PHENOLIC COMPOUNDS IN WATER AND THE IMPLICATIONS FOR RAPID DETECTION OF INDICATOR MICRO-ORGANISMS USING β-D-GALACTOSIDASE AND β-D-GLUCURONIDASE

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ABSTRACT

Faecal contamination in water is detected using appropriate microbial models such as total coliforms, faecal coliforms and *E. coli*. Beta-D-Galactosidase (β-GAL) and Beta-D-glucuronidase (β-GUD) are two marker enzymes that are used to test for the presence of total coliforms and *E. coli* in water samples, respectively. Various assay methods have been developed using chromogenic and fluorogenic substrates. In this study, the chromogenic substrates chlorophenol red β-D-galactopyranoside (CPRG) for β-GAL and *p*-nitrophenyl-β-D-galactopyranoside (PNPG) for β-GUD were used.

Potential problems associated with this approach include interference from other organisms present in the environment (e.g. plants, algae and other bacteria), as well as the presence of certain chemicals, such as phenolic compounds in water. Phenolic compounds are present in the aquatic environment due to their extensive industrial applications. The USA Enviromental Protection Agency (EPA) lists 11 Priority Pollutant Phenols (PPP) due to their high level of toxicity.

This study investigated the interfering effects of the eleven PPP found in water on the enzyme activities of both the β-GAL and β-GUD enzyme assays. The presence of these PPP in the β-GAL and β-GUD enzyme assays showed that over and under-estimation of activity may occur due to inhibition or activation of these enzymes. Three types of inhibition to enzyme activities were identified from double reciprocal Lineweaver-Burk plots. The inhibition constants (K_i) were determined for all inhibitory phenolic compounds from appropriate secondary plots.

Furthermore, this study presented a validated reverse phase high performance liquid chromatography (RP-HPLC) method, developed for the simultaneous detection,

separation and determination of all eleven phenolic compounds found in the environment. This method demonstrated good linearity, reproducibility, accuracy and sensitivity.

Environmental water samples were collected from rivers, streams, industrial sites and wastewater treatment plant effluent. These samples were extracted and concentrated using a solid phase extraction (SPE) procedure prior to analysis employing the newly developed HPLC method in this study. Seasonal variations on the presence of the PPP in the environment were observed at certain collection sites. The concentrations found were between 0.033 µg/ml for 2,4-dinitrophenol in a running stream to 0.890 mg/ml for pentachlorophenol from an tannery industrial site. These concentrations of phenolic compounds found in these environments were able to interfere with the β-GAL and β-GUD enzyme assays.

LIST OF OUTPUTS

A. POSTER PRESENTATION

Abboo, S. and Pletschke, B.I. (2008) Phenolic compounds in water and the implications for rapid detection of indicator micro-organisms using B-D-GAL and B-D-GUD. Bio-08 Conference, 1820 Settlers' Monument, Grahamstown. 21-25 January 2008.

B. ORAL PRESENTATION

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C. ANTICIPATED PUBLICATION IN PEER-REWIEWED SCIENTIFIC JOURNAL

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2,3,5-TCP	2,3,5-Trichlorophenol
2,3,5-TMP	2,3,5-Trimethylphenol
2,3-DCP	2,3-Dichlorophenol
2,4,6-TCP	2,4,6-Trichlorophenol
2,4-DCP	2,4-Dichlorophenol
2,4-DMP	2,4-Dimethylphenol
2.4-DNP	2,4-Dinitrophenol
2-C-2,4-DMP	2-Chloro-2,4-Dimethylphenol
2-CP	2-Chlorophenol
2-M-4,6-DNP	2-Methyl-4,6-Dinitrophenol
2-NP	2-Nitrophenol
3,4-DMP	2,4-Dimethylphenol
3,5-DCP	3,5-Dichlorophenol
3-CP	3-Chlorophenol
4-C-3MP	4-Chloro-3-methylphenol
4-NP	4-Nitrophenol
C_{18}	Octadecyl
C_2	Ethyl
C_8	Octyl
CE	Capillary Electrophoresis
CH_3CN	Acetonitrile
CP	Chlorophenol
CPR	Chlorophenol red
CPRG	Chlorophenol red β-D-galactopyranoside
DMSO	Dimethylsulphoxide
DNP	dinitrophenol
E. coli	*Escherichia coli*
EC	Electrochemical
ECD	Electron Capture Detection
EDM	Enzyme detection method
EPA	Environmental Protection Agency
EtOH	Ethanol
EU	European Union
FID	Flame ionisation detection
GC	Gas Chromatography
HCl	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
ICH	International Convention on Harmonization (ICH)
ID	Internal Diameter
ISH	*in situ* Hybridisation
kDa	KiloDalton
K_i	Inhibition constant
K_m	Michaelis constant
LLE	Liquid-Liquid Extraction
LOD	Limit of Detection
LOQ	Limit of Quantification
M	Molar
MAC	Maximum Permissible Concentration

MF	Membrane Filter Technique
mg	Milligram
ml	Milliliter
mm	Millimeter
mM	Millimolar
mOD	Milli optical density
MP	Methylphenol
MPN	Most Probable Number
MTF	Multiple-tube Fermentation Technique
MUG	4-methylumbelliferyl-β-D-glucuronide
nm	Nanometers
nM	Nanomolar
ONPG	o-Nitrophenol-β-D-galactopyranoside
ρ-NP	ρ-Nitrophenol
PCP	Pentachlorophenol
PCR	Polymerase chain reaction
Ph	Phenol
PNPG	p-Nitrophenol-β-D-glucuronide
PPP	Priority Pollutant Phenols
psi	Pounds Per Square Iinch
RP-HPLC	Reverse- Phase High Performance Liquid Chromatography
RSD	Relative Standard Deviation
SPE	Solid Phase Extraction
SPE-LC-MS	Solid Phase Extraction-Liquid Chromatography-Mass Spectrometry.
Tris	TRIS (hydroxymethyl) aminomethane
UV	Ultraviolet
XGLU	5-Bromo-4-Chloro-3-Indol-β-D-Glucuronide
β-GAL	β-D-Galactosidase
β-GUD	β-D-Glucuronidase
µg	Micrograms

CHAPTER 1

LITERATURE REVIEW

1.1 PATHOGENIC MICROORGANISMS IN DRINKING WATER

Life on earth cannot exist without water; hence water is the most vital natural resource for our survival (Bates, 2000). A contaminated aquatic environment becomes unsuitable for the supply of drinking water, fishing (agriculture) for human consumption or recreational uses and causes severe disturbances in the ecological functioning of aquatic ecosystems (George *et al*., 2002; Gilpin *et al*., 2003).

In 1854 a cholera outbreak in London caused 10 000 deaths and linked sewage pollution with bacterial contamination which resulted in enteric diseases (Bates, 2000). The presence of coliforms such as *Escherichia coli* (*E. coli)* in water indicates faecal pollution (Manafi, 1996), since coliforms are present in large numbers in the intestinal flora of both humans and warm- blooded animals (Rompré *et al*., 2002).

The most frequently encountered pathogens in water sources are enteric pathogens, hence faecal pollution in drinking water must be strictly monitored. Very low concentrations of entero-pathogens, such as *(E. coli*) are present in water within a diversified micro floral system (Rompré *et al.*, 2002).

1.2 DISEASES ASSOCIATED WITH FAECAL POLLUTION IN DRINKING WATER

Waterborne diseases such as cholera, typhoid, amoebic and bacillary dysentery and other diarrhoeal diseases are caused by the ingestion of water contaminated by human or animal faeces or urine containing pathogenic bacteria (Gleick, 2002). Worldwide approximately 250 million people are infected with waterborne diseases, which results in 10 to 20 million deaths annually. In South Africa about 21 million (approximately half the population) lack basic sanitation (DWAF, 1996).

Hygiene standards thus play a significant role in disease eradication (Bates, 2000). The control of these waterborne diseases is performed by monitoring the microbiological quality of drinking water by routine assesment (Sobsey *et al*., 1993). As a result, faecal pollution of drinking water, as well as water used for any human

activity must be strictly monitored, since this is a requirement for public health and safety by government regulation in the majority of, if not all countries.

1.3 COLIFORMS *(E. coli)* IN WATER – THEIR USE AS INDICATORS OF WATER QUALITY

The multiple-tube fermentation technique (MTF) defines coliforms as rod-shaped Gram-negative bacteria which are non-spore forming that ferment lactose within 48 hours at 35^{o}C with acid and gas formation.
Alternatively, using the membrane filter technique (MF), coliforms are defined as Gram-negative rod shaped bacteria when plated out on an endo-type medium that contains lactose, and when incubated at 35^{o}C for 24 hours develop red colonies.
In the enzyme detection method (EDM) coliforms test positive for beta-galactosidase and beta-glucuronidase enzyme activity using chromogenic and fluorogenic substrates (Standard Methods, 1998).

1.4 METHODS FOR DETECTING COLIFORMS

There are various methods reported for the detection of coliforms in the literature. These detection methods are divided into three main groups:
i) Cultural/classical methods,
ii) Molecular methods, and
iii) Enzymatic methods
(Venter, 2000; Rompré *et al*., 2002)

1.4.1 Cultural/ Classical method

This technique is sub-divided into two methods.

1.4.1.1 Multiple tube fermentation method (MTF)

This method consists of an inoculation process. Test tubes containing laural tryptose or lactose broths are prepared in serial dilution. These tubes are incubated at 35^{o}C for 48 hours, after which acid formation, gas production and abundant growth indicates a positive presumptive test for coliforms (Sartory and Watkins, 1999; Rompré *et al*., 2002).

The number of microorganisms present in the above technique is expressed in terms of the most probable number (MPN). This technique is enormously time-consuming, i.e. 48 hours is required for a semi-quantitative enumeration of coliforms (Standard Methods, 1998 ; Rompré *et al.*, 2002).

1.4.1.2 Membrane filter procedure

This method involves filtering a water sample through a sterile 0.45 μm filter, on which the bacteria is retained, followed by incubating the filter on selective media, and then recording the typical colonies on the filter. Media such as MacConkey agar or teepol medium are used. In cases where drinking water is chemically treated, the antimicrobial effects of such treatment results in an inability to recover, and therefore accurately account for any coliform bacteria which may have been present.

This method is not adequately specific, and a further 24 h incubation stage is required, making it a less than suitable method for enumeration of coliforms (Grabow, 1996; Van Poucke and Nelis, 2000; Farnleitner *et al.*, 2001; Rompré *et al.*, 2002).

1.4.2 Enzymatic methods

For the detection of indicator bacteria, microbial enzyme profiles are a preferred option compared to classical methods, because the reactions are more sensitive and more rapidly performed and coliforms can be detected and enumerated through specific enzyme activities (Rompré *et al.*, 2002). The bacterial enzyme β-D-glucuronidase (β-GUD) was first discovered in *E. coli*, but it is was not until Kilian and Bulow (1976) studied the Enterobacteriiaceae and reported that glucuronidase activity was mostly limited to *E .coli* and that the specificity of this enzyme to coliforms could be applied to the detection of these organisms.

The use of chromogenic and fluorogenic substrates produces a colour upon cleavage by the enzymes β-D-galactosidase (β-GAL) and β-GUD. It was observed that the use of these substrates improved accuracy as well as increased the speed of the detection method (Chróst, 1991; Manafi *et al.*, 1991).

1.4.3 Presence / absence techniques and enumeration by multi-tube procedures using enzymatic methods

A defined substrate method has been developed based on the enzyme properties of coliforms to overcome some of the limitations of the multiple-tube fermentation technique (MTF) and membrane filter technique (MF). The defined substrate

technology is based on the principle that only the target microbes i.e. *E. coli* are supplied with substrate and no provision of substrate for other bacteria, compared to the MTF and MF techniques, which eliminates the growth of non-coliform bacteria with inhibitory chemicals. To indicate the presence of target organisms a chromogen or a fluorochrome is released from the defined substrate, which occurs during the process of substrate utilization (Rompré *et al.,* 2002).

1.4.4 Membrane filter technique conjugated to enzymatic detection of coliforms

Various agar media are available commercially for the detection of *E. coli* and coliform enumeration modified with specific chromogenic and/or fluorogenic substrates for the detection of β-GAL and β-GUD (Dahlen and Linde, 1973; Brenner *et al.,* 1993; Rompré *et al.,* 2002).

1.4.5 Enzymatic activity using fluorimetry

This protocol is based on the fluorogenic substrates MUGal and MUGlu for direct enzymatic detection; however, poor limits of detection which are 20 CFU/100ml for FC and 340 CFU/100ml for TC respectively, make this method unsuitable for the screening of drinking water (George *et al.,* 2000).

1.4.6 Enzymatic methods using solid-phase cytometry

This method involves taking a sample of drinking water and filtering it through a 0.4 μm pore size filter under a vacuum. The *E. coli* retained on the filters are treated with reagents to induce the enzyme β-GUD and to label the induced cells. The fluorescence of a micro colony or a single cell is detected by the ScanRDI laser scanning device (Van Poucke and Nelis, 1999, 2000).

1.4.7 Molecular detection methods

These methods have been developed to reduce the time required for analysis. Without the need to cultivate and an additional confirmation step, the molecular methods are able to achieve a higher degree of sensitivity and specificity (Rompré *et al.,* 2002).

1.4.8 Immunological techniques

These methods are based on the specific recognition between antibodies and antigens and also permit the detection of antigens at family, genus, species or serotype levels (Rompré *et al.,* 2002).

1.4.9 Nucleic acid-based methods

Molecular hybridisation properties are used in this method, which involve the complementary sequence recognition between a nucleic probe and a nucleic target.

These methods include the polymerase chain reaction (PCR) and *in situ* hybridisation (ISH) (Rompré *et al.,* 2002).

1.5 ENZYME DETECTION METHODS (EDM)

1.5.1 Enzymatic definition of coliforms

This method makes use of the presence of the enzymes β-GAL and β-GUD in these coliforms, β-GUD catalyzes the hydrolysis of β-D-glucopyranosiduronic derivatives into D-glucuronic acid and aglycons indicating *E.coli*. β-GAL catalyzes the breakdown of lactose into galactose and glucose indicating total coliform bacteria. The detection of both these enzymes thus can be used for the enumeration of coliforms (Manafi *et al.*, 1991).

Chromogenic or fluorogenic enzyme substrates are used to detect the enzymes β-GAL and β-GUD. The chromogenic enzyme substrates are phenol based, for example enzyme substrates for β-GUD ρ-nitrophenol-β-D-glucuronide (PNPG) and 5-bromo-4-chloro-3-indol-β-D-glucuronide (XGLU), where PNPG produces *ρ*-nitrophenol, a yellow colour and XGLU produces indolyl, which is blue.

The fluorogenic substrate used to detect β-GUD is 4-methylumbelliferyl-β-D-glucuronide (MUG) (Manafi, 1996; Manafi, 2000). β-GAL is detected by using chromogenic substrates such as chlorophenol red β-D-galactopyranoside (CPRG) and *o*-nitrophenol-β-D-galactopyranoside (ONPG) (Edberg *et al.,* 1988; Cheng *et al.*, 2002).

1.6 THE ENZYMES β-GAL AND β-GUD

1.6.1 β-GAL

Beta-galactosidase is a large tetrameric enzyme produced by *E. coli,* which is made up of four identical subunits of 135, 000 Da, each with its own active site (Figure 1.1). Each subunit of this enzyme consists of five distinct domains formed by a single 1,023 residue polypeptide chain (Figure 1.2). The active site is situated at the end of the third domain. The enzyme carries out two reactions on its disaccharide substrate, lactose. This hydrolysis reaction produces two monosaccharides, glucose, and galactose which participate in various metabolic pathways.

The second reaction is the transglycosylation reaction, which produces the disaccharide *allo*lactose. This induces the production of more β-galactosidase, hence *E. coli* can utilize lactose and respond to lactose as a food source (Juers and Matthews, 2006).

The enzyme has a molecular weight of 540, 000 Da (Craven *et al.,* 1965; Cohen and Mire, 1971). The systematic name is β-D-galactosidase galactohydrolase (http://www.brenda.uni-eln.de/php/flat_result.php4?ecno=3.2.1.23&organism_list=... 18/4/2007).

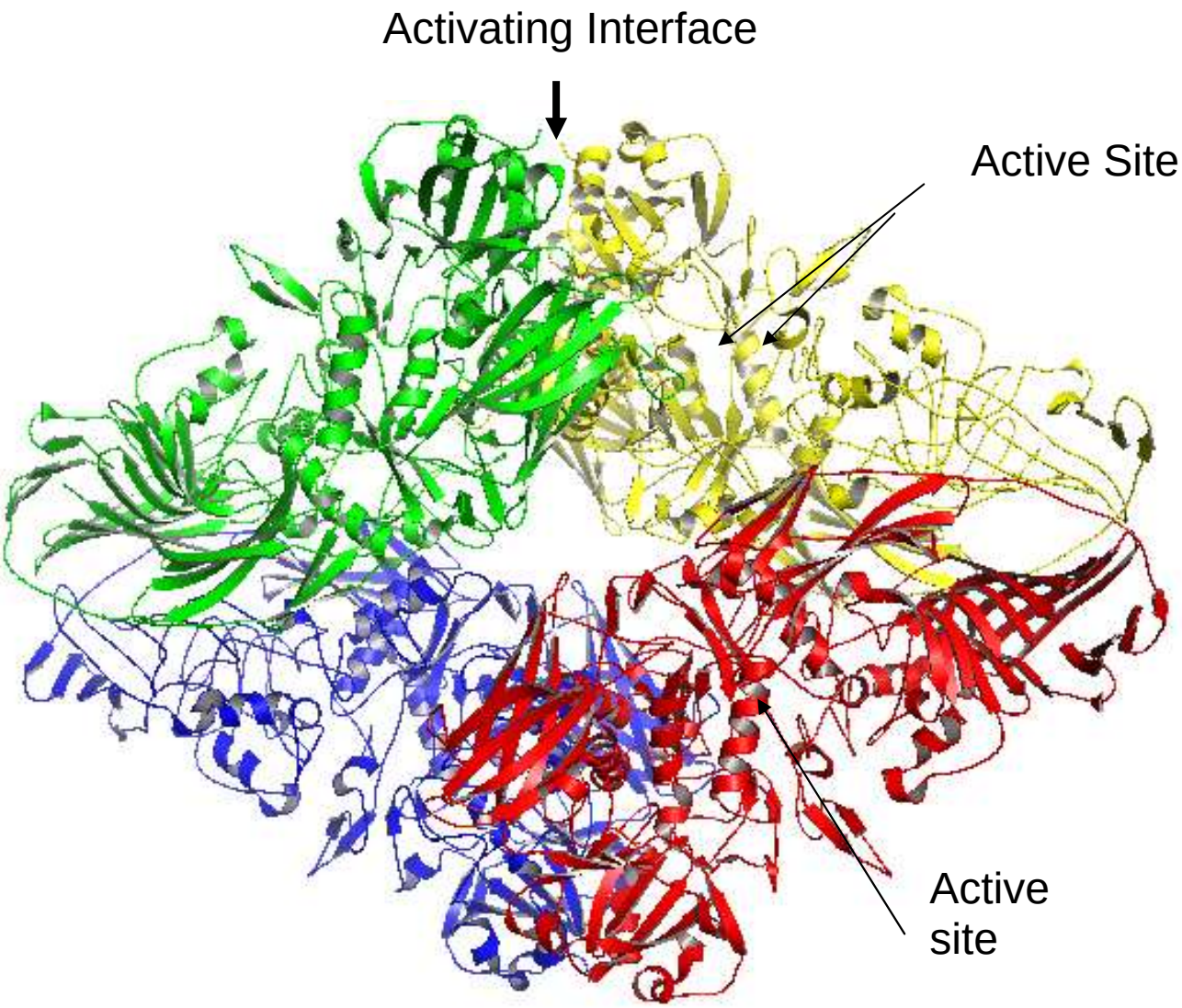


Figure 1.1: Figure of a β-GAL tetramer molecule, showing the active sites. Monomers are indicated in blue, red, green and yellow respectively (Adapted from Matthews, 2005)

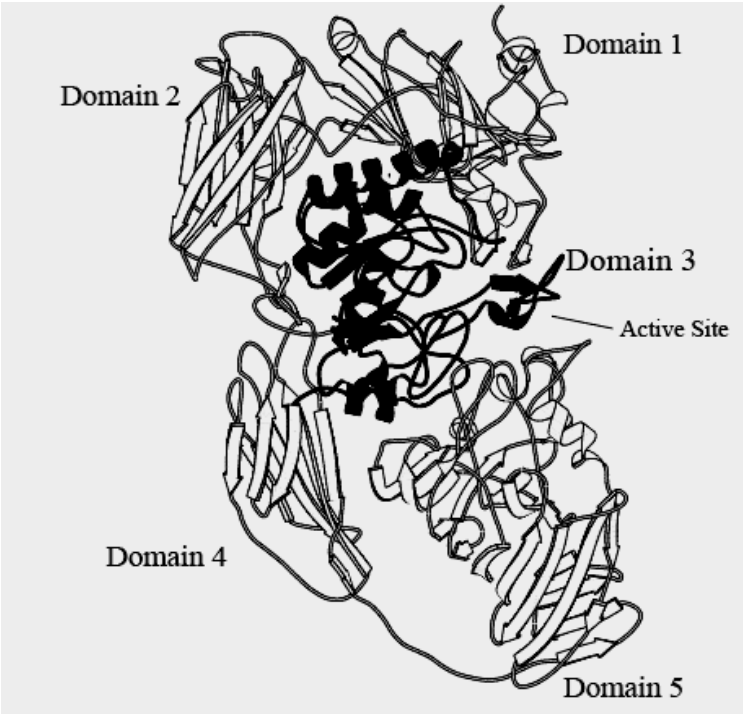


Figure 1.2: A ribbon diagram showing one subunit of the tetramer beta-galactosidase enzyme indicating the binding/active site and the five domains (Taken from Jeurs and Matthews, 2006).

1.6.1.1 pH optimum

Changes in pH have a large influence on the activity of enzymes, affecting both solubility as well as the structural stability of the molecule. Charge and charge distribution on both the enzymes and substrates are affected, which alter the bond and bond patterns, which in turn determine the rate of the enzyme–catalyzed reaction (Holme and Peck, 1998).

It was reported by Pletschke *et al*. (2006) that the enzyme activity for β-GAL was constant between pH 5.0 and pH 9.0, with an increase at alkaline pH; however, the activity was found to be less reproducible at pH 10. There was a peak in activity at pH 7.8. Pletschke *et al*. (2006) further reported that drinking water should have a pH close to 7.0 . The optimum pH for β-GAL was reported to be between pH 6.6 and 7.7 (http://www.brenda.uni-eln.de/php/flat_result.php4?ecno=3.2.1.23&organism_list=... 18/4/2007)

1.6.1.2 Temperature optimum

Temperature affects the bond vibrations within a molecule, as well as the rate of molecular collisions between enzymes and substrates. The higher rate of collision of higher temperatures increases the rate of an enzyme reaction. However increased bond vibrations induced by higher temperatures result in bond disruptions, which in turn cause the denaturing of enzymes and rapid substrate breakdown.

Therefore, the optimum temperature to perform an enzyme assay is a compromise between maximum activity and enzyme denaturation (Holme and Peck, 1998).

Studies done by Pletschke *et al.* (2006). investigated enzyme activity at temperatures between 20^{o}C and 65^{o}C. The activity was fairly stable from 20^{o}C to 60^{o}C, but at 65^{o}C decreased rapidly to almost zero and this decrease was found to be consistent at higher temperatures. The optimum temperature was reported to be 35^{o}C ((http://www.brenda.uni-eln.de/php/flat_result.php4?ecno=3.2.1.23&organism_list=... 18/4/2007)

1.6.2 β-GUD

The systematic name for β-GUD is β-D-glucuronodase glucuronosohydrolase and it has a molecular weight of 290, 000 Da. The enzyme is a tetramer made up of four subunits, each with a molecular weight of 72, 000 Da. ((http://www.brenda.uni-eln.de/php/flat_result.php4?ecno=3.2.1.31&organism_list=...18/4/2007; Kim *et al*.,

1995). The multidomain structure includes an immunoglobulin constant domain, jellyroll barrel, and a TIM barrel (Figure 1.3). This family of enzymes hydrolyses its substrate with a net retention of anomeric configuration, presumably via a double displacement mechanism involving the action of two active site carboxylic acid residues.

The first step is a nucleophillic attack on the sugar anomeric center by one of the carboxylates. The departure of the aglycone to form an alpha-D-glycosyl-enzyme intermediate occurs via general acid catalysis from the other carboxyl group.

The second step involves the hydrolysis of the intermediate by a general base-catalyzed attack of the water at the anomeric center, which results in cleavage of the glycosidic bond with net retention of the anomeric configuration (Henrissat, 1991; Wong *et al*., 1998).

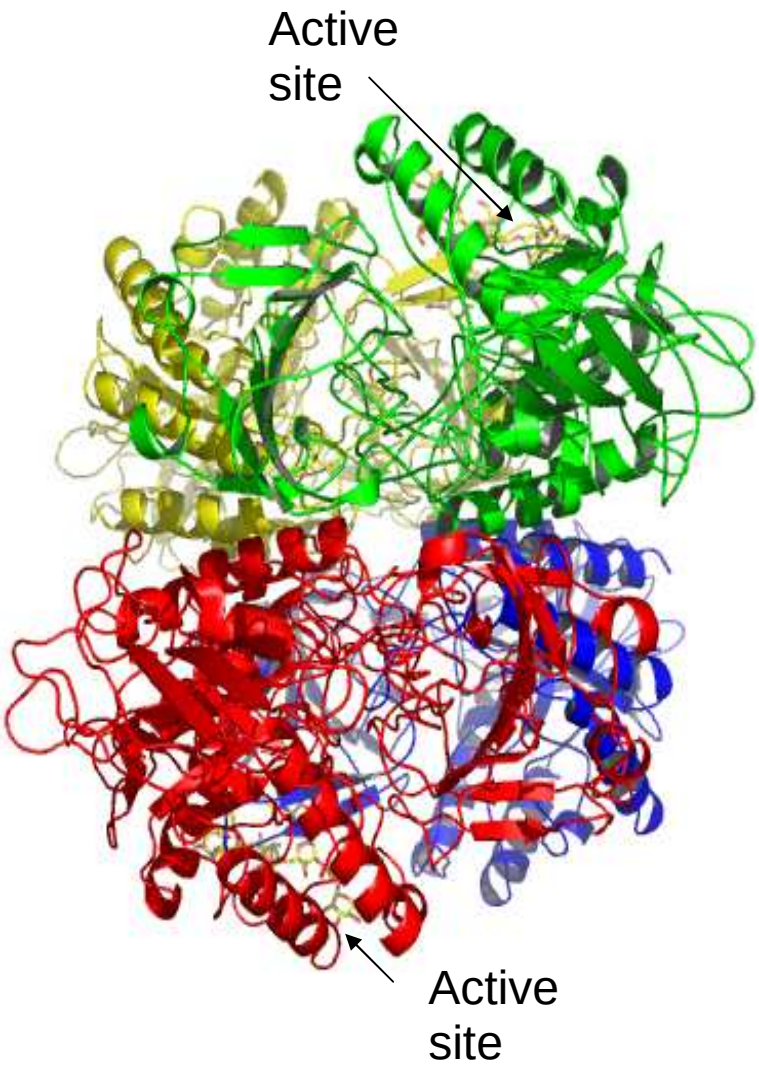


Figure 1.3. A β-GUD tetramer revealing the active sites on two of the monomers. The monomers are indicated in blue, red, green and yellow respectively (Adapted from Jain *et al.*, 1996).

1.6.2.1 pH Optimum

The optimum pH for beta-glucuronidase is between pH 6.5 and pH 6.8 (http://www.brenda.uni-eln.de/php/flat_result.php4?ecno=3.2.1.31&organism_list=... 18/4/2007). As with β-GAL, Pletschke *et al.* (2006) reported that the enzyme activity was constant between the pH 5.0 and pH 9.0, with a sudden increase in activity at pH 10-11. The increase in activity at alkaline pH was associated with poor reproducibility. There was a large degree of variability in the activity of β-GUD when comparing data at the same pH in running and stagnant water (Pletschke *et al.,* 2006). Kim *et al.* (1995) reported the optimum pH to be between pH 6.0 and 7.0.

1.6.2.2 Temperature optimum

As with pH, Pletschke *et al.* (2006) reported that the activity varied at extreme temperatures between running and stagnant water; however, there was an insignificant variation in activity between 25 ^{o}C and 50^{o}C in activity (Pletschke *et al.,* 2006). The optimum temperature is reported to be at 44.5^{o}C (http://www.brenda.uni-eln.de/php/flat_result.php4?ecno=3.2.1.31&organism_list=...18/4/2007).

1.7 INFLUENCES OF ENVIRONMENTAL FACTORS

Fiksdal and Tryland (2008) reported that an increase in detection sensitivity of β-GAL and β-GUD enzyme activities in environmental samples may be associated with accompanying interference of non-target sources such as other bacteria, algae and plants. There are chemical compounds present in the environment that can potentially interfere with the enzyme assays used to determine the microbial standard of water (Pletschke *et al*., 2006). Phenolic compounds are examples of such chemical compounds present in aquatic systems.

1.8 PHENOLIC COMPOUNDS

1.8.1 Introduction

Phenols are organic compounds which belong to the aromatic hydrocarbon group, to which an hydroxyl group (-OH) is attached (Figure 1.4). Phenols have a number of substituents that include halogenated (eg. chloro-), nitrated (nitro-), alkylated

(dimethylphenol-), bromo- and ether (methoxy -) phenol derivatives (Angelino and Gennaro, 1997; Kim and Kim 2000*;* Torres *et al.*, 2003).

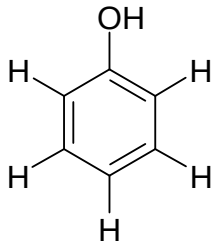


Figure 1.4 Phenol – an aromatic hydrocarbon to which a hydroxyl group (-OH) is attached.

