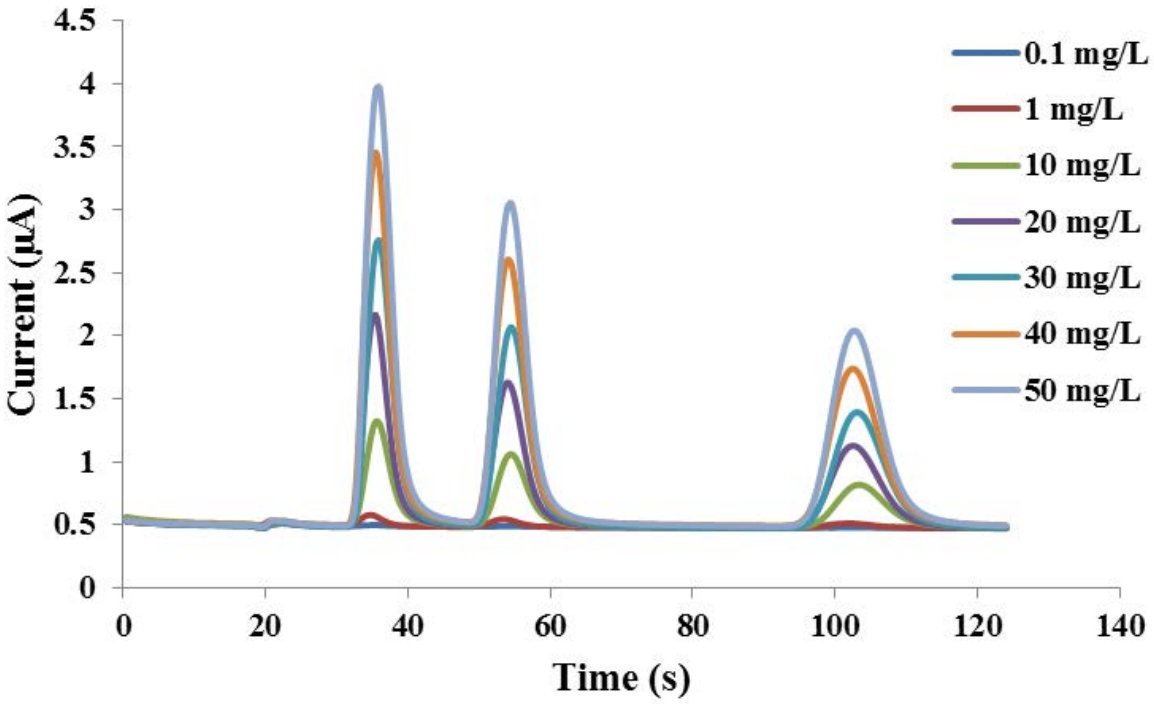


Figure 4.9 Chromatogram of the three parabens (10 mg L^{-1}) at several concentrations of 0.1 to 50.0 mg L^{-1} separated on a monolithic column at a flow rate of 2.5 mL min^{-1}. The detection potential was 1.5 V vs. Ag/AgCl using a BDD electrode.

