DEVELOPMENT OF A SIMPLE, SELECTIVE AND SENSITIVE FLUORIMETRIC METHOD FOR THE DETERMINATION OF BISPHENOL A IN WATER FROM DIFFERENT SOURCES

By

CHONG YEW WENG

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ABSTRACT

DEVELOPMENT OF A SIMPLE, SELECTIVE AND SENSITIVE FLUORIMETRIC METHOD FOR THE DETERMINATION OF BISPHENOL A IN WATER FROM DIFFERENT SOURCES

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It is suggested that in hydrochloric acid medium, there is a competition between BPA and Rh6G for the reduction of hydroxyl radicals. Fluorescence quenching of Rh6G is therefore inhibited. Combined with fluorescence spectroscopy which offers excellent detection limits in the determination of trace amounts of organic compounds, the conventional Fenton-like reagent was applied to the determination of BPA in real samples. The fluorescence spectrometer was operated at excitation wavelength 345 nm and emission wavelength at 547 nm was used for detection. An optimized reaction condition was obtained by investigating the optimum concentration of reagent concentrations and reaction condition. The optimized concentration of HCl, Fe(III), H_2O_2 and Rh6G was 9.38×10^{-4} M, 0.1953 μg/mL**,** 4.59 mM and 23.49×10^{-7} M, respectively. The optimized reaction time was 40 minutes and reaction temperature was 60 ºC. A quenching step was included in the development of methodology in this project by the addition of pentetic acid at the end of the reaction, which greatly improves the measurement precision. The calibration curve was rectilinear using the method of Least-Squares and

the linear regression formula produced is 4.3912x + 3.8842 with a coefficient of determination, R^2 value of 0.9966. The method presents an instrumental LOD and LOQ of 4.2 and 13.9 ug/L, respectively. Solid phase extraction (SPE) was developed with a two step washing step to remove as much impurities as possible employed using 20 % methanol: 80 % water solution and 5 % ethyl acetate: 95 % *n*-hexane solution. Then, a 20 % ethyl acetate: 80 % *n*-hexane solution was used for elution. The SPE procedure shows high recovery of 89 $\pm$ 5.5 %. The developed method was successfully applied to BPA residue in PC bottles and environmental waters but failed for canned foods. Then, the measured results were cross-validated with the results using HPLC/FLD and identification of BPA presence using GC/MS.

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APPROVAL SHEET

This dissertation/thesis entitled "**DEVELOPMENT OF A SIMPLE, SELECTIVE AND SENSITIVE FLUORIMETRIC METHOD FOR THE DETERMINATION OF BISPHENOL A IN WATER FROM DIFFERENT SOURCES"** was prepared by CHONG YEW WENG and submitted as partial fulfillment of the requirements for the degree of Master of Science (Hons.) in Chemistry at Universiti Tunku Abdul Rahman.

Approved by:

_____

(Assc. Prof. Dr. Hnin Pwint Aung)

Date:……..

Supervisor

Department of Chemical Science

Faculty of Science

Universiti Tunku Abdul Rahman

_____

(Asst. Prof. Dr. Leong Loong Kong)

Date:……..

Co-supervisor

Department of Chemical Engineering

Faculty of Engineering and Science

Universiti Tunku Abdul Rahma

FACULTY OF SCIENCE

UNIVERSITI TUNKU ABDUL RAHMAN

Date: _____

SUBMISSION OF THESIS

It is hereby certified that CHONG YEW WENG (ID No: 09UEM09088) has completed this final year thesis entitled "**DEVELOPMENT OF A SIMPLE, SELECTIVE AND SENSITIVE FLUORIMETRIC METHOD FOR THE DETERMINATION OF BISPHENOL A IN WATER FROM DIFFERENT SOURCES** " under the supervision of Dr. HNIN PWINT AUNG (Supervisor) from the Department of Chemical Science, Faculty of Science, and Dr. Leong Loong Kong (Co-Supervisor) from the Department of Chemical Engineering, Faculty of Chemical Engineering.

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DECLARATION

I hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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LIST OF ABBREVIATIONS

BPA	**Bisphenol A**
Rh6G	**Rhodamine 6G**
em	**emission**
ex	**excitation**
HPLC	**High Performance Liquid Chromatography**
HPLC/FLD	**High Performance Liquid Chromatography with Fluorescence Detection**
GC-MS	**Gas Chromatography-Mass Spectroscopy**
FLD	**Fluorescence Detection**
PL	**Photoluminescence**
LOD	**Limit of detection**
LOQ	**Limit of quantitation**
Lu*s	**Luminescence unit multiplied by time (seconds)**
AU	**Arbitrary unit**
ppm	**Part per million**
ppb	**Part per billion**
RP-HPLC/FLD	**Reverse Phase High Performance Liquid Chromatography/Fluorescence detection**
SPE	**Solid Phase Extraction**
TDI	**Tolerable Daily Intake**
µg/L	**Microgram per liter**
mL/min	**milliliter/minute**

CHAPTER 1

INTRODUCTION

Bisphenol A (BPA) is now deeply imbedded in the products of modern consumer society, not just as the building block for polycarbonate plastic but also in the manufacture of epoxy resins and other plastics, including polysulfone, alkylphenolic, polyalylate, polyester-styrene, and certain polyester resins. Extensive research on BPA has been conducted in the last 50 years (Nagel *et al*., 1997; Newbold *et al.,* 2009; Oehlmann *et al.,* 2009; Palanza *et al*., 2002). The pharmacological test results from major studies indicates that consumer exposure to BPA at concentrations normally experienced in daily living does not pose a risk to human health (USFDA, 2008; European Commission, 2010; EFSA, 2008b). On the other hand, minor toxicological studies indicate potential risks to human health (Petre *et al*., 2002; Vom Saal & Welhsons, 2006; Zoeller *et al*., 2005; Akingbemi *et al*., 2004).

1.1 Bisphenol A

Bisphenol A or 4,4'-dihydroxy-2,2-diphenylpropane, commonly abbreviated as BPA, is an organic compound with two phenol functional groups. It is a difunctional building block of several important polymers and polymer additives. BPA is one of the highest volume chemicals in the world

(Burridge, 2003). The molecular structure of bisphenol A is shown in **Figure 1.1**.

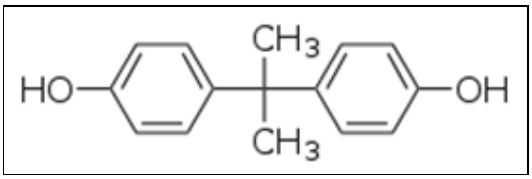

Figure 1.1: Molecular structure of bisphenol A

Bisphenol A is used primarily to make plastics, and products containing bisphenol A-based plastics have been in commerce for more than 50 years. It is a key monomer in production of epoxy resins (Ubelacker, 2008) and in the most common form of polycarbonate plastic (Alliance Polymers, Inc., 2009). Polycarbonate plastic, which is clear and nearly shatter-proof, is used to make a variety of common products including baby and water bottles, sports equipment, medical and dental devices, dental fillings and sealants, eyeglass lenses, CDs and DVDs, and household electronics. Epoxy resins containing bisphenol A are used as coatings on the inside of almost all food and beverage cans (Erickson & Britt, 2008).

1.2 Bisphenol A Based Polycarbonate

Polycarbonate is mainly a condensed polymer of bisphenol A and carbonyl chloride or diphenyl carbonate. Polycarbonates are an unusual and extremely useful class of high heat polymers known for their toughness and clarity. Since it is transparent, has excellent heat resistance and impact resistance; polycarbonate plastic is suitable to be used in both beverage and

food containers. There are two polycarbonate manufacturing methods. They are Solvent Method (Interfacial Polycondensation) and Melt Method (Ester Interchange Method). In the solvent method, polycarbonate was produced by the reaction of BPA with carbonyl dichloride and in the melting method, polycarbonate was produced by reaction of BPA and diphenyl carbonate (Polycarbonate Resin Manufacturing Group, 2007).

In the polycarbonate resin production process, there is a stage to remove non-reacted BPA and other non-reacted substances, but they cannot be removed completely. It should be the reason that trace amounts of BPA remain in polycarbonate products. During the manufacturing process of polycarbonate products, free BPA residues might be present in the product due to improper manufacturing condition standards or problems during the manufacturing process (Polycarbonate Resin Manufacturing Group, 2007).

1.3 Bisphenol A Based Polymeric Coatings

Food and beverage cans often have an internal polymeric coating to protect the food and prevent undesirable interactions between the metal from the can and the food. The polymeric coatings are usually highly cross-linked thermoset resins that can withstand typical processing conditions (1.5 hour at 121°C). BPA is a starting substance used in the manufacture of most types of epoxy resins, which are then cross-linked and used to coat food cans. However, if bisphenol A diglycidyl ether (BADGE) was used as an additive to

scavenge hydrogen chloride in these coatings, residues of BPA, as unreacted starting material in the BADGE, may be present (Goodson *et al.*, 2002).

1.4 Human Exposure and Detrimental Effects of Bisphenol A

The widespread exposure of BPA to humans is mainly due to its use in the production of a large variety of consumer products. Children, infants and unborn babies face the greatest risk from exposure to this endocrine disruptor as they do not have the biological resistance of an adult human. As polycarbonate is commonly used in making drinking equipments such as baby bottles, water bottles and water containers while bisphenol A derivative based epoxy resin is used in can line coating and dental sealants, it has been shown that BPA leaching can occur when they are being treated with high temperature or extreme pH (Le *et al*., 2008; Brede *et al*., 2003 Munguia-Lopez *et al*., 2007).

The first evidence of the estrogenicity of bisphenol A came from experiments in the 1930s in which it was fed to ovariectomized rats (Dodds & Lawson, 1936; Dodds & Lawson, 1938). The affinity of BPA for estrogen receptor is about 10,000 to 100,000 fold weaker then that of estradiol, so it has been considered a very weak environmental estrogen. However, a large number of recent *in vitro* studies have shown that the effects of BPA are mediated by both genomic and non-genomic mechanisms, with the disruption of cell functions occurring at doses as low as 1pM (Vom Saal, 2006). Recent studies also indicate the potential of BPA at part-per-trillion levels to cause

disruption of thyroid functions (Zoeller *et al*., 2005), proliferation of prostate cancer cells (Wetherill *et al*., 2002) and blocking testosterone synthesis (Akingbemi *et al*., 2004).

In its risk assessment on BPA, the European Food Safety Authority (EFSA) set a Tolerable Daily Intake (TDI) of 0.05 milligram/kg body weight for BPA. The TDI is an estimate of the amount of a substance, expressed on a body weight basis that can be ingested daily over a lifetime without appreciable risk (EFSA, 2008a). In 2008, EFSA reaffirmed this TDI, concluding that age-dependent toxicokinetics differences of BPA in animals and humans would have no implication for the default uncertainty factor (UF) of 100 and in turn for the TDI (EFSA, 2010).

The specific migration limits (SML) specify the amount of a chemical that is permitted to migrate into foodstuffs. The European Commission Scientific Committee on Food (EC SCF) has established a specific migration limit (SML) in food of 0.6 mg/ per kg of food in the Commission Directive 2004/19/EC (The Commission of the European Communities, 2004). In August 2004, the European Normalization Institute (CEN) published a testing standard containing a requirement for a migration limit of BPA of 0.03 μg/mL from thermoplastic drinking equipment (European Committee for Standardization, 2004). Debate continues on what is the safety limit of this compound.

1.5 BPA Exposure to the Environment

BPA can contaminate the environment either directly or through degradation of products containing BPA, such as ocean-borne plastic trash (Barry, 2009). As an environmental contaminant, this compound interferes with nitrogen fixation at the roots of leguminous plants associated with the bacterial symbiont *Sinorhizobium meliloti*. Despite a half-life in the soil of only 1–10 days, its ubiquity makes it an important pollutant (Fox *et al.*, 2007). Studies also indicate that it can currently be found in municipal wastewater (Barry, 2009).

A 2009 review of the biological impacts of plasticizers on wildlife published by the Royal Society with a focus on annelids (both aquatic and terrestrial), molluscs, crustaceans, insects, fish and amphibians concluded that BPA have been shown to affect reproduction in all studied animal groups, to impair development in crustaceans and amphibians and to induce genetic aberrations (Oehlmann *et al.*, 2009).

1.6 Problem Statement

At present, separation, identification and quantification are carried out using expensive chromatographic methods such as LC-MS, GC-MS, LC coupled to fluorescence or electrochemical detection. The high costs, time consuming and need for trained technicians have made it difficult for

popularization especially in the small/medium industrial sector that wants to run indoor screenings for BPA.

1.7 Objectives of the Research

The main aim of this work is to determine the amount of leaching of bisphenol A in water from polycarbonate drinking containers, canned foods and also from different water sources. For this aim, the following objectives are deduced;

- To develop the simple, selective and sensitive method for the determination of BPA.
- To develop the method of sample extraction from different matrices using SPE.
- To validate the developed method by determining the linearity, range, limit of detection (LOD), limit of quantitation (LOQ).
- To cross-validate the outcome achieved using the developed spectrofluorimetric method and SPE by RP-HPLC/FLD.

CHAPTER 2

LITERATURE REVIEW

In recent years, researchers from government agencies, academies and worldwide industries have studied the remains of BPA in the environment and the potential for low levels of BPA to migrate from polycarbonate products into foods. These studies consistently show that the detection level of BPA is extremely low. Because of the carcinogenic, aberrant and mutagenic harm to human and nature, special attention has been paid to the determination of BPA. Therefore, due to the high volume, wide spread use of BPA, further development of method should be focused in terms of simplicity, cost, sensitivity and selectivity.

2.1 Review on Methodology Used In Determination of Migrated Bisphenol A

The polarity and the low concentrations of bisphenol A that are normally encountered causes significant problem in devising appropriate analytical methods. The literatures on the analysis of bisphenol A and its derivatives reveal a wide array of techniques used depending on the type of sample. In **Figure 2.1**, the chart sums up the techniques used in determination of BPA.

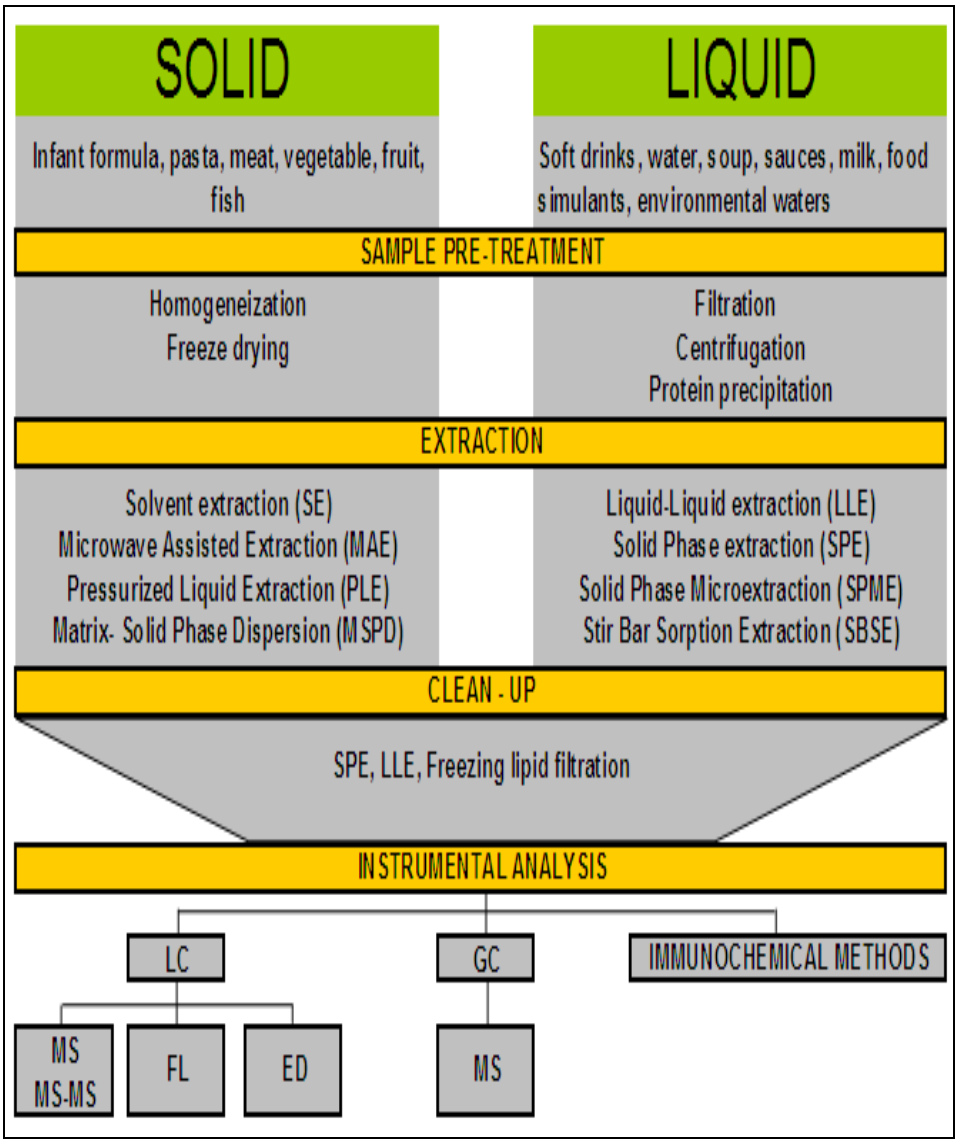

Figure 2.1: Analytical methodologies for determination of BPA

(Ballesteros-Gomez *et al.*, 2008)

2.2 Sample Pre-treatment

There are a wide variety of BPA sources including fresh food, canned solid/liquid samples and environmental waters. Special treatments can be required depending on the matrix composition; for example carbonated drinks are degassed, protein in food requires removal through precipitation and meat tissues are crushed and freeze-dried before homogenization. Canned food containing liquid and solid portions are usually filtered and treated separately (Ballesteros-Gomez *et al.*, 2008).

2.3 Extraction

Solvent extraction and solid phase extraction (SPE) are the most widely used techniques for isolation of BPA from solid and liquid samples respectively. Other techniques although scarcely used so far, may improve extraction of BPA in terms of sample size, automation and solvent consumption (Ballesteros-Gomez *et al.,* 2008). Both solvent-based extraction (e.g. SE, LLE, MAE, PLE and MSPD) and solid phase extraction will be discussed in this Section.

2.3.1 Solvent Extraction (SE) and Liquid-liquid Extraction (LLE)

Solvent extraction (SE) is still the most common technique for isolation of BPA from solid foodstuff, being the most effective way to extract BPA trapped inside the solid matrix of food using acetonitrile (Goodson *et al.,* 2002). Other solvents like acetone, methanol and ethanol may also be used efficiently

(Ballesteros-Gomez *et al.,* 2008). Goodson *et al.* (2002) proposed a method for extraction of BPA and isomers of bisphenol F from a variety of canned products, including fish, fruit, vegetables, beverages, soup, dessert, infant formula, meat and pasta, which were subsequently applied by Thomson *et al.* (2005) to a wide array of foodstuffs.

Liquid-liquid extraction is a method to separate compounds based on their relative solubilities in two different immiscible liquids, usually water and an organic solvent. Liquid foods are extracted with ethyl acetate, chloroform or dichloromethane (Ballesteros-Gomez *et al.,* 2008). Sodium sulphate anhydrous is often added to remove trace amounts of water in the organic layer. Because of the limited selectivity of LLE, samples with complex matrices often require extensive clean-up. LLE can also be used in removing lipids that may affect the stationary phase of LC columns and also accumulating in GC/MS injection port, column and ion source. Fat removal is mainly done by liquid-liquid extraction with n-heptane, trimethylpentane and n-hexane (Goodson *et al.,* 2002).

2.3.2 Microwave Assisted Extraction (MAE)

Microwave extraction (MAE) has been used for many years to extract compounds from plastics, biological samples, foods, animal feeds, paper, wastewater and many other types of samples. MAE is based on the application of microwave energy to the sample during extraction, which is subsequently agitated and heated quickly. Compared to Soxhlet extraction and sonication, it requires

lesser solvents and also is faster and cheaper compared to pressurized solvent extraction (Tatke & Jaiswal, 2011)

2.3.3 Pressurized Liquid Extraction (PLE)

Pressurized liquid extraction (PLE) involves the use of liquid solvents at elevated pressures (1000-2500psi) and temperature (40-200 °C). Under these conditions, solvents have enhanced solvation power and increased extraction rates. The use of PLE for extraction of BPA is rare, but its suitability for extraction from animal or vegetable origin has been proved. The solvents used have been dichloromethane for meat (pork, meat, rabbit, duck and chicken) (Shao *et al.*, 2007a) and acetone- n-hexane (1:1, v/v) for fish liver (Tavazzi *et al.*, 2002).

2.3.4 Solid Phase Extraction (SPE)

SPE is by far the most used technique for both the extraction of BPA from liquid foods and clean up of crude extracts after solvent extraction. The types of SPE are typically categorized by the type of stationary phase sorbents used. The selection of the appropriate type of sorbent is usually determined by the sample solution matrix (Ballesteros-Gomez *et al.*, 2008).

2.3.4.1 Non-Selective Type of Solid Phase Extraction

There are non-selective type of stationary phase sorbents which are able to separate the analyte from the matrix based on the polarity. The following are

some of the types of non-selective sorbents that has been used in extraction of BPA;

- **Reversed-phase silica (C_{18})** SPE sorbent features a highly retentive alkyl-bonded phase for nonpolar to moderately polar compounds. It has been proposed for the isolation of BPA from mineral water and wines (Lambert & Larroque, 1997) and powdered milk (Maragou *et al.*, 2006).
- **Divinylbenzene/N-vinylpyrrolidone copolymer** The hydrophilic N-vinylpyrrolidone polymer affords good wetability of the sorbent and acts as a hydrogen acceptor, while the hydrophobic divinylbenzene polymer provides reversed-phase retention of BPA. OASIS HLB cartridges from Waters has been applied to the isolation of BPA from the leachate originated from empty pet food cans with distilled water (Kang & Kondo, 2002) and drinking water and soda beverages (Shao *et al.*, 2005).
- **Multi-mode phases** (Isolute multi-mode catridges) have also been proposed for isolation of BPA from instant coffee (Kang & Kondo, 2002). Isolute multi-mode catridges combine cationic, anionic and non-polar functionalities. Recoveries in coffee ranged from 85 to 89% (Ballesteros-Gomez *et al.*, 2008).

2.3.4.2 Selective Type of Solid Phase Extraction

A variety of highly selective SPE sorbents have been developed that are suitable for determination of BPA from complex samples, thus performing extraction and clean-up in one step. The following are some of the documented selective sorbents that have been used;

- **Restricted access materials (RAMs).** RAMs combine size exclusion of protein and other macromolecules with the simultaneous enrichment of low molecular mass analytes at the inner pore, which are retained by conventional mechanisms (hydrophobic, ionic or affinity interactions) (Souverain *et al.,* 2004). A RAM (Lichrosphere RP-18 ADS from Merck) has been used for the simultaneous on-line SPE-LC-MS/MS analysis of BPA, other phenolic compounds and triclocarban in breast milk (Ye *et al,* 2005).
- **Immunosorbents (ISs)** are made by covalently bonding antibodies onto an appropriate support. They provide unique selectivity on the basis of molecular recognition, which is particularly suited to complex food matrices. Recoveries of BPA strongly depend on the food matrix, 103% in lemon soft drink (Braunrath *et al.,* 2005) and 74-81% in wines (Brenn-Struckhofova & Cichna-Markl, 2006) have been reported.
- **Molecularly imprinted polymers (MIPs)** are synthetic polymers having molecular recognition ability for a target analyte. MIPs offer some advantage over ISs such as stability against organic solvents, strong acids and bases and heating. This technique has been used by Zhu *et al.,* (2009) from Nanjing Medical University with recoveries of BPA from shampoo, bath lotion and cosmetic cream were 97.3, 92.1 and 87.3% respectively.

2.3.5 Less Common Extraction Techniques

Miniaturised sorptive extraction techniques such as solid-phase microextraction (SPME) (Chang *et al.,* 2005; Nerin *et al.,* 2002; Chang *et al.* 2005) and stir bar sorptive extraction (SBSE) (Kawaguchi *et al.,* 2004) have the

capability of improving the isolation and clean-up of contaminants from food in terms of solvent consumption, automation and sample handling reduction. Likewise, matrix solid-phase dispersion (MSPD) has the potential of simplifying the extraction of solid samples (Fernandez *et al.*, 2000; Shao *et al.*, 2007b). However, these applications on the extraction of BPA from food are still limited to date (Ballesteros-Gomez *et al.*, 2008).

2.4 Separation and Detection

Due to the trace levels at which BPA is frequently found, the detection and quantitation of BPA requires the use of highly sensitive and selective techniques. The determination of BPA is mainly carried out by HPLC/FL, HPLC/MS and GC/MS. Other techniques like LC-electrochemical detection (LC-ED) and immunoassays have been used in a lesser extend (Ballesteros-Gomez *et al.*, 2008).

2.4.1 Liquid Chromatography

Determination of BPA using HPLC is usually carried out using reversed-phase C_{18} columns. Mobile phase varies according to the type of detector used. Water in mixture with acetonitrile or methanol is the commonest solvents used for fluorescence detection. Elution conditions highly depend on the type food matrices and gradient elution is frequently used when complex matrices are encountered.

2.4.1.1 High Performance Liquid Chromatography with Fluorescence Detection (HPLC-FLD)

BPA shows native fluorescence with excitation wavelength at about 230 nm and 275 nm, and emission wavelength at about 305 nm, which keep constant in the solvents frequently used in LC mobile phases, namely water, acetonitrile and methanol. The fluorescence intensity of BPA is much higher in organic media **(Figure 2.2)**, and thus the sensitivity of the LC will be dependent on the mobile phase composition (Ballesteros-Gomez *et al.*, 2008).

