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Determination of bisphenol A in water bottles by spectrophotometric method

Dissertation submitted in partial fulfillment for the
Degree of Bachelor of Science in Forensic Science

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Nur Syuhada Abd Razak

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BpA	: Bisphenol A
BSA	: Bovine serum albumin
ELISA	: Enzyme-linked immunosorbent assay
ER	: Estrogen receptor
EU	: European Union
HPLC	: High Performance Liquid Chromatography
IBWA	: International Bottled Water Association
LOD	: Limit of detection
NHANES	: National Health and Nutrition Examination Survey
ODS	: Octadecyl
PET	: Polyethylene terepthalate
SML	: Specific migration limit
SPI	: Society of the Plastic Industry
UV-Vis	: Ultraviolet-visible
VP-ODS	: VarioPack-Octadecyl

LIST OF SYMBOLS

% : Percentage

α : Alpha

β : Beta

ε : The molar absorptivity

c : Sample concentration in moles per liter

l : Path length of light through the cell in centimetres

λ : Wavelength

°C : degree Celsius

cm^2 : centimetre squared

mg : miligrams

mL : mililitres

mM : miliMolar

nm : nanometres

oz : ounce

ng/mL : nanograms per mililitres

kJ/mol : kilojoules per moles

ABSTRACT

Bisphenol A (BpA) is a building block for the production of epoxy resins and polycarbonate plastics. These plastics are extensively employed to produce water bottles from which BpA can leach into drink. Therefore this dissertation aims to determine the trace amounts of BpA leached from water bottled samples with respect to different temperatures of water (0°C, 4°C, 27°C, 70°C and 100°C) and after being repeatedly used by using UV-Vis spectrophotometry method. A total of three plastic water bottles were randomly chosen for this purpose. The analysis of BpA was carried out by diazotizing the clenbuterol with sodium nitrite before coupled them with BpA to form an azo-compound in ammonia-ammonium chloride buffer. Then, the solution was subjected to UV Spectra at 396 nm. For the effect of different water temperatures, the trace amounts of BpA can be detected from Bottle 3 at 0°C (0.006 mM), 4°C (0.0041 mM), 100°C (0.0014 mM) and no BpA was detected at 27°C and 70°C. While, Bottle 1 and 2, there is also no BpA was detected at any temperatures of water samples. The reusability test was also conducted for all three types of bottles. Bottle 3 showed no BpA was detected for the first time of use, however about 0.0015 mM of BpA was detected after second times of use and 0.0058 mM of BpA after the third times. For Bottle 1, the trace amounts of BpA was found after had been used for three times (0.0025 mM). This study was successfully conducted to show the potential of BpA to leach from the plastic bottle at different water temperatures and after the bottle being repeatedly used.

ABSTRAK

Bisphenol A (BpA) adalah bahan asas dalam pembuatan resin epoksi dan plastik polikarbonat. Bahan plastik ini telah digunakan secara meluas untuk menghasilkan botol air, yang membolehkan BpA meresap ke dalam minuman. Oleh hal yang demikian, disertasi ini bertujuan untuk menentukan jumlah BpA yang meresap daripada sampel botol air dalam aspek suhu air yang berbeza (0°C, 4°C, 27°C, 70°C dan 100°C) dan selepas berulang kali digunakan dengan menggunakan kaedah UV-Vis spektrofotometri. Sebanyak tiga botol air plastik telah dipilih secara rawak untuk tujuan ini. Analisis BpA telah dijalankan dengan mendiazotiz clenbuterol dengan natrium nitrit sebelum ditambah pula dengan BpA untuk menghasilkan azo-sebatian dalam ammonia-ammonium klorida penampan. Kemudian, larutan tersebut didedahkan kepada spektra UV pada 396 nm. Untuk kesan suhu air yang berbeza, jumlah kesan BpA boleh didapati daripada Botol 3 pada suhu 0°C (0.0006 mM), 4°C (0.0041 mM), 100°C (0.0014 mM) dan tiada BpA yang dikesan pada suhu 27°C dan 70°C. Namun begitu, Botol 1 dan 2 juga tiada BpA yang dikesan pada mana-mana suhu sampel air. Ujian kebolehgunaan juga dijalankan terhadap ketiga-tiga jenis botol. Botol 3 tidak menunjukkan BpA telah dikesan buat kali pertama penggunaannya, bagaimanapun sebanyak 0.0015 mM BpA dikesan selepas digunakan dua kali dan 0.0058 mM BpA selepas kali ketiga. Untuk Botol 1, jumlah kesan BpA didapati setelah digunakan sebanyak tiga kali (0.0025 mM). Kajian ini telah berjaya dijalankan bagi menunjuk potensi BpA untuk meluluh daripada botol plastik dalam suhu air yang berbeza dan selepas botol tersebut digunakan berulang kali.