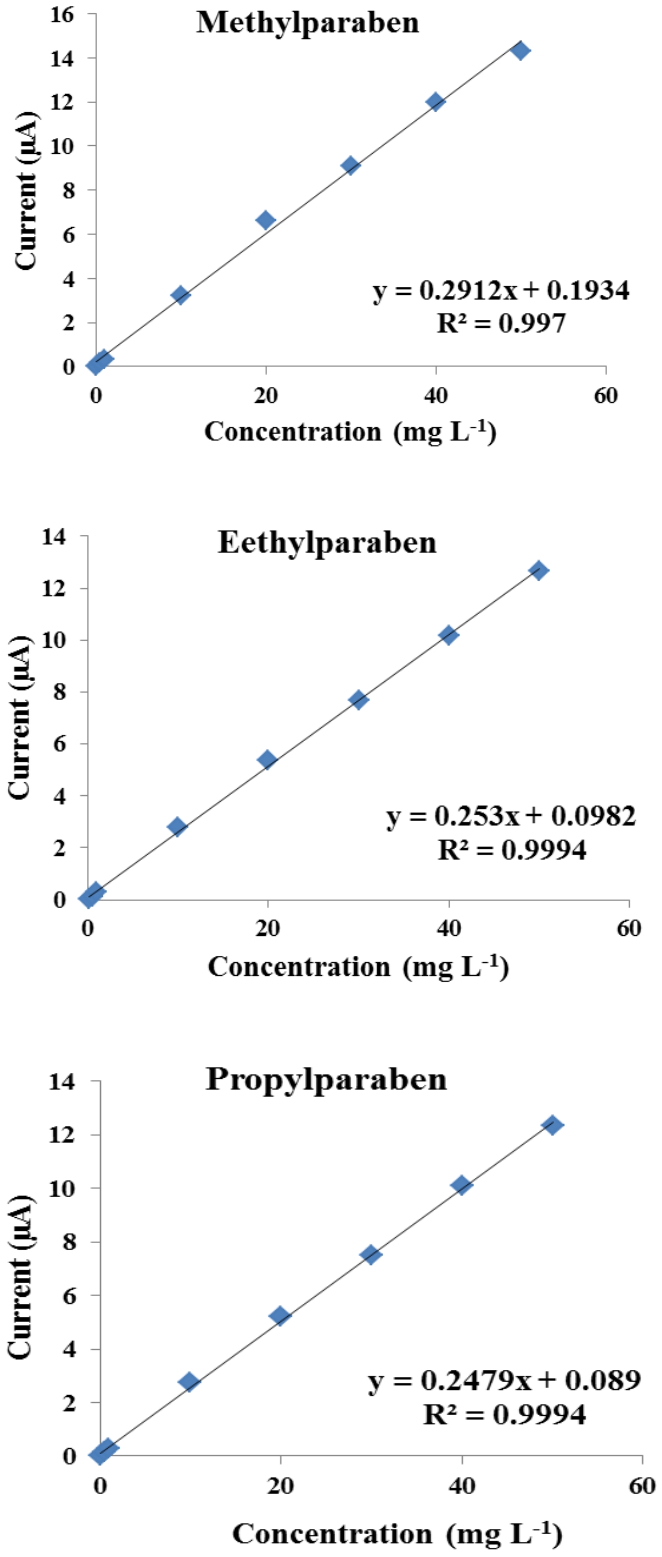


Figure 4.10 Calibration curves of the three standard parabens by UPLC-EC using a BDD electrode, concentration in a range of 0.1 to 50.0 mg L^{-1}.

4.3.2 Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection (LOD) and limit of quantification (LOQ) were obtained by experiment.

Table 4.4 Linearity range, limit of detection (LOD) and limit of quantitation (LOQ) of the UPLC-ECD method for the three parabens

Analytes	**Linearity range ($mg\ L^{-1}$)**	**Slope (peak area) ($units/mg\ L^{-1}$)**	**r^2**	**LOD ($mg\ L^{-1}$)**	**LOQ ($mg\ L^{-1}$)**
Methylparaben	0.1- 50	0.2912	0.9970	0.03	0.1
Ethylparaben	0.1- 50	0.2530	0.9994	0.03	0.1
Propylparaben	0.1- 50	0.2479	0.9994	0.03	0.1

4.3.3 Reproducibility study

The reproducibility study of the proposed method was carried out by 7 replicate measurements at three concentrations of 0.1, 10.0 and 50.0 mg L^{-1} of a mix of three parabens. The relative standard deviation (RSD) of the three concentrations were 4.0, 1.0 and 2.2% for methylparaben, 4.1, 1.1 and 2.2% for ethylparaben and 5.5, 3.7 and 2.3% for propylparaben, respectively.

4.4 Application to real samples

The proposed method was applied to the determination of methylparaben, ethylparaben and propylparaben in food, cosmetics and personal care samples. The methanol was used to extract three parabens from these samples.

4.4.1 Determination of three parabens in real samples

The proposed method was applied to six different samples, derived from food, personal care and cosmetic products (two each, see **Table 3.5**) that were purchased from local supermarkets. All samples were prepared by the extraction method using methanol. The extraction has many advantages such as: simple, low cost and high recovery. The typical chromatograms of the six real samples are presented in **Figure 4.11**, where it was demonstrated that the matrix compounds do not interfere with the analytes. As expected, this method can be used to detect all of the parabens in different matrix such as protein and lipid. The peaks were identified by a comparison with the three paraben standards, and the results are summarized in **Table 4.5.**

