1

IN-COLUMN ELECTROCHEMICAL DETECTION FOR LIQUID CHROMATOGRAPHY MICRODEVICE

Leow Pei Ling
Fauzan Khairi Che Harun
Rashidah Arsat
Danny O'Hare

1.1 INTRODUCTION

Liquid chromatography is a pressure driven separation method for chemical mixture: different affinities of the analytes for the mobile phase and the stationary phase under pressure will cause separation of analytes as diverse as proteins (Oates and Jorgenson 1990) to neurotransmitters (McKenzie *et al.*, 2002).

Liquid chromatography (LC) began in the 19th century where the first literature report of chromatography was by Tswett in 1903 (Ettre and Sakodynskii, 1993)[a] (Ettre and Sakodynskii, 1993)[b]. Classical LC was conducted using a glass column packed with the stationary phase. The column had an inner diameter ranged from 1 cm to 5 cm and a length from 50 cm up to 1 m and the size of stationary phase particles ranged between 150 μm to 200 μm.

LC is performed by having mobile phase percolating through a column packed with porous inert support (usually silica or alumina) now more usually coated with a suitable thin film, acting as the stationary phase. The mixture of chemical compounds introduced at the beginning of the column is washed through the column by the eluting mobile phase. Different affinities of the analytes for the mobile phase and stationary phase allow separation to take place. For instance, a component that is weakly adsorbed on the surface of the stationary phase will travel through the column more rapidly than compound that is more strongly adsorbed to the stationary phase surface.

However, this conventional elution method for separation process can be time consuming. Rapid liquid chromatography was developed gradually in the 1960s and became known as high performance liquid chromatography (HPLC) due to the emergence of high efficiency materials and improvements in instrumentation. The speed of the separation process has been rapidly improved by applying positive pressure to the separation column. In the past 50 years HPLC has earned its popularity in the separation science due to its wide range of separation abilities and ease of application (Lindsay, 1992, Miller, 2004, Venn, 2000, Kellner *et al.*, 2004).

1.1.1 High performance liquid chromatography

The apparatus of the HPLC system typically consists of a high-pressure pump, an autosampler and injector, a column containing a high efficiency stationary phase, and a detector and data acquisition system. Figure 1.1 illustrates a schematic diagram of the basic instrumentation of the HPLC system.

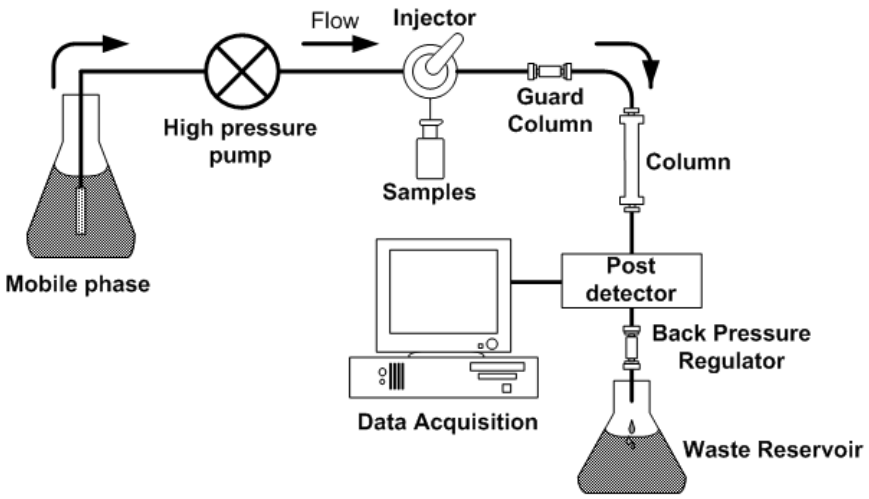


Figure 1.1: Basic HPLC instrumentation.

