THE RECOVERY OF PHENOLIC ANTIOXIDANTS FROM FRUIT PROCESSING WASTEWATERS OF SOUTH AFRICA

By

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Thesis presented for the Degree of Masters of Science

In the Department of Chemical Engineering

University of Cape Town

August 2009

Acknowledgements

I would like to express my sincere gratitude to Prof S. Burton for providing the opportunity to perform this research under her supervision. Thanks to Prof F. Deghani of the University of Sydney, Australia for her hospitality and patience in teaching me the supercritical fluid extraction technique. Special thanks also to Dr C. Garcin for showing me how to operate the supported liquid membrane system. To the BTB group, I thank you for the numerous times that you have helped during my research.

The Water Research Council, National Research Fund and the Citrus Academy are gratefully acknowledged for providing funding, without which, this project would not have been possible.

The fruit processors in the Western Cape who were willing to give me wastewater samples, as well as Dr Gorgens of Stellenbosch Univeristy for donating the silage water are gratefully thanked.

A special thanks to my sister Tendai for sacrificing your time looking after Noku, so that I could concentrate on writing my theses. I am truly indebted to you, thanks sis. Finally, to my husband and daughter, you have been my pillar of strength during the whole period of doing experiments until now. Thanks for understanding when I had to be away from you doing my research, you can have me back now!

Declaration

I know the meaning of plagiarism and declare that all the work in the document, save for that which is properly acknowledged, is my own

Abstract

Phenolic compounds are known to confer protection against free radicals through their antioxidant properties. There has been increased interest in the use of phenolic compounds from natural sources as antioxidants, because of the perceived negative effects associated with the use of synthetic antioxidants such as butylated hydroxyanisole. Fruit processing wastewaters from the processing of citrus (CW1 and CW2) and deciduous fruits (AW1, AW2 and SW) have been identified here as possible sources of phenolic antioxidants because they contain some of the phenolic compounds present in the fruits. However, limited research has been done previously to recover phenolic antioxidants from fruit processing wastewaters. Thus, this study focused on the determination of antioxidant activity and the extraction of phenolic antioxidants from fruit processing wastewaters obtained from fruit processors in the Western Cape region of South Africa, using various extraction techniques.

The chemical analysis of the fruit processing wastewaters showed that the silage water SW had the highest concentration of total phenols of 399.52 mg/L gallic acid equivalents (GAE) and apple wastewater AW1 had the lowest concentration of 7.61 mg/L GAE. The fruit processing wastewaters also contained sugars and complex carbohydrates. Determination of the antioxidant capacity of the fruit processing wastewaters was achieved using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, the 2,2'-azonobis (3-ethybenzothiazoline-6-sulphonate) ($ABTS^{+)}$ assay, the ferric reducing antioxidant power (FRAP) assay and the β-carotene linoleic acid model system (β-CLAMS). The DPPH free radical scavenging ability of the fruit processing wastewaters was measured to be in the range of 22.05% in apple wastewater AW1 to 100% in the silage water SW. The radical scavenging ability of the fruit processing wastewaters ranged from 13.8% in apple wastewater AW1 to 74.67% in the silage water SW using the $ABTS^{+}$ assay. The silage water SW exhibited the highest reducing power of 0.03 GAE followed by citrus wastewater CW2, determined by the ferric reducing antioxidant power assay. Apple wastewater AW1 had the lowest reducing power, measured by the ferric reducing antioxidant power assay. All the fruit processing wastewaters were able to inhibit β-carotene bleaching in the β-carotene linoleic acid model system. The silage water SW showed the highest % inhibition of 92.9% and apple wastewater AW1 had the lowest % inhibition of 10%.

High performance liquid chromatography (HPLC) analysis was used to identify individual phenolic compounds in the fruit processing wastewaters. Chlorogenic acid was detected in the apple wastewaters AW1 and AW2, the citrus wastewater CW2 and the silage water SW, at concentrations of 0.3, 0.33, 0.36 and 10.53 mg/L respectively. Catechin was identified in citrus wastewater CW2 and silage water SW at concentrations of 1.60 and 8.10 mg/L respectively, whilst gallic acid and resveratrol were only identified in the silage water at concentrations of 101.4 mg/L and 0.6 mg/L respectively.

Solvent extraction using ethyl acetate and hexane was investigated and ethyl acetate extracted more phenolic compounds than hexane from all the fruit processing wastewaters. Consequently, the antioxidant activity was higher in ethyl acetate extracts than in hexane extracts. As an alternative to solvent extraction, solid phase extraction using C18 Sep-Pak® cartridges was employed to separate neutral and acidic phenolics. The extraction efficiencies ranged from 44.1% to 56.9% in all the fruit processing wastewaters. Neutral phenolics exhibited higher antioxidant activity compared with acidic phenolics. The use of PVPP as a solid phase adsorbent was studied, and the extraction yielded low extraction efficiencies with negligible extraction in AW1. Supported liquid membrane extraction was then employed to extract phenolic compounds, specifically, gallic acid, from the silage water. Silage water SW was chosen because it contained the highest total phenolics content amongst the studied wastewaters. The main product of the extraction was gallic acid, which was extracted at a concentration of 45.23 mg/L, with an extraction efficiency of 44.6%. Finally, the use of supercritical fluid extraction, which is an attractive alternative for the extraction of phenolic compounds because of its use of CO_2, making it environmentally friendly, was investigated. The solubility of gallic acid in supercritical-CO_2 was determined in order to optimize the extraction conditions. The optimum operating conditions were found to be 25°C using 20ml of ethanol at a pressure of 180 bar.

The data provided here support the use of fruit processing wastewaters for the recovery of phenolic compounds. Fruit processing wastewaters studied here exhibit antioxidant activity against free radicals using different mechanisms of antioxidant action. This has important implications in the food and pharmaceutical industries in particular, as this provides a potential alternative to the use of synthetic antioxidants.

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CHAPTER 1: INTRODUCTION

This thesis reports an investigation into the extraction of phenolic compounds present in wastewaters generated during the processing of citrus and deciduous fruits, referred to as fruit processing wastewaters in this study. Such phenolic compounds have potential use as antioxidants for application in the food industry. The project involved detailed characterisation of wastewaters from the processing of citrus and deciduous fruits collected from fruit processing companies in the Western Cape region of South Africa. The antioxidant activity present in the fruit processing wastewaters themselves was determined first, using the following assays: DPPH radical scavenging assay, $ABTS^+$ assay, ferric reducing antioxidant power assay and the β-carotene linoleic acid model system. The extraction of phenolic compounds from fruit processing wastewaters, using techniques such as solvent extraction, solid phase extraction, supported liquid membrane extraction and supercritical fluid extraction, was investigated to determine the most suitable extraction technique for the recovery of phenolic compounds from fruit processing wastewaters for use as antioxidants, based on the potential use of the phenolic extracts and on the economic feasibility of the extraction process.

The fruit processing industry utilises large quantities of water in various stages, resulting in the generation of high volumes of wastewaters that require treatment before they can be disposed. The disposal of wastewaters containing organic compounds by municipalities and manufacturers not only threatens environmental quality, but also under utilises the potential of the wastewaters as a source of recovering valuable products such as phenolic antioxidants and sugars. Technologies are being developed to utilise agricultural residues for the production of fertiliser, energy, antioxidants and dietary fibre, and thus, potentially, to improve the economies of regions (Pap, 2004).

In South Africa, large volumes of wastewater are produced each year during the processing of deciduous fruits such as apples, peaches and apricots as well as during the processing of citrus fruits and grapes. The processing of apple juice takes place mainly during the months from February to June each year. The wastewaters from apple juice processing plants are generated during the stages of unloading, washing, sorting, pressing, juice extraction and packaging. South Africa's apple juice processing industry produces approximately 6.8 million m^3 of wastewaters annually and these wastewaters contain considerable amounts of organic compounds (Sigge *et al.*, 2006). The wastewaters from apple juice processing are characterised by high levels of pectin and phenolic compounds, and a pH of 3.5 to 5 (Lu and Foo, 2000; Thassitou and Arvanitoyannis, 2001).

The South African citrus industry generates 9 million m^3 of wastewaters annually from the processing of citrus fruit pulp to canned or packaged juice (Burton *et al.*, 2006). The wastewaters from citrus processing are characterised by high concentrations of organic compounds such as terpene- containing oils, flavonoids, carbohydrates and phenolic compounds (Burton *et al.*, 2006).

Wastes from the processing of fruits have recently gained interest as potential sources of antioxidants to be used as health supplements and preservatives in the food industry (Prakash *et al.*, 2007). The large quantities of wastewaters produced annually during fruit processing make them a potential source for the recovery of phenolic antioxidants. However, there are very few reports of processes in which phenolic antioxidants are extracted effectively from wastewaters. Thus, there is a need for the investigation of various extraction techniques for the recovery of phenolic compounds from fruit processing wastewaters to determine the most suitable technique. This research has potential value to fruit processors, who would benefit from reduced environmental impacts, as well as consumers, based on the health benefits associated with antioxidant phenolic compounds.

1.0 Thesis structure

This thesis consisted of five chapters. **Chapter 1** (Introduction) describes the background of the present study. **Chapter 2** (Literature Review) is a literature review of antioxidants, phenolic compounds and the extraction techniques that can be used to obtain phenolic compounds from wastewaters. The characteristics of fruit processing wastewaters are described in Section 2.1. In section 2.2 the range of valuable products that can potentially be obtained from fruit processing wastes is discussed. The chemistry, structure-activity relationships and the industrial applications of phenolic compounds and antioxidants are described in this section. Section 2.3 describes reactive oxygen species, their sources, the damage generated by reactive oxygen species in the body and possible benefits of antioxidants. Section 2.4 describes the various assays and techniques that can be used for the analysis of phenolic compounds and Section 2.5 examines the analysis of antioxidant activity using different assays. The various extraction techniques that can be employed for the recovery of phenolic compounds from wastewaters are reviewed in Section 2.6, The concluding part of this Chapter, Section 2.7, presents the objectives and motivation for this study.

A detailed description of experimental procedures and techniques used in this thesis is presented in **Chapter 3**.

In **Chapter 4**, the results from the study are presented and discussed. This includes the characterisation of the studied wastewaters and the antioxidant activity in the wastewater samples, using various assays. Further, results obtained after the extraction of phenolic compounds, using various extraction techniques, are reported. This chapter concludes with a comparative discussion of the extraction techniques and the feasibility of using each technique at a commercial scale.

Finally, a summary of the major findings, conclusions and recommendations for future work in this research field is presented in **Chapter 5**.

CHAPTER 2: LITERATURE REVIEW

Introduction

This section gives an overview, from literature, of the valuable products that can potentially be obtained from fruit processing wastewaters. The recovery of phenolic compounds from fruit processing wastewaters can be achieved by various extraction techniques; these extraction techniques are described in detail. Also reviewed in this chapter are phenolic compounds, their structure-activity relationships, antioxidant activities and their applications in various industries. The role of antioxidants and the sources of reactive oxygen species are discussed. Finally, the quantification of phenolic compounds using various methods and the analyses available to measure antioxidant capacities are discussed. There are many reports published in the literature regarding fruit phenolic compounds found in fruit juice and solid wastes. However, there is limited information on phenolic compounds present in the wastewaters produced through the processing of citrus and deciduous fruits.

2.1 Wastewaters in fruit processing

Wastewaters produced through the processing of citrus and deciduous fruits are potential sources of antioxidants because phenolic compounds found in fruits are also present in the wastewaters. Thus, there is potential for obtaining value added products in the form of phenolic antioxidants which can be used in various industries such as the pharmaceuticals, cosmetics and food. Large volumes of wastewaters containing phenolic antioxidants are produced annually in South Africa and globally as discussed in Chapter 1. Phenolic antioxidants have been extracted from other wastewaters such as the olive wastewaters at concentrations up to 2700 mg/L (De Marco *et al.*, 2007) and in by-products of fruit processing such as the pomace (Adil *et al.*, 2007). Thus, in the context of the present study, the most attractive waste management systems are those which recover phenolic antioxidants that are known to have beneficial effects on health. With regard to this, there is a need for developing an efficient extraction system that would recover phenolic antioxidants from the wastewaters. Therefore, the aim of this project was to use extractive treatment of wastewaters to obtain antioxidants, while simultaneously producing cleaner water with lower environmental impact.

2.2 Potential valuable products obtainable from fruit processing wastewaters

Value addition can be defined as the increase of technical and/or economic value of by-products and wastes that are generated in different industries (Pap, 2004). Fruit processing wastewaters contain various valuable products that can potentially be recovered at a commercial scale. Some of the valuable products that have been obtained from wastes are discussed below.

2.2.1 Antioxidants

An antioxidant can be defined as any substance which when present in low concentrations compared to those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate (Becker *et al.*, 2004). Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tert-butylhydroquinone (TBHQ) are commonly used in the prevention of lipid peroxidation in foods, since this is a major cause of food deterioration during handling, processing and storage (Baydar *et al.,* 2007). However, growing knowledge on these synthetic antioxidants has shown that they may be carcinogenic and exert negative effects on metabolic enzymes (Jayaprakash *et al.*, 2008a). This has prompted renewed interest in the search for natural antioxidants of plant origin since these compounds are thought to confer protection from hazardous reactive oxygen species (Peschel *et al.*, 2006, Marino-Martinez *et al.*, 2009). Although the protective effects have previously been attributed primarily to better known antioxidants such as Vitamins C and E and β-carotene, recent studies have shown that plant phenolics are likely to contribute significantly to the antioxidant activity of plant extracts (Soobrattae *et al.*, 2005, Chua *et al.*, 2008).

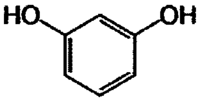
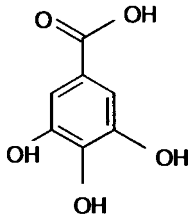
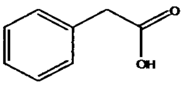
2.2.2 Phenolic compounds

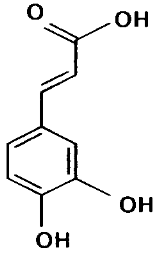
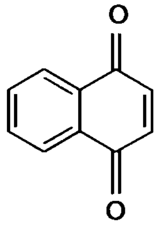
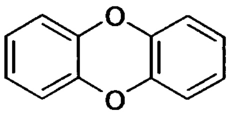
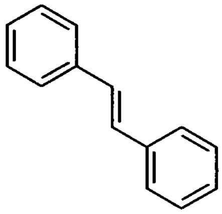
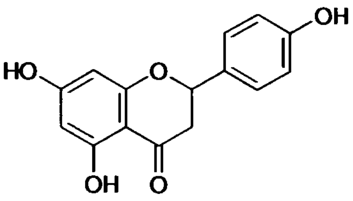
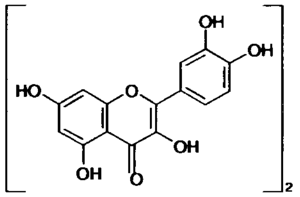
Phenolic compounds are a class of chemical compounds whose structures consist of one or more aromatic rings, with at least one hydroxyl substituent on the rings (Balasundram *et al.*, 2006). Polyphenols are phenolic compounds characterised by the presence of more than one aromatic ring (Balasundram *et al.*, 2006). Polyphenols are amongst the most widely distributed phytochemicals in the plant kingdom where, for example, they play an important role in growth and reproduction through attracting pollinating agents by exhibiting their attractive colours (Escribano-Bailon *et al.*, 2004; Soobrattae *et al.*, 2005). According to a review by Chandrashekar and Satyanayarana (2006), polyphenols contribute to the survival of the plant because they are toxic to pathogens and pests, as for example, in the grains of sorghum and millet. Biomedical research into the physiological effects of phenolic compounds has shown that different polyphenols exhibit different effects in the human body. For example, anti-allergenic, anti-viral (Chavez *et al.*, 2006), anti-bacterial (Vaquero *et al.*, 2007), anti-thrombotic, antioxidant and anti-tumor effects have been reported, largely based on epidemiological studies (Peeny *et al.*, 2002; Silva *et al.*, 2008).

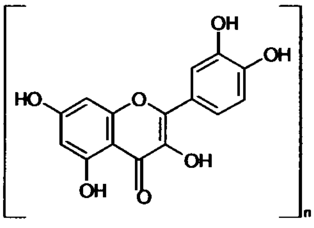
2.2.1.1 The chemistry of phenolic compounds

Phenolic compounds can be categorised into different classes as shown in Table 2.1 below. The major classes of phenolic compounds found in plants comprise the phenolic acids, flavonoids and hydrolysable tannins.

Table 2.1: Classes of phenolic compounds in plants (Balasundrum *et al.*, 2006)

Class	Example	Structure
Simple phenolics	resomicol	
Hydroxybenzoic acids	Gallic acid	
Acethophenones, phenylacetic acids	Phenylacetic acid	

Hydroxycinnamic acids	Caffeic acid	
Napthoquinones	Napthoquinone	
Xanthones	Xanthone	
Stilbenes	Stilbene	
Flavonoids	Naringenin	
Biflavonoids	Quercetin	

Condensed tannins	Proanthocyanindins	

Among the phenols, phenolic acids are commonly found in fruits and also therefore, the by-products, such as the juices and the wastewaters. Phenolic acids can be classified into two groups, namely, hydroxybenzoic acids and hydroxycinnamic acids. Hydroxybenzoic acids consist of an aromatic ring with an OH group and one carboxylic acid group attached, whereas the aromatic ring in hydroxycinnamic acids has a 3- carbon side chain (Balasundram *et al.*, 2006). Examples of hydroxybenzoic acids and hydroxycinnamic acids include gallic acid and caffeic acid respectively, and the structures are shown in Table 2.1.

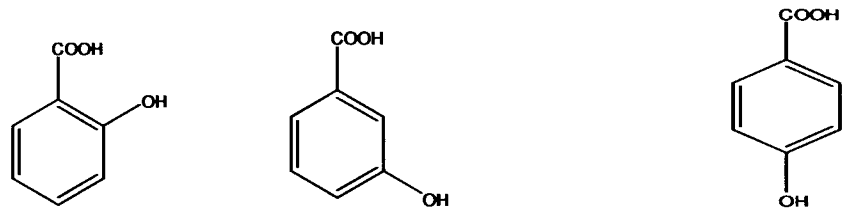
The flavonoids constitute one of the largest and most structurally diverse groups of phytochemicals. They consist of two aromatic rings, named A and B, linked by a 3-carbon heterocyclic ring C. The aromatic rings A and B are derived from the malonate and shikimate pathways respectively (Wojdylo *et al.*, 2007). The example of a flavonoid shown in Table 2.1 is naringenin. The variations in the substitution patterns on the ring structures results in the different classes of flavonoids, namely, flavanols, flavonols, flavanones, flavones, isoflavones, flavanonols and anthocyanidins (Lien *et al.*, 1999; Boots *et al.*, 2008). Most flavonoids, with the exception of flavanols, occur in plants and most foods as glycosides. Examples of flavonoid glycosides include hesperedin, naringin and rutin (Balasundram *et al.*, 2006).

The tannins are a group of high molecular weight phenolic compounds. They can be subdivided into hydrolysable and condensed tannins. Hydrolysable tannins (gallo- and ellagitannins) are esters of gallic acid while condensed tannins (proanthocyanidins) are polymers of polyhydroxyflavan-3-ol monomers (Meagher *et al.*, 2004; Haslam, 2007). Other classes of phenolic compounds such as the napthoquinones, xanthones and stilbenes are found in plants to a lesser extent and are not as widely distributed as the phenolic acids and flavonoids.

2.2.1.2 The structure-activity relationships of phenolic compounds

This section describes the relationship between a phenolic compound and the corresponding antioxidant activity. In order to gain a deeper understanding of how the different functional groups on the phenolic compound affect antioxidant activity, it is important to understand the structure of the phenolic compound in relation to its antioxidant activity. The ability of phenolic compounds to act as antioxidants depends on their structure and the position of the functional groups such as hydroxyl and carboxyl groups, a concept known as the structure-activity relationship (SAR) (Balasandram *et al.*, 2006). This information will be useful in the present study since identifying the individual phenolic compounds in the fruit processing wastewaters will help us in understanding the nature of the antioxidants, and hence determine the most suitable use of the extracted phenolic antioxidants.

The antioxidant activity of phenolic acids depends on the number and position of hydroxyl groups (-OH) in relation to the carboxyl groups (-COOH) (Podsedek, 2007). This is exemplified by the lack of antioxidant activity in hydroxybenzoic acids when the -OH moiety is at the *ortho-* (1) or *para-* (3) positions to the –COOH group, as shown in Figure 2.1. However, when the –OH moiety is at the *meta-* (2) position, hydroxybenzoic acids exhibit antioxidant activity (Podsedek, 2007).



(1) (2) (3)

Figure 2:0:1: Structure of -*ortho* (1), -*meta* (2) and -*para* (3) hydroxybenzoic acid

In contrast with mono-hydroxy phenolic acids, the dihydroxy phenolic compounds exhibit higher antioxidant activity due to the presence of an extra –OH. Hydroxycinnamic acids have even greater antioxidant activity due to the presence of the CH=CH-COOH which increases their hydrogen donating ability and radical stabilisation capacity (Balasandram *et al.*, 2006).

In the flavonoid group, the complexity and diversity of the group leads to a wide variability in antioxidant activity. A higher number of hydroxyl groups on the flavonoid results in better antioxidant activity because of the increased stability conferred by the hydroxyl groups and increased ability to donate H^+ ions which will stabilise free radicals (Balasundram *et al.*, 2006; Torres de Pinedo *et al.*, 2007). The antioxidant activity of flavonoids depends on the position of double bonds as well as hydroxyl groups. Research conducted on the structural aspects of antioxidant activity of flavonoids has revealed that the presence of a double bond between C_2 and C_3, together with a hydroxyl moiety on C_3, enhances the radical scavenging effect of flavonoids (van Acker *et al.*, 1995). Furthermore, the antioxidant activity of flavonoids in general, is enhanced by their ability to chelate metals (Tsao and Yang, 2003; Ozsoy *et al.*, 2008). Thus, the positions of the hydroxyl groups, and the degree of hydroxylation in the B ring, results in higher antioxidant activity because this gives higher stability to the aroxyl radical through electron delocalisation, thus providing good binding sites for metals on the aroxyl radical (Balasundram *et al.*, 2006).

2.2.1.3 Phenolic compounds as natural food antioxidants

There is a need for the development of new sources to provide antioxidants with the capacity to preserve diversified food systems such as emulsions or bulk oils from oxidation (Luther *et al.*, 2007). Phenolic compounds are rapidly replacing synthetic antioxidants such as BHA and BHT in the food industry because phenolic compounds of plant origin are perceived to be much safer compared to synthetic antioxidants which are reported to exhibit toxicity and inhibitory effects on metabolic enzymes (Balasundram *et al.*, 2006; Prakash *et al.*, 2007).

Yi *et al.* (2008) investigated the antioxidant and antimicrobial activities of phenolic extracts from the peels of orange (*Pericarpium citri reticulatae).* This study showed that orange peels, which constitute waste from citrus processing, exhibit high levels of antioxidant and anti-microbial activities due to the presence of terpenes and phenolic acids. This suggests that extracts obtained from wastes from the processing of citrus fruits could potentially be used as food additives as well as for the protection against degenerative and free radical induced diseases. However, few industrial processes have implemented the techniques for the recovery of valuable components of fruit processing wastewaters, such as phenolic antioxidants.

Research conducted on the antioxidant activity of white grape vinification by-products showed that extracts from the peels, stems and seeds of grape seeds had high antioxidant activity, and these extracts have potential use as commercial antioxidants because of the high antioxidant capacity they exhibit

(Makris *et al.*, 2007). Similarly, the antioxidant activity of phenolic compounds extracted from mushrooms was studied, and these compounds were also shown to be effective as antioxidants in *in vitro* assays (Tsai *et al.*, 2007).

2.2.1.4 Industrial applications of phenolic compounds

Phenolic compounds are used in industry as antioxidants, chemical intermediates, disinfectants, stabilisers and tanning agents. In the cosmetic industry, there has been a recent increase in the incorporation of phenolic compounds during preparation. This is because phenolic compounds can protect skin cells from radiation and cancer causing agents. Peschel *et al.* (2006) conducted suitability tests on the incorporation of phenolic compounds extracted from industrial vegetable and fruit wastes in crème formulations and concluded that phenolic compounds represented a potential source of antioxidants for use in dermatology. In a review paper by Lupo (2001), it was reported that catechin extracted from green tea conferred protection against photocarcinogenesis on murine skin. Furthermore, genisten, an isoflavone isolated in soybeans, exhibited anticancer properties through the inhibition of tyrosine protein kinase. Anthocyanins are being applied in the food, cosmetic and pharmaceutical industries as substitutes for synthetic colorants, and capsules are now being prepared for use in health rejuvenation and prophylaxis against cold viruses (Petri *et al.*, 1997; Luque-Rhodriguez *et al.*, 2007).

Some research has been conducted on the effect of the addition of phenolic antioxidants from agro-industrial waste to several foods and food model systems. For example, Fki *et al.* (2005) evaluated the potential application of phenolic compounds extracted from olive mill waste waters (OMWW) for the stabilisation of refined oils. These authors noted that 3, 4-dihydroxyphenyl acetic acid and hydroxytyrosol extracted from OMWW exhibited useful antioxidant properties and presented an alternative to artificial antioxidants for incorporation into foods. Luther *et al.* (2007) studied the inhibition of lipid peroxidation in fish oil by Chardonnay grape and black raspberry seed extracts. Both seed extracts were able to suppress lipid oxidation, thereby preserving important fatty acids in fish oil. These results suggest the possible use of phenolic compounds obtained from fruit processing wastewaters as food preservatives. Therefore, phenolic compounds can be used in various industries, and their recovery from natural sources such as the fruit processing wastewaters would be beneficial.

2.2.3 Other valuable products that can be recovered from fruit processing wastewaters

Although other valuable products such as pectin and sweeteners are outside the scope of the present study, they can be obtained from the fruit processing wastewaters and they are discussed briefly here.

Pectin can be recovered from fruit processing by-products, including wastewaters. Pectin is a complex carbohydrate that consists of multiple units of α-galacturonic acid and a variable number of methyl ester groups (Liu *et al.*, 2006a; Pinheiro *et al.*, 2008). This dietary fibre acts as a bulking agent aiding in intestinal mobility and increasing the moisture content of faecal matter (Sudha *et al.*, 2007). The extraction of pectin for commercial use has been reported by various authors (Sohair *et al.*, 1987; Habibi *et al.*, 2005; Liu *et al.* 2006a; Sudha *et al.*, 2007; Wang *et al.*, 2007).

The need for a safe, non-nutritive sweetener for diabetics and diet conscious people has motivated further research on finding alternative sources of sweeteners that are of plant origin (Tanaka, 1982, Sehar *et al.*, 2008). A natural sweetener is a sugar substitute that duplicates the effect of sugar or corn syrup in taste, but usually has less food energy (Sehar *et al.*, 2008). Natural sweeteners can be obtained from by-products of chicory processing and the processing of citrus fruits at concentrations of about 250mg/L (Scordino *et al.*, 2007; Sehar *et al.*, 2008). Thus, natural sweeteners are potentially valuable products that can be extracted from fruit processing wastewaters.

2.3 Reactive oxygen species (ROS) and their interaction with antioxidants

Antioxidants confer protection against various diseases in the human body. They also prevent lipid peroxidation in foods, through different mechanisms of action against free radicals such as reactive oxygen species. Reactive oxygen species (ROS) are partially reduced or activated derivatives of oxygen (Breusegem and Mittler, 2008). Inside the human body, ROS are formed during normal cellular metabolic processes such as respiration. These include, for example, the superoxide anion, hydroxyl radical, peroxyl radical and non-radical species such as hydrogen peroxide (Ardestani and Yazdanaparast, 2007)

ROS can have either beneficial or detrimental roles in the body, and the balance between ROS and antioxidants is an important strategy for maintaining health (Yen *et al.*, 2008). Under normal circumstances, ROS are used to kill micro organisms such as bacteria. For example, during the respiratory burst in immune mediated host defence, monocytes, neutrophils and phagocytes produce ROS and these ROS are used as cytotoxic agents against pathogens (Kelly *et al.*, 1998; Nindl, 2004).

The human body is well equipped to combat the negative effects of ROS through the action of enzymes such as superoxide dismutase (SOD) and antioxidants such as glutathione and vitamin E. However, when levels of ROS exceed the availability of these antioxidants, oxidative stress results, leading to tissue damage and the development of degenerative diseases (Buonocore and Groenendaal, 2007).The sources of ROS can be endogenous or exogenous, as explained in detail below.

2.3.1 Endogenous sources of ROS

ROS can be formed inside the body in cell organelles such as the mitochondria, the endoplasmic reticulum and microsomes. The formation of ROS in these organelles is discussed below.

Mitochondria are the cell organelles which are mainly responsible for energy generation through the process of oxidative phosphorylation (Pieczenik and Neustadt, 2007). Cellular oxidative phosphorylation involves the reduction of molecular oxygen to water and partially reduced intermediates such as the superoxide anion (${}^{\cdot}O_2^{-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (${}^{\cdot}OH$) (Vallyathan and Shi, 1997). The reduction of oxygen to water occurs through the process illustrated below (Kelly *et al.*, 1998).

$$O_2 + e^{-} \longrightarrow {}^{\cdot}O_2^{-} \tag{1}$$

$$ {}^{\cdot}O_2^{-} + e^{-\,2H+} \longrightarrow H_2O_2 \tag{2}$$

$$H_2O_2 + e^{-} \xrightarrow{H+} HO^{\cdot} + H_2O \tag{3}$$

$$HO^{\cdot} + e^{-H+} \longrightarrow H_2O \tag{4}$$

The activities of several oxidising enzymes may also lead to the production of ROS; for example, xanthine oxidase converts hypoxanthine to xanthine producing ${}^{\cdot}O_2^{-}$ and H_2O_2. Similarly, aldehyde oxidases found in the endoplasmic reticulum of liver cells generate H_2O_2 and $^{\cdot}O_2^-$ while acting on their aldehydic substrates. The conversion of 5-hydroxytryptamine to 5-hydroxyindol acetic acid by monoamine oxidases also leads to production of $^{\cdot}O_2^-$ and H_2O_2 (Vallyathan and Shi, 1997; Kelly *et al.*, 1998).

Another source of ROS in the body which leads to the production of the superoxide radical is due to phagocytes. These are white blood cells that protect the body by ingesting harmful foreign particles. Phagocytes are activated when they encounter foreign particles such as inhaled particles or micro-organisms. This results in a respiratory burst which increases oxygen uptake, glucose metabolism, and the utilisation of NADPH using a plasma membrane bound oxidase. This reaction leads to the generation of the superoxide radical (Vallyathan and Shi, 1997).

Metal ions such as iron are also involved in ROS generation. Iron is found in the body bound to haemoglobin, myoglobin, cytochromes, enzymes, transferrin and ferritin. Biological reducing agents such as ascorbate can promote the release of iron from its bound form, triggering ROS formation through the Fenton reaction, resulting in the further formation of other ROS as summarised below (Kelly *et al.*, 1998, Vidrio *et al.*, 2008). The Fenton reaction involves the reaction between the superoxide anion and hydrogen peroxide, producing the hydroxyl radical, catalysed by Fe^{2+}/Fe^{3+} (Kelly *et al.*, 1998).

$$O_2 + \text{cellular reductants} \longrightarrow O_2^{\cdot -} + H_2O_2 \qquad (1)$$

$$Fe^{3+} + O_2^{\cdot -} \longrightarrow Fe^{2+} + O_2 \qquad (2)$$

$$2\,O_2^{\cdot -} + 2H^+ \longrightarrow O_2 + H_2O_2 \qquad (3)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\cdot}OH + OH^- \qquad (4)$$

2.3.2 Exogenous sources of ROS

A number of foreign substances can cause ROS formation in the tissues. Some common examples are discussed below.

The presence of asbestos fibrils in the body leads to the production of ROS. Chrysotile, the most common form of asbestos, is made up of silica, magnesium and a small amount of iron (Vallyathan and Shi, 1997). Thus, the presence of asbestos in the body triggers the production of ROS through Fenton reactions.

ROS are also formed during phagocytosis of asbestos fibrils by macrophages, leading to the release of $^{\cdot}O_2^-$. Since asbestos is not degradable, it will continue to be ingested by other phagocytes, thereby promoting continuous production of $^{\cdot}O_2^-$ (Vallyathan and Shi, 1997; Nymark *et al.*, 2008).

Inhalation of coal mine dust has been implicated in the development of coal workers' pneumoconiosis (CWP), silicosis, chronic bronchitis and emphysema. When inhaled, coal mine dust leads to ROS production (Vallyathan and Shi, 1997; Armutcu *et al.*, 2007) and many studies have shown that coal contains carbon-centred free radicals which, in the presence of H_2O_2, may lead to the generation of ROS such as $^{\cdot}OH$ (Vallyathan and Shi, 1997).

