

CHAPTER III

EXPERIMENTAL

3.1 Chemicals and materials

All chemicals and materials used in these experiments are listed in Table 3.1

Table 3.1 List of chemicals and materials including their suppliers

Chemicals/materials and reagents	Suppliers
Methyl paraben (MP), analytical grade	Sigma-Aldrich, USA
Ethyl paraben (EP), analytical grade	Sigma-Aldrich, USA
Propyl paraben (PP), analytical grade	Sigma-Aldrich, USA
butyl paraben (BP), analytical grade	Sigma-Aldrich, USA
Polyaniline (PANI)	Sigma-Aldrich, USA
polyvinylpyrrolidone (PVP)	Sigma-Aldrich, USA
camphor-10-sulfonic acid (CSA)	Sigma-Aldrich, USA
Potassium ferricyanide ($K_3Fe(CN)_6$)	Sigma-Aldrich, USA
Dimethylformamide (DMF)	Merk, Germany
Chloroform ($CHCl_3$)	Merk, Germany
di-Sodium hydrogen phosphate (Na_2HPO_4)	Merk, Germany
Methanol (CH_3OH), HPLC grade	Merk, Germany
Acetonitrile (ACN), HPLC grade	Merk, Germany
Sodium hydroxide (NaOH)	Merk, Germany
ortho-phosphoric acid (85%)	Merk, Germany
Isobutyl paraben (IBP), analytical grade	Tokyo chemical industry, Japan
Graphene (G) nanopowder	SkySpring Nanomaterials, Inc., USA



Chemicals/materials and reagents	Suppliers
Carbon ink	Gwent group, United Kingdom)
Silver/silver chloride ink	Gwent group, United Kingdom)
Potassium dihydrogen phosphate (KH_2PO_4)	CarloErba Reagenti-SDS, France
Potassium chloride (KCl)	Ajax Finechem, Australia
Potassium ferrocyanide ($\text{K}_4\text{Fe}(\text{CN})_6$)	Mallinc krodtt chemical works, USA

3.2 Instruments and equipments

All instruments and equipments are listed in Table 3.2

Table 3.2 List of all instruments and equipments including their suppliers

Instruments and equipments	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)
CHI 1232A	CHI Instrument (USA)
Analytical Column	Phenomenex (USA)
Electrochemical flow cell	Bioanalytical System (USA)
Teflon cell gasket	Bioanalytical System (USA)
Platinum wire	Bioanalytical System (USA)
Stainless-steel tube	Bioanalytical System (USA)
Silver/silver chloride electrode (Ag/AgCl)	Bioanalytical System (Japan)
PEEK tubing (0.25 mm. i.d.)	Upchurch (USA)
Teflon tubing (1/10 inch i.d.)	Upchurch (USA)



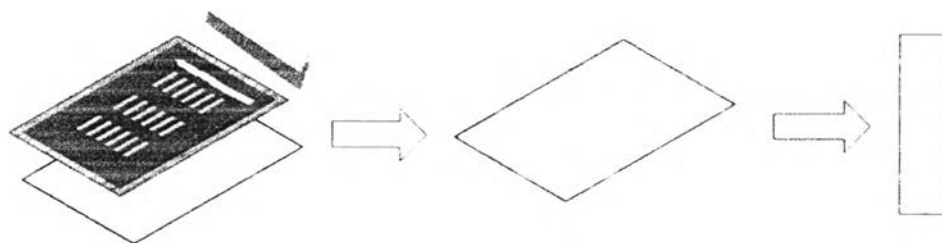
Instruments and equipments	Suppliers
Vacuum pump	GAST (USA)
Analytical balance	Mettler Toledo (Thailand)
pH meter	Mettler Toledo (Thailand)
Mili Q	Merk (Germany)
Autopipette	Eppendorf (Germany)
Nylon membrane filter papers (0.20 μm)	Vertical Chromatography (Thailand)
Nylon membrane filters (0.20 μm)	Vertical Chromatography (Thailand)
Sonicate	Elma (Germany)
Vortex mixer VTX-3000L	Mixer Uzusio LMS (Japan)