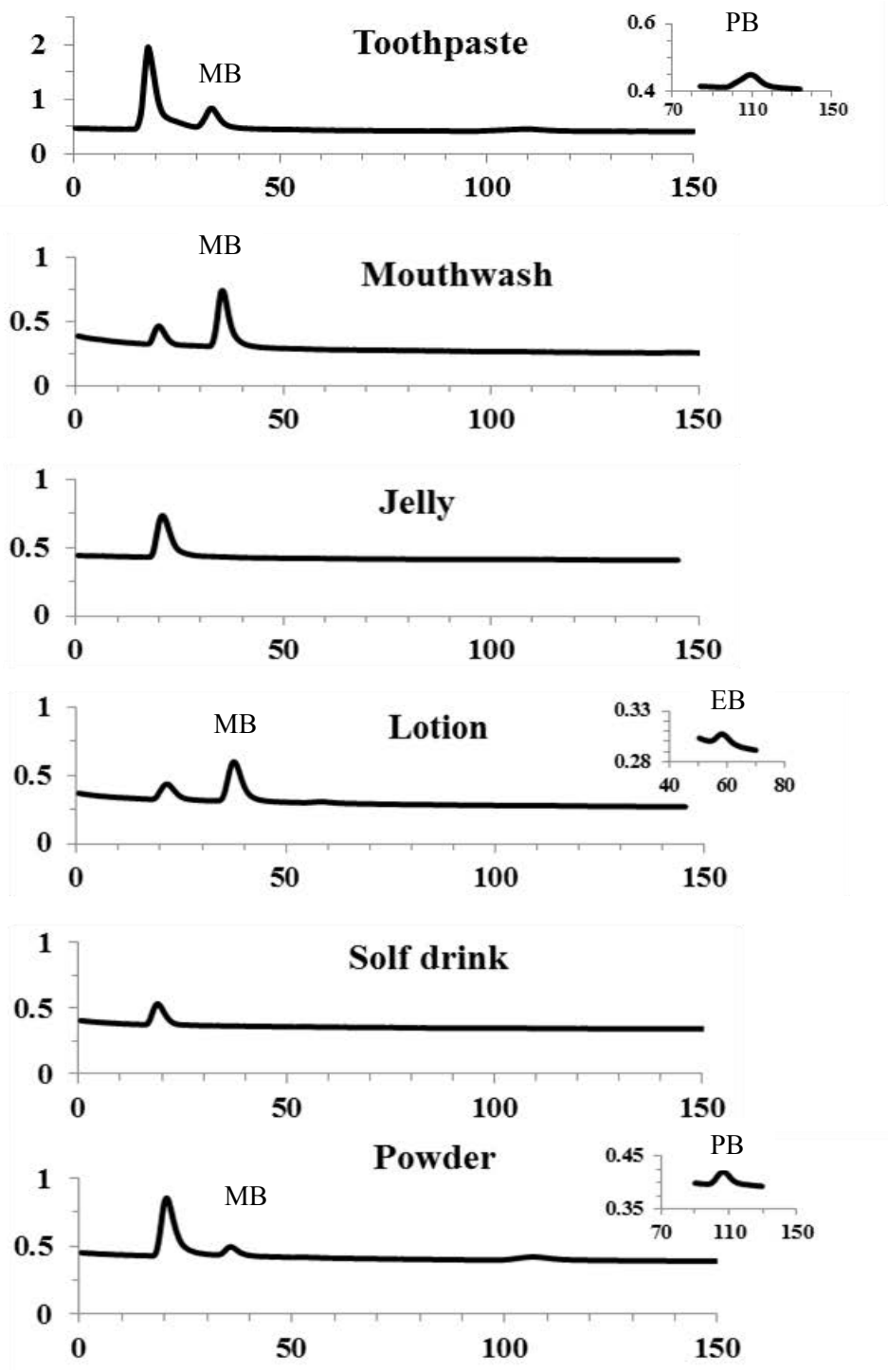


Figure 4.11 UPLC-EC derived chromatograms of the three parabens in six real samples.

Table 4.5 Determination of the paraben levels in six real samples

Analyte [a]	Concentration of parabens found	
	UPLC-EC	**UPLC-UV**
Sample A		
MB	-	-
EB	-	-
PB	-	-
Sample B		
MB	-	-
EB	-	-
PB	-	-
Sample C		
MB	240.0 ± 0.5	290.0 ± 0.5
EB	-	-
PB	130.0 ± 0.2	100.0 ± 0.2
Sample D		
MB	630.0 ± 0.4	700.0 ± 0.2
EB	-	-
PB	-	-
Sample E		
MB	437.0 ± 0.3	472.5 ± 0.2
EB	35.0 ± 0.0	52.5 ± 0.0
PB	-	-
Sample F		
MB	360.0 ± 0.0	420.0 ± 0.1
EB	-	-
PB	480.0 ± 0.0	480.0 ± 0.0

[a] Sample codes are as in Table 3.5

Data is shown as the mean $\pm$ 1 SD, and is derived from 3 independent repeats

4.4.2 Accuracy and Precision

The precision of the UPLC-EC method was then validated by calculating the relative standard deviations (RSD) of the repeated injections of working solution. To evaluate the repeatability, the three paraben concentrations as above (12.0, 22.0 and 32.0 mg. L^{-1}, or low, medium and high) were studied. The intra-day and inter-day precision were obtained by repeating in one day and on a different day. The accuracy of the UPLC-EC method was calculated from the %recovery of the spiked sampled at three concentrations (12.0, 22.0 and 32.0 mg. L^{-1}). The precision and accuracy data for the three parabens in the six samples are summarized in **Table 4.6 to 4.7**. The inter-day precision levels were in the range of 0.3-4.9%, 1.0-4.8% and 1.9-4.9% for MB, EB and PB, respectively. The intra-day precision levels were very similar, ranging from 0.4-4.9%, 2.2-4.8% and 0.7-4.9% for MB, EB and PB, respectively. The inter-day recoveries were in the range of 80.3-96.6%, 81.1-94.4% and 83.2-96.5% for MB, EB and PB, respectively, and the intra-day recovery levels were also similar at 80.3-96.0%, 80.4-96.5% and 81.6-98.9% for MB, EB and PB, respectively. According to the AOAC (in Appendix), the recovery and %RSD values for the developed method were acceptable because this method presents a recovery and %RSD less than the AOAC recommended value. Thus, this method was demonstrated to be reproducible over time.

Table 4.6 Intra-day precisions and recoveries of spiked level 12, 22 and 32 mg L^{-1}.