The process of HPLC begins with the sample mixture or analytes being injected into the system and a high-pressure pump supplying mobile phase through the column. The LC separation column is typically a stainless steel or a polyetheretherketone (PEEK) column with an inner diameter of 0.2 mm to 5 mm and length of 5 cm up to 30 cm. As columns are mainly very expensive and can be easily degraded by dust or impurities in the sample or solvent, the entrance of the main column is protected by a guard column that contains the same stationary phase as the main column. High purity, spherical, microporous particles of silica that are permeable to solvent are used as the stationary phase packed within the column. The most commonly used packing materials are microparticulate silica beads ranging in diameter, from 3 – 10 μm, which in bulk, resemble fine talcum powder. Other packing materials such as fluorocarbons, carbon, alumina or polymeric resins have been developed as silica has a limitation for pH less than 2.5 or above 9, severely limiting separations for strong acidic or basic materials which would elute with the solvent from within this pH range. Most analytical HPLC is done with chemically

modified silica as the stationary phase. The silica particles are chemically modified so as to alter the properties of the silica surface, which is important for the so-called reversed phase (RP) separation mode.

The separation of components occurs as the analytes and mobile phase are pumped through the column. The mobile phase used can be water, organic solvents or buffers. Mixture will be introduced by the sample injector at the beginning of the column. The components in the mixture are eluted from the column as a narrow band. Different affinities of the components for the stationary phase and mobile phase will cause separation of the mixture. Finally, these separated components pass through the detector and signals that are generated will be recorded. The recorder or the data acquisition system records the narrow band of analytes as peaks appearing in time. The appearance and the shape of the peak carry information about the chromatographic process.

There are five different common separation modes of chromatography based on the mechanism of interaction between the solute, stationary phase and mobile phase. Table 1.1 summarizes these five common chromatography separation modes.

Table 1.1: Summary of different separation modes in chromatography based on the retention mechanism.

	Separation mode	**Principle**
1.	Adsorption	Solute is adsorbed on the surface of the solid particles and equilibrium between the stationary and mobile phase accounts for separation of different solutes.
2.	Reverse phase	Non-polar stationary phase is used by covalently bonding alkyl chains to the support surface and reversing the elution

		order. The partition coefficient depends on the affinity of the solutes to the stationary and mobile phase.
3.	Partition	Solute molecules partitioned between the stationary liquid and a separate mobile liquid phase. The liquid stationary phase forms a thin film on the surface of a solid support and solute equilibrates between the stationary liquid and the mobile phase.
4.	Ion-exchange	Retains solute molecules based on coulombic (ionic) interactions. Stationary phase surface displays ionic function groups that interact with solute ions of opposite charge.
5.	Molecular exclusion	Does not rely on attractive interaction between solute and stationary phase, this technique separates molecules by using molecular size which determines the average path length through the column packing (gel).

Of these separation modes, reverse phase LC with bonded phases is the most widely used as it allows samples with a wide range of polarity to be separated; the mobile phase requirement is inexpensive; it can be used to separate ionic or ionisable compounds by using ion pairing or suppression agent; it also is a very reproducible, fast and easy to operate separation mode (Lindsay, 1992, Venn, 2000).

1.1.2 Reverse phase liquid chromatography

In reverse-phase liquid chromatography (LC), the polarity of the stationary phase is lower than the mobile phase. The stationary phase is prepared by chemically bonding non-polar molecules on the solid support (silanol groups) and is normally made up of hydrophobic alkyl chains into which analytes are partitioned. Due to the sizes of the hydrophobic groups such as octadecylsilane (ODS or C18), it is not possible to have the whole solid surface covered. As a consequence, non-reacted or uncovered silanol groups remain and may cause peak tailing. Therefore, end-capping process is used to reduce these non-reacted silanols to a non-polar bonded phase by supplying to the column a small silylating agent such as trimethylchlorosilane.

Ideally in reverse phase HPLC, analytes migrate through the column in a neutral form. Under condition that there are no ionisable groups, water and an organic solvent based mobile phase will be sufficient for the separation. However, if ionisable groups are present, pH adjustment is necessary for the mobile phase. Conventionally, silica based columns are only tolerant of pH in the range 2.5 – 9.0. Below pH 2.5, the bonded phase may be hydrolysed and above pH 9.0 the silica support starts to dissolve. However, some improved silica based columns are currently able to tolerate up to around pH 10.0.