3.3 Fabrication of the electrodes

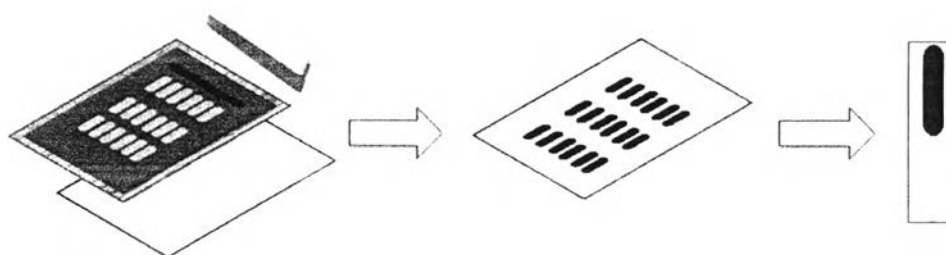
3.3.1 Screen printed-carbon electrode (SPCE)

The screen printed-carbon electrodes (SPCEs) used in this research, were produced in our laboratory by screen-printing technique. First, Ag/AgCl ink was printed onto a PVC substrate as an electrical connector and then dried in an oven at 55 °C for 1 hour. After that, carbon ink was printed as an active area of working electrode and dried in an oven under the same condition. The SPCE was obtained and subsequently for the modification of G/PVP/PANI nanocomposite. The steps of fabrication are schematically illustrated in Figure 3.1.





Step 1: An electrical connector part of Ag/AgCl on the PVC substrate



Step 2: An active area of working electrode

Figure 3.1 The printing of screen-printed carbon electrode

3.3.2 G/PVP/PANI nanocomposite-modified SPCE

3.3.2.1 Preparation of G and PVP dispersion

Twenty milligrams of graphene nanopowder and 20 mg of PVP were dispersed in 10 mL of dimethylformamide (DMF) and sonicated for 6 hours at room temperature as shown in Figure 3.2.



Figure 3.2 A dispersed graphene dispersion

3.3.2.2 Preparation of PANI

PANI was prepared by weighing 100 mg of PANI and adding 129 mg of camphor-10-sulfonic acid (CSA) for doping PANI to make it conductive and then subsequently dissolved in 15 mL of chloroform. The solution was stirred by magnetic bar for 6 hours. Finally, PANI solution was filtered through 0.45 μm Nylon membrane filter as a homogeneous PANI solution (shown in Figure 3.3).



Figure 3.3 A CSA doped PANI solution

3.3.2.3 Modification of SPCE with G/PVP/PANI nanocomposite-solution

The G/PVP/PANI nanocomposite solution was prepared by mixing of the dispersed graphene solution and the CSA doped PANI solution with a ratio of 1:1, v/v. The electro spraying apparatus consists of syringe pump, high-voltage power supply, ground collector, syringe and stainless steel needle. The G/PVP/PANI nanocomposite solution was filled in the syringe and high voltage was applied. Finally, the G/PVP/PANI nanocomposite solution was sprayed onto SPCE. The conditions for electro spraying including flow rate, the distance between the needle and the ground collector, and voltage were 1.0 mL.min⁻¹, 5 cm, and 9 kV, respectively. The fabrication of G/PVP/PANI nanocomposite-modified SPCE by electro spraying technique is illustrated in Figure 3.4.