Chromium (VI)-containing compounds lead to ROS formation through the reduction of Chromium (VI) by glutathione to Chromium (V), producing glutathione thiyl radicals in the process (Kelly *et al.*, 1998; Myers *et al.*, 2008). The glutathione thiyl radical may react with another glutathione molecule to generate O_2^- and a further glutathione thiyl radical, leading to the formation of H_2O_2 (Vallyathan and Shi, 1997).

Cigarette smoke is made up of several substances, including nicotine, tar and a gas phase that contains chemicals considered to play a major role in the development of diseases associated with cigarette smoke (Nicita-Mauro *et al.*, 2008). In the lungs, cigarette smoke can lead to the release of H_2O_2 which can in turn react with endogenous iron, producing $^{\cdot}OH$. Cigarette tar also contains iron which encourages formation of hydroxyl anions through the Fenton reaction (Kamp *et al.*, 1998; Jaimes *et al.*, 2004).

In addition, other exogenous sources of ROS include diet, stress and drugs. These lead to oxidative stress, resulting in the generation of ROS.

2.3.3 ROS-generated damage

The involvement of ROS in the pathogenesis of many human diseases such as chronic inflammation, and degenerative diseases such as cardiovascular diseases and cancer, is widely recognised (Aruoma *et al.*, 1999; Ozsoy *et al.*, 2008). Thus, in one mechanism, the excessive production of ROS leads to the disruption of membranes through lipid peroxidation. Lipid peroxidation involves three 3 steps as shown in Figure 2.2 below, namely, initiation, propagation and termination. Initiators of lipid peroxidation include

$O_2^{\bullet -}$ and H_2O_2 but more commonly, $^{\bullet}OH$. Initiation may occur through the removal of a hydrogen atom from a lipid molecule by ROS, thereby forming a lipid radical. Alternatively, metal ions can break down pre-existing lipid peroxides, generating radicals. Propagation involves reactions of molecular oxygen with lipid radicals producing lipid peroxyl radicals. In the absence of antioxidants, lipid peroxides abstract hydrogen atoms from neighbouring lipid molecules, producing more reactive carbon centred radicals, thus propagating the radical chain reaction. Termination results when two radicals react, producing a stable product or when an antioxidant is present which then quenches the free radicals (Boots *et al.*, 2008).

Initiation:

$$X^{\bullet} + LH \longrightarrow L^{\bullet} + XH \qquad (5)$$

Propagation:

$$L^{\bullet} + O_2 \longrightarrow LOO^{\bullet} \qquad (6)$$

$$LOO^{\bullet} + LH \longrightarrow L^{\bullet} + LOOH \qquad (7)$$

Termination:

$$LO^{\bullet} + LO^{\bullet} \longrightarrow \text{nonradical polymers} \qquad (8)$$

$$LOO^{\bullet} + LOO^{\bullet} \longrightarrow \text{nonradical polymers} \qquad (9)$$

$$L^{\bullet} + L^{\bullet} \longrightarrow \text{nonradical polymers} \qquad (10)$$

$$LOO^{\bullet} + L^{\bullet} \longrightarrow \text{nonradical polymers} \qquad (11)$$

Figure 2-2: An illustration of the three stages that occur during lipid peroxidation, namely, initiation, propagation and termination.

Lipid peroxidation leads to the disruption of membrane function. Membranes have a high content of polyunsaturated lipids which can be attacked by ROS leading to the formation of lipid radicals. This alters the chemical structure of the lipids, and this change in structure affects the proper functioning of the membrane (Buonocore and Groenendaal, 2007). Furthermore, lipid peroxidation products such as malondialdehyde can react with biomolecules and cause cytotoxic and genotoxic effects (Ardestani and

Yazdanaparast, 2007). Lipid peroxidation is also a major concern to the food industry, deteriorating colour, flavour, texture and nutritional value of food (Ferreira *et al.*, 2007; Qian *et al.*, 2008).

ROS also lead to DNA damage which is implicated in ageing and senescence (Pieczenik and Neustadt, 2007). The bases on the DNA strands are a site of attack by $^{\cdot}$OH. This is the only ROS which can react directly with DNA, but the attack of the $^{\cdot}$OH on DNA strands leads to hydroxylation, ring opening and fragmentation (Kelly *et al.*, 1998; Sallmyr *et al.*, 2008). The $^{\cdot}$OH radical also reacts with the sugar-phosphate backbone of the DNA strands resulting in apurinic sites where the base has been removed by oxidant-mediated reactions (Kelly *et al.*, 1998). However, the presence of antioxidants would be able to prevent DNA damage through delaying or inhibiting the propagation of free radicals. The effect of antioxidants on ROS through various mechanisms of action is described in detail in Section 2.5. Thus, investigations into the recovery of antioxidants from fruit processing wastewaters could confer significant health benefits.

2.4 The analysis of phenolic compounds

When evaluating a potential antioxidant for incorporation into food, as an example, several steps need to be considered and Figure 2.3, adopted from Becker *et al.*,(2004), illustrates these steps. The first step in the schematic diagram facilitates the identification and quantification of phenolic compounds. It is necessary to quantify phenolic compounds effectively at the same time as antioxidant capacities are measured in order to determine the contribution of phenolic compounds in the antioxidant activity of the sample (Becker *et al.*, 2004). Steps 2 and 3 involve the measurement of antioxidant activity and methods for achieving this. The final step depends on the intended use of the antioxidant, that is, whether it will be incorporated into food (making it is relevant to conduct storage condition studies) or in pharmaceuticals (where the effect on the human body is studied) (Becker *et al.*, 2004). These steps are discussed in detail in the following sections.

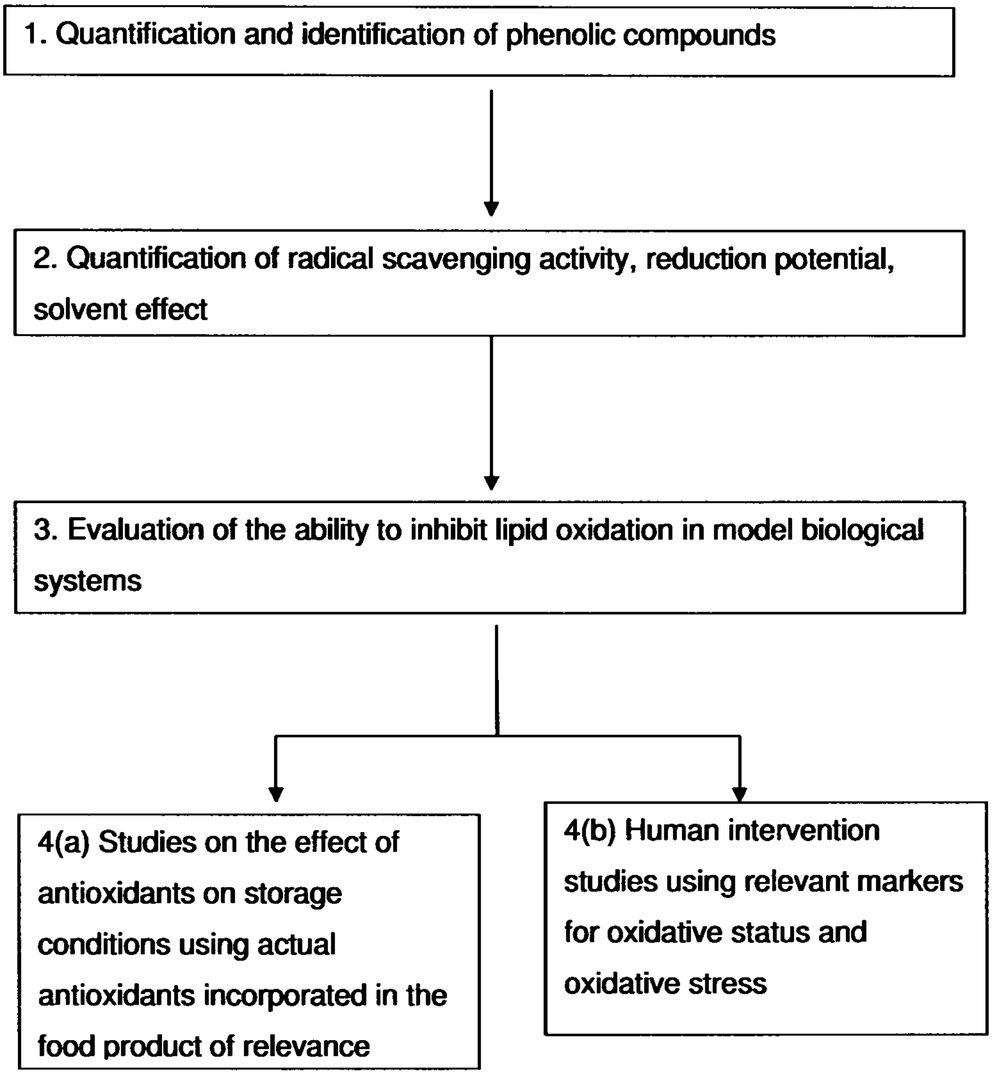


Figure 2-3: Schematic diagram showing the steps followed for investigating the potential application of antioxidants to foods (Becker *et al.*, 2004)

2.4.1 The Folin Ciocalteau assay for the determination of total phenolic concentrations

The quantification of total phenolics in fruits and their products after processing is achieved by the Folin Ciocalteau (Folin C) assay which measures the reduction of phosphomolybdic-phosphotungstic acid to a blue complex, which can be measured using a spectrophotometer at 725nm (Naczk and Shahidi, 2004). Gallic acid is usually used as a standard.

Although the Folin C assay is not selective for phenolic compounds, since the Folin C reagent may also be reduced by other reducing agents including ascorbic acid, (Becker *et al.*, 2004) it is widely used to quantify total phenolics in agricultural wastes, including fruit processing wastes. For example, using the Folin C assay, total phenolics quantified in apples (Hagen *et al.*, 2007) and apple wastes (McCann *et al.*, 2007), were measured to be 838 mg/100g fresh weight and 690 mg/100g fresh apple wastes respectively. The concentration of total phenolics in citrus wastewaters were measured to be 3.9 mg/L and 63 mg/L for different wastewater samples (Burton *et al.*, 2006) and 270 mg/L for olive mill wastewaters (De Marco *et al.*, 2007) respectively.

2.4.2 The use of chromatographic techniques to quantify phenolic compounds

The quantification of phenolic compounds in fruit processing wastewaters can be achieved by high performance liquid chromatography (HPLC) which has the advantage of separating and also allowing the identification of components (Naczk and Shahidi, 2004). The use of HPLC was important in the present study because it can facilitate the identification of individual phenolic compounds present in samples such as the fruit processing wastewater samples. HPLC can also quantify the concentration of individual phenolic compounds through the use of standard phenolic compounds that are commercially available. There are various types of HPLC, namely, normal phase, reverse phase, size exclusion, ion exchange and bioaffinity chromatography (Naczk and Shahidi, 2004). However, reverse phase HPLC (RP-HPLC) is most commonly used and is relevant to the work in this project.

A RP-HPLC system consists of a non-polar stationary phase (column), a moderately polar mobile phase, a pump, detector and recorder. Some of the commonly used columns are C18 and C8 columns which are made of silica bonded to straight chain alkyl groups. The separation of phenolic compounds in RP-HPLC depends on the principle of hydrophobic interactions between the stationary phase and the phenolic compound resulting in partitioning of the desired phenolic compound (Michalkiewicz *et al.*, 2008). The retention times of the desired solutes depend on their polarity, and thus, non-polar solutes have longer retention times, with polar solutes eluting more readily. The most useful way to maintain a reasonable retention factor range and to achieve good selectivity for the resulting separation is to vary the pH and the concentration of an organic modifier in the mobile phase. The nature of the compounds being separated in this study, particularly phenolic acids, required the use of an acidic mobile phase for satisfactory separation and peak shapes. Formic acid, acetic acid or phosphate buffers are commonly used in the separation of phenolic acids (Michalkiewicz *et al.*, 2008).

As a relevant example of the application of this method, the phenolic content of apple wastes was analysed using RP-HPLC and various phenolic compounds, including catechin, epicatechin and quercetin were identified (Mcann *et al.*, 2007). RP-HPLC was also used to identify phenolic compounds in olive mill wastewaters by comparing the retention times with those of standard solutions in a study conducted by DeMarco *et al.*, (2007). The chromatograms clearly showed several peaks corresponding to different phenolic compounds. Similarly, two hydroxybenzoic acids and five hydroxycinnamic acids were identified in orange juices in a study conducted by Kelebek *et al.*, (2008).

Other quantification techniques of note are the Vanillin-HCl assay used to measure proanthocyanidins and the pH differential method used to quantify anthocyanins (Namakura *et al.* 2003; Rossi *et al.*, 1997).

2.5 The analysis of antioxidant activity of phenolic compounds

The development and validation of analytical methods for the assessment of antioxidant capacity in food products has recently been an area of interest in research. These methods are important to nutritional researchers and the food and medical industries, since active dietary constituents, including phenolic compounds, are capable of preventing free radical induced reactions. However, the methods currently used to determine antioxidant capacity differ from each other in terms of mechanisms, substrates, free radicals involved and reaction conditions, and in the form in which results are expressed (Becker *et al.*, 2004).

The evaluation of antioxidant activity is usually achieved using a range of assays in order to determine whether the antioxidant is a good electron donor, metal chelator or an inhibitor of lipid peroxidation (Becker *et al.*, 2004). Each assay relates to the generation of a different radical, acting through a variety of mechanisms, and the different end products are measured either at a fixed time point or over a range of time. Generally, two types of approach have been taken, namely; the inhibition assays, which involve determining the extent of the scavenging by hydrogen- or electron- donation of a preformed radical as the marker of antioxidant activity. Another approach is assays involving the presence of antioxidants in the system during the generation of the free radical (Re *et al.*, 1999).

Antioxidant capacity assays may also be broadly classified as hydrogen atom transfer (HAT) and electron transfer (ET) based assays. HAT based assays measure the capability of an antioxidant to quench free radicals by hydrogen atom donation (Erkan *et al.*, 2008). The HAT mechanism of antioxidant action, in which the hydrogen atom of a phenol is transferred to a reactive oxygen species (denoted by $ROO^{\cdot}$), generated in various ways as described in Section 2.3 can be summarised by the reaction below. The AH species denotes the biological molecule, for example, lipid or protein, that is being protected from attack by reactive oxygen species ($ROO^{\cdot}$), whereas the ArOH species denotes the phenolic antioxidant conferring protection to the biological molecule against attack by ROS.

$$ROO^{\bullet} + AH/ArOH \longrightarrow ROOH + A^{\bullet} / ArO^{\bullet} \qquad (12)$$

The resulting aryloxyl radical ($ArO^{\cdot}$) formed from the reaction of an antioxidant phenol with a peroxyl radical ($ROO^{\cdot}$) is stabilised by resonance, thereby terminating the reactions that lead to the generation of more reactive oxygen species. The phenolic antioxidants need to react faster with free radicals than the rate at which the biological molecules react with the free radicals in order for the biological molecules to be protected from oxidation. Generally, HAT reactions are relatively independent of solvent and pH effects, and are completed in a short period of time (Apak *et al.*, 2007).The DPPH radical scavenging assay is a good example of a HAT based assay.

The ET mechanism of antioxidant action is based on the reactions:

$$ROO^{\bullet} + AH/ArOH \longrightarrow ROO^{-} + AH^{+}/ArOH^{+} \qquad (13)$$

$$AH^{+} + ArOH^{+} + H_2O \longleftrightarrow A^{\bullet} /ArO^{\bullet} + H_30^{+} \qquad (14)$$

$$ROO^{-} + H_30^{+} \longleftrightarrow ROOH + H_20 \qquad (15)$$

The aryloxy radical ($ArO^{\bullet}$) is subsequently oxidised to the corresponding quinone (Ar=O) and the more stabilised the aryloxyl radical is, the easier will be the oxidation from ArOH to Ar=O due to redox potential (Apak *et al.*, 2007). The ET reactions are relatively slow, compared with those of HAT-based assays and they are solvent and pH dependent.

Spectrophotometric ET based assays measure the capacity of an antioxidant in the reduction of an oxidant which changes colour when reduced. The degree of colour change (either an increase or decrease of absorbance at a given wavelength) is correlated to the concentration of antioxidant in the sample. Antioxidant assays based on the ET mechanism include the $ABTS^{+}$ and ferric reducing power (FRAP) assays (Apak *et al.*, 2007). These assays are discussed in more detail in the following sections.

2.5.1 Assays used for the quantification of radical scavenging activity and reducing power

Radical scavenging assays primarily operate by direct measurement of hydrogen atom donation (HAT) to, or electron transfer (ET) from, the potential antioxidant to free radical molecules present in simple systems (Becker *et al.*, 2004). Such assays are often available in commercial kits and are widely used. The methods are discussed below.

2.5.1.1 The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay gives an indication of the hydrogen donating ability of an antioxidant (Kubola and Siriamornpun, 2008). The ability of an antioxidant to scavenge the DPPH radical can be monitored spectrophotometrically at 515nm, and the decrease of colour intensity at 515nm, from deep purple to brown, is a quantitative indication of the quenching of the radical (Devi and Arumughan, 2007). The antioxidant activity of samples measured using the DPPH assay can be expressed in various ways; the EC_{50} value expresses the amount of antioxidant needed to decrease the radical concentration by 50% (Kelebek *et al.*, 2008). The EC_{50} value is quantified by measuring a known antioxidant at different concentrations, and calculating the antioxidant concentration required to reduce the initial concentration of DPPH by 50% at steady state. A lower EC_{50} value is associated with a stronger radical scavenging activity under the same experimental conditions (Yu, 2007). The percentage radical scavenging activity (%RSA) can be used as an alternative way of expressing the extent of quenching of the DPPH free radical by antioxidants in the sample. It is calculated by obtaining the difference in absorbance of the reaction mixture from time t= 0 to the time the reaction is stopped or when the reaction reaches steady state, then expressing this as a percentage.

The DPPH radical scavenging assay is an easy assay to perform and it allows the comparison of powerful antioxidants, such as BHT and gallic acid, with antioxidants present in the studied samples (Kelebek *et al.*, 2008). In addition, the DPPH assay has the advantage of not being affected by certain side reactions of phenolic compounds, such as metal ion chelation and enzyme inhibition (de Oliveira *et al.*, 2009). However, the DPPH assay is not representative of the *in vivo* situation because the synthetic radical used is not found in any biological system (Devi and Arumughan, 2007).

In applications relevant to antioxidant activity in plant extracts and wastes, the DPPH radical scavenging assay was used to measure the antioxidant activity of Moro and Sanguinello orange juices; here the EC_{50} values obtained for the two orange varieties were 0.18 and 0.29 mg/L respectively, and the antioxidant radical scavenging activity in pomegranate juices ranged from 10 to 67% (Teczan *et al.*, 2009). Similarly, extracts from wastes from the processing of pineapple and passion fruit exhibited antioxidant activities ranging from 10 to 100%, measured using the DPPH assay (de Oliveira *et al.*, 2009). The antioxidant capacity of 6 industrial dark fruit juices was also evaluated using the DPPH assay, and here, blackcurrant and cranberry juices exhibited the highest activity using the DPPH assay (Piljac-Zegarac *et al.*, 2009).

2.5.1.2 The 2,2'-azonobis (3-ethybenzothiazoline-6-sulphonate) ($ABTS^+$) assay

The 2,2'-azonobis (3-ethybenzothiazoline-6-sulphonate) (ABTS) assay, also referred to as the trolox equivalent antioxidant capacity (TEAC) assay, is a very common method used in analytical facilities and in industry, to determine the antioxidant activity of phenolic compounds. The assay measures the electron transfer ability of both lipid soluble and water soluble antioxidants using the 2,2'-azonobis (3-ethybenzothiazoline-6-sulphonate) ($ABTS^+$) anion, with a colour change being an indication of the reduction of ABTS (Erkan *et al.*, 2008). Trolox, a commercially available water soluble derivative of Vitamin E, is used as the standard with which the antioxidants can be compared (Magalhaes *et al.*, 2007).

The main advantages of the ABTS$^+$ assay are that it is inexpensive, rapid, and easy to apply. The reaction is also very consistent with variations in pH, and therefore can be used to study pH effects on antioxidant activity (Zulueta *et al.*, 2009). The major disadvantages include the extra step required to generate the free radical from an ABTS salt, and the free radical generated is not stable for long periods of time. In addition, the method is not readily standardised, making comparison of results from other laboratories difficult (Zulueta *et al.*, 2009).

The ABTS$^+$ assay has been used to measure the antioxidant activity of various food products, for example, orange juice and milk beverages was determined using the ABTS$^+$ assay, giving values of 9648 and 3028 trolox equivalents respectively (Zulueta *et al.*, 2009). In a broad study conducted on the total antioxidant capacity of vegetables, fruits and beverages, citrus fruits were found to exhibit intermediate antioxidant capacity, with oranges being the most effective, followed by grapefruit, with respect to the ABTS$^+$ assay (Pellegrini *et al.*, 2003). These results obtained on the antioxidant activities of fruits and vegetables using the ABTS$^+$ assay show the potential applicability of using this assay to determine the electron donating ability of the fruit processing wastewaters in the present study.

2.5.1.3 The ferric reducing power (FRAP) assay

The ferric reducing power assay measures the ability of an antioxidant to reduce the ferric tripyridyltriazine (Fe^{3+}-TPTZ) ion to the ferrous form (Benzie and Strain, 1996; Katalinic *et al.*, 2005). The colour change, from yellow to various shades of green and blue, is measured at 700nm, giving an indication of the reducing power of the antioxidant (Ferreira *et al.*, 2007; Kubola and Siriamornpun, 2008). There is no standard way of expressing the reducing power of a sample, but the most commonly used way of expressing reducing power is to measure the reducing power of different concentrations of a known antioxidant, such as gallic acid, and expressing results as antioxidant equivalents.

The main advantage of the FRAP assay lies in its relative simplicity which allows many samples to be analysed simultaneously. The FRAP assay is fast, and reproducible results can be obtained with minimal sample preparation (Shetty *et al.*, 2006). However, the reaction is not specific as any compound with a lower redox potential than Fe^{3+} and Fe^{2+} (0.77V) can reduce the complex, thereby contributing to misleadingly high FRAP values. The reaction also assumes that completion of the reduction is spontaneous. However, many biological antioxidants, especially phenolic compounds, continue to reduce the ferric complex even after completion of the reaction time (Shetty *et al.*, 2006).

The FRAP assay has been applied in measuring the antioxidant activity of various fruit products. For example, the FRAP assay was used to determine the reducing power of a new citrus cultivar and its main flavonoids, and results indicated that all samples exhibited reducing power to some degree (Yi *et al.*, 2008). Nectars of some fruits, including orange, sour cherry, peach and apricot were analysed for total antioxidant activity using the FRAP assay; results were expressed as moles of antioxidant/ml of nectars and average FRAP values were 6.54 for orange nectars, 8.01 for sour cherry nectars, 5.68 for apricot nectars and 5.19 for peach nectars (Tosun and Ustun, 2003).

The ability to analyse many samples simultaneously with minimal sample preparation makes the FRAP assay relevant for determining the reducing power of fruit processing wastewaters as they would only need to be centrifuged to remove insoluble particles, a procedure that is relatively quick and simple to perform. Furthermore, because the wastewater samples contain mixtures of antioxidants with different antioxidant characteristics, this assay will enable the identification of wastewaters with strong reducing power.

2.5.2 Assay of antioxidant activity based on the ability to inhibit or halt lipid oxidation in model systems

In contrast to radical scavenging assays described in the previous sections, lipid peroxidation assays are based on model systems involving oxidation substrates which are found in the human body, namely, lipids. The assays described below are to some extent representative of *in vivo* reactions, and thus may give a better indication of the potential action of the antioxidants in the human body.

2.5.2.1 The β-carotene linoleic acid model system

The ability of antioxidants to prevent bleaching of β-carotene by lipid peroxides is measured in the β-carotene linoleic acid model system (β-CLAMS) (Liu *et al.*, 2007b). Linoleic acid, present in the reaction mixture, undergoes auto-oxidation caused by the exposure of linoleic acid to air at high temperatures, leading to the formation of radicals such as lipid peroxides. Due to their high reactivity, lipid peroxides tend to react with highly unsaturated β-carotene (also present in the reaction mixture), leading to its discoloration. However, in the presence of an antioxidant, the lipid peroxides are scavenged by the antioxidant, thus preventing β- carotene bleaching. The action of the antioxidant in preventing the bleaching of β-carotene is monitored at 470nm using a spectrophotometer (Liu *et al.*, 2007b; Kubola and Siriamornpun, 2008). The results obtained in the β-CLAMS assay are expressed as % inhibition, whereby the total change in absorbance over a specified period of time is expressed as a ratio of the initial absorbance of the mixture before the start of the experiment with the absorbance of the mixture at the end of the reaction (Wang *et al.*, 2006).

The β-CLAMS assay has been used to measure the antioxidant activity of phenolic compounds in various products, such as apple pomace. Amongst the phenolic compounds present in the apple extracts, procyanidins and quercetin glycosides showed the highest antioxidant activity (Lu and Foo, 2000). The antioxidant activity of processed and unprocessed seed extracts, as determined by bleaching of β-carotene ranged from 5 to 85% in cowpea extracts in a study conducted by Sidhhuraju and Becker, (2007). Thus, the β-CLAMS assay will be relevant in determining the potential of using the antioxidants present in the fruit processing wastewaters to inhibit lipid peroxidation, for example in foods that contain fat.

2.5.2.2 The thiobarbituric acid reactive substances (TBARS) assay

The thiobarbituric acid reactive substances (TBARS) assay is a simple assay for measuring lipid oxidation. The TBARS assay uses spectrophotometric measurements at 532nm, of the pink pigment produced through the reaction of thiobarbituric acid and malondialdehyde (MDA), a secondary product of lipid oxidation. In the experiment, lipid oxidation is initiated by the addition of copper sulphate to the lipid source, leading to the formation of MDA (Bounatirou *et al.*, 2007; Lykkesfeldt, 2007). MDA forms a 1:2 adduct with thiobarbituric acid, as shown in Figure 2.4 below.

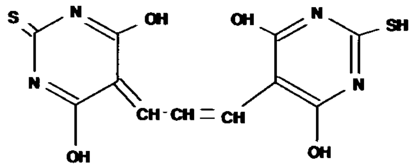


Figure 2-4: MDA-thiobarbituric acid adduct

The TBARS assay is simple and easy to perform but suffers from a lack of sensitivity because many compounds, especially the products of side reactions of lipid oxidation, also have absorption maxima in the 532-535nm range (Lykkesfeldt, 2007). Other products of lipid peroxidation, such as hydroperoxides and conjugated aldehydes, can also react with TBA, and hence may lower the sensitivity of the method. Reducing sugars and aldehydes that have a carbonyl group can also react with TBA to give misleading high readings (Shetty *et al.*, 2006).

In a study conducted on the antioxidant activity of processed cowpea seed extracts, all extracts were reported to exhibit high antioxidant activity (74.3-84.6%) using the TBARS assay (Siddhuraju and Becker, 2007). The TBARS assay was also used to measure the antioxidant activity of vegetable and fruit wastes, including apples, and % inhibition of apple juices ranged from 76.72 to 82.53% (Peschel *et al.*, 2006).

2.5.3 Summary of antioxidant activity assays

The antioxidant activity assays described above provide information on the nature of the antioxidant present in a sample. Antioxidants act in various ways, binding metal ions, scavenging radicals and decomposing peroxides (Laguerre *et al.*, 2007). Often, more than one mechanism is involved, and in a sample such as fruit processing wastewaters where more than one type of antioxidant is present, it is necessary to be able to measure and determine the characteristics of all antioxidants present. Antioxidants can be primary (chain breaking antioxidants) or secondary antioxidants, which reduce the rate of chain initiation (Moure *et al.*, 2001). In samples containing a mixture of antioxidants such as fruit processing wastewaters, they are likely to contain both primary and secondary antioxidants. Therefore, the antioxidant activity must be evaluated with different tests in order to understand the different mechanisms (Moure *et al.*, 2001). The DPPH and $ABTS^+$ assays will be relevant in the present study as they will provide information on the hydrogen- and electron donating ability of the wastewater compounds respectively. Also, the FRAP and β-CLAMS assays are important in providing information on the reducing power and the ability to inhibit lipid oxidation by antioxidants present in the wastewaters. Thus, the antioxidant activity assays described above are useful in order to gain a better understanding of the potential of the fruit processing wastewaters as a source for antioxidants.

Very little information has been reported in literature on the antioxidant activity and extraction of phenolic antioxidants from fruit processing wastewaters. Therefore, this present study presents an opportunity for research into the extraction and potential use of phenolic antioxidants in various industries, for example, the food industry.

2.6 The extraction of phenolic compounds

The extraction of phenolic compounds is the first step towards their recovery from their source, in order for them to be used as antioxidants in industries such as the food industry. The factors affecting the extraction effectiveness include the technique used for extraction, the chemical nature of the phenolic compounds, (that is, whether they are simple or complex phenolics), storage time and conditions, and the presence of interfering substances (Chirinos *et al.*, 2007).

Very little information has been published in literature on the extraction of phenolic compounds from fruit processing wastewaters, and Table 2.2 shows that there is limited information on their recovery. However, information is available on the extraction of phenolic compounds from fruits and their pomace, and some examples are shown in Table 2.2.

Table 2.2: A summary of the information available on the extraction of phenolic compounds from fruits and their by-products after processing

Source	**Concentration of total phenolics (mg/L)**	**Chemical characterisation**	**Antioxidant activity**	**Reference**
Citrus wastewaters	3.9 mg/L and 63mg/L	pH = 3.8 and 11.4 conductivity = 0.67 and 3.71 COD= 5306mg/L and 3180mg/L Reducing sugars =9.9 mg/L and 6.1 mg/L	DPPH scavenged ranged from 0.01-0.11 mg/L	Burton *et al.*, (2006)
Orange juices	43.7-291.3 mg/L	pH=3.05 to 3.35 Total sugars=103.7 to 109.5mg/L	Not reported	Kelebek *et al.*, (2006)
Apple fruit	190-838 mg/L	Soluble solids= 9.8 to 10.9 mg/L	1.9 to 91. trolox equavalents, ORAC assay	Hagen *et al.*, (2007)
Olive mill wastewaters	1150-2702 mg/L	Not reported	3.43-55.8 trolox equivalents	De Marco *et al.*, (2007)
Apple pomace	Apple pomace=143-502 mg/kg	Not reported	0.425-2.573 mgDPPH/g apple pomace	Adil *et al.*, (2007)
Peach pomace	Peach pomace=71-250 mg/kg	Not reported	0.276-1.335 mgDPPH/g peach pomace	Kelebek *et al.*, (2006)
Orange pulp	Not reported	pH-3.4	Not reported	

wash		Total soluble solids=9.4 g/L Total sugars=74.5 g/L		

**The antioxidant activity assays mentioned in Table 2.2 are discussed in detail in Section 2.6*

The extraction of phenolic compounds from fruit processing wastewaters can potentially be achieved using various extraction techniques, including solvent extraction, solid phase extraction, supported liquid membrane extraction and supercritical fluid extraction (Herrera and Luque de Castro, 2005; Beyer and Bizuik, 2008). Each of these techniques is discussed in more detail in the following sections.

2.6.1 Solvent Extraction

Solvent extraction is the most widely used technique for the extraction of phenolic compounds from biological materials, including fruit processing wastewaters (Chimuka *et al.*, 2004). Solvents such as methanol and acetone are used in their pure form, whilst others such as ethyl acetate may be used as mixtures of solvent and water, to facilitate the recovery of a wide range of phenolic compounds of diverse structure from solid wastes. Solvents that are not miscible with water, such as ethyl acetate and hexane can be used to extract phenolic compounds from aqueous samples such as wastewaters (Abad-Garcia *et al.*, 2007). The principle behind this technique is the partitioning of the phenolic compounds between the organic phase (extraction medium) and the aqueous phase (sample solution) according to their partition coefficients (Chimuka *et al.*, 2004). The recovery of phenolic compounds depends on various factors, including the type of solvent used, pH, temperature and the nature of the phenolic compounds to be extracted from the fruit processing wastewaters.

In examples of solvent extraction reported in literature, various solvents (petroleum ether, ethyl acetate and butanol) were used to extract phenolic compounds from fermented fruit juices of *Morinda citrifolia,* and the antioxidant effects of the extracted phenolic compounds on various free radicals was determined. In this study, all the solvents successfully extracted phenolic compounds (Chang-hong *et al.*, 2007). Phenolic compounds were also extracted from olive-oil mill wastewater using ethyl acetate and the extraction yield was 50 mg of total phenolics from 100 g of wastewater (De Leonardis *et al.*, 2007).

Therefore, these results show that it is possible to use organic solvents to extract phenolic compounds from fruits or the wastewaters arising during fruit processing.

The advantages of solvent extraction are that the technique is relatively inexpensive and it is simple to operate. However, solvent extraction requires large volumes of solvent, and thus, the disposal of the organic solvents may pose a threat to the environment. Furthermore, solvent extraction is time consuming, requiring large quantities of energy, and the choice of solvents is limited due to health concerns, specifically if the antioxidants are to be incorporated into foods (Adil *et al.*, 2007).