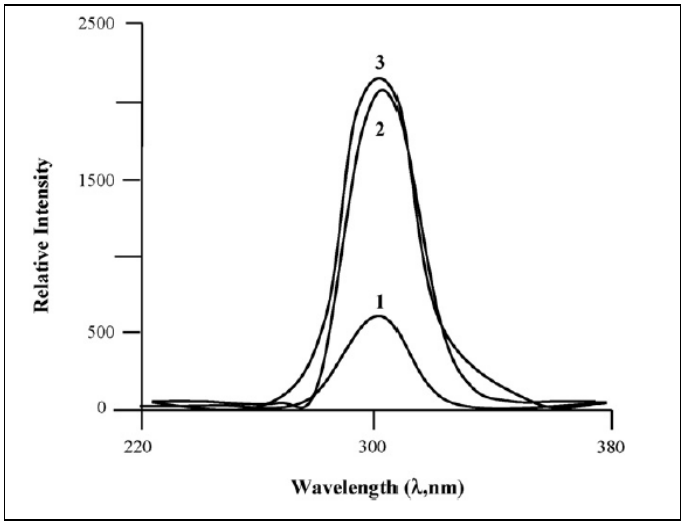

Figure 2.2: Fluorescence spectra (excitation wavelength 275 nm) for bisphenol A in (1) water, (2) acetonitrile and (3) methanol. BPA concentrations : (1) 25 mg/L and (2 and 3) 1 mg/L

(Ballesteros-Gomez *et al.*, 2008)

The identification of BPA in sample is only based on retention times, so the possibility of interference from other fluorescent species, for example,

bisphenol A diglycidyl ether (BADGE), bisphenol F diglycidyl ether (BFDGE) or novolacs glycidyl ethers (NOGE), should always be considered since they may produce false-positive results. Indeed, confirmation by LC/MS after quantification by LC-fluorescence detection has sometimes been employed (Ballesteros-Gomez *et al.*, 2008).

2.4.1.2 High Performance Liquid Chromatography with Electrochemical Detection (HPLC/ED)

Electrochemical detection (ED) of BPA is based on the well known electroactivity of the phenolic groups present in the molecule. LC-ED has been used for the determination of BPA in biological fluids (Inoue *et al*., 2000). Inoue *et al.* (2000) compared the instrumental detection limits obtained for BPA with LC coupled to electrochemical, fluorescence and UV detectors. They reported superior method detection limits for the electrochemical detector which was 3000 and 200 times lower than those obtained by UV and fluorescence, respectively using the same injection volume (50 μL). The main drawback of ED is that isocratic elution is used, otherwise rather large equilibrium times will be required for measurements if gradient elution was used (Inoue *et al*., 2000).

2.4.1.3 High Performance Liquid Chromatography with Mass Spectrometer (HPLC-MS)

The use of mass spectrometry combined with HPLC can reduce sample treatment and even may enable the extraction of an analyte at the detection stage

of the method by selection of specific ions or transitions. Furthermore, clean extracts are preferred to extend the column life and spend less time on the instrument maintenance. HPLC/MS based BPA methods offer higher confidence in identification than HPLC/FLD and HPLC/EC. Compared to GC-MS, the time consuming derivatization step of BPA is not required (Ballesteros-Gomez *et al.*, 2008).

2.4.2 Gas Chromatography with Mass Spectrometer (GC-MS)

GC/MS provides higher resolution and lower detection limits than LC-MS for the determination of BPA, although the need for a derivatization step makes the GC-based methods labour intensive and introduces new sources of errors, mainly due to contamination (Ballesteros-Gomez *et al.,* 2008). Quantitation of BPA by GC-MS requires the derivatization of the analyte in order to improve its separation and detection. However for the confirmation of the presence of BPA, GC-MS with electron ionization (EI) has widely been used and no derivatization of BPA is required for this application.

Since the presence of lipids can significantly reduce the analytical performance of GC (Dodo & Knight, 1999), extensive clean-up is required for fatty foods, such as fish. Like LC-MS methods, the use of an internal standard is common, being deuterated BPA-d_{16} and BPA-d_{14} are most common.

2.5 Fluorimetric Method for Determination Bisphenol A based on Its Inhibitory Effect on the Redox Reaction between Hydroxyl Radical and Rhodamine 6G

Fan *et al.* (2006) found experimentally that hydroxyl radical (·OH) produced by Fenton-like reagent (Fe(III) + H_2O_2) oxidized Rhodamine 6G (Rh6G), causing the fluorescence reduction of Rh6G; the addition of trace BPA has an inhibitory effect on the redox reaction. Based on this observation, an inhibitory kinetic fluorimetric method has been proposed for the determination of BPA.

In the work done by Fan *et al*. (2006), hydroxyl radical produced by Fenton-like reagent oxidized Rh6G in acid medium caused the structure destruction and the fluorescence reduction of Rh6G. It is possible that BPA was involved in the reaction and competed with hydroxyl radicals, which slows the reaction rate of hydroxyl radicals with Rh6G. The possible reaction mechanism was suggested as follows:

$$Fe(III) + H_2O_2 \rightarrow Fe(II) + HO_2^{\bullet} + H^+ \quad (1)$$

$$Fe(II) + H_2O_2 + H^+ \rightarrow Fe(III) + {}^{\bullet}OH + H_2O \quad (2)$$

$${}^{\bullet}OH + Rh6G \rightarrow P_1 \quad (3)$$

$${}^{\bullet}OH + BPA \rightarrow P_2 \quad (4)$$

Equation (1) shows the redox reaction of Fenton-like reagent, and the hydroxyl radicals (${}^{\bullet}OH$) are produced in the reaction indicated by Equation (2). P_1 is the product of the oxidized Rh6G, this oxidation effect results in the

fluorescence reduction of Rh6G. When the BPA is added, reaction (4) occurs, where P_2 stands for the products of the oxidized BPA by hydroxyl radicals (Katsumata *et al.*, 2004). In a definite condition, the concentration of adding BPA has a linear relationship with the recovery of the system's fluorescence (Fan *et al,* 2006).

The reactivity of iron is highly dependent upon its ligand environment. For example, chelators that contain oxygen ligands tend to stabilize Fe(III), while chelators that contain nitrogen or sulfur ligands tend to stabilize Fe(II). Ligation of iron by chelators that stabilize the ferrous form of iron, such as phenanthrolines, results in an increase in the reduction potential of the iron (≈ + 1.1 V). Conversely, ligation of iron by chelators that stabilize the ferric form of iron, such as deferroxamine, results in a decrease in the reduction potential of the iron (≈ -0.4 V) (Miller *et al.*, 1990). In a study done by Graf *et al.* (1983) and Yoshimura *et al.* (1992), various types of iron chelators like Ethylenediaminetetraacetic acid (EDTA), Diethylenetriamine pentaacetic acid (DTPA), phytic acid, and Desferal has been shown to increased or decreased/inhibited the generation of hydroxyl radicals. Recognizing the importance of aquo sites in transition metal catalysis, Graf *et al.* (1984) postulated that iron requires at least one coordination site that is open or occupied by a readily dissociable ligand such as water or azide for catalytic activity.

The use of Fenton or Fenton-like reagent has been extensively applied to the degradation of environmental pollutants. During the time when the work published in Fan *et al.* (2006), it was stated that to the best of their knowledge,

there is no report in the literature of Fenton-like reagent on their applications in the analytical determination of environmental organic pollutants. Later on, Fan and colleagues have also published fluorimetric methods based on the inhibitory effect of the redox reaction of methomyl and penthchlorophenol on the fluorescent reduction of Rhodamine B as reported in Fan *et al.* (2009) & Guo *et al.* (2007) respectively. It was observed that the method used in both papers also share the same principles as for the determination of BPA (Fan *et al.*, 2006), whereby methomyl and pentachlorophenol show inhibitory effect in the fluorescence reduction of Rhodamine B instead of Rhodamine 6G by Fenton reagent.

A method for determination of iron in chinese herbal medicine based on the fluorescent reduction of Rh6G was also reported in Wang *et al*. (2009), although the method uses KI instead of hydrogen peroxide to produce I_3^- anion which subsequently reacted with Rh6G, but the principle of the measurement is similar with only difference in the oxidant.

CHAPTER 3

MATERIALS & METHOD

Application of fluorescence spectrometer to determine the fluorescence intensity change of Rh6G due to the effect of redox reaction by Fenton-like reagent as well as in the presence of BPA, BPA extraction by SPE, cross validation of BPA determination by RP-HPLC and GC/MS are described in this chapter.

3.1 Reagents and Apparatus

The following are the reagents and apparatus used to conduct the experiments shown in **Table 3.1 and 3.2**.

Table 3.1: List of reagents

Reagent	Manufacturer
bisphenol A 2,2-bis(4-hydroxyphenyl)propane (> 99 % purity)	Fluka
methanol HPLC grade (> 99 % purity)	Merck
ethyl acetate analytical grade (> 98 % purity)	Merck
n-hexane analytical grade (> 98 % purity)	SYSTERM
acetonitrile HPLC grade (> 99 % purity)	Scharlau
Ultrapure water, Type I	Filtered using Sartorius Arium DI system
rhodamine 6G chloride (> 99 % purity)	Fluka
ammonium Iron(III) sulphate dodecahydrate (> 99 % purity)	SYSTERM
hydrogen peroxide (30 % in water)	Merck
sulphuric acid (97 %)	Fisher Scientific

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hydrochoric acid (37 %)	Fisher Scientific
sodium sulphate anhydrous) (> 98 % purity)	R & M Chemicals
pentetic acid (DTPA) (> 99 % purity)	Merck
phytic acid (50 % in water)	Nacalai Tesque
disodium salt of Ethylenediaminetetraacetic acid (EDTA) (> 99 % purity)	SYSTERM
phosphoric acid (Analytical grade 85 %)	R & M Chemicals

Table 3.2: List of apparatus and instruments

Apparatus	**Manufacturer**
Volumetric flask (5 mL, 10 mL, 25 mL, 50mL and 100 mL)	Witeg
Schott bottle (500 mL and 1000 mL)	SCHOTT
Measuring cylinder (100 mL, 250 mL and 500 mL)	BOMEX
Transfer pipettes (1-10 µL, 20-200 µL, 100-1000 µL, 100-5000 µL and 1000-10000 µL)	GILSON
Glass HPLC injector syringe (25 µL)	Agilent
Reverse phase HPLC column LiChroCART® 150-4,6 HPLC- Catridge *Purospher® STAR RP-18* endcapped (5 µm)	MERCK
SPE vacuum manifold set (12 port)	Phenomenex
SPE cartridge (Strata C-18E, 500 mg, 5 µm 70 A, 6 mL)	Phenomenex
Cellulose acetate membrane filter (0.45 µm)	Sartorius
Fluorescence Spectrometer LS 55 FLWinlab 4 sided Quartz cuvette (10 mm pathlength)	Perkin Elmer
High Performance Liquid Chromatography 1100 Series with Fluorescence detection (G1321A)	Agilent
Analytical Injection Valve- 20 µL loop (7725i)	Rheodyne
Gas Chromatography with Quadruple Mass Spectrometer Varian WS (3800GC/4000MS)	Varian

3.2 Sampling

Three different classes of samples were chosen to test the effectiveness of the proposed method. They are namely polycarbonate drinking bottles, canned foods and environmental water. In total, 3 types of PC drinking bottles and 3 types of canned food were purchased whereas 5 types of environmental water were collected from different localities **(Table 3.3).**

Table 3.3: Samples and their designated code

Sample picture	Description	Brand/ Location	Sampling date	Sample code
	2000 mL Polycarbonate drinking bottle	Fuguang	-	PC2000A PC2000B PC2000C
	650 mL Polycarbonate drinking bottle	Athletic Water Bottle	-	PC650A PC650B PC650C
	250 mL Polycarbonate baby bottle	-	-	PC250A PC250B PC250C

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	Green peas	TST	Manufacturing/ Expiry date: 20 Nov 2009/ 19 Nov 2011	GPA GPB
	Tuna chunks in water	TC BOY	Manufacturing/ Expiry date: 5 May 2010/ 5 May 2013	TUNAA TUNAB
	Mushroom	HOWEI	Manufacturing/ Expiry date: 6 May 2009 / 5 May 2012	SHROOMA SHROOMB
	Tap water	UTAR Laboratory	1 June 2010	TW
	Lake water	UTAR Lake	1 June 2010	UW
	River water	Lata Kinjang	3 June 2010	LK

Table continue on next page

	Lake water	Tasik Titiwangsa	24 July 2010	TT
	Sea water	Port Dickson	24 July 2010	PD

3.2.1 Food Sample Preparation

3.2.1.1 For Spectrofluorimetric method

The whole content was homogenized using a Waring blender, then kept in glass bottles and stored at 4 ºC prior to testing. Five to six grams of food were weighed and shaken with 40 mL of acetonitrile in a 25 mL conical flask for 25 minutes on an orbital shaker at 150 rpm. The weight of sample used was noted. This mixture was then filtered through a filter paper into a 250 mL separating funnel. After rinsing the conical flask with 10 mL of acetonitrile, 75mL of *n*-hexane was added. The mixture was shaken for 20 minutes and allowed to stand for 25 min. The acetonitrile layer was removed but retained in a 100 mL beaker, and the n-hexane layer was extracted twice with acetonitrile (30 mL; 20 mL). The solvent was evaporated from the acetonitrile extract using an oven preset at 70 ºC attached to vacuum suction. The residue was reconstituted with 50 mL water and

sonicated for 30 minutes. They were then loaded onto SPE cartridges. After SPE standard operating procedure **Section 3.4.2**, the eluent dried at 70 °C was reconstituted with 10 mL deionized water before application to the spectrofluorimetric method.

3.2.1.2 For RP-HPLC and GC-MS

The sampling procedure was the same as described above. After SPE standard operating procedure **Section 3.4.2,** the eluent dried at 70 °C was reconstituted with 1 mL deionized water. The reconstituted sample was equally divided into two portions. One portion 500 µL was kept at 4 °C before HPLC. For another 500 µL was dried in a vacuum oven at 70 °C and reconstituted with 500 µL ethyl acetate before GC-MS measurement.

3.2.2 BPA Migrated Water Samples from Polycarbonate Bottles Preparation

3.2.2.1 For Spectrofluorimetric method

European standard EN14350-2 (European Committee for Standardization, 2004) approach was applied to incubate water samples in the polycarbonate bottles. Each newly purchased bottle was first immersed in boiling water for 10 minutes without touching the walls of the container to remove the surface coating

arising from the manufacturing processes and ensure that the materials used are stable in boiling water. Half of volume of each bottle was filled up with deionized water and incubated for 24 hours in an oven preset at 40 ºC. After that the solution was transferred into screw capped glass bottle.

For BPA migrated water samples from PC 650 and PC 250, without using SPE, stored at 4° C and designated as “whole water samples”. For water sample from PC2000, solid phase extraction was carried out to concentrate the volume of water sample. After SPE standard operating procedure **Section 3.4.2**, the eluent dried at 70 °C was reconstituted with 10 mL deionized water and designated as “aliquot samples”.

3.2.2.2 For RP-HPLC

After incubation of water in PC bottles according to the European standard EN14350-2, 1 mL each of all water samples from PC2000, PC650 and PC250 were kept in the sample vials without treating with SPE method and it will be direct applied to the RP-HPLC.

3.2.3 Environmental Water Samples Preparation

3.2.3.1 For Spectrofluorimetric method

Eleven liters of environmental water sample from each location was collected and stored at 4 ºC prior to testing. Five hundred milliliters of the water

sample were filtered through a 0.45 μm cellulose acetate membrane filter with vacuum suction. The filtrate water samples underwent SPE to concentrate the water sample volume. After SPE standard operating procedure **Section 3.4.2**, the eluent dried at 70 °C was reconstituted with 10 mL deionized water and designated as “aliquot water samples”.

3.2.3.2 For RP-HPLC and GC-MS

The procedure was the same as described above **Section 3.2.3.1.** The dried residue from environmental water was reconstituted with 1 mL deionized water. The reconstituted sample was equally divided into two portions. One portion 500 μL was kept at 4 °C before HPLC. For another 500 μL was dried in a vacuum oven at 70 °C and reconstituted with 500 μL ethyl acetate before GC-MS measurement.

3.3 Fluorimetric Analysis

Characterization of the standard Rh6G and the effect of BPA to the fluorescence intensity of Rh6G were investigated using fluorescence spectroscopy. The investigation was carried out at room temperature using *Perkin Elmer LS 55 Fluorescence Spectrometer*. Data acquisition was performed by using FLWinlab software.

3.3.1 Preparation of Stock Solutions for Fluorimetric Analysis

Bisphenol A first stock solution (5.05 mg/mL) was prepared by dissolving 125 mg of BPA in 25 mL of methanol. Then, 245 µL of the stock solution was diluted with deionized water in a 500 mL volumetric flask to produce the **BPA second stock solution (2.578 mg/L)**.

Rh6G stock solution (0.1 mM) was prepared by dissolving 47.90 mg of Rh6G chloride powder with deionized water in a 1000 mL volumetric flask.

0.6 M HCl solution was prepared by diluting of 25 mL of 37 % HCl with deionized water in a 500 mL volumetric flask to produce 0.6 M HCl stock solution. Then, **0.06 M HCl** stock solution was prepared by diluting 10 mL of 0.6 M HCl solution with deionized water in a 100 mL volumetric flask.

0.1 M H_2SO_4 was prepared by dissolving 5.5 mL of 97 % H_2SO_4 with deionized water in a 1000 mL volumetric flask. Then, 50 mL of the H_2SO_4 solution was diluted with deionized water in another 1000 mL volumetric flask to produce 5 mM H_2SO_4 solution.

0.06 M of Iron(III) was prepared by dissolving 143.95 mg of $NH_4Fe(SO_4)_2 \cdot 12H_2O$ with 5 mM H_2SO_4 solution in a 500 mL volumetric flask.

0.1958 M H_2O_2 was prepared by diluting 2 mL of 30 % H_2O_2 with deionized water in a 100 mL volumetric flask. This solution was prepared fresh everyday.

10 mM pentetic acid stock solution was prepared by dissolving 3933.5 mg of pentetic acid with deionized water in a 1000 mL volumetric flask.

10 mM of phytic acid stock solution was prepared by diluting 9.217 mL of 50% phytic acid with deionized water in a 1000 mL volumetric flask.

10 mM Na_2-EDTA stock solution was prepared by dissolving 3722.4 mg of disodium salt of ethylenediaminetetraacetic acid (Na_2-EDTA) was dissolved with deionized water in a 1000 mL.

10 mM H_3PO_4 stock solution was prepared by diluting 0.684 mL of phosphoric acid (H_3PO_4) was dissolved with deionized water in a 1000 mL volumetric flask.

3.3.2 Characterization of Rhodamine 6G (Rh6G) with Fluorescence Spectroscopy

Rhodamine 6G solution (0.2 μM) was produced by diluting 0.05 mL of Rh6G stock solution (0.1 mM) with water in a 25 mL volumetric flask. This solution was then scanned with fluorescence spectrometer from 200 to 800 nm to obtain the excitation and emission spectra.

The emission spectrum was obtained while the solution was excited at 345 nm. The emission peak wavelength at 547 nm was found to be most intense and was chosen to investigate the optimum excitation wavelength.

Then, the excitation spectrum was obtained by excitation of the solution from 200 to 800 nm and the intensity of florescence emission at 547 nm was measured. The operating conditions for the analysis of Rh6G were stated below;

Excitation slit	:	10.0 nm
Emission slit	:	3.0 nm
Scan mode	:	Accumulation mode, average of 3 repeated scan
Scan speed	:	500 nm/min
Cuvette	:	4 clear sided quartz (Suprasil), standard volume size 10.0 mm pathlength. Cuvette holder temperature set at 25 ºC.

3.3.3 Optimization Procedure for Fluorimetric Method

For optimization of procedure, the operating reaction was set by adding the stock solutions in the following order; 1.0 mL of BPA (2.578 mg/L), 0.6 mL of Rh6G (0.1 mM), 0.40 mL of HCl (0.06 M), 0.15 mL of Fe(III) (0.06 M) stock solutions (**Section 3.3.1**). Water was added to the mark in the volumetric flask (25 mL) and it was shaken to mix well. At the same time blank solution was prepared without addition of BPA.

After the flask has been incubated in a water bath at 60 ºC for 10 minutes, 0.6 mL of H_2O_2 stock solution (0.2 M) (**Section 3.3.1**) was added to initiate the reaction process. Finally, after 40 minutes incubation at 60 ºC, 1.0 mL pentetic acid (10 mM) stock solution (**Section 3.3.1**) was added, shaken and cooled down with running water for 10 minutes. Then, the solution was scanned with fluorescence spectrometer from 200 to 800 nm within 30 minutes after cooling down to room temperature. The fluorescence intensity at 547 nm was recorded. The concentrations of the reagents and BPA were calculated based on the volume of 25.6 mL (after addition of 0.6 mL of H_2O_2 stock solution) where the reaction takes place.

3.3.4 Study of the Reagents Involvement in the Fention-like Reaction

In order to study the mechanism of the reaction in determination of BPA, the effect of the different reagents on the fluorescence intensity of Rh6G was tested. Five different mixture solutions were prepared by addition of the following reagents as shown in **Table 3.4**. The addition of the reagents is as according to the procedure described in **Section 3.3.3**. The excitation and emission spectrum of these solutions was obtained between 300-400 nm and 450-700 nm respectively.

Table 3.4: Preparation of Rh6G in different reagents

Designation	Reagents					
	Rh6G	**HCl**	**H_2O_2**	**Fe(III)**	**BPA**	**pentetic acid (DTPA)**
A-A'	√	√	O	O	O	√
B-B'	√	√	√	O	√	√
C-C'	√	√	√	O	O	√
D-D'	√	√	√	√	√	√
E-E'	√	√	√	√	O	√

√ - Sign indicates that reagent have been added
O – Sign indicates that reagent was not added

3.3.5 Optimization of Concentration of Reagents Involved in Fenton-like Reaction

The concentration of HCl, Iron (III), H_2O_2, Rhodamine 6G and reaction time were optimized by varying the individual concentration of reagent while other reagent concentrations and reaction conditions kept maintain to determine the highest fluorescence intensity difference ΔF between the reaction mixture in the presence of BPA (F) and in the absence of BPA F_0).

3.3.5.1 Optimization of HCl Concentration

Reaction mixture solutions in the presence and absence of BPA as shown in **Section 3.3.3** were prepared with the addition of various volume of 0.06 M HCl as shown in Table 3.5. The end concentrations of HCl in reaction mixtures were from 0 to 30.82 mM (in total volume of 25.6 mL). The fluorescence

intensity of reaction mixture solutions with 7 different HCl concentrations were scanned with fluorescence spectrometer in the range from 400 to 700 nm. The fluorescence intensity at 547 nm was recorded. The difference between the fluorescence intensity ΔF of reaction mixture between in the presence and absence of BPA were calculated to plot ΔF against – log [HCl].

Table 3.5: Optimization of HCl concentration for both blank and standard solutions

No.	Volume of HCl stock solution added into reaction mixture solutions	Concentration of HCl in 25.6 mL (mM)
1	0.00	0
2	0.02	0.047
3	0.10	0.234
4	0.40	0.938
5	1.20	2.813
6	4.00	9.375
7	13.15	30.82

3.3.5.2 Optimization of Fe (III) Concentration

Six set of Fe (III) solutions were prepared by dissolving ammonium iron (III) sulphate dodecahydrate (**Table 3.6**) with 5 mM sulfuric acid solution in a 500 mL volumetric flask.

Reaction mixture solutions in the presence and absence of BPA as shown in **Section 3.3.3** were prepared with the addition of various concentration of 0.15 ml HCl as shown in **Table 3.6**.

Table 3.6: Preparation of Fe (III) standard solutions

Standard solution	Amount of $NH_4Fe(SO4)_2.12H_2O$ (mg)	Concentration of Fe(III) standard solution (mg/L)
Fe 1	143.95	33.33
Fe 2	287.82	66.67
Fe 3	431.78	100.00
Fe 4	575.69	133.33
Fe 5	719.64	166.67
Fe 6	863.55	200.00

The end concentration of Fe (III) in each reaction mixture was varied between 0.1953 to 1.1719 mg/L (in 25.6 mL) as shown in Table 3.7. The fluorescence intensity was recorded at 547 nm. The difference of the fluorescence intensity, ΔF, in the presence and absence of BPA in the reaction solutions were calculated to plot against concentration of Fe (III).

Table 3.7: Optimization of Fe (III) concentration for both blank and standard solutions

No.	Addition of 0.15 mL of Fe(III) standard solution into reaction mixture solution	Concentration of Fe(III) in 25.6 mL (mg/L)
1	Fe 1	0.1953
2	Fe 2	0.3906
3	Fe 3	0.5859
4	Fe 4	0.7813
5	Fe 5	0.9766
6	Fe 6	1.1719

3.3.5.3 Optimization of H_2O_2 Concentration

A hydrogen peroxide solution with a concentration of 391.6 mM was prepared by diluting 2 mL of hydrogen peroxide 30% solution with deionized water in a 50 mL volumetric flask. In the optimization procedure shown in 3.4.3, the volume of hydrogenperoxide added to initiate the oxidation reaction was set 0.6 mL. Therefore, different concentration of hydrogen peroxide was prepared by mixing different volume of 391.6 mM H_2O_2 with appropriate volume of water as shown in Table 3.8 to obtain total volume of 0.6 mL. After addition of 0.6 mL of different concentration of H_2O_2 into the reaction mixtures prepared according to 3.4.3, the end concentrations of H_2O_2 in 6 sets of reaction mixture were varied between 1.53 to 9.18 mM (in 25.6 mL).

The difference between the fluorescence intensity, ΔF of the solutions in the presence and absence of BPA were recorded at 547 nm to plot against concentration of H_2O_2.

Table 3.8: Optimization of H_2O_2 concentration for blank and standard solution

No.	Required volume of 391.6 mM H_2O_2	Requiredvolume of water (mL) to reach 0.6 mL	Concentration of H_2O_2 in 25.6 mL (mM)
1	0.10	0.50	1.53
2	0.20	0.40	3.06
3	0.30	0.30	4.59
4	0.40	0.20	6.12
5	0.50	0.10	7.65
6	0.60	0.00	9.18

3.3.5.4 Optimization of Rh6G Concentration

The concentration of Rh6G was varied between 0.391 to 3.131 μM (in 25.6 mL) by addition of various volume of 0.1 mM Rh6G stock solution into the reaction mixture solution as shown in **Table 3.9**. The difference between the fluorescence intensity, Δ*F*, in the presence and absence of BPA of reaction mixture were recorded at 547 nm to plot against Rh6G concentration.