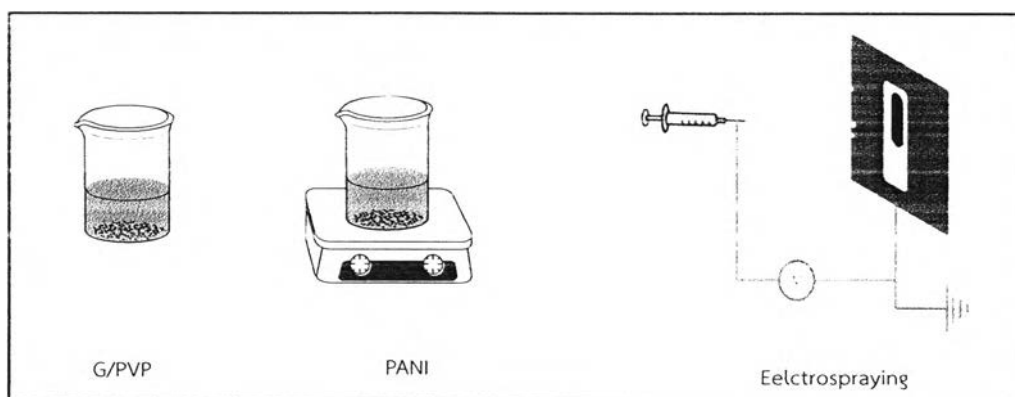


Figure 3.4 The fabrication of G/PVP/PANI nanocomposite-modified SPCE by electro spraying technique

3.4 Preparation of chemical solution

3.4.1 Cyclic voltammetry study

3.4.1.1 Potassium chloride (KCl) solution

The solution of 0.5 M KCl was prepared by weighing 3.73 g of KCl, and dissolving with Mili-Q water to final volume of 100 mL in volumetric flask.

3.4.1.2 Ferri/ferrocyanide solution

Ferri/ferrocyanide solution was used for electrochemical characterization of G/PVP/PANI modified SPCE. Ferri/ferrocyanide (1 mM) was prepared by weighing 20 mg of potassium ferricyanide ($K_3Fe(CN)_6$) and 42.241 mg of potassium ferrocyanide ($K_4Fe(CN)_6$). Finally, ferri/ferrocyanide was dissolved with 0.5 M KCl to final volume of 100 mL in a volumetric flask.

3.4.1.3 Supporting electrolyte

Phosphate buffer solution was used as supporting electrolyte to investigate the electrochemical behavior of five parabens. Phosphate buffer solution was prepared by dissolving 0.60 g of potassium dihydrogen phosphate (KH_2PO_4) and 0.08 g of di-sodium hydrogen phosphate (Na_2HPO_4) in Mili-Q water to final volume of 100 mL in a volumetric flask. The solution was then precisely



adjusted to the desired pH (pH: 6) with ortho-phosphoric acid and sodium hydroxide (NaOH).

3.4.1.4 Stock standard solutions

The stock standard solutions of five parabens ($1000 \mu\text{g ml}^{-1}$), namely methyl paraben (MP), ethyl paraben (EP), propyl paraben (PP), butyl paraben (BP) and isobutyl paraben (IBP) were prepared by dissolving 10 mg of each compound in 10 mL of Mili-Q water: acetonitrile (50:50, %v/v). The standard working solutions were diluted from these stock solutions to the desired concentrations.

3.4.1.5 Standard working solution

The standard working solutions for cyclic voltammetry study were prepared by diluting the stock standard solutions to the desired concentrations into 3 mL of electrochemical cell with phosphate buffer solution, pH 6.

3.4.2 HPLC-ECD

3.4.2.1 Mobile phase

The mobile phase including 0.05 M phosphate buffer solution and acetonitrile were used for HPLC-ECD with the ratio of 60:40 (%v/v).

3.4.2.1.1 The pH of supporting electrolyte

The 0.05 M phosphate buffer with different pH was prepared by weighing potassium dihydrogen phosphate (KH_2PO_4) and di-sodium hydrogen phosphate (Na_2HPO_4) at certain ratios according to Table 3.3. The mixture was transferred to 500 mL volumetric flask and dissolved with Mili-Q to final volume. Before making up the final volume, sodium hydroxide (NaOH) and phosphoric acid were added to adjust the pH. Finally, phosphate buffer solution was filtered through 0.22 μm Nylon membrane filter papers.