CHAPTER I

INTRODUCTION

1.1 Bisphenol A (BpA)

A carbon-based synthetic compound, Bisphenol A (BpA) is a colourless crystalline solid having mild phenolic odour with the molecular formula of $C_{15}H_{16}O_2$ (O'Neil, 2006). Figure 1.1 shows the chemical structure of BpA (National Center for Biotechnology Information, 2015).

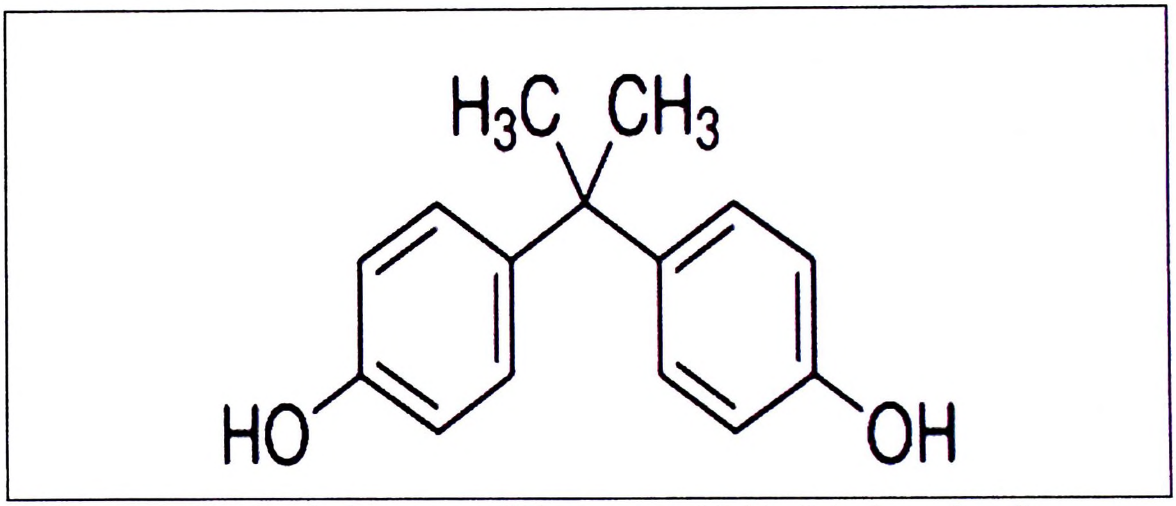


Figure 1.1. The chemical structure of Bisphenol A, 2,2-bis-(4-hydroxyphenyl)propane

BpA falls in diphenylmethane derivatives and bisphenols group. It has poor solubility in water compared to organic solvents (Shareef *et al.*, 2006). It was first synthesized by Dianin in 1891, through condensation of phenol with acetone in the presence of strong acidic ion exchange resin which acts as the catalyst (Rogers, 2014). BpA was further studied by Edward Charles Dodds, a British medical researcher at the University of London in the 1930s during a search for synthetic estrogens (Rubin & Soto, 2009). Several years after Dodds published his research on synthetic estrogens, Dr. Dan Fox of General Electric in the United States and Dr. Hermann Schnell of Bayer in

Germany developed manufacturing processes of epoxy resins and polycarbonate using BpA as the starting material (Vogel, 2009). However, commercial production of BpA only began in 1950s when large-scale uses for polycarbonate plastic and epoxy resins were developed.

The BpA-derived plastic was found to have many useful properties such as high heat resistance, shatter resistance, electrical resistance and high optical clarity in which explain their wide applications (Rykowska & Wasiak, 2006). Epoxy resins are generally used as protective coatings on metal equipment, steel drums, pipings and the interior of food cans (Sato *et al.*, 2005). BpA lining in the inner surfaces of cans, helps to extend the shelf life of canned food and beverage. The optical clarity and their light weight made them useful for eyeglasses (Musson, 2008). It is also found in a variety of other products, including paints and adhesives. BpA polycarbonate plastic is very strong and stable. It can bears exposure to high temperatures, enabling them to withstand heating in microwave ovens, and they can encounter high-impact collisions (Rogers, 2014), making them valuable as components of safety equipment, including motorcycle helmets, face guards, safety glasses and bullet-resistant windows (Musson, 2008).

1.2 Plastic bottles

The plastic bottles made from variety of materials. Plastics products are classified and coded based on their types of material. The Plastics Identification Code is designed to be easy to read and required to appear on 8 oz bottles and greater (Ebottles.com, 2013). The symbol consists of a triangle formed by three "chasing arrows", with a specific number in the center that indicates the material from which the bottle is made. The coding system was launched in 1988 by the Society of the Plastics Industry (SPI) in the United

State and Table 1.1 shows the coding system used (Plastics and Chemical Industries Association, 2011).