There are three common alkyl chain lengths commercially available for bonded phase, C4, C8, and C18, where C4 is normally used for protein separation, C8 and C18 is used for capturing small biological molecules such as neurotransmitters and drugs. The most common chromatography columns use octadecyl (C18) and this is often the default column in routine analytical chromatography.

1.1.3 State of the art

The conventional LC system is bulky with a footprint of typically 0.5 m^2. Since the introduction of microfluidics (Manz *et al.*, 1990), LC have been widely adopted in a microfluidic platform for analytical application. The technologies for miniaturizing the analytical systems have become known as micro total analysis systems (μTAS) or the 'lab-on-a-chip' (LOC). Since 1990, LOC has found applications as diverse as environmental analysis(Ohira and Toda, 2005), genomics, proteomics(Sanders and Manz, 2000), high throughput screening, single cell investigation and is still a fast growing field (Whitesides, 2006, West *et al.*, 2008).

Liquid chromatography in the early days of LOC was not as popular as electrokinetically driven separation. The high pressure of the process, materials availability and fabrication issues have caused LC to be less favoured in the microfluidic format, even though the first silicon based LOC was actually made for pressure-driven LC(Manz *et al.*, 1990). Better materials (Becker and Locascio, 2002), bonding, fabrication techniques and alternative design of channel(Yang *et al.*, 2005) have emerged to counter not all but many of these physical issues. More recently, pressure-driven fluid transport has been more widely used in LOC, for injection(Fuentes and Woolley, 2007, Xu *et al.*, 2008), mixing and valve control applications (Edwards *et al.*, 2007, Shim *et al.*, 2007). As new technologies and materials for microfabrication and column development are introduced (Eijkel, 2007), shrinking the conventional HPLC system within a chip is now feasible.

In the late 1990s, the role of LC in the microfluidic platform has increasingly important (Harris, 2003). One of the early chromatography devices using a monolithic column and electroosmotic pump was presented in 1998 (He *et al.*, 1998). Peptide on-chip separations were reported by (Yin *et al.*, 2005), using a micro LC C18 column and Jindal *et. al* used electrochromatography to separate three tryptophan-containing

peptides under isocratic elution(Jindal and Cramer, 2004). Another LC chip was fabricated to demonstrate the separation of a mixture of fluorescein isothiocyanate-lable angiotension I and II peptides in a shear-driven C18 column (Vankrunkelsven *et al.*, 2006).

The evolution of both separation techniques by coupling with the miniaturization platform promises the general advantages of LOC techniques (Dittrich *et al.*, 2006) such as

- Reduced instrument size
- Reduced analyte and chemical consumption
- Safe handling of hazardous materials due to reduced volume
- Higher throughput with short separation time
- Cheap mass production of micro analytical instruments

1.2 ELECTROCHEMICAL DETECTION

Figure 1.2 illustrates a general electrode reaction when an electroactive substrate O(∞) from the bulk solution approaches the electrode surface under Fick's law of diffusion and undergoes electron transfer at the electrode surface.

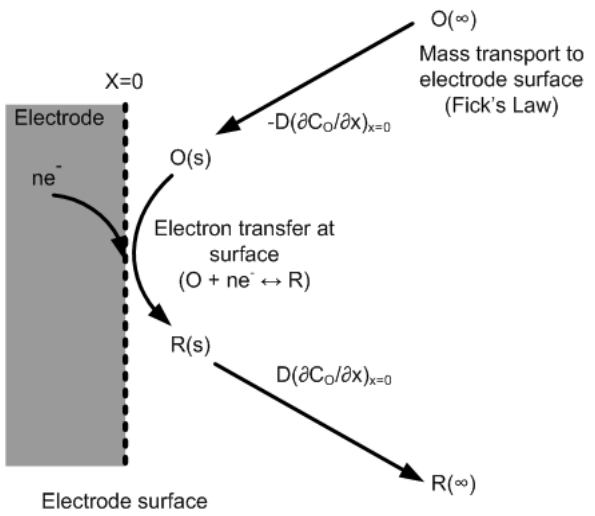


Figure 1.2: Pathway of a general electrode reaction.