Samples[a]	**Spiked level (mg L^{-1})**	**Intra-day**					
		MB		**EB**		**PB**	
		Recovery	**RSD**	**Recovery**	**RSD**	**Recovery**	**RSD**
Sample A	12	87.9 ± 4.3	4.7	86.6 ± 4.1	4.8	85.9 ± 4.3	4.3
	22	93.7 ± 3.7	4.0	94.2 ± 2.2	2.3	97.2 ± 2.1	2.1
	32	89.2 ± 2.9	3.3	92.8 ± 2.4	2.6	95.5 ± 2.1	2.2
Sample B	12	80.3 ± 0.4	0.4	82.2 ± 2.2	2.7	91.1 ± 2.8	3.1
	22	81.9 ± 2.8	3.4	86.2 ± 2.2	2.5	97.5 ± 0.8	0.9
	32	81.6 ± 0.4	0.5	83.8 ± 1.9	2.2	96.7 ± 0.7	0.7
Sample C	12	80.7 ± 0.5	0.6	80.4 ± 0.2	0.3	84.4 ± 1.6	2.0
	22	84.5 ± 3.2	3.8	81.9 ± 3.0	3.6	84.0 ± 2.5	3.0
	32	82.5 ± 2.8	3.4	80.2 ± 0.1	0.2	85.1 ± 3.2	3.8
Sample D	12	96.0 ± 1.7	1.7	80.8 ± 1.3	1.3	81.6 ± 0.6	0.8
	22	90.8 ± 4.1	4.5	86.2 ± 3.2	3.7	88.5 ± 4.4	4.9
	32	81.8 ± 1.3	1.6	81.9 ± 1.4	1.7	87.8 ± 1.6	1.8
Sample E	12	86.2 ± 0.6	0.7	94.5 ± 1.1	1.2	98.9 ± 0.9	0.9
	22	90.4 ± 3.9	4.3	96.5 ± 1.7	1.8	97.9 ± 1.4	1.5
	32	80.7 ± 0.2	0.3	83.4 ± 2.2	2.6	96.7 ± 1.3	1.3
Sample F	12	98.8 ± 1.7	1.7	95.5 ± 2.1	2.2	93.1 ± 4.2	4.5
	22	95.3 ± 3.6	3.8	89.7 ± 4.0	4.5	84.2 ± 3.3	3.9
	32	94.7 ± 4.6	4.9	95.2 ± 3.6	3.8	94.7 ± 4.5	4.8

[a]Sample codes are as in Table 1

Data is shown as the mean % $\pm$ 1 SD, and is derived from three independent repeats.

Table 4.7 Inter-day precisions and recoveries of spiked 12, 22 and 32 mg L^{-1}.

Samples[a]	**Spiked level (mg L^{-1})**	**Inter-day**					
		MB		**EB**		**PB**	
		Recovery	**RSD**	**Recovery**	**RSD**	**Recovery**	**RSD**
Sample A	12	82.7 ± 2.3	2.8	84.8 ± 3.3	3.9	85.1 ± 4.1	4.8
	22	86.1 ± 4.1	4.8	87.7 ± 3.1	3.6	88.3 ± 3.5	4.0
	32	83.2 ± 3.7	4.4	87.2 ± 4.1	4.7	89.9 ± 4.4	4.9
Sample B	12	80.3 ± 0.2	0.3	81.1 ± 0.8	1.0	89.6 ± 1.7	1.9
	22	82.1 ± 2.7	3.3	84.6 ± 3.9	4.6	94.2 ± 4.5	4.8
	32	81.3 ± 0.4	0.5	84.4 ± 3.2	3.8	92.7 ± 4.6	4.9
Sample C	12	82.2 ± 2.4	3.0	82.7 ± 3.5	4.2	83.2 ± 1.8	2.2
	22	94.4 ± 3.8	4.0	89.1 ± 3.8	4.2	92.6 ± 2.1	2.3
	32	84.5 ± 4.1	4.9	84.8 ± 4.1	4.8	89.8 ± 2.3	2.5
Sample D	12	96.6 ± 1.7	1.7	81.1 ± 0.8	1.0	83.8 ± 2.8	3.3
	22	89.5 ± 2.1	2.4	83.8 ± 2.1	2.5	84.5 ± 3.3	3.9
	32	82.2 ± 0.7	0.9	82.0 ± 1.5	1.8	83.3 ± 3.6	4.3
Sample E	12	85.9 ± 1.2	1.4	90.8 ± 4.3	4.8	93.2 ± 3.6	3.9
	22	89.0 ± 4.0	4.5	94.4 ± 3.4	3.6	96.5 ± 3.1	3.2
	32	82.1 ± 1.6	2.0	82.0 ± 1.8	2.2	94.4 ± 2.5	2.7
Sample F	12	93.2 ± 4.6	4.9	91.1 ± 4.2	4.7	91.5 ± 4.1	4.4
	22	93.0 ± 4.4	4.7	94.0 ± 2.4	2.6	90.8 ± 3.7	4.1
	32	89.9 ± 4.1	4.6	94.1 ± 4.5	4.7	93.2 ± 2.6	2.8

[a] Sample codes are as in Table 3.5

Data is shown as the mean % $\pm$ 1 SD, and is derived from three independent repeats.

4.4.3 Comparison of Methods between UPLC-EC and UPLC-UV

The validation of the proposed method was obtained by comparing with the UPLC-UV method. For a direct comparison with the UPLC-UV method, the results obtained using UPLC-EC and standard UPLC-UV were compared by a paired-t-test for six samples that were spiked with different known paraben concentrations (12, 22 and 32 mg L^{-1} to represent a low, medium and high level, respectively). The critical t-value (2.5706) was significantly higher than the experimental t-values between the two pairs of assays. Using the UPLC-ECD method, the t-values for MP, EP and PP were -0.7408, 2.4752 and 1.9383, respectively, for a spiked sample at 12 mg L^{-1}. For a spiked sample at 22 mg L^{-1}, $t_{calculated}$ value two-tail were -0.7882, 0.8953 and 2.1581, respectively, and for a spiked sample at 32 mg L^{-1}, t-values were -0.5355, -0.4202 and 2.1222, respectively. There were no significant differences between the two assay methods at all three spiked concentrations. Thus, the results obtained from the developed method can be accepted. The results obtained by both methods are shown in **Table 4.8**.