1.8.2 Toxic effects

Phenolic compounds are easily absorbed through the natural membranes of living organisms including plants, causing mutagenic, genotoxic and hepatotoxic effects and also interfering with reactions in cell respiration and photosynthesis due to their lipophilic nature (Washburn and Phillips, 1995). They are therefore highly toxic to all living organisms (Brodfuehrer *et al*., 1990; Yager *et al*., 1990). Phenolic compounds are known or suspected carcinogens (for example chlorophenols) and have been linked with non-Hodgkin's lymphomas and soft tissue sarcomas (McConnell *et al*., 1991; Kim and Kim, 2000; Peñalver *et al*., 2002). Oral exposure results in liver and kidney failure as well as diseases of the blood (http://www.eco-usa.net/toxics/phenol.shtml 2/4/2007)

It is because of their toxicity that phenols are high on the list of organic pollutants. The United States of America (USA) and the European Union (EU) have listed phenols as priority pollutants by their respective Environmental Protection Agencies (EPA's) (Angelino and Gennaro, 1997).

1.8.3 Industrial application

Phenolic compounds have extensive industrial applications and are found in several commercial products such as pesticides, bactericides, wood preservatives and dyes. They are used in pulp processing, petroleum refining, leather tannery, textiles and plastics (Lee *et al.*,1996; Angelino and Gennaro 1997; Llompart *et al*., 2002; Peñalver

et al., 2002; Asan and Isildak, 2003; Lupetti *et al.*, 2004). Phenols are also found in fertilizers and explosives (Aktas *et al.*, 2006).

1.8.4 Priority Pollutant Phenols (PPP)

The US Environmental Protection Agency (EPA) lists eleven Priority Polluted Phenols (PPP), according to their toxicity, which are characterised by the substituents chloro, nitro and methyl groups (Angelino and Gennaro 1997; Llompart *et al*., 2002). These eleven PPP compounds are:

phenol,
4-nitrophenol,
2-chlorophenol,
2-nitrophenol,
2,4-dinitrophenol,
2,4-dimethylphenol,
4-chloro-3-methylphenol,
4,6-dinitro-2-methylphenol,
2,4–dichlorophenol,
2,4,6-triclorophenol, and
pentachlorophenol

Below are the chemical structures of these compounds (Figure 1.5):

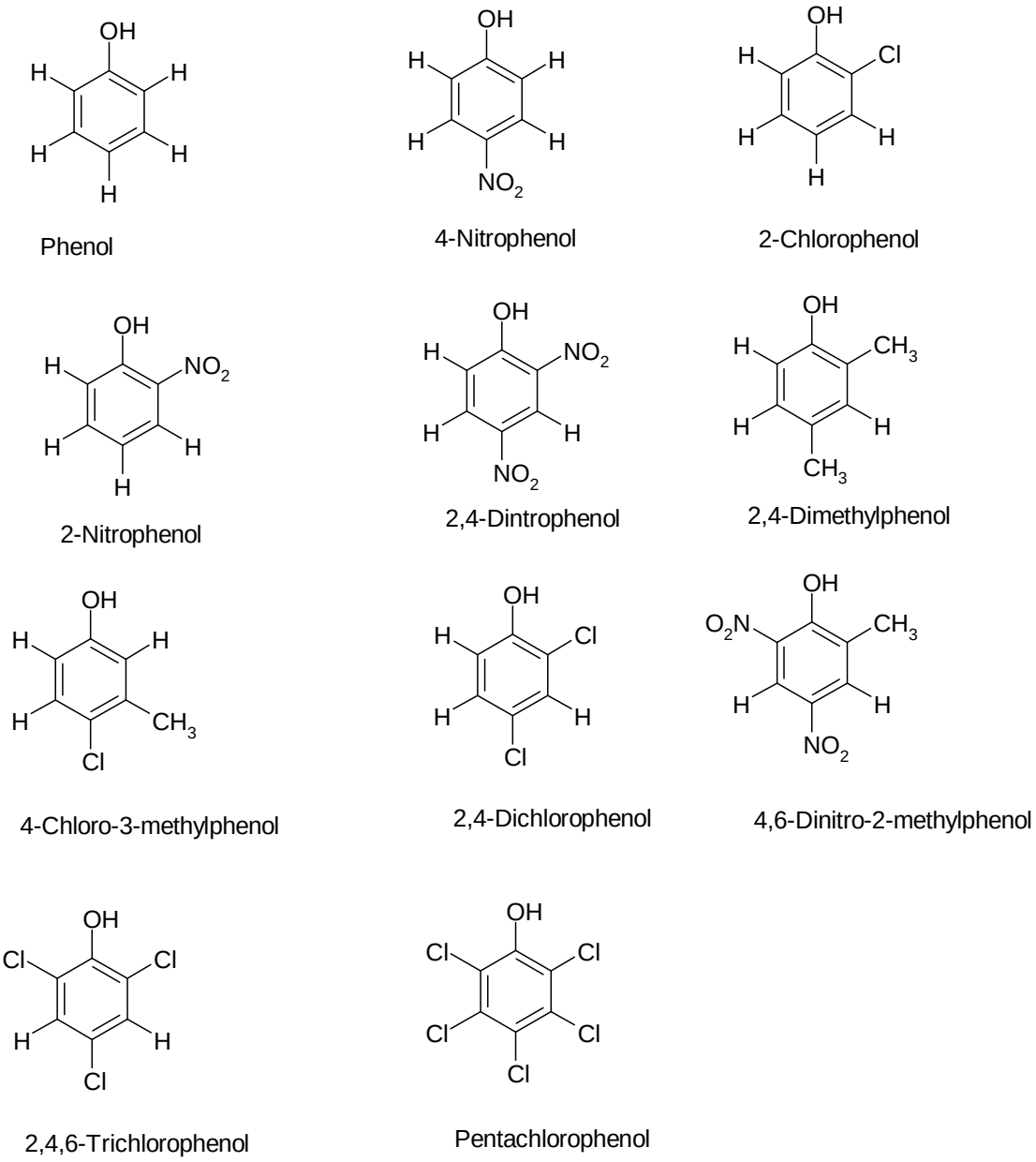


Figure 1.5 Chemical Structures of the Priority Pollutant Phenolic compounds listed by the USA Enviromental Protection Agency (EPA).

The European Union (EU) lists the following seven highly toxic phenols in their legislation: 2-amino-4-chlorophenol, 4-chloro-3-methylphenol, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, trichlorophenol and pentachlorophenol (González-Toledo *et al.,* 2001*).* The EU limits are set at maximum of 0.5 ug/l for total phenols and 0.1 ug/l for each individual phenol (Peñalver *et al.,* 2002). Many of the European countries follow the US EPA list of PPP compounds (Puig and Barceló, 1996).

The South African maximum operation limit for total phenols in drinking water is set at an upper limit of 10 ug/l and between 10-70 ug/l for a limited duration (SANS, 2005).

1.8.5 Detection of phenolic compounds in water

Various methods for the detection of phenolic compounds in drinking and wastewater have been proposed in the literature of which reversed-phase high performance liquid chromatography (RP-HPLC) is the most widely used with ultraviolet (UV) detection. Other methods are gas chromatography (GC) and capillary electrophoresis (CE) with various modes of detection.

It is imperative that a sensitive, reliable, reproducible and effective HPLC method is developed and optimised for the detection of these phenolic compounds in drinking water due to their high toxicity to humans and the environment.

The GC methods described in the literature employ both capillary and packed columns, with flame ionization (FID) or Electron Capture Detection (ECD) methods. These methods require the derivatization of the phenolic compounds before detection in order to improve sensitivity (Angelino and Gennaro, 1997).

HPLC is preferred over GC, since the derivatization of the phenolic compounds prior to analysis by GC is time consuming (Santos *et al*., 1997; Lee *et al*., 1998; Peñalver *et al*., 2002; Berhanu *et al*., 2006). The other disadvantage of using GC is that due to the high polarity of these compounds they produce broad, tailed peaks, which increase with the number of injections onto GC columns (Mußmann *et al.,* 1994; Llompart *et al.,* 2002).

In HPLC, both electrochemical (EC) and ultraviolet (UV) detection are used. Although electrochemical detection provides increased sensitivity for the detection of some of the phenolic compounds, it is less sensitive than UV detection when detecting nitrophenols and dinitrophenols (Ruana and Urbe, 1993; Pocurull *et al*., 1996; Peñalver *et al*., 2002). Electrochemical detection (EC), unlike UV detection, does not

allow for the use of gradient elution, as well as the use of all liquid chromatography (LC) solvents (Ruana and Urbe, 1993; Angelino and Gennaro, 1997).

1.9 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) METHODS FOR THE ANALYSIS OF PHENOLIC COMPOUNDS IN WATER

1.9.1 Introduction

An Italo-Russian botanist, Michael S. Tswett, coined the term "chromatography'' for the method he developed for separating plant pigments over a hundred years ago. As an modern advancement High Performance Liquid Chromatography (HPLC) is now a well-established technique. This is a popular technique due its versatility, accuracy, precision, speed and selectivity. HPLC instruments consist mainly of an analytical column, injector, mobile phase reservoir and a detector. A sample mixture of various components is injected onto the system and the different components are separated due to the partitioning behaviour between the stationary phase and the mobile phase. This technique has improved rapidly due to the development of various column packing materials as well as the attachment of various in-line detectors.

1.9.2 Reverse-Phase chromatography

Reverse-phase chromatography was developed to improve the separation of compounds with similar chemical properties. The principle of this technique is that due to the mobile phase being more polar than the stationary phase, the polar solutes are eluted first (Horváth, 1980).

The stationary phase consists of silica based packing materials with n-alkyl chains that are covalently bonded, with ligands C_8 (octyl) and C_{18} (octadecyl) in the matrix. Hydrophilic compounds elute more quickly than hydrophobic compounds due to the tendency of the column to retain hydrophobic moieties i.e. the more hydrophobic the molecule, the longer the retention time.

The mobile phase would normally consist of a mixture of various mixtures acetonitrile, methanol and water (Horváth, 1980; Hamilton and Sewell, 1982).

1.10 HPLC METHODS EMPLOYED TO DETECT PHENOLIC COMPOUNDS IN WATER

As can be seen in Table 1.1, HPLC methods used to detect phenolic compounds employ reverse phase chromatography with mainly UV detection at various wavelengths. Suliman *et al.* (2006) used diode array detection at 210-400 nm as well as fluorescent detection with excitation at 360 nm and emission at 460 nm. Although Angelino and Gennaro (1997) and Pocurull *et al*., (1996) separated all eleven phenolic compounds (listed in Figure 1.5), the retention times required to elute all eleven phenolic compounds were 27.89 minutes by Angelino and Gennaro (1997) and about 28 minutes by Pocurull *et al*. (1996). Pocurull *et al.* (1996) used a wavelength of 280 nm to detect 2,4-dinitrophenol, 2-nitrophenol and 2-methyl-4.6-dinitrophenol and 316 nm for 4-nitrophenol, the remaining of the seven compounds were detected by electrochemical detection. Gonzalez-Toledo *et al.* (2001) detected nine phenolic compounds at four different wavelengths, namely 268 nm, 311 nm, 302 nm and 278 nm. Phenol was detected at 268 nm, 4-nitrophenol at 311 nm, pentachlorophenol at 302 nm and 2-nitrophenol, 2-chlorophenol, 2,4 dimethylphenol, 4-chloro-3-methylphenol, 2,4-dichlorophenol and 2,4,6-trichlorophenol at 278 nm. Angelino and Gennaro (1997) used a detection wavelength of 285 nm while Zhao and Lee (2001) used 220 nm. Grynkiewicz *et al.* (2002) used diode array detection (DAD).

Table 1.1 HPLC methods available to determine phenolic compounds in water.

Reference	**Detection mode**	**Mobile phase**	**Analytical column**	**Phenolic compounds Detected**
Suliman *et al.*, (2006)	DAD 210-400 nm and Fluorescence Exciting 360 nm Emission 460 nm	Buffer pH12/Acetonitrile 50:50	Supelco resin based ODP-50	Ph 2-CP 4-CP 2,3DCP 3,5-DCP 2.3,5-TCP
Angelino and Gennaro (1997)	UV 285 nm	6.0 mM octylammonium orthophosphate in water/acetonitrile 65:35	Lichrospher C18 5 um	2- CP 2,4-DCP 2,4,6-TCP PCP 2-NP

				4-NP 2,4-DNP 4-C-3MP 2,4-DN-6-MP 2,4-DMP
Pocurull *et al. (*1996)	UV 280 nm and EC 1.0 V	Gradient Solvent A: 1% (v/v) acetic acid and 0.5 g/l KCl Solvent B: MeOH	Sperisorb ODS2 5µm	Ph 4-NP 2,4-DNP 2-CP 2-NP 2,4-DMP 2-M-4,6-DNP 4-C-3-MP 2,4-DCP 2,4,6-TCP PCP
Gonzalez *et al*. (2001)	Ph-268 nm 4-NP- 311 nm PCP 302 nm Remaining 5 compounds at 278 nm	Gradient Solvent A; 1% (v/v) acetic acid(aqueous) Solvent B: 1% (v/v) acetic acid (acetonitrile)	Hypersil 5 µm	Ph 4-NP 2-NP 2-CP-2,4-DMP 4-C-3MP 2,4-DCP 2,4,6-TCP PCP
Grynkiewicz *et al*. (2002)	DAD	Gradient Solvent A: 0.1% (v/v) H_3PO_4 in MeOH Solvent B: 0.1% (v/v) H_3PO_4 in water	Li Chrisper 100 RP-18 5 µm	Ph 0-cresol p-NP DNP CP 2,4,6-TCP PCP 3,4-DMP 2,4-DMP
Zhao and Lee (2001)	UV 220 nm	Isocratic Acetonitrile / 0.05% (v/v) trifluoroacetic acid 20:80	Novapak C18 4µm	m-Cresol 2-CP 3-CP 2,4-DMP 2,3,5- TMP 2,4-DCP

2,3,5-TCP	2,3,5-trichlorophenol
2,3,5-TMP	2,3,5-trimethylphenol
2,3-DCP	2,3-dichlorophenol
2,4,6-TCP	2,4,6-trichlorophenol
2,4-DCP	2,4-dichlorophenol
2,4-DMP	2,4-dimethylphenol

2.4-DNP	2,4-dinitrophenol
2-C-2,4-DMP	2-chloro-2,4-dimethylphenol
2-CP	2-chlorophenol
2-M-4,6-DNP	2-methyl-4,6-dinitrophenol
2-NP	2-nitrophenol
3,4-DMP	2,4-dimethylphenol
3,5-DCP	3,5-dichlorophenol
3-CP	3-chlorophenol
4-C-3MP	4-chloro-3-methylphenol
4-NP	4-nitrophenol

1.11 EXTRACTION OF PHENOLIC COMPOUNDS FROM ENVIRONMENTAL WATER SAMPLES

In order to clean up sample matrices and improve the detection limits of the analytes of interest, sample preparation is required. This is a fundamental step in the analysis of environmental samples. Solid phase extraction (SPE) is a popular technique for the extraction and concentration of analytes from complex matrices such as environmental samples. This replaces the liquid-liquid extraction (LLE) procedure.

Phenolic compounds are among the most challenging organic compounds to extract from the environment due to their high polarity. These compounds have low breakthrough volumes on most commercially available solid phase extraction columns. Compounds such as phenol, nitro- and 2-chlorophenol present the greatest practical limitations since they give the lowest breakthrough volumes of the phenolic compounds found in water (Bagheri and Saraji, 2003; Fontanals *et al.*, 2007).

1.11.1 Solid Phase Extraction (SPE) methods

SPE is a sorptive technique based on ionic and polar interactions between the sorptive material and the analyte. The retention of the analyte on the packing material is due to reversible hydrophobicity.

This method of extraction is an effective technique used in the field of separation science for the clean up and pre-concentration of analytical samples in various matrices. Pre-concentration is required in order to reach the required sensitivity for detection. This technique is performed using a commercially obtained small column with the required packing material. The most commonly used material for this purpose is silica, usually chemically bonded with C_2, C_8 or C_{18} organic group or

polymeric material. However the use of these silica based materials is problematic, due to pH instability, as well as poor reproducibility. This can be attributed to the presence of free silanol groups in amounts that cannot be reproduced or controlled. Hence there is a tendency to use more stable organic materials such as polymer sorbents, examples of which are Lichrolut EN; Hysphere-1; Isolute ENV+; Porapak RDX and PLRPS. Polymeric sorbents are more suitable than silica based materials due to their chemical stability and the wide range of their physico-chemical properties (Bagheri and Saraji, 2003; Fontanals *et al*., 2004; Fontanals *et al.*, 2007).

A new generation of polymers have been designed to extract a wide variety of analytes, examples of which are Oasis from Waters® and Absolute from Varian®. These types of polymers are able to extract analytes, which are acidic, basic or neutral, or whether these compounds are polar or non-polar (Hennion, 1999). The ability of these sorbents to retain the high polar phenolic compounds longer on the SPE columns is due to the polymeric skeleton which supports the π-π interactions as well as the reverse phase mechanism and the presence of the functional groups on the packing material (Bagheri and Mohammadi, 2003; Fontanals *et al*., 2007).

CHAPTER 2

INTRODUCTION TO THE PRESENT STUDY

2.1 PROBLEM STATEMENT

The detection of faecal contamination in water has long been an essential component in the monitoring and management of water systems. When determining the microbial standard of water the degree of pollution is tested using appropriate microbial models such as total coliform counts. The direct measurement of the activities of certain of the enzymes produced by indicator organisms is a potentially viable approach to quantifying the level of faecal contamination. Since indicator microorganisms like coliforms produce the enzymes β-GAL and β-GUD, the measurement of the activity of these enzymes can be used as a quantitative measurement of the potential presence of pathological organisms in drinking water.

Various assay methods have been developed using chromogenic and fluorogenic substrates, based on the assumption that β-GAL and β-GUD are metabolic markers for coliforms and *E. coli*, respectively.

Potential problems associated with this approach include interference on enzyme activity from other organisms present in the environment (plants, algae and other bacteria) (Davies *et al*., 1994), as well as the presence of certain chemical compounds in the water, such as phenols, which if they affect the activities of marker enzymes, may result in the inaccurate estimation of the presence of coliforms or *E. coli* (Pletschke *et al*., 2006). Previous studies in our group and those of other researchers have investigated the potential of interference by other chemicals. However, the interference of phenolic compounds on β-GAL and β-GUD activity has not been studied. This study therefore investigated the effects of these phenolic compounds.

2.2 HYPOTHESIS

The presence of phenols in water can affect the activities of β-GAL and β-GUD present in the water environment, thereby leading to an over- or underestimation of indicator microorganisms.

2.3 APPROACH

The purpose of this study was to investigate the effects of phenolic compounds in water on the enzyme activities of both β-GAL and β-GUD and to establish whether these compounds have a stimulatory or inhibitory effect on the activity of the enzymes. Another aim of this study was to develop and optimize a method of extraction and detection for priority pollutant phenolic (PPP) compounds in drinking water. This study involved HPLC fingerprinting of phenols present in water samples and correlated this with changes in enzyme activities of β-GAL and β-GUD in the presence of these phenolic compounds.

2.4 AIMS

The aims of this study were:

1) to examine the effects of the eleven PPP compounds listed by US EPA on the β-GAL and β-GUD enzyme assays / activities,
2) to perform enzyme inhibition / activation studies with both the β-GAL and β-GUD assays, and assess the effects of a range of phenolic compounds that are known to be present in water samples,
3) to develop, optimise and validate an HPLC method for the simultaneous detection and quantification of the eleven PPP compounds in water,
4) to develop and optimise a method to extract phenolic compounds from environmental water samples using SPE, and
5) to analyse environmental samples and correlate the effects of the phenolic compounds detected in these samples to the β-GAL and β-GUD enzyme activities.

CHAPTER 3

β-GAL AND β-GUD ENZYME ASSAY OPTIMIZATION

3.1 INTRODUCTION

3.1.1 Enzyme assay optimization

When designing an enzyme assay method to measure the specific activity of an enzyme, the optimal conditions for the enzyme have to be determined in order for the enzyme to perform at its optimum. These include parameters such as pH, temperature, assay time, assay volume, enzyme and substrate concentration as well as buffer ionic strength. It is also important to determine the optimal wavelength for the detection of the enzyme-substrate product. This is normally established by performing wavelength scans.

Once the above parameters are established for the enzyme assay, the method must show stability and show a linear response to an increasing range of enzyme concentrations, as well as consistent reproducibility under the same conditions. This is monitored by drawing up a calibration curve, which is a graphical plot of product formation versus time for the enzyme being assayed (Figures 3.1 and 3.2).

Starting guidelines for assay parameters can be obtained from the literature. An enzyme assay for a novel enzyme can be determined by looking at enzymes of similar characteristics for which stable assay methods have already been established. The parameters can then be adjusted accordingly.

3.1.2 pH Optimum

pH has an effect on the solubility as well as the structural stability of an enzyme, thus influencing its activity. Changes in pH lead to changes in charge and charge distribution on both the enzymes and substrates, which alters the bonds, and bond patterns, which then determine the rate of the enzyme–catalyzed reaction (Holme and Peck, 1998).

3.1.3 Temperature Optimum

Temperature affects the bond vibrations as well as the rate of molecular collisions, which increase as the temperature increases. This increases the number of collisions between the enzymes and substrates, which in turn increases the rate of the reaction. However, the increase in bond vibrations result in bond disruptions, which in turn causes denaturation of enzymes and rapid substrate breakdown. Therefore the optimum temperature at which to perform an enzyme assay would be a compromise between maximum activity and enzyme denaturation (Holme and Peck, 1998).

3.2 β-GAL AND β-GUD ENZYME ASSAYS

Various assay methods have been developed to detect both β-GAL and β-GUD enzymes using chromogenic and fluorogenic substrates. In this chapter the chromogenic substrates chlorophenol red β-D-galactopyranoside (CPRG) for β-GAL and ρ-nitrophenol-β-D-galactopyranoside (PNPG) for β-GUD were optimized.
Stated below are optimized enzyme activity assay methods developed for both β-GAL and β-GUD adapted from Cheng *et al*. (2002) and Fisher and Woods (2000), respectively.

3.2.1 β-GAL assays

3.2.1.1 pH

The selection of pH 7.0 for the β-GAL enzyme assay is based upon results obtained by Pletschke *et al.* (2006), where the enzyme activity for β-GAL was reported to be constant between pH 5.0 and pH 9.0. The main focus of this study was on drinking water and Pletschke *et al*. (2006) ascertained that the pH of water intended for drinking purposes would normally be around neutral confirms this pH as the most suitable for water environments. Furthermore, Wutor (2006) performed pH optimum studies and found 7.2 as the pH at which the β-GAL showed the highest activity. The pH range used by Wutor (2006) was from 5.0 to 11.0, when samples collected from running and stagnant river water samples were compared.

3.2.1.2 Temperature Selection

A temperature of 37^{o}C was selected since this is the temperature at which coliforms are present in the intestines of humans. Furthermore, studies performed by Pletschke *et al.* (2006) indicated that the activity of β-GAL increased between 30 and 60^{o}C, with inconsistent results obtained at the higher temperatures. An optimum temperature of 37 ^{o}C was reported by Wutor (2006) for commercial β-GAL.

3.2.1.3 Substrate Selection

The choice of using CPRG (chlorophenol red-β-galactopyranoside) as the chromogenic substrate in the β-GAL assay was based on substrate comparison studies between CPRG and ONPG (*o*-nitrophenyl-β-D-galactopyranoside) performed by Watson *et al*. (2000). This comparison considered sensitivity and kinetic parameters which revealed CPRG to be the best substrate for this enzyme assay. Furthermore, Wutor (2006) compared the fluorogenic substrate MuGAL with CPRG and reported interferences with the fluorogenic technique.

The β-GAL enzyme hydrolysed the substrate CPRG to produce the product chlorophenol red (CPR).

3.2.2 β-GUD Enzyme assays

3.2.2.1 pH

The β-GUD assay was adapted from the method of Fisher and Woods (2000), in which pH 8.0 was used, and hence the option to use pH 8.0 was selected for this study. This pH range was supported by studies performed by Pletschke *et al.* (2006) who reported that β-GUD activities were constant between pH 5.0 and pH 9.0. Furthermore, pH 8.0 is not far from neutral pH at which most drinking water is likely to be found (Pletschke *et al.*, 2006).

3.2.2.2 Temperature selection

A temperature of 37^{o}C was chosen, as this was the temperature used by Fisher and Woods (2000). Pletschke *et al.* (2006) reported little difference in β-GUD activity between temperatures of 25 to 50 ^{o}C.

3.2.2.3 Substrate Selection

Fluorogenic substrates for the detection of β-GUD activity are more sensitive than chromogenic substrates; however, fluorometric techniques are less desirable as they are prone to interference by naturally occurring fluorogenic components in the environment. Furthermore, fluorogenic substrates and associated instrumentation are more expensive than those required for colorimetric methods (Marxsen and Witzel, 1991; John, 2002; Pletschke *et al*., 2006). The chromogenic substrate PNPG is considered to be more feasible, as it is the least expensive of the chromogenic substrates (Aich *et al*., 2001). Therefore, PNPG was the substrate chosen for the detection for β-GUD activity in this study. The β-GUD enzyme hydrolysed the substrate PNPG to form D-glucuronic acid and ρ-nitrophenol (PNP).

3.3 MATERIAL AND METHODS

3.3.1 β-GAL assays

The CPRG assay performed in this study was adapted from Cheng *et al*. (2002). The assay buffer used was 0.1 M sodium phosphate buffer, pH 7.0. A substrate solution of 0.75 mM CPRG (Roche Molecular Biochemicals.) was prepared using the assay buffer. A 100 µl aliquot of commercial B-GAL enzyme (Sigma Aldrich G-2519, EC 3.2.1.23) was prepared at appropriate concentrations, added to wells in a microtitre plate, followed by 100 µl of the CPRG solution to initiate the reaction. The change in absorbance was detected at a wavelength of 550 nm at 37^{o}C for 30 minutes, reading at one minute intervals on a Powerwave$_{x}$ microtiterplate reader (Bio-Tek Instruments, Inc., USA). The reaction was performed in triplicate. Since this was a continuous assay, the initial rates were monitored for each reaction.

3.3.1.1 Calibration curve

Various concentrations of commercial β-GAL (Sigma-Aldrich G-2519, EC 3.2.1.23) were prepared in 0.1 M sodium phosphate buffer, pH 7.0: 0.5; 1; 2; 2.5; 3 and 3.5 µg/ml.

3.3.2 β-GUD assays

The enzyme assay used to determine β-GUD activity was adapted from Fisher and Woods (2000). The substrate PNPG was prepared at a concentration of 10 mM in assay buffer consisting of 0.1 M Tris-HCl, pH 8.0 containing 0.6 mM $CaCl_{2.}$ The commercial β-GUD was obtained from Sigma-Alrich (G 7396, EC 3.21.31) and prepared in the above assay buffer at appropriate concentrations. A 100 µl aliquot of enzyme solution was placed in a microtiterplate well after which 100 µl of PNPG (substrate) was added to initiate the reaction which was detected at a wavelength of 405 nm for 10 min reading every 30 seconds at 37^{o}C on a Powerwave$_{x}$ microtiterplate reader (Bio-Tek Instruments, Inc., USA).

3.3.2.1 Calibration curve

Various concentrations of commercial β-GUD were prepared in a 20 mM Tris-HCl, pH 8.0 containing 0.6 mM $CaCl_2$, as follows: 0; 6; 9; 12; 18; and 24 µg/ml.

3.4 RESULTS AND DISCUSSION

3.4.1 β–GAL Calibration curve

Figure 1 below represents a β-GAL enzyme calibration curve with a correlation coefficient (r^2) of 0.9849.

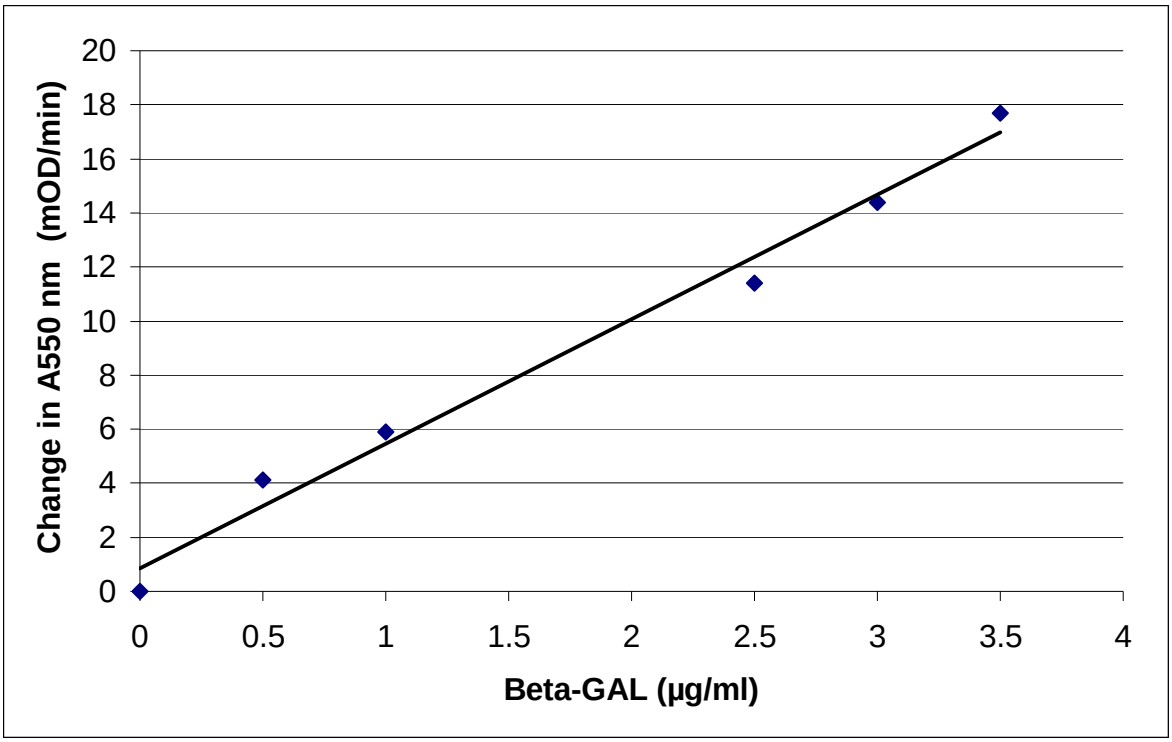


Figure 3.1 Calibration curve showing a linear response of the β-GAL enzyme assay.