2.6.2 Supercritical fluid extraction of phenolic compounds

Among recent developments in extraction techniques, supercritical fluid extraction has been a focus of interest and is a potential choice for the isolation of valuable constituents such as phenolic compounds from biological material (Daintree *et al.*, 2008). A supercritical fluid is formed when a fluid is forced to a pressure and temperature above its critical point as illustrated in Figure 2.5 below. The critical temperature of a material is the temperature above which distinct liquid and gas phases do not exist and the critical pressure is the vapour pressure at the critical temperature. The point at which both critical temperature and critical pressure are reached is called the critical point (Herrero *et al.*, 2006).

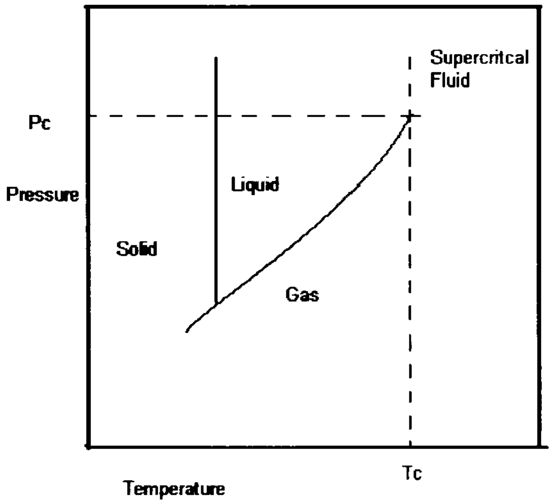


Figure 2-5: Typical phase diagram for a pure compound (Herrero *et al.*, 2006)

Supercritical fluids have better mass transfer properties than conventional liquid solvents because of their low viscosity and relatively high diffusivity, which lead to better extraction yields. The solvating power of a supercritical fluid is very dependent on its density, and the density of a supercritical fluid can be modified by altering the temperature or pressure. Thus, it is possible to extract specific phenolic compounds of interest by adjusting the values of temperature and pressure to favour the extraction of the specific compound (Daintree *et al.*, 2008). For example, by varying the temperature and pressure of the extraction system, maximum yields for the extraction of lycopene from tomato skins were observed (Yi *et al.*, 2009).

Carbon dioxide is preferred as a supercritical fluid for use in the extraction of phenolic compounds because of its favourable critical temperature and pressure of 31.1°C and 73.8 bars respectively, which are relatively low and easy to achieve. Other advantages of using supercritical CO_2 as a solvent for extracting phenolic compounds are that it is relatively cheap and readily available in large quantities with high purity, it is non-toxic, non-corrosive and non-flammable. This makes it the solvent of choice for the extraction of natural products (Shi *et al.*, 2005). Investigations into finding alternative solvents to CO_2 have been conducted. However, most of them had several disadvantages. For example, water has high critical temperature and pressure, and hence, its use in the extraction of valuable phenolic compounds that are heat sensitive could potentially damage the compounds of interest. Nitrous oxide, ethers and hydrocarbons have high flammability, and ammonia is very corrosive, making them hazardous to use in extraction processes (Mendiola *et al.*, 2007).

2.6.3.1 The use of co-solvents during supercritical fluid extraction

A problem may arise when supercritical CO_2 is used to extract very polar compounds such as phenolic and glycosidic compounds from natural matrices; these compounds are often not extractable using CO_2 supercritical fluid because of their poor solubility. However, their solubility can be enhanced through the addition of other compounds, known as co-solvents or modifiers (Herrero *et al.*, 2006). The modifiers are highly polar compounds which, when present in small amounts, can alter the solvating power of the supercritical fluid. Co-solvents include alcohols such as ethanol, methanol, isopropanol, acetonitrile and dichloromethane, and they act by increasing the density of the solute or through chemical interactions such as acid-base interactions, with the solute. The choice of a co-solvent for a particular solute is usually determined through trial and error, although a more theoretical approach can be used based on liquid-liquid solvent extraction studies (Daintree *et al.*, 2008).

2.6.3.2 The principles and instrumentation for a supercritical fluid extraction (SFE) system

The design of a supercritical fluid extraction (SFE) system can be relatively simple, or highly complex, depending on the specific requirements, and SFE can be performed at analytical or pilot/industrial scale. The difference is in the quantities of the analyte produced, with analytical scale operation yielding milligrams to grams, whereas preparative scale operations can produce kilograms of extract. In preparative systems, two different configurations are used for the processing of solid or liquid samples. Solid processing is always conducted in a batch or semi-continuous process while liquid processing is usually carried out under counter current conditions in a continuous mode (Daintree *et al.*, 2008). Figure 2.6 hows an example of a preparative SFE system on a pilot scale plant. The system consists of a solvent pump that delivers the supercritical fluid throughout the whole system, a modifier pump (if necessary) for the delivery of a co-solvent, an extraction cell or column, depending with the system configuration, and one or more separators for the collection of the extract and for the recovery of the solvent. The separators have independent temperature and pressure controls to allow for the fractionation of the extracted compounds through stepwise depressurisation (Herrero *et al.*, 2006).

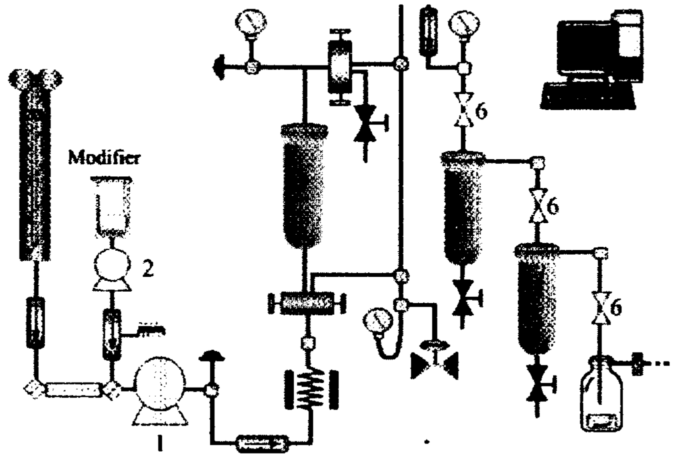


Figure 2-6: Diagram of SFE pilot plant equipped with 2 fractionation cells. (1) CO_2 pump; (2) modifier pump; (3) solid samples extraction cell; (4) fractionation cell 1; (5) fractionation cell 2; (6) valve (Herrero *et al.*, 2006).

2.6.3.3 The extraction of antioxidants using supercritical fluid extraction (SFE)

The need for natural antioxidants has led to research into the use of SFE for the extraction of antioxidants because of its environmentally friendly approach that uses CO_2 for extraction. The extracts obtained by SFE from various plants have shown to exhibit higher antioxidant activities compared with the extracts obtained using other extraction techniques. For example, Vitamin E (tocopherol) is a lipid soluble antioxidant produced only by photosynthetic organisms. The extraction of Vitamin E from *Spirulina platensis* yielded 12 times the concentration of Vitamin E extracted using other extraction techniques (Mendiola *et al.*, 2008). Similarly, Cavero *et al.*, (2006) identified 9 flavonoid antioxidants in the herb oregano using SFE, and natural pigments and antioxidants were extracted effectively using SFE with subcritical propane (Hamdan *et al.*, 2008). Polyphenols from grape fruit skin such as catechin, epicatechin and gallic acid have been extracted successfully with SFE (Palma and Taylor, 1999).

The efficiency of extraction of phenolics, and the antioxidant efficiencies of the extracts from apple and peach pomaces was higher after supercritical fluid extraction than with conventional methods (Adil *et al.*, 2007). Similarly, the antioxidant activity of rosemary extracts obtained by SFE increased with an increase in temperature and pressure of the supercritical fluid (Carvalho *et al.*, 2005). In this latter study, different extraction techniques, namely, microwave assisted extraction; soxhlet extraction and SFE, were compared for their extraction efficiencies. Although one step microwave-assisted extraction gave the most concentrated extract, followed by one step SFE, two step SFE was concluded to be the preferred method based on environmental considerations.

2.6.3 Supported Liquid Membrane (SLM) Extraction

An alternative extraction technique, based on the combination of solvent extraction and membrane separation is supported liquid membrane (SLM) extraction. In SLM extraction, a donor aqueous phase is separated from an acceptor organic phase by a porous membrane impregnated with an organic solvent. There are various liquid membrane types, including bulk, emulsion and thin sheet supported liquid membranes. However, a hollow fibre supported liquid membrane was used in the present study and will thus be discussed in detail in this section (Chimuka *et al.*, 2004). The hollow fibre supported liquid membrane (HFSLM) consists of an outer shell, which is a single layer of non-porous material, and a lumen. Inside the shell, there are thin fibres that span the length of the shell. The wastewater sample moves through the system in the lumen, and the pores in the fibre of the outer shell are filled with the organic phase (Garcin, 2005). The extraction of the desired solute is dependent on its concentration gradient, causing it to move from a region of higher concentration in the donor phase, through the organic membrane, to a region of lower concentration in the acceptor phase (Chimuka *et al.*, 2007).

Advantages of SLM include the high degree of selectivity, quick and straightforward clean-up, and the use of relatively small quantities of solvent (Chimuka *et al.*, 2007). The membrane surface area and thickness provide rapid transportation of phenolic compounds. Leakages and contamination are easily contained. Also, the donor and acceptor phases are more easily recoverable when compared with traditional liquid-liquid extraction. However, hollow fibre systems must be cleaned between uses in order to avoid aqueous and contaminant build up. Furthermore, membrane fouling often occurs due to surface effects and the presence of particles in the system. In addition, SLM is associated with high capital costs (Garcin, 2005).

2.6.4 Solid phase extraction (SPE)

Solid phase extraction (SPE) has become an established technique in the concentration of desired solutes, such as phenolic compounds. Non-polar, moderately polar and polar analytes present in samples can be extracted by SPE using different sorbents (Niu *et al.*, 2007). SPE uses a stationary phase often packed in a column, and a liquid phase, to isolate the desired solute from a solution. The solution containing the desired solute is loaded onto the column; unwanted components are washed away by one solvent, followed by addition of a different solvent which then dissolves (elutes) the desired solute off the stationary phase.

SPE is commonly used to purify a sample before quantification of the desired solute by chromatography or other analytical methods. Selection of a suitable eluting solvent can provide clean samples in the SPE process and, therefore, good selectivity. For the complete elution of all analytes, the composition of the eluting solvent and washing solvents needs to be established so as to elute as much of the desired solute as possible, thereby reducing losses during recovery (Michalkiewicz *et al.*, 2008).

2.6.5 Summary of the techniques that can be used to extract phenolic compounds

The extraction of phenolic compounds from fruit processing wastewaters could be achieved through various techniques such as solvent extraction, solid phase extraction, supported liquid membrane extraction and supercritical fluid extraction. These techniques have been discussed in detail in the sections above. Each extraction technique has its own potential advantages and disadvantages when considered for extracting phenolic compounds from fruit processing wastewaters. For instance, solvent extraction using ethyl acetate would result in the extraction of mainly polar phenolic compounds, whereas solid phase extraction, supported liquid membrane extraction and supercritical fluid extraction could all achieve a high degree of selectivity. All these techniques are likely to be suitable for the extraction of phenolic compounds in diluted samples. Thus, for samples such as fruit processing wastewaters, containing a dilute mixture of phenolic compounds, it would be of benefit to compare the quantities and composition of the extracts obtained after using various extraction techniques. This will then lead to the selection of the most suitable extraction technique to use for the recovery of phenolic antioxidants at large scale. The composition of the phenolic compounds present in an extract obtained using a specific extraction technique can be achieved through the analysis of phenolic compounds using either spectrophotometric assays or chromatographic techniques, or both, as discussed in Section 2.4.

2.7 Research aims and objectives

The main objective of this project was to investigate methods which could be used to extract phenolic compounds present in the wastewaters generated in the processing of citrus and deciduous fruits, and to determine the activity of these phenolic compounds as natural antioxidants, with a view to determining which might be valuable in the food, cosmetic, or pharmaceutical industry. Thus, the specific objectives were:

1. Detailed characterisation of wastewaters obtained from the local fruit processing industries to provide information on their typical composition, and to compare with information in the literature.
2. An investigation into the extraction of phenolic compounds using different extraction methods (solvent extraction, SPE, SLM and SFE) to evaluate each technique in terms of maximal phenolics extraction and suitability for a potential commercial process.
3. The analysis of the antioxidant activity of the phenolic extracts obtained to assess the feasibility of obtaining antioxidants from the wastewaters.

2.7.1 Problem statement

Wastewaters from the processing of citrus and deciduous fruits may cause pollution to the environment due to relatively high organic loads that result from the presence of phenolic compounds and other organic compounds. However, potentially useful products such as phenolic compounds can be recovered from these fruit processing wastewaters. The phenolic compounds might potentially be used in the food, cosmetic or pharmaceutical industries as antioxidant additives, thereby replacing synthetic antioxidants that are thought to have negative effects on the human body. Thus, a method is needed that will extract useful compounds such as phenolic compounds, thereby reducing the organic load of the wastewaters and isolating the phenolic compounds as extracts. In addition, the antioxidant activities of extracted phenolic compounds need to be investigated by different assays so that the nature of the antioxidants present in the fruit processing wastewaters can be established.

The following hypotheses were formulated, based on the problem statement above:

2.7.2 Hypotheses

1. The wastewaters from the fruit processing industries contain phenolic compounds at concentrations which allow the extraction of useful antioxidants to be economically feasible at commercial scale.
2. Phenolic compounds in fruit processing wastewaters can be extracted using appropriate technologies such as solvent extraction, solid phase extraction, supercritical fluid extraction or supported liquid membrane extraction, which allow for the recovery of concentrated extracts of phenolic compounds that can either be used as an additive in various industries`or consumed as a dietary supplement.
3. The extracted phenolic compounds exhibit antioxidant activity that can be useful in the quenching of free radicals or the inhibition of lipid peroxidation in biological systems.

CHAPTER 3: MATERIALS AND METHODS

Introduction

Chapter 3 describes the detailed experimental methodologies and materials used in this study. The experimental approach included chemical characterisation of the fruit processing wastewaters, determination of the antioxidant activity of the fruit processing wastewaters using various assays and the extraction of phenolic compounds using various extraction techniques.

3.1 Information on the fruit processing wastewaters

Wastewaters from the processing of apples were collected from two different fruit processing manufacturers in the Western Cape region of South Africa and are named AW1 and AW2. AW1 wastewaters were collected a few days after the start of the processing season and this wastewater was collected at a point where primary treatment had been done. AW2 wastewaters were a mixture of the cleaning water and residual liquid after pressing of the apples. Citrus wastewaters were collected from two fruit processing manufactures in the Western Cape region of South Africa and are named CW1 and CW2. The silage water (SW), a richer waste than the wastewaters, is seepage collected from the pomace of deciduous fruits, which was a kind donation from Dr Johann Gorgens, Stellenbosch University.

3.2 Storage of the fruit processing wastewaters and general procedures

For long term storage, wastewater samples were kept at -20°C, and for short term storage, at 4°C. Reagents and standard compounds were all of analytical or HPLC grade as required. Distilled and de-ionised reagent water (ddH_2O) was obtained from a Millipore Elix 3 purification system. All analyses were performed at room temperature unless otherwise stated. Analytical determinations were performed in triplicate where possible, with the results presented as the mean, including the standard error of the mean

(SEM). Common analyses were performed according to American Public Health Association's (APHA) Standard Methods for the Examination of Water and Wastewater (1998).

3.3 Analytical equipment used in conducting the present study

A Cyberscan 1000 pH meter was used for pH measurements. The instrument was calibrated with appropriate buffers before use. A Lutron CD-4301 conductivity meter was used to measure electrical conductivity. All spectrophotometric analyses were performed on a Unicam Helios α UV/Visible spectrophotometer. A Buchi R200 Rotavapor was used for *in vacuo* solvent removal.

3.4 The measurement of total, suspended and dissolved solids in the fruit processing wastewaters

Total solids were determined by evaporating 100 ml of well mixed wastewater samples in an oven at 90°C until constant weight was reached. The samples were evaporated in pre-weighed aluminium dishes and were cooled to room temperature before weighing. Dissolved solids were determined in the same manner after sample filtration. Suspended solids were determined as the difference between the total and dissolved solids.

3.5 Determination of the chemical oxygen demand (COD) of fruit processing wastewaters

The chemical oxygen demand (COD) of the fruit processing wastewater samples was determined using a Merck reagent kit (1.14555 HR). The wastewater sample (0.4 ml) was mixed with Reagent A (0.4 ml), followed by the addition of 3.4 ml of Reagent B. The reaction mixture was then put in a digestion block for 2 hours at 150°C. Readings were taken using a Nova Spectroquant photometer. Potassium hydrogen phthalate was used as the standard, at concentrations of 425 and 850 mg/L in distilled water, corresponding to COD values of 500 and 1000 mg/L respectively. Distilled water was used as the reagent blank.

3.6 Determination of the total organic carbon (TOC) present in the fruit processing wastewaters

The total organic carbon (TOC) of the fruit processing wastewater samples was measured using a SGE Anatoc Series II analyser, after filtration and appropriate dilution of samples. The organic carbon in samples was oxidised using TiO_2 catalyst and UV light. The resulting CO_2 was quantified using infra-red detection. Potassium hydrogen phthalate in the range 0-100 mg/L was used as standard. TOC analyses were performed by the Minerals Processing Research Unit at UCT.

3.7 Quantification of reducing sugars in fruit processing wastewaters using the dinitrosalicylic (DNS) assay

The concentrations of reducing sugars present in the fruit processing wastewater samples were determined using the dintrosalicylic acid (DNS) colorimetric method, according to the method of Frost, (2004) and Miller (1959). The DNS reagent (3 ml), containing 1g of 3,5-dinitrosalicylic acid (DNS), 1.6 g NaOH and 30 g of sodium sulphite in a litre of water, was added either to the wastewater sample or the glucose standard (3 ml) and incubated at 90°C for 15 minutes. Thereafter, 40% of Rochelle salt (potassium sodium tartrate) (1 ml) was added. The reaction was cooled at room temperature and absorbance was read at 540nm. Distilled water was used as a reagent blank while glucose in the range of 0 – 1000 mg/L was used as the standard (Refer to Appendix A-2).

3.8 Quantification of total carbohydrates present in the fruit processing wastewaters

The determination of carbohydrates was conducted according to the method of Mecozzi *et al*, (1999) and Miller (1959) with modifications. To 0.5 ml of the wastewater sample was added 5% phenol (0.5 ml) followed by concentrated sulphuric acid (2.5 ml). This was incubated at 30°C for 20 minutes followed by cooling at room temperature for 30 minutes. Distilled water was used as a reagent blank. The absorbance of the reaction mixture was corrected for the contribution of the colour of the wastewater sample by measuring the absorbance of the wastewater at 490nm and subtracting from the absorbance of the reaction mixture. Glucose in the range of 0- 1000 mg/L was used as standard (Refer to Appendix A-3). Absorbance was read at 490nm.

3.9 Quantification of the total phenolics in fruit processing wastewaters using the Folin Ciocalteau assay

The total phenolics content of wastewaters was determined colorimetrically using the Folin Ciocalteau assay according to the method of Celiktas *et al.*, (2007). The method was scaled down so it could be performed directly in 4 ml cuvettes. 400 µl sample was added to 2.5 ml of distilled water and mixed. This was followed by adding an equal volume of Folin Ciocalteau reagent and 20% sodium carbonate. This was stored in a dark place for 90 minutes to allow the reaction to go to completion and absorbance readings were taken thereafter at 765nm. Gallic acid in the range 0- 100 mg/L was used as a standard (Refer to Appendix A-1) and all total phenolic assays are thus reported as gallic acid equivalents (GAE). Samples were diluted where necessary to fall within the standard range and distilled water was used as reagent blank.

3.10 Antioxidant activity assays

3.10.1 Determination of the radical scavenging activity of fruit processing wastewaters using the DPPH radical scavenging assay

The determination of the radical scavenging activity was done according to the method of Devi and Arumughan, (2007). The wastewater sample or control (0.5 ml) was added to 3.5 ml methanolic DPPH (6.25 mg/L) and the reaction was monitored over time at 515nm until steady state was reached. Readings were taken at one minute intervals. For the silage water, 0.1 ml was used for the reaction because at 0.5 ml, the reaction was too fast to be monitored. The blank consisted of absolute methanol. The radical scavenging activity (RSA) was calculated using the following formula:

$$\% \text{ RSA} = \frac{\text{Initial Absorbance- Final Absorbance}}{\text{Initial Absorbance}} \text{ X } 100$$

3.10.2 Determination of antioxidant activity of fruit processing wastewaters using the 2,2'-azonobis (3-ethybenzothiazoline-6-sulphonate) ($ABTS^+$) assay

The determination of antioxidant activity of fruit processing wastewaters using the 2,2'-azonobis (3-ethybenzothiazoline-6-sulphonate) ($ABTS^+$) assay, also known as the Trolox Equivalent Antioxidant Capacity (TEAC) assay, was done according to the method of Magalhaes *et al.*, (2007). The $ABTS^+$ free radical was generated by reacting 7 mM ABTS and 2.45 mM potassium persulphate in water. The solution was stored in the dark for 18 hours and the free radical solution was diluted with water so that the absorbance was less than 1.The $ABTS^+$ free radical (3 ml) was added to 30 µl of the wastewater sample or trolox standard and readings were taken at 1 minute intervals for 15 minutes at 734nm (Magalhaes *et al.*, 2007). The radical scavenging activity of the fruit processing wastewaters was expressed as a percentage using the following formula:

$$\% \text{ RSA} = \frac{\text{Initial Absorbance- Final Absorbance}}{\text{Initial Absorbance}} \text{ X } 100$$

3.10.3 Determination of reducing power of fruit processing wastewaters using the ferric reducing antioxidant power (FRAP) assay

To 1 ml of the wastewater sample in a test tube was added 2.5 ml potassium phosphate buffer and 2.5 ml of potassium ferricyanide. This was incubated at 50°C for 20 minutes. 10% TCA was added immediately afterwards to stop the reaction. 2.5 ml of the above mixture was then added to 2.5 ml of distilled water and $FeCl_3$ (0.5 ml). The reaction was left to stand for 30 minutes at room temperature. Absorbencies were read at 700nm. The method was according to Ferreira *et al.*, (2007). Gallic acid was used as the standard (Refer to Appendix A-4). The reducing power of gallic acid at varying concentrations (0-100 mg/L) was determined and a graph was plotted of concentration against absorbance. Thus, the reducing power of the wastewater samples was expressed as gallic acid equivalents.

3.10.4 Determination of the ability to inhibit lipid peroxidation by fruit processing wastewaters using the β-carotene linoleic acid model system (β-CLAMS)

The β-CLAMS assay was conducted using the method of Liu *et al.*, (2007c). β-carotene (2 mg) was dissolved in 10 ml chloroform. A 1 ml aliquot of this solution was taken and the chloroform removed in vacuo in a round bottom flask. Linoleic acid (40 μl) and Tween 80 (400 μl) were added to the round bottom flask and made up to 100 ml with distilled water. 3 ml aliquots of this mixture were added to 1 ml of wastewater sample. Absorbencies were measured immediately and at 15 minute intervals for 2 hours (Liu *et al.*, 2007b). The ability to inhibit bleaching was expressed as a percentage and calculated as follows:

$$\% \text{ Inhibition} = \frac{\text{Initial Absorbance- Final Absorbance}}{\text{Initial Absorbance}} \text{ X } 100$$

3.11 Analysis of the fruit processing wastewaters using high performance liquid chromatography (HPLC)

Reversed phase HPLC was used to identify and quantify individual phenolic compounds present in the fruit processing wastewaters. This was performed on filtered and centrifuged wastewater samples, and on the wastewater extracts. A Merck Hitachi L-7000 series chromatograph was used. Monophenolics were separated by isocratic elution using a mobile phase of 80:20:2.5 ddH2O:-methanol-acetic acid. UV detection was used to quantify compounds, at a wavelength of 280nm. Individual compounds were identified by comparison of retention times of external standards with peaks detected in the wastewater samples (Ting, 2004).

3.12 Use of solvent extraction to obtain phenolic compounds from fruit processing wastewaters

Organic solvent extraction was performed to recover low molecular weight phenolic compounds from wastewater samples, which were then quantified by the total phenolics assay as described in Section 3.9. Wastewaters (100 ml) were extracted three times with equal volumes of ethyl acetate or hexane. The separated organic phases were pooled and dried over anhydrous sodium sulphate followed by filtration. The organic solvent in the combined organic phase was removed *in vacuo* at 30°C and the residual dried extract was redissolved in minimal volumes of methanol for analysis (Abad-Garcia *et al.*, 2007). The antioxidant activity of the organic solvent extracts was determined using the DPPH free radical scavenging assay.

3.13 Extraction of phenolic compounds from fruit processing wastewaters using solid phase extraction (SPE)

The procedure was conducted according to Suarez *et al.*, (1996). Figure 3.1 illustrates the experimental procedure used and each step is explained below.

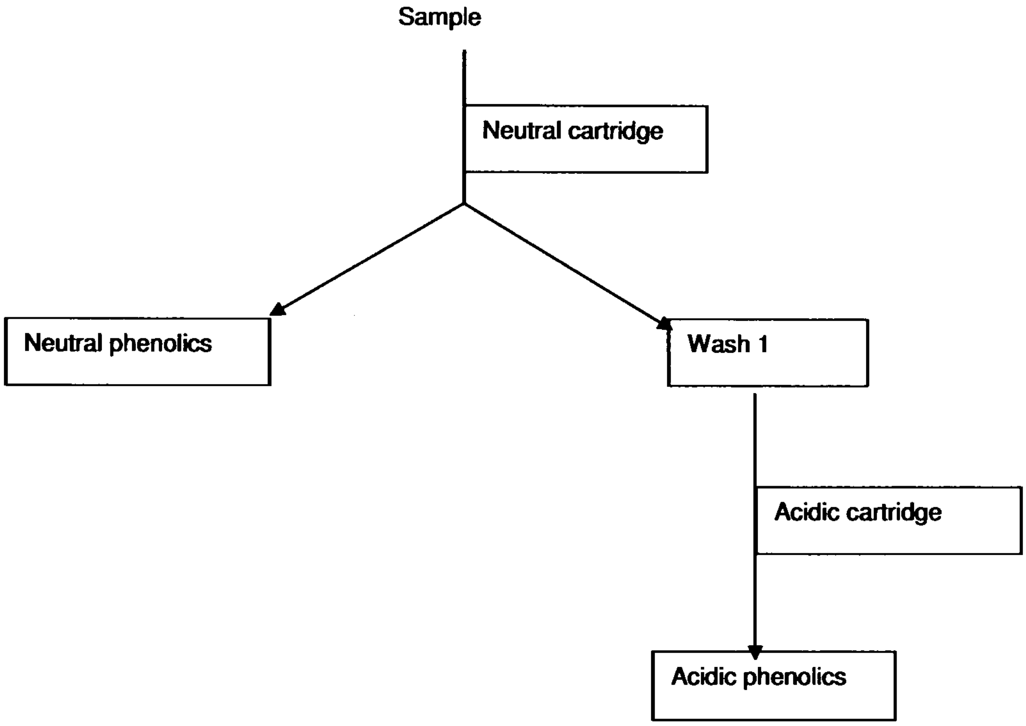


Figure 3-0:1: Experimental procedure for solid phase extraction

Preconditioning for neutral phenolics

Each Sep-Pak cartridge was preconditioned by passing through 8 ml methanol followed by 4 ml distilled water. The wastewater sample was adjusted to pH 7 using 1M NaOH and was passed through the preconditioned column. This was followed by a washing stage using 10ml distilled water. The adsorbed phenolic compounds were eluted with 12 ml methanol.

Preconditioning for acidic phenolics

The Sep-Pak cartridge was preconditioned with 8 ml methanol followed by 4 ml 0.01M HCl. The effluent from the separation of neutral phenolics was adjusted to pH 2 using 2M HCl and passed through the preconditioned column. The column was washed with 5 ml 0.1M HCl and the adsorbed phenolics eluted with 12 ml methanol.

Extracts of the neutral and acidic phenolics were filtered using 0.45 µm filters and the filtrates were analysed by HPLC. Total phenolics content in the residual water wash was determined using the Folin C assay. The antioxidant activity of the neutral and acidic phenolics was determined by the DPPH radical scavenging assay.

3.14 Extraction of phenolic compounds from fruit processing wastewaters using Polyvinylpolypyrolidone (PVPP)

The procedure for extracting phenolic compounds from fruit processing wastewaters was adapted from O'Reilly and Merrow (1998). PVPP was mixed with the respective wastewater sample at a ratio of 5 g/L of wastewater, for 10 minutes, to allow the phenolic compounds to bind to PVPP. This was followed by filtration and washing of the PVPP with distilled water (15 ml) 3 times. The bound phenolic compounds were eluted with 1 mM NaOH (10 ml). The concentrations of total phenolics were determined before and after extraction with PVPP using the Folin C assay. The stripped wastewater (residual remaining after PVPP extraction), water wash and NaOH eluant were analysed using HPLC. The antioxidant activity of the sodium hydroxide eluant was determined using the DPPH radical scavenging assay.

3.15 Extraction of phenolic compounds from fruit processing wastewaters using supported liquid membrane (SLM) extraction

The wastewater sample with the highest total phenolics content, in this case, the silage water, was selected and used for further investigations using supported liquid membrane (SLM). The SLM extraction was conducted according to Gonzalez-Munoz *et al.*, (2003) and Garcin (2005). Various organic solvents were considered for use in the SLM extraction system and this is discussed further in the next section. A Microdyne polypropylene hollow fibre membrane module (MD20CP2N) was used as contactor, with the following properties:

Membrane specifications	n = 40
	ID = 1.8MM, OD = 2.0MM
Pre size	0.2 µm
Total membrane surface area	$A_m = 0.1\ \mathrm{m}^2$

Lumen cross-section area	$A_l = 2.5 \text{ X } 10^{-6} \text{ m}^2$
Shell cross section area	$A_s = 1.8 \times 10^{-4} \text{ m}^2$
Membrane length	$L = 0.5$ m

A schematic diagram of the experimental procedure used for the SLM extraction system is shown in Figure 3.2 below. Reservoirs of the silage water and the organic solvent were placed on magnetic stirrers to ensure proper mixing of the bulk phases. The reservoirs were 1 L Schott bottles, and were sealed to prevent evaporative losses. The silage water (feed) was placed in the vessel labelled TT-01 which was pumped via pump PP-01 through the lumen of the membrane extraction module (MM-01) where it was passed through a pressure gauge and a pressure regulation valve which was used to set and adjust the trans-membrane pressure. The solvent was pumped via pump PP-02 counter currently through the shell side MM-01 and returned to its source vessel TT-02. The entire extraction rig was operated at ambient temperature and pressure in a fume hood.

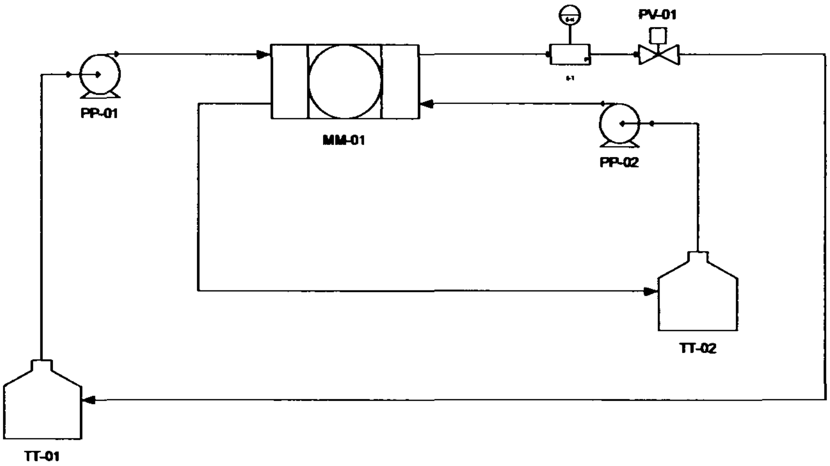


Figure 3-2: A schematic diagram of the membrane extraction system used to extract phenolic compounds from fruit processing wastewaters

3.15.1 Determination of the distribution coefficients of various organic solvents to determine the most suitable solvent for use in the SLM extraction system

The distribution coefficients of the phenolic compounds in the silage water between the aqueous and organic phase were first determined. The organic solvents used were amyl acetate, toluene, methyisobutylketone and ethyl acetate. Six dilutions of the silage water (0-100%) were each mixed with an equal volume (20 ml) of the organic solvent, shaken for 30 minutes and left to settle. This allowed for the concentrations of phenolic compounds in the two phases to reach equilibrium. After separation, the concentrations of phenolic compounds in the organic phase were determined by first removing the organic solvent *in vacuo*, and redissolving the residue in an equal volume of water. HPLC was used to monitor the concentration of gallic acid and determine the distribution coefficient. The analysis was conducted according to the procedure described in Section 3.11.