Table 3.9: Optimization of Rh6G concentration for both blank and standard solutions

No.	Volume of Rh6G stock solution added into the reaction mixture solutions (mL)	Concentration of Rh6G (μM)
1	0.10	0.391
2	0.20	0.783
3	0.30	1.174
4	0.40	1.566
5	0.50	1.957
6	0.60	2.349
7	0.70	2.740
8	0.80	3.131

3.3.5.5 Optimization of Reaction Time

Seven standard and blank solutions were prepared as explained in **Section 3.3.3.** The reaction time was varied between 10 to 70 minutes with 10 min intervals. The difference between the fluorescence intensity, Δ*F* of the standard solution and the blank solution at 547 nm was plotted against time.

3.3.5.6 Selection of Chelating Agents

As chelating agents disodium salt of EDTA, pentetic Acid, phytic Acid and phosphoric acid were selected to determine the best chelating agent to stop the catalytic function of Fe^{3+} ion (**Section 2.5**). Stock solutions of disodium salt of EDTA, pentetic acid, phytic acid and phosphoric acid at 10 mM concentration was prepared as described in **Section 3.3.1.**

A standard solutions containing 100 μg/L of BPA was prepared and the reaction was undergone as shown in **Section 3.3.3**. Then, 1.0 mL of the 10 mM pentetic acid was added into the volumetric flask, shaken and cooled down to room temperature under running water. The solution was then scanned with fluorescence spectrophotometer at the range between 400 – 700 nm. The intensity at 547 nm was recorded at every 30 minutes intervals until 150 minutes. The intensity at 547 nm was plotted against time for each solution.

The same procedure was repeated for phytic acid, disodium salt of EDTA, and phosphoric acid standard solution. As experimental control, the same procedure was repeated with addition of water only, instead of chelating agent.

3.3.5.7 Optimization of the Concentration of Selected Chelating Agent

Pentetic acid was found to be most suitable because it can slow down the reduction of florescence intensity compared to other chelating agents. The effect of the selected chelating agent concentration was investigated by varying the

concentration of solution at 10, 1 and 0.1 mM. Then, the same experiment was repeated using the different concentrations of pentetic acid.

3.3.6 Sample Analysis Procedure for Spectrofluorimetric Method

This section describes the analysis procedure using spectrofluorimetric method for each type of sample after preparation in **Section 3.2.1.1, 3.2.2.1 and 3.2.3.1**.

3.3.6.1 For Food Samples

The dried residue from food sample prepared as described in **Section 3.2.1.1** were reconstituted with 10 ml water and sonicated. This "aliquote sample" was transferred into 25 mL volumetric flasks that was previously added with 0.6 mL of Rh6G (0.1 mM) , 0.40 mL HCl (0.06 M) and 0.15 mL of Fe(III) stock solution (0.06 M), topped up to the 25 mL mark with water and shaken. After the flask has been heated in a water bath at 60 ºC for 10 minutes, 0.6 mL of H_2O_2 stock solution was added to initiate the reaction process. Finally, 1.0 mL pentetic acid stock solution (10 mM) was added after 40 minutes, shaken and cooled to room temperature with running water, scanned with fluorescence spectrometer and the emission intensity at 547 nm was recorded**.** The procedure was repeated to obtain triplicate results.

3.3.6.2 For Water samples from PC bottles (PC650 and PC250)

Firstly, 0.6 mL of Rh6G, 0.40 mL HCl and 0.15 mL of Fe(III) stock solutions were transferred into 25 mL volumetric flasks, then the "whole water sample" from PC650A (described in **Section 3.2.2.1**) was used to top up to 25 mL mark and shaken. After the flask has been heated in a water bath at 60 ºC for 10 minutes, 0.6 mL of H_2O_2 stock solution was added to initiate the reaction process. Finally, after 40 minutes, 1.0 mL pentetic acid stock solution was added, shaken and cooled to room temperature with running water, scanned with fluorescence spectrometer and the emission intensity at 547 nm was recorded**.** The same procedure was repeated to obtain a triplicate result. The overall procedure was repeated for water sample from PC650B, PC650C, PC250A, PC250B and PC250C .

3.3.6.3 For Water samples from PC bottles (PC2000)

The dried residue from PC2000 samples prepared as described in **Section 3.2.2.1** were reconstituted with 10 ml water and sonicated. This "aliquote sample" was used for spectrofluorimetric method as described in **Section 3.3.6.1**.

3.3.6.4 For Environmental Water

The dried residue from environmental sample prepared as described in **3.2.3.1** were reconstituted with 10 ml water and sonicated. This "aliquote sample" was used for spectrofluorimetric method as described in **Section 3.3.6.1.**

3.3.7 Preparation of Calibration Solutions to Produce Calibration Curve of Bisphenol A for Fluorimetric Method

Six standard solutions with different concentrations of BPA were prepared as described in **3.3.3.** Standard solutions containing BPA at concentrations of 10.0, 20.1, 40.3, 60.4, 80.5, and 100.7 μg/L were prepared by addition of 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mL respectively of BPA second stock solution (2577.5 μg/L). Five separate preparations for each standard solution was measured. Eleven replicates of blank solutions (without BPA) were also prepared as described in **Section 3.3.3.**

All the solutions were then scanned with fluorescence spectrophotometer at the range between 400 – 700 nm. The difference between the fluorescence intensity, ΔF of the standard solution and the blank solution at 547 nm was plotted against BPA concentration.

The limit of detection (LOD) is defined as LOD = $3S_b/k$, where S_b is the standard deviation of the regent blank (n = 11) and k is the slope of the calibration curve. The limit of quantitation (LOQ) is defined as LOQ = $10S_b/k$ (Fan *et al.*, 2006).

3.4 Solid Phase Extraction (SPE)

The SPE cartridges with 500 mg of C_{18} (endcapped) packing were purchased from Phenomenex. A 12 port SPE vacuum manifold attached to a

vacuum pump was used to process up to 12 samples at once. The sample loading volume of the SPE cartridges was extended using 60 mL syringe tubes attached together with adaptor.

Standard BPA solutions used for the development of SPE were 20, 200 and 500 µg/L. To prepare these standard solutions of BPA, aliquots of the second stock solution (1026 µg/L) were diluted with deionized water in 25 mL volumetric flasks as shown in **Table 3.10**. Weight of bisphenol A stock solution transferred to prepare these standard solutions is shown in **Table 3.11 (Appendix A)**. Each of the solution was injected into HPLC/FLD thrice before SPE extraction (operating condition of HPLC/FLD in **Section 3.5**).

Table 3.10: Preparation of bisphenol A standard solutions for development of solid phase extraction

Standard solutions	Volume of BPA (1026 µg/L)	Final BPA concentration (µg/L)
SPE 20	**0.5 mL**	**20.0**
SPE 200	**5.0 mL**	**204.9**
SPE 500	**12.0 mL**	**499.8**

3.4.1 Development of Sample Pre-treatment using Solid Phase Extraction (SPE)

There are 4 steps in SPE extraction procedure which are CONDITION, LOAD, WASH and ELUTE. The method development of the SPE focuses on the optimization of the WASH and ELUTE step.

For the CONDITION step, initially the conditioning of the packing involves activating the sorbent ligands by flowing through 6 mL of methanol, then conditioning the sorbent with 6 mL of water, both steps at a flow rate of 1-2 mL/min under vacuum suction. Then, 1.0 mL of SPE 500 was transferred into a 50 mL beaker and diluted up to 50 mL with water. The weight of SPE 500 transferred was noted. This solution was then loaded into the cartridge at a flow rate of 2-3 mL/min for the LOAD step.

The development of the WASH step involves using methanol: water and ethyl acetate: n-hexane solution. methanol: water solutions at a ratio of 0, 10, 20, 30, 40 and 50 % (v/v) were prepared by dissolving 0.0, 1.0, 2.0, 3.0, 4.0 and 5.0 mL of methanol, respectively with deionized water in a 10 mL volumetric flask, respectively. ethyl acetate: n-hexane solutions at a ratio of 0, 5, 10, 15, 20, 30 and 40 % (v/v) were prepared by dissolving 0.0, 0.5, 1.0, 1.5, 2.0, 3.0 and 4.0 mL of ethyl acetate, respectively with n-hexane in a 10 mL volumetric flask.

For the first WASH step, 6 SPE cartridges pre-loaded with 1.0 mL of SPE 500 were washed with 5 mL of 0, 10, 20, 30, 40 and 50 % methanol: water solution, respectively at a flow rate of 1-2 mL/min. The cartridges was then dried under vacuum air flow for 40 minutes, then BPA was eluted into 10 mL glass vials with 5 mL ethyl acetate at a flow rate of 1-2 mL/min. The eluate was then dried in an oven set at 70 ºC attached to vacuum suction. One milliliter of deionized water was used to reconstitute the residue and shaken in a sonicator until the residue completely dissolved and the weight of water was noted. Each

sample was then injected into the HPLC/FLD and the recovery percentage from the procedure was calculated.

It was found that cartridges washed with 20 % methanol solution still retains > 90 % percentage recovery of BPA and was chosen to proceed to study the second WASH step.

For the second stage of the WASH step and ELUTE step, another 7 cartridges washed with 20 % methanol: water solution. Then, BPA was eluted with 5 mL of each 0, 5, 10, 15, 20, 30 and 40 % ethyl acetate: n- hexane solution at a flow rate of 1-2 mL/min. The eluates were then dried in an oven set at 70 ºC attached to vacuum suction. One milliliter of deionized water was used to reconstitute the residue and shaken in a sonicator until completely dissolved and the weight of water was noted. Each sample was then injected into the HPLC/FLD and the recovery percentage from the procedure was calculated

3.4.2 Standard Operation of SPE Procedure

Samples solution was loaded onto SPE cartridges at a flow rate of 2-3 mL/min that were previously conditioned with 6 mL of methanol and equilibrated with 6 mL water at a flow rate of 1-2 mL/min. After washing the cartridge with 5 mL of 5 % ethyl acetate in n-hexane (v/v), the analyte were eluted with 5mL of 20% ethyl acetate in n-hexane (v/v) into 10 mL screw-capped glass vials at a flow rate of 1-2 mL/min. Then, the solvent was evaporated using a vacuum oven preset

at 70 ºC and attached to vacuum suction. The dried residue in screw capped vials was stored at 4 ºC prior to testing.

3.5 Reverse Phase High Performance Liquid Chromatography with Fluorescence detector (RP-HPLC/FLD)

The aqueous sample from the PC bottles or reconstituted after SPE extraction were analyzed using a High Performance Liquid Chromatography with fluorescence detection. The following column and operating conditions have been found to be suitable for the determination of bisphenol A.

Column	:	Reverse phase HPLC column LiChroCART® 150-4,6 HPLC Catridge Purospher® STAR RP 18 endcapped (5μm)
Column temperature	:	30 ºC
Mobile phase	:	methanol : Deionized water (65 : 35) (isocratic)
Flow rate	:	1.0 ml/min
Pressure	:	Approximately 163 bar
Injection volume	:	20 μL
Detection	:	Fluorescence detector (FLD); excitation wavelength Ex=228 nm, emission wavelength Em = 313 nm
PMTGain for FLD	:	12
Detector response	:	2 seconds

The peak for bisphenol A has been found to have a retention time of approximately 5.1 $\pm$ 0.2 minutes (refer to **Figure 4.16** in **Section 4.7)**.

3.5.1 Optimization of RP-HPLC/FLD Operating Conditions

Adjustments to the HPLC such as flow rate, mobile phase ratio and column temperature were performed. The settings to the fluorescence detector were done to achieve the lowest instrumental detection limit of BPA.

The most sensitive detection wavelength in detecting the presence of BPA was investigated. A standard BPA solution of 100 μg/L was injected into the HPLC to determine the most intense excitation and emission wavelength in the 200-400 nm range. To determine the wavelength of maximum emission, an emission scan was obtained in the range of 280-500 nm by setting the excitation wavelength at 230 nm. Then, an excitation scan was obtained in the range of 200-285 nm with 313 nm as the emission wavelength. The excitation and emission scan spectrum were analyzed using 3D Plot in the Chemstation software.

For determination of suitable detector response time, the peak width at half height of the BPA peak was measured. A detector time constant that is approximately 10% of the minimum peak width at half height in a normal chromatogram was chosen to obtain the highest signal-to-noise ratio of the analyte peak, (Hinshaw, 2001).

3.5.2 Preparation of BPA Stock and Standard Solutions for HPLC Analysis

To prepare the first BPA stock solution, 12.50 mg of bisphenol A was dissolved with HPLC grade methanol in a 25 mL volumetric flask. A second BPA

stock solution was prepared by diluting 50 µL of the first stock solution with water in a 25 mL volumetric flask. The first stock solution (0.5014 mg/mL) and second stock solution (1.025 mg/L) were stored at 4 ºC and free from light. In order to determine the accurate concentration of the BPA solution prepared, all volumes of liquid transferred were weighed and the room temperature was measured.

To produce the calibration graph of peak area against concentration of BPA, 14 sets of standard BPA solutions ranging from 1.1 to 501.1 µg/L were prepared by transferring aliquots from the second stock solution (1025.6 µg/L) into 10 mL volumetric flasks and topped up with deionized water as shown in **Table 3.12.** The weight of liquid transferred and calculations to determine the concentration of the standard solutions are shown in **Appendix A (Table 3.11).**

Table 3.12: Preparation of bisphenol A standard solutions

Standard solutions	Volume of BPA stock 2 solution diluted to 10 mL with water (µL)	Final BPA concentration (µg/L)
Cal 1	**10**	**1.1**
Cal 2	**30**	**3.2**
Cal 3	**50**	**5.2**
Cal 4	**70**	**7.2**
Cal 5	**100**	**10.2**
Cal 6	**200**	**20.6**
Cal 7	**400**	**41.8**
Cal 8	**800**	**81.9**
Cal 9	**1400**	**143.3**
Cal 10	**2000**	**204.5**
Cal 11	**2650**	**268.8**
Cal 12	**3700**	**380.0**
Cal 13	**4900**	**501.1**

3.5.3 Determination of Standard BPA Concentration as Peak Area using HPLC/FLD

The standard solutions from 1.1 to 501.1 μg/L of BPA in deionized water (as described in **Section 3.5.2**) were injected into the RP-HPLC/FLD. Each of the solution was injected 5 times into the HPLC/FLD.

A calibration curve for the quantitation of bisphenol A was generated based on peak area measurement against BPA concentration. The peak area measurement from BPA standard solution with concentrations of 5.2, 10.2, 20.6, 41.8, 81.9, 143.3, 204.5, 268.8, 380.0, and 501.1 μg/L were used.

The limit of detection, LOD was determined using the calibration curve constructed from BPA standard solutions of five different concentrations near the estimated LOD (1.1, 3.2, 5.2, 7.2 and 10.2 μg/L). Using these data, a regression equation $Y = mX + C$ of the area under peak Y versus concentration X is calculated. The limit of detection, LOD is calculated by the formula $LOD = 3.3(SD/b)$, where SD is the standard deviation of the free term, C and b is the slope of the main calibration curve (Epshtein, 2004). The SD was obtained by using the LINEST function in MS Excel 2007. The limit of quantification, LOQ was calculated by the formula $LOQ = 10(SD/b)$.

3.6 Validation of Sample Preparation Procedure

After the optimized SPE procedure was established in **Section 3.4.1**, the SPE extraction efficiency and precision were investigated when applied to environmental, food and PC bottle samples.

3.6.1 Validation of the Sample Preparation Procedure for Spectrofluorimetric Method

To validate the extraction procedure for environmental water samples, 200 μL of second BPA stock solution (2.578 mg/L) were transferred into a 500 mL beaker and diluted up to 500 mL with deionized water and filtered through 0.45 μm cellulose acetate membrane.

To validate the extraction procedure for canned foods, 200 μL of second BPA stock solution (2.578 mg/L) was added to 40 mL of acetonitrile with 10 g of anhydrous sodium sulphate in a 25 mL conical flask, then shaken for 25 minutes on an orbital shaker at 150 rpm. The mixture was then filtered through a filter paper into a 250 mL separating funnel. After rinsing the conical flask with 10 mL of acetonitrile, 75mL of *n*-hexane were added. The mixture were shaken for 20 minutes and allowed to stand for 25 minutes. The acetonitrile layer was removed but retained in a 100 mL beaker, and the hexane layer was washed twice with acetonitrile (30 mL; 20 mL). The solvent was removed from the acetonitrile extracts using an oven preset at 70 ºC attached to vacuum suction. The residue was reconstituted with 50 mL water and sonicated for 30 minutes.

To validate the extraction procedure for water incubated in PC bottles (PC2000), 200 μL of second BPA stock solution (2.578 mg/L) were transferred into a 100 mL beaker and diluted up to 100 mL with deionized water.

The procedure for each type of sample as mentioned for environmental water, canned foods and PC bottles were repeated using 0, 500 and 1000 μL of second BPA stock solution (2.578 mg/L). Each sample was produced in triplicate.

For solid phase extraction, standard operating procedure 3.7.2 was conducted. After elution, the eluate was dried using an oven preset at 70 ºC attached to vacuum suction. Then, 10 mL of deionized water were added to re-dissolve the residue. It was shaken in a sonicator until completely dissolved. The solutions were then processed for spectrofluorimetric method as described in **3.3.3,** and then scanned with *Perkin Elmer LS 55 Fluorescence Spectrometer* and the recovery percentage of BPA was calculated.

3.6.2 Validation of the Sample Preparation Procedure for HPLC method

The procedure is same as stated in **Section 3.2.1.2, 3.2.2.2 and 3.2.3.2**, except that the entire procedure for both type of samples (canned food and environmental water) was tested with the sample replaced with equivalent volume of deionized water to investigate if there is any interfering peak arises at the same retention time for BPA.

3.7 Gas Chromatography with Mass Spectrometer Analysis

All the samples that were identified to contain detectable amounts of BPA were confirmed with GC/MS analysis. As only a small portion of the sample was used during HPLC analysis, 500 μL of the same sample was transferred into 10 mL glass vials and dried at 70˚C under vacuum suction. The dried residue was then re-dissolved in ethyl acetate for GC/MS analysis. A *Varian WS GC-MS (3800GC/4000MS)* gas chromatograph coupled to a quadropole mass spectrometer with an electron impact ionization source was used. The following **Table 3.13** shows the operating conditions of the GC/MS.

Table 3.13: Operating conditions of GC/MS

Component		Parameter
GC	**Column**	VF -5ms fused silica column (5% phenyl, 95% dimethylpolysiloxane, 30m ´ 0.25 mm I.D., 0.25 µm)
	Injection mode	Split ratio 20 at initial, split ratio off after 0.01 min and split ratio 50 after 1 min
	Injection temperature	250°C
	Oven temperature	60°C for 1 min, raised to 120°C (30°C min^{-1}) for 3 min, then raised to 320°C (15 °C min^{-1}) for 21.33 min and held for 5 min
	Carrier gas	Helium gas
	Flow rate	1 mL/min
	Transfer line (GC-MS interface) temperature	280 °C
Auto sampler	**Injection volume**	2 µL
	Syringe size	10 µL
	Injection mode	split mode
Quadrupole mass spectrometer	**Ionization mode**	external electron impact ionization positive
	Ion source temperature	220 °C
	Emission current	25 mAmp
	Scan type	Full scan m/z 100:300
	Scan time	1.00 seconds/scan (8 mcans)
	Scan speed	Normal
	Run time	23.33 min
	Library	NIST MS search 2.0

CHAPTER 4

RESULTS & DISCUSSION

4.1 Characterization of Rhodamine 6G with Fluorescence Spectrometry

Rh6G emits a very strong yellow-green fluorescence and has a remarkably high photostability, high quantum yield (0.95) (Kubin & Fletcher, 1982). The excitation and emission spectra of Rhodamine 6G were obtained as described in **Section 3.3.2** and are shown in **Figure 4.1**. The characteristic excitation and emission peak of Rhodamine 6G at 345 nm and 547 nm respectively was identified to be almost similar as the detection wavelength used by Fan *et al.* (2006), which uses 345 nm and 551 nm as the excitation and emission wavelengths, respectively. In the excitation spectrum, it was observed that there were 3 peaks at around 240 nm, 270 nm and 345 nm. The wavelength at 345 nm was more specific for Rh6G and thus chosen as the excitation wavelength to produce the emission spectrum. The emission spectrum showed a maximum intensity peak at 547 nm and was chosen as the emission spectrum.

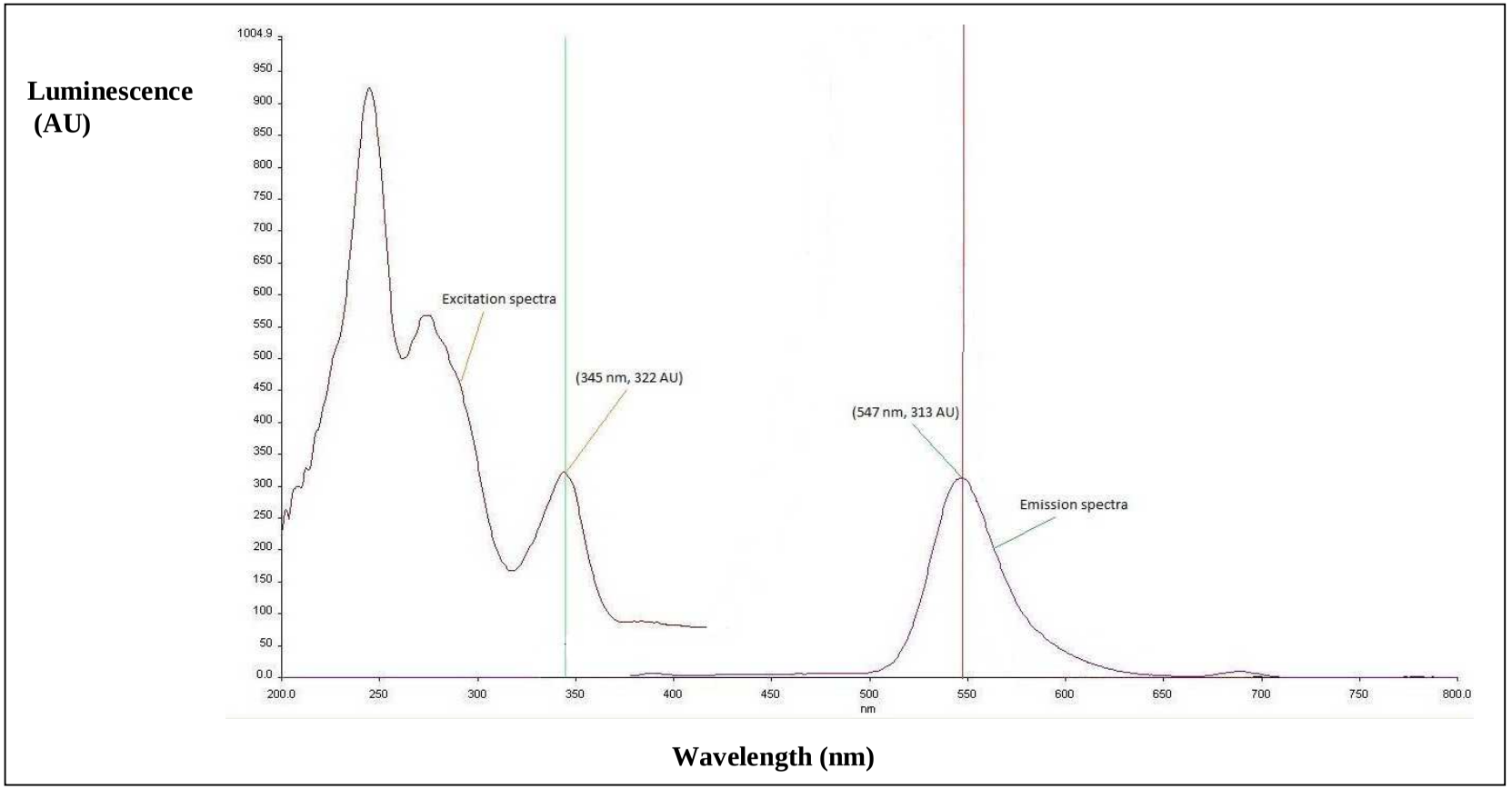


Figure 4.1: Excitation and Emission spectrum of Rhodamine 6G (2×10^{-7} M) at wavelength 200 – 800 nm using *Perkin Elmer LS 55 Fluorescence Spectrometer*

4.2 Reaction Mechanism of the Fluorescent Reduction of Rh6G by Fenton-like Reagent

When Rh6G is oxidized, its molecular structure is destroyed and the fluorescence intensity decreases. From the excitation and emission spectra shown in **Figure 4.2 (A-A', B-B' and C-C')**, it can be observed that BPA has little inhibitory effect on the redox reaction between H_2O_2 and Rh6G. In **Figure 4.2 (D-D' and E-E')**, after iron(III) was added to the mixture, a significant fluorescence reduction of Rh6G was observed, which indicated that Fe(III) influences the production of hydroxyl radicals significantly. Comparing the spectra of **D-D'** with **E-E'**, it is clear that BPA has significant inhibitory effect on the redox reaction of Rh6G with Fenton-like reagent. These results are in agreement with the results from Fan *et al.* (2006).