3.4.2 β-GUD Calibration curves

Figure 2 below representing the β-GUD calibration curve with a correlation coefficient (r^2) of 0.9995.

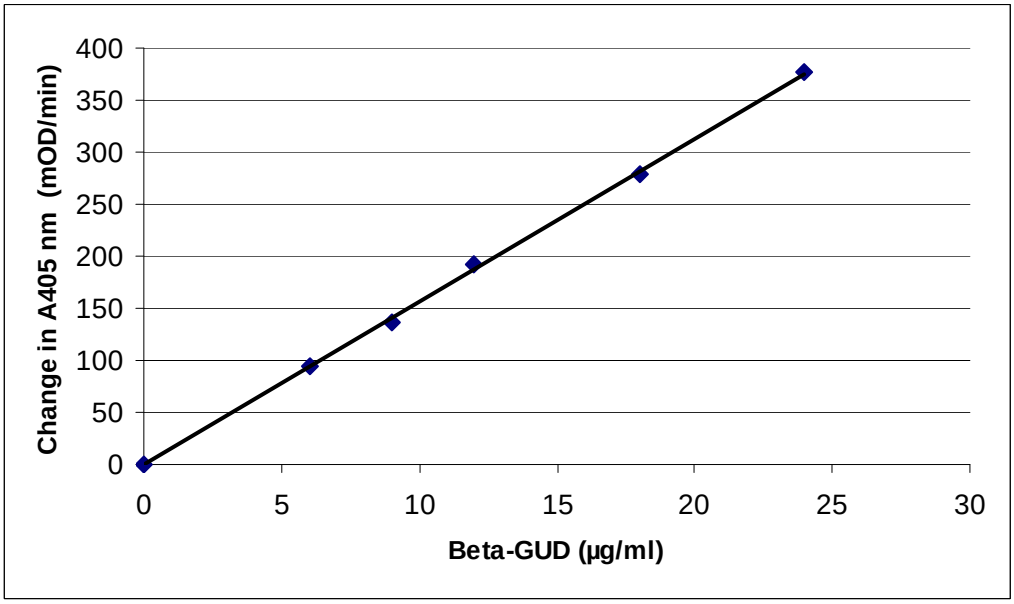


Figure 3.2 Calibration curve showing a linear response of the β-GUD enzyme assay.

3.5 CONCLUSION

The above enzyme assay parameters for both β-GAL and β-GUD demonstrated the method to be stable, reproducible and consistent and produced a linear response to an increasing range of enzyme concentrations. The correlation coefficients (r^2) for β-GAL and β-GUD were 0.985 and 0.999, respectively. This was clearly represented in both calibration curves (Figures 3.1 and 3.2) when the product formation was plotted against absorbance. Since the established protocol for both β-GAL and β-GUD enzyme assays are in place, the adjustment of assay volumes, enzyme concentration and substrate concentration could be performed easily. Furthermore, the effects of the addition of inhibitory or activating compounds to the stable enzyme assay could be observed with ease, which was desirable, since this was the main focus of the present study.

In the next chapter (Chapter 4) the eleven PPP were added individually at various concentrations to the β-GAL and β-GUD enzyme assays and the effects were observed and discussed.

CHAPTER 4

INTERFERENCE STUDIES: ENZYME INHIBITION AND ACTIVATION EFFECTS OF PHENOLIC COMPOUNDS FOUND IN WATER ON β-GAL AND β-GUD ENZYME ASSAYS

4.1 INTRODUCTION

Since the enzyme assay methods for both β-GAL and β-GUD had been optimised (Chapter 3), the addition of the eleven PPP compounds to each of these assays could now be investigated.

As mentioned previously, β-GAL and β-GUD are two enzymes that are used to test for the presence of total coliforms and *E. coli*, respectively, in water samples. Various assay methods have been developed using chromogenic and fluorogenic substrates, based on the assumption that β-GAL and β-GUD are metabolic markers for coliforms and *E. coli*.

Potential problems associated with this approach include interference from other organisms present in the environment (plants, algae and other bacteria), as well as the presence of certain chemical compounds, such as phenolic compounds in water. Pletschke *et al*. (2006) reported that ferulic acid, a phenolic compound produced during lignin degradation, inhibited β-GAL and β-GUD activities, and implied that the use of direct enzyme assays may produce false negatives. There are also other phenolic compounds present in the environment which (due to their industrial applications) may also have a similar effect, such as the eleven PPP compounds listed by US EPA (see Chapter 1, Figure 1.5).

This chapter investigated the interference of these PPP compounds commonly found in water, on the activities of β-GAL and β-GUD, which are used as enzymatic indicators of water quality.

The types of inhibition as well as inhibition constant (K_i) values were also determined.

4.2 ENZYME KINETICS

Enzyme kinetic studies enable researchers to observe and report the rates at which chemical reactions take place. Through these studies the binding affinities of an enzyme to its substrates and inhibitors are determined and the maximum catalytic rate of an enzyme reaction can also be established.

In this chapter, the effects of adding each of the eleven PPP (listed by the US EPA as being found in water) to either of each of the β-GAL and β-GUD enzyme assays were examined. These interfering effects were characterized using suitable enzyme kinetics. The K_i values for the inhibitors were calculated and discussed. Kinetic parameters are briefly defined below.

4.2.1 K_m

This is referred to as the Michaelis constant, which is the concentration of the substrate that gives an initial velocity equal to half the maximum velocity ($_{1/2}V_{max}$).

4.2.2 V_{max}

The maximum initial velocity that occurs at a substrate concentration that saturates the enzyme.

4.2.3 k_{cat}

The number of substrate molecules converted to product per enzyme molecule per unit time at $V_{max.}$ It is the kinetic efficiency of the enzyme.

4.2.4 k_{cat}/K_m

The catalytic constant is a measure of catalytic efficiency. This value can be used to compare substrates to determine which substrate has the highest specificity for its enzyme (Garrett and Grisham, 1999; Voet and Voet, 2004).

4.2.5 K_I (Inhibition constant)

This value refers to the strength of the binding of an inhibitor to the enzyme. The lower the K_i value the greater the affinity of the inhibitor for the enzyme. K_i is expressed in the same unit as inhibitor concentration. The extent to which an inhibitor affects the rate of an enzyme depends on both the strength of its complex with the

enzyme and the concentration of the inhibitor added to the enzyme assay when performing kinetic analysis (Wilson and Walker, 2000).

4.3 ENZYME INHIBITION

Enzyme inhibition occurs when substances (inhibitors) influence the binding of a substrate to its enzyme, decreasing the velocity of an enzymatic reaction. This effect may be reversible, where inhibitors interact with the enzyme through noncovalent association, or irreversible where inhibitors form covalent associations with the enzyme. There are various ways that these inhibitors affect the enzyme substrate complex, resulting in either competitive, non-competitive or mixed inhibition, and uncompetitive inhibition.

4.3.1 Competitive inhibition

This occurs when the substrate and the inhibitor compete for the same active site on the enzyme.

4.3.2 Un-competitive inhibition

This occurs when the inhibitor combines to the enzyme substrate complex and not to the enzyme. This is a theoretical situation, not really found in real practical terms.

4.3.3 Mixed inhibition

This occurs when the inhibitor combines with both the enzyme and enzyme- substrate complex. Non-competitive inhibition is a special form of mixed inhibition (Garrett and Grisham, 1999; Voet and Voet, 2004).

4.4 INTERFERENCE STUDIES

Nistor *et al.* (2002) measured the amount of phenolic compounds in environmental water samples at different stages of wastewater treatment. The three sources of effluent were at raw, primary and final stages. The concentrations of phenolic compounds were as high as 0.114 mg/ml for nitrophenol at the primary effluent stage

and 0.083 mg/ml for 2,3-dichlorophenol at the raw effluent stage. These results were obtained by solid phase extraction-liquid chromatography-mass spectrometry (SPE-LC-MS) analysis. Based on these findings, the concentrations of phenolic compounds used in this study to examine the effect of interference of these phenolic compounds on the B-GAL and B-GUD assays were set at 0.1 mg/ml and 0.2 mg/ml respectively.

A concentration of 0.1 mg/ml of each of the eleven phenolic compounds was added individually to the β-GAL and β-GUD enzyme assays. The results are represented in Figure 4.1 for β-GAL and Figure 4.2 for β-GUD, respectively.

All assays performed were blanked with the relevant buffers and the positive control consisted of enzyme and buffer without any phenolic compounds present.

4.5 MATERIALS AND METHODS

4.5.1 β-GAL assays

The CPRG assay performed in this study was adapted from Cheng *et al*. (2002). The assay buffer used was a 0.1 M sodium phosphate buffer, pH 7.0. A substrate solution of 0.75 mM CPRG (Roche Molecular Biochemicals), diluted in the sodium phosphate buffer, was prepared. A 100 µl aliquot stock solution in buffer of commercial β-GAL enzyme (Sigma Aldrich G-2519, EC 3.2.1.23) was then added to a well in a microtiter plate to give a final concentration of 6.88×10^{-5} nM, followed by 100 µl of the CPRG solution to initiate the reaction. The change in absorbance was detected at 550 nm at 37^{o}C for 30 minutes, reading at one minute intervals on a Powerwave$_x$ microtiterplate reader (Bio-Tek Instruments, Inc., USA). All assays were performed in triplicate.

4.5.2 β-GUD assays

The enzyme assay used to determine β-GUD activity was adapted from Fisher and Woods (2000). The substrate PNPG was prepared in assay buffer at a concentration of 10 mM. The assay buffer consisted of 0.1 M Tris-HCL, pH 8.0 containing 0.6 mM $CaCl_2$. Commercial β-GUD was obtained from Sigma-Alrich (G 7396, EC 3.21.31) and prepared in the assay buffer above. A 100 µl aliquot stock solution in buffer of enzyme was placed in a microtiterplate well to give a final concentration of 6.88×10^{-5} nM, after which 100 µl of PNPG substrate was added to initiate the reaction which was monitored at 405 nm for 10 min taking readings every 30 seconds at 37^{o}C on a Powerwave$_x$ microtiterplate reader (Bio-Tek Instruments, Inc., USA).

4.5.3 β-GAL and β-GUD assays in the presence of phenolic compounds

In order to observe the effects of the eleven PPP listed above on β-GAL and β-GUD enzyme activity, the same assay parameters were used as described above but with the addition of each individual phenolic compound. Individual stock solutions (0.2 mg/ml) of each phenolic compound were prepared by dissolving the compounds in either acetonitrile or dimethylsulfoxide, and then diluting using an appropriate amount of HPLC grade water to the required volume. A mixture of the respective enzyme and individual phenolic compound was prepared to give final concentrations of 0.1 mg/ml and 0.2 mg/ml of each phenolic compound, added to the wells in the microtitre plate, and placed in the Powerwave$_x$ microtiterplate reader until a temperature of 37^{o}C was reached. The respective substrates were then added and the reactions were monitored. Controls for the respective buffer and enzyme, and a second control of substrate and buffer were performed at each phenol concentration used. All assays were performed in triplicate.

In this field of water research, concentrations are more commonly expressed in mg/l than molarity and therefore all phenolic concentrations in this thesis have been expressed as mg/l. For the various concentration levels used in initial studies to test the effects of the eleven PPP compounds on the β-GAL and β-GUD assays, a stock solution of 2.5 mg/ml was made and diluted to a final concentration of 1 mg/ml, 2.5 µg/ml and 0.2 µg/ml, respectively.

4.6 RESULTS AND DISCUSSION

4.6.1 Selection of organic solvents to dissolve the phenolic compounds.

Since phenolic compounds are not totally soluble in water, an organic solvent was used to dissolve these compounds prior to adding HPLC grade water to the desired volume for stock solutions. It was therefore crucial that the organic solvent used to dissolve the phenolic compounds would not interfere with the enzyme assays, so that only the effects of the eleven PPP being monitored would be observed.

Acetonitrile was selected as the organic solvent for this purpose. Initial studies were performed to establish whether or not acetonitrile contributed to any background interference. These experiments were performed with both enzyme assays using only the enzyme (β-GAL or β-GUD) and acetonitrile without any of phenolic compounds present. The organic solvent was tested at the same concentrations as those present

during the investigation of the effects of the eleven PPP on the β-GAL and β-GUD assays.

4.6.1.1 Effects of Acetonitrile

4.6.1.2 Solvent selection for the B-GUD enzyme assay

When tested in the β-GUD assay acetonitrile was shown to be a compatible solvent, resulting in little background interference, as can be seen in Figure 4.1 below.

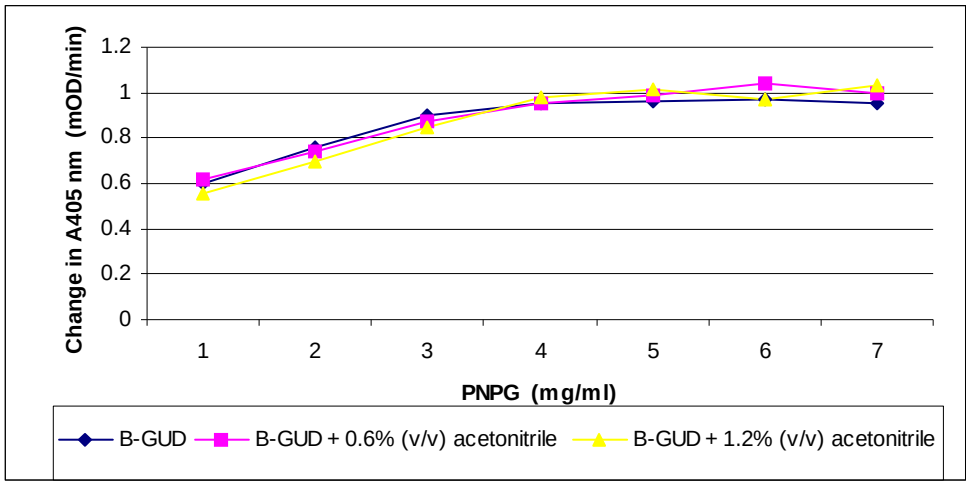


Figure 4.1 Effect of acetonitrile on β-GUD enzyme assay. Data points represent the means ± SD (n=3).

4.6.1.3 Solvent selection for the β-GAL enzyme assay

When tested in the β-GAL assay; however, acetonitrile contributed to some background interference, at a concentration of 1% (v/v) (see Figure 4.2).

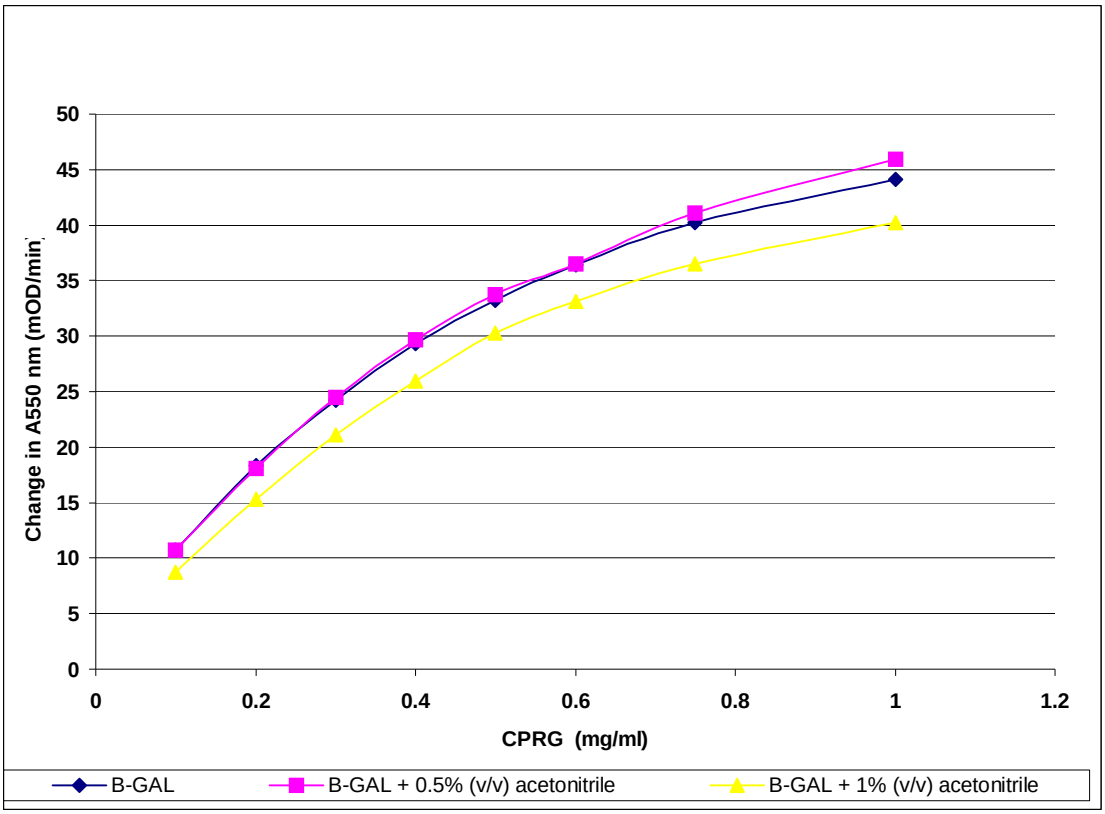


Figure 4.2 Effects of acetonitrile on β-GAL assay. Data points represent the means ± SD (n=3).

For this reason, alternative organic solvents were therefore also investigated. The following additional organic solvents were tested to determine which would be the most compatible: dimethyl sulfoxide (DMSO), ethanol and acetone.

Once again, organic solvents at the same concentrations as those used to dissolve the phenolic compounds were tested with the β-GAL assay. The results are represented in the graphs below. The organic solvent DMSO (Figure 4.3) was found to give the least background interference in the β-GAL assay when compared to acetonitrile (Figure 4.2), and ethanol and acetone (Figures 4.4 and 4.5). The most compatible solvent which was selected to dissolve the phenolic compounds for the β-GAL assay was therefore DMSO.

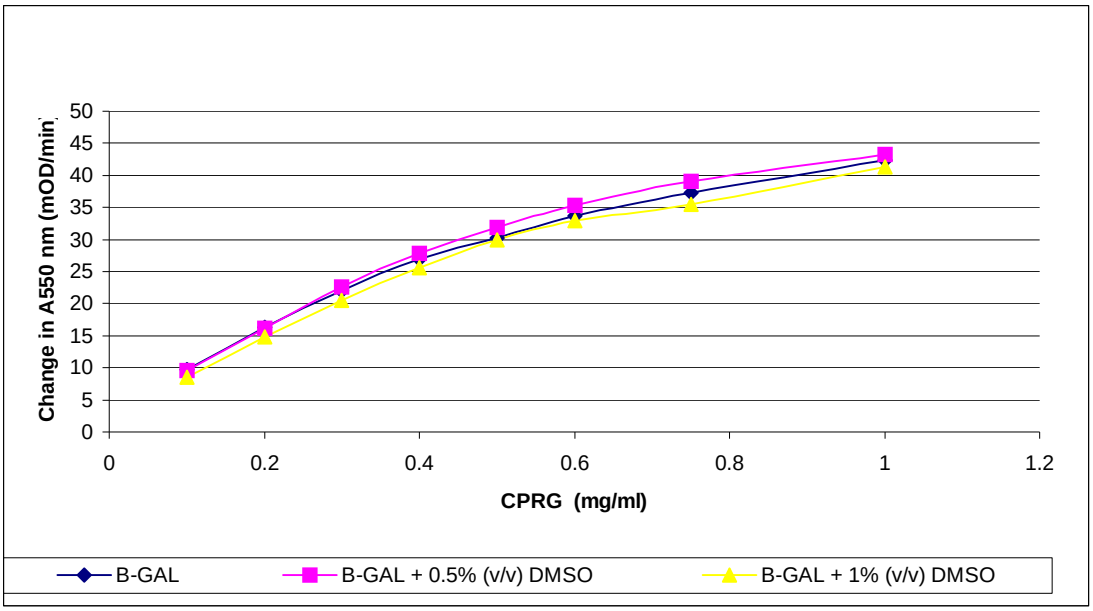


Figure 4.3 Effect of DMSO on the β-GAL assay. Data points represent the means ± SD (n=3).

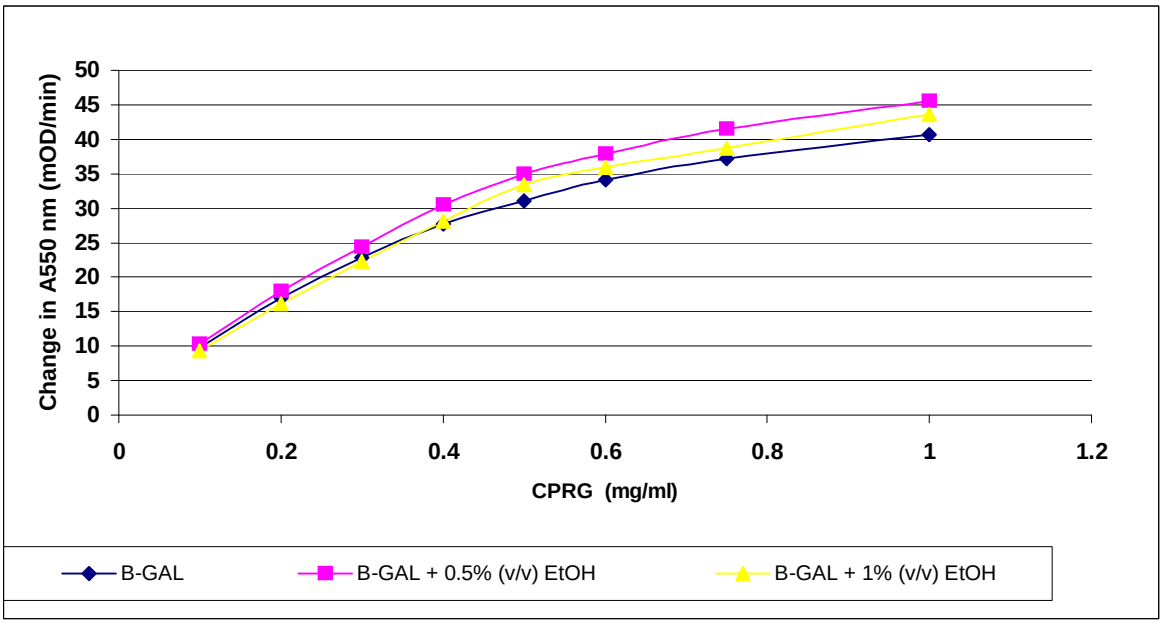


Figure 4.4 Effect of Ethanol on β-GAL enzyme assay. Data points represent the means ± SD (n=3)

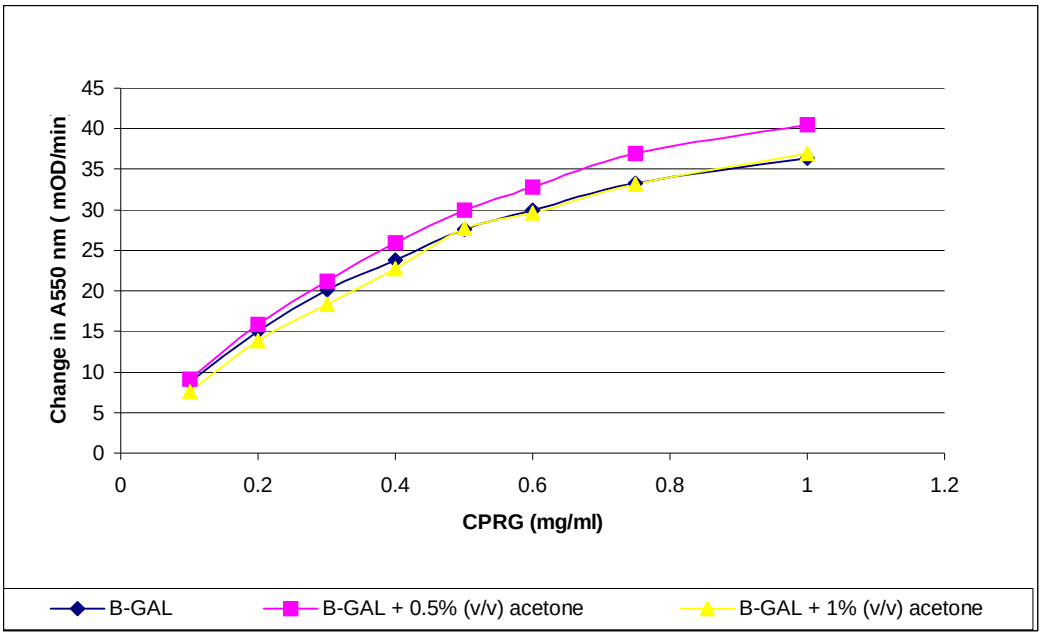


Figure 4.5 Effect of acetone on β-GAL enzyme assay. Data points represent the means ± SD (n=3).

4.6.2 Effects of eleven PPP on the β-GAL and β-GUD enzyme assays

A concentration of 0.1 mg/ml of each of the phenolic compounds was added individually to the β-GAL and β-GUD assays. These were done in triplicate in microtiter 96 well plates and read in the Powerwave$_x$ under the above conditions for the respective assays. The blank consisted of the buffer and substrate. The positive control consisted of buffer, enzyme and substrate and this represented 100% enzyme activity.

4.6.2.1 Effects of 0.1 mg/ml of each PPP on the β-GUD assay

As can be seen in Figure 4.6, two compounds (namely 4-nitrophenol and 2,4-dinitrophenol) activated β-GUD enzyme activity. For both of these compounds the mean value exceeded the maximum absorbance of 4 mOD. Eight of the compounds produced an inhibitory effect, with the eleventh compound (2-nitrophenol) exhibiting a large negative absorbance and interfering with the β-GUD assay.

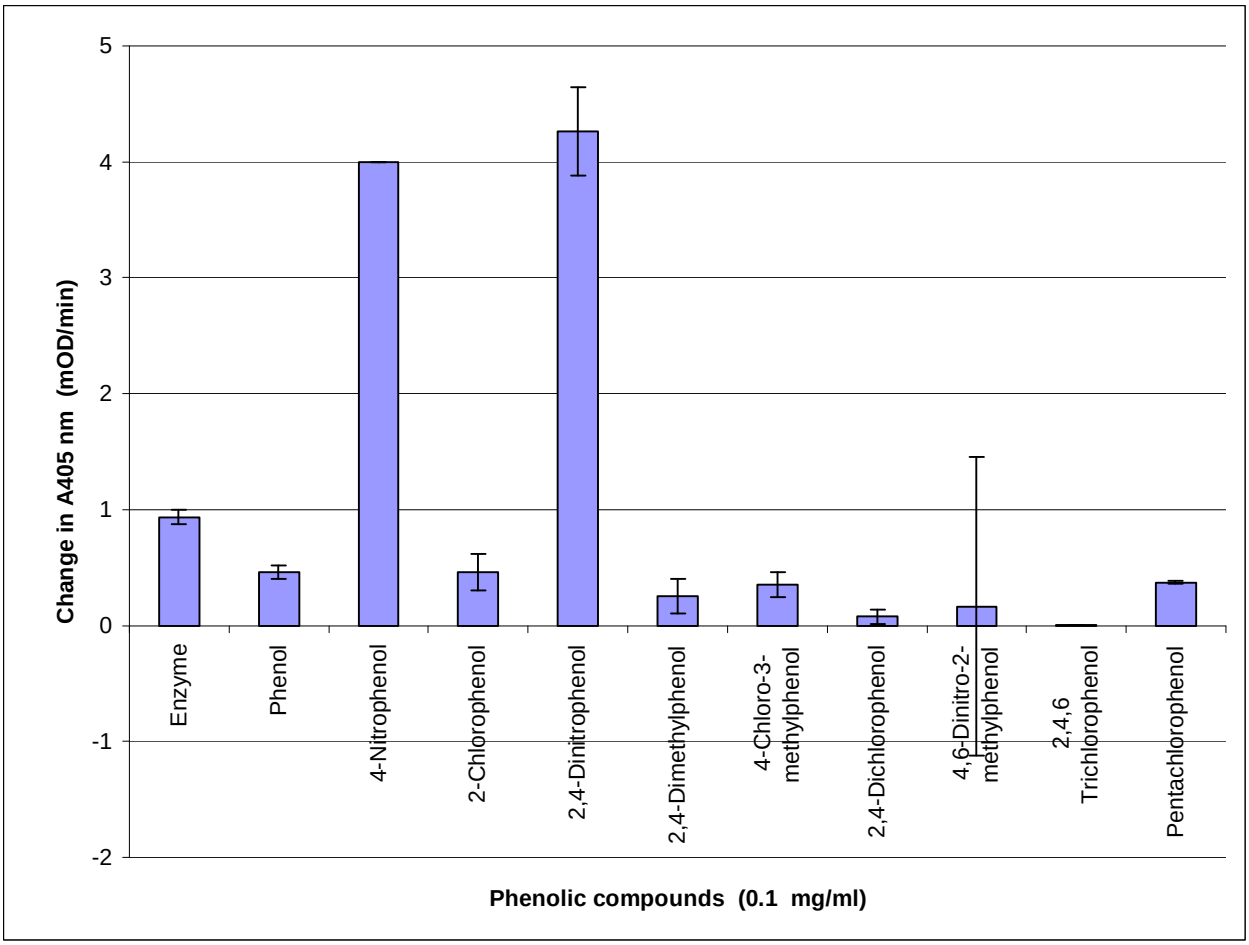


Figure 4.6 Inhibition and activation of β-GUD activity in the presence of various phenolic compounds at a concentration of 0.1 mg/ml. Data points represent the means ± SD (n = 3).