Table 1.1. The Plastics Identification Code, types of material and their applications

Codes	Types of material	Applications
1 PETE	Polyethylene Terepthalate	Carbonated soft drink bottles, fruit juice bottles
2 HDPE	High Density Polyethylene	Milk bottles and bleach bottles
3 V	Polyvinyl Chloride	Bottles for salad oil, mineral oil, shampoos and cosmetic products
4 LDPE	Low Density Polyethylene	Squeeze bottles
5 PP	Polypropylene	Kitchen wares and plastic jars
6 PS	Polystyrene	Yoghurt & dairy containers, vending cups, jars for vitamins, petroleum jellies, and spices.
7 OTHER	All other resins	Bottles for insecticides, agricultural chemicals, household cleaners, waxes, paint thinner and gasoline.

Generally, water bottles are made from polyethylene terepthalate (PET), a strong and light material. PET is a thermoplastic polymer, produced from petroleum hydrocarbons, through a reaction between terephthalic acid and ethylene glycol (ThomasNet, 2015). According to Krulik and Waggoner (1981), the first stage in bottle manufacturing is stretch blow molding. The assumption of the shape of a long, thin tube is generated by heating the PET and placed in a mold. This process is known as injection molding. Then, the tube of PET or parison is transferred into a second, bottle-shaped mold. A mandrel (thin steel rod) is slid inside the parison. The parison is now fills with highly pressurized air, and stretch blow molding takes place. A separate component of plastic is simultaneously joined to the bottle during blow molding, to make sure that the bottom of the bottle retains a flat shape. After that, the mold is cooled quickly either through direct or indirect methods. Krulik and Waggoner also added that carbon dioxide or pressurized air can directly cools the mold and plastic. Indirect methods include using water coursing through pipes surrounding the mold. Once the bottle has cooled and set, it is removed from the mold. After removing the bottle from the mold and removing excess plastic, the bottles are ready for transportation. Figure 1.2 illustrates the process to produce plastic bottle (Savillex Corporation, 2013).