From its chemical structure and the characteristics of phenolic compounds, it is known that BPA could be oxidized by strong oxidizing agent. It is possible that BPA was involved in the reaction and competed with hydroxyl radicals, which slowed down the reaction rate of hydroxyl radicals with Rh6G (Fan *et al.*, 2006). The possible reaction mechanism was suggested by Fan *et al.* (2006) as follows:

$$Fe(III) + H_2O_2 \rightarrow Fe(II) + HO_2^{\bullet} + H^+ \quad (1)$$

$$Fe(II) + H_2O_2 + H^+ \rightarrow Fe(III) + {}^{\bullet}OH + H_2O \quad (2)$$

$${}^{\bullet}OH + Rh6G \rightarrow P_1 \quad (3)$$

$${}^{\bullet}OH + BPA \rightarrow P_2 \quad (4)$$

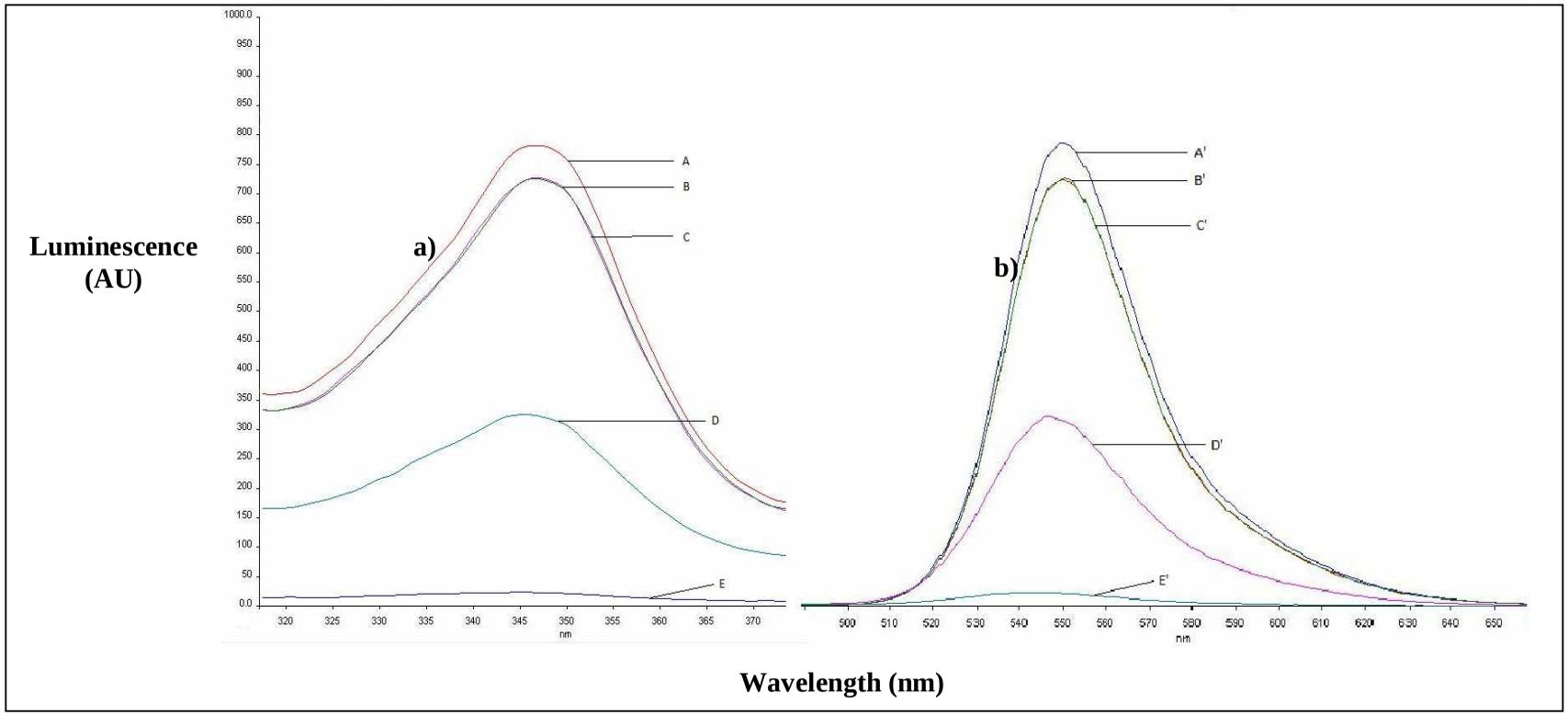


Figure 4.2: **a) Excitation and b) emission spectra of Rh6G in the presence of different reagents: (A-A'), Rh6G + HCl + DTPA; (B-B'), Rh6G + HCl + H_2O_2 + BPA + DTPA; (C-C'), Rh6G + HCl + H_2O_2 + DTPA; (D-D'), Rh6G + HCl + H_2O_2 + Fe(III) + BPA + DTPA; (E-E'), Rh6G + HCl + H_2O_2 + Fe(III) + DTPA**

Equation (1) shows the redox reaction of Fenton-like reagent, and the hydroxyl radicals (˙OH) are produced in the reaction indicated by Equation (2). P_1 is the product of the oxidized Rh6G, this oxidation would affect the results in the fluorescence reduction of Rh6G. When the BPA is added, reaction (4) occurs, where P_2 stands for the products of the oxidized BPA by hydroxyl radicals (Katsumata *et al*., 2004).

4.3 Optimization of Reaction Conditions for Fluorimetric Method

In order to obtain an optimized system, which is sensitive and selective in detection and quantitation of BPA, various experimental parameters have been investigated. The reagent concentrations and reaction conditions were optimized by setting all parameters to be constant and optimizing one at a time. The concentration of BPA was kept constant at 100.7 μg/L. Each parameter was tested twice to confirm the results.

4.3.1 Optimization of HCl Concentration

Several types of reaction medium have been tried by Fan *et al.* (2006) such as hydrochloric acid, sulfuric acid, phosphoric acid and perchloric acid. Among them, only hydrochloric acid shows a striking effect on the inhibition effect of BPA. Since Fe(III) hydrolyzes at pH > 3.0, Fan *et al.*, (2006) studied the effect of HCl concentration at a range of 2.4×10^{-3} to 2.88×10^{-2} M.

In this study, the effect of HCl was studied in the range of 0.00 to 308.20 x 10^{-4} M. The difference between the F and F_o is insignificant when no HCl was added which indicates that HCl were needed as the reaction medium for BPA to have any inhibitory effect on the fluorescence reduction of Rh6G (**Table 4.1 and 4.2 in Appendix B**).

By observing the trend in **Figure 4.3**, it shows the highest ΔF (F-F_o) at – log [HCl] = 3.0, which is when 0.4 mL of 0.06 M HCl added to the reaction mixture (9.38 x 10^{-4} M). As the H^+ concentration increases, the rate of hydroxyl radicals produced increases and Rh6G oxidation also increases as shown in the **Equation** (**1**) and (**2**) (**Section 4.2**), however when the pH value is very low, the excess H^+ quenches the produced hydroxyl radicals (Fan *et al.*, 2006). Thus, the concentration of 9.38 x 10^{-4} M of HCl was selected for the next study. The duplicate results match each other and thus confirm the results.

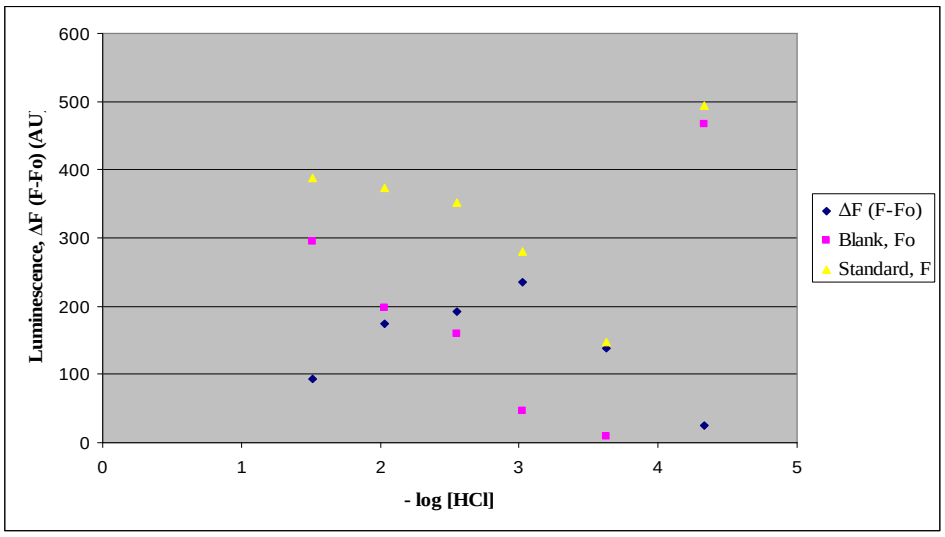


Figure 4.3: Effect of HCl concentration on the fluorescence reduction of Rh6G (first replicate, refer to Table 4.1 in Appendix B)

4.3.2 Optimization of Fe(III) Concentration

In the present system, Fe(III) is used as the catalyst to drive the production of hydroxyl radicals, there should be a close relationship between its concentration and hydroxyl radicals generated (Fan *et al.*, 2006). It was noted by Fan *et al*. (2006) that Fe(III) has the similar characteristics as Fe(II), but it offers advantages such as good stability and no particular protection is needed. The effect of Fe(III) was studied in the range of 0.2 – 1.0 μg/mL using $NH_4Fe(SO_4)_2 \cdot 12H_2O$ as the source for Fe(III) by Fan *et al.* (2006). In this study, the concentration of Fe(III) was studied in the range of 0.1953 to 1.1719 mg/L (in 25.6 mL)**.** The intensity of the emission at 547 nm for each standard solution is shown in **Table 4.3 and Table 4.4 (Appendix B)**.

By observing the trend in **Figure 4.4**, increase in Fe(III) concentration results in the decrease in ΔF (F-F_o). The figure also shows that F and F_o decreases with an increase of Fe(III) concentration. As the concentration of Fe(III) increases, the generation of hydroxyl radicals also increased which accelerated the fluorescence reduction of Rh6G, as shown in the **Equation** (**1**) and (**2**) (**Section 4.2**). The concentration of 0.1953 mg/L of Fe(III) was selected for the next study as it gives the highest ΔF (F-F_o) value. Although lower concentrations of Fe(III) could be used, the concentration of Fe(III) would inevitably affects the other parameters. For example, the reaction time has to be lengthened as Fe(III) concentration would affect the rate of reaction. As a screening method, it would be impractical for the reaction time to be too long as it would hinder the number

of samples that can be tested in a day. The duplicate results match each other and thus confirm the results.

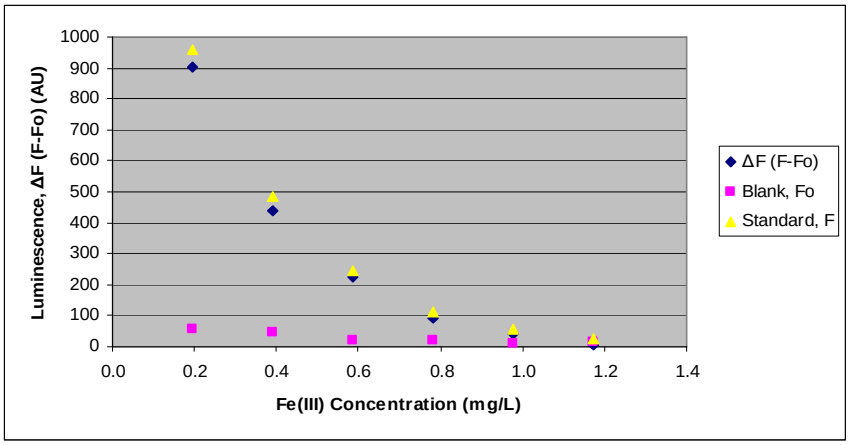


Figure 4.4: Effect of Fe(III) concentration on the fluorescence reduction of Rh6G (first replicate, refer to Table 4.3 in Appendix B)

4.3.3 Optimization of H_2O_2 Concentration

As the precursor of the hydroxyl radicals, the concentration of hydrogen peroxide may greatly influence the hydroxyl radical concentration (Fan *et al.*, 2006). The effect of H_2O_2 was studied in the range of 0.012 – 0.0192 % by Fan *et al.* (2006). In this study, the influence of hydrogen peroxide has been investigated in the range of 1.53 to 9.18 mM (in 25.6 mL)**.** The intensity of the emission at 547 nm for each standard solution is shown in **Table 4.5 and Table 4.6 (Appendix B)**.

Figure 4.5 shows that an increase in H_2O_2 concentration results in the decrease of ΔF (F-F_o). The graph also shows that F and F_o decreases with increase in H_2O_2 concentration. As the concentration of H_2O_2 increases, the

generation of hydroxyl radicals also increased which accelerated the fluorescence reduction of Rh6G, as shown in the **Equation** (**1**) and (**2**) (**Section 4.2**). The concentration of 4.59 mM of H_2O_2 was selected for the next study. Although lower concentrations of H_2O_2 could be used, it would be impractical for the same reasons as explained for Fe(III). The duplicate results match each other and thus confirm the results.

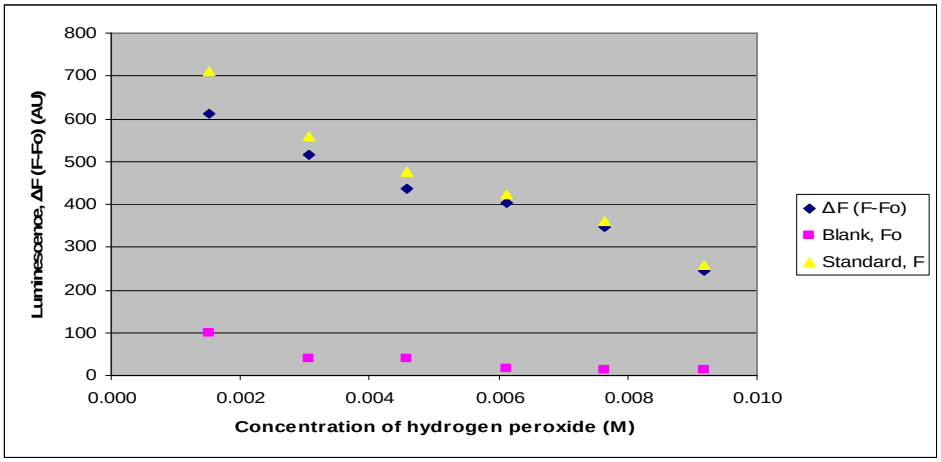


Figure 4.5: Effect of H_2O_2 concentration on the fluorescence reduction of Rh6G (first replicate, refer to Table 4.5 in Appendix B)

4.3.4 Optimization of Rh6G Concentration

The effect of Rh6G starting concentration was studied in the range of 3.91 x 10^{-7} to 31.31 x 10^{-7} M (in 25.6 mL) compared to 4.0 x 10^{-7} to 4.0 x 10^{-6} M by Fan *et al.* (2006). The intensity of the emission at 547 nm for each standard solution is shown in **Table 4.7 and Table 4.8 (Appendix B)**. The F and F_o values increase with the increase of Rh6G concentration as shown in **Figure 4.6.** A maximum in ΔF (F-F_o) at 23.49 x 10^{-7} M was observed and was selected for the

next study. The duplicate results match each other and thus confirm the results (**Table 4.7 and Table 4.8 in Appendix B**).

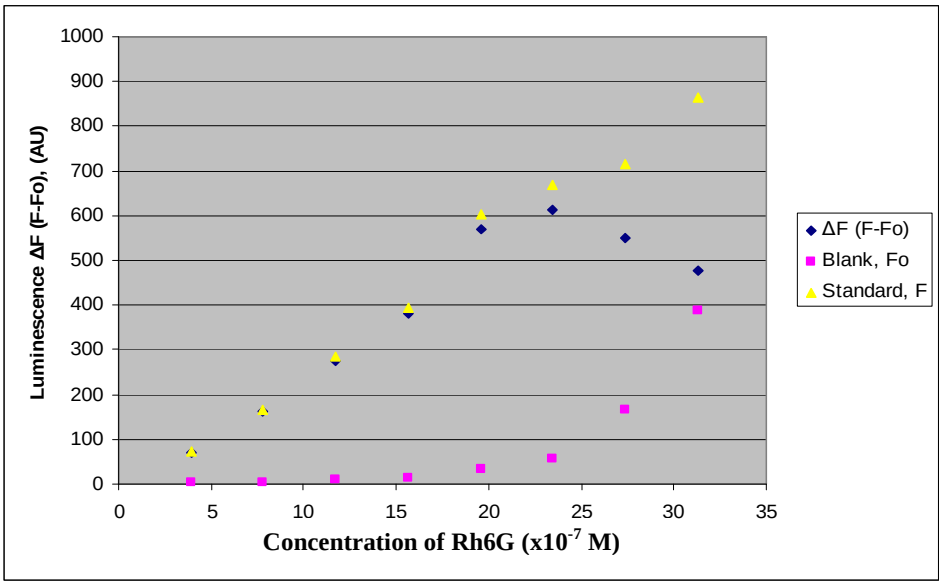


Figure 4.6: Effect of Rh6G concentration on the fluorescence reduction of Rh6G (first replicate, refer to Table 4.7 in Appendix B)

4.3.5 Optimization of Reaction Time

The effect of reaction time was investigated in the range of 10 to 70 minutes compared to 1-16 min as studied by (Fan *et al.*, 2006). The intensity of the emission at 547 nm for each standard solution is shown in **Table 4.9 and Table 4.10 (Appendix B).** It was observed that an increase in reaction time caused a decreased in fluorescence intensity for both F and F_o in **Figure 4.7**. This was anticipated as a longer reaction time would cause more Rh6G to be oxidized. But, since both blank and standard solutions have different rates at which the Rh6G was oxidized, a point in which the ΔF (F-F_o) is maximum was observed at

40 minutes and was selected for the next study. The duplicate results match each other and thus confirm the results (**Table 4.9 and Table 4.10 in Appendix B**).

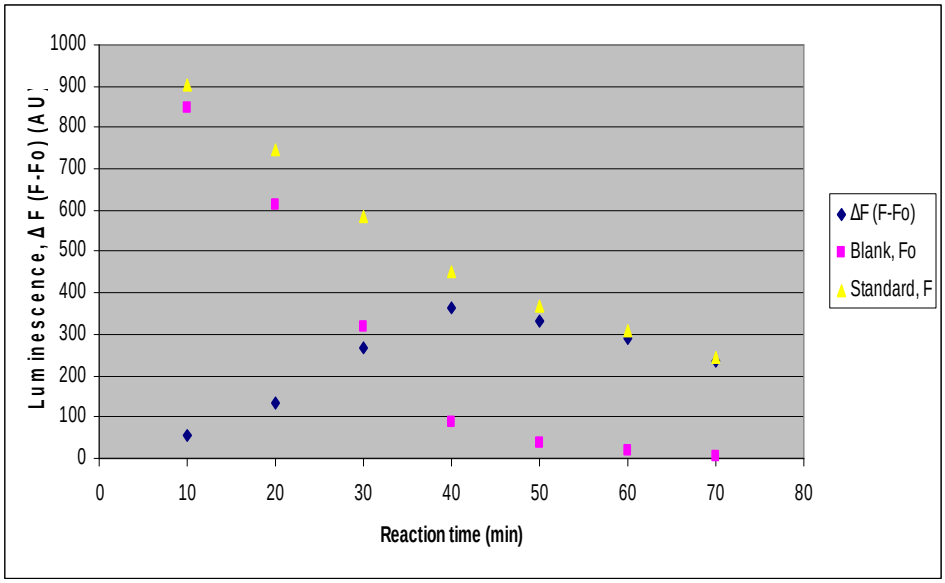


Figure 4.7: Effect of reaction time on the fluorescence reduction of Rh6G (first replicate, refer to Table 4.9 in Appendix B)

4.3.6 Optimization of Reaction Temperature

In Fan *et al.* (2006), the effect of temperature was studied in the range of 30 to 65 ºC and the temperature at 60 ºC was chosen as the temperature dependence of ΔF shows a maximum at that temperature. In our study, 60 ºC was found to be hot enough for the reaction to take place rapidly enough yet it is still safe for bare hands handling of the volumetric flasks in/out of the water bath. Higher or lower temperatures can be used to control the reaction time, in this case, 40 minutes was the desired reaction time.

4.3.7 Further Optimization of Reaction Condition

During the initial testing phase using the method used by Fan *et al.* (2006), attempts to produce precise results were unsuccessful. The fixed time method that was used by Fan *et al.* (2006) would have to include exact time sequence in which the reaction was initiated, quenched and subsequently scanned with fluorescence spectrofluorometer which the fixed time sequence time sequences was not specified in his journal. In consequence, error in time accuracy for each step is inevitable. To improve this situation, extra steps in the initiation, and quenching part of the reaction was included to enable us to attain a better level of precision.

4.3.7.1 Optimization of Initiation of Reaction

For the initiation step, after HCl, Rh6G, Fe(III) and sample were added into the 25 mL volumetric flask, the flask was first heated (60 ºC) in the water bath for 10 minutes. This will allow the flask and the content to reach temperature equilibrium with the water bath before the reaction was initiated with the addition of H_2O_2. This would prevent inconsistent heating rates due to small variations in mass and shape of the volumetric flask as well as initial room temperature variations from causing inconsistent results.

4.3.7.2 Optimization of Chelating Agents

In the methodology used by Fan *et al.* (2006), after the mixture was reacted for 12 minutes at 60 ºC, the flask was cooled to room temperature by

running water and then the fluorescence intensity was determined under a fixed time method. Previous attempts to use the same method were unsuccessful at getting precise results. It is because precise cooling rate was not attainable due to the same reasons as mentioned for the initiation stage.

Other attempts to reduce the reaction rate also includes by cooling down the reaction in ice bath to quench the reaction and also by adding excess NaOH to convert Fe(II) and Fe(III) into hydroxide form. The latter method decolourized Rh6G instantly. These attempts were failed for quantitation.

For catalytic activity, iron requires at least one coordination site that is open or occupied by a readily dissociable ligand such as water (Graf *et al*., 1983). To fully inactivate iron from reacting with hydrogen peroxide to perform radical formation, the chelating agent would have to occupy all the coordination sites of the iron ion.

In the study by Graf *et al*., it was shown that chelation of iron by phytate (Graf *et al*., 1983; Graf et al., 1987) and pentetic acid (Graf *et al*., 1983) excludes iron-associated water and uncouples the oxidation of Fe^{2+} from the formation of $^{\bullet}OH$. However, EDTA was shown to be unable to fully suppress the formation of $^{\bullet}OH$ as aquo coordination sites are still available. EDTA possesses 6 centres to form coordination bonds with metals; however it is known that the structure of Fe^{3+}.EDTA complex includes a seventh coordination site occupied by water (Lind *et al*., 1964). Pentetic acid can be viewed as an expanded version of EDTA and it is used similarly (**refer to Figure 4.8**). The conjugate base of pentetic acid

(DTPA) has a high affinity for metal cations. Thus, the penta-anion $DTPA^{5-}$ is potentially an octadentate ligand (Lind *et al*., 1964). The formation constants for DTPA (pentetic acid) complexes are also about 100 greater than those for EDTA (Hart, 2005).

Chelation is the formation or presence of two or more separate bindings between a polydentate (multiple bonded) ligand and a single central atom (IUPAC, 1997). The effects of several iron chelators were tested namely ethylenediaminetetraacetic acid (EDTA) in its disodium salt form, phytic acid, pentetic acid (DTPA) and phosphoric acid. Their chemical structures is shown in **Figure 4.8.**

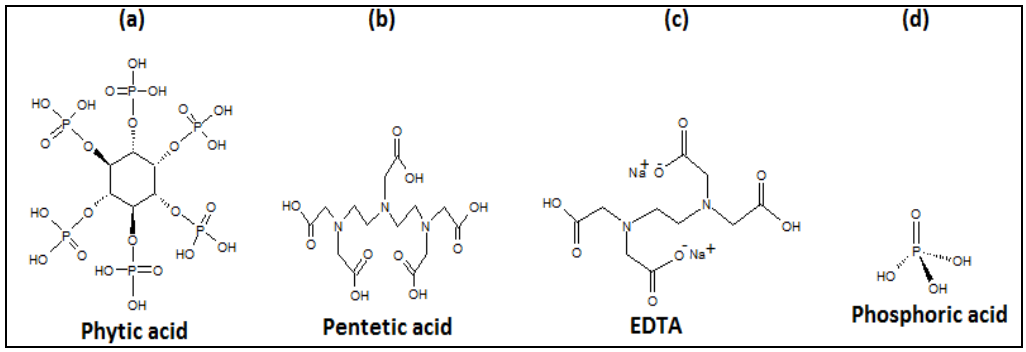


Figure 4.8: Chemical structure of (a) Phytic acid, (b) Pentetic acid, (c) disodium salt of EDTA & (d) Phosphoric acid

Firstly, these chelating agents were tested at the same concentration dose. When 1.0 mL of the chelating agent solution (0.01 M) was added into the 25.6 mL reaction solution, the volume would increase to 26.6 mL and the chelators concentration would be 3.76×10^{-4} M. This concentration is about 100 times

higher than the concentration of Fe ions (0.1880 mg/L or 3.37 x 10^{-6} M) in the resulting solution. The intensity of the emission at 547 nm for each standard solution is shown in **Table 4.11 (Appendix B).** In **Figure 4.9**, all the 4 types of chelators still show the trend of fluorescence intensity decreasing with time, but the slope of the graphs shows that the rate of decrease has slowed down when chelators were added. The slope with the lowest gradient was observed for pentetic acid and it was chosen to be used throughout this project. For a period of 60 minutes, the fluorescence intensity only drops by about 9 AU and it equals to about 2.1 µg/L of BPA.

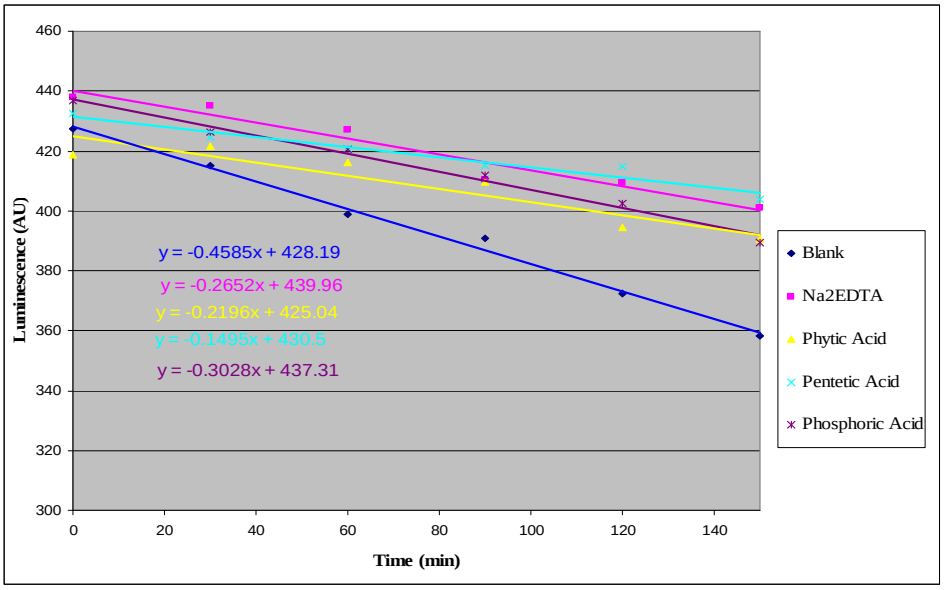


Figure 4.9: Effect of different chelating agents on the fluorescence intensity with time

The effect of the concentration of pentetic acid was also studied at pentetic acid/Fe(III) ratio of 111, 11, and 1 times. The results are shown in **Table 4.12 (Appendix B)** and plotted in **Figure 4.10**. The figure shows that the slope of the

graphs for 11x remains the same compared to 111x when the concentration of pentetic acid was reduced from 111 times to 11 times higher than the concentration of Fe(III), but becomes steeper when the ratio was reduced to only about 1 times (1x compared to 111x). This shows that the concentration of pentetic acid was high enough and had reached equilibrium.