4.6.2.2 Effects of 0.1 mg/ml of each PPP on the β-GAL assay

The results observed with the eleven phenolic compounds and β-GAL showed that all the compounds (with the exception of 2-chlorophenol) had an inhibitory effect on the enzyme assay (see Figure 4.7). The compound 2-chlorophenol resulted in a similar absorbance to the positive β-GAL control (i.e. no phenolic compound present - "Enzyme"). The compound 2,4-dichlorophenol exhibited the greatest degree of inhibition, followed closely by 4-chloro-3-methylphenol; 4,6-dinitro-2-methylphenol and pentachlorophenol.

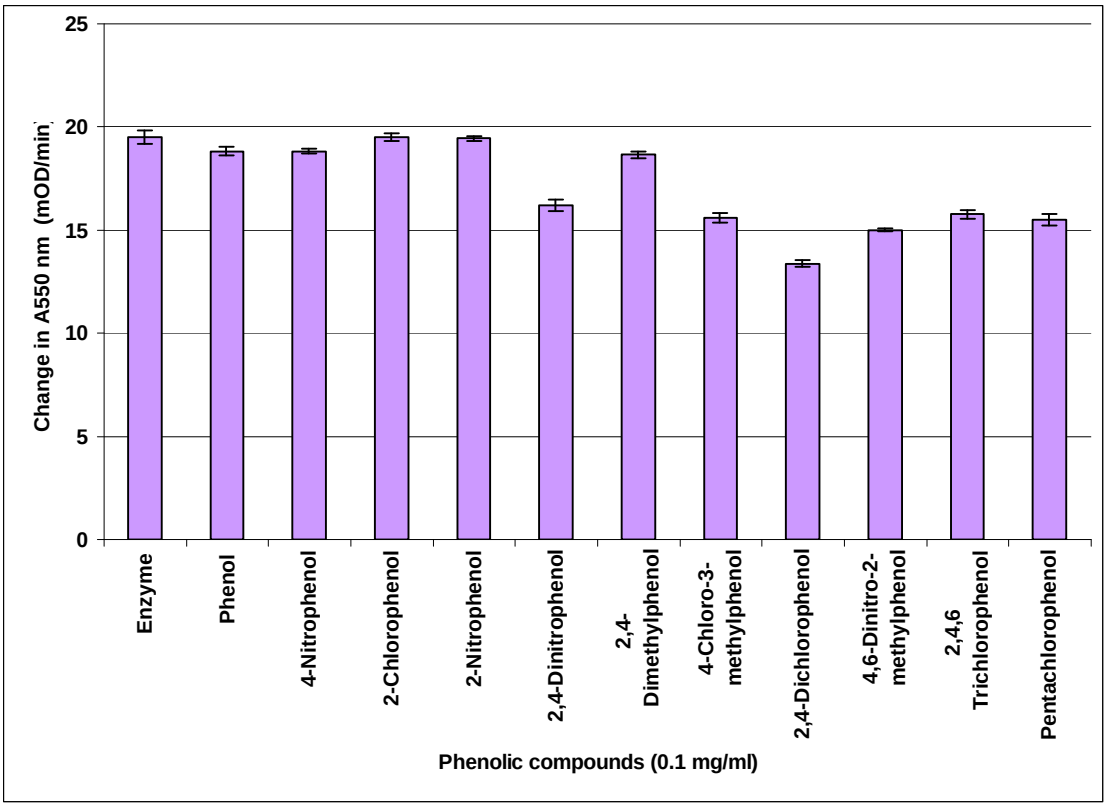


Figure 4.7 Inhibition of β-GAL activity in the presence of the eleven PPP listed by the USA EPA at a concentration of 0.1 mg/ml. Data points represent the means ± SD (n = 3).

4.6.3 The effects of each of the PPP at concentrations higher and lower than 0.1 mg/ml on the β-GAL and β-GUD enzyme assays

Studies were performed at higher and lower concentrations than 0.1 mg/ml for the phenolic compounds in order to observe the effects of various concentration levels. For the β-GAL assays four different concentrations were used, namely 0.2 μg/ml, 2.5 μg/ml, 1 mg/ml and 2.5 mg/ml. The three different concentrations used for the B-GUD assays were 0.2 μg/ml, 1 mg/ml and 2.5 mg/ml.

4.6.3.1 The effects of the eleven PPP on the β-GAL enzyme assay at concentration levels of 0.2 μg/ml, 2.5 μg/ml, 1 mg/ml and 2.5 mg/ml

The graphs illustrating the results of the effects of the eleven PPP in the β-GAL assays at concentrations of 0.2 μg/ml (Figure 4.8) showed little or no effect on the enzyme activity. There was, however, a slight activation with the addition of the

compounds 2,4-dichlorophenol, 4,6-dinitro-3-methylphenol, 2,4,6-trichlorophenol and pentachlorophenol at the level of 0.2 µg/ml (Figure 4.8). The addition of 2.5 µg/ml (Figure 4.9) of each the PPP to the β-GAL assay demonstrated inhibitory effects on the β-GAL enzyme activity by all eleven compounds.

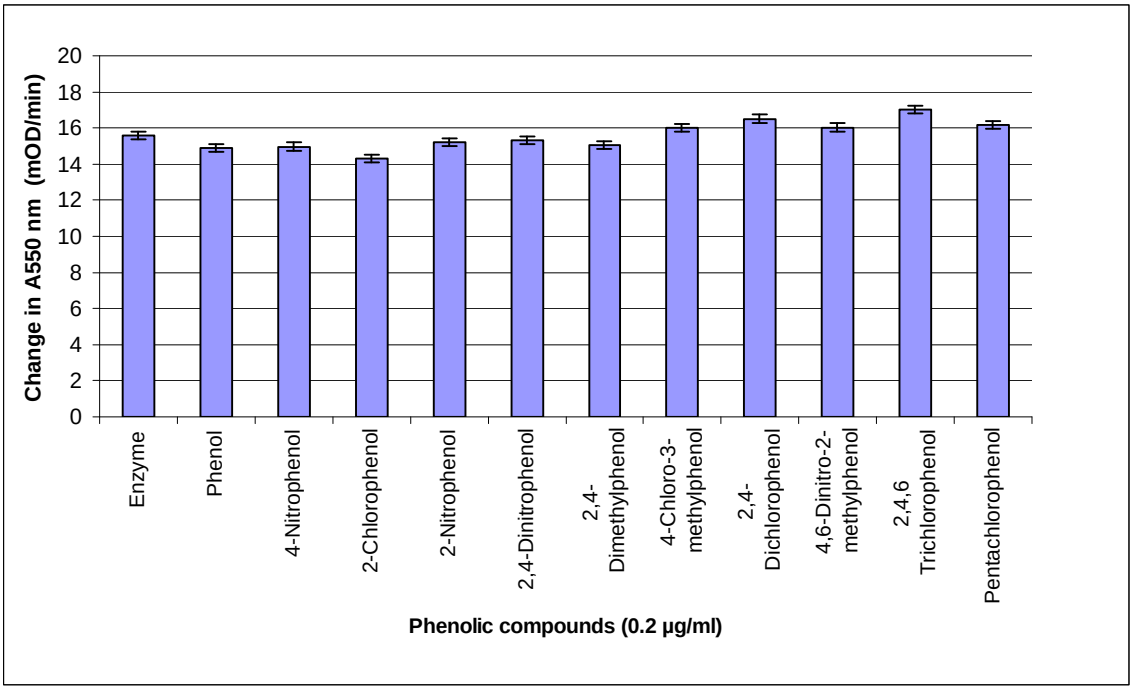


Figure 4.8 Effect of 0.2 µg/ml of each of the eleven PPP on the B-GAL enzyme assay. Data points represent the means ± SD (n = 3).

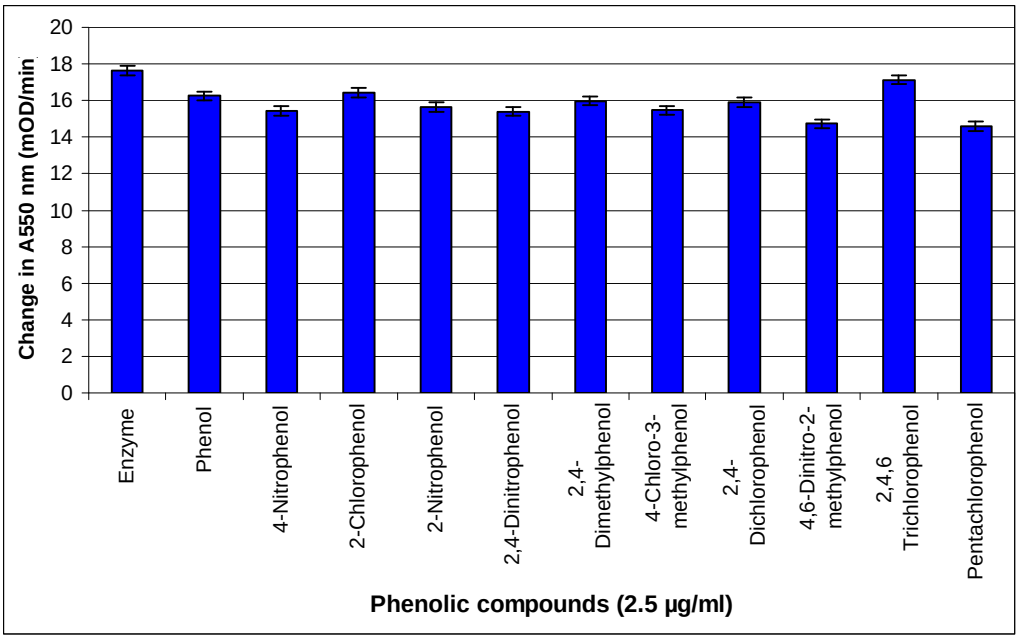


Figure 4.9 The effect of 2.5 µg/ml of each the eleven PPP on β-GAL enzyme assay. Data points represent the means ± SD (n = 3).

At higher concentrations of the PPP i.e. at 1 mg/ml (Figure 4.10) and 2.5 mg/ml (Figure 4.11) the interference observed was even greater, implying that the higher concentrations of the eleven PPP resulted in even greater inhibition of the β-GAL enzyme assays.

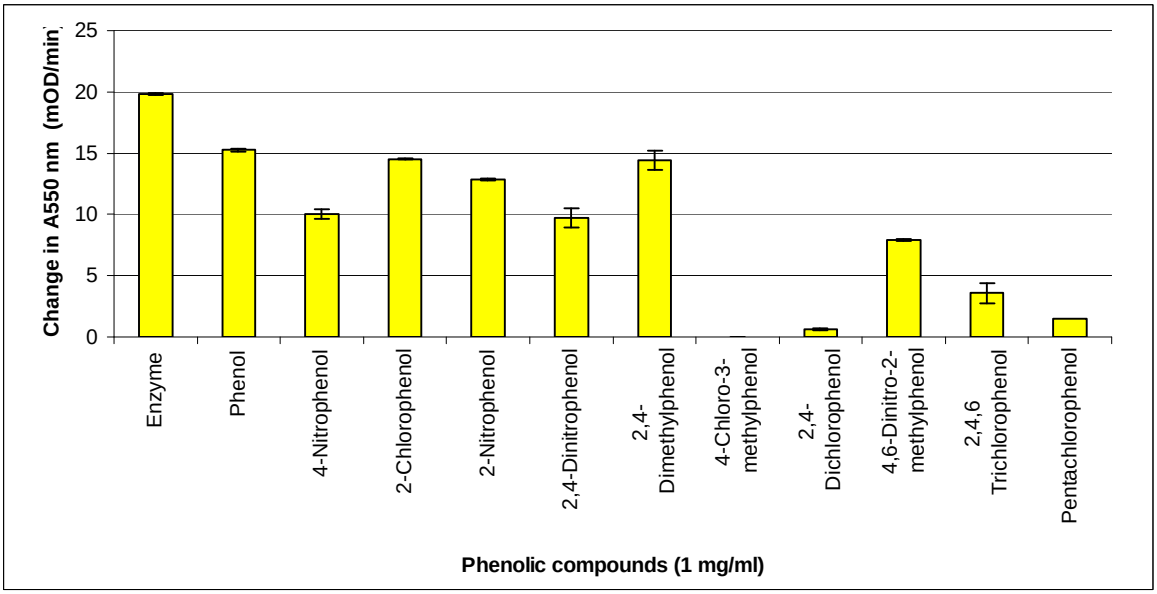


Figure 4.10 The effect of 1 mg/ml of each of the eleven PPP on β-GAL enzyme assay. Data points represent the means ± SD (n = 3).

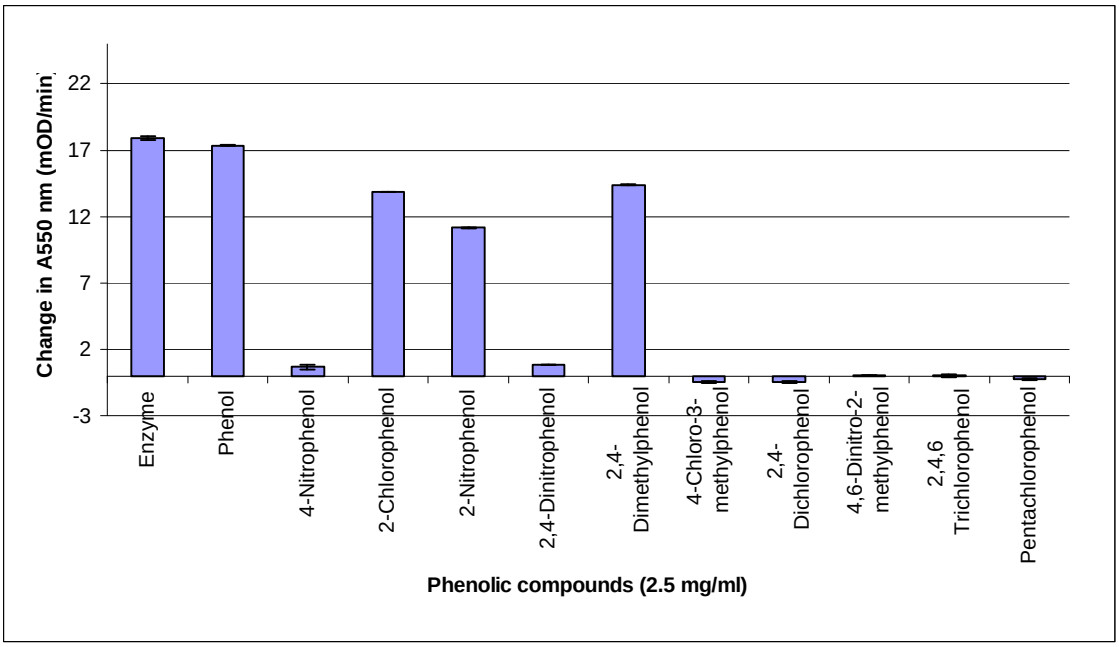


Figure 4.11 The effect of 2.5 mg/ml of each of the eleven PPP on the β-GAL enzyme assay. Data points represent the means ± SD (n=3).

4.6.3.2 The effects of the eleven PPP on the β-GUD enzyme assay at concentration levels of 0.2 µg/ml, 1 mg/ml and 2.5 mg/ml

As with β-GAL, the β-GUD assays were performed at various concentrations to determine the affects of various concentration levels of the eleven PPP as well as reproducibility. As seen in Figure 4.12 below, at phenolic compound concentrations of 0.2 µg/ml, the effect of the PPP compounds on the β-GUD enzyme assay varied. The effects were either activating or inhibitory to the β-GUD enzyme assay.

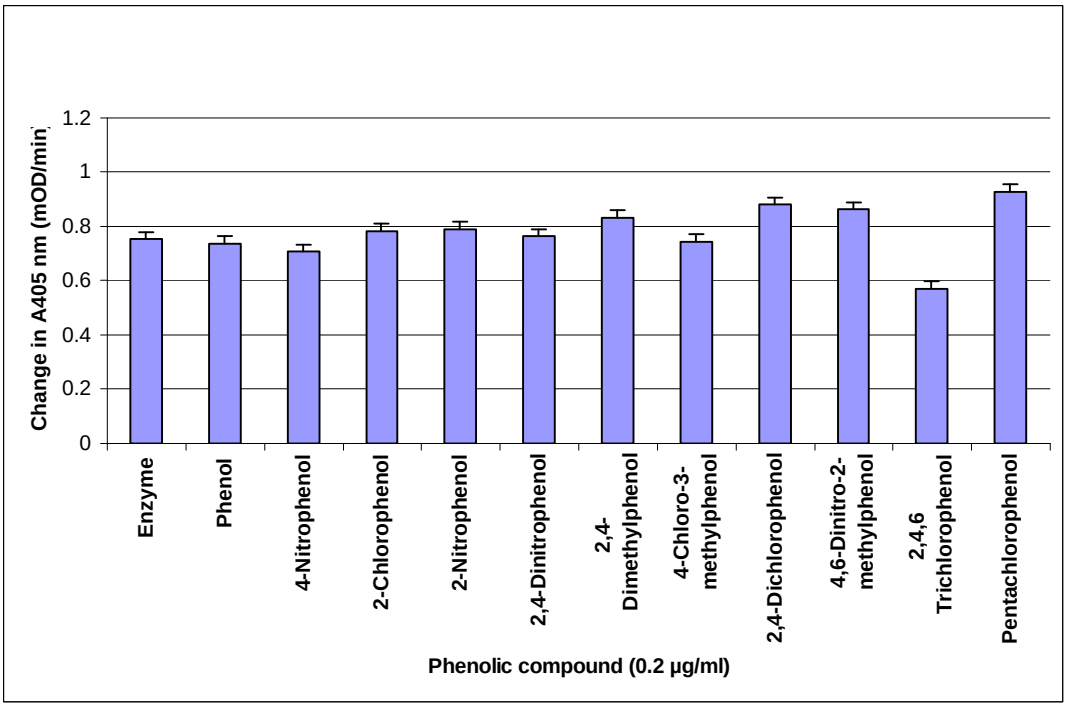


Figure 4.12 The effect of 0.2 µg/ml of each of the eleven PPP on β-GUD enzyme assay. Data points represent the means ± (n=3).

At the higher concentrations, i.e. 1 and 2.5 mg/ml, the effects on the β-GUD assay were much more pronounced.

At a concentration of 1 mg/ml (Figure 4.13) the effects of the phenolic compounds on the β-GUD enzyme assay varied greatly with four compounds namely 4-nitrophenol, 2-nitrophenol, 2,4-dinitrophenol and 4,6-dinitro-2-methylphenol giving readings above the maximum of 4 mOD, and 2-chlorophenol a reading at 3.269 mOD. These readings indicate a large activating effect by these compounds.

The effect of 2,4,6-trichlorophenol was different, contributing to a negative mOD reading, as did pentachlorophenol and 2,4-dichlorophenol, indicating an inhibitory effect.

At 2.5 mg/ml (Figure 4.14) the phenolic compounds showed a similar pattern of activation. The reading for five of the compounds, 4-nitrophenol, 2-nitrophenol, 2,4-dinitrophenol, 4,6-dinitro-2-methylphenol and 2,4,6-trichlophenol exceeded the maximum of 4mOD while 2-chlorophenol was slightly lower at 3.754 mOD. Interestingly, 2,4,6-trichlorophenol and pentachlorophenol went from inhibitory at

1 mg/ml to activating at 2.5 mg/ml. The two compounds 4-chloro-3-methylphenol and 2,4-dichlorophenol also showed greater inhibitory effects at 1 mg/ml and 2.5 mg/ml compared to the concentration of 0.2 µg/ml.

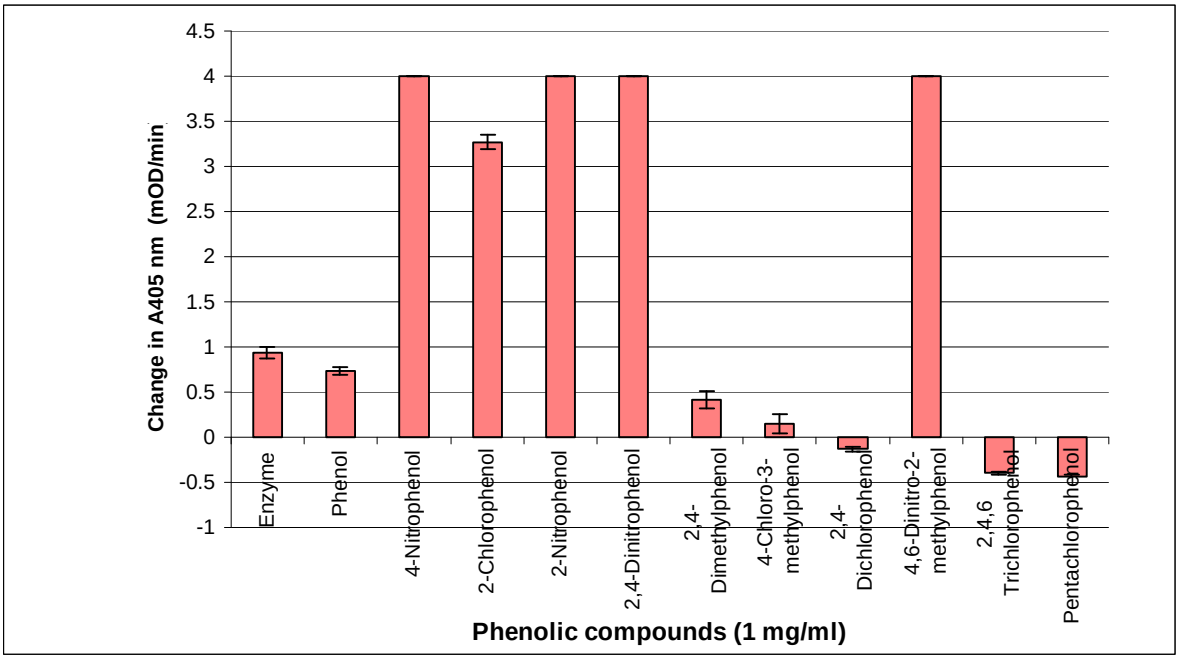


Figure 4.13 The effect of 1 mg/ml of each of the eleven PPP on β-GUD enzyme assay. Data points represent the means ± (n=3).

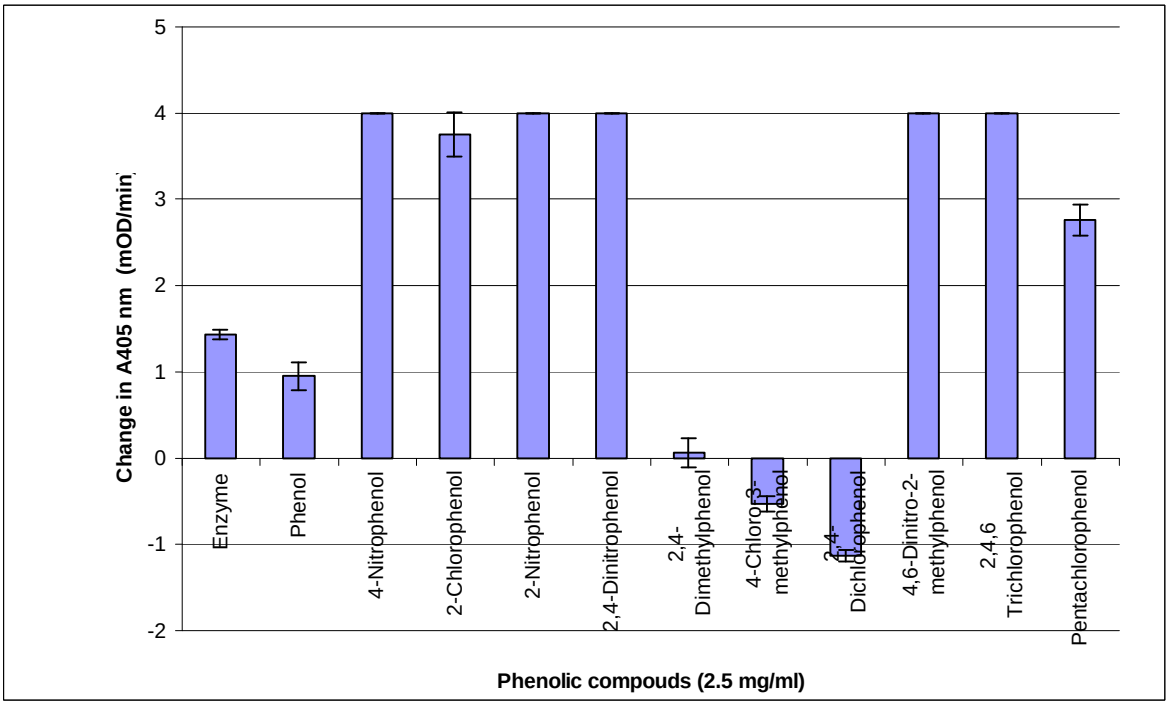


Figure 4.14 The effect of 2.5 mg/ml of each of the eleven PPP on β-GUD enzyme assay. Data points represent the means ± SD (n=3).

4.7 INHIBITION AND ACTIVATION STUDIES OF THE FIVE SELECTED PHENOLIC COMPOUNDS ON THE β-GAL AND β-GUD ENZYME ASSAYS

Further inhibition and activation studies were performed, selecting five of the original PPP compounds used.

For the B-GAL enzyme assay, four of the compounds namely 4-chloro-3-methylphenol, 2,4-dichlorophenol, 4,6-dinitro-2-methylphenol and pentachlorophenol were selected on the basis of exhibiting the most inhibition on the activity of the β-GAL enzyme assay when the eleven PPP were added. The fifth compound (2-chlorophenol) was also selected based on the fact that the chemical structure is similar to chlorophenol red, which is the product formed in the β-GAL enzyme assay when the substrate CPRG is hydrolysed. This selection process was based on the results in Figure 4.7 representing the effects of the eleven PPP added at a concentration of 0.1 mg/ml to the β-GAL enzyme assay. Statistical analysis revealed that the effects of these phenols were indeed significant ($P < 0.05$) using Microsoft Excel 2003 statistical tool at 5% level of significance. Although the effect of 2-chlorophenol was not significant ($P>0.05$), this compound was also selected for further study since this product is formed in the CPRG assay.

The selection for the β-GUD enzyme assay was based on two of the compounds 4-nitrophenol and 2,4-dinitrophenol showing a dramatic activation on the β-GUD enzyme assay. Furthermore, 4-nitrophenol is the product formed during the β-GUD enzyme assay when the substrate PNPG is added. The remaining three compounds 2,4-dimethylphenol, 4-chloro-3-methylphenol and 2,4-dichlorophenol, were selected because these compounds showed a high degree of inhibition to the β-GUD enzyme assay. As with the β-GAL enzyme assay, these findings were observed when the eleven PPP were added individually at a concentration of 0.1 mg/ml to the β-GUD enzyme assay (Figure 4.6). Statistical analysis revealed all five of these phenolic compounds that were selected for further study with regards to the potential for interference of phenolic compounds to have a significant effect on the activity of β-GUD ($P<0.05$).

In order to confirm reproducibility, the above five selected PPP compounds for both the β-GAL and β-GUD enzyme assays were added individually at a concentration of 0.1 mg/ml to each of the enzyme assay. The results are represented in Figures 4.15 and 4.16 below.

As can be seen from these graphs (when compared to the earlier studies performed at this concentration), the trends were very similar for the selected phenolic compounds in both assays.

For the β-GAL assay (Figure 4.15) all five selected PPP showed inhibition. Interestingly, 2-chlorophenol exhibited an inhibitory effect compared to an insignificant effect in Figure 4.7 at the same concentration.

For the β-GUD assay (Figure 4.16), the two compounds 4-nitrophenol and 2,4-dinitrophenol revealed an activating effect which corresponded to the earlier studies performed in Figure 4.6 at the same concentration. The two compounds 2,4-dimethylphenol and 4-chloro-3-methylphenol again showed an inhibitory effect as in Figure 4.6. The fifth compound (2,4-dichlorophenol) showed no significant effect; however, this compound showed an inhibitory effect at the same concentration in Figure 4.6.

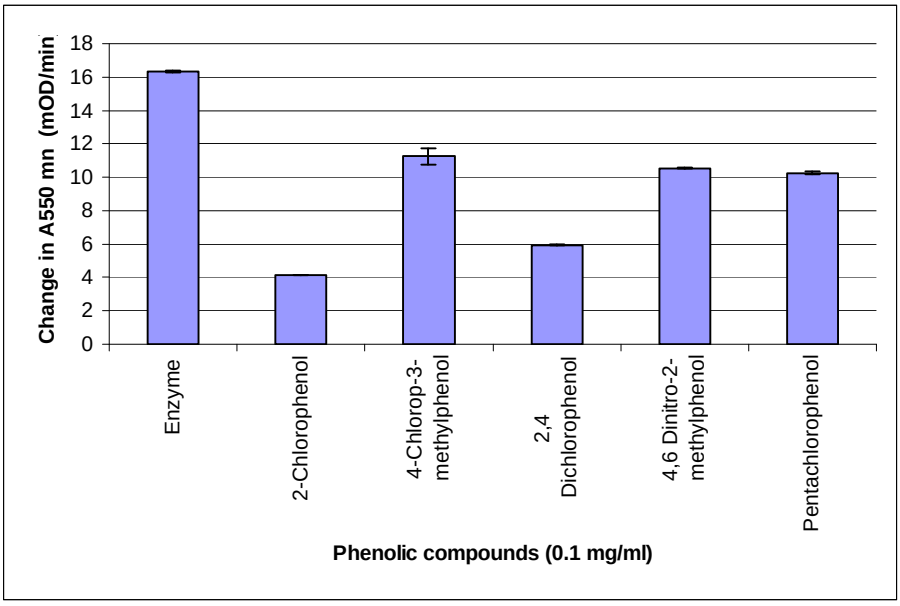


Figure 4.15 Effect of five selected phenolic compounds at 0.1 mg/ml on the β-GAL enzyme assay. Data points represent the means ± SD (n=3).