Table 4.8 The $t_{calculated}$ values of methylparaben, ethylparaben and propylparaben at three spiked level of six samples.

Spiked level ($\mu g\ L^{-1}$)	**$t_{calculated}$ value two-tail**		
	MB	**EB**	**PB**
12	-0.7408	2.4752	1.9383
22	-0.7882	0.8953	2.1581
32	-0.5355	-0.4202	2.1222

CHAPTER V

CONCLUSIONS

5.1 Conclusions

In this research, the method for the determination of the three paraben preservatives including, methylparaben, ethylparaben and propylparaben using Ultra-performance liquid chromatography coupled with electrochemical detection on BDD electrode was developed. The electrochemical behaviors of the three parabens were investigated by cyclic voltammetry, which showed the oxidation peak on a BDD electrode in 0.05 M phosphate buffer pH 5.0. The method for the rapid analysis of three commonly used paraben preservatives (MP, EP and PP) by UPLC coupled with amperometric detection using a BDD electrode was successfully developed. Particular attention was paid to the use of a monolithic column coupled with a UPLC instrument for fast separation. A silica-based monolithic column was used for the separation of common parabens because of its high tolerance to organic solvents, high polarity and low backpressure relative to the traditional column that arose from an increased flow rate (reducing evaluation time) without a loss of resolution. The optimal UPLC-EC conditions were found to be a 0.05 M phosphate buffer (pH 5.0) and acetonitrile in a ratio of 25:75 (v/v) as the mobile phase on a monolithic column. The flow rate was 2.5 mL min^{-1}.

In addition, the innovative electrochemical detection system using a BDD electrode, with an optimal potential of 1.5 V vs. Ag/AgCl, yielded a high degree of reproducibility, based on % RSDs for the intra- and inter-assays below 5%. Analysis time was 2 min. For the validation of this method, the linearity was found to be in the range of 0.1-50.0 mg L^{-1} all analytes. The correlation coefficient of the three parabens were all greater than 0.997 (r^2 values of 0.9970, 0.9994 and 0.9994 for MP, EP and

PP, respectively). The LOD and LOQ of this method were in a range at 0.03 mg L^{-1} and 0.10 mg L^{-1}, respectively. This method was successfully applied to determine the concentrations of each paraben in six real samples with the recoveries ranging from 80.3 - 98.9% for all three parabens from samples spiked at 12, 22 and 32 mg L^{-1}. The precision of the UPLC-ECD method was then validated by calculating the relative standard deviations (RSD) from triplicates using the same three paraben concentrations as above (12.0, 22.0 and 32.0 mg. L^{-1}, or low, medium and high). The inter-day precision levels were in the range of 0.3-4.9%, 1.0-4.8% and 1.9-4.9% for MB, EB and PB, respectively. The intra-day precision levels were very similar, ranging from 0.4-4.9%, 2.2-4.8% and 0.7-4.9% for MB, EB and PB, respectively. The percentage recoveries and %RSDs of the proposed method were acceptable according to AOAC International. Thus, this method was proven to be reproducible over time.

However, the actual paraben levels in the samples were unknown. For a direct comparison with the UPLC-UV method, the results obtained using UPLC-ECD and standard UPLC-UV were compared by a paired-*t*-test for three samples that were spiked with different known paraben concentrations (12, 22 and 32 mg L^{-1} to represent a low, medium and high level, respectively). The critical *t*-value was significantly higher than the experimental *t*-values between the two pairs of assays. There were no significant differences between the two assay methods at all three spiked concentrations. Thus, the results obtained from the developed method can be accepted. Overall, the proposed method successfully provided a rapid analysis, was highly sensitive and precise for the determination of parabens in several kinds of products.

5.2 Suggestion for further work

In this work, the UPLC coupled with the electrochemical detection on the BDD electrode was successfully applied to the determination of the three parabens in several kinds of samples. In addition, the determination of other preservatives, such as sorbic acid, benzoic acid and propinoic acid could also be considered.

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APPENDICES

APPENDIX A

Table A1 The peak height of each parabens in concentration of phosphate buffer pH 5.0 at 0.05 to 0.3 M

Concentration of phosphate buffer pH 5 (M)	Peak Height (μA)		
	Methylparaben	Ethylparaben	Propylparaben
0.05	11.26	8.38	8.17
0.1	10.76	8.42	8.11
0.2	9.16	8.78	8.07
0.3	10.59	8.57	8.01