Following the mass transport (by only diffusion or convection and diffusion), electrons are transferred at the electrode surface with the overall electrode reaction described as $\mathbf{O} + \mathbf{ne}^- \leftrightarrow \mathbf{R}$. The electron transfer reaction causes the conversion of the oxidised species in the solution to a reduced form, **R**. This electrode reaction rate is controlled by the electron transfer rate on the surface and the mass transfer rate. Similar arguments apply for the reverse process of oxidation.

Mass transfer is the movement of a volume of ions of an element within a solution that evolves from either electrical or chemical potential. The mass transfer by the diffusion is described by Fick's first law, where the diffusive flux, J can be related to current in the external circuit, i

$$J = \frac{i}{nFA} = \frac{j}{nF} \qquad \textbf{(1. 1)}$$

j is the current density (A cm^{-2});

n is the number of electrons transferred;
F is Faraday's constant (96485.3 C mol^{-1});
J is the diffusive flux of the substance through a small area (mol cm^{-2} s^{-1});
A is the area of the electrode (cm^{-2}).

Following Fick's law of diffusion, the Nernst-Planck equation extends Fick's first law to further describe the flux of ions under the influence of the electric potential (V) expressed as

$$J_{\mathrm{O}}(x) = -D_{\mathrm{O}}\frac{\partial C_{\mathrm{O}}(x)}{\partial x} - \frac{z_{\mathrm{O}}F}{\mathrm{RT}}D_{\mathrm{O}}C_{\mathrm{O}}\frac{\partial \varphi_{\mathrm{O}}(x)}{\partial x} + C_{\mathrm{O}}v(x) \qquad \textbf{(1. 2)}$$

$J_O(x)$ is the flux of species *O* (mol cm^{-2} s^{-1}) at distance *x* from the surface;
D_O is the diffusion coefficient (cm^2 s^{-1});
is concentration gradient at distance *x*;
$\partial C_O(x)/\partial x$ is the concentration gradient at distance *x*;
$\partial \varphi_O(x)/\partial x$ is the potential gradient;
Z_O is the charge (dimensionless) of species *O*;
C_O is the concentration (mol cm^{-3}) of species *O*;
v is the velocity (cm s^{-1}) with which a volume element in solution moves along the axis;
R is gas constant (8.3144 J K^{-1} mol^{-1});
and *T* is the absolute temperature (K).

The three terms from this equation represents three modes of mass transfer which are diffusion, migration and convection respectively.

Most electrochemical methods of analysis suppress ion migration of the species of interest by having a high concentration of inert electrolyte such as potassium chloride. In addition, the interpretation of the experiment is usually simplified by either having controlled convection for which exact solution exist (e.g. the rotating disc electrode, cuvette flow or wall jet) or by using an

electrode so small that any natural convection boundary layer is large compared with the Nernst layer, so called microelectrodes or ultramicroelectrodes.

A simple system for electrochemical detection consists of a reference electrode, a working electrode and a counter electrode. However, multi-point electrochemical detection is feasible by adding additional working electrodes connected to current followers.

1.2.1 Electrochemical techniques

There are several electrochemical techniques used for electrochemical detection and the most generally used are cyclic voltammetry and amperometry(Bard and Faulkner, 2001).

Cyclic voltammetry is a technique frequently used for initial electrochemical investigation of new systems. In a cyclic voltammetry experiment, the potential of the working electrode is ramped linearly. The electrochemical reaction is represented by the current. The resulting voltammogram is plotted as current measured between the working electrode and the counter electrode versus the potential applied between the reference electrode and the working electrode.