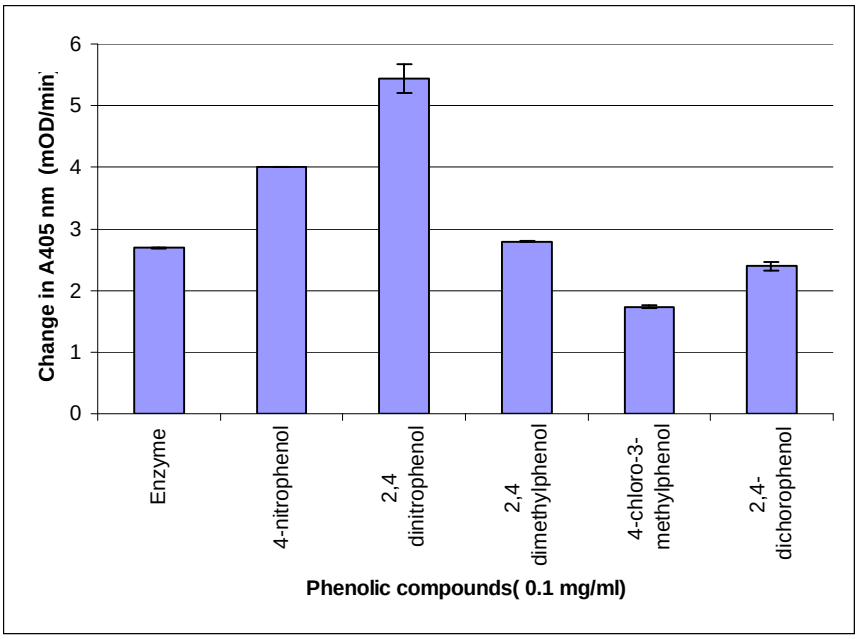


Figure 4.16 The effect of five selected phenolic compounds at 0.1 mg/ml on the β-GUD enzyme assay. Data points represent the means ± SD (n=3).

4.8 THE EFFECTS OF THE SELECTED PPP COMPOUNDS ON β-GAL AND β-GUD ENZYME ASSAYS

Detailed investigations were performed on the five selected compounds for β-GAL and β-GUD. These were inhibition studies performed on the interference of the selected phenolic compounds on the β-GAL and β-GUD assays by investigating the effects of 0.1 mg/ml and 0.2 mg/ml of each phenolic compound on the enzyme assays at various levels of substrate varying from 0 to 2.5 mg/ml (for PNPG) and 0 to 1 mg/ml (for CPRG).

Double-reciprocal Lineweaver-Burk plots for each phenolic compound were then constructed using the data obtained. From these graphs, the type of inhibition was elucidated. Three types of inhibition were identified; competitive, non-competitive and mixed inhibition. Secondary plots were then constructed in order for the inhibitor constant K_i to be calculated.

4.8.1 β-GAL assays effected by the five selected PPP

In the β-GAL enzyme assays, the types of inhibition exhibited by four of the phenolic compounds tested were as follows: 4-chloro-3-methylphenol (competitive); 4,6-dinitro-2-methylphenol (competitive); 2,4-dichlorophenol (non-competitive) and pentachlorophenol (competitive). The fifth phenolic compound, 2-chlorophenol, exhibited no significant effect on the normal reaction observed in the absence of inhibitor. These results are listed in Table 4.1.

The inhibition described above was attributed to 4-chloro-3-methylphenol, 4,6-dinitro-2-methylphenol and pentachlorophenol competing for the same binding site as the substrate CPRG, since this study revealed competive inhibition. The compound 2,4-dichlorophenol also bound to an alternative binding site on the B-GAL enzyme molecule, as in case of the non-competitive inhibition. This resulted in a decrease in velocity of the enzyme reaction.

The fifth compound, 2-chlorophenol, did not show any significant effects on the β-GAL enzyme assay.

4.8.2 β-GUD assays effected by the five selected PPP

Three of the phenolic compounds were tested in the β-GUD enzyme assay. Both 4-chloro-3-methylphenol and 2,4-dichlorophenol demonstrated mixed inhibition; while 2,4-dimethylphenol showed competitive inhibition (Table 4.2).

The inhibitory effects on the β-GUD enzyme assay was attributed to 2,4-dimethylphenol competing for the same binding site as the substrate PNPG on the β-GUD molecule. The compounds 4-chloro-3-methylphenol and 2,4-dichlorophenol combined to the same active site as the substrate PNPG as well as an alternate binding site on the β-GUD enzyme molecule.

4.8.3 Activation of the β-GUD enzyme assay

Figure 4.17 below shows activation of the β-GUD enzyme assay with increasing concentrations of 4-nitrophenol and 2,4-dinitrophenol. At 100 µg/ml addition 4-nitrophenol exceeded the maximum absorbance.

This activation occurred due to the fact that the product formed in the β-GUD enzyme assay was also 4-nitrophenol, hence the activation when 4-nitrophenol and 2,4-dinitrophenol were added to the assay. These phenolic compounds are identical (4-nitrophenol) and similar (2,4-dinitrophenol) to the product (4-nitrophenol) formed during the β-GUD enzyme assay. The colour formed in the β-GUD enzyme assay with PNPG as the substrate was yellow in colour. This solution absorbed at 405 nm; the yellow colour of these phenolic compounds also absorbed at this wavelength resulting in false positive results.

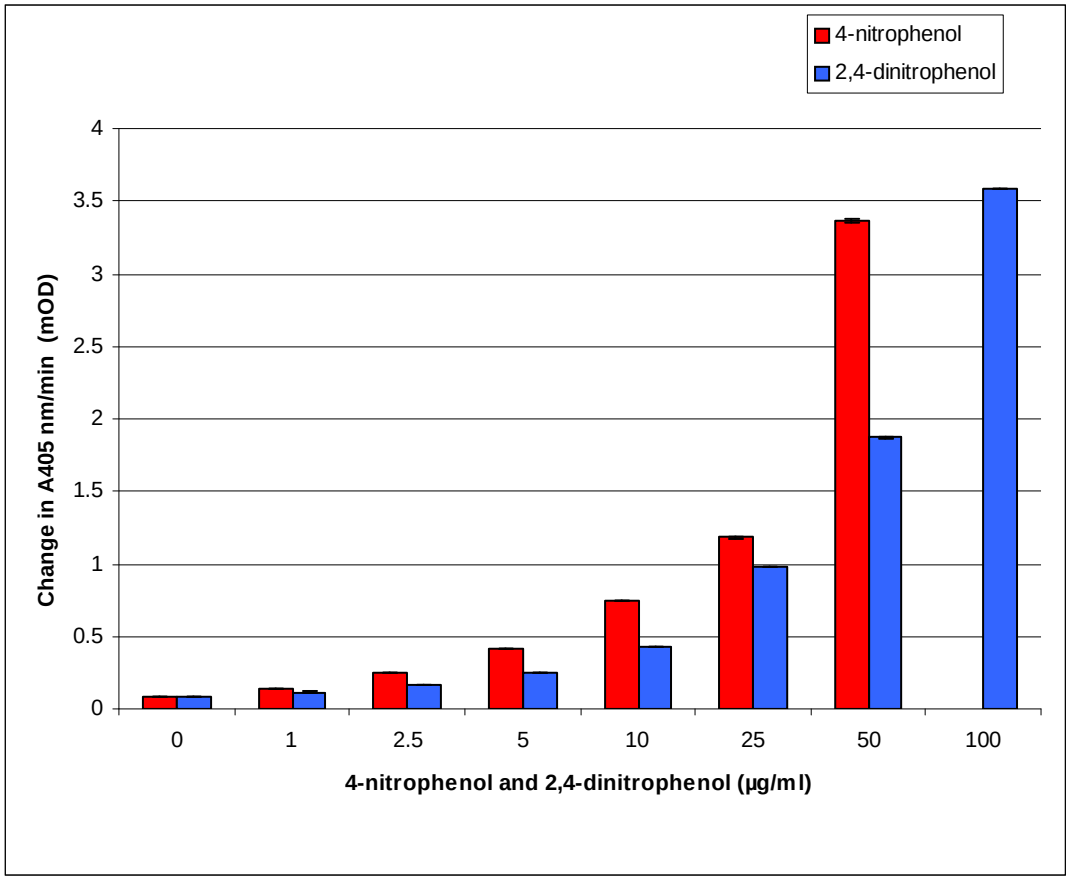


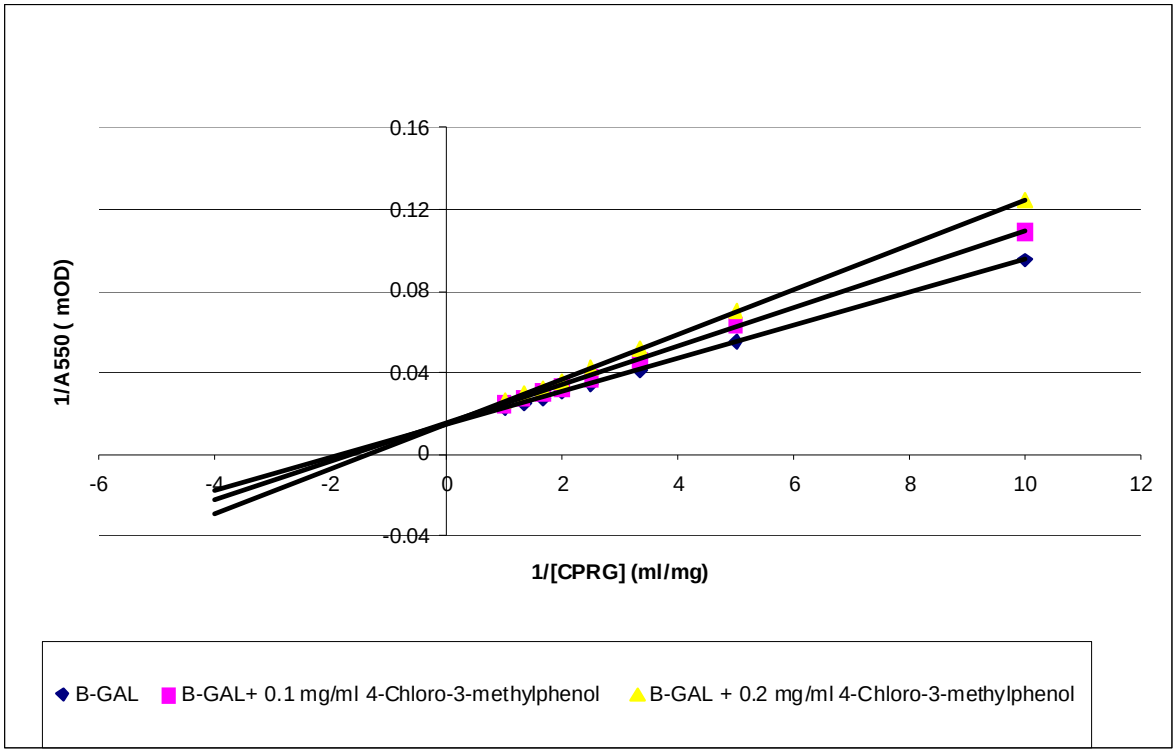
Figure 4.17 Activation of the β-GUD enzyme assay by the addition of 4-nitrophenol and 2,4-dinitrophenol. Data points represent the means ± SD (n=3).

4.9 LINEWEAVER-BURK PLOTS FOR THE INHIBITORY COMPOUNDS

Although the Hanes-Woolf, Woolf-Augustinsson-Hofstee and Eadie-Scatchard plots are more accurate these highly specialized plots are unfamiliar to researchers in the water field. We therefore decided to use the traditional Lineweaver-Burk plots to identify the type of inhibition and to determine inhibition constant values.

Figures 4.18 to 4.24 represent Lineweaver-Burk plots illustrating types of inhibition occuring due the addition of phenolic compounds to the β-GAL and β-GUD enzyme assays. Secondary plots of the Lineweaver-Burk plots were constructed in order to determine the respective K_i values.

(A)



(B)

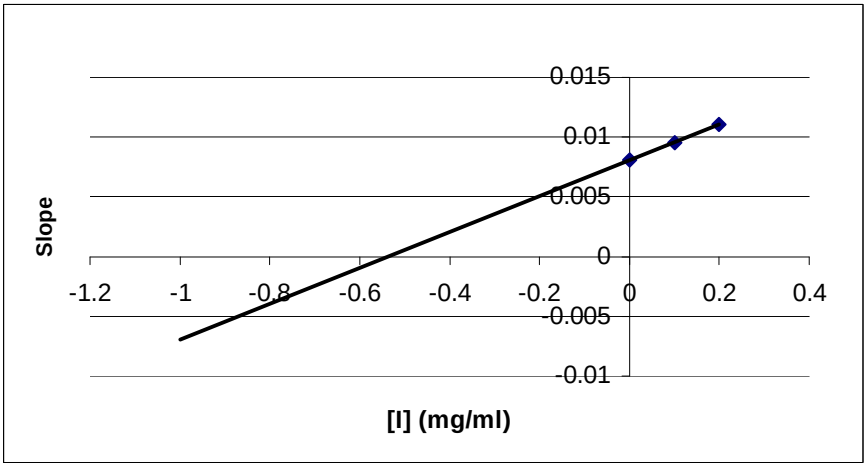
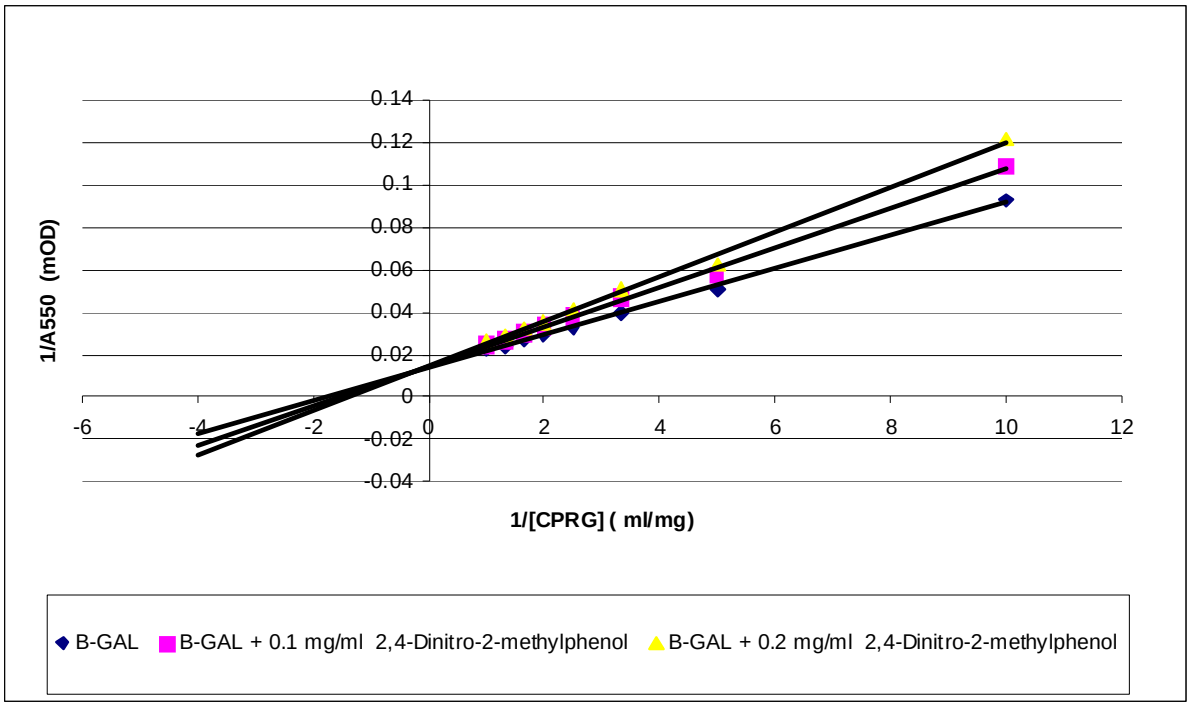


Figure 4.18 (A) Primary Lineweaver-Burk plot showing competitive inhibition of the β-GAL enzyme at 6.88 x 10^{-5} nM and 4-chloro-3-methylphenol at 0.1 mg/ml and 0.2 mg/ml. Data points represent the means ± SD (n=3). **(B)** The corresponding secondary plot indicating a K_i value of 0.540 mg/ml.

(A)



(B)

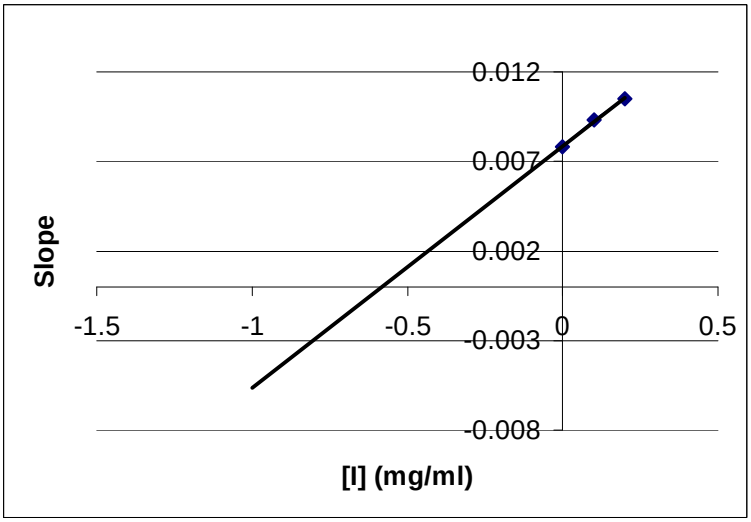
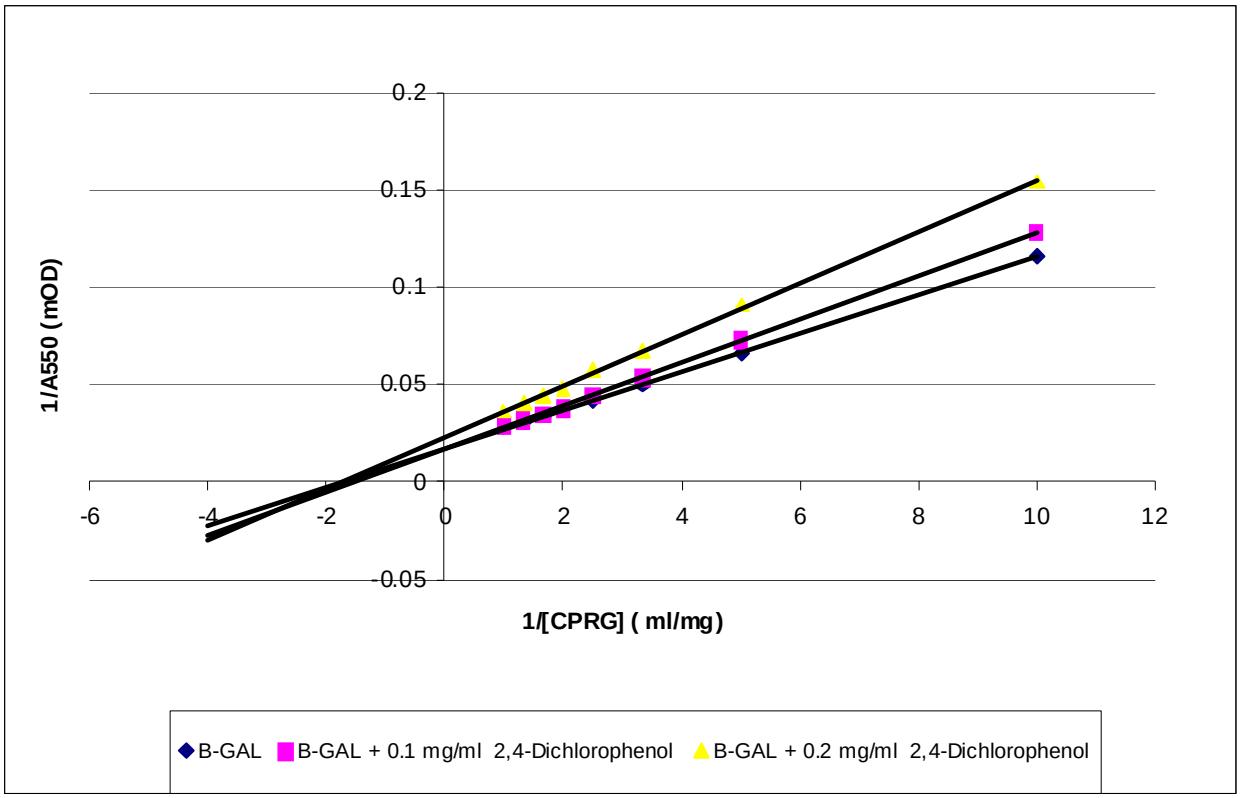


Figure 4.19 (A) Primary Lineweaver-Burk plot showing competitive inhibition of the β-GAL enzyme at 6.88 x 10^{-5} nM and 4,6-dinitro-2-methylphenol at 0.1 mg/ml and 0.2 mg/ml. Data points represent the means ± SD (n=3).**(B)** The corresponding secondary plot indicating a K_i value of 0.585 mg/ml.

(A)



(B)

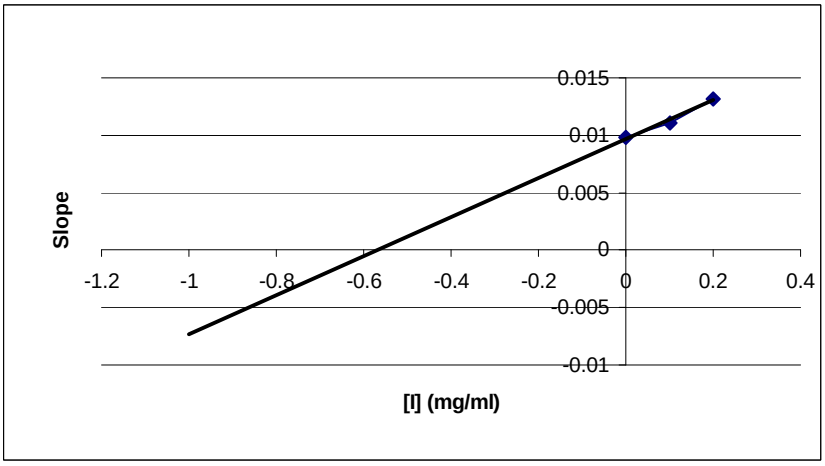
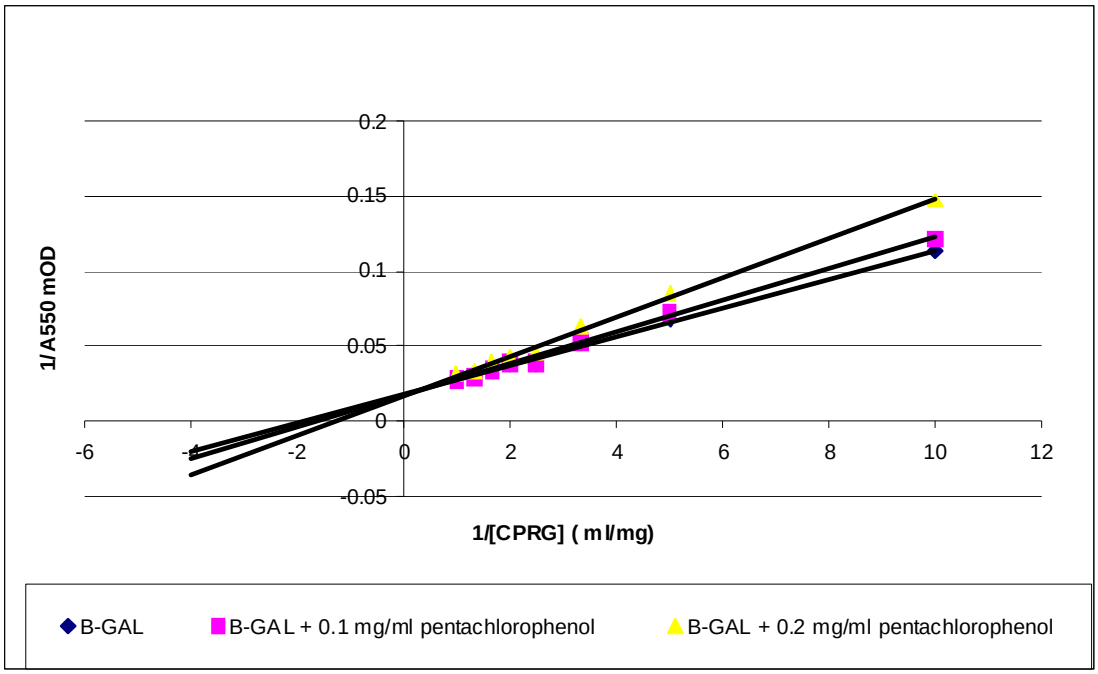


Figure 4.20 **(A)** Primary Lineweaver-Burk plot showing non-competitive inhibition of the β-GAL enzyme at 6.88 x 10^{-5} nM and 2,4-dichlorophenol at 0.1 mg/ml and 0.2 mg/ml. Data points represent the means ± SD (n=3). **(B)** The corresponding secondary plot indicating a K_i value of 0.571 mg/ml.

(A)



(B)

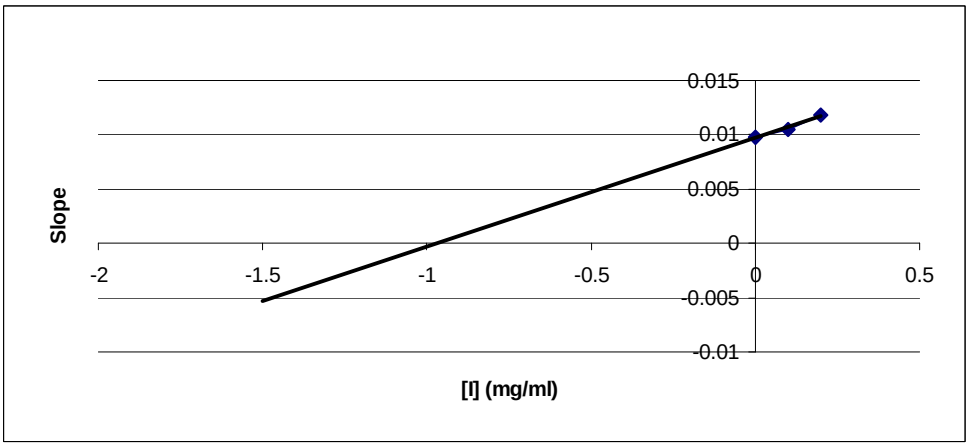
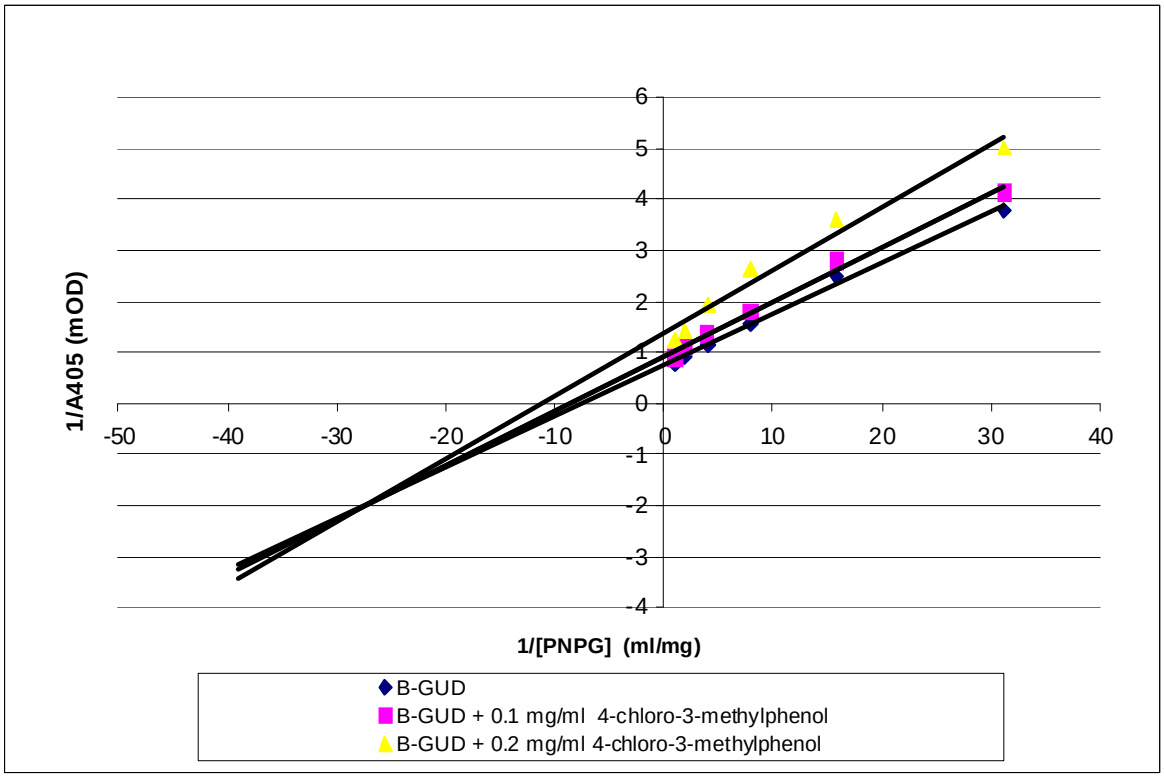


Figure 4.21 (A) Primary Lineweaver-Burk plot showing competitive inhibition of the β-GAL enzyme at 6.88 x 10^{-5} nM and pentachlorophenol at 0.1 mg/ml and 0.2 mg/ml. Data points represent the means ± SD (n=3). **(B)** The corresponding secondary plot indicating a K_i value of 0.970 mg/ml.