The Scan rate dependence study of Methylparaben on BDD electrode

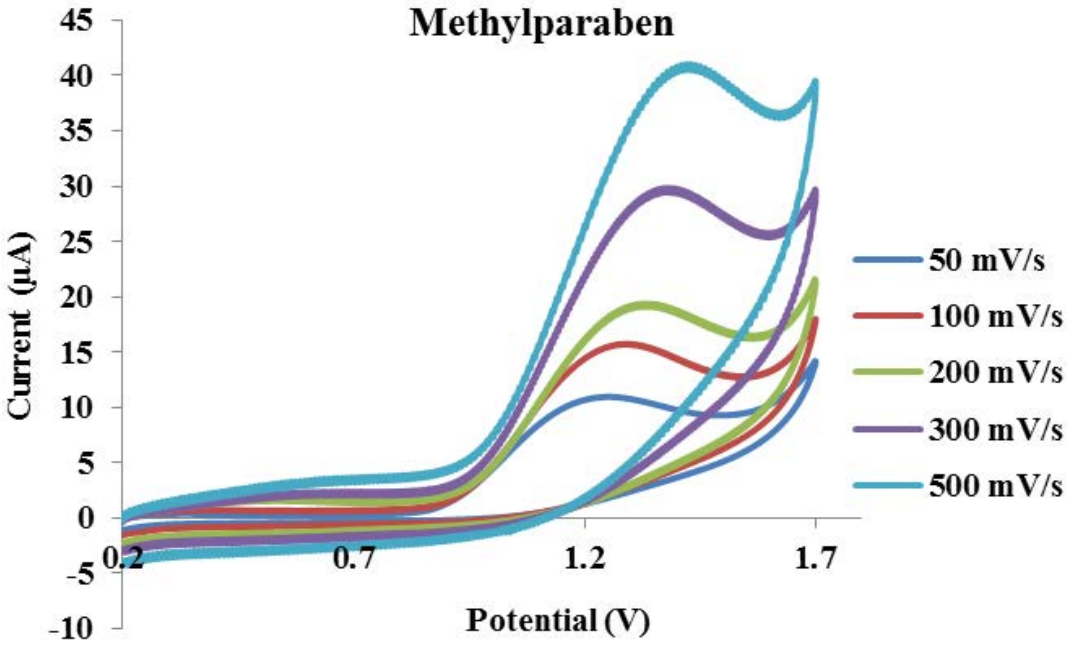

Figure A1 Cyclic voltammetry of 50 mg L^{-1} methylparaben in 0.05 M phosphate buffer pH 5.0 at the BDD electrode.

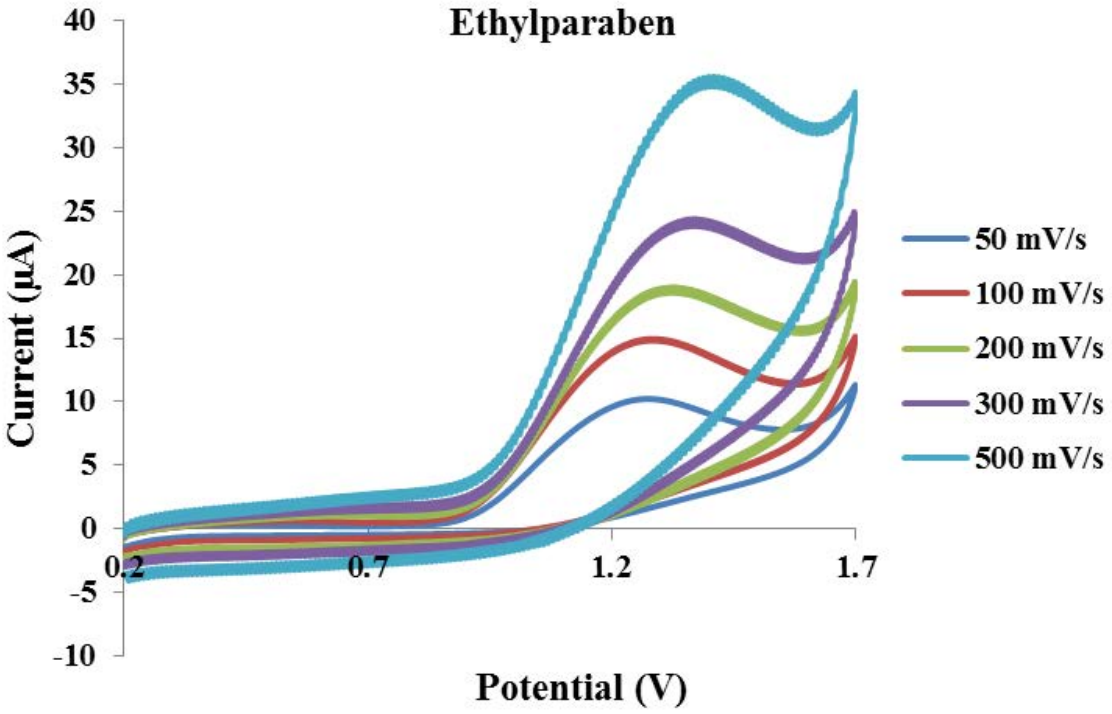

Figure A2 Cyclic voltammetry of 50 mg L^{-1} ethylparaben in 0.05 M phosphate buffer pH 5.0 at the BDD electrode.