None of the studied chelating agents was able to completely quench the oxidation of Rh6G as shown by the slope of the graph, a flat graph would be shown if otherwise. Nonetheless, the use of chelating agent in combination with cooling the volumetric flask with running water greatly improved the precision of the results as compared to just cooling with running water alone to stop the reaction.

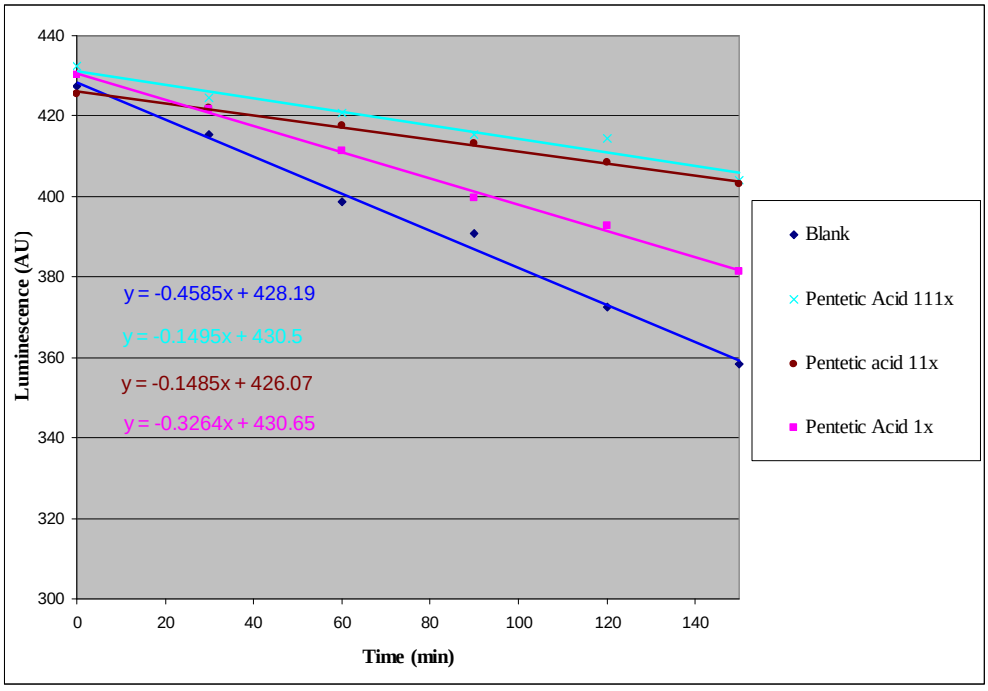


Figure 4.10: Effect of pentetic acid concentration on the change in fluorescence intensity with time

4.4 Calibration Curve for Detection of BPA using Fluorimetric Method

In **Table 4.13 (Appendix B)**, the results for the 11 blank replicates are shown. The mean value (28.0605 AU) was used as the F_o value throughout this project and was used to deduct the F value of standards. The standard deviation value (6.1157 AU) was used for calculation of LOD and LOQ as shown in **Table 4.13 (Appendix B)**. The LOD and LOQ calculated were 4.2 and 13.9 μg/L, respectively.

The results for the 10.0, 20.1, 40.3, 60.4, 80.5, and 100.7 μg/L standard solutions are shown in **Table 4.14 (Appendix B)**. The calibration curve shown in **Figure 4.11** was generated from 5 times replicate preparation of standard solutions at each concentration level. The calibration curve was rectilinear using the method of Least-Squares. The linear regression formula produced is $y = 4.3912x + 3.8842$ with an acceptable coefficient of determination, R^2 value of 0.9966 considering all the replicate standard solutions was separately prepared. The linear concentration range is 10.1 – 100.7 μg/L. The error bars on the points of the calibration curve was shown as the standard error of the mean estimate (SEM).

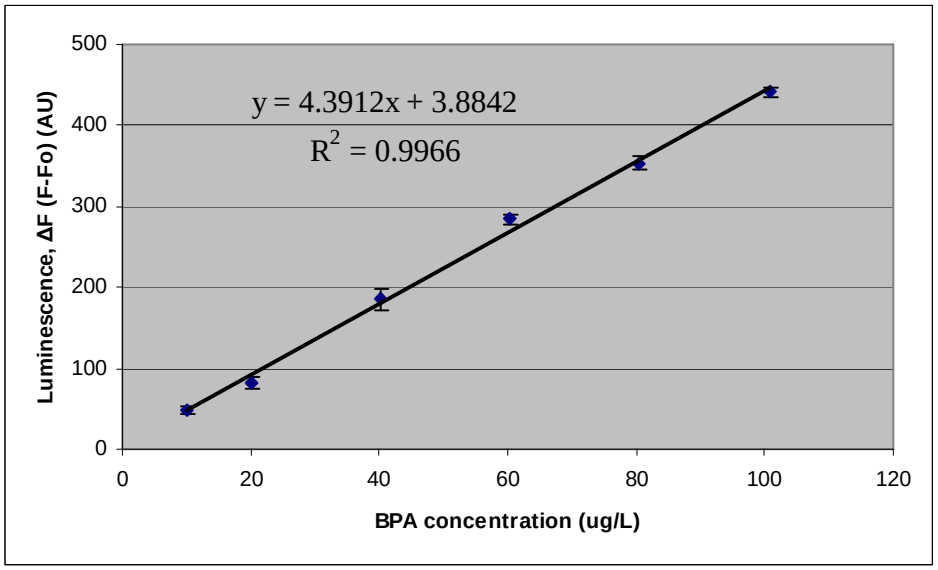


Figure 4.11: Graph of ΔF ($F-F_0$) against concentration of bisphenol A standard solutions

4.5 Overview of Optimized Reaction Conditions

The optimized conditions and range of study in our project as well as in comparison with the method used by Fan *et al*. (2006) are shown in **Table 4.15**. Compared to the method in Fan *et al*. (2006), the method proposed in this project uses lower concentrations of acid and Fe(III) which gives a longer reaction time.

Reaction is initiated at every 1 minute interval for each sample in this project, so a 40 minutes reaction time would allow up to 39 samples to be processed at a time. The longer reaction time is more ideal as more samples can be tested at a time in this project and might be more suitable for industrial application. The use of chelating agent in the quenching step also greatly improves the precision of the method in this project.

Table 4.15: Comparison of optimized conditions and range of study

Parameters of study	Fan *et al*. (2006)		Current study*	
	Range of study	Optimum condition	Range of study	Optimum condition
Hydrochloric acid (mol/L)	2.4×10^{-3} to 2.88×10^{-2}	8.4×10^{-3}	0.00 to 308.20×10^{-4}	9.38×10^{-4}
Fe(III) (µg/mL)	0.2 to 1.0	0.6	0.1953 to 1.1719	0.1953
H_2O_2	0.0048 to 0.0336 %	0.0144 %	1.53 to 9.18×10^{-3} M	4.59×10^{-3} M
Rh6G (mol/L)	4.0×10^{-7} to 4.0×10^{-6}	1.6×10^{-6}	3.91×10^{-7} to 3.13×10^{-6}	23.49×10^{-7}
Temperature (ºC)	30 to 65	60	-	60
Reaction time (min)	1 to 16	12	10 to 70	40
Use of chelating agent	-	-	Pentetic acid, Phytic acid, Na2EDTA, and Phosphoric acid	Pentetic acid (3.76×10^{-4} M)**
LOD (µg/L)	-	2.0	-	4.3
LOQ (µg/L)	-	6.7	-	14.2
Linear concentration range (µg/L)	-	24 - 400	-	10.1 – 100.7

* Concentration in 25.6 mL mixture

** Concentration in 26.6 mL mixture (after addition of 1.0 mL chelating agent solution)

4.6 Development of Sample Pre-treatment using SPE

Solid-phase extraction (SPE) is a separation process by which compounds that are dissolved or suspended in a liquid mixture are separated from other compounds in the mixture according to their physical and chemical properties. Determination at the trace level requires a pre-concentration step and sufficient purification especially for food and environmental samples.

The use of SPE cartridges were considered to be suitable to be used in this project because it is less labour intensive and time saving method which has been widely used in the purification of a wide range of samples for determination of BPA. The use of SPE in the purification of samples for determination of BPA has mainly been used prior to analysis with chromatographic techniques like HPLC/FLD, LC/MS or GC/MS but it has not been applied prior to determination using a fluorimetric method like the one proposed by Fan *et al*. (2006).

In the work done by Fan *et al*. (2006), the purification of samples has been accomplished by pre-separation using strong anionic and cationic exchange resins. The removal of interfering phenolic compounds by the use of XDA-1 (Xi'an, China) absorption resin was also mentioned. Based on this study, a comparison between the use of SPE compared to ion exchange resins used by Fan *et al*. (2006) is shown in **Table 4.16**.

Table 4.16: Comparison of sample preparation method

Fan *et al*. (2006)	Current study
Pre-separation using strong anionic and cationic exchange resins, the use of XDA-1 absorption resin was also mentioned	Pre-separation using SPE cartridges C-18E (endcapped)
Requires acidification prior to loading into resin	No acidification required
Requires manual packing of resins which could lead to inconsistent packing and compromise the precision of the method	Pre-packed cartridges are used
Only single sample could be processed at one time	Up to 12 samples can be processed at one time using 12 port vacuum manifold significantly improving the automation and efficiency of the method.
Does not use organic solvents	Methanol, n-hexane and ethyl acetate is used in this study. But the consumption of organic solvents in SPE method is relatively low compared to other techniques like liquid-liquid extraction.
Anionic and cationic resins can be regenerated for re-use	Single use only
Sample was not pre-concentrated in the study	Has the ability for pre-concentration of samples, offering higher sensitivity in detecting BPA in very trace amounts

SPE cartridges with 500 mg of Octadecyl C_{18} (end-capped) functionalized silica sorbent were selected for study in this project. Compared to ion exchange resins which are based on the electrostatic attraction of charged functional groups of the analyte to oppositely charged functional groups on the sorbent, the stationary phase of a reversed phase SPE cartridge

is derivatized with hydrocarbon chains which use hydrophobic (van der Waals) interactions to retain compounds of mid to low polarity. The use of ion exchange resins by Fan *et al*. (2006) was for removal of interfering ions as some metal ions are known to catalyze the Fenton reaction. The use of a reversed- phase sorbent will only retain mid to low polarity compounds while metal ions and highly polar molecules will not be retained. But, since the reverse phase coating also retains many mid to non- polar compounds that could also be oxidized by the Fenton reagent, steps to remove these interferences was included during WASH step prior to elution of BPA from the sorbent.

A reversed- phase SPE sorbent is commonly activated with methanol or acetonitrile then conditioned with water. Then, the aqueous sample is loaded and the interferences were washed with 5-20% methanol/acetonitrile in water without prematurely eluting compounds of interest. Then elution of the analyte was accomplished with organic solvents of sufficient non-polar character.

A two step procedure in the WASH step of the solid phase extraction was employed using a highly polar solvent mixture (methanol in water) for the first step and a non polar solvent mixture (ethyl acetate in n-hexane) for the second step. Then, BPA was eluted by using an increased polarity solvent mixture from the second wash step. The volume of solvent needed for sufficient activation of sorbent and elution is dependent on the volume of sorbent used. A general guide of a minimum 3 mL for conditioning, wash and

elution was recommended by the manufacturer (Phenomenex Inc., 2010). The results are shown in **Table 4.17 – 4.19 (Appendix D)**.

The graph in **Figure 4.12** shows the effect of increasing methanol concentration as the wash solvent on the recovery of BPA. The aqueous solvent could remove residual metal ions and some of the polar organic compounds. The recovery of BPA remains stable above 90% when up to 20 % methanol were used but the recovery reduces with further increase of methanol concentration. Thus, 20 % methanol in water was chosen to be used for further development of the method.

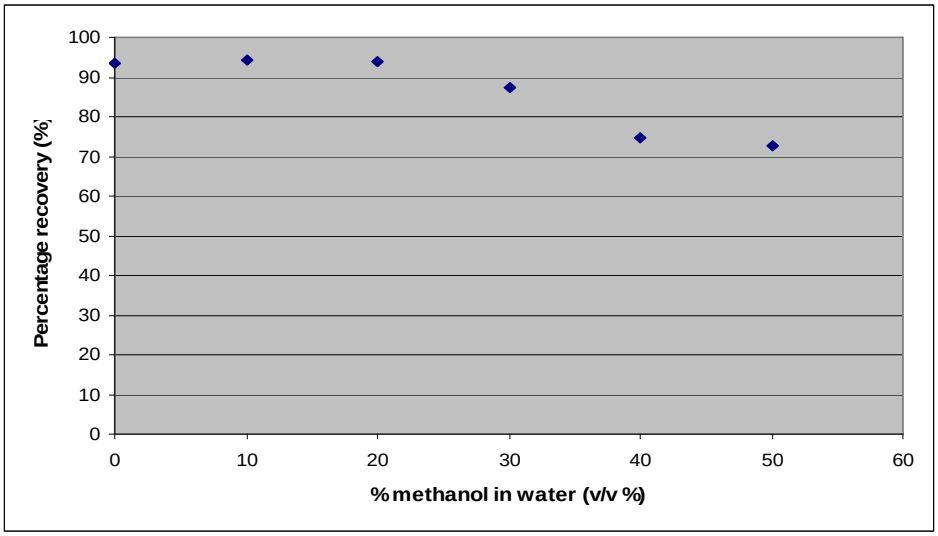


Figure 4.12: Graph of percentage recovery of BPA against concentration of methanol for first WASH step

It was observed that BPA has low solubility in a non-polar solvent like n-hexane but highly soluble in a more polar solvent like ethyl acetate. Thus, a second step of the WASH step was included to remove non-polar organic

compounds using non-polar solvent. In **Figure 4.13**, it can be observed that the % recovery was still above 90 % when up to 5 % ethyl acetate was used but BPA was almost fully eluted when the concentration of ethyl acetate were increased to 20 %. Thus, 5 % ethyl acetate was chosen as the second WASH solvent and 20 % ethyl acetate is used for elution of BPA. This will lessen the possible interfering compounds from being co-eluted with BPA.

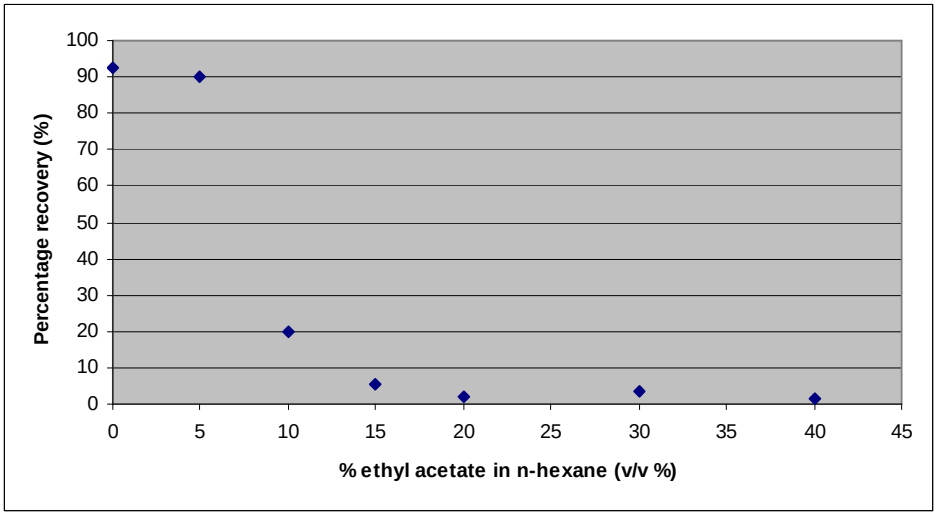


Figure 4.13: Graph of percentage recovery of BPA against concentration of ethyl acetate for second WASH step

4.7 Optimizing RP-HPLC/FLD System for BPA Separation and Detection

Bisphenol A is a nonionic polar molecule and a stationary phase C_{18} column [LiChroCART® 150-4,6 HPLC Catridge *Purospher® STAR RP-18 endcapped (5µm)*] was suitable for use. In order to choose the right elution condition to obtain well resolved bisphenol A separation, initially some trial and error were done by testing with different mobile phase composition such

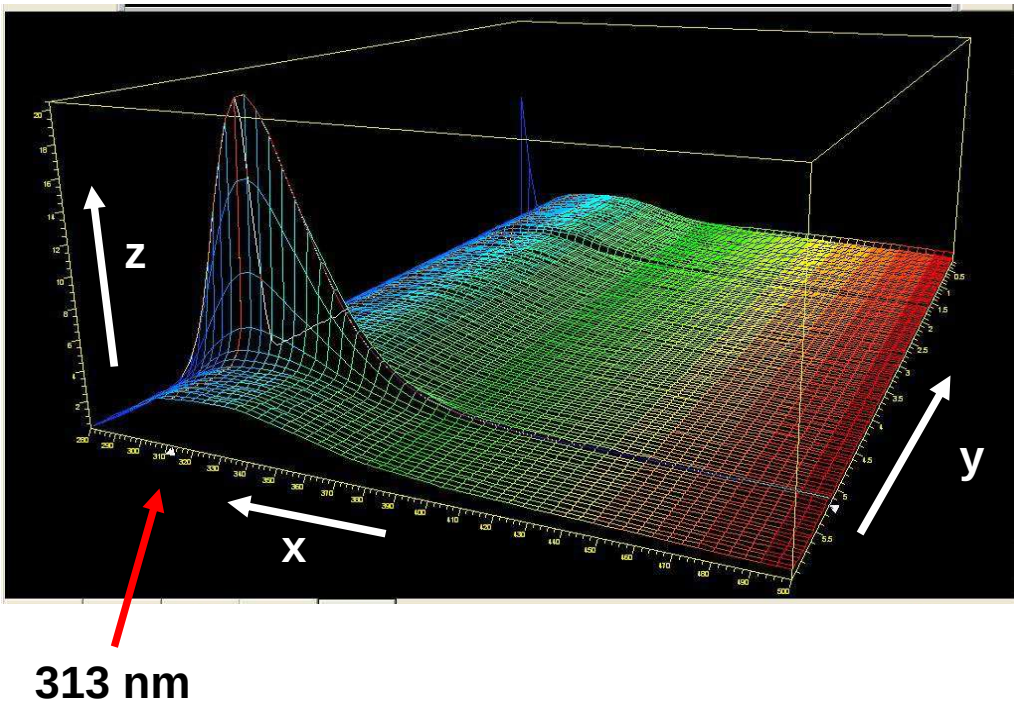
as methanol:deionized water in the ratio of 60:40, 65:35 and 70:30 with the flow rate 1.0 mL/min at ambient temperature. An isocratic mobile phase consisting of methanol: deionized water at a ratio of 65:35 was chosen as all the compounds eluted were well resolved and were fully eluted in a reasonable time. **Table 4.20** shows the complete sequence of mobile phase composition and flow rate for elution, column flushing and equilibration. The retention time for the peak corresponding to bisphenol A appeared at approximately 5.1 minutes.

Table 4.20: Elution conditions for HPLC/FLD

Time (min)	Methanol (%)	Water (%)	Flow rate (mL/min)	Condition
0.0 – 6.0	65	35	1.0	Isocratic elution
6.1 – 8.0	100	0	1.0	Column flushing
8.1 – 10.0	100	0	1.5	
10.1 - 20	100	0	2.0	
20.0 – 30.0	65	35	1.0	Equilibration

Fluorescence detection is highly suitable in detecting bisphenol A as the 2 benzene rings and a symmetrical structure of bisphenol A give it an exceptional fluorescing ability. As most of the compounds do not fluoresce, this becomes an advantage to detect limited species of compound. The optimization of detection wavelengths and response time of the detector is crucial to attain the most sensitive detection capability.

The excitation and emission spectrum of BPA was obtained using 3D Plot in the Chemstation software. To determine the most sensitive emission wavelength, 228 nm was set as the excitation wavelength with the emission scanned in the range of 280-500 nm. The 3D plot (**Figure 4.14**) shows the chromatogram with the highest intensity between the peak maxima compared to the baseline was observed when the emission wavelength is at 313 nm.

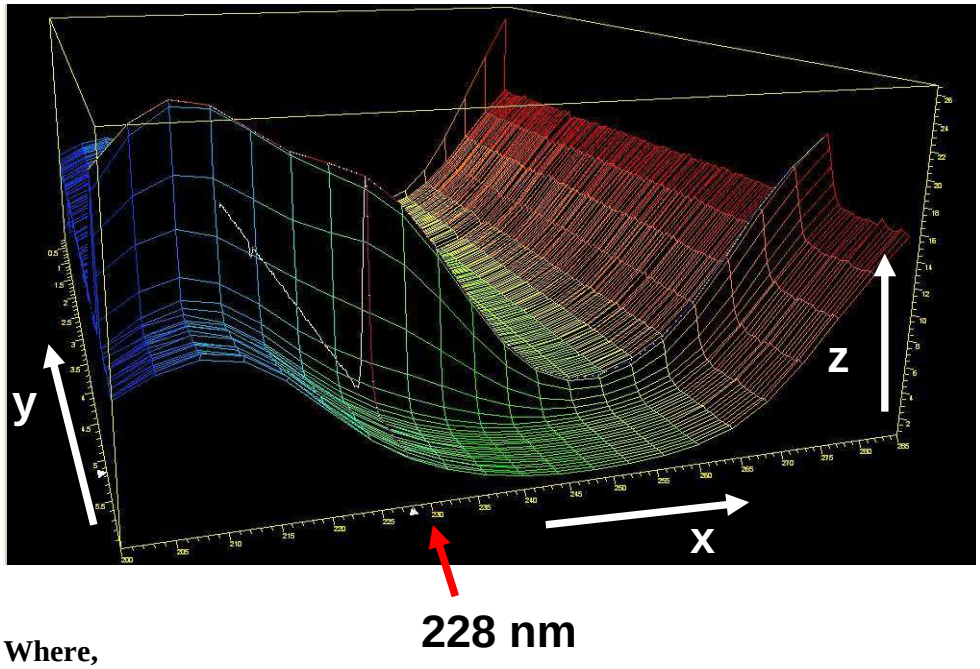


Where,

x-axis = emission wavelength (nm),
y-axis = time (min)
z-axis = intensity(LU)

Figure 4.14: 3D Plot of emission scan (280- 500 nm), excitation wavelength 230 nm

To determine the most sensitive excitation wavelength, the emission intensity at 313 nm was scanned with the excitation wavelength varied between 200- 285 nm. The 3D plot in **Figure 4.15** shows the chromatogram with the highest intensity between the peak maxima compared to the baseline was observed when the excitation spectrum is at 228 nm.



Where,

x-axis = excitation wavelength (nm),
y-axis = time (min)
z-axis = intensity(LU)

Figure 4.15: 3D Plot of excitation scan (200- 285 nm), emission wavelength 313 nm

A too-fast response time permits excessive noise in the chromatographic signal, and a too-slow response time distorts peak shapes, potentially reduces peak resolution, and makes peak start- and end-point identification more difficult. In order to obtain the highest signal-to-noise ratio

of the analyte peak, a detector time constant that is approximately 10% of the minimum peak width at half height in a normal chromatogram should be chosen (Hinshaw, 2001). The peak width at half height for the BPA peak is about 0.13 to 0.14 min and a detector response time of 2 seconds was suitable according to the Chemstation software.

A chromatogram of 501.1 µg/L BPA solution is shown in **Figure 4.16**. The column and operating conditions is shown in **Section 3.5**.

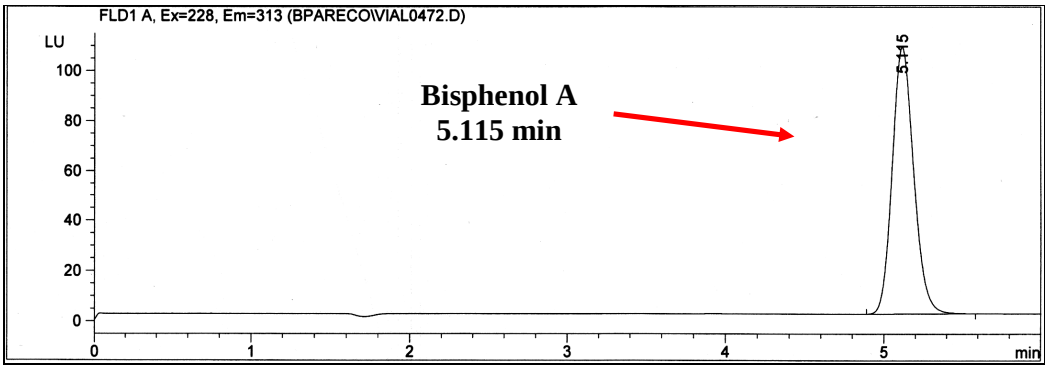


Figure 4.16: Chromatogram of 501.1 µg/L BPA solution

4.8 Calibration Curve for Determination of BPA using HPLC/FLD

The calibration curve shown in **Figure 4.17** was generated from five times replicate injections at each concentration and the peak area from the injections are shown in **Table 4.21 (Appendix C)**. The calibration curve were generated using BPA standard in the range of 10.2 to 5001.1 µg/l and rectilineared using the method of Least-Squares with the linear regression formula of Y=2.1444X + 1.5981 and coefficient of determination, R^2 value of 0.9995 which shows excellent linearity of the analytical method under examination.