(A)



(B)

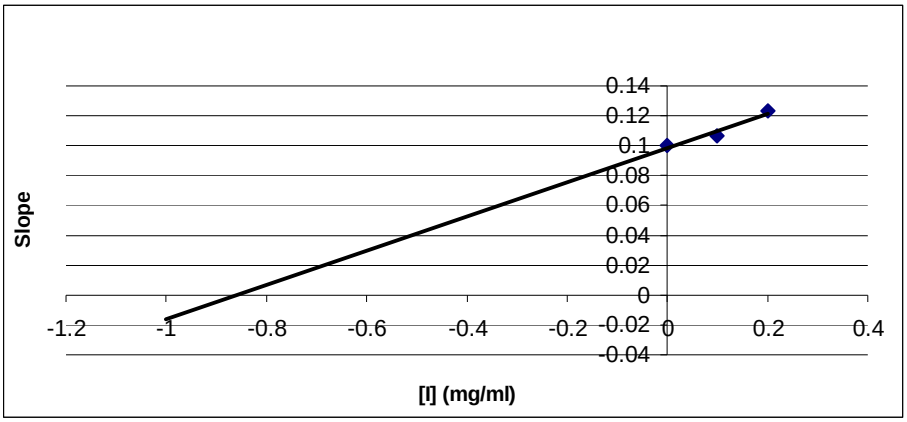
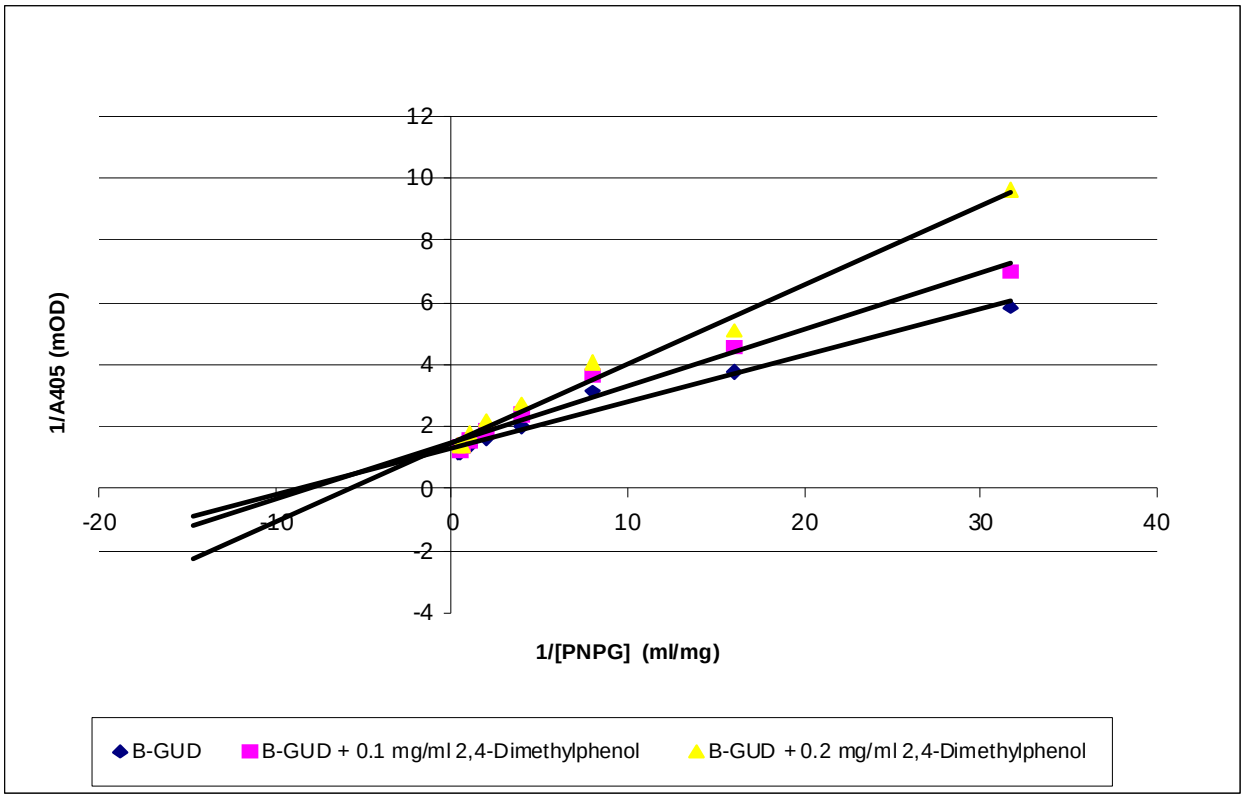


Figure 4.22 (A) Primary Lineweaver-Burk plot showing mixed inhibition of the β-GUD enzyme at 6.88 x 10^{-5} nM and 4-chloro-3-methylphenol at 0.1 mg/ml and 0.2 mg/ml. Data points represent the means ± SD (n=3). **(B)** The corresponding secondary plot indicating a K_i value of 0.857 mg/ml.

(A)



(B)

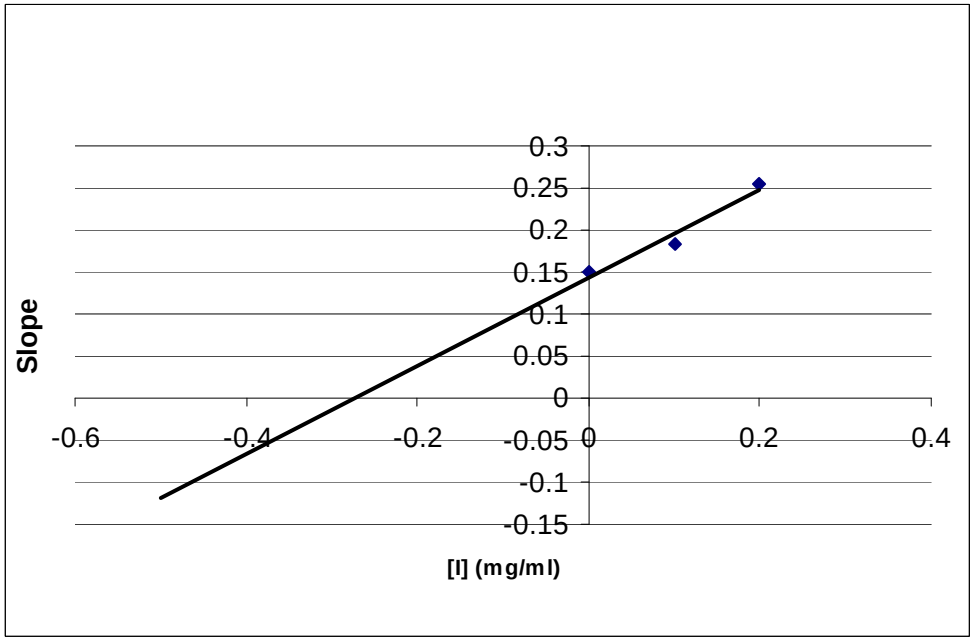
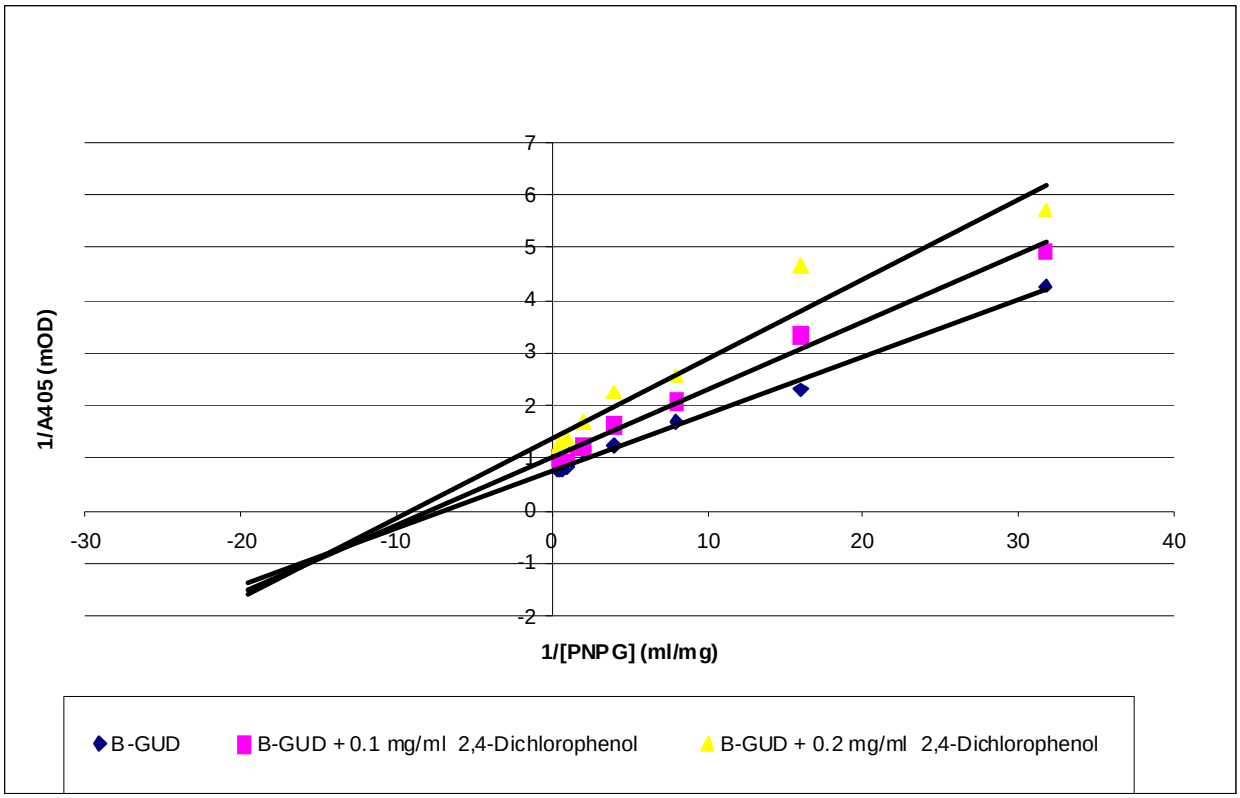


Figure 4.23 (A) Primary Lineweaver-Burk plot showing competitive inhibition of the β-GUD enzyme at 6.88 x 10^{-5} nM and 2,4-dimethylphenol at 0.1 mg/ml and 0.2 mg/ml. Data points represent the means ± SD (n=3). **(B)** The corresponding secondary plot indicating a K_i value of 0.274 mg/ml.

(A)



(B)

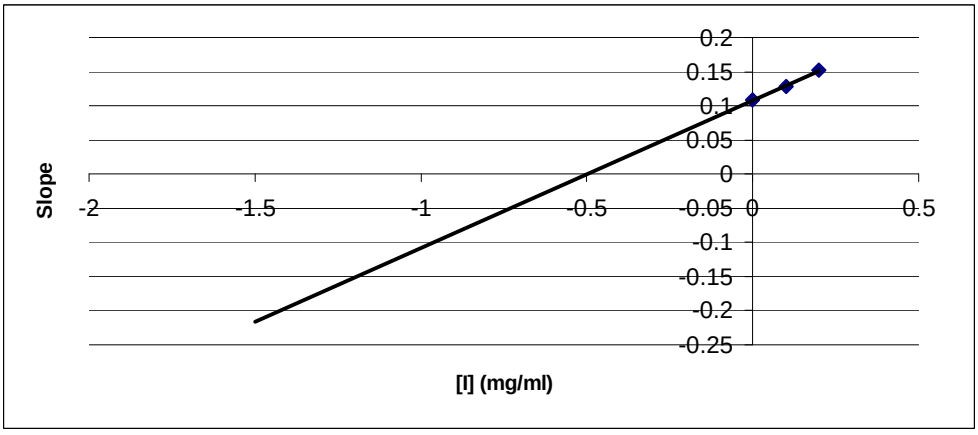


Figure 4.24 (A) Primary Lineweaver-Burk plot showing mixed inhibition of the β-GUD enzyme at 6.88 x 10^{-5} nM and 2,4-dichlorophenol at 0.1 mg/ml and 0.2 mg/ml. Data points represent the means ± SD (n=3).**(B)** The corresponding secondary plot indicating a K_i of value of 0.500 mg/ml.

Table 4.1 Type of inhibition exhibited and K_i values by five PPP compounds when added to the β-GAL assay.

B-GAL ENZYME ACTIVITY		
PHENOLIC COMPOUND	**TYPE OF INHIBITION**	**K_i (mg/ml)**
4-chloro-3-methylphenol	competitive	0.54
4,6-dinitro-2-methylphenol	competitive	0.585
2,4-dichlorophenol	non-competitive	0.571
pentachlorophenol	competitive	0.970
2-chlorophenol	no-inhibition	

Table 4.2 Type of inhibition exhibited and K_i values by three PPP compounds when added to the β-GUD assay.

B-GUD ENZYME ACTIVITY		
PHENOLIC COMPOUND	**TYPE OF INHIBITION**	**K_i (mg/ml)**
4-chloro-3-methylphenol	mixed	0.857
2,4-dimethylphenol	competitive	0.274
2,4-dichlorophenol	mixed	0.500

4.10 CONCLUSION

The eleven phenolic compounds listed by the US EPA present in the environment were indeed able to affect β-GAL and β-GUD enzyme activity. There is therefore a possibility that an over or under estimation of β-GAL and β-GUD enzyme activities can be obtained when these phenolic compounds are present in the environment.

The different concentrations clearly had varied effects on the activities of both enzymes. These effects were also concentration dependent. These phenolic compounds competed for the same active sites as the substrates used in these assays as well as combined to alternate sites on the enzyme molecules. In mixed inhibition the inhibitors could also be binding to the enzyme-substrate complex, thereby reducing the velocity of the reaction.

As revealed in this chapter the interference of the eleven PPP compounds on the β-GAL and β-GUD assays were concentration dependent. It was therefore important to detect and quantify each of the eleven PPP present in the water environment where the enzymes β-GAL and β-GUD (i.e. from coliform and *E. coli* source) are expected to be found. The following chapter (Chapter 5) therefore involved HPLC method development and validation for the simultaneous detection and quantification of the eleven PPP compounds found in the contaminated water environment.

CHAPTER 5

HPLC ANALYSIS: METHOD DEVELOPMENT, VALIDATION AND SIMULTANEOUS QUANTIFICATION OF ELEVEN PPP COMPOUNDS IN WATER

5.1 INTRODUCTION

An HPLC method has to be validated in order show reproducibility as well as reliability in the data and results produced. A validated method demonstrates effectiveness and therefore allows confidence in the method (Buick *et al.*, 1990; Edwardson *et al*., 1990).

The phenolic compounds analyzed in this study are found in the environment, and HPLC was the most suitable method of analysis for the detection of these compounds (Pocurull *et al.*, 1996). It was therefore critical that the method is specific for the analytes of interest and had the ability to detect phenolic compounds at the levels required by legislation. In the process of validation, the method was required to meet a series of criteria.

The method validation process requires a series of tests to be performed. These are reproducibility, accuracy, precision, linearity, robustness, ruggedness, specificity, limit of detection (LOD), limit of quantification (LOQ) as well as recoverability. (Inman and Rickard, 1988; Buick *et al.*,1990; Edwardson *et al*., 1990).

In this chapter the above parameters were discussed and applied to the analytical method used to simultaneously detect eleven phenolic compounds in water and furthermore, to demonstrate that the proposed method was suitable for its intended purpose.

This chapter demonstrated that the development and validation of an HPLC method to successfully and simultaneously separate the eleven PPP compounds found in water. The collected data and related calculations were presented and discussed.

5.2 VALIDATION PARAMETERS

5.2.1 Analytical standards

Before proceeding with the method validation process, authentic reference standards with known purity, of HPLC grade, were obtained. Standards of known concentration were prepared and the decomposition over time observed under specific storage conditions was observed. In this study, pure compounds were purchased from Sigma-Aldrich and Merck chemical companies.

5.2.2 Reproducibility

The reproducibility of a method is tested by multiple sample injections of the pure standard under the same operational parameters and observing the fluctuations in retention time, peak height or peak area. According to the International Convention on Harmonization (ICH) the number of injections should be a minimum of nine injections over a specific range (Buick *et al*., 1990).

5.2.3 Accuracy

Accuracy is the closeness of agreement between the true or reference value that is found by the test method. This is an absolute measurement and is dependent upon precision and specificity. To report accuracy, data must be collected from a minimum of nine determinations over at least three concentration levels over a specified range (Buick *et al*., 1990; Swartz and Krull, 1997).

5.2.4 Precision

Precision is an indication of reproducibility. Under the same analytical conditions precision is a measure of closeness of agreement of the data values from multiple sampling or sample injections onto an HPLC system. This must be from the same homogenous sample and at a minimum of three different concentrations. Precision is calculated as the relative standard deviation (RSD) of the peak areas or peak heights (Buick *et al*., 1990; Edwardson *et al*., 1990; Swartz and Krull, 1997).

5.2.5 Range

The range of a method is the concentration intervals between the upper and lower levels of the analyte that have been demonstrated to be analysed with accuracy, precision and linearity (Buick *et al*., 1990; Edwardson *et al.*, 1990).

5.2.6 Linearity

The linearity of a method is demonstrated when the response is directly proportional to the analyte concentration over a given working range (Buick *et al.*, 1990; Edwardson *et al*., 1990). The range at which the experiments are performed should be 25-200% of the nominal concentration of analyte (Buick *et al.*, 1990, Edwardson *et al*., 1990). According to Edwardson *et al*. (1990) the proportional relationship between the concentration of the analyte and the response ensures confidence in the method and confirms that a single reference standard can be used to perform calculations, rather than using the equation of the calibration line. Linearity is the variance of the slope of the regression line, the least-square linear regression mathematically defines the calibration line. The ICH guidelines state that a minimum of five concentration levels should be used.

5.2.7 Specificity

Specificity is the ability of the chromatographic equipment to detect and resolve the peaks of interest; it is a measure of a method's sensitivity to potential sample-related hindrance. It is the ability of the methods ability to detect the analyte of interest in the presence of other compounds in the same sample. In this specific study, the method developed to detect the eleven phenolic compounds in water should demonstrate its specificity for each compound. It is critical that the peak of interest is due to a single component response and not due to co-elution of two or more compounds.

5.2.8 Robustness and ruggedness

Robustness is the ability of the method to remain unaffected by small variations in the method. These include assaying of the same set of results by two analysts (Edwardson *et al.,* 1990), changing brands or batches of compounds and varying makes of equipment.

5.2.9 Limit of Detection (LOD)

The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected above the baseline noise. This measurement is dependent on background signal that could be due to electronic noise or an endogenous sample. This parameter cannot be quantified, but should be at least at a 3:1 ratio of signal to noise (Buick *et al.*,1990; Edwardson *et al*., 1990; Swartz and Krull, 1997).

5.2.10 Limit of Quantification (LOQ)

The LOQ is defined as the lowest concentration of an analyte in a sample that can be determined above the baseline noise with acceptable reproducibility, precision and accuracy by the method used (Buick *et al*., 1990; Edwardson *et al*., 1990; Swartz and Krull, 1997).

5.3 HPLC METHOD DEVELOPMENT PROCEDURES FOR THE SIMULTANEOUS DETECTION OF ELEVEN PPP COMPOUNDS.

Introduction

Literature revealed reverse phase high-performance liquid chromatography (RP-HPLC) to be the most commonly used method for the analysis of phenolic compounds in the environment. Ultraviolet detection with a mobile phase at acidic pH is most widely used (Pocurull *et al.*, 1996; Angelino and Gennaro, 1997; Zhao and Lee, 2001; Grynkiewcz *et al.*, 2002; Asan and Isildak 2003).

Suliman *et al*. (2006) used fluorescent detection in conjunction with DAD. Angelino and Gennaro (1997) and Pocurull *et al.* (1996) used wavelengths of 285 and 280 nm, respectively. While Gonzalez - Toledo *et al*., (2001) used four different wavelengths (278 nm, 268 nm, 311 nm and 302 nm) to detect nine of the eleven priority pollutant phenols. Zhao and Lee (2001), however, used a wavelength of 220 nm to detect six of the phenolic compounds.

The mobile phases used were most frequently composed of either acetonitrile or methanol as the organic phase with the aqueous phase at an acidic pH using a gradient mode of elution. Zhao and Lee (2001), however, used an isocratic elution method.

The analytical columns used were mainly reverse phase columns with C18 packing material and 5 µm partical size (Pocurull *et al.,* 1996; Angelino and Gennaro, 1997; Gonzalez - Toledo *et al*., 2001; Grynkiewicz *et al*., 2002; Suliman *et al*., 2006).

Some of these methods did not detect all eleven priority pollutant phenols (see Chapter 1, Table 1.1), while methods that detected all eleven phenolic compounds had total retention times of about 27 minutes.

The official US EPA analytical method to analyze phenolic compounds is a GC method with various detection techniques. This method requires derivatization of these compounds which is labour intensive. The samples are extracted using liquid-liquid extraction (LLE), which requires the use of large volumes of expensive solvents. These procedures require more sample preparation time, increased workload for the analyst as well an increased risk of error (Knutsson *et al*., 1996; Berhanu *et al*., 2006).

The US EPA method 528 (Munch, 2000) made use of a SPE method with analysis by GC-MS, but reported interference by contaminants from solid phase sorbent.

The LOQ reported by Gonzalez-Toledo *et al*. (2001) for phenolic compounds found in water was between 1 µg/l and 10 µg/l. Both Peñalver *et al.* (2002) and Zhao and Lee (2001) reported LOQ's from 0.5 to 2.5 µg/l, while Pocurull *et al*. (1996) reported LOQ's from 0.02 to 0.05 µg/l. Angelino and Gennaro (1997) indicated a LOQ from 5 to 15 µg/l, while Suliman *et al*. (2006) reported LOQ's of 0.3 to 3.0 µg/l and LOD's between 0.1 and 3.0 µg/l for these phenolic compounds.

In this chapter the development of a reproducible, reliable method with a reasonable retention time for the simultaneous detection of all eleven PPP was developed and described.

5.4 METHOD DEVELOPMENT PROCEDURES

5.4.1 Analytical column selection

Literature revealed that the type of analytical columns used for the separation of phenolic compounds consisted of either C_8 or C_{18} column packing material with 5 or 10 µm particle size. Zhao and Lee (2001) used a 4 µm particle size Novapak C18 column (Pocurull *et al.,* 1996; Angelino and Gennaro, 1997; Grynkiewicz *et al.,* 2001; Zhao and Lee, 2001; Asan and Isildak, 2003).

The column selected for this specific study was a Waters Symmetry® column with C8 packing material and 5.0 µm particle size. The dimensions of the column were 3.9 mm internal diameter (ID) and 150 mm in length. The column was obtained from Waters Corporation, Massachusetts, USA Part no. WAT046970.

The guard column used was a Sentry Symmetry® C8 5 µm guard column, with dimensions of 3.9 mm ID and 20 mm in length which was also obtained from Waters.

5.4.2 Mobile phase selection

The optimum mobile phase selected for the simultaneous detection of the eleven phenolic compounds in water, in the present study, consisted of an initial isocratic application, followed by an additional gradient step.

The initial mobile phase that was selected consisted of an acetonitrile- water (40:60) mixture. Seven of the eleven phenolic compounds, namely: phenol , 4-nitrophenol, 2-chlorophenol, 2-nitrophenol, 2.4-dichlorophenol, 4-chloro-3-methylphenol and 2,4-dichlorophenol compounds, were detected and resolved; however, baseline resolution was not achieved between phenol and 4-nitrophenol. Another four compounds (2,4-dinitrophenol, 4,6- dinitro-2-methylphenol, trichlorophenol and pentachlorophenol) were analysed but the resolution was very poor.

Adjustment of the pH of the mobile phase to an acidic pH was attempted, in order to protonate the analytes resulting in the compounds becoming more polar. The addition of 1% (v/v) acetic acid to the water component of the mobile phase (now referred to as mobile phase A) achieved the desired resolution between all eleven compounds. The acidic pH was necessary as this enhanced the lipophilic properties of the phenolic compounds analysed (Angelino and Gennaro, 1997).

However, the retention time for pentachlorophenol was 47 minutes, which was not optimal. This long retention time was due to pentachlorophenol being far less polar than the other PPP compounds tested, and therefore remaining on the analytical column for a longer period. A gradient step was implemented at 18 minutes once the other ten compounds had been eluted off the column. To employ the gradient step, a solvent of 100% acetonitrile (mobile phase B) was introduced over a duration of 2 minutes, until a ratio of 10% mobile phase A: 90% mobile phase B was reached, and this composition was maintained until the end of the run. This reduced the run time to 22.9 minutes for pentachlorophenol, and this was an acceptable run time for the elution of all eleven compounds.

5.4.3 Wavelength selection

A wavelength of 280 nm was selected for the detection of all eleven compounds, although 300 nm could be used to increase the sensitivity of pentachlorophenol detection.

5.4.4 Flow rate

A standard flow rate of 1 ml/min was employed.

5.4.5 Temperature selection

The column was maintained at ambient temperature in a controlled air-conditioned room at 22^{o}C.

5.5 OPTIMUM CHROMATOGRAPHIC PARAMETERS DEVELOPED FOR THE SIMULTANEOUS SEPARATION OF THE ELEVEN PPP COMPOUNDS

Mobile phase:

Mobile phase A: Acetonitrile and 1% (v/v) acetic acid in a ratio of 40:60

Mobile phase B: Acetonitrile 100%

Gradient: 100% mobile phase A for 18 minutes.

At 18 minutes changed to 90% mobile phase B over a duration of 2 minutes and remained at 10% mobile phase A and 90% mobile phase B until the end of the run.

Analytical column: Waters Symmetry® C8 5µm (3.9 x 150 mm)

Guard column: Waters Sentry guard column C8 5µm (3.9 x 20 mm)

Column back-pressure: 1680 psi

Wavelength Detection: 280 nm

Column temperature: Ambient

Injection volume: 20 µl (fixed loop)

5.6 STANDARD PREPARATIONS

Standards

phenol (Sigma-Aldrich, P 5566)
4-nitrophenol (Sigma-Aldrich, 1048)
2-chlorophenol (Sigma-Aldrich, 18 577-9)
2-nitrophenol (Sigma-Aldrich, V1970-2)
2,4-dinitrophenol (Riedel de-Haen, 34334)
2,4-dimethylphenol (Sigma-Aldrich, D174203)
4-chloro-3-methylphenol (Sigma-Aldrich, C5 540-2)
2,4-dichlorophenol (Sigma-Aldrich, 10 595-3)
4,6-dinitro-2-methylphenol (Fluka, 42120)
2,4,6-trichlorophenol (Sigma-Aldrich, T5, 530-1)
pentachlorophenol (Sigma-Aldrich, P260-4)

Individual stock solutions of 0.1 mg/ml of the above eleven PPP compounds were prepared by weighing approximately 10 mg in "A" grade 100 ml volumetric flasks and dissolved in 100 ml acetonitrile. The final concentrations were calculated for each individual phenolic compound. Working solutions of 1 µg/ml were prepared in HPLC grade water by performing serial dilutions of the above stock solutions. Sample volumes of 20 µl were injected onto the column to determine the retention time of each phenolic compound.

After the retention time of each phenolic compound was determined all eleven compounds were mixed and 20 µl of the mixture was injected onto the column.
In order to check the robustness of the method, a commercially available phenol mix of the eleven compounds was obtained from BDH (EPA604, product no. 12474R) and injected onto the column using optimal chromatographic conditions.

5.6.1 Calibration Curves

The following eighteen concentrations for each of the eleven phenolic compounds listed above were prepared in HPLC grade water:

Table 5.1: List of PPP compounds concentrations in the calibration curves

Calibration Mixture	Concentration (µg/ml)
1	1.465×10^{-3}
2	2.93×10^{-3}
3	5.86×10^{-3}
4	0.012
5	0.023
6	0.047
7	0.094
8	0.188
9	0.375
10	0.750
11	1.500
12	3
13	6.2
14	12
15	25
16	50
17	100
18	500

5.7 INSTRUMENTATION, MATERIALS AND REAGENTS

5.7.1 Instrumentation

HPLC

The HPLC system consisted of the following:

1) Beckman System Gold 126 Solvent module (Beckman Coulter, Inc., Fullerton, California, USA).
2) Beckman System Gold 166 detector (Beckman Coulter, Inc., Fullerton, California, USA) .
3) Beckman 32 Karat^{TM} Software Version 7.0 (Beckman Coulter, Inc., Fullerton, California, USA) .
4) Rheodyne 7725i injector with a 20 µl fixed loop.
5) Hamilton 100µl syringe (Hamilton Company, Reno, USA).
6) Symmetry® C8 5µm 3.9 x 150mm analytical column, Part no. WAT04970 (Waters, Milford, Massachusetts, USA)
7) Symmetry® C8 5µm 3.9 x 20mm Guard column, Part no. WAT054250 (Waters, Milford, Massachusetts, USA)

5.7.2 Additional equipment

1) Milli-Q Academic water purification system (Millipore, Bradford, USA).
2) Shimadzu Analytical Balance AUW120D (Shimanzu Corporation, Kyoto, Japan).
3) IonLab pH meter Level 1 (WTW, Meilheim, Germany).

5.8 MATERIALS AND REAGENTS

5.8.1 Mixture of phenols

Phenol mix, EPA 604, 0.5 mg/ml in methanol (BDH, Merk Chemicals)

Phenol mix, Supelco SS EPA Phenols Mixture 500-2500 µg/ml in methanol (Sigma-Aldrich)

5.8.2 Mobile phase

Acetonitrile (BDH 152516Q)

Acetic Acid (Sigma-Aldrich A 6283)

Purified Milli-Q HPLC grade water was purified through a Milli-Q system which used a process of reverse osmosis (Millipore Corporation, Bedford, USA).

5.9 RESULTS

The chromatogram below (Figure 5.1) shows the simultaneous detection and separation of the eleven PPP found in water, using the optimum parameters developed and validated in this study. The compounds eluted at the following retention times: phenol (3.283 min), 4-nitrophenol (3.917 min), 2-chlorophenol (5.083 min), 2,4-dinitrophenol (5.983 min), 2-nitrophenol (6.400 min), 2,4-dimethylphenol (6.983 min), 4-chloro-3-methylphenol (8.250 min), 2,4-dichlorophenol (9.850 min), 4,6-dinitro-2-methylphenol (10.767 min), 2,4,6-trichlorophenol (16.383 min) and pentachlorophenol (22.933 min).