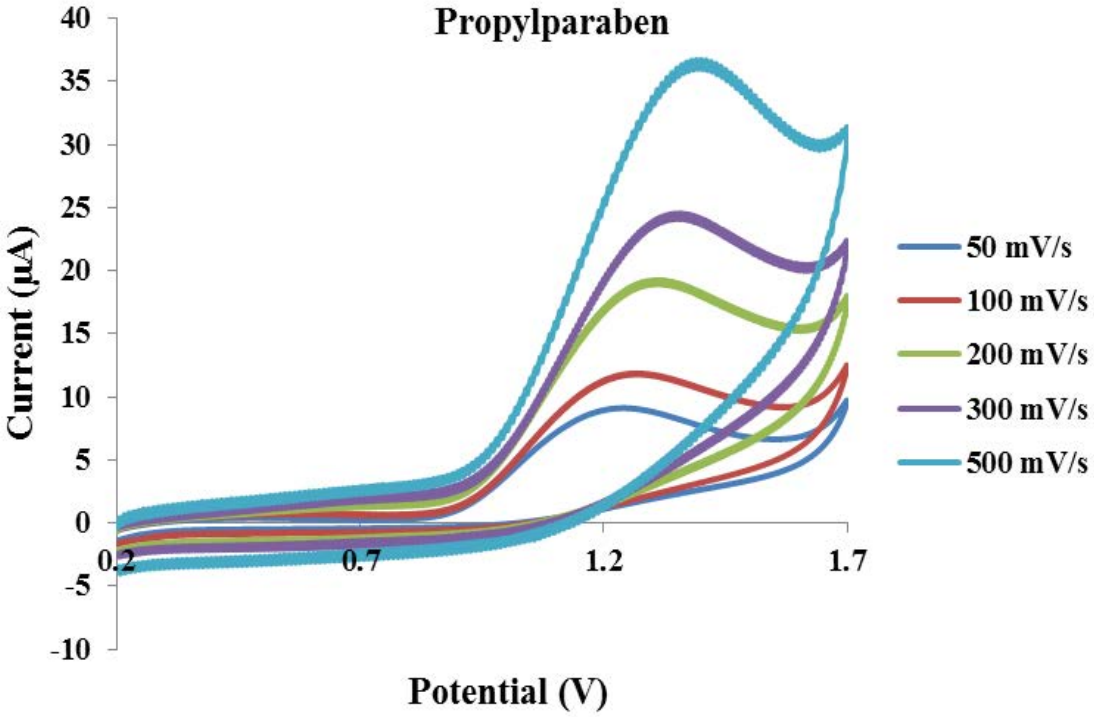

Figure A3 Cyclic voltammetry of 50 mg L^{-1} propylparaben in 0.05 M phosphate buffer pH 5.0 at the BDD electrode.

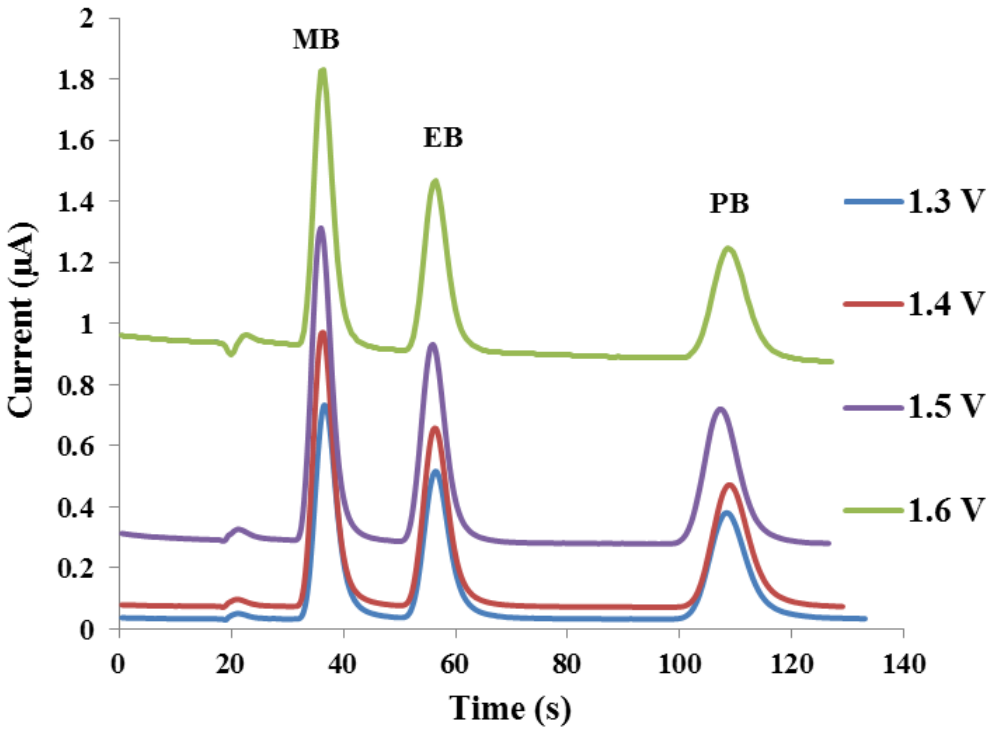

Figure A4 UPLC-ECD chromatogram of the three parabens at 10 mg L^{-1} separated on a C18 monolithic column (**A**) at a flow rate of 2.5 mL min^{-1} with 25:75 ratios of acetonitrile (ACN): 0.05 M phosphate buffer (pH 5.0) as the mobile phase. The detection potential was 1.3 to 1.5 V versus Ag/AgCl using a BDD electrode.