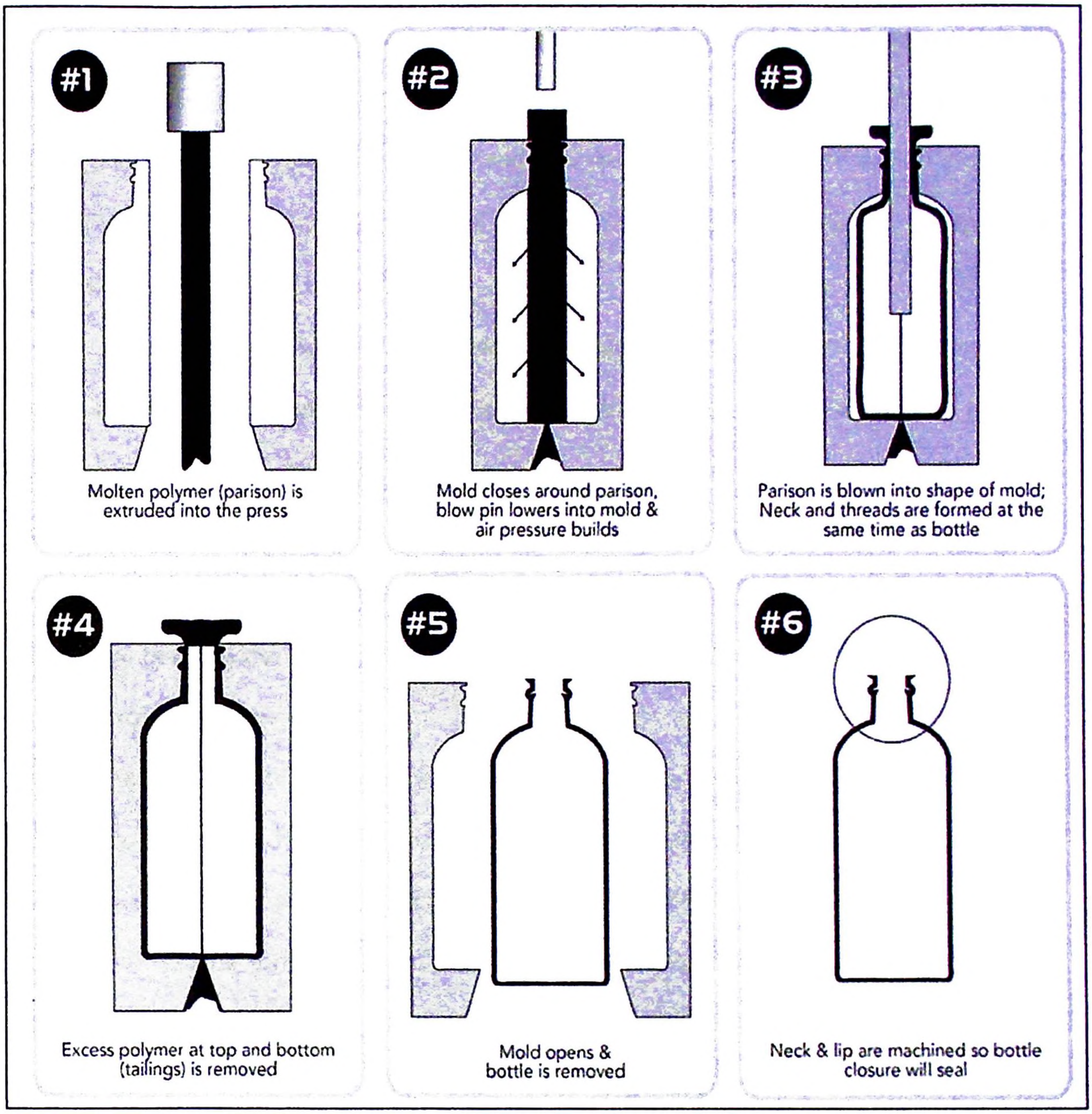


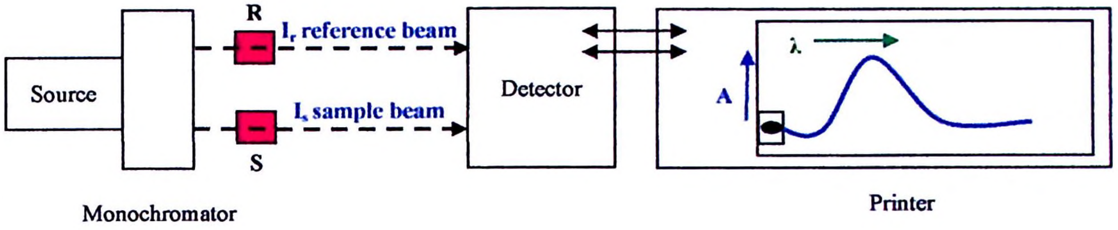
Figure 1.2. The process of plastic bottle manufacturing.

1.3 UV-Vis Spectroscopy

Ultraviolet-Visible (UV-Vis) absorption spectroscopy is used to provide information about the structure and length of the conjugated part of a molecule by detecting the electronic transitions of that conjugated system (Wade, 2013). Ultraviolet (UV) frequencies correspond to shorter wavelengths and having higher energy than infrared. The UV region is a range of frequencies just beyond the visible. Common UV spectrometers function in the range of 200 to 400 nm or about 300 to 600 kJ/mol in photon energies. These spectrometers often extend into the visible region (400 to 800 nm or 150 to 300 kJ/mol of photon energies) and therefore is known as UV-visible spectrometers (Royal Society of Chemistry, 2015).

As stated by Wade (2013), in order to measure the UV spectrum of a compound, the sample is usually dissolved first in a solvent that does not absorb above 200 nm such as water and diethyl ether. The sample solution is place in quartz cell and some of the solvent is placed in reference cell. The spectrometer will compare the amount of light transmitted through the sample with the amount of light in the reference beam. The reference beam passes through the reference cell to compensate for any absorption of light by the solvent and the cell.

In the ultraviolet spectrometer, a monochromator selects one wavelength of light, by using prism or diffraction grating to spread all frequencies of UV light that comes from a source into a spectrum. This single wavelength of light is split into two beams, with one beam passing through the reference cell, and the other passing through the solvent cell. The detector measures the ratio of the two beams, and the printer draws a graph of absorbance of the sample as a function of wavelength. Figure 1.3 shows the schematic of UV-Vis Spectrophotometer (Wade, 2013).



- R : Reference cell with solvent
- S : Sample dissolved in solvent

Figure 1.3. The schematic of UV-Vis spectrometer

The absorbance, A of the sample at a particular wavelength is governed by the Beer's Lambert Law. The law states about the linear relationship between absorbance and concentration of an absorber of electromagnetic radiation (Anderson *et. al.*, 2004), which given as:

$$\mathbf{A = \log (I_r/I_s) = \varepsilon cl}$$

where

A : absorbance

ε : the molar absorptivity

c : sample concentration in moles per liter

l : path length of light through the cell in centimetres

1.4 Problem statement

According to a report from International Bottled Water Association (IBWA), global bottled water consumption estimated to have neared 70.4 billion gallons in 2013, and Asia makes up 26% of the world's bottled water consumption with Malaysia consumed 24 liters per capita in 2013. In addition, the report also stated that plastic bottled water packaging is preferred over glass in almost every country.