3.16 Preliminary studies on the extraction of phenolic compounds in fruit processing wastewaters using supercritical fluid extraction (SFE)

From the chemical characterisation of wastewaters, gallic acid in the silage water was found to be present in quantities sufficient to make the silage water a potential source of this antioxidant. SFE was then employed to determine the solubility and the extraction efficiency of gallic acid. The work described in this section was done in collaboration with Professor F. Deghani at the Department of Biosciences and Chemical Engineering, University of Sydney, Australia.

3.16.1 The determination of the solubility of gallic acid in supercritical CO_2

The solubility of gallic acid in supercritical CO_2 was measured using the procedure by Yi *et al.*, (2009) with modifications. The experimental apparatus that was used is shown in Figure 3.3 below. CO_2 gas cylinders were provided by BOC and stored at room temperature.

The SFE system consisted of two pumps. Pump 1 (Fike Cooperation) was a high pressure system pump used to provide an uninterrupted flow of supercritical CO_2. Pump 2 (Fike Cooperation) was used for cooling CO_2 through an ice water bath. Cooling increases the density of CO_2 thereby modifying CO_2 in to a liquid like state. This is desirable because it results in an increase in the solvating power of CO_2. The employed apparatus was specially designed to operate at pressures up to 200 bar and temperatures up to 120°C. The flow rate of the supercritical fluid used in all experiments was 3 ml/min. The system was preheated to the desired temperature before CO_2 was compressed to the desired operating pressure of 180 bar. This was done because increasing temperature after pressurisation would lead to a rapid expansion of CO_2 thereby increasing the pressure leading to an explosion. Pressure was monitored by 2 pressure gauges and a digital controller and the system was closed after 180 bar, stopping the flow of supercritical CO_2 to the extraction vessel. In all extractions, 1 g of gallic acid was used. After extraction, the system was washed with supercritical CO_2 for 30 minutes at pressures of 150 bar. The extract was redissolved in 10 ml Millipore water. Analysis was conducted using a UV-Vis spectrophotometer (Varian Cary). During each experiment, the system pressure was maintained to within ± 1 bar while the system temperature was controlled to within ± 1°C of the desired value.

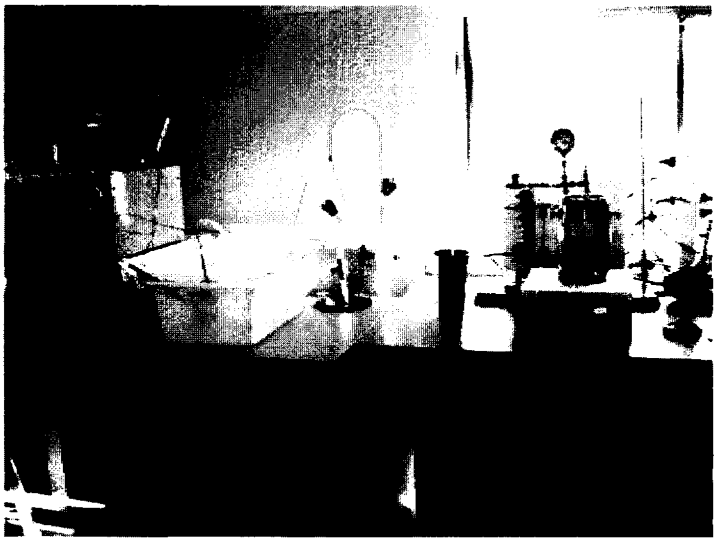


Figure 3-3: The laboratory-scale SFE system that was used for determining the solubility of gallic acid in supercritical CO_2

Four experimental systems were set up and are summarised in Table 3.1 below. Experiment 1 was designed according to data collected from a literature review and experiments 2 to 4 were done in order to see the effect of different parameters on the solubility and extraction of gallic acid thereby optimising the extraction conditions.

Table 3.1 Experimental designs with the operating conditions of temperature, pressure, volume of co-solvent and time

Experiment	Temperature (°C)	Pressure (bar)	Volume of ethanol used (ml)	Time (hours)
1	25	180	10	2
2	40	180	10	2
3	25	180	20	2
4	25	180	20	3

To determine the effect of temperature on the solubility and extraction of gallic acid, the temperature was increased from 25°C in experiment 1 to 40°C in experiment 2, under the same pressure, time and co-solvent volume. The optimum temperature was determined to be 25°C, and the rest of the experiments were conducted at 25°C. The effect of the volume of co-solvent added was studied in experiment 3. Finally, the effect of exposure time of gallic acid to both the co-solvent and supercritical CO_2 was investigated in experiment 4. An absorbance spectrum of gallic acid was run using a UV-Vis spectrophotometer from 100 to 800 nm to determine the absorbance maxima, which was found to be 270 nm. A gallic acid standard curve (refer to Appendix B-1) with concentration range of 0.0005 to 0.0125 g/L was obtained at 270 nm and this was used to determine the amount of gallic extracted.

CHAPTER 4: RESULTS AND DISCUSSION

Introduction

This chapter describes the results achieved in the investigations into the extraction of phenolic compounds from wastewaters obtained during the processing of citrus and deciduous fruits, using various extraction techniques. The citrus wastewaters (CW1) and (CW2) had been collected from fruit processors in the Western Cape, while the apple processing wastewaters (AW1) and (AW2) were collected from apple fruit processors located in the Western Cape. The silage water (SW) was collected from a fruit processor in Stellenbosch.

4.1 Experimental approach and the methods used in the study of the extraction of phenolic compounds from fruit processing wastewaters

Below in Figure 4.1 is a summary of the experimental approach and the methods used in this study. The aim of the initial investigations was to determine the composition of the wastewater samples, and hence the quantities of potential value-added products that could be recovered from wastewaters resulting from the processing of citrus and deciduous fruits. This was achieved through detailed chemical characterisation of the wastewaters to measure pH, conductivity, solids (total, dissolved and suspended), chemical oxygen demand (COD), total phenolics content and reducing sugars, total carbohydrates and total organic carbon contents. After the chemical characterisation of the fruit processing wastewaters had been completed, the antioxidant activities of the wastewaters were determined by various assays, namely, the DPPH radical scavenging assay, the 2,2'-azonobis (3-ethybenzothiazoline-6-sulphonate) ($ABTS^+$) assay, the ferric reducing antioxidant power (FRAP) assay and the β- carotene linoleic acid model system (β-CLAMS) assay.

The identification and quantification of individual phenolic compounds in the fruit processing wastewaters was achieved using high performance liquid chromatography (HPLC). Thereafter, the extraction of phenolic compounds from fruit processing wastewaters was investigated using various extraction techniques, namely, solvent extraction, solid phase extraction, extraction using PVPP and supported liquid membrane (SLM) extraction. Gallic acid was used as a model compound to assess the suitability of the supercritical fluid extraction (SFE) technique in obtaining phenolic compounds from fruit processing wastewaters. Gallic acid was chosen because it is known to be present in the silage water at concentrations that make its extraction potentially feasible at large scale. After the extraction of phenolic compounds from the fruit processing wastewaters using these extraction techniques, the quantification of the total phenolics in the extracts was conducted using the Folin Ciocalteau assay, and the antioxidant activity of the extracts was determined by the DPPH radical scavenging assay.

The various extraction techniques were compared in order to determine the extraction technique that would be most suitable for the recovery of phenolic compounds from fruit processing wastewaters. Factors that were considered were the cost of the extraction process, the feasibility of the extraction at a large scale, as well as the potential application of the extracted phenolic compounds in the food industry.

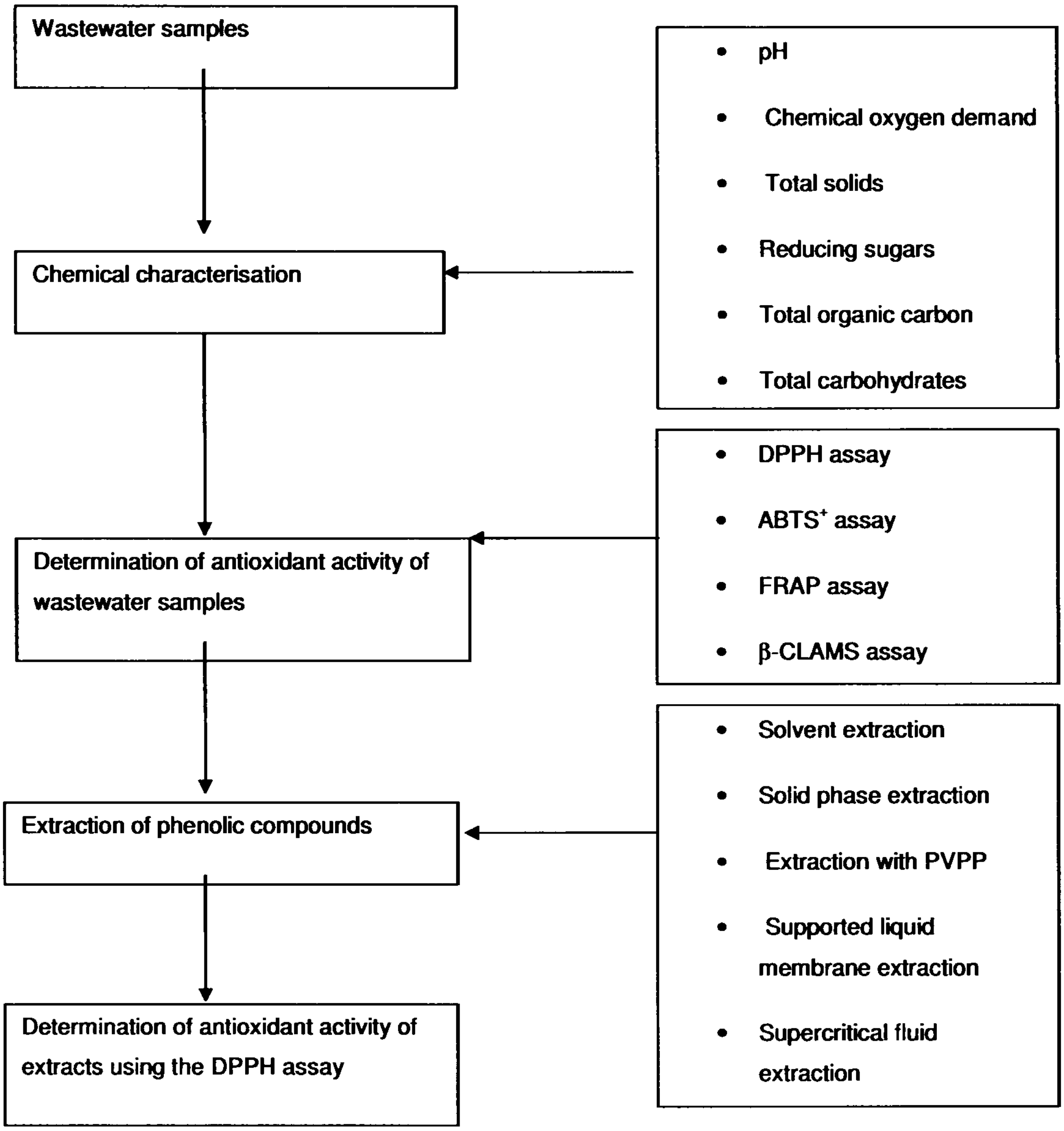


Figure 4-0:1: Flow diagram showing the experimental procedure and techniques used in the present study

4.2 Chemical characterisation of wastewaters from the processing of citrus and deciduous fruits

Detailed chemical analyses were conducted on fruit processing wastewaters using the methods described in Sections 3.4 to 3.9. This chemical characterisation was conducted in order to establish the composition of the fruit processing wastewater samples, thereby providing an indication of the presence and concentrations of potentially valuable compounds (such as phenolic compounds). The results of the chemical analyses of the fruit processing wastewater samples are summarised in Table 4.1 below, and the results are discussed in the following sections.

4.2.1 Chemical analyses of wastewaters from citrus and deciduous fruit processing

The pH of the fruit processing wastewater samples is important because it affects chemical and biological reactions, as well as the stability of phenolic compounds in solution. For example, the stability of anthocyanins in citrus fruits and their by-products is dependent on pH, with most anthocyanins being more stable at low pH. This was shown in a study conducted by Laleh *et al.*, (2006), where an increase in pH caused greater destruction of anthocyanins. In addition, pH also affects the ionisation of phenolic compounds, and this is an important contributor to the extractability of the phenolic compounds (Laleh *et al.*, 2006). The pH values measured for the wastewater samples were generally acidic, with the apple processing wastewater samples AW1 and AW2, having pH values of 6.4 and 5.1 respectively, whilst the citrus processing wastewater sample CW1, and the silage water SW, had pH values of 3.6 and 3.9 respectively (see Table 4.1). These wastewaters are generally acidic due to the presence of phenolic compounds and organic acids. The citrus processing wastewater CW2 was the only exception, having a value of 11. The high pH observed for CW2 may have most likely arisen from the processing procedures which may involve the use of chemicals that increase pH.

The term total solids (TS) refers to matter that is suspended (solids retained by a filter) or dissolved (the portion that passes through the filter) in wastewaters. The determination of total, dissolved and suspended solids was relevant in this study because the extraction of phenolic compounds is affected by the composition of the wastewater samples. The presence of significant quantities of solids in the wastewater samples decreases the mass transfer capacity of phenolic compounds, which then affects the extraction process, particularly during supported liquid membrane extraction (Turhan *et al.*, 2006). Furthermore, high quantities of solids can impact negatively on the aquatic life present in the receiving water where the fruit processing wastewaters are discharged. All the fruit processing wastewater samples characterised in this study contained a higher proportion of dissolved solids than suspended solids (see Table 4.1). These suspended and dissolved solids would have arisen during the processes of extraction, filtration and concentration, where the juice is squeezed from the fruit, leaving the rind and pulp suspended in water (Morris, 1996).

Electrical conductivity is an appropriate and convenient measure of dissolved ionic species in a solution. The wastewaters from any secondary biological oxidation treatment may contain inorganic ions such as phosphates and nitrates, and measuring the electrical conductivity of these wastewaters gives an indication of the purity of the water, which in turn reflects the effectiveness of any wastewater treatment procedure. In this study, the electrical conductivity measured for the wastewaters (using an electrical conductivity meter), was generally low (0.3 to 4.22 $mScm^{-1}$), as shown in Table 4.1, compared with data reported by Achak *et al.*, (2008) which showed a value of 6.9 $mScm^{-1}$ in citrus processing wastewaters (Achak *et al.*, 2008).

4.2.2 Determination of the chemical oxygen demand (COD) of wastewaters from the processing of citrus and deciduous fruits

Quality assessments of effluents and wastewaters prior to discharge are commonly based on the chemical oxygen demand (COD) value of the samples. The COD test is a measure of the capacity of water to consume oxygen during the decomposition of organic matter and the oxidation of inorganic chemicals. It is widely used as an indicator in monitoring discharges and for assessing treatment plant performance. The impact of an effluent on the water body receiving the effluent can be predicted by its chemical oxygen demand, based on the removal of oxygen from the natural water, reducing its ability to sustain aquatic life (Saravacos and Iredale, 1971).

The chemical oxygen demand of the wastewater samples was determined using a COD Merck reagent kit which consisted of Reagent A and B; a known volume of reagent A was mixed with a known volume of the wastewater sample, followed by the addition of reagent B. This mixture was then subjected to a two hour digestion at 120°C. Potassium hydrogen phthalate was used as a standard as described in Section

3.5 (Garcin, 2005). All the wastewaters used in this study would be considered as low strength wastewaters because of their low COD values. Values ranged from 328 mg/L in AWI to 5760 mg/L in CW2, as shown in Table 4.1. The apple wastewaters AW1 and AW2 had COD values of 328 mg/L and 2200 mg/L respectively, whilst the citrus wastewaters CW1 and CW2 had values of 3170 mg/L and 5760 mg/L respectively. The silage water SW had a COD value of 4750 mg/L. COD values obtained for other industrial wastewaters such as the olive mill wastewater are often greater than 10 000 mg/L (Garcin, 2005).

4.2.3 Quantification of total phenolics from the processing of citrus and deciduous fruits

The total phenolics content of wastewaters from the processing of citrus and deciduous fruits was determined using the Folin Ciocalteau method as described in Section 3.9 (Celiktas *et al.*, 2007). This method is based on the colour change measured spectrophotometrically at 765nm, which is caused by the reduction of the Folin Ciocalteau reagent by phenolates. These phenolates are produced from the phenolics in the presence of sodium carbonate (Adil *et al.*, 2007). Gallic acid was used as a standard compound and the concentration of total phenolics is expressed as milligrams gallic acid equivalents per litre of wastewater, denoted by mg/L GAE (Celiktas *et al.*, 2007).

A wide range of total phenolics concentrations was found in the wastewaters studied, ranging from 7.61 to 399.52 mg/LGAE as shown in Table 4.1 below. Much research on the characterisation and potential use of citrus and deciduous fruits to obtain phenolic antioxidants has been reported in literature, and this has been discussed in Sections 2.2.1 and 2.2.2. However, there is insufficient data on studies conducted on fruit processing wastewaters, as discussed in Section 2.6. Thus, comparison of the present work was done with studies conducted on other industrial wastewaters, including the olive oil mill wastewaters, winery wastewaters, cork processing and tannery wastewaters, as well as with deciduous and citrus fruits or pomace. For example, the wastewaters studied here had concentrations of total phenolics comparable with winery wastewaters studied by Burton *et al.*, (2007) where values ranging from 13.1 to 247 mg/L were reported. However, these values are relatively low when compared with values obtained in other agricultural wastewaters such as the cork processing and tannery wastewaters where total phenolics content of 358 and 1400 mg/L GAE respectively were reported (Minhalma *et al.*, 2006; Marino-Martinez *et al.*, 2009).

The apple processing wastewater samples, AW1 and AW2 studied here had total phenolics contents of 7.61 and 42.45 mg/L GAE respectively. The low concentrations of phenolic compounds in the apple processing wastewaters would be expected, because during processing, most of the phenolic compounds are retained in the apple pomace which consists of the peel, pulp and seeds, where most of the phenolic compounds are located. For example, apple pomace, studied by Schieber *et al.*, (2003), had total phenolics content of 2408 mg/kg, and the total phenolics content of whole fruit apples was found to be between 6240 and 10 530 mg/kg GAE in a study conducted by Hagen *et al.*, (2007). This shows that at least 20% of the total phenolics end up in the pomace, whilst the fruit juice contains most of the phenolics, meaning that relatively low proportions would be expected to be present in the wastewaters.

Citrus processing wastewater samples CW1 and CW2 contained low amounts of total phenolics, namely 18.6 and 137 mg/L respectively. The results obtained in the present study are comparable with results obtained in a study conducted by Burton *et al.*, (2006) where the total phenolics content of citrus wastewaters from two different sources were 3.9 and 63 mg/L GAE. However, these values are relatively low when compared to quantities present in the peel and pulp of sour orange, where total phenols were reported to be 569 mg/L GAE in the juice and 4871 mg/kg in the pulp (Ersus and Cam, 2007). The distribution of phenolic compounds in fruits is higher in the pulp (which constitutes the pomace after fruit processing) than in the juice and peel (Marinova *et al.*, 2005), and this explains why only low amounts of phenolic compounds would be present in the wastewaters, since the residual pomace containing the peel and pulp is separated from the washing water.

In general, the concentrations of total phenolics from fruit processing wastewaters vary depending on the point of collection of the wastewater samples for analyses. In the present study, the low phenolics content for AW1 was attributed to the wastewater samples having been collected after the secondary biological treatment stage, which reduces the organic load of the wastewaters. The total phenolics content in the silage water, SW, was higher (399.52 mg/L) than the apple and citrus processing wastewaters studied here, also as expected. This is because the silage water SW, consisted of the seepage liquid that arises from the storage of residues of various deciduous fruits such as peaches, pears and plums which are known to contain relatively high concentrations of total phenolics. Generally, peaches have total phenolic contents of 846 mg/kg fresh weight and plums have 1740-3750 mg/kg fresh weight (Balasundram *et al.*, 2006). Although the low concentrations of phenolics measured are not surprising, this is important because it will have an impact on the amounts recoverable by extraction.

Table 4.1 Chemical analysis of wastewaters from the processing of citrus and deciduous fruits

Analysis	Wastewater Sample				
	AW1	AW2	CW1	CW2	SW
pH	6.4	5.1	3.6	11	3.9
Conductivity (mScm^{-1})	0.80	1.00	0.30	2.34	4.22
Total solids (g/L)	1.52± 0.02	2.44±0.01	5.10±0.02	4.90±0.01	6.87±0.3
Dissolved solids (g/L)	0.70± 0.01	1.721±0.01	4.7±0.02	4.46±0.2	5.42±0.01
Suspended solids (g/L)	0.828±0.01	0.716±0.01	0.4±0.01	0.44±0.01	1.45±0.01
Chemical oxygen demand (mg/L)	Less than 500 (328.33)	2 200	3 170	5 760	4 750.00
Total phenolics content (GAE mg/L)	7.61±0.01	42.45±0.02	18.6±0.02	137±0.03	399.52±0.05
Reducing sugars (mg/L)	126.45±0.02	215.330±0.03	230.3±0.03	396.3±0.02	234.8±0.01
Total carbohydrates (mg/L)	388.6±0.01	688.6±0.02	5 793.65±0.04	2 828.57±0.03	7 000±0.05
Total organic carbon (mg/L)	500	1 170	1 025	2 081.25	31 625

Key: AW1 and AW2 are apple processing wastewaters obtained from apple processing companies in the Western Cape; CW1 and CW2 are citrus processing wastewaters obtained from citrus processing companies in the Western Cape; Silage water, SW, is seepage from deciduous fruits wastes collected from a local canning plant

4.2.4 Quantification of reducing sugars in wastewaters from the processing of citrus and deciduous fruits

Measurement of reducing sugars in a wastewater is necessary because it gives an indication of the quantities of sugars that can potentially be obtained from the wastewater. In this study, the quantification of reducing sugars was achieved using the dinitrosalicyclic acid (DNS) colorimetric method (Frost, 2004; Miller, 1959). The respective wastewater samples were each mixed with the DNS reagent and heated. This was followed by the addition of potassium sodium tartrate and the measurement of absorbance at 540nm. Glucose was used as the standard reducing sugar. The results were expressed as mg of glucose per litre of wastewater. The method is described in detail in Section 3.7.

Values ranging from 126.5 to 396.3 mg/L were measured for the reducing sugars in the wastewater samples, as shown in Table 4.1. The low concentration of reducing sugars in the silage water SW (234.8 mg/L) is likely to be due to the sugars having already been fermented by the microbial population present in the wastewater. Sugars were recovered from orange pulp wash in a study conducted by Scordino *et al.*, (2007), where 1185.6 mg/L of total sugars were recovered. Thus, the wastewaters studied here are a potential source of reducing sugars that could be used as a carbon source in media for microbial growth or used for biomass production. Alternatively, they may find use as sugar syrup that can be used as a natural sweetener for food drinks and fruit nectars. This would require further investigations into the incorporation of the reducing sugars in food products. However, this is outside the scope of this project as the focus of the present research is on recovering antioxidants.

4.2.5 Quantification of total carbohydrates in wastewaters from the processing of citrus and deciduous fruits

The fruit processing wastewaters contain considerable properties of complex carbohydrates as well as simple sugars, which contribute to the chemical oxygen demand of the wastewaters. Therefore, the quantities of carbohydrates in these wastewaters need to be determined.

The determination of total carbohydrates present in the wastewater samples was conducted by a method involving hydrolysis using sulphuric acid to produce simple sugars and measuring the absorbance at

490nm spectrophotometrically (Mecozzi *et al.*, 1999). The experimental procedure is described in detail in Section 3.8. Glucose was used as a standard simple sugar and the results obtained were expressed as mg glucose per litre of wastewater. The total carbohydrates concentration measured in the wastewaters ranged from 388.6 mg/L in AW1 to 7000 mg/L in SW as shown in Table 4.1.

The determination of total carbohydrates by acid hydrolysis may have some discrepancies, since absorbance of samples at 490nm can also be attributed to naturally occurring coloured wastewater components or coloured substances formed by the non-specific action of concentrated sulphuric acid on organic material (Safarik and Santruckova, 1992). In the present study, the colour of the wastewaters was corrected for by subtracting the absorbance of the wastewater sample at 490nm from the absorbance of the reaction mixture. Thus, this assay gives an indication of the total carbohydrates present in the wastewaters and other methods, for example, the determination of the total organic carbon, should be used together with the determination of total carbohydrates in order to measure the total carbohydrates concentration more accurately. This was investigated, as described in the next section.

4.2.6 Quantification of the total organic carbon (TOC) in wastewaters from the processing of citrus and deciduous fruits

After the quantification of the total phenolics and total carbohydrates, the total organic carbon (TOC) was determined in order to assess the contribution of these carbon containing compounds in the wastewaters to the total organic carbon content. TOC is a sum measurement of all the organic carbon present in a sample. This includes organic carbon in the form of sugars, carbohydrates and phenolic compounds and others.

The measurement of total organic carbon (TOC) gives a specific and absolute measure of the organic carbon in samples such as the fruit processing wastewaters studied here (Mara and Horan, 2003). TOC was measured by acidifying the sample, followed by chemical oxidation and measuring the organic carbon content as carbon dioxide. The measurement of TOC is automated and in the present study, a SGE Anatoc Series Π analyser was used to measure TOC after appropriate dilution of samples. Potassium hydrogen phthalate was used as a standard compound and the results were expressed as mg of potassium hydrogen phthalate per litre of wastewater. The experimental procedure is described in detail in Section 3.6.

The wastewaters studied here had TOC values ranging from 500 to 31625 mg/L. The apple processing wastewater samples AW1 and AW2 had TOC values of 500 and 1170 mg/L respectively, whereas the citrus processing wastewater samples CW1 and CW2 had TOC values of 1025 mg/L and 2081.25 mg/L respectively. The silage water SW had a TOC value of 31 625 mg/L.

The results obtained in the present study show that the sum of the values obtained for total phenolics, and total carbohydrates did not add up to the TOC value of the wastewater sample (Table 4.2). For apple processing wastewater AW1, the value obtained after adding up the concentrations of the carbon sources studied was 73.79 mg/L less than the value for the TOC. The sum of all the carbon sources in apple processing wastewater sample AW2 was less than the TOC content by 438.95 mg/L. The TOC value for citrus processing wastewater sample CW1 was less than the sum of all the carbon sources in CW1 by 4787.25 mg/L, whereas in CW2, the sum of all the carbon sources was more than the TOC value by 884.32mg/L. Also, the silage water SW, TOC value was four times more than the value of the sum of all the carbon sources. In all cases, experiments were done in triplicate and the relative error was determined as the percentage of the mean value, and values within 5% of the relative error were considered.

This may be attributed partly to inaccuracies during the measurement of the phenolic compounds, reducing sugars and total carbohydrates. It has been noted that the Folin Ciocalteau assay may not be a completely reliable measurement of phenolic compounds because some other substances such as organic acids, residual sugars, amino acids, proteins and other hydrophilic compounds interfere with this assay (Cetkovic *et al.*, 2008). Investigations into the use of other techniques such as high performance liquid chromatography could be conducted in order to obtain more accurate concentrations for total phenolics and sugars. In addition, the discrepancy noted between the total organic carbon content and the sum of the carbon sources measured here is more likely due to the presence of other carbon containing sources such as lignocelluloses and essential oils in citrus and deciduous fruits, which were not measured directly in the present study. The most notable discrepancy was observed with the silage water (SW) and this may be attributed to the higher levels of total solids (6.87 mg/L) compared with the other wastewaters, implying higher levels of other organic compounds not measured directly in the present study. However, this was not pursued further because phenolic compounds were the focus of this project and not total organic carbon, which would be more relevant for fermentation potential

Table 4.2: A summary of the concentrations of all the carbon sources and their summations, compared with the values for the total organic carbon measurements

Sample	Parameter				
	Total phenolics (mg/L)	Total carbohydrates (mg/L)	Sum of all the carbon sources (mg/L) assayed	Total organic carbon (mg/L)	Difference (mg/L)
AW1	7.61±0.01	388.6±0.01	426.21	500	73.79
AW2	42.45±0.02	688.6±0.02	731.05	1170	438.95
CW1	18.6±0.02	5793.65±0.04	5812.25	1025	4787.25
CW2	137±0.03	2828.57±0.03	2965.57	2081.25	884.32
SW	399.52±0.05	7000±0.05	7399.52	31625	24225.48

4.3 Antioxidant activities of antioxidant components in wastewaters derived from the processing of citrus and deciduous fruits

The work reported in the previous sections involved determinations of the chemical composition of the wastewater samples, including concentrations of reducing sugars, total carbohydrates and total phenolics, which were obtained for each wastewater sample through detailed chemical characterisation. However, the main aim of this project was to recover potential value added products, particularly phenolic antioxidant compounds, in the fruit processing wastewaters samples. Hence, this section describes work conducted in the determination of antioxidant activity present in the wastewater samples.

The determination of the antioxidant capacity of a sample such as a wastewater cannot be achieved comprehensively and consistently by any single method because of the complex nature of phytochemicals (Du *et al.*, 2009). Because multiple reaction characteristics and mechanisms are involved, for example the HAT and ET based mechanisms (Section 2.5), it is generally agreed that at least two different assays are required to accurately assess antioxidants in mixed or complex systems such as the fruit processing wastewaters (Erkan *et al.*, 2008).

In this study, the antioxidant activities of fruit processing wastewaters were determined by using the DPPH and $ABTS^+$ (also known as the trolox equivalent antioxidant capacity (TEAC) assay) free radical scavenging capacity assays. These assays are useful in determining whether the wastewater samples are effective free radical scavengers, and they also provide information on the electron donating ability of the wastewater samples. However, the DPPH radical scavenging assay and the $ABTS^+$ assay use synthetic free radicals that are not found in any biological system. Thus, there is need to conduct antioxidant capacity assays that involve reactions and free radicals that would be found in the human body, such as the ferric reducing antioxidant power (FRAP) assay and the β- carotene linoleic acid model system (β-CLAMS). The FRAP assay provides information on the ability of antioxidant samples to reduce metal ions from the Fe^{3+} state to the Fe^{2+} state. The β-CLAMS assay determines the potential of the antioxidants to inhibit β-carotene bleaching. Thus, the FRAP and β-CLAMS assays are more relevant in determining the potential effectiveness of putative antioxidants in the removal of free radicals *in vivo* (Alen-Ruiz *et al.*, 2009).

The results gained from these assays provide simple data that make it possible to classify organic compounds and mixtures with respect to their antioxidant capacity. Because antioxidant activity does not always correlate with the presence of large quantities of polyphenolics, the phenolic content and antioxidant data need to be examined together. In the context of the present study, assays of the wastewaters would give indications of the amounts of antioxidant components that they contain, and could therefore be extractable.

4.3.1 Determination of the free radical scavenging capacity of fruit processing wastewaters using the DPPH radical scavenging assay

The DPPH radical scavenging assay is commonly used in antioxidant studies and offers a rapid technique to screen the radical scavenging activity of pure synthetic compounds, isolated natural compounds, crude plant extracts and foods (Becker *et al.*, 2004). Unlike free radicals such as the hydroxyl and superoxide radicals that need to be generated in the laboratory, DPPH is a commercially available free radical. In addition, the DPPH radical has the advantage of not being affected by certain side reactions such as metal-ion chelation brought about by various additives during free radical synthesis in other assays such as the FRAP assay (Amarowicz *et al.*, 2004).

In order to apply the DPPH assay in the present study, methanolic DPPH was mixed with each respective wastewater sample, and the reaction was monitored by measuring the absorbance at 515nm with readings taken at one minute intervals until steady state was reached (Devi and Arumughan, 2007). The results of the DPPH assay were expressed as % radical scavenging activity, which was calculated as the change in absorbance after the reaction was complete, divided by the initial absorbance at time t=0 and expressing the result as a percentage. The experimental procedure and the calculations used are described in more detail in Section 3.10.1. The reactions were conducted in triplicate.

The results obtained in the DPPH radical scavenging assays are shown in Figure 4.2. The silage water SW sample exhibited the highest radical scavenging activity compared with the rest of the wastewater samples investigated, as shown by a more rapid decrease in absorbance. The rate of decrease in absorbance over time at 515nm is an indication of the rate of scavenging of the DPPH radical by antioxidant components in the wastewater samples. The silage water SW completely decolourised DPPH, indicating strong hydrogen donating ability (the HAT mechanism, Section 2.5). This corresponds with the higher total phenolics content of the silage water SW (399.52 mg/L) in comparison with other wastewater samples (Table 4.1). Apple wastewater AW1 exhibited the lowest radical scavenging activity of 22.05% and the lowest total phenolics content of 7.61 mg/L GAE. This observation was expected, since phenolic compounds are believed to account for a major portion of antioxidant activity in many plants, and thus, there would be a correlation between concentration of phenolic compounds and antioxidant activity (Ciou *et al.*, 2008).