Limit of detection, LOD is the lowest quantity of a substance that can be distinguished from the absence of that substance within a stated confidence limit (95%). Limit of quantification, LOQ is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. The standard deviation of the y- intercept measured according to **Section 3.3.4** is 1.2909 Lu*s. The LOD and LOQ calculated is 2.0 and 6.0 µg/l respectively (**Appendix C**).

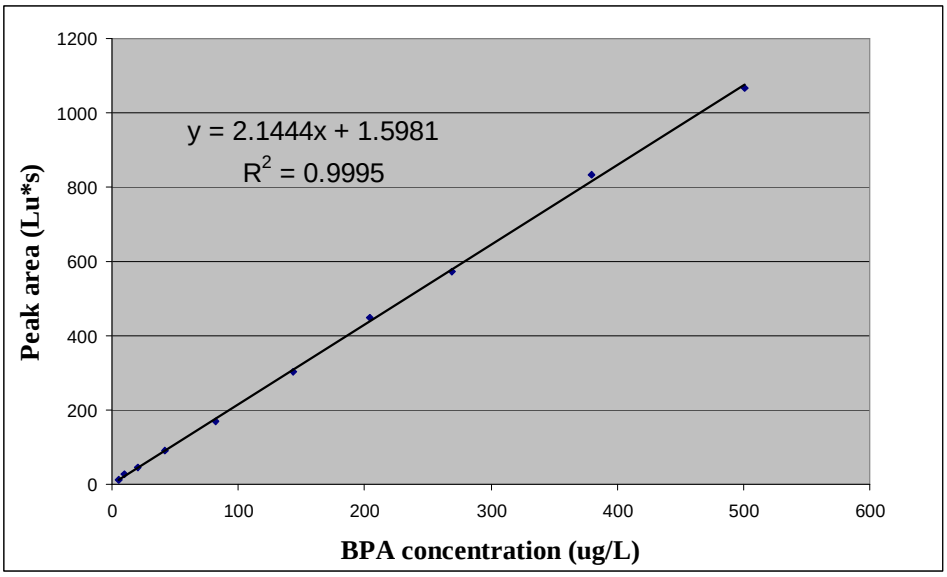


Figure 4.17: Graph of Peak Area against Concentration of bisphenol A Standard Solutions

4.9 Determination of Samples

The results from the PC bottles, canned foods and environmental waters determined using the fluorimetric method and HPLC/FLD are

summarized in **Table 4.21**. All the data recordings and calculations are shown in **Appendix E**.

Three types of polycarbonate drinking bottles were tested, among them; only the water sample from PC2000 which has a volume of 2000 mL contained enough sample volume to be tested using the SPE procedure in the fluorimetric method. All the other samples were determined directly using both methods. As can be observed in **Table 4.22**, the concentration of BPA tested using both methods are quite similar for PC bottles. The results from direct determination of PC650 and PC250 using fluorimetric method show slightly higher concentrations compared to HPLC determination which suggests that other compounds may have leached out from the polycarbonate that also shows inhibition to the fluorescent reduction. But since the reaction condition has been optimized to show highest selectivity towards BPA, the effect of other interferences was minimized. Using SPE for the PC2000 sample, the concentrations detected were very close using both methods. However, we could not conclude that the use of SPE would provide more accurate results as comparative results between samples treated with SPE and without SPE using identical samples was not available for these samples. Nonetheless, the use of SPE is advantageous to provide pre-concentration capability to the method and enabled the concentration of SPE2000 be determined at higher than LOQ level.

Table 4.22: Determination of BPA using Fluorimetric and HPLC method

	Sample	BPA concentration ± Standard deviation	
		Fluorimetric	HPLC/FLD
PC Bottles (µg/L)	**PC650A**	7.9 ± 1.5	5.8 ± 0.7
	PC650B	8.9 ± 0.4	4.5 ± 1.0
	PC650C	5.9 ± 0.8	3.1 ± 1.0
	PC250A	11.7 ± 2.7	4.2 ± 0.4
	PC250B	8.6 ± 0.8	2.5 ± 0.4
	PC250C	5.6 ± 2.6	ND ± -
	PC2000A	9.4 ± 0.1	9.1 ± 0.5
	PC2000B	9.6 ± 0.3	9.0 ± 0.7
	PC2000C	10.5 ± 0.5	8.5 ± 0.3
Canned foods (µg/kg)	**GPA**	142.02 ± 7.31	12.38 ± 0.28
	GPB	147.26 ± 2.47	11.41 ± 0.24
	TUNAA	412.72 ± 9.72	17.25 ± 0.70
	TUNAB	419.72 ± 10.39	17.64 ± 0.36
	SHROOMA	176.65 ± 10.37	3.22 ± 0.29
	SHROOMB	186.41 ± 8.89	2.96 ± 0.07
Environmental waters (µg/L)	**TW**	ND ±	0.085 ± 0.009
	UW	0.543 ± 0.055	ND ± -
	LK	ND ±	ND ± -
	TT	0.642 ± 0.045	ND ± -
	PD	0.377 ± 0.110	0.023 ± 0.003

ND - Not detected
* Concentrations stated after corrected with pre-concentration factor

Three types of canned foods were tested in this experiment. The major components like protein, fat, carbohydrate, minerals and fiber composition are varied for each type of food. These samples were blended to ensure homogeneity. Then, 5 grams of sample was extracted into acetonitrile and water residue was dried off with anhydrous sodium sulfate. Lipid removal in food samples is essential since it affects the active surface of the stationary phase in HPLC and degrades the resolution power of the column. In GC/MS,

lipids accumulate in the injection port, column and ion source (Ballesteros-Gomez *et al.*, 2008). Lipid removal is made by liquid- liquid extraction (LLE) of the acetonitrile extract with n- hexane (Sun *et al*., 2006; Munguia- Lopez *et al*., 2005; Yoshida *et al*., 2001). Due to the moderately polar nature of BPA, it is highly soluble in polar solvents like acetonitrile but poorly soluble in non-polar solvents like n- hexane. The results for the canned foods show a large difference between the two methods. As the matrix in foods is very rich and complex, the SPE extraction method was not capable to purify the sample enough. The impurities had competed with Rh6G along with BPA to be oxidized by hydroxyl radicals and contributed to the high concentration in the measurement. In addition to that, there are other reasons such as the presence of co-eluted compounds that emit light at the same wavelength as Rh6G at 547nm contributing to the high concentration in the measurement.

As observed in the results for canned foods and environmental waters, the fluorimetric method is highly sensitive to impurities which the SPE could not fully removed. As the method was only developed using deionized water to represent the wide range of samples, bias in determination caused by uncertainties in the sample matrix was unavoidable. To prevent this, the samples could be developed in a representative blank that resembles closely to the specific type of sample. Thus, this method is only recommended to be used as a screening procedure unless it was developed to be used in one specific type of known sample. So far, the results only showed higher concentration compared to determined using HPLC, thus it suggests that the compounds that co-eluted were compounds that competed with Rh6G along with BPA to be oxidized by hydroxyl radicals. In addition to that, there are other reasons such

as the presence of co-eluted compounds that emit light at the same wavelength as Rh6G at 547nm contributing to the high concentration in the measurement.

The spiking recoveries using both methods are shown in **Table 4.23**. The percentage recoveries for all the samples tested is close to 100% and means that there was no interference or factors that will cause the results to deviate from the accurate value. Results and calculations are shown in **Appendix E**.

Table 4.23: Spiking recovery of BPA using Fluorimetric and HPLC method

	Sample	Percentage recovery ± Standard deviation (%)	
		Fluorimetric	HPLC/FLD
PC Bottles	PC650A	92.64 ± 7.35	
	PC650B	88.38 ± 4.57	
	PC650C	93.73 ± 3.30	
	PC250A	95.90 ± 5.84	
	PC250B	94.38 ± 2.67	
	PC250C	93.43 ± 5.55	
	PC2000A	90.78 ± 7.86	
	PC2000B	89.01 ± 4.11	
	PC2000C	92.71 ± 3.55	
Canned foods	GPA	97.87 ± 2.90	86.26 ± 2.39
	GPB	104.24 ± 1.91	91.13 ± 2.34
	TUNAA		101.14 ± 4.54
	TUNAB		97.38 ± 4.37
	SHROOMA	96.99 ± 4.19	84.67 ± 2.59
	SHROOMB	92.00 ± 3.58	87.65 ± 5.05
Environmental waters	TW	103.91 ± 3.22	89.42 ± 4.12
	UW	101.00 ± 4.84	91.76 ± 4.24
	LK	98.27 ± 6.42	93.93 ± 7.37
	TT	102.48 ± 7.22	88.96 ± 1.91
	PD	91.98 ± 0.89	87.10 ± 3.15

* No result for TUNA A and B for fluorimetric method as the concentration detected is above the calibration range

4.10 Validation of the Sample Preparation Procedure for Spectrofluorimetric Method

The precision and accuracy of the sample preparation and SPE extraction for canned foods, environmental water and water incubated in PC bottle (**refer to Section 3.6.1**) were validated at different concentration levels covering the range of the calibration curve. **Table 4.39** shows the percentage recovery of the sample preparation method for canned foods, environmental waters and water incubated in PC bottle (PC2000) and the accuracy of these methods was similar with an average recovery of 88.05 %, 87.95 % and 88.60 % respectively. The standard deviations of the methods are within 5.41 % which shows good precision.

Table 4.40 shows that the found concentration was close to zero and that the deviation can be assumed to be caused by random errors that occurred in the measurement process. Thus, it can be concluded that the preparation procedure for all 3 methods does not contribute any bias in the quantification of BPA. The raw result and calculations are shown in **Table 4.41 and 4.42** (**Appendix D).**

Table 4.39: Percentage recovery of canned food, environmental waters and water incubated in PC bottle sample preparation method

Added BPA concentration (in 25.6 mL) (µg/L)	Percentage recovery, mean ± std. deviation (%)		
	Sample preparation for canned foods	Sample preparation for environmental waters	Sample preparation for PC bottle (PC2000)
20.1	89.71 ± 5.41	85.92 ± 4.74	87.94 ± 2.90
60.4	88.31 ± 1.70	89.63 ± 2.10	90.39 ± 2.35
100.7	86.12 ± 2.13	88.29 ± 2.47	87.47 ± 1.93

Table 4.40: Concentration found without BPA added

Added BPA concentration (in 25.6 mL) (µg/L)	BPA concentration founds, mean ± std. deviation (µg/L)		
	Sample preparation for canned foods	Sample preparation for environmental waters	Sample preparation for environmental waters
0	-0.8 ± 0.6	-0.8 ± 1.2	0.3 ± 1.8

4.11 Validation of the Sample Preparation Procedure for HPLC Method

The sample preparation procedure for canned food and environmental water (refer to **Section 3.7.2**) was evaluated to check if any impurities that arise from the sample preparation method, may affect the determination of

BPA. **Figure 4.18** shows that there are no overlapping impurity peaks between 4.5 to 5.3 min with the retention time of BPA (4.95 – 5.25 min).

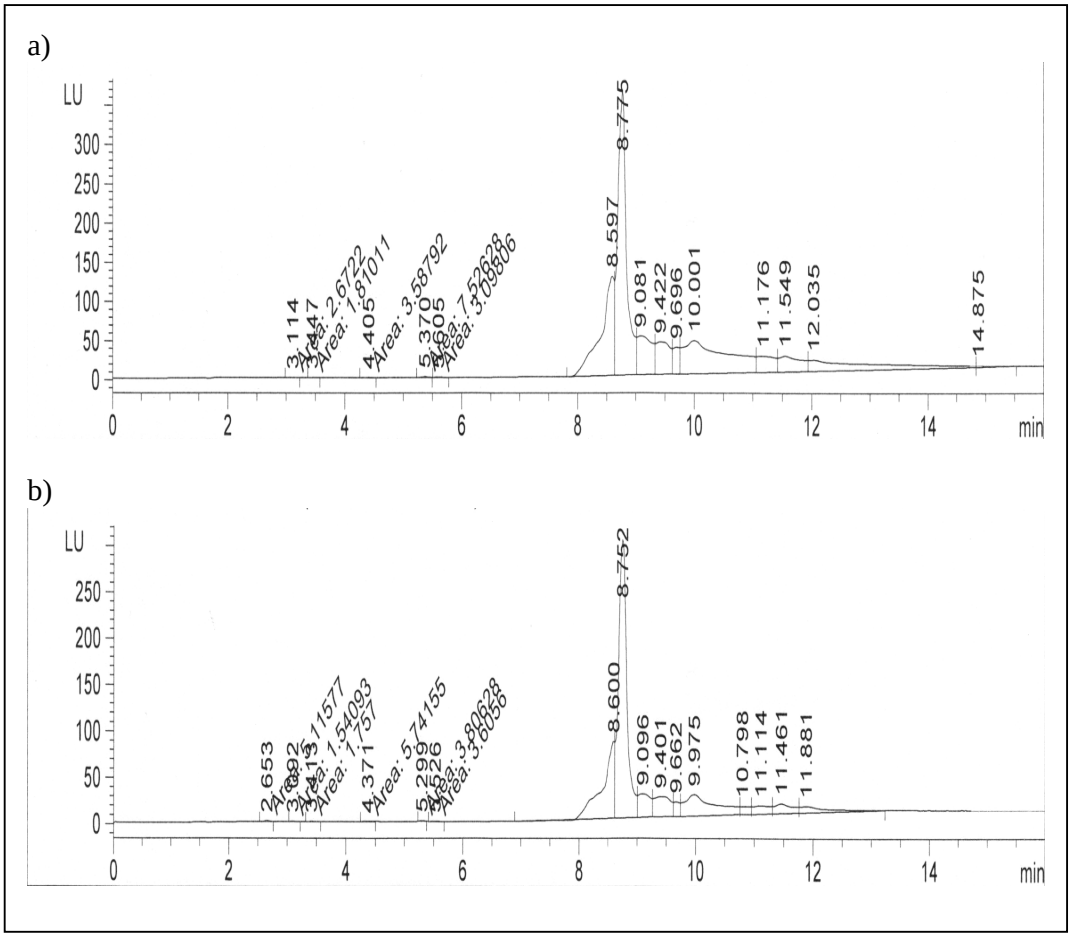


Figure 4.18: Chromatogram of blank samples using sample preparation procedure for a) canned food and b) environmental waters

4.12 Confirmation of BPA using Gas Chromatography with Mass Spectrometer (GC/MS)

The canned foods and environmental waters that were tested positive for detectable levels of BPA were also tested using GC/MS. Detection using scanning mode between m/z 100 to 300 was used. The spectrum of a standard 390 pg/µl of BPA in ethyl acetate is shown in **Figure 4.19 (Appendix F)**. **Figure 4.19a** shows the Total ion chromatogram (TIC) which represents the summed intensity across the entire range of m/z = 100 - 300 being detected at every point in the analysis. In the Extracted Ion Chromatogram (EIC) (**Figure 4.19b)**, the total intensity at m/z = 213.0 is plotted at every point in the analysis. By obtaining the EIC at m/z = 213.0, the retention time for BPA was found to be at 18.000 to 18.050 min.

The mass spectrum in the range of m/z 100 – 300 was obtained at the retention time of BPA (18.026 min) **(Figure 4.19c)**. Only a very small relative abundance (%) for the molecular ion was found at m/z = 228.3 amu. The loss of a methyl radical generates the base peak at m/z = 213.3 amu (**Figure 4.20a**). The second highest intensity peak was observed at m/z = 119.2 amu which is equivalent to the ion structure proposed in **Figure 4.20b**. By calculating the molecular weight in **Figure 4.20b**, a molecular weight of 119.14 was obtained and is very close to the m/z = 119.2 obtained. For identification of BPA in the samples, the presence of the peaks at m/z at 213.3 and 119.2 had to be found and the ratio of % relative abundance of the peak at m/z 119.2 to 213.3 is also used for identification which is 365/1207 x 100 % = 30 % (refer to **Figure 4.19c**).

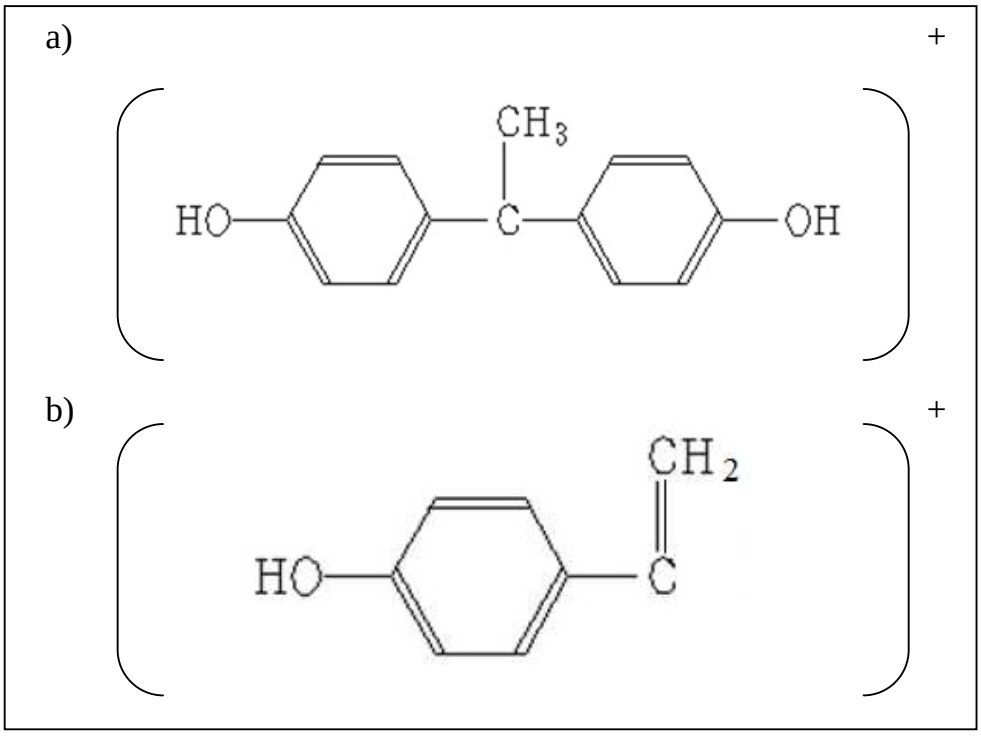


Figure 4.20: Proposed ion structure of mass fragment at m/z a) 213.3 and b) 119.2.

The GC/MS results for the samples are shown in **Appendix F (Figure 4.21)**. All the samples showed characteristic peak in the range of 18.000 to 18.050 min with the EIC measured at m/z = 213.0. Each of the TIC also showed both the peaks at m/z 213.3 and 119.2. The ratio of % relative abundance of the peaks at m/z 119.2 to 213.3 was also between 25 to 34 %. Thus, the samples detected to contain BPA using the HPLC was also validated using GC/MS.

CHAPTER 5

CONCLUSIONS AND FUTURE STUDIES

It is suggested that in Fenton-like reaction at hydrochloric acid medium, there is a competition between BPA and Rh6G for the reduction of hydroxyl radicals. Fluorescence reduction of Rh6G is therefore inhibited. Fenton-like reagent was applied to the determination of BPA in real samples, combined with fluorescence spectroscopy which offers excellent detection limits.

The fluorescence spectrometer was operated at excitation wavelength of 345 nm and emission wavelength at 547 nm was used for detection. An optimized reaction condition was obtained by investigating the optimum concentration of reagent concentrations and reaction condition. The optimized concentration of HCl, Fe(III), H_2O_2 and Rh6G was 9.38×10^{-4} M, 0.1953 μg/mL**,** 0.0141 % and 23.49×10^{-7} M, respectively. The optimized reaction time was 40 minutes and reaction temperature at 60 ºC. A quenching step was included in the development of this project by the addition of pentetic acid at the end of the reaction which greatly improves the measurement precision.

Compared to the method in Fan *et al*. (2006), the method proposed in this project uses a longer reaction time. The longer reaction time is more ideal as more samples can be tested at a time and are more suitable for industrial application. The linear regression formula produced is 4.3912x + 3.8842 with

a coefficient of determination, R^2 value of 0.9966. The method presents an instrumental LOD and LOQ of 4.2 and 13.9 μg/L, respectively.

Due to the high sensitivity and selectivity of RP-HPLC with fluorescence detector, it is a widely used instrument to detect the very small concentration of bisphenol A migrated into the various types of samples without undergoing derivatisation. It serves as a good benchmark for the fluorimetric method to be compared with. The excitation and emission wavelength for fluorescence detector were operated at 228 nm and 313 nm respectively. An isocratic elution of HPLC grade methanol: deionized water of 65: 35 as mobile phase and a flow rate of 1.0 ml/min at 30℃ were used. The injection volume was 20 μl. A reverse phase C_{18} column [LiChroCART® 150-4,6 HPLC Catridge *Purospher® STAR RP-18 endcapped (5μm)*] was used for elution of bisphenol A. The retention time for the peak corresponding to bisphenol A in the calibration curve has been identified to be eluted at approximately 5.1 minutes.

The calibration curve were rectilinear using the method of Least-Squares and the linear regression formula produced which is Y=2.1444X + 1.5981 with a coefficient of determination, R^2 value of 0.9995. The instrumental LOD and LOQ is 2.0 and 6.0 μg/l, respectively.

Solid phase extraction (SPE) provides a fast and easy way to purify large numbers of samples prior to determination. A two step washing step to remove as much impurities as possible was employed using 20 % methanol:

water solution and 5 % ethyl acetate: n-hexane solution. Then, to limit the co-elution of impurities with BPA, a 20 % ethyl acetate: n-hexane solution was used for elution. The relatively low amount of organic solvent used in SPE technique means it is also friendlier to the environment. The SPE procedure has good accuracy with high recovery of about 89 % and the standard deviations are within 5.5 % when applied with the fluorimetric method. The SPE technique also provides the ability to pre- concentrate samples that otherwise contain too low amounts of BPA to be detected especially for BPA migrated from PC bottles into water and environmental waters.

The fluorimetric method is sensitive to impurities in which without purification using SPE it would not be feasible to test real samples. The results for PC bottles show very close results between the 2 methods. For environmental waters, some of the samples were detected positive for BPA but in actual fact the BPA levels were too low to be detected using the fluorimetric method. But the concentrations detected were very low (< LOQ level), so it was still acceptable. As the sample becomes more complex and highly dense with interferences like food samples, the SPE was not adequate to purify the samples enough for the fluorimetric method. Although SPE removed most of the interferents, there is still a small amount that co-eluted during elution of BPA. It was observed that the results determined using the fluorimetric method would frequently shows a slight higher concentration compared to using HPLC which was able to separate the compounds for quantitation. Thus, this method is only recommended to be used as a screening procedure unless it was developed to be used in one specific type of known sample.

Another shortcoming of the fluorimetric method is the lower pre-concentration factor it could achieve as the sample has to be reconstituted to 25 mL after SPE extraction compared to using less than 1 mL for injection into HPLC. However, for the purpose of screening, an ultra trace detection capability may not be necessary as the method already posses a competitive instrumental detection level comparable to HPLC with fluorescence detection.

On the other hand, the SPE procedure works excellently for all the sample types tested when using HPLC/FLD as the chromatograms showed that the peak for BPA was able to be separated from nearby impurity peaks and baseline integrations were attained even using only isocratic elution. A SPE procedure prior to determination of BPA with HPLC/FLD that was suitable for a wide range of sample matrices was successfully developed. The percentage recoveries for all the samples tested using both method were close to 100% and means that there was no interference or factors that will cause the results to deviate from the accurate value.

For further studies, SPE technique using selective type of sorbent like immunosorbents (ISs) could be used. Imunosorbents provides unique selectivity on the basis of molecular recognition and can be designed for targeting just BPA, which is particularly suited to complex matrices. The method has been tested on food samples by Braunrath & Cichna (2005). Another type of class of sorbent called molecularly imprinted polymers (MIPs) also has the recognition ability for a target analyte. MIPs permits

larger sample volumes and reusability compared to ISs. An application has been developed for the MIP-based extraction of BPA from food by Martin-Esteban & Tadeo (2006).