BpA is a key monomer in the production of plastics. BpA can leach from the plastic containers due to the breakdown of polymer upon heating (Poorahong, *et al.*, 2011). Thus, people may regularly consume trace amounts of BpA. Several studies have shown that BpA is an endocrine-disrupting chemical, that able to mimic or induce the effects of endogenous hormones in organism.

It was first recognized to have estrogenic activity as a synthetic drug in 1936 and able to exhibit either *in vitro* or *in vivo* (Gaido *et al.*, 1997; Steinmetz *et al.*,1998; Bergeron *et al.*, 1999). BpA can binds to human estrogen receptors and stimulates the transcriptional activity of both estrogen receptor (ER) subtypes, ERα and ERβ (Perez *et al.*, 1998). It also can exhibit xenoestrogenic effects *in vitro* at very low concentrations of 6 ppb or 25 nM (Brotons *et al.*, 1995). In addition, BpA could possibly cause neural and behavioural changes in infants and children, low sperm counts, hyperactivity, testicular cancer, obesity and breast cancer (Li *et al.*, 2010).

In 1993, the United States Environmental Protection Agency sets a safety standard for BpA, but only at very high doses (50 micrograms of BPA per kilogram of body weight per day), despite studies showing that even low doses may be linked to cancer, reproductive problems, and other health issues (The Washington Post, 2008). A cross sectional National Health and Nutrition Examination Survey (NHANES) conducted by the U.S. Centers for Disease Control in 2003 revealed that 92.6% of 2500 participants, age six and above, had detectable BpA in their urine with males had slightly lower levels than females (Calafat *et al.*, 2008).

As stated in European Union (EU) Commission Press Release (2011), Canadian regulators announced a ban on the use of BpA plastics in baby bottles in April 2008, followed by Denmark and France in 2010. France targeted on baby bottles only, while Denmark considered other food contact materials intended for children. The EU took another step in March 2011 by announcing that union had banned the production of baby bottles containing BpA. Malaysia also took the preventive measure by ordering all of plastic food and beverage containers to comply with the Malaysian Standard MS 2234 : 2009 - The Plastic Materials and Articles Intended to Come into Contact with Food , in which the specific migration limit (SML) for BpA is at a level of 0.6 mg/kg, equivalent to EU countries (Ministry of Health Malaysia, 2011).

Concerning about the harmful effects of BpA to humans, therefore a simple, rapid and low cost detection methods to quantify the trace amounts of BpA in water bottle samples is needed.

1.5 Objectives

1.5.1 General objective

i. To determine the trace amounts of leached BpA from water bottle samples

1.5.2 Specific objectives

i. To measure the concentrations of leached BpA from water bottle samples in different temperatures of water
ii. To measure the concentrations of leached BpA from repeatedly-used water bottle samples.

1.6 Significance of the study

This study will be a significant endeavour in providing information whether temperature of water and repeated used water bottles may leach BpA from water bottle samples. As public awareness on BpA has risen, many plastic manufacturing companies have shifted to BpA-free products. So the study is important to determine whether claimed BpA-free products are actually free from BpA or vice versa.

CHAPTER 2

LITERATURE REVIEW

2.1 Analytical techniques to determine the trace amounts of BpA

A growing interest has been recently focused on quantitative determination of BpA, due to its ubiquitous nature and endocrine-disrupting potential. Several analytical techniques have been used to this aim, including UV-visible spectroscopy, electrochemical, fluorimetric and liquid chromatography with various detectors.

Previous study conducted by Sun *et al.* (2000) had focused on detection of BpA leached from hot water in contact with polycarbonate baby bottles using HPLC with peroxyoxalate chemiluminescence. The octadecyl (ODS) column was used with a mixture of imidazole-HNO_3 buffer : acetonitrile (17:83, v/v) as the mobile phase. The detection limit was obtained at 0.38ppb with linear standard curve in the range of 0.57 to 22.8 ppb. From this study, Sun et al. (2000) stated that BpA seem to leach at trace amount, the concentration of BpA was detected at 0.59-0.75 ppb level during the first use of baby bottles. Sun (2000) also stated that there was no significant change observed in the concentration of BpA, after the bottles were washed with a brush to create scratches on the inner surface, so as to facilitate the migration of BpA.

Sun et al. (2003) also conducted another study on determination of BpA in year 2003. However, in this study, Sun aimed to quantify the concentration of BpA in human breast milk samples from 23 healthy lactating Japanese women by using HPLC with column-switching and fluorescence detection. BpA was derivatized with fluorescent labelling reagent, 4-(4,5-diphenyl-1*H*-imidazol-2-yl) benzoyl chloride (DIB-Cl) after being extracted. The separation of DIB-BpA from endogenous materials in milk was carried out using two C_{18} columns, and the detector wavelength which was set at 475nm.