Table 3.3 The preparation of pH in a range 4 to 8 of phosphate buffer solution

pH	Potassium dihydrogen phosphate (KH_2PO_4) (g)	di-Sodium hydrogen phosphate (Na_2HPO_4) (g)
4*	-	3.4051
5	3.3750	0.0283
6	3.0246	0.3939
7	1.4051	2.0832
8	0.1258	3.4176

* For pH 4, di-Sodium hydrogen phosphate was dissolved with 6.11 mL of phosphoric acid

3.4.2.2 Stock standard solution

The stock standard solution of mixed parabens was prepared by dissolving 10 mg of each compound in 10 mL of Mili-Q water: acetonitrile (50:50, %v/v), and the solutions was stored at 4 °C during the experiments.

3.4.2.3 Standard working solution

The standard working solution for HPLC-ECD was prepared by diluting the stock standard solution to the desired concentrations with Mili-Q water: acetonitrile (50:50, %v/v). Finally, the standard working solution was filtered through 0.22 μm Nylon membrane filter paper.

3.4.3 Sample preparation

Samples in this work are soft drink and cosmetic products. 1 mL of each sample was extracted by 2.5 mL of methanol. The solution was sonicated for 15 minutes in an ultrasonic bath. Finally, all sample solutions were filled through 0.22 μm Nylon membrane filters.

3.5 Procedure

3.5.1 Cyclic voltammetry study

Cyclic voltammetry (CV) was chosen to investigate the electrochemical characteristic of ferri/ferrocyanide and electrochemical behavior of parabens using G/PVP/PANI modified SPCE. CV was examined by a potentiostat with



three-electrode system; G/PVP/PANI nanocomposite-modified SPCE as working electrode, silver/silver chloride (Ag/AgCl) with a salt bridge as reference electrode, and platinum wire as counter electrode. The electrochemical measurement was obtained in a home-made cell and all experiments were carried out at room temperature. The electrochemical cell is shown in Figure 3.5.

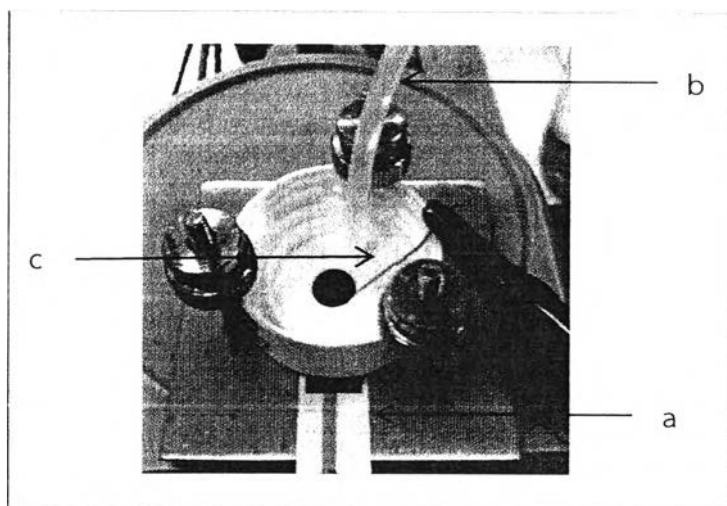


Figure 3.5 Electrochemical cell including; G/PVP/PANI nanocomposite-modified SPCE (a), silver/silver chloride (Ag/AgCl) with a salt bridge (b), and platinum wire (c)

3.5.1.1 Characterization of G/PVP/PANI nanocomposite-modified SPCE

The electrochemical characterization of G/PVP/PANI nanocomposite-modified SPCEs was investigated by ferri/ferrocyanide redox couple. The potential was scanned from -0.6 V to +1.0 V with a scan rate of 100 mV s^{-1} . Moreover, to investigate the mass transfer process of the modified electrode, the potential scan rates of CV in the ferri/ferrocyanide solution were varied, and the currents were measured. The potential scan rates studied were in the range of 20 to 100 mV s^{-1} .