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APPENDIX A

Calculations for the preparation of standard BPA solution for calibration (section 3.5.2) and standard BPA solutions for development of SPE (section 3.4)

Table 3.11 Weight of liquid transferred in preparing BPA standard solutions

Standard solutions	Weight of second stock solution (mg)	Weight of water added (mg)	Final BPA concentration (µg/L)
Cal 1	**10.21**	**9880.84**	**1.1**
Cal 2	**31.10**	**9907.89**	**3.2**
Cal 3	**50.55**	**9887.39**	**5.2**
Cal 4	**70.08**	**9871.87**	**7.2**
Cal 5	**99.89**	**9904.02**	**10.2**
Cal 6	**199.37**	**9735.78**	**20.6**
Cal 7	**403.18**	**9497.44**	**41.8**
Cal 8	**792.48**	**9137.24**	**81.9**
Cal 9	**1389.10**	**8554.44**	**143.3**
Cal 10	**1992.16**	**8001.25**	**204.5**
Cal 11	**2646.72**	**7451.35**	**268.8**
Cal 12	**3702.10**	**6289.33**	**380.0**
Cal 13	**4876.21**	**5103.76**	**501.1**
SPE 20	**495.97**	**25001.26**	**20.0**
SPE 200	**4998.23**	**20023.54**	**204.9**
SPE 500	**11545.93**	**12148.62**	**499.8**

Example of calculation to prepare Cal 14,

Room temperature = 28.5 ºC

Density of water at 28.5 ºC = 996.0926593 mg/mL*

Weight of second stock solution = 4876.21 mg

Volume of second stock solution $= \frac{\text{Weight of second stock solution}}{\text{Density of water}}$

= 4876.21 mg / 996.0926593 mg/mL

= 4.895338 mL

BPA added = Volume of second stock solution x concentration of second stock solution

= 4.895338 mL x 1.0256128 μg/mL

= 5.020721 μg

Weight of water added = 5103.76 mg

Total volume ** $= \frac{\text{Weight of second stock solution + water added}}{\text{Density of water}}$

$= \frac{4876.21 \text{ mg} + 5103.76 \text{ mg}}{996.092659 \text{ mg/mL}}$

= 10.019118 mL

Concentration of BPA second stock solution $= \frac{\text{Weight of BPA}}{\text{Total volume}}$

= 5.020721 μg / 10.019118 L x 1000

= 501.1 μg/L

* Density data is provided by National Institute of Science & Technology standard reference database

APPENDIX B

Table 4.1: Optimization of HCl concentration for blank and standard solution (first replicate)

Concentration of HCl (x 10^{-4} M)	- log[HCl]	Fluorescence intensity at 547nm (AU)		
		Blank, F_o	Standard, F	ΔF (F-F_o)
0	-	607.7071	637.1499	29.4428
0.47	4.3	467.2682	493.1499	25.8817
2.34	3.6	8.7282	147.4525	138.7243
9.38	3.0	45.8111	280.7835	234.9724
28.13	2.6	159.6565	351.6253	191.9688
93.75	2.0	198.0501	373.0593	175.0092
308.2	1.5	295.0370	388.0662	93.0292

Table 4.2: Optimization of HCl concentration for blank and standard solution (second replicate)

Concentration of HCl (x 10^{-4} M)	- log[HCl]	Fluorescence intensity at 547nm (AU)		
		Blank, F_o	Standard, F	ΔF (F-F_o)
0	-	595.1949	607.2196	12.0247
0.47	4.3	460.0627	471.2195	11.1568
2.34	3.6	8.2938	140.1225	131.8287
9.38	3.0	38.9897	266.1688	227.1791
28.13	2.6	154.1295	317.8574	163.7279
93.75	2.0	213.5283	329.3896	115.8613
308.2	1.5	299.3593	371.0442	71.6849

Table 4.3: Optimization of Fe(III) concentration for blank and standard solution (first replicate)

Concentration of Fe(III) (mg/L)	Fluorescence intensity at 547nm (AU)		
	Blank, F_o	Standard, F	ΔF (F-F_o)
0.1953	55.7941	959.3125	903.5184
0.3906	45.6202	486.4374	440.8172
0.5859	19.8563	245.5474	225.6911
0.7813	21.5192	111.3149	89.7957
0.9766	10.4839	53.8166	43.3327
1.1719	17.8349	24.1166	6.2817

Table 4.4: Optimization of Fe(III) concentration for blank and standard solution (second replicate)

Concentration of Fe(III) (mg/L)	Fluorescence intensity at 547nm (AU)		
	Blank, F_o	Standard, F	ΔF (F-F_o)
0.1953	93.4605	922.5626	829.1021
0.3906	45.4534	468.2264	422.7730
0.5859	20.9649	244.5000	223.5351
0.7813	18.2287	123.5719	105.3432
0.9766	12.0552	61.1986	49.1434
1.1719	12.6016	51.1436	38.5420

Table 4.5: Optimization of H_2O_2 concentration for blank and standard solution (first replicate)

Concentration of H_2O_2 (x 10^{-3} M)	Fluorescence intensity at 547nm (AU)		
	Blank, F_o	Standard, F	ΔF (F-F_o)
1.53	104.2928	715.5162	611.22
3.06	47.1835	563.0168	515.83
4.59	46.8528	480.3423	433.49
6.12	23.0929	428.0851	404.99
7.65	17.9013	366.9416	349.04
9.18	18.0380	262.2748	244.24

Table 4.6: Optimization of H_2O_2 concentration for blank and standard solution (second replicate)

Concentration of H_2O_2 (x 10^{-3} M)	Fluorescence intensity at 547nm (AU)		
	Blank, F_o	Standard, F	ΔF (F-F_o)
1.53	100.9961	712.7986	611.80
3.06	55.1516	559.5753	504.42
4.59	44.7440	473.2737	428.53
6.12	21.0170	418.3238	397.31
7.65	16.6794	352.0140	335.33
9.18	16.7194	261.4438	244.72

Table 4.7: Optimization of Rh6G concentration for blank and standard solution (first replicate)

Concentration of Rh6G (x 10^{-7} M)	Fluorescence intensity at 547nm (AU)		
	Blank, F_o	Standard, F	ΔF (F-F_o)
3.91	4.1980	73.8207	69.62
7.83	2.5536	166.3098	163.76
11.74	9.3904	283.2980	273.91
15.66	13.3996	393.3771	379.98
19.57	33.7289	604.1320	570.40
23.49	56.5352	668.2846	611.75
27.40	165.7955	715.1650	549.37
31.31	386.9185	864.8554	477.94

Table 4.8: Optimization of Rh6G concentration for blank and standard solution (second replicate)

Concentration of Rh6G (x 10^{-7} M)	Fluorescence intensity at 547nm (AU)		
	Blank, F_o	Standard, F	ΔF (F-F_o)
3.91	3.9045	66.7769	62.87
7.83	3.6002	157.8171	154.22
11.74	7.0737	278.2838	271.21
15.66	12.4127	379.4090	367.00
19.57	31.2985	523.2187	557.59
23.49	52.8311	632.1631	609.33
27.40	159.4602	707.8232	548.36
31.31	375.0320	827.6089	452.58

Table 4.9: Optimization of reaction time for blank and standard solution (first replicate)

Reaction time (min)	Fluorescence intensity at 547nm (AU)		
	Blank, F_o	Standard, F	ΔF (F-F_o)
10	847.9182	901.6939	53.7757
20	611.0358	746.2637	135.2279
30	319.3050	584.7097	265.4047
40	89.4799	451.6427	362.1628
50	36.4771	368.8679	332.3908
60	16.9606	308.8925	291.9319
70	6.2974	243.3819	237.0845

Table 4.10: Optimization of reaction time for blank and standard solution (second replicate)

Reaction time (min)	Fluorescence intensity at 547nm (AU)		
	Blank, F_o	Standard, F	ΔF (F-F_o)
10	837.7484	877.8161	40.0677
20	601.7501	732.1296	130.3795
30	308.5431	606.8216	298.2785
40	82.9844	443.4993	360.5149
50	33.5067	370.1925	336.6858
60	14.8585	303.9278	289.0693
70	4.9978	234.2929	229.2951

Table 4.11: Fluorescence intensity of 100.7 µg/L BPA standard solution added with different chelating agents

	Luminescence (AU)				
Time (min)	Water (without chelating agent)	EDTA (0.01 M)	Phytic Acid (0.01 M)	Phosphoric acid (0.01 M)	Pentetic Acid (0.01 M)
0	427.27	437.81	418.82	436.92	432.36
30	415.28	435.07	421.75	426.48	424.56
60	398.7	427	416.33	420.59	420.9
90	390.94	410.37	409.7	411.71	415.27
120	372.37	409.15	394.35	402.36	414.58
150	358.29	400.99	390.46	389.59	404.06

Table 4.12: Fluorescence intensity of 100.7 µg/L BPA standard solution added with pentetic acid at different ratios

	Luminescence (AU)		
Time (min)	Pentetic Acid (0.01 M)	Pentetic acid (0.001 M)	Pentetic acid (0.0001 M)
0	432.36	425.39	430.28
30	424.56	421.96	421.58
60	420.9	417.48	411.38
90	415.27	413.22	399.73
120	414.58	408.36	392.57
150	404.06	403.22	381.47

Table 4.13: Results for 11 blank replicates

Replicate	Luminescence, F_0 (AU)
1	25.2165
2	32.6770
3	27.5097
4	24.9388
5	18.7720
6	40.5449
7	34.5863
8	23.4114
9	30.7861
10	26.4174
11	23.8059
Mean	28.0605
Std. Deviation	6.1157

The limit of detection (LOD), LOD = $3S_b/k$, where S_b is the standard deviation of the regent blank (n=11) and k is the slope of the calibration curve.
LOD = 3 (6.1157)/4.3912
= 4.2 ug/L

The limit of quantitation (LOQ), is defined as LOQ = $10S_b/k$.
LOQ = 10 (6.1157)/4.3912
= 13.9 ug/L

Table 4.14: Results for BPA calibration solutions

Concentration (ug/L)	Luminescence, ΔF (F−F0) (AU)							
	Replicate					Mean	Std. Deviation	Std Error of Mean
	1	2	3	4	5			
10.1	40.6355	62.1571	56.9904	37.3014	49.4864	49.3142	10.53	4.7
20.1	57.7618	85.0933	92.245	73.2234	99.5798	81.5807	16.4865	7.4
40.3	164.092	176.933	219.136	154.597	209.265	184.804	28.1979	12.6
60.4	291.524	261.477	280.504	288.753	298.467	284.145	14.2119	6.4
80.5	376.263	348.1592	359.06	355.352	325.273	352.8213	18.5435	8.3
100.7	459.978	430.292	445.464	428.721	441.626	441.216	12.7052	5.7

APPENDIX C

Table 4.21: Peak area measurement of BPA standard solutions

[BPA] (µg/L)	Peak area (LU*s)					
	1	2	3	4	5	Mean ± SD
1.1	3.18497	2.80186	3.07368	2.87926	3.01945	2.99184 ± 0.15291
3.2	8.85673	7.46137	7.61131	7.30707	6.94828	7.63695 ± 0.72499
5.2	11.15286	13.93479	11.15467	11.58694	11.28944	11.82374 ± 1.19330
7.2	17.99257	19.20901	16.65650	15.71113	17.41356	17.39655 ± 1.32560
10.2	26.83229	26.08972	27.25895	28.04032	32.65588	28.17543 ± 2.60225
20.6	47.68242	46.14629	45.22810	45.18847	49.98298	46.84565 ± 2.02453
41.8	87.11948	93.08643	87.11600	85.53171	98.68282	90.30729 ± 5.50059
81.9	166.90596	170.28175	164.88303	172.84306	167.81416	168.54559 ±
143.3	295.22742	308.23074	307.04111	302.32419	305.61371	303.68743 ±
204.5	465.66406	453.89175	442.13559	437.53857	445.78555	449.00310 ±
268.8	561.82391	574.66101	583.75171	566.65753	578.19470	573.01777 ±
380.0	846.46063	825.16119	822.23016	817.11810	855.18091	833.23020 ±
501.1	1056.99207	1108.47168	1099.86096	1048.26196	1013.41559	1065.40045 ±

To calculate Limit of Detection (LOD) and Limit of Quantitation (LOQ),

LOD = 3.3(SD/b)
= 3.3 (1.2909 Lu*s / 2.1444 Lu*s L /µg)
= 2.0 µg/L

LOQ = 10(SD/b)
= 10 (1.2909 Lu*s / 2.1444 Lu*s L /µg)
= 6.0 µg/L

APPENDIX D

Table 4.17: Peak area of SPE20, SPE200 and SPE500 standard solutions

Standard recovery solution	BPA concentration (µg/L)	Retention time	Peak area (Lu*s)	Average Peak area (Lu*s)
SPE20	20.0	5.083	42.5273	40.54779
		5.089	38.71493	
		5.054	40.40113	
SPE200	204.9	5.041	445.99905	440.26805
		5.036	432.39871	
		5.054	442.4064	
SPE 500	499.8	5.062	1045.86951	1042.43420
		5.059	1030.02502	
		5.044	1051.40808	

Table 4.18: Development of first WASH step

% Methanol	Peak area (Lu*s)	Average peak area	SPE500 added (mg)	Water reconstituted (mg)	% Recovery
0	970.10114 984.15906	977.13010	1001.72	1003.17	93.60
10	977.70013 987.60492	982.65253	1001.14	1001.58	94.22
20	989.03662 974.49298	981.76480	1000.83	1002.18	94.05
30	886.58337 933.21100	909.89719	1002.55	999.53	87.55
40	779.69965 774.87708	777.28837	1001.61	1001.45	74.58
50	755.33679 762.25989	758.79834	1000.87	1001.73	72.73

Table 4.19: Development of second WASH step

% Ethyl acetate	Peak area (Lu*s)	Average peak area	SPE500 added (mg)	Water reconstituted (mg)	% Recovery
0	970.59491 987.37329	978.98410	992.42	1005.23	92.72
5	923.56097 934.81042	929.18570	1004.26	993.55	90.10
10	211.13725 205.60287	208.37006	999.28	999.91	19.98
15	56.04556 60.28495	58.16526	1006.3	1000.57	5.61
20	20.36334 20.42659	20.39497	1003.9	1000.94	1.96
30	39.08356 29.56052	34.32204	1004.76	1001.75	3.30
40	16.64519 13.3386	14.99190	992.83	997.65	1.43

Table 4.41 Concentration found without BPA added (Fluorimetric method)

Sample preparation method	Added [BPA] (µg/L)	Fluorescence intensity (AU)		Found [BPA] (µg/L)	mean ± std. deviation (µg/L)
		F	**Δ*F*** **(*F*−*F*$_0$)**		
Canned foods	0.0	31.2686	3.2081	-0.2	
	0.0	26.3163	-1.7442	-1.3	-0.8 ± 0.6
	0.0	27.1075	-0.9530	-1.1	
Environmental waters	0.0	30.8434	2.7829	-0.3	
	0.0	31.5873	3.5268	-0.1	-0.8 ± 1.2
	0.0	22.2960	-5.7645	-2.2	
Water incubated in PC bottle	0.0	24.2486	-3.8119	-1.8	
	0.0	37.7581	9.6976	1.3	0.3 ± 1.8
	0.0	37.7581	9.6976	1.3	

Table 4.42: Percentage recovery of canned food, environmental waters and water incubated in PC bottle sample preparation method (Fluorimetric method)

Sample preparation method	Added [BPA] (µg/L)	Fluorescence intensity (AU)		Found [BPA] (µg/L)	% Recovery (%)	mean ± std. deviation (%)
		F	ΔF $(F-F_0)$			
Canned foods	20.1	115.4822	87.4217	19.0	94.50	
	20.1	112.1921	84.1316	18.3	90.78	89.71 ± 5.41
	20.1	106.0608	78.0003	16.9	83.85	
	60.4	271.0488	242.9883	54.5	90.14	
	60.4	262.0914	234.0309	52.4	86.76	88.31 ± 1.70
	60.4	265.4963	237.4358	53.2	88.04	
	100.7	401.9316	373.8711	84.3	83.69	
	100.7	416.6591	388.5986	87.6	87.02	86.12 ± 2.13
	100.7	419.4822	391.4217	88.3	87.66	
Environmental waters	20.1	104.6815	76.6210	16.6	82.29	
	20.1	106.3576	78.2971	16.9	84.18	85.92 ± 4.74
	20.1	112.6346	84.5741	18.4	91.28	
	60.4	263.3161	235.2556	52.7	87.22	
	60.4	273.5640	245.5035	55.0	91.08	89.63 ± 2.10
	60.4	272.2306	244.1701	54.7	90.58	
	100.7	434.7039	406.6434	91.7	91.10	
	100.7	414.0966	386.0361	87.0	86.44	88.29 ± 2.47
	100.7	418.0313	389.9708	87.9	87.33	
Water incubated in PC bottle	20.1	109.3792	81.3187	17.6	87.60	
	20.1	107.2846	79.2241	17.2	85.23	87.94 ± 2.90
	20.1	112.3786	84.3181	18.3	90.99	
	60.4	265.7293	237.6688	53.2	88.13	
	60.4	278.1882	250.1277	56.1	92.83	90.39 ± 2.35
	60.4	271.2847	243.2242	54.5	90.22	
	100.7	427.3789	399.3184	90.1	89.44	
	100.7	418.2887	390.2282	88.0	87.39	87.47 ± 1.93
	100.7	410.2878	382.2273	86.2	85.58	

APPENDIX E

Table 4.24: Concentration of BPA from water incubated in PC bottles determined using Fluorimetric method

Samples	Standard, F	ΔF $(F-F_0)$	[BPA] (µg/L)	Mean (µg/L)	Corrected (µg/L)	Standard deviation (µg/L)
PC650A	57.1211	29.0605	5.7			
PC650A	67.3192	39.2586	8.1	7.4	7.9	1.5
PC650A	68.2554	40.1948	8.3			
PC650B	68.1010	40.0404	8.2			
PC650B	67.0127	38.9521	8.0	8.3	8.9	0.4
PC650B	69.8992	41.8386	8.6			
PC650C	54.0183	25.9577	5.0			
PC650C	53.8211	25.7605	5.0	5.5	5.9	0.8
PC650C	59.9048	31.8442	6.4			
PC250A	85.6625	57.6019	12.2			
PC250A	86.4707	58.4101	12.4	10.9	11.7	2.7
PC250A	67.0860	39.0254	8.0			
PC250B	66.7483	38.6877	7.9			
PC250B	64.3185	36.2579	7.4	8.0	8.6	0.8
PC250B	70.7483	42.6877	8.8			
PC250C	43.0520	14.9914	2.5			
PC250C	58.1006	30.0400	6.0	5.2	5.6	2.6
PC250C	63.8191	35.7585	7.3			
PC2000A	194.8325	166.7719	37.1			
PC2000A	190.6801	162.6195	36.1	36.7	9.4	0.1
PC2000A	193.4656	165.4050	36.8			
PC2000B	193.0312	164.9706	36.7			
PC2000B	195.1458	167.0852	37.2	37.5	9.6	0.3
PC2000B	201.6248	173.5642	38.6			
PC2000C	210.4877	182.4271	40.7			
PC2000C	220.0512	191.9906	42.8	40.9	10.5	0.5
PC2000C	204.4217	176.3611	39.3			

Table 4.25: Percentage recovery of BPA from water incubated in PC bottles determined using Fluorimetric method after spiked with 40.3 ug/L of BPA

Samples spiked with 40.3 (ug/L)	Standard, F	ΔF $(F-F_0)$	[BPA] (µg/L)	Recovery (%)	Mean (%)	Standard deviation (%)
PC650A	242.0790	214.0184	47.9	100.80		
PC650A	217.3896	189.3290	42.2	86.55	92.64	7.35
PC650A	224.4813	196.4207	43.8	90.55		
PC650B	234.0635	206.0029	46.0	93.65		
PC650B	219.5247	191.4641	42.7	85.43	88.38	4.57
PC650B	220.6386	192.5780	43.0	86.06		
PC650C	219.5807	191.5201	42.7	92.48		
PC650C	217.3896	189.3290	42.2	91.25	93.73	3.30
PC650C	228.4087	200.3481	44.7	97.47		
PC250A	256.9896	228.9290	51.2	100.16		
PC250A	237.6690	209.6084	46.8	89.24	95.90	5.84
PC250A	253.6690	225.6084	50.5	98.28		
PC250B	230.6429	202.5823	45.2	92.32		
PC250B	239.6278	211.5672	47.3	97.40	94.38	2.67
PC250B	232.5782	204.5176	45.7	93.41		
PC250C	219.2667	191.2061	42.7	92.83		
PC250C	230.6429	202.5823	45.2	99.26	93.43	5.55
PC250C	211.0705	183.0099	40.8	88.20		
PC2000A	369.6869	341.6263	76.9	99.85		
PC2000A	345.0883	317.0277	71.3	85.95	90.78	7.86
PC2000A	346.1648	318.1042	71.6	86.55		
PC2000B	361.5979	333.5373	75.1	93.24		
PC2000B	353.6771	325.6165	73.3	88.76	89.01	4.11
PC2000B	347.0670	319.0064	71.8	85.03		
PC2000C	368.4738	340.4132	76.6	88.62		
PC2000C	379.3391	351.2785	79.1	94.76	92.71	3.55
PC2000C	379.3666	351.3060	79.1	94.77		

Table 4.26: Concentration of BPA from canned foods determined using Fluorimetric method

Samples	Standard, F	ΔF ($F-F_0$)	Concentration (µg/L)	Sample used (mg)	Amount of BPA per kg of sample (µg/kg)	Mean (µg/kg) A	Standard deviation (µg/kg)
	169.4651	141.4045	31.3	5329.36	150.43		
GPA	166.9834	138.9228	30.8	5732.48	137.33	142.02	7.31
	160.6809	132.6203	29.3	5427.41	138.28		
	161.1328	133.0722	29.4	5198.43	144.88		
GPB	176.7532	148.6926	33.0	5739.22	147.09	147.26	2.47
	168.9964	140.9358	31.2	5333.26	149.81		
	455.8201	427.7595	96.5	5839.48	423.18		
TUNAA	437.2411	409.1805	92.3	5849.30	403.95	412.72	9.72
	435.8449	407.7843	92.0	5728.44	411.05		
	421.8447	393.7841	88.8	5285.41	430.06		
TUNAB	437.6612	409.6006	92.4	5634.16	419.81	419.72	10.39
	427.7640	399.7034	90.1	5637.97	409.29		
	200.7546	172.6940	38.4	5218.43	188.59		
SHROOMA	180.7082	152.6476	33.9	5058.30	171.45	176.65	10.37
	182.9897	154.9291	34.4	5182.40	169.92		
	192.8029	164.7423	36.6	5320.43	176.26		
SHROOMB	200.0819	172.0213	38.3	5083.47	192.82	186.41	8.89
	197.4316	169.3710	37.7	5073.50	190.16		

Table 4.27: Concentration of BPA from canned foods determined using Fluorimetric method after spiking

Samples	Standard, F	ΔF ($F-F_0$)	Concentration (µg/L)	Sample used (mg)	Amount of BPA per kg of sample (µg/kg) B	Mean (µg/kg)	Standard deviation (µg/kg)
GPA	327.976	299.9154	67.4	5287.2	326.41		
	331.292	303.2310	68.2	5032.9	346.75	334.38	10.86
	339.656	311.5957	70.1	5436.3	329.99		
	347.239	319.1786	71.8	5332.4	344.71		
GPB	356.093	328.0328	73.8	5423.4	348.44	345.17	3.07
	357.13	329.0696	74.1	5537.5	342.35		
	347.384	319.3236	71.8	5029.5	365.64		
SHROOMA	364.4	336.3394	75.7	5198.4	372.84	371.96	5.93
	364.001	335.9405	75.6	5129.4	377.40		
	350.868	322.8078	72.6	5038.5	369.01		
SHROOMB	355.121	327.0607	73.6	5089.3	370.20	373.00	5.91
	365.518	337.4569	76.0	5120.4	379.79		

Table 4.28: Spiking recovery of BPA from canned foods determined using Fluorimetric method

Sample	Volume of BPA added (2577.5 µg/L) (mL)	Amount of BPA used for spiking C	Amount of BPA added per kg of sample (µg/kg) A	B	Spiking recovery (%)	Mean (%)	Standard deviation (%)
GPA	0.4	195.00		326.41	94.56		
	0.4	204.85	142.02	346.75	99.94	97.87	2.90
	0.4	189.65		329.99	99.11		
	0.4	193.35		344.71	102.12		
GPB	0.4	190.10	147.26	348.44	105.83	104.24	1.91
	0.4	186.19		342.35	104.78		
	0.4	204.99		365.64	92.19		
SHROOMA	0.4	198.33	176.65	372.84	98.92	96.99	4.19
	0.4	201.00		377.40	99.88		
	0.4	204.62		369.01	89.24		
SHROOMB	0.4	202.58	186.41	370.20	90.72	92.00	3.58
	0.4	201.35		379.79	96.04		

To calculate spiking recovery for first result in **Table 4.40**,

Spiking recovery = ([BPA] in spiked sample – [BPA] in sample) / [BPA] added for spiking x 100 %
= (B – A) / C x 100 %
= (326.41 µg/kg – 142.02 µg/kg) / 195.00 µg/kg x 100 %
= 94.56 %

Same formula is used to calculate spiking recovery in **Table 4.43**

Table 4.29: Concentration of BPA from environmental waters determined using Fluorimetric method

Samples	Standard, F	ΔF ($F-F_0$)	Concentration (µg/L)	Amount of BPA per Liter of sample (µg/L)	Mean (µg/L) A	Standard deviation (µg/L)
TW	41.5385	13.4779	2.2	0.112		
	36.33	8.2694	1.0	0.051	0.078	0.031
	37.9257	9.8651	1.4	0.070		
	73.0539	44.9933	9.4	0.479		
UW	80.7159	52.6553	11.1	0.569	0.543	0.055
	81.6609	53.6003	11.3	0.580		
	37.4272	9.3666	1.2	0.064		
LK	45.0273	16.9667	3.0	0.153	0.132	0.061
	47.4357	19.3751	3.5	0.181		
	83.6501	55.5895	11.8	0.603		
TT	86.0558	57.9952	12.3	0.631	0.642	0.045
	91.2345	63.1739	13.5	0.691		
	75.0474	46.9868	9.8	0.503		
PD	57.643	29.5824	5.9	0.300	0.377	0.110
	60.2256	32.1650	6.4	0.330		

Table 4.30: Concentration of BPA from environmental waters determined using Fluorimetric method after spiking

Samples	Standard, F	ΔF ($F-F_0$)	Concentration (µg/L)	Amount of BPA per Liter of sample (µg/L) B	Mean (µg/L)	Standard deviation (µg/L)
TW	225.7544	197.6938	44.1	2.260		
	215.789	187.7284	41.9	2.144	2.220	0.066
	225.5202	197.4596	44.1	2.257		
	266.497	238.4364	53.4	2.735		
UW	255.0541	226.9935	50.8	2.601	2.625	0.100
	249.7481	221.6875	49.6	2.540		
	211.2297	183.1691	40.8	2.090		
LK	209.8544	181.7938	40.5	2.074	2.159	0.132
	230.1859	202.1253	45.1	2.311		
	269.6182	241.5576	54.1	2.771		
TT	280.2311	252.1705	56.5	2.895	2.755	0.149
	254.8046	226.7440	50.8	2.598		
	225.1699	197.1093	44.0	2.253		
PD	227.7229	199.6623	44.6	2.283	2.274	0.018
	228.0291	199.9685	44.7	2.286		

Table 4.31: Spiking recovery of BPA from environmental waters determined using Fluorimetric method