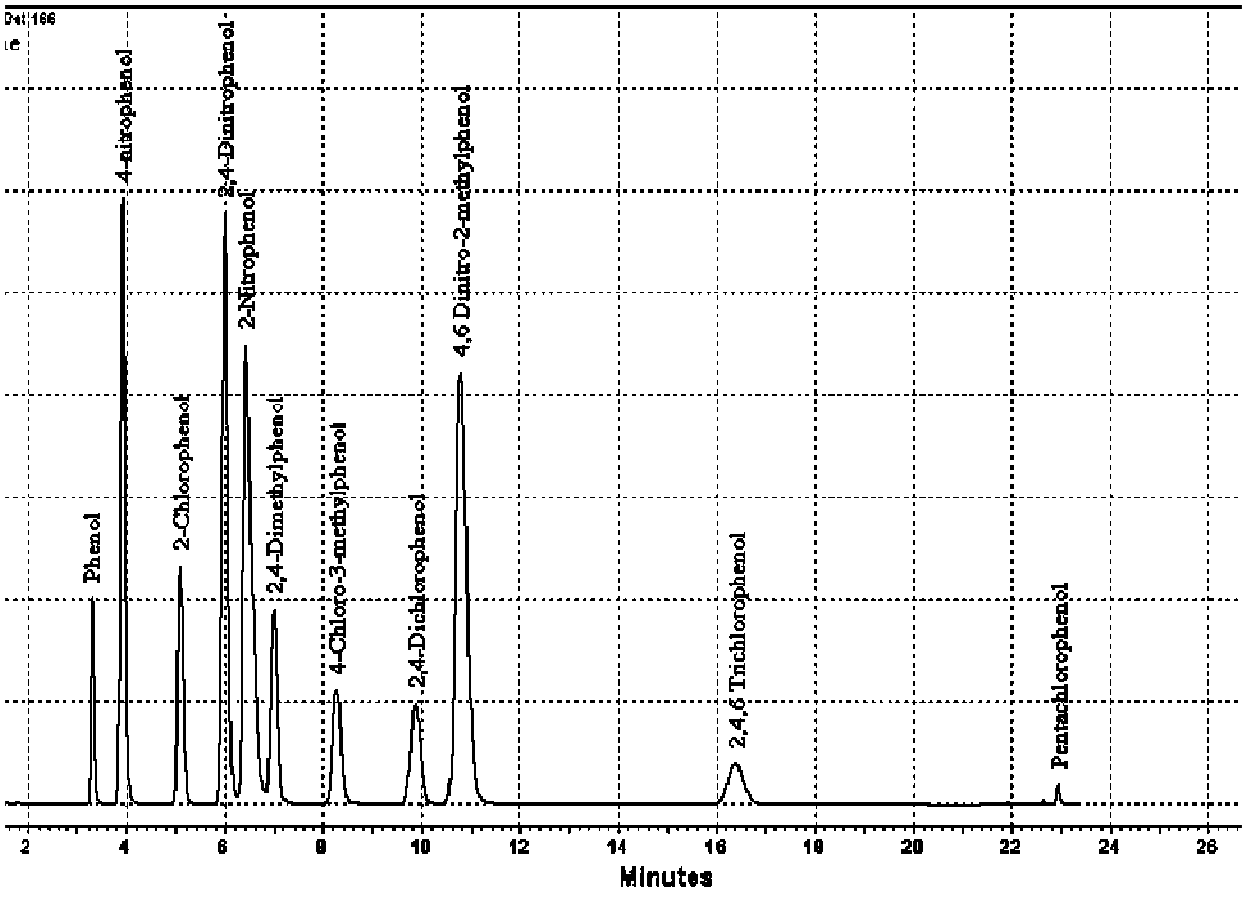


Figure 5.1 Chromatogram showing the simultaneous detection of eleven priority pollutant phenolic compounds listed by EPA USA separated using the optimum parameters described**.**

5.9.1 Linearity

For this study the linearity of the eleven phenolic were demonstrated by injecting standards in the concentration range of 0.0015 µg/ml-500 000 µg/l. The calibration plots were drawn by plotting the peak area versus standard concentration between 0.0030-500 000 µg/l. Straight lines were always obtained as demonstrated by the regression parameters (Table 5.2).

As can be seen in Table 5.2 below, all eleven of the phenolic compounds showed good linearity with squared regression coefficients (r^2) ranging from 0.997 for pentachlorophenol to 0.999 for 2,4-dichlorophenol.

Table 5.2 Linear range , regression and correlation coefficient of eleven PPP**.**

Phenolic compounds	Linearity range µg/l	Linear regression	Coefficient of correlation (r^2)
phenol	0.0030 - 500 000	Y = 1.267x – 1.664	0.999
4-nitrophenol	0.006 - 500 000	Y = 4.361x – 7.656	0.999
2-chlorophenol	0.012 - 500 000	Y = 2.141x -17.223	0.998
2,4-dinitrophenol	0.012 - 500 000	Y = 6.270x – 5.208	0.997
2-nitrophenol	0.006 - 500 000	Y = 6.967x –16.623	0.998
2,4-dimethylphenol	0.003 - 500 000	Y = 2.285x – 5.373	0.998
4-chloro-3-methylphenol	2.35 - 500 000	Y = 1.634x – 3.985	0.998
2,4-dichlorophenol	2.35 - 500 000	Y = 1.706x – 2.436	0.999
4,6-dinitro-2-methylphenol	2.35 - 500 000	Y = 8.258x – 17.895	0.998
2,4,6-trichlorophenol	2.35 - 500 000	Y = 1.242x – 4.384	0.996
pentachlorophenol	0.002 - 500 000	Y = 0.404x – 3.466	0.967

5.9.2 Limits of Detection (LOD) and Limits of Quantification (LOQ)

Good limits of detection (LOD) and Limits of Quantification (LOQ) were obtained during the method validation compared to the literature as can be seen in Table 5.3 below. The LOD was from 0.0015 µg/l for phenol and 4-nitrophenol to 0.012 µg/l for 2,4-dimethylphenol, 4-chloro-3-methylphenol, 2,4-dichlorophenol and 2,4,6-trichlorophenol. The LOQ was found to be between 0.003 µg/l for phenol and 2,4-dimethylphenol and 2.35 µg/l for 4-chloro-3-methylphenol, 2,4-dichlorpohenol, 4,6-dinitrol-2-methylphenol and 2,4,6-trichlophenol.

Table 5.3 LOD and LOQ of the eleven PPP using the validated HPLC method.

Phenolic compounds	Limit of detection (LOD) µg/l	Limit of Quantification (LOQ) µg/l
phenol	0.0015	0.003
4-nitrophenol	0.0015	0.006
2-chlorophenol	0.0059	0.012
2,4-dinitrophenol	0.0029	0.012
2-nitrophenol	0.0029	0.006
2,4-dimethylphenol	0.0117	0.003
4-chloro-3-methylphenol	0.0117	2.350
2,4-dichlorophenol	0.0117	2.350
4,6-dinitro-2-methylphenol	0.0029	2.350
2,4,6-trichlorophenol	0.0117	2.350
pentachlorophenol	0.0059	0.012

5.9.3 Reproducibility, Accuracy and Precision

The reproducibility of the method was demonstrated using a multiple of nine injections of three different concentration ranges, i.e. three injections at each standard concentration. The results represented as %RSD in Table 5.4 below demonstrated good reproducibility, accuracy and precision. All compounds at all levels showed %RSD below 4%, with the exception of 2,4,5-trichlorophenol at the concentration of 0.001 mg/ml which was at 7%.

Table 5.4 Summary of % RSD accuracy and precision studies. Data represent the means (n=3).

PHENOLIC COMPOUNDS	0.001mg/ml (n=3)	0.005 mg/ml (n=3)	0.01mg/ml (n=3)
phenol	1.294	1.463	0.747
4-nitrophenol	0.632	1.928	0.730
2-chlorophenol	1.769	2.669	2.967
2,4-dinitrophenol	1.091	1.960	1.998
2-nitrophenol	1.000	3.716	1.970
2,4-dimethylphenol	1.639	2.394	0.472
4-chloro-3-methylphenol	1.267	1.324	1.218
2,4-dichlorophenol	1.862	2.763	2.477
4,6-dinitro-2-methylphenol	0.264	3.264	1.545
2,4,6-trichlorophenol	7.001	3.113	2.824
pentachlorophenol	0.516	0.557	0.463

% RSD, *n*=3

5.9.4 Specificity

This method demonstrated the ability to detect and quantify the analytes of interest. This was shown by mixing all eleven phenolic compounds and determining the linearity. Each individual phenolic compound showed a linear relationship between peak area and concentration. Furthermore, no interfering peaks were observed when HPLC grade water, which was used for preparation of samples was injected onto the HPLC system. No interfering peaks were also observed when a blank solid phase extraction (SPE) was performed.

5.9.5 Robustness of the HPLC method

The robustness and ruggedness of the method were tested by obtaining commercial mixtures of the eleven PPP from Merck (BDH EPA604-Phenol mix 0.5 mg/ml in methanol, product no. 124742R) and Sigma-Aldrich (Supelco SS EPA Phenols Mixture 500-2500 μg/ml in methanol). Both the commercial mixtures were prepared in methanol and not HPLC grade water and acetonitrile as in this study. The chromatograms of the commercial mixtures compared favourably to the mixtures made in this study. The resolution and retention times of the peaks of the commercial mixtures were exactly identical to those dissolved in the mixture of acetonitrile / HPLC grade water used to dissolve the phenolic compounds in this study as the calibration and external standards. This method demonstrated good robustness to variations in the sample mixture. The method was thus able to detect the analytes of interest in the presence of different matrices.

5.10 CONCLUSION

A reproducible, accurate, precise and sensitive method was developed and validated for the detection and quantification of the eleven PPP listed by the US EPA. The method was successfully applied to the simultaneous detection and quantification of the eleven PPP compounds found in water. This method could thus be used with confidence for its intended purpose.

In the next chapter (Chapter 6), this newly developed HPLC method was employed to detect and quantify the eleven PPP in various water environments, including rivers, streams, and industrial site and effluent waste water treatment plants in Grahamstown, Eastern Cape, South Africa.

CHAPTER 6

ANALYSIS OF ENVIRONMENTAL WATER SAMPLES USING SOLID PHASE EXTRACTION (SPE) AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

6.1 INTRODUCTION

Phenolic compounds are prevalent as pollutants in water as they have huge industrial applications. These toxic compounds are widely used in pesticides, bactericides, wood preservatives, synthetic intermediates, textiles, pulp processing and leather tanneries and are listed as priority pollutants because of their toxicity to the environment (Angelino and Gennaro 1997; Ohlenbusch *et al*., 2000; Peñalver *et al.*, 2002).

In order to detect these compounds at the sensitivity required using analytical techniques such as HPLC, the environmental water sample must first be concentrated. This will enable the HPLC method to detect the phenolic compounds at trace levels, which is how they mostly occur in the environment.

These compounds are polar in nature and are difficult to remove from aqueous solutions and this represents an analytical challenge. One of the earlier methods used to concentrate the environmental samples is liquid-liquid extraction. This method requires the use of large quantities of organic solvents (EPA, 1974; Angelino and Gennaro, 1997).

More recently, solid phase extraction (SPE) has been used more frequently as a preconcentration technique as an alternative to liquid-liquid extraction. Among the type of SPE materials used are silica C18 microcolumns, Sep Pak cartridges, polyurethane foams, porous graphic carbons, lipophilic lipidex gel and macroreticular polymetric resins (Norén and Sjövall, 1987; Coquart and Hennion, 1992; Angelino and Gennaro, 1997).

As mentioned in Chapter 1, there is a new generation of polymers available for the extraction and concentration of a wide spectrum of analytes. These are Oasis® and Absolute®, which are obtainable from Waters® and Varian® respectively. These SPE columns can be used to extract both polar and non-polar compounds, irrespective of whether they are acidic, basic or neutral. This is due to the interaction between the polymeric skeleton of the column and the functional groups (Hennion, 1999; Bagheri and Mohammadi, 2003).

For this present study, the Oasis SPE columns from Waters were selected to extract the eleven PPP (listed below) from environmental water samples.

Water samples were collected from rivers, streams, and industrial and wastewater treatment plant effluents. For sake of comparison, samples were obtained from both running and stagnant streams at various times of the year in order to observe seasonal variations in the amounts of phenolic compounds found in these sources.

6.2 METHODOGY

Materials

6.2.1 Phenolic compound standards

These were as listed in Chapter 1:

phenol (Sigma-Aldrich, P 5566)
4-nitrophenol (Sigma-Aldrich, 1048)
2-chlorophenol (Sigma-Aldrich, 18 577-9)
2-nitrophenol (Sigma-Aldrich , V1970-2)
2,4-dinitrophenol (Riedel de-Haen, 34334)
2,4-dimethylphenol (Sigma-Aldrich, D174203)
4-chloro-3-methylphenol (Sigma-Aldrich, C5 540-2)
2,4-dichlorophenol (Sigma-Aldrich, 10 595-3)
4,6-dinitro-2-methylphenol (Fluka, 42120)
2,4,6-trichlorophenol (Sigma-Aldrich, T5, 530-1)
pentachlorophenol (Sigma-Aldrich, P260-4)

6.2.2 Diethyl ether

Obtained from Burdick and Jackson Cat. 106-4, for HPLC, GC, pesticide residue analysis and spectrophotometry.

6.2.3 Extraction columns

Extraction columns used were Waters Oasis HLB 3cc 60mg, part no. WAT094226, Waters Corporation, Massachusetts, USA.

6.2.4 Methanol

The methanol grade was LiCrosolv. for liquid chromatography, cat no. 1.06007.2500., purchased from Merk, Darmstadt, Germany.

6.2.5 Nitrogen (Instrument grade)

The instrument grade nitrogen was used for evaporation and was obtained from Afrox.

6.2.6 Equipment

6.2.6.1 Waters 20 position Extraction manifold

The manifold used for the SPE columns was obtained from Waters Corporation, Massachusetts, USA, Part no. WAT 200606

6.2.6.2 Six-port Mini-Vap

The mini-vap was obtained from Chromatography Research supplies, KY, USA, Catologue no. 201006.

6.2.7 Methods

6.2.7.1 Standard solution preparation

A stock solution of 100 μg/ml of each of the eleven phenolic compounds was prepared and appropriate amounts of each phenolic compound were added to a mixture to give a final concentration of 1 μg/ml. This standard was used as an external standard for HPLC analysis, as well as for percentage recovery studies.

The environmental samples were extracted using Waters® Oasis extraction columns (Waters Oasis HLB 3cc 60 mg, part no. WAT094226), prior to injection onto the HPLC system.

All environmental samples were collected in glass Schott bottles, followed by filtration using a 0.45 μm Milliipore filter to remove any particulate material. The samples were acidified to pH 2.0 with phosphoric acid in order to increase the lipophilic nature of the analytes.

The extraction procedure was adapted from the Waters SPE Applications manual (2003) (Figure 6.1). Prior to loading the environmental samples onto the extraction columns, the columns were equilibrated with 3 ml methanol: diethyl ether (10:90) rinsed with 2 ml methanol and followed with a wash step of 2 ml of HPLC grade

water. A volume of 50 ml of environmental sample was loaded onto the extraction column. This was followed by a second wash step of 1 ml of water. The analytes were eluted with 2 ml 10:90 methanol:diethyl ether, evaporated under a stream of nitrogen to 0.2 ml and reconstituted with 0.3 ml HPLC grade water. A volume of 20 µl was injected onto the HPLC column using the optimised HPLC parameters as discussed in Chapter 5.

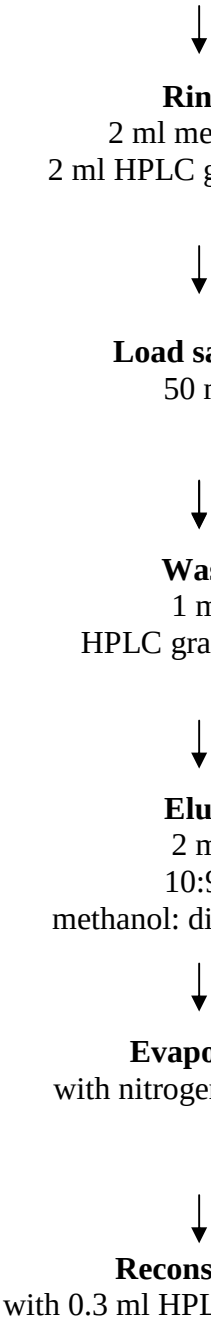


Figure 6.1 Flow diagram representing the extraction procedure for phenol compounds from environmental water samples using a Waters Oasis SPE columns.

6.3 RESULTS AND DISUSSION

6.3.1 Recovery studies and SPE validation

Aqueous standard samples were prepared containing all eleven PPP at a concentration of 1 µg/ml. These were then extracted using the Oasis SPE columns (method adapted from Waters, 2003) applying the method described (see Figure 6.2). The percentage recovery of all eleven phenolic compounds was determined (see Table 6.1). The recovery percentages ranged from 63% for 2,4-dichlorophenol to 88.4% for 4,6-dinitro-2-methylphenol. The lower recoveries at 63% could be due to evaporation of the phenolic compound under the stream of nitrogen.

Blank extractions were performed (Figure 6.3) in order to ensure that only the analytes of interest were eluted from samples. Samples of HPLC grade water used in the extraction process were also injected onto the HPLC system to ensure that no other phenolic compounds except those from the environmental samples were present (Figure 6.4). As can be seen in Figure 6.4 below no peaks appeared at any of the expected retention times of the eleven PPP.

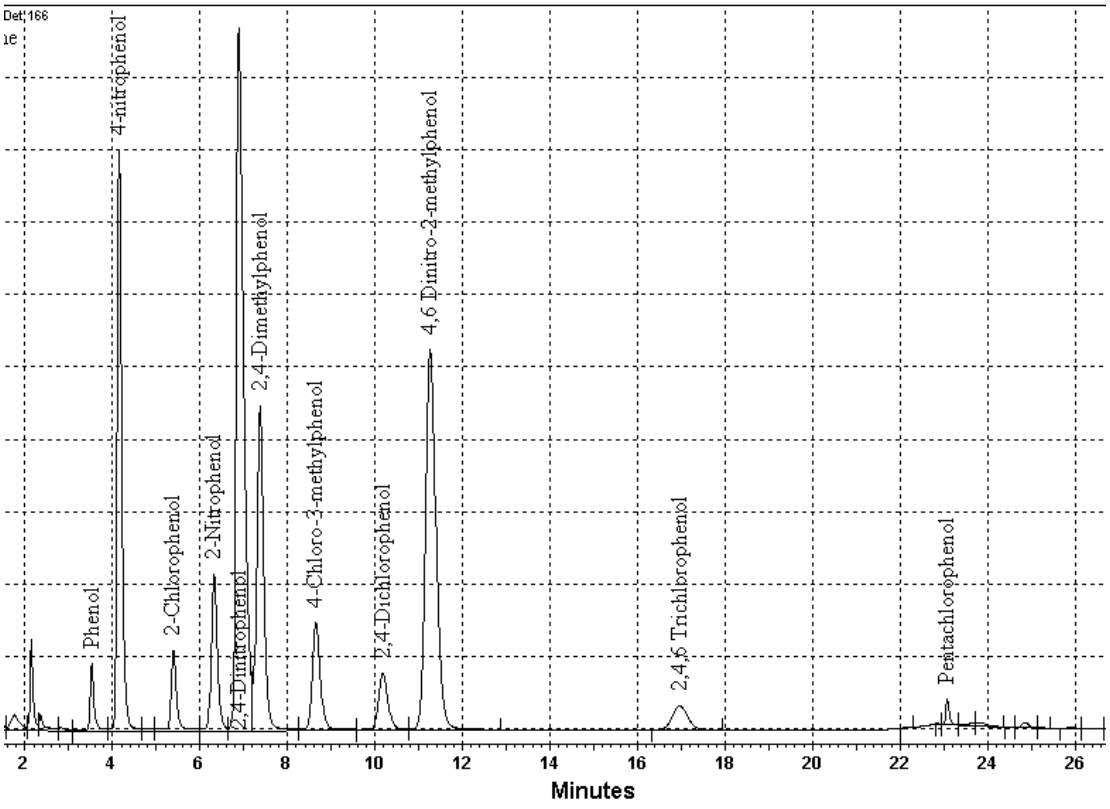


Figure 6.2 Extraction of a standard mixture to determine percentage recovery.

Table 6.1 Recovery percentages from a standard mixture

PHENOLIC COMPOUNDS	PERCENTAGE RECOVERY (%)
phenol	80.0
4-nitrophenol	88.0
2-chlorophenol	70.4
2,4-dinitrophenol	87.0
2-nitrophenol	84.0
2,4-dimethylphenol	75.0
4-chloro-3-methylphenol	87.0
2,4-dichlorophenol	63.0
4,6-dinitro-2-methylphenol	88.4
2,4,6-trichlorophenol	64.0
pentachlorophenol	74.0

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6.3.2 Environmental sample analyses

Environmental samples were collected in and around Grahamstown, Eastern Cape, South Africa. These were from rivers, streams (stagnant and running) as well as from industrial sites and waste water treatment plant effluent. The environmental samples were collected and analysed according to the methods described earlier.

Phenolic compounds were found to be present in all of the samples analysed. As can be seen in Table 6.2, the concentrations of most phenolic compounds were above the maximum admissible concentration (MAC) (Angelino and Gennaro, 1997) for drinking purposes, which is 0.1 μg/l for individual phenolic compounds.

Environmental water samples from the Belmont Valley site were collected from both running and stagnant streams. Samples were collected in June 2007 (winter) and November 2007 (late spring/early summer). Three phenolic compounds (4-nitrophenol, 2,4-dinitrophenol and 2,4-dimethylphenol) were detected in both stagnant and running streams in June 2007, compared to November 2007, where only 4-nitrophenol was detected in both running and stagnant streams (Table 6.2). This variation can be attributed to the spring/ summer rainfall experienced this area, leading to more rapid flow and possible dilution of pollutants in these streams during November. During the dry winter months, there would be less water to dilute the pollutants, hence the greater number and concentration of phenolic compounds detected in samples collected in June.

Samples were collected from the exit of an leather tannery in the area. High concentrations of phenolic compounds were found. As can be seen in Table 6.2, concentrations of pentachlorophenol as high as 0.890 mg/ml were observed.

Samples from the final effluent of the Grahamstown wastewater treatment plant were collected and analyzed. The following compounds were detected: phenol, 4-nitrophenol, 2,4-dimethylphenol, 2,4-dichlorophenol, 4,6-dinitro-2-methylphenol and pentachlorophenol (See Table 6.2). Once again, the concentration of pentachlorophenol was the highest, at concentrations of 225.22 µg/ml (0.225 mg/ml).

Table 6.2 Mean **c**oncentrations of phenolic compounds found in six environmental samples collected in Grahamstown, Eastern Cape.

PHENOLIC COMPOUND	Belmont Valley Stagnant June 07 µg/ml	Belmont Valley Running June 07 µg/ml	Belmont Valley Stagnant November 07 µg/ml	Belmont Valley running November 07 µg/ml	Waste Water treatment Plant µg/ml	Leather Tannery mg/ml
phenol					2.900	0.095
4-nitrophenol	0.209	0.278	0.791	0.593	0.220	0.005
2-chlorophenol						0.012
2,4-dinitrophenol	0.043	0.033				0.003
2-nitrophenol						0.008
2,4-dimethylphenol	0.084	0.124			0.129	0.005
4-chloro-3-methylphenol						0.018
2,4-dichlorophenol					0.047	0.298
4,6-dinitro-2-methylphenol					0.049	0.007
2,4,6-trichlorophenol						0.087
pentachlorophenol					225.22	0.890

Interestingly, the phenolic compound 4-nitrophenol was found in all six of the environmental samples collected for analysis. From Figure 6.5 it was observed that

the Leather Tannery sample had the highest concentration of PPPs; however this sample was collected at the immediate outlet of the tannery, which explains this high concentration. This sample would be further diluted in the environment. The lowest concentration was found in the final effluent at the Waste Water Treatment Plant and this was expected since this site is the furthest from the source of origin of 4-nitrophenol. The Belmont Valley samples concentrations were slightly higher than the Waste Water Treatment plant since these samples were collected further upstream and had not yet been subjected to treatment. In the Belmont Valley samples, the concentration of 4-nitrophenol was also higher in the rainy summer season (November) than in the dry winter season (June).

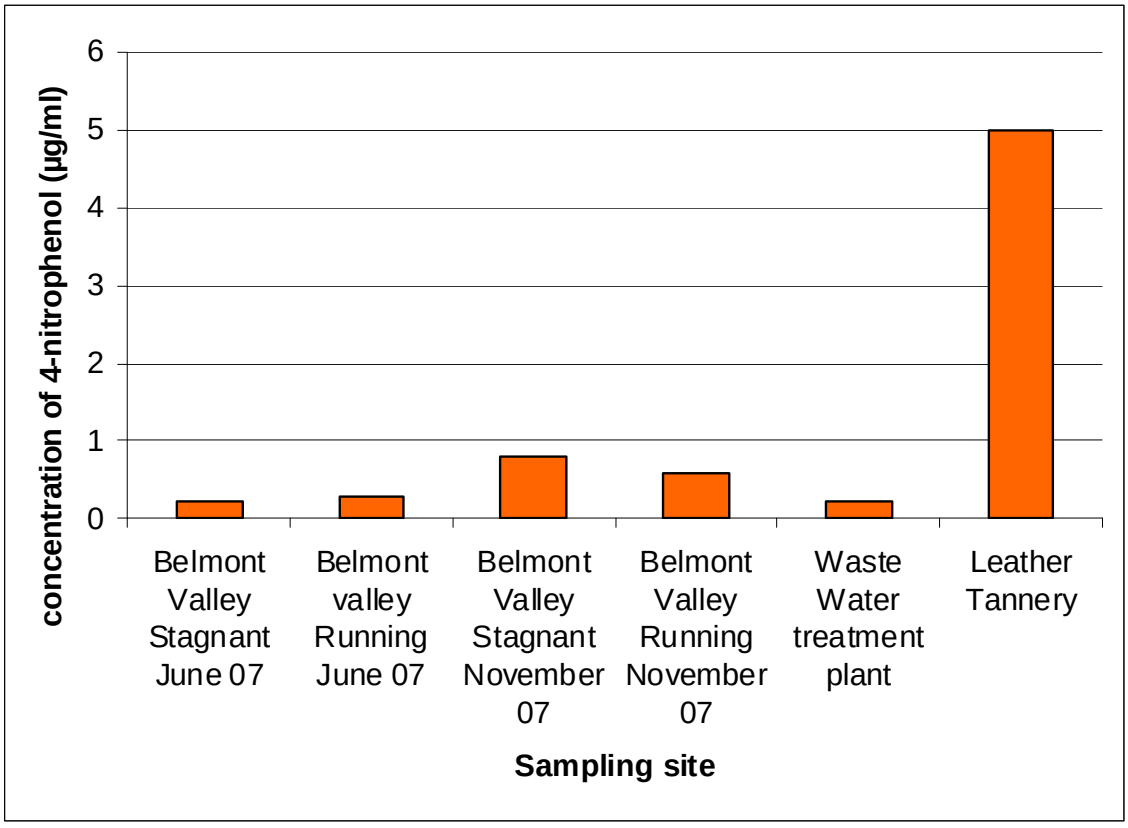

Figure 6.3 A comparison the concentrations of 4-nitrophenol found in all six environmental samples analysed.

Figures 6.4, 6.5 and 6.6 illustrate the sampling sites for the extraction and detection of phenolic compounds in this study. These sites are as follows: Belmont Valley running stream (Figure 6.6), Belmont Valley stagnant stream (Figure 6.7) and final effluent of a Waste Water treatment plant (Figure 6.8).

Figure 6.4 Belmont Valley running stream, Grahamstown, Eastern Cape.

Figure 6.5 Belmont Valley stagnant stream, Grahamstown Eastern Cape.

Figure 6.8 Waste Water Treatment plant final effluent before release into a river in the background.

6.4 CONCLUSION

Some the eleven PPP compounds listed by the US EPA were present in rivers, streams and effluents in the Grahamstown area. The concentrations were above the MAC values listed in EU Countries, which is set at 0.5 μg/l for total phenols and 0.1 μg/ml for individual phenols. Seasonal changes and rainfall patterns may have affected the detection of phenolic compounds in water. As observed in the Belmont Valley streams, not all the phenolic compounds present in the dry season sample were detected during the rainy season. This was the same for both the stagnant and running streams.

CHAPTER 7

7.1 GENERAL DISCUSSION

The aim of this research project was to investigate the interference of phenolic compounds in the water environment on the enzyme activities of β-GAL and β-GUD, and to develop and validate a sensitive HPLC method for the simultaneous detection and quantification of priority pollutant phenols (PPP) compounds in water. This study also involved obtaining water samples from the environment and extracting phenolic compounds present in these matrices, using an SPE method. The phenolic compounds were then identified and quantified using the newly validated HPLC method for the detection of these compounds.

A problem with using an enzymatic approach for the detection of coliforms in water is the potential presence of interfering compounds. Phenolic compounds are potentially one group of interfering compounds. Eleven PPP found in water are listed by the US-EPA (some of these compounds are also listed by the EU). These phenols are potentially able to affect the activities of both β-GAL and β-GUD, resulting in the inhibition and activation of these enzyme assays.