Amperometry describes a technique where a fixed potential is applied, usually in the diffusion limited regime and where the current generated is recorded *versus* time. The applied fixed-potential is normally decided according to the analytes which are desired to be oxidised or reduced. Current is recorded when electrochemical reaction takes place as the analyte reaches the surface of the electrode. This technique offers the best temporal resolution of electrochemical techniques and is a popular technique to detect rapid changes of chemical concentration within solution.

These cyclic voltammetry and amperometry techniques are used to characterize electrodes and electrochemical detections respectively for the LC devices.

1.3 MICROFLUIDIC DEVICE MICROFABRICATION

The microfluidic device was designed using polyethylene terephthalate (PET) substrates. Gold electrode array was printed on the substrate for detection purposes. This electrode array was aligned underneath a separation column.

The array of gold band electrode was patterned on the substrate using photolithography technique. Figure 1.3 shows the patterned electrode array on a PET substrate.

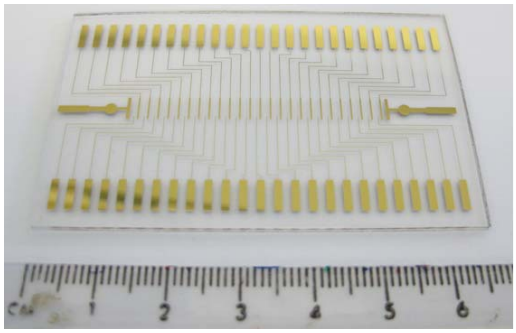


Figure 1.3: Inlaid gold electrode array on PET substrate.

To ensure good adhesion, these patterned electrodes were reversely embedded towards the substrate by hot-embossing a blank mould on the electrodes printed surface. Microfluidic channel was fabricated using thermal casting technique. The dimension of the separation channel is 1 mm × 1mm × 50 mm was cast and semicured in the oven at 60 ˚C for 10 minutes. This prebake TPE channel was demould and a stailess steel frit with 0.2 μm pore size

(Phenomenex®) was inserted at the outlet of the channel. This channel was aligned on the electrode array substrate and was irreversibly thermal bonded in the oven at 76 ˚C for 2 hours. Figure 1.4 illustrates the fabrication protocol of fabricating the microchannel.

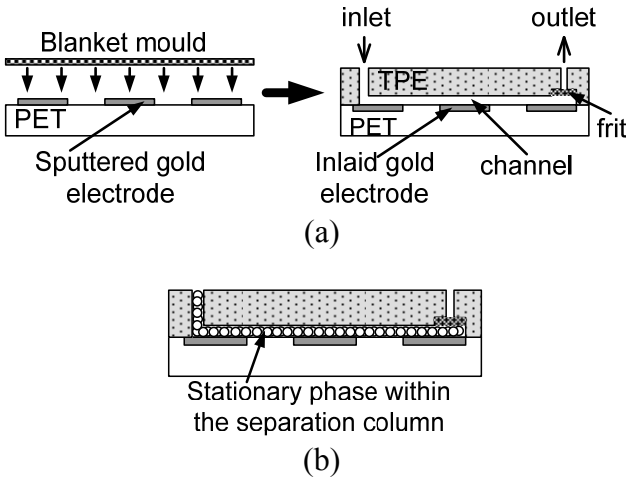
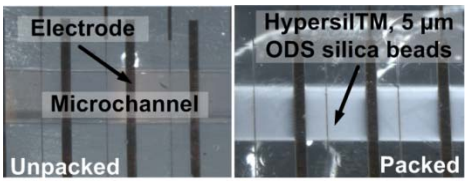


Figure 1.4: Schematic of cross section for the fabrication protocol of the whole device (a) microchannel with electrode array embedded (b) packed C18 silica beads within the microchannel

After the microchannel was fully cured and bonded, the microchannel was packed with 5 µm octadecyl silane (ODS, Hypersil, Phenomenex®) beads to prepare the chromatography column. The slurry solvent was prepared by suspending 2 mg of ODS into 2 ml of methanol. The slurry solvent was sucked from the outlet of the PET column using a vacuum pump. Frit at the outlet retained the beads within the column and caused the silica beads to remain in the column and the solvent was sucked out from the channel. Figure 1.5 shows the comparison of channel before and after packing with the stationary phase.