The % radical scavenging activities (RSA) of the wastewaters were calculated and Table 4.3 shows values ranging from 22.05% in AW1 to 100% in the silage water SW. The citrus processing wastewaters, CW1 and CW2, exhibited radical scavenging activities of 29.31 and 43.37% respectively. The radical scavenging activity of the studied citrus processing wastewaters was comparable with values obtained in a study conducted on the antioxidant capacity of citrus fruits cultivated in China where the greatest radical scavenging activity was 61.62% (Xu *et al.*, 2008). Although a direct comparison of the antioxidant activity of the citrus processing wastewaters and citrus fruit can not be made, these values give an indication of the potential of the wastewaters to exhibit radical scavenging activity that is comparable to whole fruits.

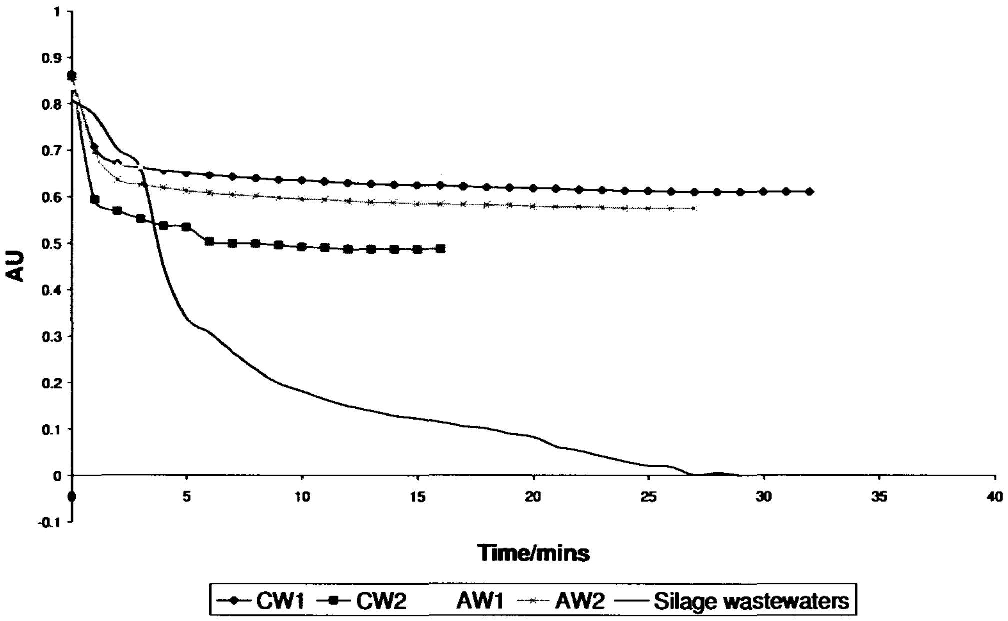


Figure 4-2: DPPH radical scavenging assay of antioxidant activity in the fruit processing wastewater samples

Table 4.3 shows the % DPPH radical scavenging activity, total phenolics content and the ratio of the radical scavenging activity to total phenolics for each of the wastewater samples studied. The ratio of the radical scavenging activity of the wastewater to the total phenolics content of the wastewater sample gives an indication of the potency of the phenolic antioxidants present in the sample. The apple processing wastewater samples AW1 and AW2 had ratios of 2.9 and 0.69 respectively whilst the citrus processing wastewaters CW1 and CW2 had values of 1.58 and 0.32 respectively. The silage water SW had the lowest ratio of 0.25. According to these results, the phenolic antioxidants present in the apple processing wastewater AW1 were the most potent, followed by CW1, AW2 and CW2, with silage water SW phenolic antioxidants exhibiting the least potency.

Table 4.3: Analysis of the rates of radical scavenging of the fruit processing wastewaters

Sample	Parameter		
	Total phenolics (mg/L GAE)	% DPPH radical scavenging activity	Ratio of %RSA to Total phenolics (%RSA/mg/L wastewater)
AW1	7.61±0.01	22.05±0.01	2.89
AW2	42.45±0.02	29.32±0.01	0.69
CW1	18.60±0.02	29.31±0.01	1.58
CW2	137.00±0.03	43.37±0.02	0.32
SW	399.52±.05	100.00±0.01	0.25

The concentration of total phenolics in AW2 was 42.45 mg/L whilst the total phenolics content of CW1 was less than half of this, at 18.6 mg/L. However, the %RSA of AW2 and CW1 were 29.32 and 29.31 respectively, a very small difference in scavenging activity. This may be attributable to some discrepancies in the results obtained using the Folin C assay, which is known to be subject to interference and variability (see Section 2.4.1). Also, the total phenolics content does not necessarily indicate all the antioxidants that may be present in the wastewater sample as the Folin C assay does not measure, for example, vitamins that may also contribute to the antioxidant activity of the wastewater sample (Tawaha *et al.*, 2007). Hence, this may explain the unexpected trend observed on the total phenolics content and antioxidant activity of AW2 and CW1. The determination of the concentration of vitamins could be conducted and the contribution of these vitamin antioxidants could then be established.

Since the DPPH assay was used as a preliminary free radical scavenging evaluation, analysis on the electron donating, reducing power, and potential to inhibit lipid oxidation were subsequently performed, to confirm the free radical scavenging effect of the fruit processing wastewaters.

4.3.2 Determination of the electron donating ability of the fruit processing wastewaters using the $ABTS^+$ assay

The $ABTS^+$ assay has been recently proposed as a standardised method to measure antioxidant capacity of food products and dietary supplements (Magalhaes *et al.*, 2007). The $ABTS^+$ assay is based on the ability of antioxidant molecules to quench the $ABTS^+$ free radical, a blue-green chromophore with characteristic absorption at 734nm. The $ABTS^+$ free radical is generated *in situ* using potassium persulfate to oxidise ABTS, and the ability to quench the $ABTS^+$ free radicals by the antioxidant samples is compared with that of Trolox, a water soluble Vitamin E analog. The addition of antioxidants to the preformed radical cation reduces it to ABTS, resulting in decolourisation (Pellegrini *et al.*, 2003). The results of the $ABTS^+$ assay are expressed as % radical scavenging activity, which is calculated as the change in absorbance after the reaction is complete, divided by the initial absorbance at time t=0. The procedure and the calculations used are described in detail in Section 3.10.2.

In the investigation using the $ABTS^+$ assay, the silage water SW and the citrus wastewater CW2 exhibited the highest radical scavenging activity among all the samples tested, as shown in Figure 4.3. The sharp decrease in absorbance of 1mM Trolox showed that Trolox was able to quench the $ABTS^+$ radical faster than the wastewater samples, as would be expected, since Trolox is a powerful antioxidant. The results shown in Figure 4.3 demonstrate that the reaction with $ABTS^+$ radical was complete after 10 minutes for most of the wastewater samples, except for the silage water SW which showed a more prolonged scavenging effect, of up to 15 minutes. This is a desirable characteristic for an antioxidant, since it would then potentially continue scavenging free radicals for longer periods.

Table 4.4 shows the total phenolics contents, % radical scavenging activity and the ratio of the radical scavenging activity to total phenolics of the wastewater samples. The silage water SW exhibited the highest radical scavenging activity of 74.67% and the lowest ratio of radical scavenging activity to total phenolics content of 0.19. The citrus wastewater samples generally exhibited higher scavenging capacities of 21.63 and 55.49% for CW1 and CW2 respectively, compared with the apple wastewater samples that exhibited values of 13.80 and 30.30% for AW1 and AW2 respectively.

Statistical analysis using the Pearson's test was conducted to determine the correlation coefficient between total phenolics content and antioxidant capacity obtained from the DPPH and $ABTS^+$ assays. A positive linear correlation between antioxidant activity and total phenolics content was demonstrated, where R = 0.995 and R = 0.937 were obtained between total phenolics and DPPH and $ABTS^+$ assays respectively. These results show that phenolic compounds contributed significantly to the antioxidant capacity of investigated wastewaters, and are consistent with the findings of Tawaha *et al.*, (2007) who also reported a positive correlation of R = 0.892 between total phenolics present in Jordanian plant species and antioxidant activity.

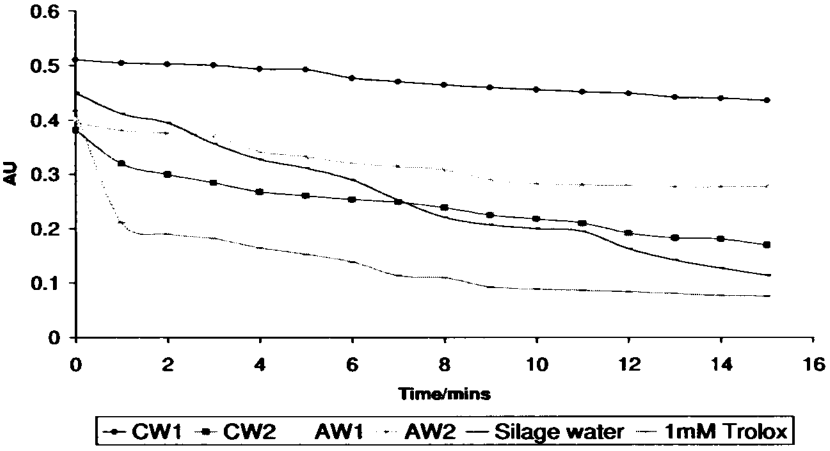


Figure 4-3: $ABTS^+$ radical scavenging activities of fruit processing wastewaters using the $ABTS^+$ assay

Table 4.4: Total phenolics concentration and radical scavenging activities of fruit processing wastewaters

Sample	Parameter		
	Total phenolics (mg/L GAE)	% $ABTS^{\cdot +}$ radical scavenging assay	Ratio of %RSA to Total phenolics (%RSA/mg/L wastewater)
AW1	7.61±0.01	13.80±0.01	1.81
AW2	42.45±0.02	30.30±0.02	0.71
CW1	718.60±0.02	21.63±0.01	1.16
CW2	137.00±0.03	55.49±0.01	0.41
SW	399.52±0.05	74.67±0.02	0.19

The results obtained in the DPPH and $ABTS^+$ assays show that the fruit processing wastewater samples could potentially be used as antioxidants that exhibit both hydrogen donating and electron donating capacities. In order to gain a deeper understanding of the antioxidant nature and mechanism of action of the phenolic antioxidants present in the wastewaters, the ferric reducing antioxidant power assay was conducted and the results obtained are described below.

4.3.3 Determination of antioxidant activity of fruit processing wastewaters using the Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power (FRAP) assay was used to provide information on the extent to which the antioxidants present in the wastewater samples would be able to reduce ferric ions to ferrous ions, this being somewhat closer to a physiological reaction than the assays described in Section 4.3.1 and 4.3.2

The reducing ability of antioxidants present in the fruit processing wastewaters was determined by the FRAP assay. This assay measures the ability of the antioxidant to reduce the ferric tripyridyltriazine (Fe^{3+} - TPTZ) to the Fe^{2+} form shown by an increase in absorbance at 700nm (Jayaprakash *et al.*, 2008b; Katalinic *et al.*, 2005). The FRAP assay offers a method for the determination of dilute concentrations of phenolic compounds which participate in the redox reactions (Amarowicz *et al.*, 2004). Antioxidants in the wastewater samples react with ferric ion free radicals converting them to more stable products thereby terminating radical chain reactions (Bounatirou *et al.*, 2007). The yellow colour of the test solution changes to various shades of green or blue depending on the reducing power of the sample.

The FRAP assay was conducted according to the method of Ferreira *et al.*, (2007) and the method is described in detail in Section 3.10.3. Gallic acid was used as a standard antioxidant compound. Thus, the reducing power of the wastewater samples is expressed as gallic acid equivalents (GAE). Figure 4.4 shows the reducing power of the wastewater samples. The reducing power of ascorbic acid, a known antioxidant, was also determined and used to compare the potency of the antioxidants in the wastewater samples with that of a known antioxidant.

In the FRAP assay, the silage water SW showed the highest reducing power of 0.03 GAE. Generally, the citrus wastewaters exhibited higher reducing power compared with the apple wastewaters. The reducing power of CW2 (0.023 GAE) was found to be similar to that of ascorbic acid (0.025 GAE), a known antioxidant, whilst the reducing power of CW1 was 0.016 GAE. AW2 exhibited reducing power of 0.005

GAE whilst the reducing power of AW1 was almost negligible with a value of 0.0001 GAE. Jayaprakash *et al.*, (2008a) studied the reducing power of navel oranges. At 250 mg/L total phenolics, the reducing power of navel orange juice ranged from 0.058 to 0.418 GAE. These values are higher than the reducing power of the citrus wastewater samples studied here as would be expected.

There was a good correlation (R= 0.81), determined by the Pearson's correlation test, between the reducing power and the total phenolics content, suggesting that total the phenolics in the sample wastewaters contribute significantly to the reducing power. Thus, the FRAP assay provides information on the ability of the antioxidants present in the wastewater samples to reduce Fe^{3+} to Fe^{2+}.

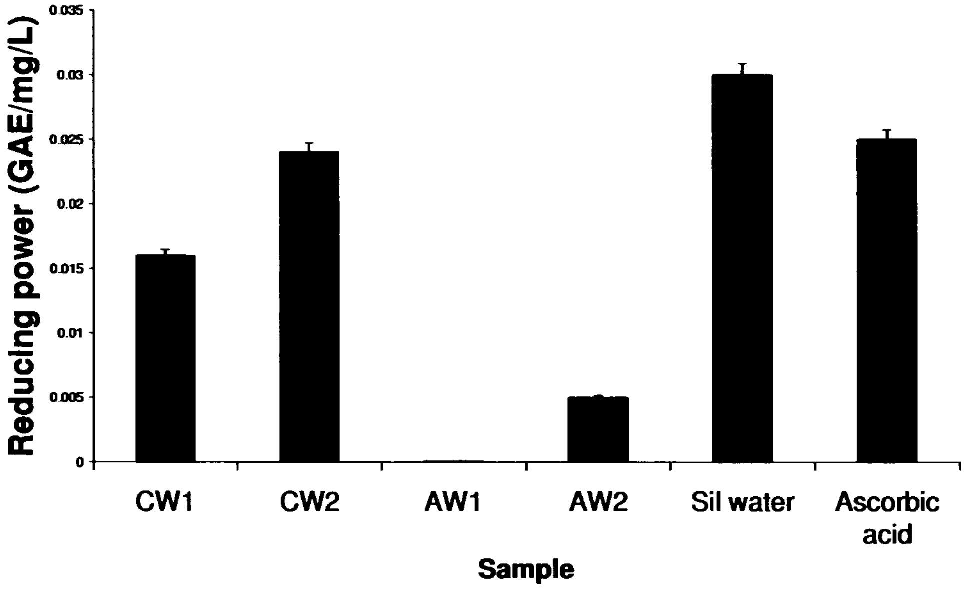


Figure 4-4: The ferric ion reducing activity of antioxidants in the fruit processing wastewaters measured using the FRAP assay using gallic acid as a standard for comparison. Reactions were conducted in triplicate and the mean values are shown with SEM.

The results obtained in the study of the reducing power of the wastewater samples give an indication of the ability of the antioxidants in the wastewater samples to reduce metal ions such as the ferric ions, a characteristic that is desirable for antioxidants that may be incorporated into foods as they will prevent the propagation of free radicals through the Fenton reaction which is described in Section 2.3.1

4.3.4 Determination of the inhibition of lipid oxidation by fruit processing wastewaters using the β-carotene bleaching assay

The oxidation of lipids in the human body results in the formation of lipid peroxides and other lipid radicals that lead to oxidative damage through the alteration of the lipid chemical structure. The β-carotene linoleic acid model system (β-CLAMS) assay provides information on the ability of the antioxidants present in the wastewater samples to inhibit lipid peroxidation. This is measured by the ability of antioxidants present in the fruit processing wastewaters to prevent bleaching of β-carotene by the lipid peroxides. The β-CLAMS method is based on the de-colouration of β-carotene by the peroxides generated during the oxidation of linoleic acid at elevated temperatures (Liu *et al.*, 2007c).

In the experimental procedure, a known quantity of β-carotene is dissolved in chloroform and an aliquot of this solution is taken and the chloroform removed *in vacuo*. Linoleic acid and Tween 80 are then added and the solution is diluted to a standard volume. Thereafter, known volumes of this solution are mixed with the antioxidant sample and the absorbance changes are monitored for 2 hours. In this study, the procedure was conducted according to Liu *et al.*, (2007c) and is described in detail in Section 3.10.4. This oxidative destruction of β-carotene by the radicals of linoleic acid degradation is measured by the decrease in absorbance at 470nm. Gallic acid was used as a standard antioxidant to compare the effect of inhibition of bleaching by a standard antioxidant, with the inhibition of bleaching conferred by the antioxidants in the fruit processing wastewaters.

Figure 4.5 shows the percentage inhibition of β-carotene bleaching demonstrated in the β-CLAMS assay of the sample wastewaters. A low % inhibition would be indicative of poor inhibition of β-carotene bleaching. Amongst the wastewater samples studied, the silage water SW exhibited the highest inhibition of β-carotene of 86%, followed by the citrus wastewaters CW1 and CW2 (49.2 and 69.04% respectively). However, the apple wastewater samples exhibited the lowest ability to inhibit β-carotene bleaching, with AW1 and AW2 having values of 15.2 and 29.3% respectively. SW and CW2 demonstrated inhibition capacities that were comparable with 100 mg/L gallic acid. The marked activity of the silage water (greater than 80%) is mainly attributed to its higher phenolics content. The relatively high inhibition capacities observed for the citrus wastewaters and silage water may be indication that they contain more hydrophobic antioxidants compared with the apple wastewaters.

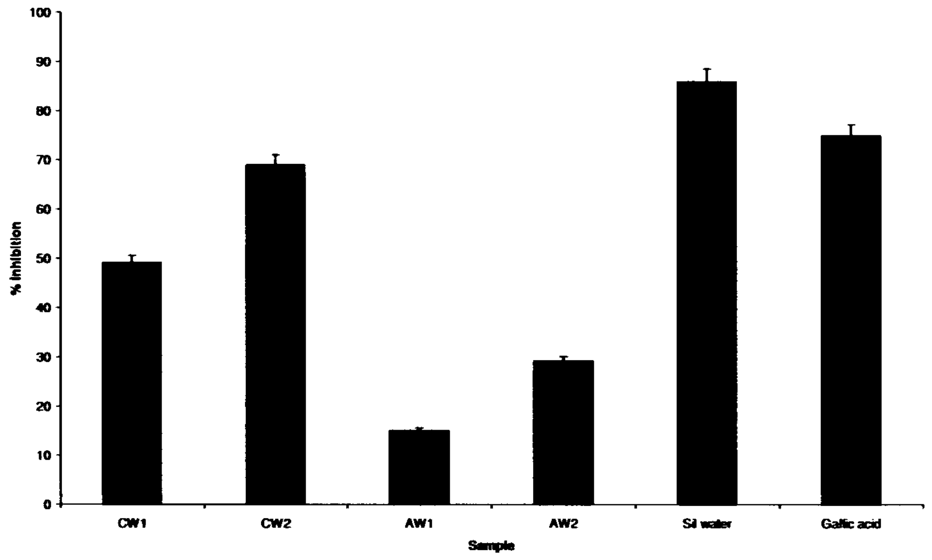


Figure 4-5: Antioxidant activity in fruit processing wastewaters measured using the of β-carotene linoleic acid model system. Experiments conducted in triplicate and the mean values are shown with SEM

Pearson's correlation coefficient test showed there is a strong linear relationship between the total phenolics content and the ability to inhibit β-carotene bleaching, since R= 0.85, again confirming the role of total phenolics on the antioxidant action of the wastewater samples. In the present study, the results from the β-CLAMS assay reveal that the wastewater samples contain antioxidants that are capable of stabilising the reactive lipidic radicals that arise during the oxidation of linolenic acid, thereby terminating the propagation of free radicals. This will be useful in the food industry, since a major cause of rancidity in foods that contain fat is lipid oxidation (Reyes-Caudillo *et al.*, 2008).

4.3.5 Summary of the antioxidant activity of the fruit processing wastewaters using various assays

Various antioxidant activity assays were used to determine the antioxidant activity of the fruit processing wastewaters as discussed in the previous section. The results obtained are summarised in Table 4.5. The antioxidant activity of a sample measured depends on the assay used and on which free radical or oxidant used in the measurement. Therefore, the evaluation of the different analytical methods was helpful for better understanding and interpreting the results obtained. In order to understand the contribution of phenolic compounds to the antioxidant activity of the wastewater samples, high performance liquid chromatography (HPLC) was used to identify and quantify individual phenolic compounds as explained in the following section.

Table 4.5: Summary of the antioxidant activities of the fruit processing wastewaters using various antioxidant activity assays

Sample	% Antioxidant activity			
	DPPH assay (%RSA)	ABTS^{+} assay (%RSA)	FRAP assay* (GAE)	β-CLAMS assay (%inhibition)
AW1	22.05±0.01	13.80±0.01	0.0001±0.0	15.20±0.01
AW2	29.32±0.01	30.30±0.02	0.005±0.0001	29.30±0.01
CW1	29.31±0.01	21.63±0.01	0.016±0.001	49.20±0.01
CW2	43.37±0.02	55.49±0.01	0.023±0.002	69.04±0.02
SW	100.00±0.01	74.67±0.02	0.03±001	86.00±0.02

** Reducing power in the FRAP assay was measured as gallic acid equivalents*

4.4 Characterisation of phenolic compounds present in fruit processing wastewaters by HPLC

The results of the antioxidant assays discussed above showed capacities for radical scavenging, reducing power and the lipid peroxidation inhibition in the fruit processing wastewater samples. However, these assays gave no indication of the individual phenolic compounds and their concentrations in the wastewater samples. High performance liquid chromatography (HPLC) analysis was used to identify the individual phenolic components of fruit processing wastewaters. This analysis was performed using detection at 280nm because all aromatic polyphenolic compounds absorb at 280nm, whereas at this wavelength, aliphatic compounds such as sugars and organic acids do not absorb (Alen-Ruiz *et al.*, 2009).

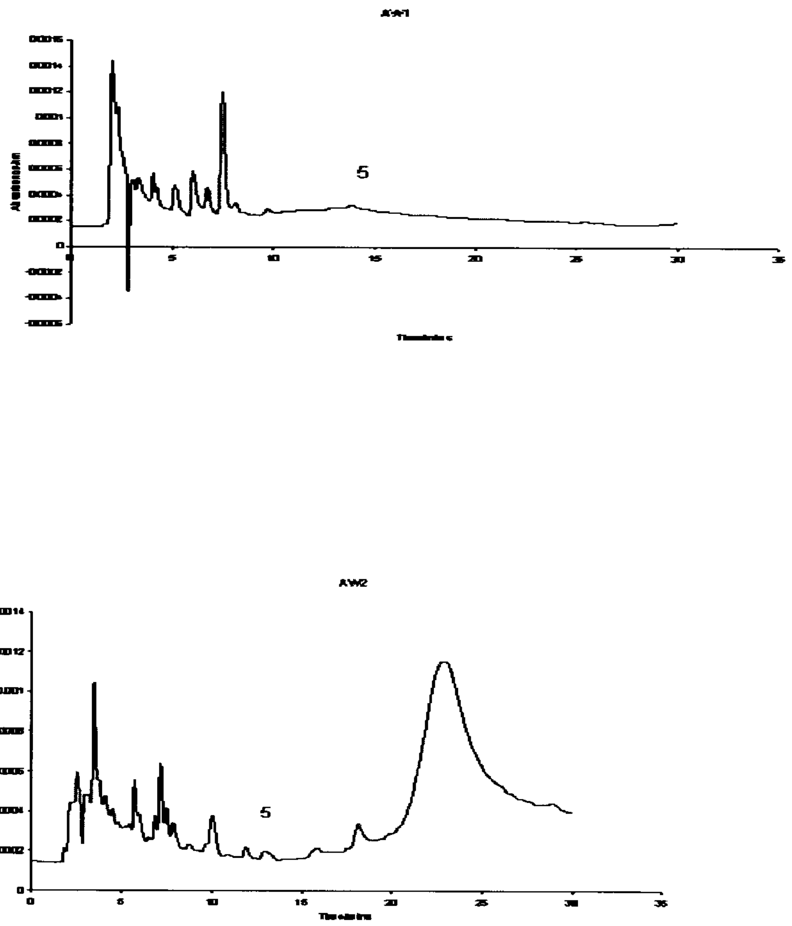
HPLC allows for analysis of phenolic compounds with high precision, sensitivity and within a reasonable time. Resveratrol, gallic acid, catechin, caffeic acid, chlorogenic acid, epicatechin and p-coumaric acid were used as standards. The standards were chosen based on the results published in literature where gallic acid, caffeic acid, chlorogenic acid, p-coumaric acid were identified in orange juices (Kelebek *et al.*, 2008) and resveratrol, catechin and epicatechin were also detected in deciduous fruits (Suarez *et al.*, 1996; Lu and Foo, 2000). The choice of standards was limited by the availability of the standards at the time the analysis was conducted. Spiking experiments were conducted where appropriate. The retention times of the standards was used to identify peaks of the standard compounds in the sample wastewaters.

Figures 4.6 to 4.8 show the HPLC profiles of the wastewater samples studied here. The concentrations of individual phenolic compounds that were identified in the fruit processing wastewaters are summarised in Table 4.6. All major phenolic compounds were separated with good resolution. Chlorogenic acid was found to be a common component in most of the wastewater samples. However, epicatechin and p-coumaric acid were not detected in any of the wastewaters. It was expected that p-coumaric acid would be detected in both the citrus and apple wastewaters as these were previously detected in studies conducted by other researchers in the citrus and apple fruits (Goristein *et al.*, 2001; Hagen *et al.*, 2007).

Table 4.6: Concentrations of respective phenolic compounds in fruit processing wastewaters as determined by HPLC analysis

Sample	Standard/concentration (mg/L)						
	Resveratrol	Gallic Acid	Catechin	Caffeic Acid	Chlorogenic Acid	Epicatechin	p-coumaric acid
AW1	-*	-	-	-	0.30	-	-
AW2	-	-	-	-	0.33	-	-
CW1	-	-	-	-	-	-	-
CW2	-	-	1.60	-	0.36	-	-
SW	0.60	101.40	8.10	-	10.53	-	-

** -denotes that the phenolic compound was not identified*



(A)

(B)

Figure 4-6: HPLC chromatogram for apple processing wastewater AW1 (A) and AW2 (B). The peak with retention time of 13.43 minutes corresponds to chlorogenic acid

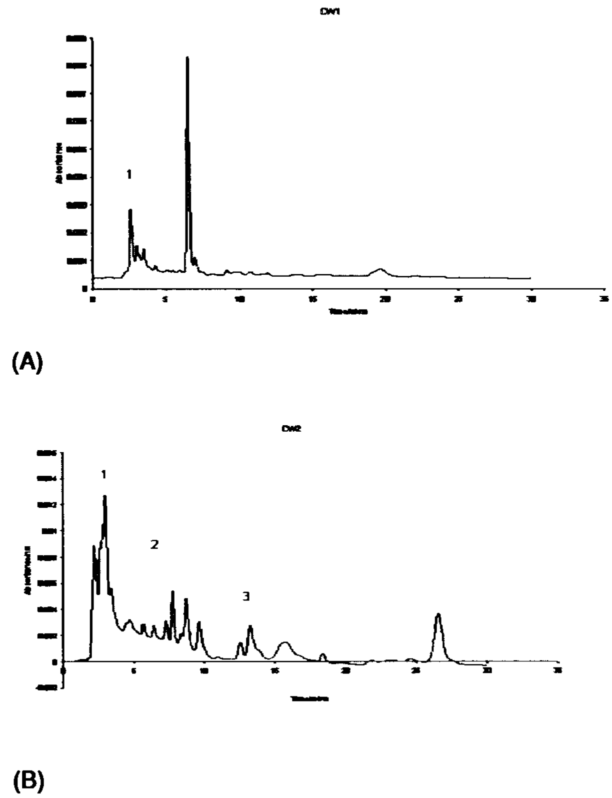


Figure 4-7: HPLC chromatogram for citrus processing wastewater CW1 (A) and CW2 (B). The peaks shown in (B) with the retention times of 7.38 and 13.43 minutes correspond to catechin and chlorogenic acid respectively.

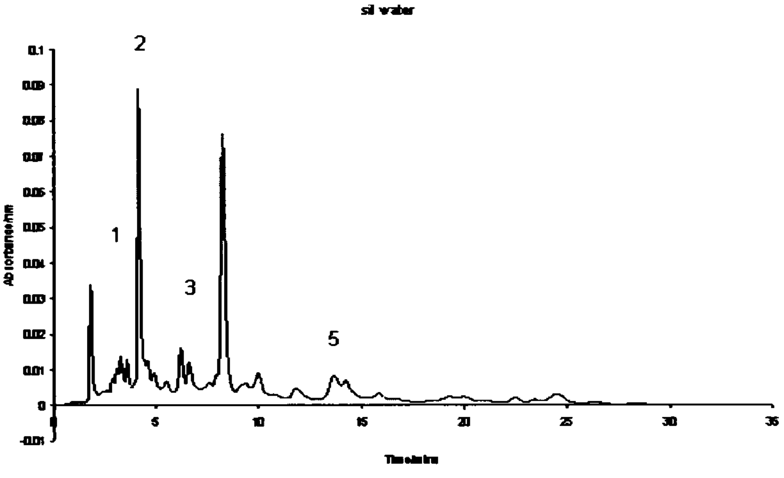


Figure 4-8: HPLC chromatogram of silage water SW. The peaks with the retention times of 2.95, 4.16, 7.38 and 13.43 minutes correspond to resveratrol, gallic acid, catechin and chlorogenic acid respectively

Phenolic compounds in apples include hydroxycinnamic acids such as caffeic, p-coumaric, ferulic and chlorogenic acid; flavonols such as quercetin glycosides and flavanols such as catechin and epicatechin (Adil *et al.*, 2007; Hagen *et al.*, 2007). However, chlorogenic acid was the only phenolic compound identified in apple wastewaters AW1 and AW2, at concentrations of 0.3 and 0.33 mg/L respectively. The concentrations were very low in both wastewaters, presumably due to dilution that occurs during processing. Chlorogenic acid and p-coumaric acid were also identified as two of the major phenolic compounds in apple juices studied by Suarez *et al.*, (1996) at concentrations ranging from 151 to 154 mg/L.

Only a few phenolic compounds in very low concentrations were identified in the citrus wastewaters CW1 and CW2. Catechin and chlorogenic acid, at concentrations of 1.6 and 0.36 mg/L respectively, were identified in CW2, whereas no phenolic compounds were detected in CW1 using the available standards. The low concentrations of phenolic compounds identified in the citrus wastewaters may be because the wastewaters were collected at a point where primary treatment (to remove insoluble particles) and secondary biological treatment (to remove dissolved organics) had been done. In addition, the pulp wash was further diluted with water collected from cleaning the production equipment, further diluting the phenolic compounds. Chlorogenic acid was reported in blood orange juices at concentrations ranging from 12.87 to 15.08 mg/L (Kelebek *et al.*, 2008), these concentrations being higher than the citrus wastewater samples studied here as would be expected (Kelebek *et al.*, 2008). Other phenolic compounds that were expected to be detected in citrus wastewaters were caffeic acid, p-coumaric acid and gallic acid as these were also identified in blood orange juices (Kelebek *et al.*, 2008).

The silage water was found to contain gallic acid, at a concentration of 101.4 mg/L. The silage water also contained chlorogenic acid, at a concentration of 10.53 mg/L. Resveratrol and catechin were also detected in the silage water at concentrations of 0.6 and 8.1 mg/L respectively.

The information obtained on the HPLC analyses of the fruit processing wastewater samples give an indication of the concentrations of phenolic antioxidants that could be extracted from them. Thus the next step in the study was to investigate the extraction of phenolic compounds from the wastewaters, as described in the following sections.

4.5 The extraction of phenolic compounds from fruit processing wastewaters using various extraction techniques

The previous sections reported the antioxidant activity of the antioxidants present in the wastewater samples determined using various antioxidant assays. Furthermore, HPLC analyses gave information on the concentrations of individual phenolic compounds present in the wastewater samples. The antioxidant activity was attributed to the presence of phenolic compounds. However, in order to make use of the phenolic antioxidants present in the fruit processing wastewaters at a commercial scale, it is necessary for them to be extracted from the wastewaters. The extraction of phenolic compounds requires special care because they are easily oxidised and rapidly degraded by light (Herrera and de Castro, 2005). Several different sample preparation and extraction techniques are available for the recovery of phenolic compounds as discussed in Section 2.6. The following sections describe five different extraction techniques that were employed in this study for the recovery of phenolic antioxidants from the fruit processing wastewaters. These techniques are: solvent extraction, solid phase extraction, extraction using PVPP as a solid phase adsorbent, supported liquid membrane extraction and supercritical fluid extraction. The choice of these methods and the antioxidant capacity of the extracts obtained using each extraction technique, are also discussed in the following sections.