3.5.1.2 An electrochemical behavior of parabens

To study the electrochemical behavior of MP, EP, PP, BP and IBP onto G/PVP/PANI nanocomposite-modified SPCE, all $10 \mu\text{g ml}^{-1}$ parabens were separately examined in 0.05 M phosphate buffer (pH 6) with a scan rate 100 mV s^{-1} . The potential was scanned from +0.4 V to +1.3 V.

3.5.2 HPLC-ECD

The separation of parabens was carried out by HPLC system (Shimadzu LC-20AD XR UFLC Shimadzu, Japan). The chromatographic column was a Luna $5 \mu\text{m}$ C18 column (15 mm \times 4.6 mm i.d.) from Phenomenex (CA, USA). The electrochemical detection part was a thin-layer flow cell (GL Sciences Inc., USA) comprised of three electrodes; a G/PVP/PANI-modified SPCE as working electrode, a Ag/AgCl as reference electrode (Bioanalytical System Inc., USA) and a stainless steel tube as counter electrode. A potentiostat (CHI 1232A, CHI Instrument, USA) was used to measure and control the signal. The thin-layer flow cell is shown in Figure 3.6.

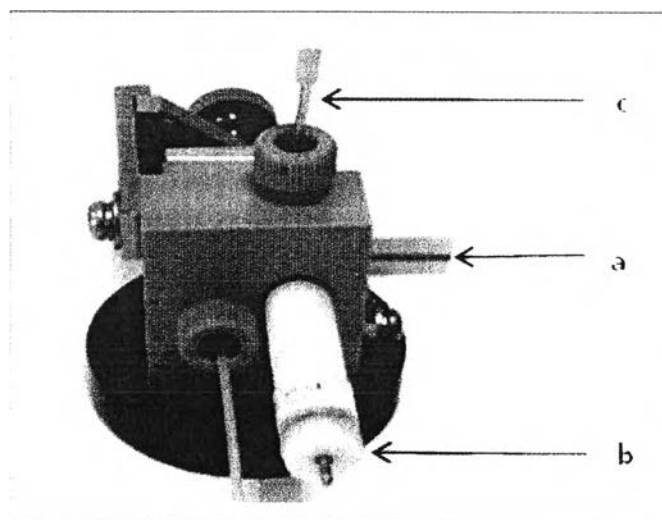


Figure 3.6 Electrochemical flow cell including; G/PVP/PANI nanocomposite-modified SPCE (a), silver/silver chloride (Ag/AgCl) (b) and a stainless steel tube (c)

3.5.2.1 Ratio of mobile phase

MP, EP, PP, BP and IBP were isolated using HPLC with a packed column C18 in an isocratic system for the simultaneous determination of five parabens. The various ratios of mobile phase were applied to optimize the separation of five parabens. The ratios of mobile phase between 0.05 M phosphate buffer solution (pH 6) and acetonitrile were examined by different percentages of acetonitrile at a flow rate of 1.5 mL min^{-1} . The ratios of mobile phase were optimized from 65:35, 60:40, 55:45, 50:50 and 45:55 (%v/v) of 0.05 M phosphate buffer pH 6:acetonitrile).

3.5.2.2 The Optimization of pH

The effect of pH of the phosphate buffer solution to the electrochemical behavior of five parabens was also investigated. The pH values were from 4 to 8. The mixture of five parabens was determined; the mobile phase was 0.05 M phosphate buffer solution and acetonitrile (60:40, %v/v) at a flow rate of 1.5 mL min^{-1} with the amperometric detection at the potential $+1.2 \text{ V vs Ag/AgCl}$.

3.5.2.3 The Optimization of detection potential

The electrochemical behavior of five parabens was investigated with amperometric detection. The mixture of five parabens was examined at the potential in the range of $+1.0$ to $+1.4 \text{ V vs Ag/AgCl}$; the mobile phase was 0.05 M phosphate buffer solution (pH 6) and acetonitrile (60:40, %v/v) at the flow rate of 1.5 mL min^{-1} .