Validation of method was checked from 0.2 - 0.5 ng mL^{-1}, and the correlation coefficient, R^2 was 0.994 and its limit of detection (LOD) was 0.11 ng mL^{-1}. For the result, the concentration of BpA in milk samples were determined to be in the range of 0.28-0.97 ng mL^{-1} with the mean concentration of 0.61± 0.20 ng mL^{-1} and there was no correlation with lipid content of milk samples. Sun also stated in this study, there was unclear relationship between milk sampling before or after meal (lipid content of milk) and BpA concentrations, therefore further investigations are needed.

Other than that, a study aimed to determine the trace amounts of BpA by using enzyme-linked immunosorbent assay (ELISA) method was conducted by Kim *et al.* in 2007. Bovine serum albumin (BSA), a carrier protein and 4,4-Bis(4-hydroxyphenyl) valeric acid (BHPVA), a reagent to make antigen were used in this study. BpA was quantified using a modified competitive ELISA method. This system was based on BHPVA–BSA for polyclonal antibody production against bisphenolic structure, and BHPVA– Horseradish peroxidase (HRP) for determination of BpA substituting detection antibody in competitive reaction. The recovery range was from 96.3% to 107.2%. The detectable concentration of BpA was found to be between 2 and 1000 ng/ml. Kim stated that the modified competitive ELISA method has proven to be a very useful tool for quantification of BpA without the unexpected interaction of BSA and antiBSA polyclonal antibody. However, Hegnerova and his colleagues (2010) stated that ELISA method was laborious and time consuming for the detection of BPA in environmental samples despite its low limits of detection.

Mozaz *et al.* (2004) carried out a study on monitoring BpA in natural waters & drinking water treatment plants by solid-phase extraction- liquid chromatography- mass spectrometry from February until August 2002. The performance of the method was evaluated in which, the method had 81% of BpA recovery, linearity was good with 0.999

and its limit of detection was 6.30 ng/L. From the study, it was found that BpA was detected in all surface water sample analysed. He also stated the content of BpA detected in the groundwater did not change significantly during the period of study. In other words, the pollution of the groundwater is rather persistent but the degradation rate of BpA in that medium is very low.

Another study involving river water samples, conducted by Tanigawa *et al.* (2011) to determine the presence of BpA using automated column-switching HPLC with fluorescence detection. Shim-pack VP-ODS column was used with 10mM phosphate buffer: acetonitrile (65:35) % (v/v) as the eluent. The method was reported successfully in detection of BPA, in which its limit of detection was <0.7ng/L, the linearity was 0.999 with 1.4 % of repeatability and more than 97% of recovery.

Huang *et al.* (2011) studied on electrochemical sensor for BpA detection from plastic products based on molecularly imprinted polymers and gold nanoparticles. In this study, Huang concluded that fabricated MIP-NG sensor offered strong specific binding activity toward BpA, a remarkable stability and quantitatively repeatable analytical performance. The linearity of this method was between 8.0μ- 6.0μmol L^{-1} with a detection limit of 0.14μmol L^{-1} and the average recovery value was 95.6%.

While, Li *et al.* (2011) in his study to determine BpA in food packaging by using a glassy carbon electrode that was modified with carboxylated multi-walled carbon nanotubes. He found that the trace levels of BpA can be detected over a concentration range that is linear from 10nM to 10^4nM, with detection limit of 5.0nM and the correlation coefficient, R^2 of 0.9983. The recovery of this method was excellent in which the recoveries of BpA standard added into the samples were in the range of 98.4%- 102.8%.

Moreover, a UV-Vis spectrophotometry method was used in a study performed by Zhuang *et al.* (2013) to determine the trace amounts of BpA from table-water bottle samples. As BpA is colourless, Zhuang used clenbuterol to diazotize with sodium nitrite, then coupled with BpA to form an azo compound, which showed maximum absorption at 410nm. The determination linear range of BpA was between 0.24 and 8.4µg/mL, correlation coefficient was 0.9905 and the limit of detection was 0.15µg/mL. The recoveries was found between 95.6% and 106.1% indicate that no serious interference was observed in the sample.

There were a few methods to determine trace amounts of BpA as stated above. In this study, UV-Vis spectrophotometry method was chosen. Table 2.1 summarized various analytical techniques of determination of trace amounts of BpA.

Table 2.1. Summary of various analytical techniques of determination of trace amounts of BpA.