4.5.1 The extraction of phenolic compounds from fruit processing wastewaters using organic solvent extraction

This section describes the extraction of phenolic compounds from fruit processing wastewaters using two different organic solvents. The total phenolics concentrations obtained after solvent extraction were then compared with concentrations of total phenolics obtained using other extraction techniques such as solid phase extraction, supported liquid membrane extraction and extraction using PVPP as a solid phase adsorbent, (which are discussed in subsequent sections). Also, the antioxidant activities of the solvent extracts were determined and compared with the antioxidant activities of extracts obtained using other extraction techniques.

In the present study, solvent extraction was conducted using ethyl acetate and hexane. Ethyl acetate was chosen because it produces non-toxic extracts that can be used in the food industry. Hexane was chosen in order to compare the effect of solvent polarity on the extraction yield, as well as providing an alternative solvent for use in producing non-consumable extracts that can be used in products or materials requiring protection against oxidation (Germanis, 2006). The fruit processing wastewaters were mixed with the organic solvent at a ratio of 1:3 and extracted three times. The extracts were then pooled and the solvent was removed using low temperature evaporation, leaving a concentrated extract of the phenolic compounds that was redissolved in standardised volumes of methanol (Abad-Garcia *et al.*, 2007). The antioxidant activity of the solvent extracts was determined using the DPPH radical scavenging assay. The details of the method are discussed in detail in Section 3.12. Table 4.7 shows the total phenolics contents (measured using the Folin C method) before and after ethyl acetate or hexane extraction. The total phenolic contents of the extracts were corrected for variation in volumes and the results are expressed as mg/L of wastewater.

Generally, ethyl acetate was a better solvent for the extraction of phenolic compounds from fruit processing wastewaters, as compared with hexane; with the exception of AW1, the extracts obtained using ethyl acetate showed higher concentrations of total phenolics than the hexane extracts. For AW1, the ethyl acetate extract contained 0.93 mg/L total phenolics whilst the hexane extract contained 1.13 mg/L. The ethyl acetate extract of AW2 had a total phenolics content of 4.39 mg/L whilst the hexane extract had 1.74 mg/L. Also, for the citrus wastewaters CW1 and CW2, the ethyl acetate extracts exhibited higher quantities of total phenolics of 5.23 mg/L and 51.3 mg/L respectively, whereas the hexane extracts had values of 0.81 mg/L and 12.84 mg/L respectively. For the silage water SW, the ethyl acetate extract had total phenolics concentrations of 147.66 mg/L whilst the hexane extract contained only 3.34 mg/L.

Table 4.7: The total phenolics contents of the fruit processing wastewaters and amounts obtained after extraction with ethyl acetate or hexane

Sample	Parameter		
	Total phenolics content in original wastewater (GAE mg/L)	**Total phenolics obtained by ethyl acetate extraction (GAE mg/L)**	**Total phenolics obtained by hexane extraction (GAE mg/L)**
AW1	2.8 ± 0.2	0.93 ± 0.1	1.13 ± 0.1
AW2	9.2 ± 0.2	4.39 ± 0.7	1.74 ± 0.6
CW1	25.3 ± 0.1	5.23 ± 0.2	0.81 ± 0.2
CW2	137 ± 0.3	51.3 ± 0.1	12.84 ± 0.3
SW	727.8 ± 5.8	147.66 ± 5.7	3.34 ± 0.2

The extraction efficiencies of the ethyl acetate and hexane extracts are shown in Figure 4.9. The extraction efficiency was defined as the ratio of the amount of phenolic compounds obtained by solvent extraction, to the amount of phenolic compounds in the same volume of wastewater sample before extraction, and expressing the result as a percentage. Generally, ethyl acetate exhibited higher extraction efficiencies compared with hexane in all the wastewater samples studied except in AW1, as illustrated in Figure 4.9. Apple wastewater AW2 gave the highest extraction efficiency of 47.73% and the silage water SW gave the lowest extraction efficiency of 20.29% using ethyl acetate as the solvent for the extraction of phenolic compounds. The citrus wastewaters CW1 and CW2 gave extraction efficiencies of 20.65% and 37.45% using ethyl acetate. AW1 gave an extraction efficiency of 33.04% using ethyl acetate. When hexane was used, the extraction efficiencies ranged from 0.46% in SW to 40.18% in AW1. The citrus wastewaters CW1 and CW2 likewise exhibited lower extraction efficiencies of 3.21% and 9.37% respectively, using hexane. AW2 exhibited an extraction efficiency of 18.9% using hexane. This indicates that ethyl acetate is a better organic solvent compared with hexane for the extraction of phenolic compounds in the studied wastewater samples. Ethyl acetate, a more polar solvent compared with hexane, showed higher extraction efficiency due to its polarity and better solubility for phenolic compounds present in the fruit processing wastewaters. This shows that the fruit processing wastewaters contain polar compounds such as phenolic acids since compounds which have a polarity similar to the extracting solvent are able to dissolve more than those with differing polarities (Mohsen and Ammar, 2009). The silage water, which had gallic acid as its major phenolic compound as discussed in Section

4.4, exhibited the lowest extraction efficiency using hexane because gallic acid is a very polar phenolic compound and thus its solubility in a non-polar solvent like hexane is very poor.

Various studies similar to this have been reported in the literature. In a study conducted by de Leonardis *et al.*, (2007), the extraction yield of total phenolics from olive mill wastewaters was 500 mg/L wastewater, using ethyl acetate as the solvent for extraction. This extraction yield is relatively high compared with the results obtained in the present study where extraction yields of total phenolics ranged from 0.93 mg/L wastewater, to 147.66 mg/L wastewater, using ethyl acetate as the solvent for extraction. The extraction efficiency of ethyl acetate on extracting phenolic compounds from rice koji in a study conducted by Yen *et al.*, (2003) was only 0.84%, a value significantly lower than the results reported in the present study where extraction efficiency using ethyl acetate ranged from 20.3% to 47.7%. Ethyl acetate is frequently used to extract bio-phenols from aqueous matrices such as olive mill waste waters (De Marco *et al.*, 2007). In this example, hexane, a low polarity solvent, showed much lower extraction efficiencies compared with ethyl acetate, a more polar solvent. This was also shown in a study conducted on the extraction of total phenolics in corn tassel extracts where hexane extracted the least amounts of total phenolics, only managing to extract 0.01% of the total phenolics (Mohsen and Ammar, 2009). These results are also in agreement with a previous study conducted on mashua tubers, which reported that solvents with very low polarity, such as chloroform and hexane, did not give good extraction results (Chirinos *et al.*, 2007; Pinelo *et al.*, 2009).

However, these results differ from results obtained in a study conducted by Li *et al.*, (2007) where the solvents, ethyl acetate, hexane and water were used to extract phenolic compounds from microalgae. It was found that hexane extracted the highest concentration of total phenolics compared with either ethyl acetate or water. The total phenolics content of hexane extracts ranged from 2.12 to 39.87 mg/g GAE, whilst the ethyl acetate and water extracts ranged from 0.01 to 9.8 mg/g GAE and 0.95 to 10.65 mg/g GAE (Li *et al.*, 2007). These results emphasize the fact that the extraction of phenolic compounds depends on, amongst other factors, the polarity of the phenolic compounds in the extract and the solvent being used.

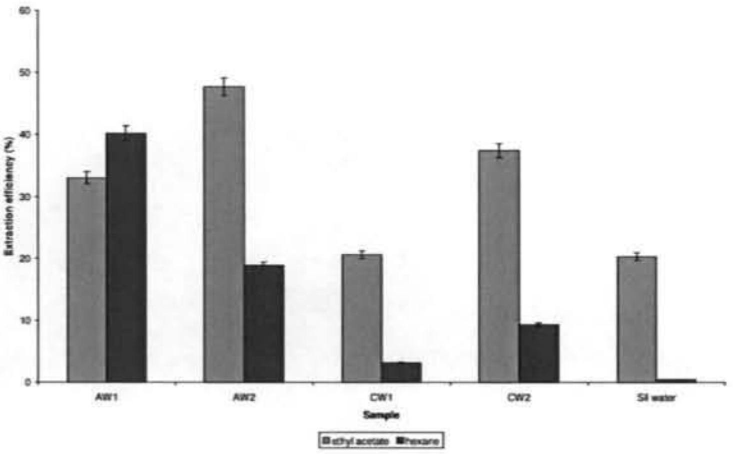


Figure 4-9: Extraction efficiencies of the solvents ethyl acetate and hexane, used for the extraction of phenolic compounds from fruit processing wastewaters. Extraction efficiency is calculated as the ratio of the amount of phenolic compounds obtained by solvent extraction, to the amount of phenolic compounds in the same volume of wastewater sample before extraction, and expressing the result as a percentage. Error bars indicate SEM of duplicate samples.

4.5.1.1 Determination of the antioxidant activity of the extracts obtained after solvent extraction of the fruit processing wastewaters

The free radical quenching potentials of the ethyl acetate and hexane extracts were evaluated using the DPPH radical scavenging assay and the results are shown in Table 4.8. Generally, the ethyl acetate extracts exhibited better scavenging activity than the hexane extracts. Among the ethyl acetate extracts, the silage water extract SW exhibited the highest antioxidant activity for the ethyl acetate extracts (> 80%); and the apple wastewater sample AW1 showed the lowest antioxidant activity of 26%. The AW2 ethyl acetate extract exhibited a radical scavenging activity of 46.7% whilst the citrus wastewaters CW1 and CW2 ethyl acetate extracts showed radical scavenging activities of 54.4% and 38.5% respectively. The antioxidant activity of hexane extracts was generally low with the silage water extract SW having the highest radical scavenging activity of 15.13%. The apple wastewaters AW1 and AW2 hexane extracts exhibited radical scavenging activities of 5.55% and 9.9% respectively whilst the citrus wastewaters CW1 and CW2 hexane extracts showed radical scavenging activities of 4.21% and 11.89% respectively.

Table 4.8: Antioxidant activities of ethyl acetate and hexane extracts using the DPPH assay

Sample	Wastewater (original)		Ethyl acetate extracts		Hexane extracts	
	%RSA	TP*	%RSA	TP	%RSA	TP
AW1	22.05	7.61	26.7 ± 0.1	0.93 ± 0.1	5.55 ± 0.3	1.13 ± 0.1
AW2	29.32	42.45	46.7 ± 0.6	4.39 ± 0.7	9.9 ± 0.5	1.74 ± 0.6
CW1	29.31	18.60	38.5 ± 0.1	5.23 ± 0.2	4.21 ± 0.1	0.81 ± 0.2
CW2	43.37	137.00	54.4 ± 0.3	51.3 ± 0.1	11.89 ± 0.2	12.84 ± 0.3
SW	100	399.52	86.3 ± 0.4	147.66 ± 0.20.15.7	15.13 ± 0.1	3.34 ± 0.2

TP denotes total phenolics content in mg/L GAE (based on 1L of wastewater)*

In order to determine the potency of the phenolic antioxidants in the extracts to scavenge for free radicals, in this case, the DPPH free radical, the ratio of % RSA of the extracts to the concentration of total phenolics in the extracts was calculated, and the results are shown in Table 4.9, and the higher the ratio, the higher the scavenging effect and potency of the phenolic antioxidants in the extract. From Table 4.9, the ethyl acetate extract of AW1 is the most potent extract compared with the other extracts. This is followed by AW2 ethyl acetate extract with a value of 10.64. CW2 hexane extract showed the least potency in scavenging for the DPPH free radical with a value of 0.63.

Generally, the ethyl acetate extracts exhibited higher ratios compared with the hexane extracts, except in the case of the silage water where the ratio was 1.71 for the ethyl acetate extract and 4.53 for the hexane extract. This observation made on the silage water is comparable with the results observed in an investigation into the scavenging effect of phenolic compounds extracted from seeds of *C. chinesis.* using hexane and ethyl acetate as the solvents for extraction, where the hexane extract showed a higher scavenging effect compared with ethyl acetate (Yen *et al.*, 2008). Such results would indicate that hexane extracted phenolic antioxidants from the silage water containing a higher proportion of primary antioxidants that are radical scavengers, compared with the phenolic antioxidants extracted by ethyl acetate. The results obtained in the present study show that both ethyl acetate and hexane extracts contain powerful free radical scavengers compared with results obtained in a study conducted by Yen *et al.*, (2003) where the ratio of radical scavenging activity to the concentration of total phenolics using ethyl acetate as the solvent for extraction, ranged from 0.1 to 0.15 at concentrations ranging between 200 mg/L and 800 mg/L.

Table 4.9: Ratios of the radical scavenging activity to the concentration of total phenolics for fruit processing wastewater samples after solvent extraction using ethyl acetate or hexane

Sample	Ratio of % RSA to TP*		
	Original wastewater	Ethyl acetate extract	Hexane extract
AW1	2.90	28.7	8.76
AW2	0.69	10.64	3.19
CW1	1.88	7.37	5.20
CW2	0.32	1.06	0.63
SW	0.25	1.71	4.53

TP denotes total phenolics content in mg/L GAE originating from 1L wastewater*

4.5.1.2 Summary of the extraction of phenolic compounds from fruit processing wastewaters using solvent extraction

The extraction of phenolic compounds from fruit processing wastewaters using ethyl acetate and hexane showed that ethyl acetate is a better solvent for the recovery of phenolic antioxidants in these samples. Investigations into the antioxidant activities of the extracts also showed that ethyl acetate extracts exhibited higher antioxidant activity compared with hexane extracts.

Since the extracts obtained after solvent extraction may contain traces of organic solvent, which may make the use of these extracts in the food industry impossible, it was necessary to further investigate other extraction techniques, such as solid phase extraction, which uses relatively small amounts of solvent.

4.5.2 The extraction of phenolic compounds from fruit processing wastewaters using solid phase extraction

The use of organic solvents for the extraction of phenolic compounds as described in the previous section has the main disadvantage of using large quantities of organic solvents that would need to be recycled or would be costly to dispose of. An alternative to solvent extraction is solid phase extraction. Solid phase extraction (SPE) is an efficient technique that utilises minimal quantities of organic solvent and thus could be a better technique for the recovery of phenolic compounds from fruit processing wastewaters, compared with solvent extraction. SPE is particularly well adapted to the extraction, separation and/or analysis of samples containing compounds with a wide range of polarity, such as phenolic compounds (Pietrogrande and Basaglia, 2007). The aim of this investigation was thus to determine the quantities of phenolic compounds that could be obtained from fruit processing wastewaters using solid phase extraction and also to determine the antioxidant activity of the fractions obtained after such extraction.

In the present study, SPE was conducted for the fractionation of acidic and neutral phenolic compounds from fruit processing wastewaters by using two C-18 Sep-Pak® cartridges. The principle by which the cartridges work is described in Section 2.6.4. The method used was adapted from Suarez *et al.*, (1996). Neutral cartridges were pre-conditioned with methanol and distilled water, while acidic cartridges were pre-conditioned with methanol and dilute HCl, consecutively. In each run, a known volume of the wastewater sample was adjusted to pH 7 by drop-wise addition of sodium hydroxide before being loaded onto the neutral cartridge. The effluent from the separation of neutral phenolics was then adjusted to pH 2 using HCl and passed through the acidic cartridge. The adsorbed phenolic compounds on the neutral and acidic pre-conditioned cartridges were eluted with methanol and the extracts analysed by HPLC. The method is described in detail in Section 3.13. The total phenolics contents of the neutral and acidic fractions were determined by the Folin C assay. The antioxidant activities of the extracts were determined by the DPPH radical scavenging assay.

The total phenolics contents (recorded as the mean with the standard error of the mean) of the neutral and acidic fractions of the fruit processing wastewater samples measured after SPE are shown in Table 4.10. Figure 4.10 to 4.14 show the HPLC profiles of neutral and acidic phenolics separated using C-18 Sep-Pak® cartridges. In all the wastewater samples, the neutral phenolics fraction contained higher concentrations of total phenolics compared with the acidic fraction. The neutral fractions of the apple wastewaters AW1 and AW2 gave total phenolics contents of 3.46 mg/L and 12 mg/L respectively, whilst their corresponding acidic fractions gave values of 2.34 mg/L and 0.2 mg/L respectively. For the citrus wastewater samples, CW1 and CW2, the neutral fractions had concentrations of 11.36 mg/L and 21.59 mg/L of total phenolics respectively, whilst the acidic fractions contained 9.34 mg/L and 22 mg/L total phenolics. Also, the silage water SW neutral fraction contained 298.49 mg/L whilst the acidic fraction contained 27 mg/L total phenolics.

Table 4.10: Total phenolics contents of the neutral and acidic fractions of wastewater samples after SPE using C-18 Sep-Pak ® cartridges

Sample	Total phenolics (mg/L) GAE	
	Neutral fraction	Acidic fraction
AW1	3.46± 0.2	2.34± 0.1
AW1	12.00± 0.1	0.20± 0.1
CW1	11.36± 0.3	9.34± 0.2
CW2	21.59±0.1	22.00±0.3
SW	298.49± 0.2	27.00± 0.1

HPLC analysis of the silage water SW showed the presence of gallic acid in the neutral fraction and catechin in the acidic fraction (Figure 4.10). Gallic acid was also detected in the neutral fraction of citrus wastewater sample CW1 as shown in Figure 4.12. The extractions were repeated to confirm these results. The results were not as predicted because gallic acid was expected to be detected in the acidic fraction, and catechin in the neutral fractions only. These extractions were not efficient in separating neutral and acidic phenolics in the wastewater samples.

The C-18 Sep-Pak® cartridges were better able to fractionate neutral and acidic phenolics in citrus wastewater sample CW2 than in the silage water SW. This is reflected by the detection of catechin in the neutral fraction only whilst the phenolic acids gallic acid and chlorogenic acid were only identified in the acidic fraction (Figure 4.11). No phenolic compounds were detected in the acidic fraction of CW1 wastewater sample (Figure 4.12). The phenolic compounds detected in the neutral and acidic fractions of apple wastewater AW1 could not be identified using the standards available.

The extraction efficiencies (recorded as the mean with the standard error of the mean) obtained for the wastewater samples using C-18 Sep-Pak® cartridges are shown in Table 4.11. The extraction efficiencies were tabulated with the standard deviations. It was observed that 56.9% of the total phenolics present in the silage water SW were extracted using SPE. The apple wastewater samples AW1 and AW2 showed extraction efficiencies of 52.1% and 51.9% respectively, whilst the citrus wastewater samples CW1 and CW2 showed extraction efficiencies of 49.5% and 44.1% respectively.

Table 4.11: Total phenolics content before and after SPE and extraction efficiencies

Sample	Parameter		
	TP before SPE	TP after SPE	Extraction efficiency (%)
AW1	11.13± 0.1	5.80± 0.3	52.1± 0.3
AW2	23.51± 0.1	12.20± 0.2	51.9± 0.1
CW1	41.81± 0.2	20.70± 0.1	49.5± 0.7
CW2	98.84±0.3	43.59± 0.3	44.1± 0.5
SW	1014.92± 0.2	577.49± 0.1	56.9± 0.9

The mechanism of retention of phenolic compounds by C18 cartridges is dependent on non-covalent and polar interactions between the phenolic compounds and the sorbent (Michalkiewicz *et al.*, 2008). Thus, the presence of gallic acid in the neutral fractions can be attributed to its high polarity, making its dissociation during the elution stage difficult. The higher carbon loading and pore diameter of cartridges supplied by Waters might also account for the greater retention of the ionised form of phenolic acids such as chlorogenic acid in the neutral fraction (Chen *et al.*, 2001).

In the solid phase extraction investigation, the extraction efficiencies for apple wastewaters AW1 and AW2 were found to be 52.3 and 51.9% respectively. The results reported in a study conducted on the determination of neutral phenolic compounds in apple juices showed recoveries ranging from 90 to 100% using C18 Sep Pak cartridges (Gomis *et al.*, 2001) and, in another study, recoveries of phenolics from different apple products ranged between 90% and 100% for all compounds (Suarez *et al.*, 1996). In both cases, the extraction efficiencies were higher compared with the results obtained in the wastewater samples studied here. Although the wastewaters were more dilute than the fruit juices or by products, it would be expected that the fruit juices and wastewater samples would have similar compositions with regards to the nature of the phenolic compounds present, and would differ only in their concentrations. The solid phase extraction technique is based on adsorption of the phenolics as they elute through the cartridge, and this is not strictly a concentration-dependent process. Thus, the extraction efficiency for phenolic compounds from either fruit juices or fruit processing wastewaters should be similar. The differences may be attributable to the stability of phenolic compounds on the adsorbent which is affected by storage conditions prior to extraction. For example, it was found that the stability of phenolic compounds depended on the water matrix, storage temperature and other physical and chemical properties (Leo and Nollet, 2000).

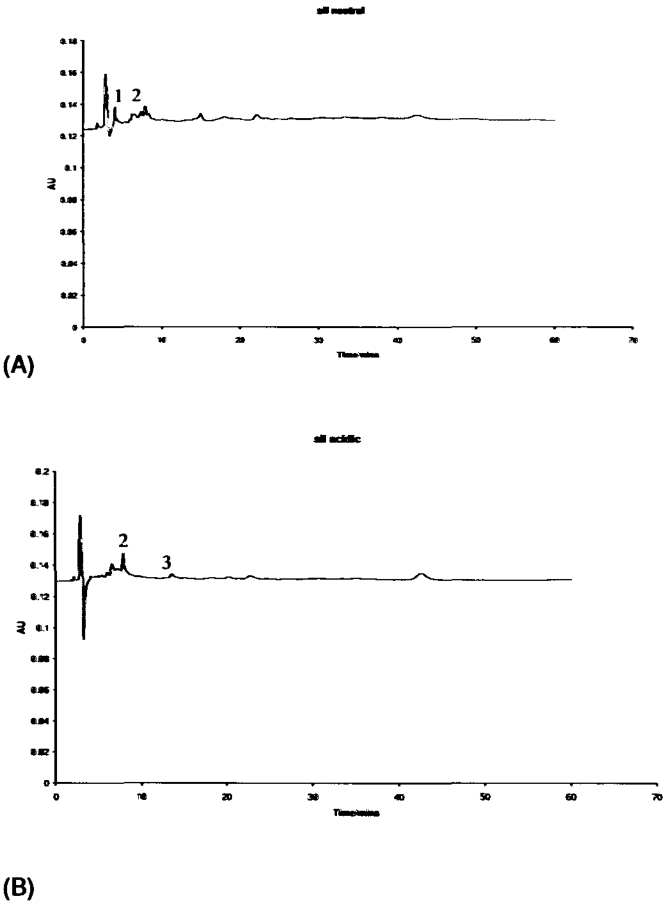
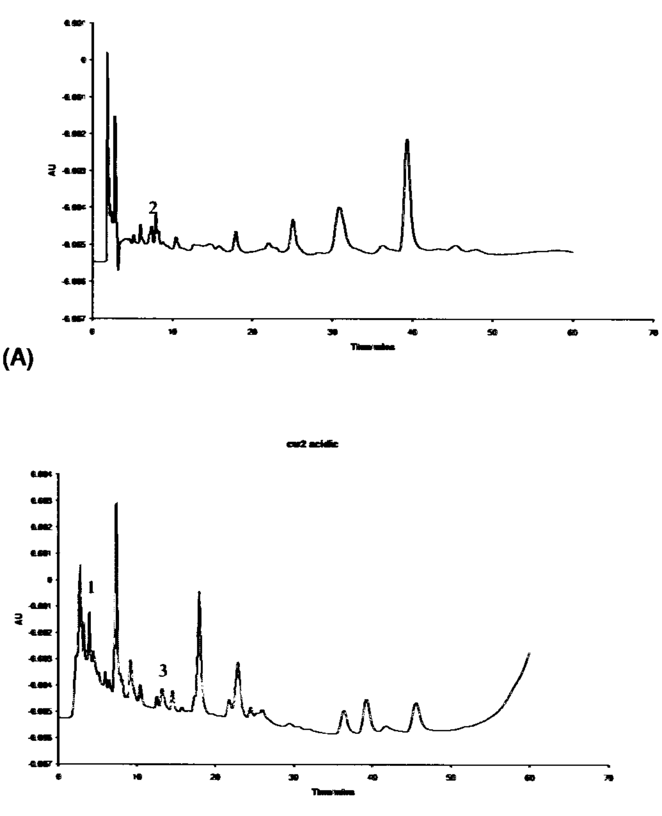


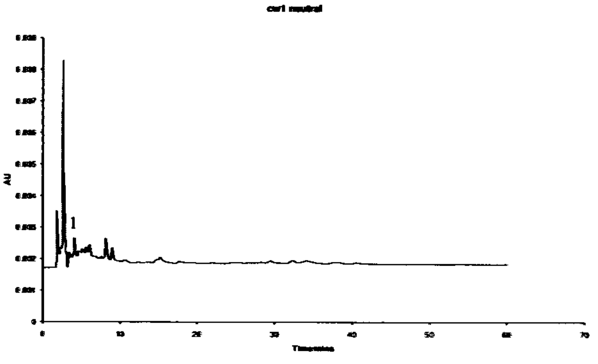
Figure 4-10: HPLC chromatogram of the neutral phenolics (A) and acidic phenolics (B) present in the silage water SW after SPE and elution using methanol. The peaks labelled 1, 2 and 3 correspond to gallic acid, catechin and chlorogenic acid respectively.



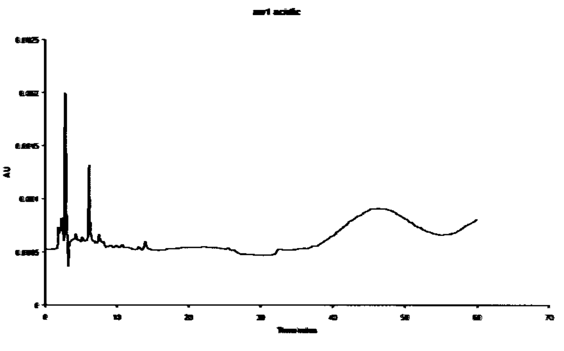
(A)

(B)

Figure 4-11: HPLC chromatogram for the neutral phenolics (A) and acidic phenolics (B) extracted from CW2 citrus wastewater sample after SPE and elution with methanol. The peaks labelled 1, 2 and 3 correspond to gallic acid, catechin and chlorogenic acid respectively.

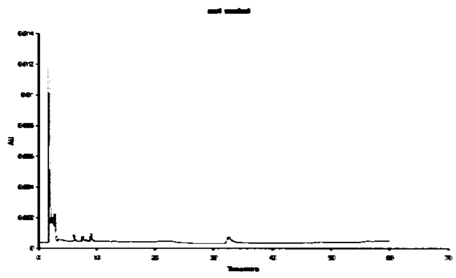


(A)

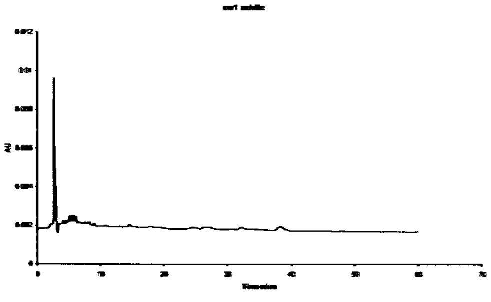


(B)

Figure 4-12 : HPLC chromatogram of neutral phenolics (A) and acidic phenolics (B) extracted from CW1 citrus wastewater sample and elution with methanol. The peak labelled 1 corresponds to gallic acid



(A)



(B)

Figure 4-13: HPLC chromatogram for neutral phenolics (A) and acidic phenolics (B) extracted from AW1 apple wastewater samples using SPE and elution with methanol. No peaks could be identified.

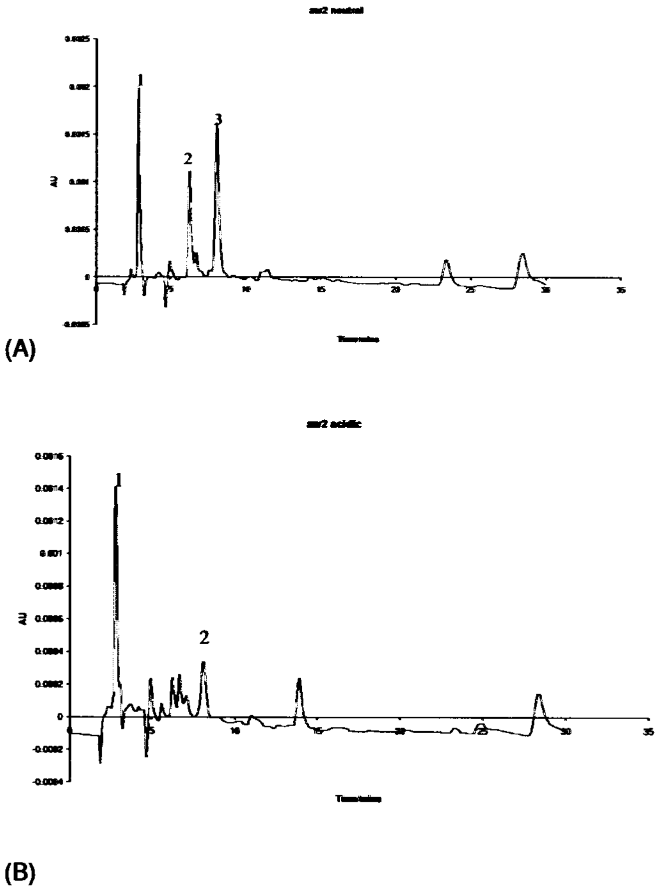


Figure 4-14: HPLC chromatogram for neutral (A) and acidic (B) phenolics extracted from AW2 apple wastewater sample. No peaks could be identified.

4.5.2.1 Determination of the antioxidant activity of the neutral and acidic extracts obtained after SPE

The aim of this part of the study was to determine the antioxidant activity of the neutral and acidic phenolics obtained after SPE, thereby identifying the fraction that contributes more to the antioxidant activity of phenolic compounds present in the fruit processing wastewaters. After SPE, the neutral and acidic phenolics were eluted from the respective neutral and acidic preconditioned C18 Sep-Pak® cartridges using methanol. The neutral and acidic extracts (0.1 ml) were each mixed with methanolic DPPH (3.9 ml) and the reaction was monitored at 515nm until steady state was reached. Figure 4.15 shows the radical scavenging activities of the neutral and acidic phenolics.

In general, the neutral phenolics fraction exhibited higher antioxidant activity compared with the acidic fraction, with the exception of CW2. This exception may be attributed to the observed high pH of 11 for CW2 which would influence the extraction of phenolic compounds using an adsorbent. The neutral fractions of the apple wastewater samples AW1 and AW2 had radical scavenging activities of 24.7 and 12.26% respectively, whilst the acidic fractions had values of 16.7 and 0.2% for AW1 and AW2 respectively. The neutral fractions of the citrus wastewater samples CW1 and CW2 exhibited radical scavenging activities of 10.78 and 55.9% respectively, whilst the acidic fractions had values of 8.87 and 46.56% respectively. The neutral fraction of the silage water SW exhibited higher radical scavenging activity of 85.48% compared with the acidic fraction (79.9%).

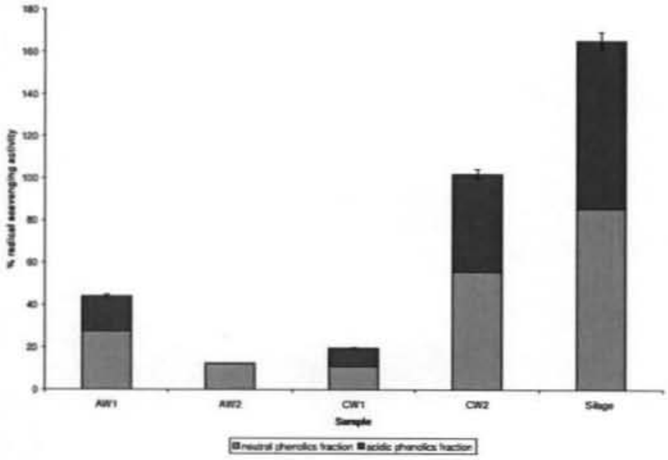


Figure 4-15: % DPPH radical scavenging assay for neutral and acidic phenolics of fruit processing wastewaters after SPE using C18 Sep Pak® cartridges.

In the citrus wastewater CW2 and silage water SW, the combined total antioxidant activities measured for the neutral and acidic fractions were 102.45% and 165.38% respectively, values greater than 100%. This may be because the antioxidant activity of the wastewater extracts depends on the synergistic action of the constituents of the neutral and acidic fractions, with the relative contribution of the neutral phenolic fraction to the antioxidant activity being greater than the acidic fraction. Significant antioxidant activity (89.5%) was also observed in the neutral fractions of cranberry extracts (Tumbas *et al.*, 2007). Thus, this shows the potential of using SPE to obtain extracts of neutral phenolics that exhibit high antioxidant activity.

4.5.2.2 Summary of the extraction of phenolic compounds from fruit processing wastewaters using solid phase extraction

Solid phase extraction of the fruit processing wastewaters using C18 Sep Pak® cartridges resulted in the separation of the phenolic compounds into neutral and acidic phenolic fractions. However, in some instances, neutral phenolics were found to be present in the acidic fraction, or acidic phenolics were detected in the neutral fraction. The neutral fractions of the wastewaters generally exhibited higher antioxidant activity compared with the acidic fraction. Further investigations into optimising the solid phase extraction technique or using a different solid phase adsorbent, would make this technique potentially useful in areas such as the research and development of antioxidants. Assessment of the feasibility of using solid phase extraction to recover phenolic antioxidants will be discussed in later sections.