(a) (b)

Figure 1.5: Photo for (a) the unpacked and (b) packed column. After packing the column, PEEK connectors (Perkin Elmer) were adhered on the inlet and outlet of the device.

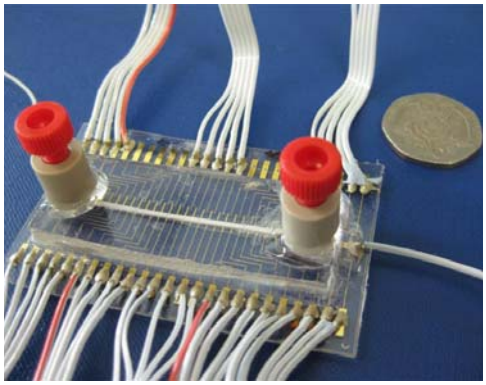


Figure 1.6: Microfluidic chromatography device with embedded electrode array

Wires were connected to the connecting pad of the electrode array using conductive silver paste. For storage, the packed column was filled up with 75:25 (v/v), methanol to water solvent and sealed with finger tight nuts.

1.4 EXPERIMENTAL SETUP

1.4.1 Chemical

Adrenaline (epinephrine, AD), 3-hydroxytyramine (dopamine, DA), 5-hydroxyindole-3-acetic acid (5-HIAA) and 3,4-

dihydroxyphenylacetic acid (DOPAC) were obtained from Sigma and used as received. All other chemicals used were obtained from VWR International, BDH Prolabo and used as received. All standards were prepared in class A volumetric glassware.

1.4.2 Mobile phase

For the separation of the neurotransmitters, a mobile phase similar to Patel et al. was utilized (Patel *et al.*, 2005). The stock citric acid buffer was prepared as follows: 25 mM sodium dihydrogenorthophosphate, 50 mM sodium citrate, 10 mM of diethylamine, 10 mM sodium chloride and 2 mM of decane-sulfonic acid sodium salt were made up to in 1 L with deionised, distilled water and buffered to pH 3.2 using concentrated sodium hydroxide.

The mobile phase consisted of, stock citric acid buffer (pH 3.2) was mixed with UV- grade methanol (HiPerSolv for HPLC, BDH Prolabo) in a ratio of 97.5: 2.5 (v/v) and filtered through a 0.20 µm membrane filter and degassed under vacuum after mixing. Samples were freshly prepared using the citric acid buffer and store in fridge.

1.4.3 Instrumentation and chromatography

The HPLC system consisted of an Agilent HP1050 pump, autosampler and column heater. PET column with geometry 50.0 mm × 1 mm × 1 mm with embedded microband electrodes array was assessed.

The in-column of microband gold electrode array with 1 mm × 250 µm that were connected to a potentiostat CHI 1030 (CHI Instruments, Inc.). The electrode configuration of the connections to the control unit is shown in Figure 1. 7.

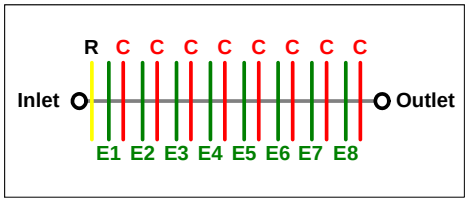


Figure 1. 7: Electrode configuration used connected to potentiostat CHI 1030

Pairing electrodes were used where each working electrode (E) has adjoined to a counter electrode (C). The gap between the working electrode and counter electrode is 1.6 mm and each pair of electrodes is 4.8 mm apart from each other. The potential and current for the in-column detectors were controlled by an 8 channel CHI 1030 and set at + 700 mV vs. gold pseudo reference electrode (R) and gold auxiliary for amperometric technique. The flow rate of mobile phase was at 80 µl min-1 for all experiments and column's temperature was set to 25 ± 0.15 ˚C.