3.5.2.4 The Optimization of injection volume

The injection loop of the instrument was selectable in the range of 0.1 to 50 μL . Therefore, the injection volumes, 10, 20, 30, 40 and 50 μL , were optimized to obtain a suitable volume for separation. The mobile phase was 0.05 M phosphate buffer solution (pH 6) and acetonitrile (60:40, %v/v) at the flow rate of 1.5 mL min^{-1} with the amperometric detection at the potential $+1.2 \text{ V vs Ag/AgCl}$.

3.5.2.5 The optimal conditions for HPLC-ECD

The separation was carried out by HPLC system (Shimadzu LC-20AD XR UFLC Shimadzu, Japan). The chromatographic column was a Luna 5 μm C18 column (15 mm \times 4.6 mm i.d.) from Phenomenex (CA, USA) with the mobile phase of 0.05 M phosphate buffer (pH 6): acetonitrile (40:60, %v/v). A flow rate of 1.5 mL min⁻¹ and an injection volume of 50 μL were employed. An applied potential was +1.2 V vs Ag/AgCl. All the experiments were performed at room temperature (~ 25 °C). The optimal conditions were shown in Table 3.4

Table 3.4 The optimal conditions for HPLC-ECD

Parameter	Optimal condition
Column	Luna 5 μm C18 column (15 mm \times 4.6 mm i.d.)
Mobile phase	0.05 M phosphate buffer pH 6:ACN (60:40, %v/v)
Flow rate	1.5 mL min ⁻¹
Injection volume	50 μL
Temperature	room temperature (~ 25 °C)
Amperometric detection	+1.2 V vs. Ag/AgCl
Working electrode	G/PVP/PANI nanocomposite-modified SPCE

3.5.3 Analytical performance

3.5.3.1 Calibration curve and linear range

The mixtures of five parabens were investigated in various concentrations at G/PVP/PANI nanocomposite-modified SPCE. Under the optimal conditions, the calibration curves and linear ranges were established from the average of peak area current.



3.5.3.2 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The slopes, intercepts and correlation coefficients were determined from calibration curves. The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated from $3S_b/S$ and $10S_b/S$, respectively, where S_b is standard deviation of blank measurement ($n = 10$) and S is slope of calibration curve.

3.5.3.3 Reproducibility and precision study

The stability of the G/PVP/PANI composite-modified SPCE in multiple analyses 30 replicates without the replacement of the electrode, under the optimal conditions was evaluated. The reproducibility of G/PVP/PANI nanocomposite-modified SPCE was investigated from 10 different electrodes. To determine the precision of electrode, intra-day and inter-day repeated measurements were investigated using the same electrode. The precision was estimated from %RSD

$$\%RSD = \frac{\text{standard deviation (SD)}}{\text{mean}} \times 100 \quad (3.1)$$

3.5.4 Application in real samples

3.5.4.1 The simultaneous determination of parabens in real samples

Two real samples, i.e. a soft drink and a cosmetic product (makeup remover) were purchased from the local supermarket. The standard addition method was used to evaluate the quantitation of MP, EP, PP, BP and IBP in real samples.

3.5.4.2 Accuracy

Accuracy was estimated from the %Recovery and also estimated by comparison with UV detection, the traditional detection using a student's t-test with the equation as shown below.



$$\% \text{Recovery} = \left| \frac{(A-B)}{C} \right| \times 100 \quad (3.2)$$

Where A is concentration of analyte found in spiked sample, B is concentration of analyte found in unspiked sample and C is concentration of spiked standard in final solution.

$$t = \frac{x_1 - x_2}{s_{x_1 x_2} \sqrt{\frac{2}{n}}} \quad (3.3)$$

Where

$$s_{x_1 x_2} = \sqrt{\frac{(n_1-1)s_{x_1}^2 + (n_2-1)s_{x_2}^2}{n_1+n_2-2}} \quad (3.4)$$

Where \bar{x} = mean, n = number of participants, 1 = group one, 2 = group two, n_1+n_2-2 is the number of degrees of freedom for either group.