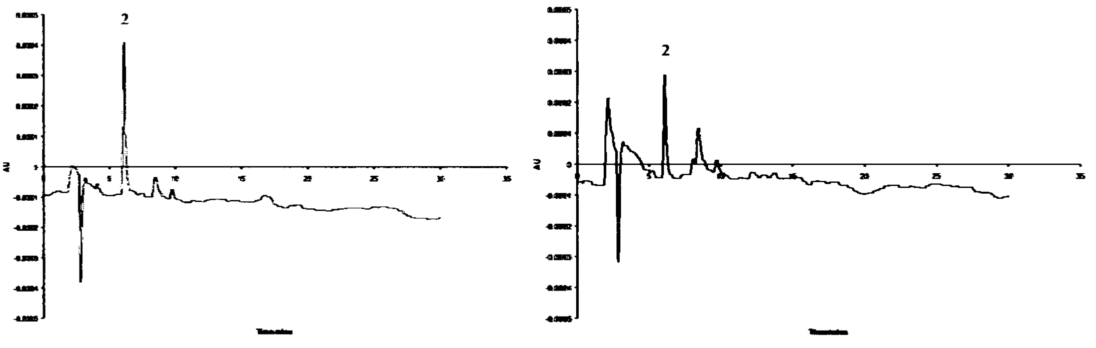
4.5.3 The extraction of phenolic compounds from fruit processing wastewaters using Polyvinylpoly pyrrolidone (PVPP) as a solid phase adsorbent

The previous section described the use of solid phase extraction for the extraction of phenolic compounds in fruit processing wastewaters. In this section, polyvinylpoly pyrrolidone (PVPP) was used as a solid phase adsorbent to extract phenolic compounds from fruit processing wastewaters. PVPP is a water-insoluble cross-linked polymer of vinyl pyrrolidone which is often used in the treatment of certain beverages to remove small quantities of unwanted substances such as phenolic compounds (Sarioglu, 2007). An important industrial application of PVPP is the stabilisation of apple juices in order to enhance their storage life. Apple juices are clear after clarification, however, during storage, changes in colour and development of undesirable haze and turbidity are common quality-degrading reactions, and these seriously compromise the acceptability of commercial juice. The development of such colloidal hazes is linked to the presence of low molecular weight polyphenolic compounds in the apple juice (Sarioglu, 2007). Thus, to reduce the formation of haze, small quantities of PVPP are added to the apple juice to adsorb phenolic compounds. After a sufficient length of time, PVPP, with the adsorbed phenolic compounds can be removed (Sarioglu, 2007). PVPP is used in a similar way to reduce haziness in beer and stabilise its colour. Further, PVPP is used in the processing of citrus to remove the bitter flavour caused by the phenolic compound naringin (O'Reilly and Merrow, 1998). In the present study, these examples provided justification for investigating the use of PVPP to extract phenolic compounds from fruit processing wastewaters using the same principle. The adsorption of phenolic compounds by PVPP occurs through hydrogen bonding between the proton donor from the phenolic compound and the carbonyl group from PVPP, as well as polar and hydrophobic interactions (Sarioglu, 2007).

The investigation into the extraction of phenolic compounds using PVPP as the solid phase adsorbent was conducted by adding a known amount of PVPP to known volumes of the respective wastewater samples, and mixing for 10 minutes. This was followed by filtration, and washing the phenolic compounds off the PVPP using dilute NaOH solution. After the pH had been readjusted using dilute HCl, the antioxidant activity of the extract for each wastewater sample was then determined by the DPPH radical scavenging assay. The analysis of the extracts obtained after the extraction of phenolic compounds from fruit processing wastewaters using PVPP as the solid phase adsorbent was conducted using HPLC and the profiles of the extracts and water wash fractions of all the wastewater samples studied here are illustrated from Figure 4.16 to Figure 4.20. It was generally expected that the individual phenolic compounds that had been detected in the wastewater samples before extraction (Figure 4.6 to Figure 4.8, Section 4.4), would also be detected in the extract obtained after using PVPP as the solid phase adsorbent. However, this was not the case in some of the wastewater samples, as is discussed below.

The HPLC profiles for the apple processing wastewater AW1 extract obtained after extracting using PVPP as the solid phase adsorbent (hereafter referred to as the extract) and the fraction collected after washing the PVPP binding the phenolic compounds with distilled water (hereafter referred to as the water wash) are shown in Figure 4.16. Catechin was detected in both the extract and the water wash. However, it had been anticipated that catechin would be adsorbed by PVPP and thus would only be detected in the extract. An experiment was conducted whereby the quantity of PVPP used for the extraction was increased from 5 g/L to 10 g/L using the same volume of NaOH (10 ml) for eluting the bound phenolics, but this resulted in no phenolic compounds being detected in either the extract or the water wash. Further, the volume of NaOH was increased from 10 ml to 20 ml using 10 g/L PVPP and this resulted in the detection of catechin in both the extract and the water wash. Unfortunately, due to time constraints, further investigations could not be conducted. Further studies to optimise the amount of PVPP to be used as well as the volume of NaOH that will result in the elution of all the bound phenolic compounds are recommended. The HPLC profile of the apple processing wastewater AW1 before extraction is shown in Figure 4.6 (Section 4.4) and only the phenolic compound chlorogenic acid was detected. However, chlorogenic acid was not detected after the extraction of phenolic compounds using PVPP as the solid phase adsorbent. This might be because chlorogenic acid did not bind to PVPP and instead eluted in the stripped wastewater. Catechin, which had not been identified in the wastewater sample before extraction, was now identified. This may be attributed to the concentration of analytes that occurs during extraction which results in concentrations that can be detected by HPLC.

Figure 4.17 shows the HPLC chromatograms of apple processing wastewater AW2 after extraction of phenolic compounds using PVPP and the water wash. No peaks were identified in either the extract or the water wash. It had been expected that chlorogenic acid would be detected in the extract since it had been present in the wastewater sample as shown by the HPLC profile of AW1 apple processing wastewater in Figure 4.6(B). Figure 4.18 illustrate the HPLC chromatograms for citrus processing wastewater CW1 obtained after extraction with PVPP and the water wash respectively. Gallic acid, initially not identified in CW1 wastewater sample, (Figure 4.7A) was now identified in both the extract and the water wash. Figure 4.19 show the HPLC chromatograms of citrus processing wastewater CW2 for the extract obtained after extraction using PVPP as the solid phase adsorbent and the water wash respectively. Catechin was the only phenolic compound extracted that could be identified. However, chlorogenic acid was not identified in the extract. Figure 4.20 show the HPLC profiles for the silage water SW of the extract and water wash respectively, obtained after the extraction of phenolic compounds from the silage water SW using PVPP as a solid phase adsorbent. Gallic acid, the major phenolic compound in silage water SW was identified in the extract.



(A) (B)

Figure 4-16: HPLC chromatogram of the AW1 apple wastewater sample (A), after extraction using PVPP and (B) the fraction collected from washing PVPP with water following the extraction of phenolic compounds from AW1 apple wastewater sample using PVPP as the solid phase adsorbent. The peak labelled 2 corresponds to catechin

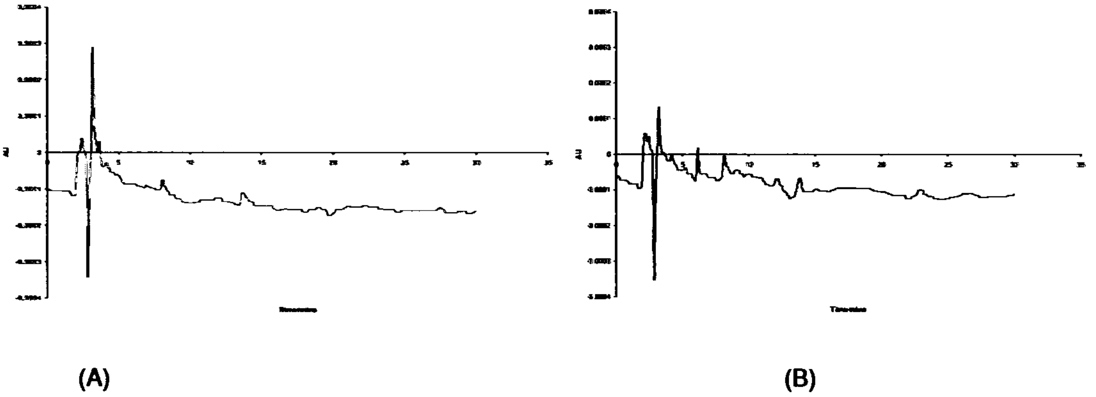
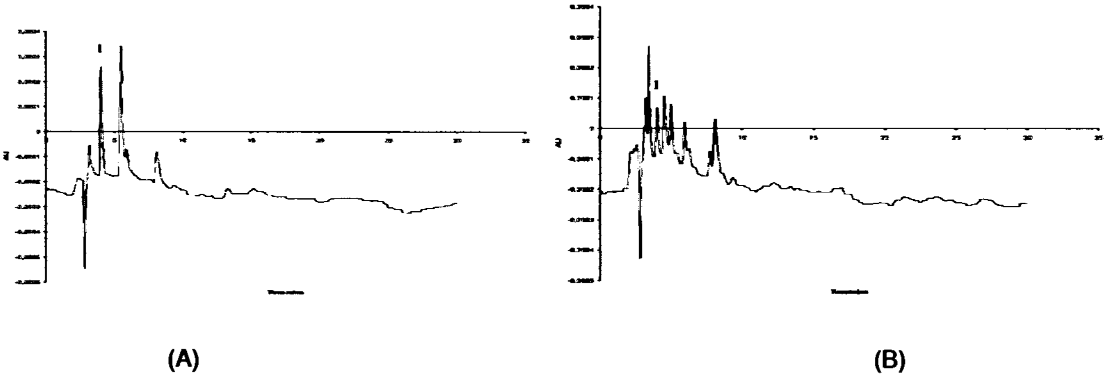
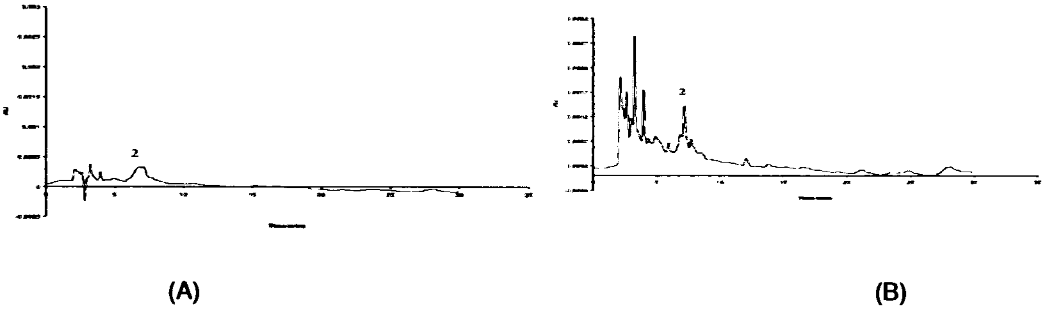

(A) (B)

Figure 4-17: HPLC chromatogram of the AW2 apple wastewater sample (A), after extraction using PVPP and (B) the fraction collected from washing PVPP with water following the extraction of phenolic compounds from AW2 apple wastewater sample using PVPP as the solid phase adsorbent.



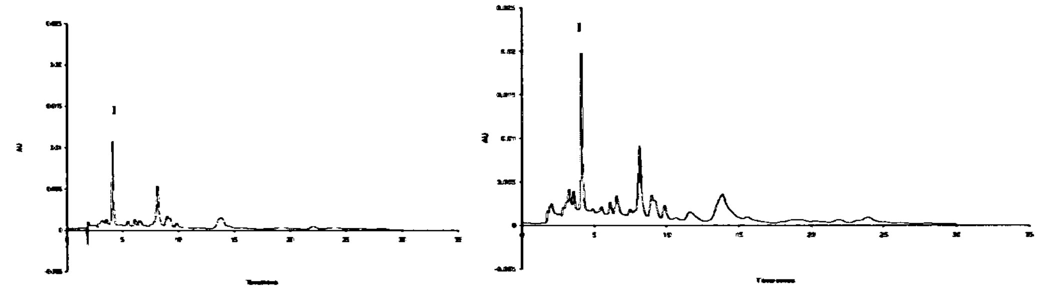
(A) (B)

Figure 4-18: HPLC chromatogram of the CW1 apple wastewater sample (A), after extraction using PVPP and (B) the fraction collected from washing PVPP with water following the extraction of phenolic compounds from CW1 apple wastewater sample using PVPP as the solid phase adsorbent. The peak labelled 1 corresponds to gallic acid.



(A) (B)

Figure 4-19: HPLC chromatogram of the CW2 apple wastewater sample (A), after extraction using PVPP and (B) the fraction collected from washing PVPP with water following the extraction of phenolic compounds from CW2 apple wastewater sample using PVPP as the solid phase adsorbent. The peak labelled 2 corresponds to catechin.



(A) (B)

Figure 4-20: HPLC chromatogram of the silage water sample (A), after extraction using PVPP and (B) the fraction collected from washing PVPP with water following the extraction of phenolic compounds from the silage water sample using PVPP as the solid phase adsorbent. The peak labelled 1 corresponds to gallic acid

After PVPP extraction, the total phenolics contents of the extracts were determined using the Folin C assay. In order to determine the efficiency of PVPP extraction, the extraction efficiency was calculated by expressing the concentration of the total phenolics in the extract as a percentage of the total phenolics content in the wastewater sample before extraction, and the results (recorded as the mean with the standard error of the mean) are shown in Table 4.12. The silage water SW was found to give the highest extraction efficiency of 39.58%, followed by the apple processing wastewater AW2, which had an extraction efficiency of 36.49%. The citrus processing wastewaters CW1 and CW2 exhibited low extraction efficiencies of 6.53 and 6.18% respectively. The quantity of total phenolics present in the extract after the extraction of phenolic compounds from apple processing wastewater AW1 could not be established using the Folin C assay as no phenolic compounds were detected.

Table 4.12: Total phenolics content and extraction efficiencies after PVPP extraction of phenolic compounds in fruit processing wastewaters

Sample	Parameter			
	Total phenolics before extraction (mg/L)	Total phenolics in the extract (mg/L)	Extraction efficiency (%)	%DPPH RSA for the extract
AW1	0.82 ± 0.1	-*	-	0.65
AW2	5.37 ± 0.3	1.96 ± 0.2	36.49	5.10
CW1	15.01 ± 0.1	0.98 ± 0.3	6.53	1.39
CW2	52.08 ± 0.4	3.22 ± 0.1	6.18	2.50
SW	401.05 ± 0.2	158.72 ± 0.5	39.58	32.71

** - denotes that the total phenolics could not be quantified using the Folin C assay*

The distribution of the phenolic compounds during extraction with PVPP is shown in Table 4.13 (recorded as the mean with the standard error of the mean) Generally, most of the phenolic compounds remained in the stripped wastewater, (which was the residual filtrate obtained after extracting and filtering off PVPP with bound phenolic compounds). In apple processing wastewater AW1, the total phenolics content in the wastewater sample before extraction was 0.82mg/L and the stripped wastewater contained 0.54 mg/L. For apple processing wastewater AW2, the total phenolics content of the wastewater before extraction was 5.37 mg/L whilst the stripped wastewater had a value of 1.23 mg/L. In the citrus processing wastewater samples CW1 and CW2, the wastewater samples had total phenolics contents of 15.01 and 52.08 mg/L respectively before extraction, whilst the stripped wastewater fractions had total phenolics contents of 10.58 and 40.78 mg/L. The total phenolics content of the silage water SW before extraction was 401.05 mg/L and the stripped wastewater fraction had a value of 198.12 mg/L.

It was also observed that the summation of the total phenolics contents in the extract obtained after extraction, stripped and water wash differed from the total phenolics content of the wastewater. In the case of the apple processing wastewater samples AW1 and AW2, the difference between the total phenolics content of the wastewater samples and the summed up value was 0.28 and 1.72 mg/L respectively, whilst for the citrus wastewater samples, the difference observed was 0.39 and 2.78mg/L respectively. For the silage water, the difference observed was 38.94 mg/L. The observations described above suggest that some of the phenolic compounds remained bound to the PVPP and were not eluted during the recovery stage.

Table 4.13: Comparison of the distribution of phenolic compounds during PVPP extraction

Sample	Concentration of total phenolics (mg/L GAE)				
	Original wastewater	Stripped wastewater	Water wash	Extract	Difference
AW1	0.82±001	0.54±0.001	-	-	0.28
AW2	5.37±0.001	1.23±0.001	0.46±0.001	1.96±0.001	1.72
CW1	15.01±0.02	10.58±0.02	3.06±0.001	0.98±0.001	0.39
CW2	52.08±0.01	40.78±0.01	10.86±0.01	3.22±0.001	2.78
SW	401.05±0.3	198.12±0.03	6.77±0.01	158.71±0.02	38.94

** - denotes that the total phenolics could not be quantified using the Folin C assay*

4.5.3.1 The determination of the antioxidant activity of the extracts obtained after the extraction of phenolic compounds from fruit processing wastewaters using PVPP

In order to determine the antioxidant activity of the extracts obtained after PVPP extraction, the DPPH radical scavenging assay was used. After the phenolic compounds bound to PVPP had been recovered using sodium hydroxide, the sodium hydroxide eluant was then readjusted using hydrochloric acid and the extract of each wastewater sample was used to determine the radical scavenging activities. The results are shown in Table 4.12 above. The apple wastewater extract AW1 exhibited the lowest scavenging capacity of 0.65% while the silage water SW gave the highest value of 32.71%. AW2 had a radical scavenging activity of 5.1% whilst the citrus wastewaters CW1 and CW2 had radical scavenging activities of 1.39% and 2.5%. These radical scavenging capacities were all low, as expected; because of the low extraction efficiencies achieved using the extraction with PVPP technique.

4.5.3.2 Problems associated with the use of PVPP as the solid phase adsorbent for the extraction of phenolic compounds from fruit processing wastewaters

The absence of some phenolic compounds in the extracts obtained after the PVPP extraction of phenolic compounds from the wastewaters may be attributed to the low concentrations of the individual phenolic compounds present in the wastewater samples. During extraction, some of the phenolic compounds may have remained bound to the PVPP, thereby further reducing the concentration of these phenolic compounds to levels that could not be detected by HPLC. It will be necessary to conduct further studies on optimising the extraction of phenolic compounds from fruit processing wastewaters using PVPP as the solid phase adsorbent. Factors that may be optimised include determining the quantities of PVPP that need to be used in order to obtain maximum yields of phenolic compounds, as well as the most suitable volumes of NaOH that need to be added to elute all the phenolic compounds bound to PVPP. The low extraction efficiencies observed in the present study can be due to inevitable losses of PVPP that occurred during the extraction process. For example, the recovery of PVPP with the bound phenolics involved transferring the PVPP from one container to a different container for vacuum filtration, and not all of the PVPP with the bound phenolics could be recovered, leading to some losses of phenolic compounds bound to PVPP.

The regeneration of PVPP involved using NaOH to disrupt the hydrogen bonds and other hydrophobic interactions binding the phenolic compounds to PVPP. Sodium hydroxide had a stronger affinity for the unbound PVPP and only started displacing phenolic compounds bound to PVPP after it had saturated the unbound PVPP. However, it could be that the amount of NaOH used was insufficient to saturate the unbound PVPP before displacing phenolic compounds leading to their recovery. A study was conducted whereby the volume of sodium hydroxide used to regenerate the PVPP was increased from 10 ml to 20 ml. However, there was no difference observed in terms of the extraction efficiencies and the detection of phenolic compounds in the extracts using HPLC. For future studies, it is suggested that different volumes of NaOH at varying concentrations be used in order to establish the most suitable volume to use so that the maximum quantities of phenolic compounds may be recovered from PVPP. Also, the potential negative effect of NaOH on phenolic compounds is a factor to be considered for further studies, necessitating the need for investigating alternative solvents to use for regenerating PVPP, thereby releasing phenolic compounds. In addition, the low extraction efficiencies may have been due to the short exposure time of the PVPP to the wastewaters, a factor that requires further investigations.

4.5.3.3 Summary of the extraction of phenolic compounds in fruit processing wastewaters using PVPP as the solid phase adsorbent

PVPP was used as a solid phase adsorbent to recover phenolic compounds from fruit processing wastewaters. The extraction of phenolic compounds from wastewater samples was generally poor as seen by the low extraction efficiencies ranging from 0 to 40%. Antioxidant activities of the extracts were determined using the DPPH assay and radical scavenging activities ranged from 0.65 to 33% in all the wastewater samples. The use of PVPP to extract phenolic compounds from wastewaters has great potential. However, further investigations to optimise the technique are required. Another technique that can be employed to extract phenolic compounds from wastewaters is membrane assisted extraction, and this technique is discussed in detail in the following section.

4.5.4 The extraction of phenolic compounds from fruit processing wastewaters using the supported liquid membrane (SLM) extraction technique

The previous sections discussed the use of adsorbents for the extraction of phenolic compounds from fruit processing wastewaters; however, solid phase extraction is generally regarded as a sample preparation technique. In this regard, supported liquid membrane extraction, which has the potential to be used at large scale, was investigated for the extraction of phenolic compounds from fruit processing wastewaters. Thus, the focus of the work described in this section was a membrane assisted solvent extraction process for the recovery of low molecular weight phenolic compounds and particularly, gallic acid from the silage water SW. Based on the results described in Section 4.4, gallic acid was found in the silage water SW at concentrations of 101.4 mg/L, and it was considered possible that the extraction of gallic acid from the silage water might be economically feasible. This technique is based on a similar principle to liquid-liquid extraction, except that the aqueous and organic phases are not mixed; they are separated by a hydrophobic membrane which is non-permeable to liquids at low pressures. The SLM extraction was conducted according to Gonzalez-Munoz *et al.*, (2003) and Garcin (2005), and the method is described in detail in Section 3.15. The investigation of the extraction of phenolic compounds from the fruit processing wastewaters involved the use of a Microdyne polypropylene hollow fibre membrane module to separate the wastewater samples from the organic solvent. A pump was used to pump the wastewater sample in to the lumen of the membrane extraction module. Samples were collected regularly from both the solvent and the wastewater streams and HPLC was used to monitor the concentration of gallic acid during extraction.

The initial selection of the organic solvents to be investigated in determining the distribution coefficients of phenolic compounds in the respective aqueous and organic phases was based on two important properties of the system; the boiling point and miscibility in water of the organic solvent as shown in Table 4.14 (Gonzalez-Munoz *et al.*, 2003). These two factors were considered because of their effect on extraction of phenolic compounds from aqueous samples. Boiling point of the solvent is important in the extraction of phenolic compounds because some phenolic compounds are heat sensitive, and the use of high temperatures to remove the organic solvent after extraction may damage the phenolics, leading to loss of antioxidant activity. Solubility in water of the solvent is also important since the source of the phenolics is aqueous, thus the most suitable solvent would be one that is immiscible with water. The final choice of the organic solvent that was used in SLM extraction was based on the distribution coefficient of phenolic compounds between the aqueous (wastewater) and organic (solvent) phases, which were obtained after experiments had been conducted. The distribution coefficient is the ratio of concentrations of phenolic compounds in the aqueous and organic phases at equilibrium. This value gives an indication of the maximum achievable concentration of phenolic compounds that can be extracted under normal conditions of temperature and pressure (Garcin, 2005). Using gallic acid as a model solute, the organic solvents used for the determination of the distribution coefficients were ethyl acetate, toluene, methylisobutylketone (MIBK) and amyl acetate. Six serial dilutions of the wastewater samples were each mixed with an equal volume of the organic solvent, shaken, and left to settle to allow for the concentrations of phenolic compounds in the two phases to reach equilibrium. HPLC was used for the determination of the distribution coefficient of gallic acid. The distribution coefficients obtained experimentally for the various organic solvents are shown in Table 4.14 below.

Table 4.14: Properties of organic solvents considered for use in the SLM extraction of phenolic compounds from fruit processing wastewaters and the distribution coefficients obtained after experiments had been conducted

Solvent	Characteristic		
	Boiling point (°C)	Solubility in water (mg/L)	Distribution coefficient
Amyl acetate	149.1	800	-
Toluene	110.6	50	0.6556
Methyisobutylketone (MIBK)	117.4	1700	0.6014
Ethyl acetate	77.1	8300	0.5569

Generally, the distribution coefficients of gallic acid in all the selected organic solvents showed that reasonable quantities of gallic acid could be extracted. The use of toluene resulted in the highest distribution coefficient of 0.6556, followed by MIBK with a distribution coefficient of 0.6014, and ethyl acetate giving a distribution coefficient of 0.5569. However, the solubility of ethyl acetate in water is 83 g/L and this is problematic since an ideal solvent should have a very low solubility in water and should be readily removed. The distribution coefficient of gallic acid in amyl acetate could not be established because, since amyl acetate has a very high boiling point of 149.1°C, it was difficult to evaporate the organic solvent from the extracts using a rotary evaporator at low temperatures. An alternative would be to increase the temperature at which the evaporation was occurring; however, since phenolic compounds are sensitive to high temperatures, this could have led to losses of antioxidant activity. Amyl acetate was therefore discarded as a possible solvent, and the SLM investigation was focussed on extraction using toluene, as described below.

Since the toluene-water system gave the highest distribution coefficient for gallic acid among the solvents stated, it was used for the extraction of phenolic compounds in the silage water SW using the SLM extraction system. The silage water sample contained gallic acid as the major phenolic compound (Section 4.4), and the concentration of gallic acid was monitored by HPLC analysis at specific time intervals, and examples of the HPLC profiles for the extracts obtained at 90 minutes are illustrated in Figure 4.21.

During SLM extraction using toluene as the solvent for extraction, a gradual decrease in the concentration of gallic acid in the feed stream was observed where the concentration in the aqueous phase decreased from 101.4 mg/L at the start of the extraction, to 11.41 mg/L after 90 minutes as shown in Table 4.15. A significant drop in the concentration of gallic acid occurred during the first 30 minutes from 101.4 mg/L initially to 13.19 mg/L. Thereafter, the change in the concentration of gallic acid was much slower. This suggests that the system was approaching equilibrium after 30 minutes, implying that the extraction process required a relatively short time.

The concentration of gallic acid in the solvent stream increased from 0 mg/L at the beginning of the extraction process to 45.23 mg/L after 90 minutes. This increase in the concentration of gallic acid was slow during the first 60 minutes as shown by the concentration of gallic acid which was 7.66 mg/L at 30 minutes, increasing slightly to 7.98 mg/L after 60 minutes as shown in Table 4.15. However, a sharp increase in the concentration of gallic acid was observed after 90 minutes whereby the concentration increased from 7.98 mg/L observed at 60 minutes, to 45.23 mg/L.

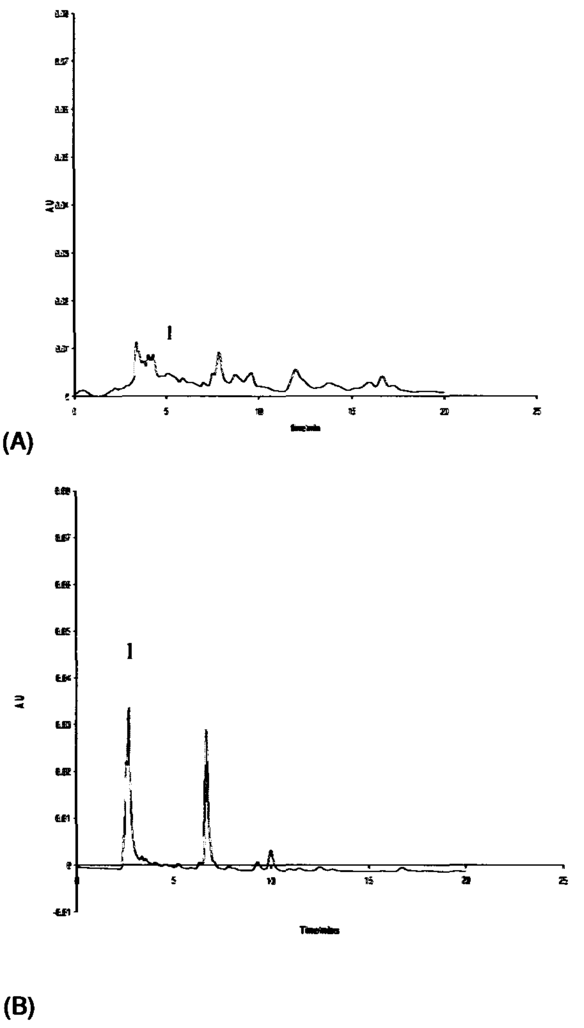


Figure 4-21: HPLC profile showing the concentration of gallic acid in (A), the feed stream and (B), the solvent stream, at t=90 minutes during supported liquid membrane extraction of the silage water SW using toluene as the solvent for extraction. The peak labelled 1 corresponds to gallic acid

Table 4.15: Concentration of gallic acid in the feed and solvent streams at various intervals during SLM extraction using toluene as the solvent for extraction

Time (minutes)	Concentration of gallic acid (mg/L)	
	Feed stream	Solvent stream
0	101.4±0.02	0
30	13.19±0.01	7.66±0.01
60	11.85±0.01	7.98±0.01
90	11.41±0.02	45.23±0.04

The extraction efficiency of the SLM system, calculated by dividing the concentration of gallic acid in the solvent stream at t=90 minutes, by the concentration of gallic acid in the feed stream at t=0 minutes and expressing the results as a percentage, was 44.6%. A factor that may have led to relatively low extraction efficiency is the effect of pressure. Since the membrane used was hydrophobic, it was necessary to have a slight overpressure of the aqueous phase (0.6-0.8 bar) to stabilise the interface in the pores so as to avoid bulk mixing of the two phases (Gonzalez-Munoz *et al.*, 2003). However, in this work, pressures of 10 to 30 kPa were used and this led to bulk mixing of the silage water and toluene. Lower pressures could not be attained probably due to possible inefficient cleaning of the system leaving residues from previous extractions inside pores which provided additional resistance resulting in increased pressures being unavoidable.

Temperature has a direct effect on the physico-chemical properties of the solvent system used for SLM extraction, that is; density, viscosity, interfacial tension and mutual solubility. Therefore, temperature would have an indirect influence on the overall transfer of phenolic compounds. Gonzalez-Munoz *et al.*, (2003) observed an increase of 60% in the overall mass transfer coefficient as temperature increased to 40°C in the extraction of phenol from aqueous synthetic solutions. In the present study, the extraction of gallic acid was done at room temperature and this may not necessarily be the most suitable temperature to ensure the maximum extraction. Similarly, pH would affect the extraction of phenolic compounds such as gallic acid, and this should be investigated in future studies.

4.5.4.1 Summary of the extraction of phenolic compounds using the supported liquid membrane extraction technique

The extraction of gallic acid from the silage water was investigated using the supported liquid membrane extraction technique, using toluene as the solvent for extraction. This resulted in 45.23 mg/L gallic acid being extracted in 90 minutes. The procedure is relatively simple and in terms of process design, the rate-limiting step was the removal of the organic solvent. Further investigations into the use of other organic solvents that are accepted in the food industry, as well as optimisation studies to increase the efficiency of the process need to be conducted. Factors to be studied include the effect of pH and temperature on the extraction of phenolic compounds from wastewaters.

4.5.6 The determination of the solubility of gallic acid in supercritical CO_2 using the supercritical fluid extraction technique

In order to identify an extraction technique that is environmentally friendly and uses minimum quantities of organic solvent, supercritical fluid extraction (SFE) was identified in literature as a good alternative to other techniques such as supported liquid membrane (SLM) extraction, for the extraction of phenolic compounds. SFE uses little or no solvents, and its use of CO_2 during extraction make it a relatively safe technique. Therefore, this part of the theses discusses the use of SFE for the extraction of a model phenolic antioxidant, gallic acid. Gallic acid was chosen because of two reasons, namely; amongst the fruit processing wastewater samples studied here, the silage water was found to contain gallic acid at a concentration of 101.4 mg/L and this presented a potential source for the extraction of gallic acid. Secondly, this part of the project was done at Sydney University, Australia, and, because of the environmental and safety regulations of Australia, which prohibit the entrance of biological samples such as wastewaters, the intention was to make a synthetic wastewater, containing gallic acid, which had previously been identified in relatively large amounts in the silage water, and this would thus serve to ascertain the feasibility of using SFE in the extraction of phenolic compounds from fruit processing wastewaters. Prior to this however, it was necessary to determine the solubility of pure gallic acid in the solid state. Supported liquid membrane (SLM) was investigated, as was discussed in Section 4.5.4 and resulted in the extraction of 45.23 mg/L of gallic acid. Thus, the aim of the work described in this section was to determine the feasibility of using supercritical fluid extraction for the recovery of gallic acid, initially by determining the solubility of pure gallic acid in supercritical CO_2.