The enzyme activities for β-GAL and β-GUD were inhibited when phenolic compounds were added to the enzyme assays.
In the case of the β-GAL enzyme assay, the phenolic compounds 4-chloro-3-methylphenol, 4,6-dinitro-2-methylphenol and pentachlorophenol competed for the same active site as the substrate CPRG on the β-GAL enzyme molecule. This resulted in a decrease in the velocity of the enzyme reaction, thus inhibiting enzyme activity. The phenolic compound 2,4-dichlorophenol bound to an active site on the enzyme-substrate complex and thus also caused a decrease in β-GAL enzyme activity.
The K_i values for the inhibiting compounds ranged from 0.540 mg/ml for 4-chloro-3-methylphenol to 0.970 mg/ml for pentachlorophenol, indicating that there was little or no variation in the affinity of the phenolic compounds for the β-GAL enzyme. Of the four phenolic compounds that inhibited the β-GAL enzyme activity, 4-chloro-3-methylphenol had the greatest affinity for the β-GAL enzyme molecule, followed by

4,6-dinitro-3-methylphenol and 2,4-dichlorophenol. Pentachlorophenol showed the lowest affinity for the enzyme.

In the case of the β-GUD assay, the activity was inhibited by 4-chloro-3-methylphenol and 2,4-dichlorophenol, both of which demonstrated mixed inhibition, where these compounds competed for the same binding site as the substrate PNPG, as well as binding to the substrate-enzyme complex. In contrast, the compound 2,4-dimethylphenol displayed competitive inhibition, competing for the same binding site on the β-GUD molecule as the PNPG substrate.

There was a larger variation in the K_i values for the three inhibiting phenolic compounds in the β-GUD assay as opposed to the β-GAL enzyme assay. These values ranged from 0.274 mg/ml for 2,4-dimethylphenol to 0.857 mg/ml for 4-chloro-3-methylphenol. The phenolic compound 2,4-dimethylphenol exhibited the highest affinity for the enzyme, almost double that of 2,4-dichlorophenol and more than three times the affinity than 4-chloro-3-methylphenol.

The phenolic compounds in the environment could therefore effectively compete with the substrates CPRG and PNPG for the active sites on the β-GAL and β-GUD enzyme molecules, because these substrates are phenol based, thus being similar in chemical composition to the phenolic compounds.

Activation of enzyme activity was mainly observed in the β-GUD assay. Five of the eleven phenolic compounds tested, namely 4-nitrophenol; 2-chlorophenol; 2-nitrophenol; 2,4-dinitrophenol and 2,4-dinitro-3-methylphenol activated the β-GUD enzyme activity. This activation resulted in a false positive test for the enumeration of coliforms in water. The chemical structures of these activating phenolic compounds (with the exception of 2-chlorophenol) have the substituent nitro- attached to the aromatic hydrocarbon.

The product formed in the β-GUD enzyme assay is 4-nitrophenol. Therefore the pollutant phenolic compounds which cause activation of the β-GUD enzyme assay have a very similar structure to the product of the enzyme reaction. This will result in an over-estimation of the product formed when recording absorption at the wavelength of 405 nm, leading to false positive results when testing for the presence of *E. coli* in a polluted environment.

The second phase of this study involved HPLC finger printing of the eleven PPP found in the environment. The newly developed HPLC method was able to simultaneously detect all eleven phenolic compounds listed by US-EPA. Once the optimum parameters were established, the analytical method was validated. The method demonstrated good linearity, specificity, accuracy and precision. The method showed good reproducibility and was thus reliable. LOQ values ranged from 0.003 μg/l to 2.35 μg/l. LOD values ranged from 0.0015 μg/l to 0.012 μg/l. These limits are an improvement over most of the LOD and LOQ limits reported in literature. This reverse-phase HPLC detection method is therefore well suited for its intended purpose.

For the environmental analysis component of this study, samples were collected from streams (stagnant and flowing), industrial sites and wastewater treatment plant effluent. Phenolic compounds were present in all of these samples.

Wutor (2006) observed interference by the naturally occurring phenolic compound, ferulic acid, between 0.05 mg/ml and 0.2 mg/ml for the β-GAL enzyme assay. Togo (2006) investigated the effects of concentrations of ferulic acid from 0.2 mg/ml to 1 mg/ml on the β-GUD enzyme assay. These studies confirmed the interference by this compound on the β-GAL and β-GUD enzyme assays.

The decision to investigate the interference of industrial pollutant phenols on the β-GAL and β-GUD enzyme assays, rather than focusing on naturally occurring phenols such as lignins and tannins in this study, was based on the levels of industrial pollutant phenols far outweighing the levels of naturally occurring phenols in the environment (Marcheterre *et al.,* 1988; Nistor *et al.,* 2002).

In the Belmont Valley stream three phenolic compounds were detected in both the stagnant and running streams, respectively. The concentrations of the individual compounds found were found to be similar. These samples were collected in June which corresponded to the dry winter season.

The samples collected from the same area in late November, which is in the spring/summer rainy season, revealed different results. Two of the compounds found in June earlier the same year (2,4-dinitrophenol and 2,4-dimethylphenol) were not detected. 4-Nitrophenol was the only compound detected at higher concentrations, i.e. 0.791 μg/ml for the stagnant stream and 0.593 μg/ml for the running stream, than in

June the same year. The absence of 2,4-dinitrophenol and 2,4-dimethylphenol in the November sample was probably due to the higher rainfall, which diluted the concentration of the compounds. The higher concentration of 4-nitrophenol in November could have been due to greater runoff during the rainy season from its point of source.

The concentration range of the three phenolic compounds in the Belmont Valley stream were found to be well within those concentration ranges observed to have a significant effect when the eleven PPP were individually added to the β-GAL assays, i.e. between 0.2 μg/ml (Fig. 4.8) and 2.5 μg/ml (Fig. 4.9). From this we may conclude that the three phenolic compounds found in the Belmont Valley stream and other similar rivers in the Eastern Cape, will significantly inhibit the β-GAL enzyme activity assays.

The effluent collected at the final stage of the Grahamstown Municipal Wastewater Treatment plant contained six of the eleven phenolic compounds, i.e. phenol at 2.9 μg/ml, 4-nitrophenol at 0.220 μg/ml, 2,4-dimethylphenol at 0.129 μg/ml; 2,4-dichlorophenol at 0.047 μg/ml, 4,6-dinitro-2-methylphenol at 0.049 μg/ml and pentachlorophenol at an extremely high concentration of 225.22 μg/ml. From Figures 4.6 and 4.7 (Chapter 4) representing the effects of the addition of the eleven PPP at 0.1 mg/ml for both the β-GUD and β-GAL enzyme assays, it was obvious that pentachlorophenol had a significant inhibitory effect on both the enzyme assays. Pentachlorophenol revealed competitive inhibition in the β-GAL enzyme assay competing for the same binding site as the subtrate CPRG on the β-GAL enzyme molecule. The resulting reduction in velocity of the β-GAL activity assay would therefore contribute to an underestimation of the presumptive test for coliforms.

The environmental sample collected from the final effluent of a tanning industry revealed very high concentrations of PPP released into the environment. These ranged from 5.00 μg/ml for 4-nitrophenol to 890 μg/ml for pentachlorophenol (Table 6.2, Chapter 6).

The concentrations of phenolic compounds found in rivers and streams in Grahamstown, Eastern Cape, South Africa were in the same concentration range as elsewhere in the world. In Barcelona, Spain, Nistor *et al*. (2002) measured the concentrations of phenolic compounds in two waste water treatment plants in local

municipalities and obtained the following concentrations for phenols: phenol-17 µg/ml, 2-chlorophenol-20 µg/ml, 3-chlorophenol-16 µg/ml, 2,4-dichlorophenol-83 µg/ml, 2,4,6-trichlorophenol-17 µg/ml and nitrophenol-114 µg/ml (see Table 7.1). The concentrations marked in bold (in table 7.1) are in the same range than those used in this study for the enzyme inhibition assays for both β-GAL and β-GUD (Chapter 4).

Table 7.1 Compounds that were detected by Nistor *et al.* (2002) using SPE-LC-MS and SSPE-LC-MS at two Waste Water treatment Plants.

Compound	WWTP Igualada			WWTP La liagosta		
	Raw influent	Primary effluent	Final effluent	Raw influent	Primary effluent	Final effluent
	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml
phenol	0.006	0.017	n.f	n.f	n.f	n.f
2-chlorophenol	n.f	n.f	0.02	0.006	0.02	n.f
3-chlorophenol	n.f	n.f	n.f	0.015	16	n.f
4-chlorophenol	n.f	n.f	n.f	n/f	n.f	n.f
2,4-dichlorophenol	**0.083**	0.023	16	n.f	n.f	n.f
2,4,6-trichlorophenol	n.f	n.f	n.f	0.017	n.f	n.f
pentachlorophenol	n.f	n.f	n.f	n.f	n.f	n.f
nitrophenol	0.011	0.021	n.f	0.034	**0.114**	n.f
2,4-dinitrophenol	n.f	n.f	n.f	n.f	n.f	n.f

n.f: not found

WWTP: Waste Water Treatment Plant

These concentrations are above the concentrations of phenolic compounds found in rivers and streams in this study. In Poland, Grynkiewicz *et al*. (2002) measured the concentration of phenols collected from five sites in Gdańsk city during a rainy period and found the following concentrations of phenols: phenol-3.651 µg/l, 4-nitrophenol-0.660 µg/l, dinitrophenol-0.554 ug/l and chlorophenol-3.026 µg/l. In China, Jin *et al.* (2006) studied river water which may have been polluted by the local chemical industry and found 4-chlorophenol at a level of 0.58 µg/l and 2,4-dichlorophenol at 1.53 ug/l, respectively. The concentrations of phenols found in the latter two countries were below that found in this present study.

The data obtained from the quantitative analysis of the environmental samples in the Eastern Cape can be used to correct for the degree of under-estimation of β-GAL- and β-GUD enzyme activities. In chapter 6, Table 6.2, we observed that the concentrations of 4-nitrophenol were between 0.209 to 0.791 µg/ml. Based on the findings on the effects of 4-nitrophenol at 0.2 µg/ml on the β-GAL and β-GUD assays (Figures 4.8 and 4.12), we can predict that the observed enzyme activity is about 95.98 % of the actual activity for β-GAL and 93.89 % of the actual activity of β-GUD. The observed activities of the enzymes in the presence of a high level of pentachlorophenol (0.225 mg/ml) present in the wastewater treatment plant, based on studies done at 0.1 mg/ml on both enzyme assays (Figures 4.6 and 4.7), is expected to represent 79.51% of the actual activity for β-GAL and 39.93 % of the actual activity for β-GUD.

Finally, this study demonstrated that phenolic compounds present in the water environment are able to interfere with the β-GAL and β-GUD enzyme assays. This may result in an over- and/or under- estimation of faecal contamination in water.
The eleven phenolic compounds listed by the US EPA present in the water environment affected β-GAL and β-GUD enzyme activities in a concentration dependent manner. Rivers and streams can potentially contain these phenolic compounds, therefore when using rapid and direct enzymatic measurement of faecal contamination, results should be stated with caution. We demonstrated that phenolic compounds are in fact present in environmental samples in the Eastern Cape. Verification by the more traditional microbiological water quality methods, like the cultural based methods described on page 2 in chapter 1, may then be required. *The rapid direct enzyme assay methods; however, still serve as an early warning and detection method for the potential presence of faecal contamination in water used for human consumption or recreation.*

Future studies in this area should include a study of naturally occurring phenols such as lignins and tannins, as well as the effects of biodegradation of humic acids on the β-GAL and β-GUD enzyme assays. Furthermore, X-ray crystallography may provide further insight into the binding of the inhibitory phenolic compounds on the β-GAL and β-GUD enzyme assays.

REFERENCES

Aich S., Delbaere L.T.J. and Chen R. (2001). Continuous spectrophotometric assay for β-glucuronidase. BioTechniques. **30:** 846-851.

Aktas A.H., Sanli N. and Pekcan G. (2006). Spectrophotometric determination of pK_a Values for some phenolic compounds in acetonitrile-water mixture. Acta Chim. Slov. **53:** 214-218.

Angelino S. and Gennaro M.C. (1997). An ion-interaction RP-HPLC method for the determination of the eleven EPA priority pollutant phenols. Anal. Chim. Acta. **346:** 61-71.

Asan A. and Isildak I. (2003). Determination of major phenolic compounds in water by reversed-phase liquid chromatography after pre-column derivatization with benzoyl chloride. J. Chromatogr. A. **988:** 145-149.

Bagheri H. and Mohammadi A. (2003). Pyrrole-based conductive polymer as the solid-phase extraction medium for the preconcentration of environmental pollutants in water samples followed by gas chromatography with flame ionozation and mass spectrometry detection. J. Chromatogr. A. **1015:** 23-30.

Bagheri H. and Saraji M. (2003). Conductive polymers as new media for solid-phase extraction: Isolation of chlorophenols from water sample. J. Chromatogr. A. **986:** 111-119.

Bates A.J. (2000). Water as Consumed and its impact on the consumer-do we understand the Variables? Food Chem. Toxicol. **38:** S29-S36.

Berhanu T., Liu J-F., Romero R., Megersa N and Jönsson Å. (2006). Determination of trace levels of dinitrophenolic compounds in environmental water samples using hollow fiber support liquid membrane extraction and high performance liquid chromatography. J. Chromatogr. A. **1103:** 1-8.

Brenner K.P., Rankin C.C., Roybal Y.R., Stelma G.N., Scarpino P.V. and Dufour A.P. (1993). New medium for the simultaneous detection of total coliforms and *Escherichia coli* in water. Appl. Environ. Microbiol. **59:** 3534 – 3544.

Brodfuehrer, J.I., Chapman, D.E., Wilke, T.J. and Powis, G. (1990). Comparative studies of the *in vitro* metabolism and covalent binding of ^{14}C-benzene by liver slices and microsomal fraction of mouse, rat, and human. Drug. Metab. Dispos. **18:** 20-27.

Buick, A.R., Doig, M.V., Jeal, S.C., Land, G.S. and McDowall, R.D. (1990). Method validation in bioanalytical laboratory. J. Pharmaceut. Biomed. **8**: 629-637.

Cheng X., Munoz M.G., Zhou H. and Jin H. (2002). Expression of β-galactosidase by recombinant respiratory syncytial viruses for microneutralization assay. J. Virol. Methods. **105:** 287-296.

Chróst R.J. (1991). Enviromental control of the synthesis and activity of aquatic microbial ectoenzymes. In: Chróst, R.J. (Ed.). *Microbial Enzymes in Aquatic Enviroments*. Springer-Verlag, Berlin, pp. 29-59.

Cohen R and Mire M. (1971). Analytical-Band centrifugation of an active enzyme substrate-substrate complex determination of active units of various enzymes. Eur. J. Biochem. **23:** 276.

Coquart V. and Hennion M.C. (1992). Trace level determination of polar phenolic compounds in aqueous samples by high-performance liquid chromatography and on-line preconcentration on porous graphitic carbon. J. Chromatogr. **600:** 195-201.

Craven G., Steers E. and Anfinsen C. (1965). Purification, composition and molecular weight of the ß-galactosidase of *Escherichia coli* K12. J. Biol Chem. **240:** 2468-2477.

Dahlen G. and Linde A., (1973). Screening plate method for detection of bacterial β-glucuronidase. Appl. Microbiol. **26:** 863-866.

Davies C.M., Apte S.C., Peterson S.M. and Stauber J.L. (1994). Plant and algal interference in bacterial β-D-galactosidase and β-D-glucuronidase assays. Appl. Environ. Microbiol. **60:** 3959-3964.

Department of Water Affairs and Forestry (DWAF) (1996). *South African Water Quality Guidelines.* Vol 1: Domestic use Department of Water Affairs and Forestry, Pretoria, South Africa.

Edberg S.C., Allen M.J., Smith D.B. and National Collaborative Study (1988). National field evaluation of a defined substrate method for the simultaneous enumeration of total coliforms and *Escherichia coli* from drinking water: comparison with the standard multiple tube fermentation method. Appl. Environ. Microbiol. **54:** 1595-1601.

Edwardson P.A.D., Bhaskar G. and Fairbrother J.E. (1990). Method validation in pharmaceutical analysis. J. Pharmaceut.Biomed. **8:** 929-933.

Environmental Protection Agency (1974). *Manual of Methode for Chemical Analysis of Water and Wastes.* Office of Technology Transfer, Washington, DC, USA.

Fan Y., Feng Y-Q. and Da S-L. (2003). On-line selective solid-phase extraction of 4-nitrophenol with β-cyclodextrin bonded silica. Anal. Chimica. Acta. **484:** 145-153.

Farnleitner A.H., Hocke I., Beiwl C., Kavka G.G., Zeichmeister T., Kirschner, A.K.T. and Mach, R.L. (2001). Rapid enzymatic of *Escherichia coli* contamination in polluted water. Lett. Appl. Microbiol. **33:** 246-250.

Fiksdal L. and Tryland I. (2008). Application of rapid enzyme assay techniques for monitoring of microbial water quality. Curr. Opin. Biotech. **19**: 289-294.

Fisher K.L and Woods J.P. (2000). Determination of ß-D-glucuronidase enzymatic function of the *Histoplasma capsulatum H* antigen using a native expression system. Gene. **247:**191-197.

Fontanals N., Galiá M., Marcé R.M. and Borrull F. (2004). Solid-phase extraction of polar compounds with a hydrophilic copolymeric sorbent. J. Chromatogr. A **1030:** 63-68.

Fontanals N., Marcé R.M. and Borrull F. (2007). New materials in sorptive extraction technique for polar compounds. J. Chromatogr. A, **1152:** 14-31.

Garrett R.H and Grisham C.M. (1999). *Biochemistry.* 2nd Edition. Harcourt Brace College Publishers, USA.

George I., Crop P. and Servais P. (2002). Fecal coliform removal in wastewater treatment plants studied by plate counts and enzymatic methods. Water Res. **36:** 2607-2617.

Gilpin B.J., James T., Nourozi F., Saunders P., Scholes P. and Savill M. (2003). The use of chemical and molecular indicators for faecal source identification. Water Sci Technol. **47:** (3) 39-43.

Gleick P.H., (2002). Dirty Water: Estimated Deaths from Water-Related Diseases 2000-2020. Pacific Institute Research Report. (http:www.pacinst.org).

Gonzàlez-Toledo E., Prat M.D. and Alpendurada M.F. (2001). Solid-phase micro extraction coupled to liquid chromatography for the analysis of phenolic compounds in water. J. Chromatogr. A. **923**: 45-52.

Grabow W.O.K (1996). Waterborne diseases: update on water quality assessment and control. Water SA. 193-202.

Grynkiewicz M., Polkowska Ż., Kot-Wasik A. and Namieśnik J. (2002). Determination of phenols in runoff. Pol J Environ Stud. **11:** 85-89.

Hamilton R.J and Sewell P.A (1982). Introduction to High Performance Liquid Chromatography, 2nd Edition. Chapman and Hall, New York, New York.

Horváth C. (1980). High-Performance Liquid Chromatography Advances and perspectives. Vol. 2. Academic Press, Inc., New York, New York.

Hennion, H-C. (1999). Solid-phase extraction: method development, sorbents, and coupling with chromatography. J. Chromatogr. A**.856:** 3-54.

Henrissat, B. (1991). A classification of glucosyl hydrolases based on amino acid sequence similarities. Biochem. J. **280**: 309-316.

Holme D.J. and Peck H. (1998). Analytical Biochemistry. Longman, Essex.

http://www.brenda.uni-oeln.de/php/flat_result.php4?ecno=3.2.1.23&organism_list=... 18/4/2007

http://www.brenda.uni-oeln.de/php/flat_result.php4?ecno=3.2.1.31&organism_list=... 18/4/2007

http://www.eco-usa.net/toxics/phenol.shtml (2/4/2007)

Inman E.L. and Rickard E.C. (1988). Chromatographic detection limits in pharmaceutical method development. J. Chromatogr. **447:** 1-12.

Jain S., Drendel W.B., Chen Z.W., Mathews F.S., Slu W.S. and Grubb J.H. (1996). Structure of human beta-glucuronidase reveals lysoomal targeting and active-site motifs. **3:** 375-381.

Janik F. and Wolf H.U. (1992). The calcium transport-ATPase of human erythrocytes as an *in vitro* system for acute affects of some chlorinated compounds. J. Appl. Toxicol. **12:** 17-26.

Jin M., Chen X. and Pan B. (2006). Simultaneous determination of 19 chlorophenols in water by liquid chromatography-mass spectrometry with solid-phase extraction. J. Liq. Chromatogr. R. T. **29:** 1369-1380.

John. R.A. (2002). Photometric assays. In: Eisenthal, R. and Danson, M.J. (Eds.). *Enzyme Assays: Practical Approach.* Oxford University Press, Oxford University press, Oxford, pp. 49-78.

Juers D.H. and Matthews B.W. (2006). Structural Enzymology on *E. coli* Beta-Galactosidase. Institute of Molecular Biology, University of Oregon. USA.

Kilian M. and Bulow P. (1976). Rapid diagnosis of Enterobacteriaceae: detection of bacterial glycosidase. Acta Pathol Microbiol. Scand. B 84, 245-251.

Kim D-H., Jin Y-H., Jung E-A., Han M.J. and Kobashi K. (1995). Purification and characterization of β-glucuronidase from *Escherichia coli* HGU-3, a human intestinal bacterium. Biol. Pharm. Bull. **18.** 1184-1188.

Kim K-R. and Kim H. (2000). Gas chromatographic profiling and screening for phenols as isobutoxycarbonyl derivatives in aqueous samples. J. Chromatogr A. **566**: 87-96.

Knutsson M., Nilvé G., Mathiasson L. and Jönsson J.Å. (1996). Supported liquid membrane for sampling and sample preparation of pesticides in water. J. Chromatogr. A **754:** 197-205.

Lee H.K., Li S.F.Y. and Tay Y.H. (1988). High-performance liquid chromatography of eleven priority substituted phenols by isocratic elution. J. Chromatogr. **438:** 429-432.

Lee M-H., Yeh Y-C., Hsiang W-S. and Hwang B-H. (1998). Solid-phase microextraction and gas chromatography-mass spectrometry for determining chlorophenols from landfill leaches and soil. J. Chromatogr. A. **806:** 317-324.

Lee S-G., Hong S-P. and Sung M-H., (1996). Removal and Bioconversion of phenol in water by a thermalstable β-tyrosinase. Enzyme Microb. Technol. **19:** 374-377.

Llompart M., Lourido M., Landín P., García-Jares C. and Cela R. (2002). Optimization of a derivatization-solid-phase micro extraction method for analysis of thirty phenolic pollutants in water samples. J. Chromatogr. A. **963:** 137-148.

Lupetti K.O., Rocha F.R.P. and Fatibello-Filho O. (2004) An improved flow system for phenols determination exploiting multicommutation and long pathlength spectrophotometry. Talanta **62**: 463-467.

Manafi M. (2000). New development in chromogenic and fluorogenic culture media. Int. J. Food Microbiol. **60**: 205-218.

Manafi M. (1996). Fluorogenic and chromogenic enzyme substrates in culture media and identification tests. Int. J. Food Microbiol. **31:** 45-58.

Manafi M., Kneifel W. and Bascomb S., (1991). Fluorogenic and chromogenic substrates used in bacterial diagnostics. Microbiol. Rev. **55:** 335-348.

Marcheterre L., Choudhry G.G. and Webster G.R.B. (1988). Environmental photochemistry of herbicides. Rev. Environ. Contam. Toxicol. **103:** 61-126.

Marxsen J. and Witzel K. P. (1991). Significance of extracellular enzymes for organic matter degradartion and nutrient regeneration in small streams. In: Chróst, R.J. (Ed.) *Microbial Enzymes in Aquatic Environments.* Springer-Verlag, Berlin, pp. 270-285.

Matthews B.W. (2005). The structure of *E. coli* β-galactosidase. C.R. Biologies, **328:** 549-556.

McConnell E.E., Huff J.E., Hejtmancik M., Peters A.C. and Persing R. (1991). Toxicology and carcinogenesis studies of two grades of pentachlorophenol in B6C3F1 mice. Fund. Appl. Toxicol. **17**, 519-532.

Munch J.W. (2000). EPA Method 528: Determination of phenols in drinking water by solid phase extraction and capillary column gas chromatography/mass spectrometry (GC/MS). U.S. EPA.

Mußmann P., Levsen K. and Radeck W. (1994). Gas-chromatographic determination of phenols in aqueous samples after solid phase extraction. J. Anal. Chem. **348:** 654-659.

Nistor C., Rose A., Farre M., Stoica L., Wollenberger U., Ruzgas T., Pfeiffer D., Barcelo D., Gorton L. and Emnéus J. (2002). In-field monitoring of clean efficiency in waste water treatment plants using two phenol-sensitive biosensors. Anal. Chim. Acta **456**: 3-17.

Norén K. and Sjövall J. (1987). Analysis of pentachlorophenol in water and urine by enrichment with lipidex® 5000. J. Chromatogr. **414** :55-63.

Ohlenbusch G., Kumke M.U. and Frimmel F.H. (2000). Sorption of phenols to dissolve organic matter investigated by solid phase microextraction. Sci. Total Environ. **253:** 63-74.

Peñalver A.P., Pocurull E., Borrull F. and Marcé R.M. (2002). Solid-phase microextraction coupled to high-performance liquid chromatography to determine phenolic compounds in water samples. J. Chromatogr. A. **953**: 79-87.

Pletschke, B.I., Togo, C.A. and Wutor, V.C. (2006). On-Line Real-Time Enzyme Diagnostic System for the Detection and Monitoring of Faecal Contamination of Water Intended for Drinking purposes. *Technical Report for Water Research Commission project No. 1446/1/06.* Water Research Commission (ISBN No:1-77005-463-4), South Africa.

Pocurull E., Marce R.M., Borrull F. (1996). Determination of phenolic compounds in natural waters by liquid chromatography with ultraviolet and electrochemical detection after on-line trace enrichment. J. Chromatogr. A. **738**: 1-9.

Puig D. and Barceló D. (1996). Comparison of different sorbent materials for on-line liquid-solid extraction followed by liquid chromatographic determination of priority phenolic compounds in environmental waters. J. Chromatogr. A. **733:** 371-381.

Rompré A., Servias P., Baudart J., de-Roubin M-R. and Laurent P. (2002). Detection and enumeration of coliforms in drinking water: current methods and emerging approaches. J. Microbiol. Methods **49:**31-54.

Ruana J. and Urbe I. (1993). Determination of phenols at the ng/l level in drinking and river waters byliquid chromatography with UV and electrochemical detection. J. Chromatogr. A. **655:** 217-226.

SANS 241. (2005). *South African National Standard.* Annexture 2. South Africa.

Santos, F.J., Sarrión, M.N. and Galceran M.T. (1997). Analysis of chlorobenzene in soils by headspace solid-phase microextraction and gas chromatography-ion trap mass spectrometry. J. Chromatogr. A. **771:** 181-189.

Sartory F.W. and Watkins J. (1999). Conventional culture for water quality assessment: Is there a future? J. Appl. Microbiol. *Symposium Supplement*. **85:** 225S – 233S.

Sobsey MD., Dufour AP., Gerba C.P., LeChevallier, M.W. and Payment P. (1993). Using a conceptual framework for assessing risks to health from microbes in drinking water. J. AWWA **85**: 44 – 48.

Standard Methods (1998). *Standard Methods for the examination of Water and Wastewater*. American Public Health Association (APHA), Washington, DC.

Suliman F.E.O., Al-Kindi S.S., Al-Kindy S.M.Z. and Al-Lawati H.A.J. (2006). Analysis of phenols in water by high-performance liquid chromatography using coumarin-6-sulfonyl chloride as a fluorogenic precolumn label. J. Chromatogr. A. **1101:** 179-184.

Swartz M.E. and Krull I.S (1997). *Analytical Method Development and Validation.* Marcell Dekker Inc., New York. 17-83.

Togo C.A (2006). PhD Thesis, Development of an *in situ* β-D-glucuronidase diagnostic *Moraxella*-based biosensor for potensial application in the monitoring of water polluted by faecal material in South Africa. Rhodes University, South Africa.

Torres R.A., Torres W., Peringer P. and Pulgarin C. (2003). Electrochemical degradation of ρ-substituted phenols of industrial interest on Pt electrodes. Attempt of a structure-reactivity relationship assessment. Chemosphere **50:** 97-104.

Van Poucke S.O. and Nelis H.J., (1999). Detection of total coliforms and *E. coli* in water within 3.5 hours by solid phase cytometry. Proceedings of the General Meeting of the American Society for Microbiology, Chicago, May 1999. American Society for Microbiology.

Van Poucke S.O. and Nelis H.J., (2000). A 210-min solid phase cytometry test for the enumeration of *Escherichia coli* in drinking water. J. Appl. Microbiol. **89:** 390-396.

Venter, S.N. (2000). Rapid microbiological monitoring methods: The status quo. *IWA the Blue Pages*. London.

Voet D. and Voet J.G. (2004). *Biochemistry.* 3rd Edition. USA.

Washburn, K.S and Phillips T.D. (1995). Development of a field-practical assay for water-solvated chlorophenols. J. Hazard. Mater. **41:** 371-381

Waters (2003). Phenols in drinking water, *Oasis Applications note book, Page 122.*

Watson S. (2003). (Honours thesis) Enzymes used for the rapid detection and enumeration of indicator microorganisms in water-A pilot study. Department of Biochemistry, Microbiology and Biotechnology, Rhodes University, Grahamstown, South Africa.

Wilson K. and Walker J. (2000). *Principles and Techniques of Practical Biochemistry.* Fifth edition, Cambridge University Press, UK.

Wong A.W., He S., Grubb J.H., Sly W.S. and Withers S.G. (1998). Identification of Glu-540 as the catalytic nucleophile of human β-glucuronidase using electrospray mass spectrometry. J. Biol. Chem. **273:** 34057-34062.

Wutor V.C. (2006). PhD Thesis, Development of a novel *in situ* CPRG-based biosensor and bioprobe for monitoring coliform β-D-galactosidase in water polluted by faecal matter. Rhodes University, South Africa.

Yager J.W., Eastmond D.A., Robertson M.L., Paradisin W.M. and Smith M.T. (1990). Characterization of micronuclei induced in human lymphocytes by benzene metabolites. Cancer Res. **50:** 393-399.

Zhao L. and Lee H.K. (2001). Determination of phenols in water using liquid phase microextraction with back extraction combined with high-performance liquid chromatography. J. Chromatogr A. **931:** 95-105.