Mode of determination	**Author(s) & Year**	**Sample(s)**	**Limit of detection**
HPLC with peroxyoxalate chemiluminescence detection	Sun *et al.* (2000)	Baby bottles	0.38 ppb
HPLC with column-switching and fluorescence detection	Sun *et al.* (2004)	Human breast milk	0.11 ng/mL
	Tanigawa *et al.* (2011)	Environmental water samples	< 0.7 ng/L
Modified competitive ELISA method	Kim *et al.* (2007)	Polyclonal antibody	< 2 ng/mL
Liquid chromatography–mass spectrometry	Mozaz *et al.* (2004)	Natural waters and drinking water treatment plants	15.0 ng/L
Electrochemical sensor - Based on molecularly imprinted polymers and gold nanoparticles	Huang *et al.* (2011)	Plastic product	1.389 x 10^{-7} mol L-1
Electrochemical sensor - By a glassy carbon electrode modified with carboxylated multi-walled carbon nanotubes	Li *et al.* (2011)	Food package	5.0 nM
Spectrophotometric and high performance liquid chromatographic methods for sensitive determination of bisphenol A	Zhuang *et al.* (2013)	Beverage bottles	0.15 µg/mL

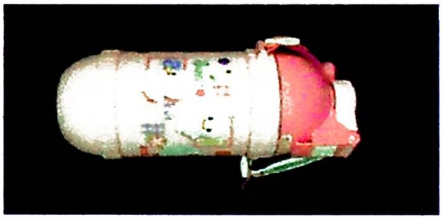
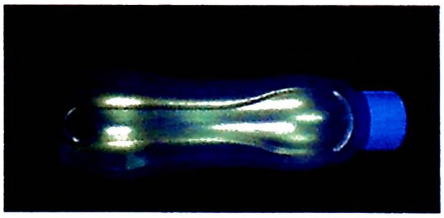
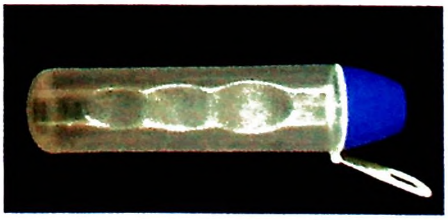
CHAPTER 3

METHODOLOGY

3.1 Sample

There are three types of water bottles that were analyzed for trace amounts of BpA as shown below in Table 3.1. According to the bottles packaging, Bottle 3 is a BpA-free product, while Bottle 1 and 2 were not claimed by the manufacturing companies as BpA-free product. Therefore, Bottle 1 and 2 can be classified as non BpA-Free product.

Table 3.1. List of water bottles

No.	Types of bottle	BpA-Free product
1	Bottle 1 	No
2	Bottle 2 	No
3	Bottle 3 	Yes

3.2 Chemicals and reagents

Table 3.2 shows the reagents used in this study.

Table 3.2. List of chemicals and reagents used in this study

No.	Chemicals	Brands/ States of manufacturer
1	Clenbuterol	Purity Solutions/ Los Angeles, United States
2	Ethanol	Merck KGaA/ Darmstadt, Germany
3	Bisphenol A	Sigma Aldrich/ Missouri, United States
4	Sodium nitrite	Sigma Aldrich/ Missouri, United States
5	Ammonia	Merck KGaA/ Darmstadt, Germany
6	Hydrochloric Acid	Merck KGaA/ Darmstadt, Germany

3.3 Apparatus

Table 3.3 displays the list of apparatus that had been used in this study.

Table 3.3. List of apparatus used in this study

No.	Apparatus	Brands/ States of Manufacturer
1	50 mL Beaker	Schott/ New York, United States
2	Glass pipette	Hirschmann/ Neckartenzlingen, Germany
3	1000 µL Micropipette	Chemopharm/ Petaling Jaya, Malaysia
4	5 mL Volumetric flask	Pyrex/ Massachusetts, United States
5	50 mL Volumetric flask	Pyrex/ Massachusetts, United States
6	Amber glass	-
7	Quartz cuvette	IT Tech Research/ Subang Jaya, Malaysia

3.4 Instruments

The instrument that had been used were listed in the Table 3.4.

Table 3.4. List of instruments used in this study

No.	Instruments	Brands/ States of Manufacturer
1	Cary 100 Spectrophotometer	Varian/ California, United States
2	Hot plate EMS HP-7000	ERLA/ Seri Kembangan, Malaysia
3	Electronic balance	Shimadzu/ Kyoto, Japan

3.5 Preparation of standard stock solution

The standard stock solution was prepared by dissolving 57.5 mg of Bisphenol A in 0.5 mL of ethanol in 50 mL volumetric flask. The solution was diluted to a final concentration of 5 mM with distilled water.