Sample	Volume of BPA added (2577.5 µg/L) (mL)	Amount of BPA added per Liter of sample (µg/L) A	B	Amount of BPA used for spiking C	Spiking recovery (%)	Mean (%)	Standard deviation (%)
	0.4		2.260	2.062	105.83		
TW	0.4	0.078	2.144	2.062	100.19	103.91	3.22
	0.4		2.257	2.062	105.70		
	0.4		2.735	2.062	106.32		
UW	0.4	0.543	2.601	2.062	99.85	101.00	4.84
	0.4		2.540	2.062	96.85		
	0.4		2.090	2.062	94.96		
LK	0.4	0.132	2.074	2.062	94.18	98.27	6.42
	0.4		2.311	2.062	105.68		
	0.4		2.771	2.062	103.27		
TT	0.4	0.642	2.895	2.062	109.27	102.48	7.22
	0.4		2.598	2.062	94.90		
	0.4		2.253	2.062	90.96		
PD	0.4	0.377	2.283	2.062	92.41	91.98	0.89
	0.4		2.286	2.062	92.58		

Table 4.32: Concentration of BPA from water incubated in PC bottles determined using HPLC/FLD

Sample	Retention Time (min)	Peak area (Lu*s)	Cencentration (µg/L)	Mean (µg/L)	Standard deviation (µg/L)
PC650A	5.215	13.00869	5.3	5.8	0.7
	5.090	15.19687	6.3		
PC650B	5.080	12.81092	5.2	4.5	1.0
	5.076	9.66300	3.8		
PC650C	5.072	6.85678	2.5	3.1	1.0
	5.132	9.82236	3.8		
PC250A	5.082	11.16207	4.5	4.2	0.4
	5.085	9.93655	3.9		
PC250B	5.076	6.42872	2.3	2.5	0.4
	5.033	7.66835	2.8		
PC250C	5.010	5.56740	1.9	1.9	0.1
	5.022	5.86772	2.0		
PC2000A	5.010	21.92856	9.5	9.1	0.5
	5.014	20.41390	8.8		
PC2000B	5.083	19.96763	8.6	9.0	0.7
	5.049	21.98329	9.5		
PC2000C	5.004	19.22685	8.2	8.5	0.3
	5.013	20.27775	8.7		

Table 4.33: Concentration of BPA from canned foods determined using HPLC/FLD

Sample	Replicate	Average Peak area (Lu*s)	Concentration (µg/L)	Water used for reconstitution (mg)	Sample used (mg)	Amount of BPA per kg of sample (µg/kg)	Mean (µg/kg) A	Standard deviation (µg/kg)
	1	136.73925	63.02	996.83	5231.72	12.05		
GPA	2	148.16287	68.35	998.37	5454.95	12.56	12.38	0.28
	3	137.90028	63.56	999.47	5096.13	12.51		
GPB	1	122.31025	56.29	998.61	5038.73	11.20		
	2	122.92784	56.58	1000.59	5003.57	11.36	11.41	0.24
	3	136.60462	62.96	1000.87	5422.66	11.67		
	1	231.91312	107.40	999.38	5982.41	18.01		
TUNAA	2	179.98456	83.19	1000.34	5026.88	16.62	17.25	0.70
	3	208.17267	96.33	1000.55	5648.89	17.13		
	1	211.20932	97.75	1001.27	5587.34	17.59		
TUNAB	2	187.76178	86.81	999.43	5031.36	17.31	17.64	0.36
	3	226.82267	105.03	999.85	5845.73	18.03		
	1	39.47488	17.66	989.72	5487.79	3.20		
SHROOMA	2	46.65930	21.01	999.67	5973.23	3.53	3.22	0.29
	3	33.25332	14.76	999.79	5029.45	2.95		
	1	34.79357	15.48	1001.15	5175.92	3.01		
SHROOMB	2	39.39839	17.63	1002.25	5948.41	2.98	2.96	0.07
	3	32.77731	14.54	1001.30	5070.68	2.88		

Table 4.34: Concentration of BPA from canned foods determined using HPLC/FLD after spiking

Sample	Replicate	Average Peak area (Lu*s)	Concentration (µg/L)	Water used for reconstitution (mg)	Sample used (mg)	Amount of BPA per kg of sample (µg/kg) B	Mean (µg/kg)	Standard deviation (µg/kg)
	1	240.63064	111.47	998.12	5473.91	20.40		
GPA	2	224.24624	103.83	998.31	5034.67	20.67	20.58	0.15
	3	234.52542	108.62	999.57	5276.64	20.66		
GPB	1	234.66970	108.69	1000.00	5646.79	19.32		
	2	238.98660	110.70	1001.20	5764.24	19.30	19.42	0.18
	3	239.26123	110.83	1000.40	5673.46	19.62		
	1	408.45233	189.73	995.53	5023.10	37.75		
TUNAA	2	392.68920	182.38	998.48	5017.31	36.44	37.21	0.69
	3	415.08296	192.82	999.39	5164.82	37.46		
	1	398.45918	185.07	995.65	5018.39	36.86		
TUNAB	2	430.86128	200.18	998.27	5539.44	36.22	36.42	0.38
	3	392.82544	182.44	995.36	5038.26	36.18		
	1	126.95538	58.46	993.48	5164.38	11.29		
SHROOMA	2	125.79055	57.91	995.13	5083.62	11.38	11.46	0.22
	3	130.20628	59.97	996.87	5129.45	11.70		
	1	139.94982	64.52	997.91	5982.30	10.80		
SHROOMB	2	126.81494	58.39	998.62	5167.27	11.33	11.15	0.30
	3	123.29960	56.75	998.69	5026.23	11.32		

Table 4.35: Spiking recovery of BPA from canned foods determined using HPLC/FLD

				Amount of BPA added per kg of sample (µg/kg)					
Sample	**Replicate**	**Amount of SPE500 (499.8 µg/L) spiked (mg)**	**Sample used (mg)**	**Amount of BPA used for spiking C**	**A**	**B**	**Spiking recovery (%)**	**Mean (%)**	**Standard deviation (%)**
GPA	1	99.32	5473.91	9.10		20.40	88.19		
	2	99.55	5034.67	9.92	12.38	20.67	83.58	86.26	2.39
	3	100.09	5276.64	9.52		20.66	87.01		
	1	100.60	5646.79	8.94		19.32	88.54		
GPB	2	98.85	5764.24	8.60	11.41	19.30	91.75	91.13	2.34
	3	99.72	5673.46	8.82		19.62	93.10		
	1	198.87	5023.10	19.87		37.75	103.18		
TUNAA	2	199.96	5017.31	20.00	17.25	36.44	95.93	101.14	4.54
	3	199.38	5164.82	19.37		37.46	104.30		
	1	200.02	5018.39	20.00		36.86	96.09		
TUNAB	2	200.51	5539.44	18.16	17.64	36.22	102.26	97.38	4.37
	3	198.48	5038.26	19.77		36.18	93.80		
	1	99.73	5164.38	9.69		11.29	83.23		
SHROOMA	2	99.43	5083.62	9.81	3.22	11.38	83.11	84.67	2.59
	3	98.85	5129.45	9.67		11.70	87.66		
	1	100.21	5982.30	8.41		10.80	93.37		
SHROOMB	2	100.58	5167.27	9.77	2.96	11.33	85.72	87.65	5.05
	3	99.93	5026.23	9.98		11.32	83.84		

Table 4.36: Concentration of BPA from environmental waters determined using HPLC/FLD

Sample	Replicate	Average Peak area (Lu*s)	Concentration (µg/L)	Water used for reconstitution (mg)	Amount of BPA per Liter of sample (µg/L)	Mean (µg/L) A	Standard deviation (µg/L)
TW	1	100.34271	46.05	990.91	0.092		
	2	81.61283	37.31	991.96	0.074	0.085	0.01
	3	97.51340	44.73	993.73	0.089		
UW	1 2 3	-	ND	-	-	ND	-
LK	1 2 3	-	ND	-	-	ND	-
	1	2.99952	0.65	997.52	0.001		
TT	2	2.88981	0.60	997.22	0.001	0.001	0.00
	3	3.51950	0.90	995.44	0.002		
	1	29.33134	12.93	996.81	0.026		
PD	2	25.86639	11.32	993.50	0.023	0.023	0.00
	3	22.98294	9.97	995.19	0.020		

Table 4.37: Concentration of BPA from environmental waters determined using HPLC/FLD after spiking

Sample	Replicate	Average Peak area (Lu*s)	Concentration (µg/L)	Water used for reconstitution (mg)	Amount of BPA per Liter of sample (µg/L) B	Mean (µg/L)	Standard deviation (µg/L)
TW	1	291.67845	135.27	998.15	0.271		
	2	276.62429	128.25	998.98	0.257	0.267	0.008
	3	293.33330	136.05	996.54	0.272		
	1	190.49622	88.09	998.52	0.177		
UW	2	205.92112	95.28	999.34	0.191	0.187	0.009
	3	206.95513	95.76	997.63	0.192		
	1	200.78322	92.89	994.89	0.186		
LK	2	196.59578	90.93	996.31	0.182	0.192	0.015
	3	226.63640	104.94	995.20	0.210		
	1	194.50101	89.96	996.87	0.180		
TT	2	202.79255	93.82	998.27	0.188	0.184	0.004
	3	198.29718	91.73	995.32	0.183		
	1	208.96767	96.70	998.40	0.194		
PD	2	219.86904	101.79	997.57	0.204	0.201	0.006
	3	221.99563	102.78	993.65	0.205		

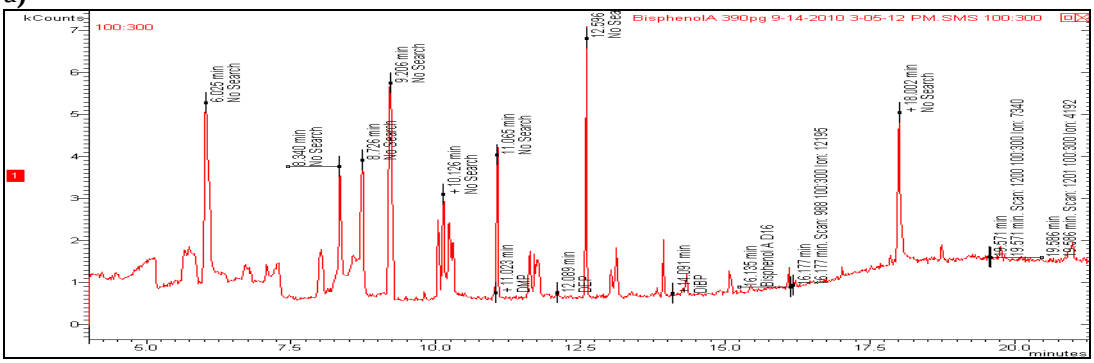
Table 4.38: Spiking recovery of BPA from environmental waters determined using HPLC/FLD

			Amount of BPA added per Liter of sample (µg/L)					
Sample	**Replicate**	**Amount of SPE200 (204.9 µg/L) spiked into 500 mL (mg)**	**Amount of BPA used for spiking C**	**A**	**B**	**Spiking recovery (%)**	**Mean (%)**	**Standard deviation (%)**
TW	1	495.21	0.204	0.085	0.271	91.32	89.42	4.12
	2	494.22	0.203		0.257	84.69		
	3	493.16	0.203		0.272	92.24		
UW	1	494.19	0.203	0.000	0.177	86.86	91.76	4.24
	2	493.58	0.203		0.191	94.15		
	3	494.70	0.204		0.192	94.25		
LK	1	497.24	0.205	0.000	0.186	90.70	93.93	7.37
	2	498.35	0.205		0.182	88.72		
	3	497.91	0.205		0.210	102.37		
TT	1	498.37	0.205	0.001	0.180	87.12	88.96	1.91
	2	498.84	0.205		0.188	90.93		
	3	497.73	0.205		0.183	88.82		
PD	1	498.22	0.205	0.023	0.194	83.45	87.10	3.15
	2	495.46	0.204		0.204	88.84		
	3	497.78	0.205		0.205	89.00		

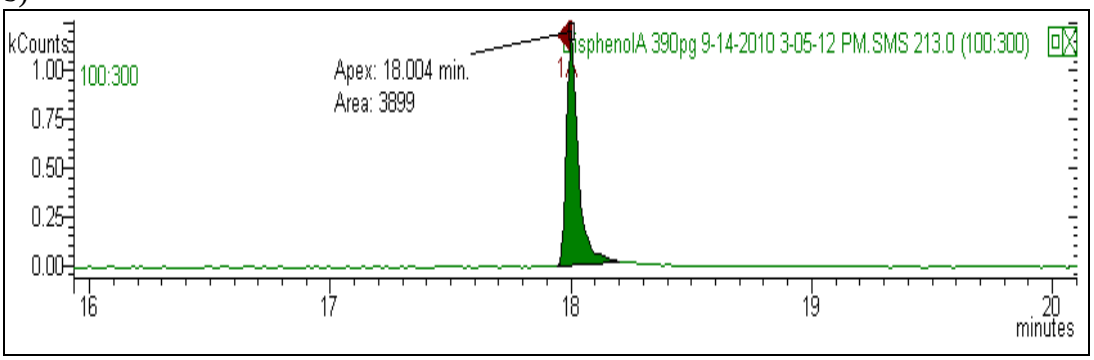
APPENDIX F

Figure 4.19: GC/MS analysis of standard BPA 390 pg/µl in ethyl acetate a) Total ion chromatogram (TIC) at m/z 100 - 300, b) Extracted ion chromatogram (EIC) at m/z 213.0 and c) MS spectrum at retention time 18.004 min (m/z 100 – 300)

a)



b)



c)

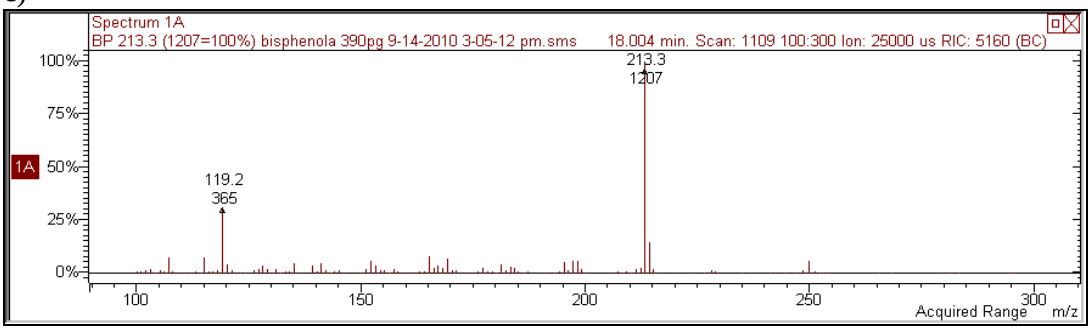
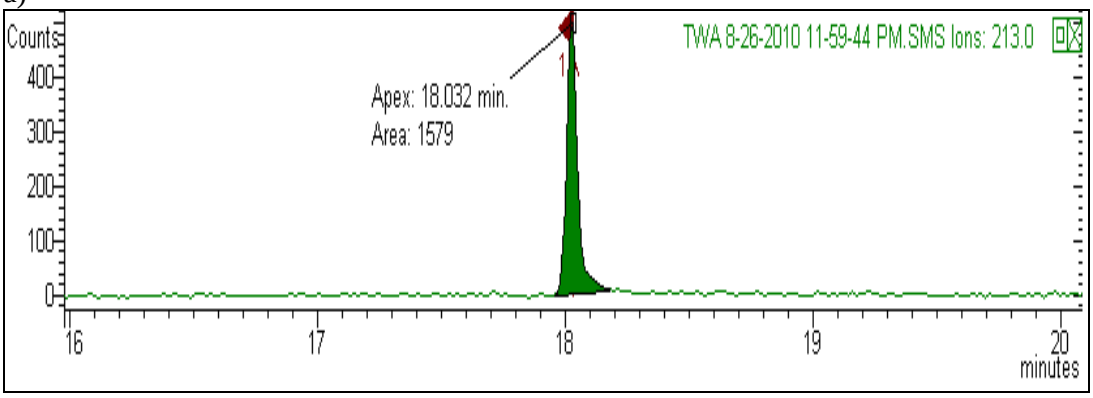
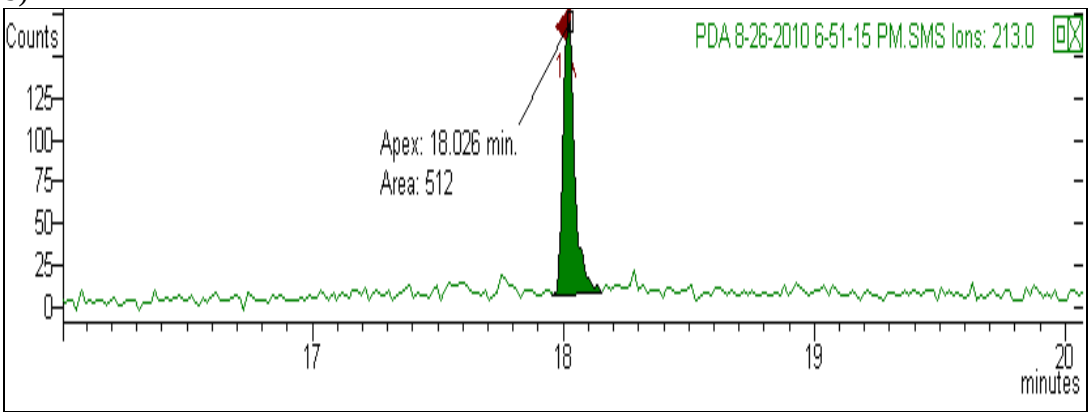


Figure 4.21: GC/MS Extracted ion chromatogram (EIC) at m/z 213.0 for sample a) TW, b) PD, c) GPA, d) GPB, e) SHROOM A, f) SHROOM B, g) TUNA A, and h) TUNA B

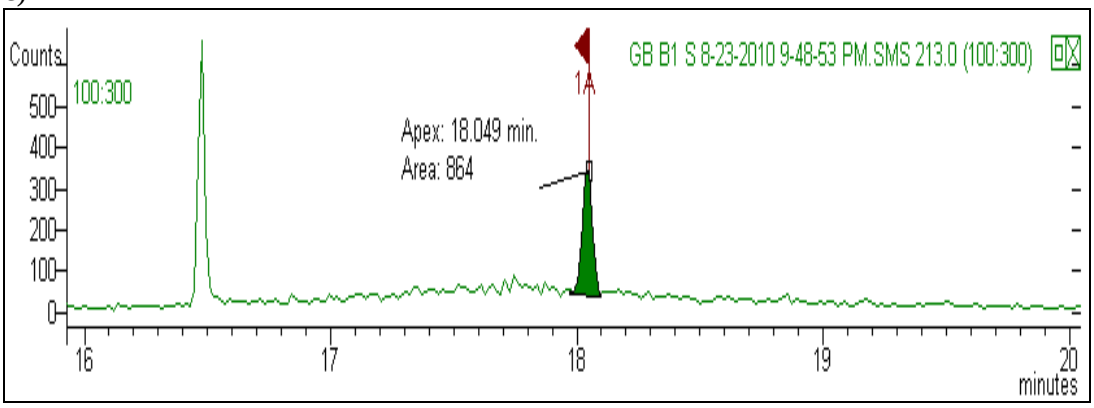
a)



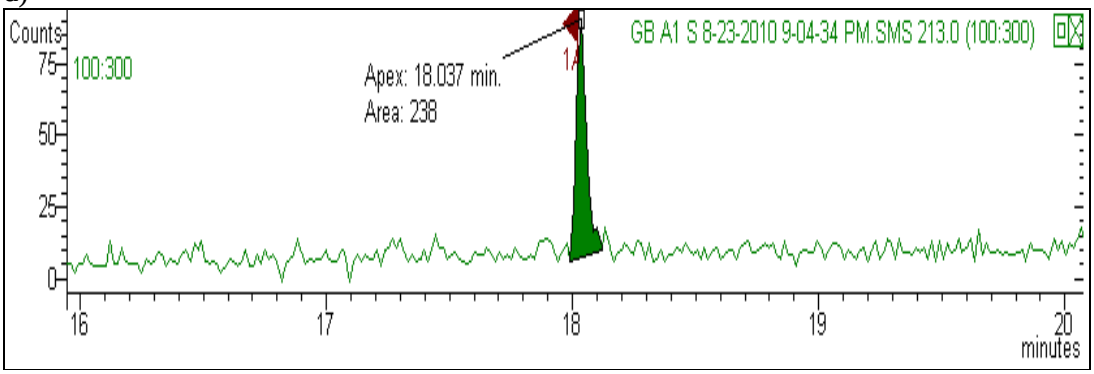
b)



c)



d)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100:300
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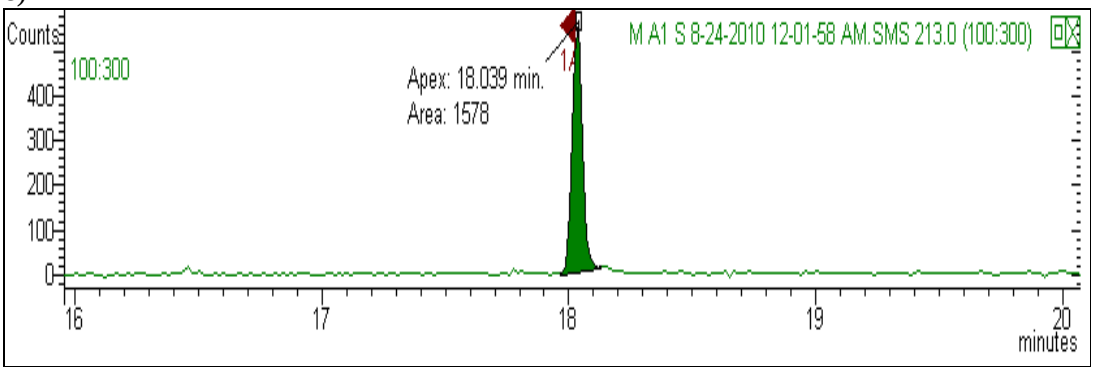
16
17
18
19
20
minutes

e)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300
200
100
0
100:300
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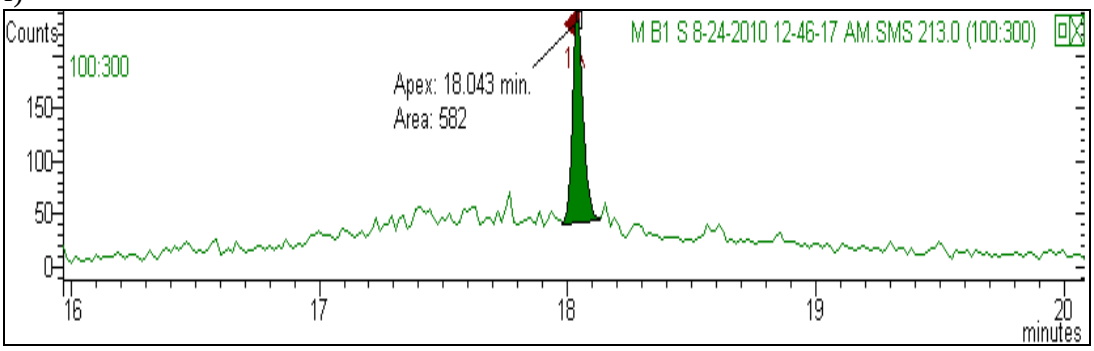
M A1 S 8-24-2010 12-01-58 AM.SMS 213.0 (100:300)
16
17
18
19
20
minutes

f)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100
50
0
100:300
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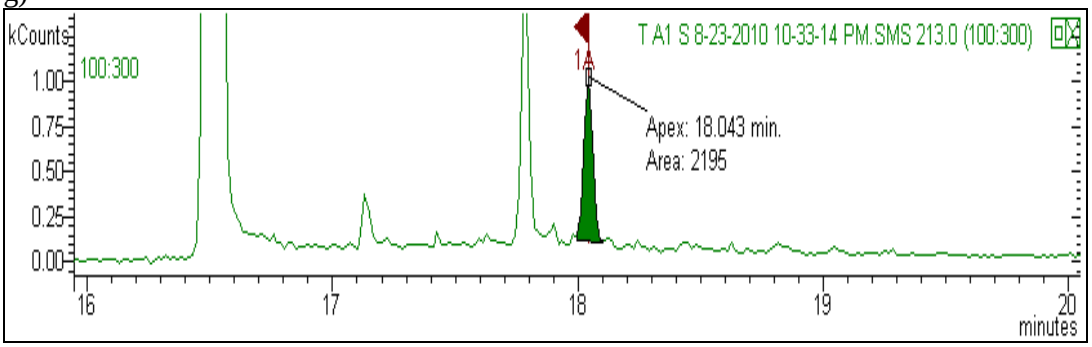
16
17
18
19
20
minutes

g)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0.00
100:300
T A1 S 8-23-2010 10-33-14 PM.SMS 213.0 (100:300)
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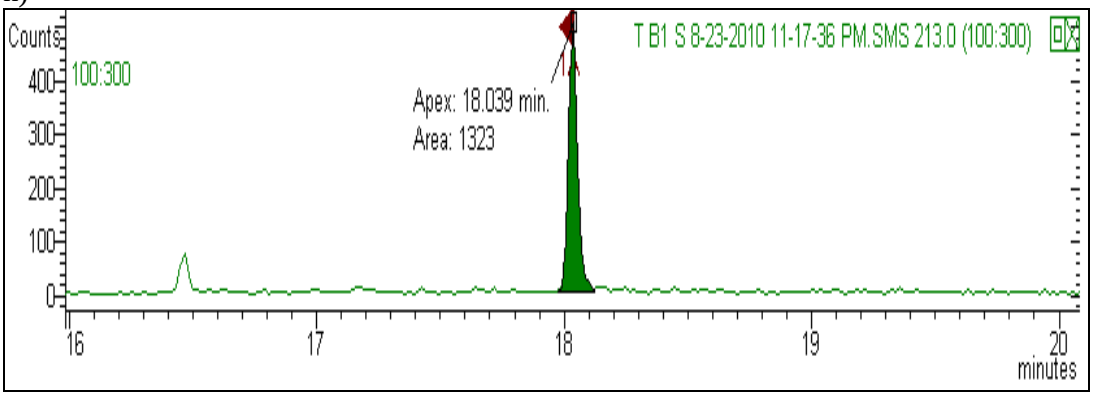
Apex: 18.043 min.
Area: 2195
16
17
18
19
20
minutes

h)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200
100
0
100:300
T B1 S 8-23-2010 11-17-36 PM.SMS 213.0 (100:300)
1
Apex: 18.039 min.
Area: 1323
16
17
18
19
20
minutes