APPENDIX B

Table B1 Paired-t-test value

df	95%	99%
1	12.71	63.66
2	4.30	9.93
3	3.18	5.84
4	2.78	4.60
5	2.52	4.03
10	2.23	3.17
15	2.13	2.95
20	2.09	2.85
30	2.04	2.75
α	1.96	2.58

Table B2 T-test: paired two sample for means at 12 mg L^{-1} concentration levels of methylparaben

	UPLC-EC	**UPLC-UV**
Mean	12.11833333	12.345
Variance	4.830256667	5.90927
Observations	6	6
Pearson Correlation	0.952513286	
Hypothesized Mean Difference	0	
df	5	
t Stat	-0.740786452	
P(T<=t) one-tail	0.246061741	
t Critical one-tail	2.015048373	
P(T<=t) two-tail	0.492123482	
t Critical two-tail	2.570581836	

Table B3 T-test: paired two sample for means at 12 mg L^{-1} concentration levels of ethylparaben

	UPLC-EC	**UPLC-UV**
Mean	10.535	10.15666667
Variance	0.31255	0.318466667
Observations	6	6
Pearson Correlation	0.777890037	
Hypothesized Mean Difference	0	
df	5	
t Stat	2.475211806	
P(T<=t) one-tail	0.028084955	
t Critical one-tail	2.015048373	
P(T<=t) two-tail	0.056169911	
t Critical two-tail	2.570581836	

Table B4 T-test: paired two sample for means at 12 mg L^{-1} concentration levels of propylparaben

	UPLC-EC	**UPLC-UV**
Mean	10.845	10.465
Variance	0.46495	0.53999
Observations	6	6
Pearson Correlation	0.772690751	
Hypothesized Mean Difference	0	
df	5	
t Stat	1.93833827	
P(T<=t) one-tail	0.05515271	
t Critical one-tail	2.015048373	
P(T<=t) two-tail	0.110305421	
t Critical two-tail	2.570581836	

Table B5 T-test: paired two sample for means at 22 mg L^{-1} concentration levels of methylparaben

	UPLC-EC	**UPLC-UV**
Mean	22.25333333	22.64833333
Variance	7.824626667	12.06481667
Observations	6	6
Pearson Correlation	0.945976516	
Hypothesized Mean Difference	0	
df	5	
t Stat	-0.788155248	
P(T<=t) one-tail	0.233155615	
t Critical one-tail	2.015048373	
P(T<=t) two-tail	0.46631123	
t Critical two-tail	2.570581836	

Table B6 T-test: paired two sample for means at 22 mg L^{-1} concentration levels of ethylparaben

	UPLC-EC	**UPLC-UV**
Mean	20.625	20.15666667
Variance	0.60759	2.430946667
Observations	6	6
Pearson Correlation	0.574676771	
Hypothesized Mean Difference	0	
df	5	
t Stat	0.895332267	
P(T<=t) one-tail	0.205817382	
t Critical one-tail	2.015048373	
P(T<=t) two-tail	0.411634764	
t Critical two-tail	2.570581836	

Table B7 T-test: paired two sample for means at 22 mg L^{-1} concentration levels of prothylparaben

	UPLC-EC	**UPLC-UV**
Mean	20.55833333	19.49166667
Variance	0.491736667	1.255456667
Observations	6	6
Pearson Correlation	0.17905048	
Hypothesized Mean Difference	0	
df	5	
t Stat	2.158057638	
P(T<=t) one-tail	0.041695693	
t Critical one-tail	2.015048373	
P(T<=t) two-tail	0.083391386	
t Critical two-tail	2.570581836	

Table B8 T-test: paired two sample for means at 32 mg L^{-1} concentration levels of methylparaben

	UPLC-EC	**UPLC-UV**
Mean	28.81666667	29.16666667
Variance	2.025666667	4.858666667
Observations	6	6
Pearson Correlation	0.688724018	
Hypothesized Mean Difference	0	
df	5	
t Stat	-0.535512196	
P(T<=t) one-tail	0.307624168	
t Critical one-tail	2.015048373	
P(T<=t) two-tail	0.615248335	
t Critical two-tail	2.570581836	

Table B9 T-test: paired two sample for means at 32 mg L^{-1} concentration levels of ethylparaben

	UPLC-EC	**UPLC-UV**
Mean	27.33333333	27.68333333
Variance	1.962666667	3.469666667
Observations	6	6
Pearson Correlation	0.243208382	
Hypothesized Mean Difference	0	
df	5	
t Stat	-0.420184922	
P(T<=t) one-tail	0.345898262	
t Critical one-tail	2.015048373	
P(T<=t) two-tail	0.691796524	
t Critical two-tail	2.570581836	

Table B10 T-test: paired two sample for means at 32 mg L^{-1} concentration levels of propylparaben

	UPLC-EC	**UPLC-UV**
Mean	29.63333333	28.31666667
Variance	2.154666667	1.393666667
Observations	6	6
Pearson Correlation	0.357400332	
Hypothesized Mean Difference	0	
df	5	
t Stat	2.122153591	
P(T<=t) one-tail	0.043633564	
t Critical one-tail	2.015048373	
P(T<=t) two-tail	0.087267129	
t Critical two-tail	2.570581836	

APPENDIX C

Table C1 The acceptable precision, the data from AOAC manual for peer verified methods program, VA, NOV 1993

Analyte concentration	%RSD
100%	± 1.3
10%	± 2.7
1%	± 2.8
0.1%	± 3.7
100 ppm	± 5.3
10 ppm	± 7.3
1 ppm	± 11
100 ppb	± 15
10 ppb	± 21
1 ppb	± 30

Table C2 The acceptable accuracy, the data from From AOAC manual for peer verified methods program, VA, NOV 1993

Analyte concentration	% recovery
100%	98-102
10%	98-102
1%	97-103
0.1%	95-105
100 ppm	90-107
10 ppm	80-110
1 ppm	80-110
100 ppb	80-110
10 ppb	60-115
1 ppb	40-120

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