3.6 Preparation of working standard solution

Working standard solutions were prepared by further diluting the appropriate amount of stock solution in distilled water to yield solutions with final concentration of 4 mM, 3 mM, 2 mM and 1 mM respectively. Table 3.5 demonstrates the serial dilution process of BpA stock solution.

Table 3.5. The preparation of BpA working solutions

M_1, Initial concentration of BpA (mM)	M_2, Final concentration of BpA (mM)	V_2, Final volume of BpA solution (mL)	V_1, Initial volume of BpA solution (mL)
5	4	50.0	40.0
4	3	50.0	37.5
3	2	50.0	33.3
2	1	50.0	25.0

3.7 Samples preparation

3.7.1 Temperature test

The sample solution was prepared by washing the water bottles with distilled water and left to dry at room temperature, 27°C. The bottles were cut into pieces of 3.0 cm^2, weighed and placed in 5 different amber glasses labelled A, B, C, D, and E. 10.0 mL of distilled water having different temperatures were added into each of the glasses. Table 3.6 shows the temperature of distilled water between different amber glasses.

Table 3.6. Amber glasses and their respective water temperatures

Amber glass	Temperature of distilled water
A	0°C
B	4°C
C	27°C
D	70°C
E	100°C

The solutions were left for 1 hour in that particular temperature. The amber glass A was put in the freezer, while amber glass B was in the fridge area. For amber glass C, it was left at the room temperature, and amber glass D and E were put on the hot plates. The temperatures of all solutions had been monitored throughout that period. After 1 hour, the amber glass A and B were taken out and left to stand at room temperature. While amber glass D and E were then cooled to room temperature.

In a different, dry and clean 5 mL volumetric flask, 0.8 mL of 0.10 mg/mL of clenbuterol solution was added. 0.2 mL of 0.10 mol/L of hydrochloric acid solution and 0.6 mL of 0.20 mg/mL sodium nitrite were transferred into the flask. The mixture was shaken and placed in ice bath (0-5°C) for 10 minutes. After that, 0.35 mL of the unknown solution and 0.25 mL of ethanol were added to the mixture and shaken, followed by 0.7 mL of 8.8 mg/mL of ammonia solution. The solution was then diluted to the mark with distilled water and mixed well.

As BpA is a colourless compound, clenbuterol was first diazotized with sodium nitrite in acidic solution to form diazonium salt. In this system, sodium nitrite is a diazotizing reagent. Diazonium salts are important synthetic intermediates that can undergo coupling reactions to form azo dyes which will absorb longer wavelengths of light than the reactants because of increased conjugation. The mixture solution was then coupled with BpA to form an azo-compound in ammonia-ammonium chloride buffer which shows maximum absorption at 396 nm. The blank reagent was prepared by adding all of the chemicals without any BpA solution. The UV spectra of unknown solution and the blank reagent were recorded using the Cary 100 UV-Vis Spectrophotometer at 396nm. Figure 3.1 shows the spectrophotometer that was used in this study.

Figure 3.1. The Cary 100 UV-Vis Spectrophotometer used in this study

3.7.2 Reusability test

The sample solution was prepared by washing the water bottles with distilled water and left to dry at room temperature, 27°C. The bottles were cut into pieces of 3.0 cm^2, weighed and placed in an amber glass labelled X. 10.0 mL of distilled water having 27°C in temperature was added into the glass. The solution were left to stand for 1 hour. After that, the solution was transferred into another new and clean amber glass and proceed for the preparation of detection by UV Spectra at 396 nm. The whole steps were repeated for two times.

3.8 Calibration curve

The calibration curve was constructed by measuring the absorbances of different known concentrations of BpA standard solutions by using spectrometer. The values of absorbance were recorded and a graph between absorbance and concentration of BpA standards was generated. Linearity was confirmed by using linear regression analysis and the coefficient of determination was then obtained. Table 3.7 summarized the preparation of BpA standard solutions that had been used to plot the calibration curve.

Table 3.7. The preparation of known concentration of BpA solutions

Initial [BpA] (mM)	**Volume of 0.1 mg/mL clenbuterol solution (mL)**	**Volume of 0.10 mol/L HCl (mL)**	**Volume of 0.20 mg/mL sodium nitrate (mL)**	**Volume of BpA solution (mL)**	**Volume of ethanol (mL)**	**Volume of 8.8 mg/mL ammonia (mL)**	**Total volume (mL)**	**Final [BpA] (mM)**
5	1.6	0.4	1.2	0.7	0.5	1.4	10	0.35
4	1.6	0.4	1.2	0.7	0.5	1.4	10	0.28
3	1.6	0.4	1.2	0.7	0.5	1.4	10	0.21
2	1.6	0.4	1.2	0.7	0.5	1.4	10	0.14
1	1.6	0.4	1.2	0.7	0.5	1.4	10	0.07