1.5 RESULTS AND DISCUSSION

Eight pairing in-column microband electrodes (working – auxiliary) were used for these measurements. The working electrodes located along the separation column with a constant distance 4.8 mm apart. 1 µl sample of a mixture consists of the 4 analytes (5 µM each), adrenaline, dopamine, 5-HIAA and DOPAC was injected via the autosampler.
Figure 1.8 shows the 8 traces of the separation progress within the column in an isocratic mobile phase with 97.5: 2.5 (v/v) buffer to methanol ratio.

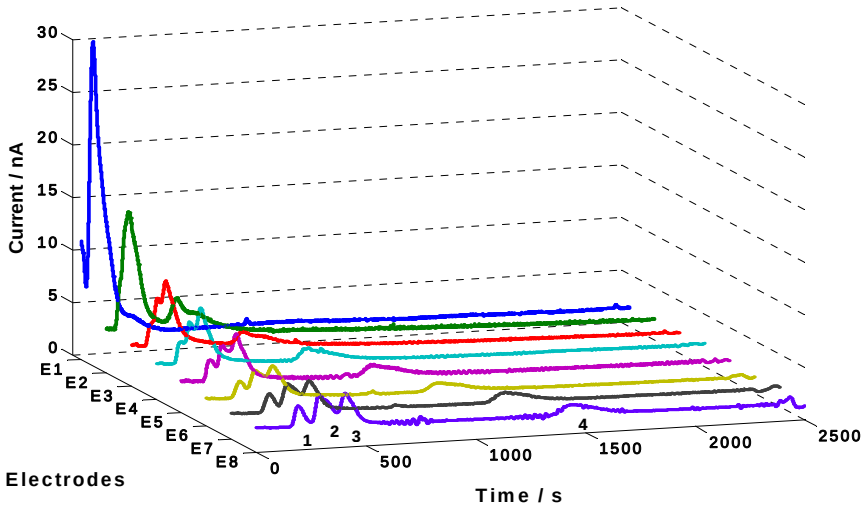


Figure 1.8: Full traces of 8 in-column detectors for the separation of 4 analytes on an isocratic mobile phase in a ratio 97.5:2.5 (v/v) citric buffer: MeOH, analytes eluted in a sequence (1) DOPAC, (2) 5-HIAA, (3) AD and (4) dopamine

From the traces, full separation of these 4 analytes can be obtained less than 1500 s at the electrode E6.
Figure 1.8 too shows that electrode 1 (E1) detected a peak for 1 µl of 1 µM mixture of all the analytes. Throughout the column, 4 peaks were slowly developed and from E6 onward, full separation of the 4 analytes was achieved in a sequence begin with DOPAC, 5HIAA, adrenaline and dopamine. However, when using the conventional end-column detector the separation of analytes can only be detected after 2000 s after the last analyte eluted from the column.

Therefore, the advantages of having multi in-column detections are firstly, experiment and analysis could be completed once 'fit-for-purpose' results are obtained; secondly, analytes can be traced once injected to the column to allow online monitoring of instantaneous separation information. Electrodes can be easily

integrated within a microfluidic channel and are sensitive for detecting analytes in small amount and low concentration.

1.5.1 Future works

Device can be used for detecting biological samples from brain. Smaller size of channel can be fabricated to enhance the separation efficiency and also shorten the separation time. Besides that, monolithic column might be fabricated to replace silica beads for better microcolumn. However, further experiments and studies needed to be done for the compatibility of monolithic column with in-column detection.

1.6 CONCLUSION

In-column electrochemical detection was achieved for the first time in a chromatography column. This in-column detection offers improved detection outcomes compared with conventional post-column and end-column detection methods as it allows simultaneous detection along the entire length of the separation column. This microfluidic device was successfully fabricated using reverse imprinting and high temperature bonding methods. The in-column electrodes show higher current response under the packed column as compared to unpacked column. The separation process of 4 analytes within the column was monitored instantaneously using the embedded microband electrodes and the optimal separation can be obtained at electrode 6 in less than 1500 s.

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