The design of supercritical processes requires knowledge of the solute solubility in the supercritical phase and the ability to efficiently model and predict it (Chafer *et al.*, 2007). The present work investigated the solubility of gallic acid in supercritical CO_2 and ethanol as co-solvent. Ethanol was chosen as a modifier because it is a polar solvent, its use is allowed in the food industry, and it can be easily removed from the extract by evaporation at relatively low temperatures (Chafer *et al.*, 2007). The solubility of gallic acid in supercritical CO_2 was measured using a supercritical fluid extraction system that consisted of a high pressure pump used to supply an uninterrupted flow of CO_2, and a second pump that was used for cooling the CO_2 (Figure 3.3, Section 3.16). The pressure of the supercritical CO_2 was closely monitored through a pressure gauge that was connected to the extraction vessel. In the first experiment, the solubility of gallic acid in supercritical CO_2 was investigated at 25°C using 10ml of ethanol at a pressure of 180 bar for 2 hours. The effect of temperature was then studied in the second experiment, whereby the temperature was increased from 25°C to 40°C using the same conditions of pressure, time and volume of co-solvent as in the first experiment. Thereafter, the effect of the volume of the co-solvent was studied by increasing the volume of ethanol used from 10 ml to 20 ml but maintaining the same conditions of time and pressure and temperature. Finally, the effect of time was investigated by increasing the extraction time from 2 hours to 3 hours at constant temperature, pressure and co-solvent volume. A standard curve for gallic acid concentration was obtained by measuring the absorbance of increasing concentrations of gallic acid using a spectrophotometer reading at a wavelength of 270 nm, and this was used to determine the amount of gallic acid extracted. The results were reported as % extraction efficiency. The experimental procedures are explained in detail in Section 3.16. The experimental results are given in Figure 4.22 below

In the investigations into the solubility of gallic acid in supercritical CO_2, the solubility of gallic acid and the extraction efficiency decreased with an increase in temperature as shown in Figure 4.22 where extraction efficiency increased from 4.02% at 25°C to1.85% at 40°C. The results obtained on the effect of temperature in the present study corresponds with results obtained by Chafer *et al.*, (2007) who also studied the solubility of natural gallic acid in supercritical CO_2 and ethanol as co-solvent. The low solubility of solutes caused by increasing temperature was also observed by Cortesi *et al.*, (1999) where ascorbyl palmitate's solubility decreased with an increase in temperature. This observation however, was unexpected because increasing temperature should increase the solvating power of CO_2 thereby resulting in more solute being transferred to the supercritical phase. However, the effect of temperature on the solute solubility in supercritical CO_2 is complex and cross-over phenomena have been observed, where, the solubility decreases with increasing temperature (Chafer *et al.*, 2007).

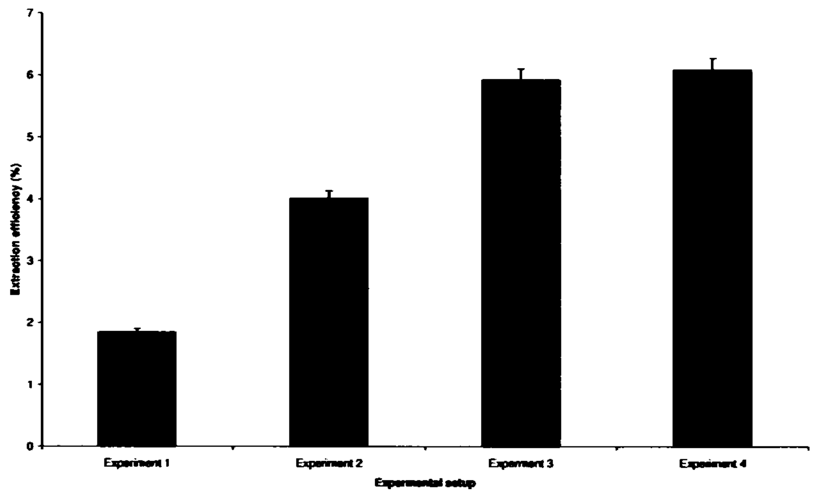


Figure 4-22: Extraction efficiencies of gallic acid in CO_2 using ethanol as co-solvent. The experiments were conducted at a constant pressure of 180bar and the specific conditions of each experiment are: Experiment 1- 25°C, 10ml ethanol, 2 hours; Experiment 2- 40 °C, 10ml ethanol, 2 hours; Experiment 3- 25 °C, 20ml ethanol, 2 hours; Experiment 4- 25 °C, 20ml ethanol, 3 hours.

The effect of the volume of the co-solvent used for extraction was studied by increasing the volume of ethanol used from 5 ml to 10 ml per gram of gallic acid respectively, resulting in an increase in the extraction efficiency from 4.02% to 5.2% . CO_2, a low polarity solvent, is not effective in extracting more polar compounds such as gallic acid. Thus ethanol, which is a polar solvent, was added resulting in substantial changes of the solvent properties of supercritical CO_2. Thus, increases in extraction efficiency with increased volumes of ethanol were expected.

The effect of the extraction time was studied by increasing the extraction time from 2 hours to 3 hours. However, only a slight increase from 5.2% to 6.09% was observed, indicating that increased extraction time did not contribute greatly to the extraction efficiency.

Other factors that affect solubility are the melting point, size and polarity of the solute, flow rate and chemical structure (Cortesi *et al.*, 1999; Murga *et al.*, 2002). Pressure also contributes significantly to the extraction efficiency as an increase in pressure results in an increase in CO_2 density, increasing the solvating power of the supercritical fluid (Yi *et al.*, 2009). The effect of pressure on the solubility of gallic acid was studied by Chafer *et al.*, (2007) where an increase in pressure resulted in increased gallic acid solubility. However, the system used in the present investigation could not go beyond pressures of 200 bars, thus the effect of pressure could not be determined. The low extraction efficiencies observed in the present study may also be due to the high polarity of gallic acid that is being dissolved in a non-polar solvent such as CO_2.

4.5.6.1 Applicability of the supercritical fluid extraction technique to obtain phenolic compounds from fruit processing wastewaters

The results obtained in the study of the investigation of the solubility of gallic acid in supercritical CO_2 show that this technique offers an alternative for the extraction of phenolic compounds present in samples such as fruit processing wastewaters and Section 2.6.3.3 discusses the applicability of the supercritical fluid extraction for obtaining antioxidants from various plant sources, including samples containing relatively low concentrations of phenolic compounds. In this study, gallic acid in its solid state was dissolved in ethanol before extraction. However, the purpose of investigating the supercritical fluid extraction technique was to assess the feasibility of using it in aqueous samples such as the fruit processing wastewaters. Though the research on the supercritical fluid extraction technique conducted in this study was still preliminary, the implication of applying supercritical fluid extraction to the fruit processing wastewaters would mean modifying the extraction conditions to suit wastewater samples. This would also mean investigating the most suitable organic modifier which is not miscible with water to enhance the extraction of phenolic compounds. An alternative would be to dry the wastewater samples using techniques such as spray drying followed by extracting the phenolic compounds from the dried product, using ethanol as a modifier. Given the increased antioxidant activity observed after extraction with supercritical fluid extraction reported in literature (Mendiola *et al.*, 2008), this would be useful for the wastewater samples studied here because of their low concentrations of phenolic compounds.

The technique requires minimum sample preparation and optimisation studies need to be conducted in order to increase the efficiency of extracting phenolic compounds. The parameters to be investigated include optimising the temperature, pressure and volumes of co-solvent to be used. Supercritical fluid extraction is already being used at large scale. However, further investigations into the extraction of gallic acid in an aqeous mixture of phenolic compounds could not be conducted due to time constraints and will be necessary to assess whether the presence of other phenolic compounds in a mixture would affect the extraction of gallic acid. Nevertheless, SFE is a promising alternative and should be considered in future studies.

4.6 Comparative discussion of the extraction of phenolic compounds from fruit processing wastewaters by various extraction techniques for the success of a process to produce antioxidant extracts

In order to determine which of the extraction techniques discussed in the previous sections would be most suitable for the recovery of phenolic antioxidants, a comparison of all the extraction techniques, namely: solvent extraction, solid phase extraction, PVPP extraction, supported liquid membrane extraction and supercritical fluid extraction was done and the results obtained from the extraction procedures are summarised in Table 4.16. This table also gives an estimate on the scalability of the extraction processes together with the implications of scaling up in terms of the costs. It is acknowledged that these extraction processes have not been fully optimised and further research would need to be conducted to make more realistic cost estimates. The scale-up costs were calculated using quotations obtained from various manufacturers of chemicals and extraction equipment. These prices reflect the costs of the major chemicals or equipment required for each technique and do not include other costs associated with the extraction technique, such as the cost of solvent disposal after solvent extraction. These are very approximate first attempts to quantify the costs of extraction using the various extraction techniques. Pilot-scale extraction systems will be necessary in order to evaluate more accurately the economic feasibility of the processes studied here.

Table 4.16: A summary of the results obtained using the various extraction techniques and the potential for application at large scale

Method	Extraction Efficiency (%)	Scale used in this study	Requirements/L of wastewater	Scalability^1	Cost Implication^2 (R)^2	Comments
Solvent extraction	Sample Ethyl acetate Hexane AW1 25.45 31.21 AW2 38.04 15.08 CW1 16.57 2.57 CW2 29.95 7.49 SW 15.13 0.366	100 ml wastewater + 300 ml solvent	3 L solvent	Industrial >1000 L	600 000 to 2 million (Sigma)	• disposal of residual organic solvents is hazardous and costly to dispose of • technique is time consuming and requires large space • organic solvent can be recycled
Solid phase extraction	Sample AW1 52.1 AW2 51.9 CW1 49.5 CW2 44.1 SW 56.9	20 ml wastewater/cartridge	10 Sep Pak® cartridges or a higher capacity cartridge	Analytical ≤1L	200 000 (Promo Chrom Technologies)	• at large scale, large quantise of sorbents required are expensive making the process uneconomical • generally used as a preconcentration step at small scale
PVPP extraction	Sample AW1 AW2 36.49 CW1 6.53 CW2 6.18 SW 39.58	5 g PVPP + 1 L wastewater	5 g PVPP	Industrial >1000L	20 000 (Sigma)	• technique is relatively simple • cheap, PVPP can be regenerated up to 20 times • technique has great potential for use in industry

SLM extraction	Sample SW 44.60	1 L wastewater+ 1 L solvent	1 L	Industrial >1000L	100 000	• better alternative to traditional techniques • relatively inexpensive
Supercritical fluid extraction	Sample Gallic acid 7.70	2 g Gallic acid + 20 ml solvent		Industrial >1000L	800 000 to 1.5 million	• initial set up costs are high, however, maintenance costs are low • uses no or minimal quantities of organic solvent

[1] *- Scalability expresses the ability of the process to be used with volumes larger than those used in this study;*

[2] *- Cost estimates were based on rough estimates of the major materials required for the extraction process and applying the quantities used in the present study to estimate the cost at large scale.The cost implications used the extraction conditions employed in the current study and the conditions used here were not optimised. Further optimisation will most likely reduce these cost estimates significantly.*

4.6.1 Advantages and disadvantages of the various extraction techniques in recovering phenolic compounds from fruit processing wastewaters

Phenolic compounds can be extracted from foods or wastewaters by solvent extraction. In the work done in this project ethyl acetate and hexane were used and extraction efficiencies ranging from 15% to 38% using ethyl acetate and from 0.3% to 31% using hexane were measured. However, solvent extraction consumes large volumes of organic solvents that are hazardous to the environment and expensive to dispose of. Based on the present study done here, the ratio of wastewater to solvent used is 1:3 and 3 L of solvent is required to extract 1 L of wastewater, but this is not optimised to minimise the quantities of organic solvent, and optimisation studies will be necessary. At large scale, large quantities of the organic solvent will be required to obtain an extract of phenolic compounds and residues of these organic solvents will be costly to dispose of (Abad-Garcia *et al.*, 2007). Furthermore, the process can be time consuming, requiring sequential extractions followed by a purification step. Purification is essential to completely remove all the organic solvent which, if trace amounts are left in the product, can be harmful when consumed. Thus, at large scale, this technique is unlikely to be economically feasible and because of the possible presence of traces of organic solvent in the extracts, the use of the extracts as food additives would not be recommended. These extracts may however be used in cosmetics for preservation or enhancement of the cosmetics. Also, the extracts obtained using solvent extraction can be used in plastics.

The disadvantages of solvent extraction methods, together with the concern over solvent residues in extracted products, has stimulated a search for alternative processing methods such as solid phase extraction. Solid phase extraction, using cartridges filled with various sorbents has been extensively used due to its advantages of rapid and simple manipulation as well as the small amounts of solvent required, compared with classical solvent extraction (Ahn *et al.*, 2007). In the present study, solid phase extraction was found to require short time periods, while using only small volumes of solvent for elution. The low solvent volumes used make this method attractive for obtaining extracts that can be applied in foods. Thus, to extract the phenolic compounds from 1 L of wastewater by solid phase extraction required 0.6 L of solvent, as compared with 3 L required in the classical solvent extraction system.

Solid phase extraction also resulted in better extraction when compared to solvent extraction with extraction efficiencies of 44% to 57%, as compared with solvent extraction giving extraction efficiencies of 2% to 38%. However, solid phase extraction is generally recognised as a pre-concentration technique. Most polar compounds, including phenolic compounds have a low affinity for reversed phase sorbents (Niu *et al.*, 2007). Furthermore, for industrial-scale operations, large quantities of sorbent are required which tends to make the process uneconomical compared to solvent extraction, because the sorbents are costly. During this study of solid phase extraction, the pH of the wastewaters had to be adjusted between 2 and 7 to facilitate elution of neutral and acidic phenolic compounds. This means that an additional neutralisation stage would be necessary if extracts were to be considered for application as food additives or nutraceuticals. Because of the poor retention capacities for polar compounds, C_{18} cartridges are not the most suitable cartridges to extract trace levels of phenolic compounds that occur in wastewaters, and alternatives should be investigated. Solid phase extraction can be useful for recovering phenolic compounds from wastewaters for research purposes since this technique is relatively cheap and fast to perform at small scale.

In the investigation of adsorption extraction using PVPP as an adsorbent, the extraction efficiencies measured ranged from 0% to 40%. This is relatively low compared with solvent extraction and solid phase extraction and, investigation in to the optimum conditions necessary for phenolics recovery will increase the extraction efficiency. However, extraction with PVPP has good potential as a method for obtaining phenolic antioxidants. Firstly, the beverage-processing industries already use PVPP for the removal of unwanted phenolic compounds that lead to haze formation. In this application, after phenolic compounds bind to PVPP, the main concern of beverage manufacturers is to regenerate PVPP for reuse. This involves the use of extreme conditions to achieve the removal of phenolic compounds, and thus, a good method is needed to recover phenolic compounds without degrading them. Although the maximum achievable recovery of total phenolics was 39.58% in the studied wastewaters, this technique could potentially be used at commercial scale. Secondly, the extraction of phenolic compounds with PVPP is a relatively inexpensive method. In this study, only 5 g of PVPP were required for every 1 L of wastewater for extraction, but this also requires further optimisation. Based on these considerations, it may not be difficult to convince fruit processors to use this technique, not only to prevent haze formation in beverages, but also to obtain value-added products from these wastewaters at minimal costs.

As an alternative approach to extraction of phenolic compounds from fruit processing wastewaters, supported liquid membrane (SLM) extraction was investigated using the relatively high concentration silage water. The recovery of gallic acid, which was found to be the major phenolic compound in the silage water, was of particular interest because it was in concentrations of 101.4 mg/L that could potentially make the process economically feasible. SLM extraction showed an extraction efficiency of 44.6% and 0.5 L solvent was required to extract 1 L of wastewater. SLM extraction is a good alternative to solvent extraction and solid phase extraction because, under normal circumstances, the aqueous and organic phases are not mixed since they are separated by a hydrophobic membrane. This means that no additional steps are required to separate the aqueous and organic solvents, as is the case in classical solvent extraction. The system design is simple and energy costs are minimised due to the use of small pumps. However, a major problem noted in this study was the mixing of the aqueous and organic phases and this was attributed to several factors. The membrane module used had previously been used for other applications and may have been modified or clogged, and the system blocked several times during extraction. It would be important that a suitable membrane module is used, and then SLM would be a better option for the recovery of phenolic compounds that can be incorporated into food stuffs. The use of toluene is prohibited in the food industry and thus extracts obtained from this process will not be applied as food additives. It is therefore necessary to select appropriate solvents and this requires further investigations.

Another substitute to the extraction techniques discussed so far, supercritical fluid extraction (SFE) was investigated and, preliminary studies were conducted to determine the solubility of gallic acid and, consequently, the extraction efficiency. This approach gave an indication of the amount of gallic acid that could be extracted from the silage water and whether further research on the use of SFE for the recovery of phenolic compounds from fruit processing wastewaters would be useful. The extraction efficiency measured was only 6%. However, a direct comparison with other methods can not yet be made because the SFE study was only very preliminary; pure gallic acid was used, and it was not possible, in the time available, to perform further experiments. Considering the cost of setting up and operating a SFE system, this technique may not always be economically feasible. However, if high solubilities of phenolic compounds, such as those reported by Murga *et al.* (2002) for p-coumaric acid and protocatechuic acid in supercritical CO_2 can be obtained, then the value of the extracted products, coupled with relatively low maintenance costs of SFE, could make this the method of choice.

The best recovery of total phenolics was observed with solid phase extraction (57%). The wide range is partly explained by the heterogeneous matrix of the wastewaters at the time of extraction. Some of the methods, namely the extraction with PVPP, and solvent extraction, consisted of several steps compared with other extraction techniques and this would increase the possibility of loss of phenolic compounds due to the transfer of samples to different containers. SLM and SFE are relatively new technologies that have great potential for the extraction of phenolic compounds in fruit processing wastewaters. However, further investigations are needed to increase the efficiencies of these technologies and recommendations are discussed further in Chapter 5.

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

The primary aim of the investigations conducted in this study was to extract potentially valuable products, specifically phenolic antioxidants, from fruit processing wastewaters produced in the Western Cape region of South Africa, for use as additives in foods, cosmetics or pharmaceuticals. Phenolic antioxidants were selected as the potential value added products to be extracted because they are perceived to confer protection against various degenerative diseases such as cancer and heart disease (Jayaprakash *et al.*, 2008). In addition, interest on the use of natural antioxidants compared with synthetic antioxidants, such as butylated hydroxyl anisole, has increased because synthetic antioxidants may have adverse effects, impacting negatively on human health (Balasundram *et al.*, 2006; Prakash *et al.*, 2007). The wastewaters used were apple wastewaters from two different fruit processors (AW1 and AW2), citrus wastewaters from two different sources (CW1 and CW2), as well as silage water (SW), which is strictly not a wastewater, but rather seepage collected from pomace of deciduous fruits. (Silage water presented a richer source of value added products compared with the other wastewaters.) Fruit processing wastewaters were chosen as a source of the phenolic antioxidants because during fruit processing, some phenolic compounds are transferred to the wastewaters. There is very little information published on the recovery of phenolic antioxidants from fruit processing wastewaters, and this presented an opportunity to explore the possibility of using fruit processing wastewaters as a source of phenolic antioxidants. Given the industrial importance of natural phenolic antioxidants and the broader environmental implications of disposing fruit processing wastewaters containing phenolic compounds, it was of interest to further investigate this.

The overall objectives of the project have been met, in that, firstly, the fruit processing wastewaters were characterised and analysed. Value added products, namely, phenolic compounds, were identified, quantified and compared with information in literature. Secondly, various extraction techniques, namely, solvent extraction, solid phase extraction, supported liquid membrane extraction and supercritical fluid extraction were employed to recover phenolic compounds from the wastewaters. In addition, extraction using PVPP as a solid phase adsorbent was also investigated. Finally, the antioxidant activity of the original wastewaters and extracts was analysed using various antioxidant activity assays. The major findings from this study are discussed below.

In the chemical characterisation of the fruit processing wastewaters, it was shown that the wastewaters contained phenolic compounds at concentrations of 7.61 mg/L and 42.45 mg/L in apple wastewaters AW1 and AW2 respectively, 18.6 mg/L and 137 mg/L in citrus wastewaters CW1 and CW2 respectively, and 399.52 mg/L in silage water SW. The wastewaters studied here had relatively low concentrations of phenolic compounds when compared with other industrial wastewaters such as cork processing and tannery wastewaters which reported values of 358 mg/L and 1400 mg/L respectively (Minhalma *et al.*, 2006; Marino-Martinez *et al.*, 2009). Thus, the silage water had the greatest potential as a source for the recovery of phenolic antioxidants.

Other potential value added products identified in the wastewaters were the reducing sugars, which were found in concentrations ranging from 126.5 mg/L to 396.3 mg/L. The values obtained in the work done here was comparable to those obtained by Scordino *et al.* (2007) who reported values of 250 mg/L in orange pulp wash. Reducing sugars have potential applications in the food industry as sugar syrup, and in research and development, where they can be used as a carbon source in media for microbial growth. However, further investigation into the extraction of sugars could not be carried out because this was outside the scope of this project. The determination of the chemical oxygen demand showed that all the wastewaters studied here can be considered as low strength wastewaters because of the low values ranging between 328 mg/L and 5760 mg/L. Therefore, in terms of the environmental impact, these wastewaters do not pose a significant negative impact; however, for the purposes of the present study, extracting phenolic compounds would further lower the COD and thus, the negative impact to the environment.

Since one of the major objectives of this research was to recover value added products exhibiting antioxidant activity, it was necessary to determine the antioxidant activity of the wastewaters before extraction of the phenolic antioxidants. Antioxidants from different sources exhibit different antioxidant action, thus it was necessary to use various assays in order to accurately determine the nature of the antioxidants present in the wastewaters (Becker *et al.*, 2004). The assays used were the DPPH, $ABTS^{+}$, FRAP and β-CLAMS assays. The silage water exhibited the highest radical scavenging activity in all the assays. There was a strong correlation between total phenolics concentration and antioxidant activity observed in all the assays, suggesting that total phenolics contribute significantly to antioxidant activity. This correlation was also observed by other researchers (Tawaha *et al.*, 2007; Ciou *et al.*, 2008).

In order to gain a deeper understanding of the antioxidants present in the wastewaters and relate them to the activity that they exhibit, HPLC analysis was conducted to identify the individual phenolic compounds in the wastewaters, and the results showed the presence of resveratrol, catechin, and chlorogenic acid in the wastewaters at very low concentrations ranging from 0 mg/L to 101 mg/L. An important observation was the presence of gallic acid in the silage water at a concentration of 101.4 mg/L, and this showed that gallic acid could potentially be recovered from the silage water for use as an antioxidant.

After the determination of antioxidant activity and the identification of the individual phenolic compounds, the next step was to extract the phenolic antioxidants from the wastewaters. Conventional solvent extraction was investigated to provide a basis for comparison with other techniques, as well as to study the effect of solvent polarity on the extraction of phenolic antioxidants by using ethyl acetate and hexane. Ethyl acetate was generally a better solvent than hexane for the extraction of phenolic compounds from fruit processing wastewaters, with extraction efficiencies ranging between 15 to 38% using ethyl acetate and 0 to 31% using hexane. The observation made in this study that ethyl acetate is a better solvent than hexane was also reported by other researchers (Chirinos *et al.*, 2007; Mohsen and Ammar, 2009; Pinelo *et al.*, 2009) Consequently, the ethyl acetate extracts exhibited better antioxidant activities between 26% to 86% compared with hexane extracts which showed values in the range of 4% to 15%. However, a problem encountered was the trace amounts of organic solvents that were in the final antioxidant extract, and because these solvents are prohibited in the food industry, this would be hazardous. Therefore, there is need for a method that completely removes the organic solvents or an alternative technique that does not require the use of organic solvents. Recommendations on the solvent extraction technique are provided later in this chapter.

In an attempt to find a technique that uses less volumes of solvent, solid phase extraction using C18-Sep Pak® cartridges, was investigated. The phenolic compounds in the wastewaters were separated into neutral and acidic phenolic fractions. The extraction efficiencies observed ranged from 44% to 57%. Extraction efficiencies reported by other researchers such as Suarez *et al.*, (1996) and Gomis *et al.*, (2001) on the extraction of phenolic compounds from various apple products using C18-Sep Pak® cartridges, showed values of 90% to 100%, values that are higher than what was observed in the current study. However, a problem was encountered when some neutral phenolic compounds were detected in the acidic fraction. This showed that the technique was not effective in separating neutral and acidic phenolics since neutral phenolics were expected to be detected only in the neutral fraction, likewise with the acidic phenolics. Also, some phenolic compounds that were detected in the original wastewaters were not detected in either the neutral or acidic fractions, suggesting that they may have remained bound to the cartridge. Further investigations are necessary in order to increase the efficiency of this technique as is discussed later in the chapter. The neutral phenolics exhibited higher antioxidant activity compared with the acidic phenolics as was expected. This observation was in agreement with a study conducted by Tumbas *et al.*, (2007). Consequently, this technique could be useful in recovering concentrated mixtures of neutral or acidic phenolics for use in various industries. Further, this technique could be used as a preliminary step towards the purification of individual phenolic compounds.

As an alternative to solid phase extraction, PVPP was used as a solid phase adsorbent to extract phenolic antioxidants from fruit processing wastewaters. The wide use of PVPP in the beverage industry to remove phenolic compounds from beverages that cause the development of haze, presented an opportunity to investigate its use in the recovery of phenolic compounds for use as antioxidants. The concentration of total phenolics after extraction were 1.96mg/L in the apple wastewater AW2, 0.98 mg/L and 3.22 mg/L in the citrus wastewaters CW1 and CW2 respectively. The extraction efficiencies ranged from 0% in AW1 to 39.58% in the silage water. Likewise, the radical scavenging activity as determined by the DPPH assay was very low, ranging from 0.65% to 32.71%. A number of challenges were encountered during the extraction of phenolic antioxidants from fruit processing wastewaters using PVPP as the solid phase adsorbent, which contributed to the low extraction efficiencies observed. Since this study was preliminary, the optimal quantities of PVPP used for extraction and the optimal volumes of NaOH required for elution had to be determined. Some experiments to optimise these variables were conducted. However, due to time constraints, the technique could not be fully optimised. Nevertheless, this technique is simple and less time consuming and thus merits further research and development.

The silage water SW showed the greatest potential as a source for the recovery of phenolic compounds, with gallic acid present at concentrations of 101.4 mg/L. Thus, the silage water was used in the investigation of the supported liquid membrane (SLM) extraction technique to extract gallic acid. After 90 minutes, 45.23 mg/L gallic acid had been extracted, indicating an extraction efficiency of 44.6%, using toluene as the solvent for extraction. The major problem encountered during SLM extraction was the mixing of the organic and aqueous phases. This problem was also encountered in previous work conducted by Garcin, (2005). Also, several times during the extraction, the system blocked. This was attributed to the fact that the module had previously been used in other research, and thus, may have contained deposits inside the module, leading to blockages. Filtration was also necessary before the silage water could be extracted using SLM extraction. Thus, an additional filtration step to minimise system blockage was necessary. The use of toluene poses a problem of residual solvent contamination in the final extract since toluene is toxic to the human body, thus an alternative solvent must be sought. However, the technique is relatively simple and easy to operate once the parameters such as solvent and feed rates have been determined and a suitable organic solvent used. SLM extraction offers a better alternative compared to traditional methods, and further research would be useful.

Information published in literature on the extraction of phenolic compounds suggested the use of supercritical fluid extraction (SFE) for the recovery of phenolic antioxidants from fruits and their products, using little or no solvent (Adil *et al.*, 2007; Mendiola *et al.*, 2007). However, due to the environmental laws of Australia that prohibit the entrance of wastewaters, it was not possible to use this technique on the wastewaters studied in the present research. Thus, a synthetic wastewater, containing gallic acid, was used in order to determine the solubility of gallic acid in supercritical CO_2 The results obtained from this study would give information on the feasibility of this technique to extract gallic acid in wastewaters. The optimum operating conditions for the extraction of gallic acid using supercritical fluid extraction were 25°C using 20ml ethanol for 3 hours at 180 bars with an extraction efficiency of 6%. The major drawback of this technique was the inability to adjust the pressure due to the nature of the pump used which could only operate at pressures up to a maximum of 180bars. Nevertheless, SFE has great potential for the extraction of phenolic compounds because of its use of CO_2 for extraction and is already being used for the large scale extraction of phenolic compounds from various sources (Mendiola *et al.*, 2007; Daintree *et al.*, 2008).

On a more general note, the fruit processing wastewaters studied here contained phenolic compounds in low concentrations that would compromise the economic feasibility of the process, with the exception of silage water. For example, some individual phenolic compounds could not be detected in extracts obtained after solid phase extraction due to the low concentrations, which, together with the inefficient extraction technique, led to these phenolic compounds not to be detected. However, no full process the design was done on these processes as this was outside the scope of the present study, only broad estimates done 'at the back of envelope' were done to determine the cost of extraction using the techniques employed here.

Thus, looking at the objectives that were stated at the beginning of this study, it can be deduced that solvent extraction is not the most suitable technique for recovering phenolic compounds from fruit processing wastewaters, mainly because of the relatively large quantities of organic solvents used. Although solid phase extraction resulted in relatively good extraction efficiencies, it is mainly used at analytical scale and given that the main purpose of the present study is to assess the suitability of an extraction technique at large scale, this technique would not be suitable. However, extraction using PVPP as a solid phase adsorbent has been identified as a potential technique for recovering phenolic compounds from fruit processing wastewaters, because of its relative simplicity. Thus, with optimisation, this technique could potentially be suitable. Supported liquid membrane extraction also presents a potentially suitable technique for the extraction of phenolic compounds from fruit processing wastewaters and would need to be further investigated. Supercritical fluid extraction on the other hand, may be an alternative to consider for recovering phenolic compounds, however, based on the preliminary nature of the research conducted here, a decisive conclusion can not be made on its suitability in the extraction of phenolic compounds from fruit processing wastewaters, and studies that will be conducted later would then make a concrete conclusion on the feasibility of using supercritical fluid extraction at large scale.

In conclusion, this thesis has described the extraction and antioxidant activity of phenolic compounds present in fruit processing wastewaters. This study was intended to determine the most suitable extraction technique through the investigation of various extraction techniques, and the data generated here show that it would be probably feasible, after further investigations, to recover phenolic antioxidants for commercial use, using some of the techniques described in this thesis. This project has set the stage for further research in several directions at a more detailed level, as described in the next section. Such research will add to the growing body of knowledge and will help in the development of systems to recover phenolic antioxidants derived from fruit processing at commercial scale. In order to apply the extraction techniques described in the present study in scaled-up, cost effective industrial process, certain areas would need to be addressed in future work which is recommended in the next section.

5.1 Recommendations

Due to the preliminary nature of some of the work reported in this study, further research may be conducted in several directions that remain open for investigation. Future work is recommended in the following areas:

In the recovery of phenolic compounds using solvent extraction, ethyl acetate and hexane were used. Further investigations into other approaches to solvent extraction, such as microwave assisted solvent extraction and the use of other solvents, should be conducted to determine whether this will be beneficial in reducing the quantities used in solvent extraction. Also, investigations into the recycling of the used organic solvent would reduce the costs of purchasing the organic solvents and disposing the solvents.

In solid phase extraction, detailed research into the rate of flow of the wastewater is recommended in order to optimise this process. In this study, a rate of 3ml/min was used and investigations into determining the effect of rate of flow will also help in increasing the efficiency of this process. Also, investigations in to the use of other cartridges need to be conducted because of the poor retention of phenolic compounds in fruit processing wastewaters by the C18 Sep-Pak® cartridges.

The extraction using PVPP has potential in the recovery of phenolic compounds from fruit processing wastewaters and is already being used in industry to prevent haze formation caused by phenolic compounds. It is recommended that further investigations be done in order to optimise the recovery of phenolic compounds bound to PVPP. This method is cheap and easy to operate. Factors to be considered are: the concentration and volume of NaOH that will ensure the maximum recovery of phenolic compounds, the amount of PVPP necessary to bind all phenolic compounds in the wastewaters, and the most suitable exposure time of wastewaters to PVPP.

The SLM technique has potential advantages over conventional extraction techniques, but needs to be further developed and optimised, and parameters such as the effect of pH and temperature will need to be investigated. It is recommended that other membrane modules such as the

LiquiCell membrane be used since Microdyne module used in this work is too porous and leads to mixing of phases. It is further recommended that studies be conducted to identify an alternative solvent for use since toluene is prohibited in products that will be used as food additives.

Gallic acid was identified as a potential antioxidant phenolic compound to be extracted from fruit processing wastewaters. It is recommended that other sources rich in gallic acid such as olive mill wastewaters and winery wastewaters be investigated to determine the feasibility of using them as alternative sources of gallic acid.

The recovery of phenolic compounds using SFE is dependent on the solubility of the phenolic compound in supercritical CO_2. In the present study, it was seen that solubility of gallic acid in supercritical CO_2 is poor. However, it is recommended that the solubilities of other phenolic compounds such as p-coumaric acid, be investigated, and their antioxidant activity be quantified as well. SFE has great potential in the recovery of phenolic compounds mainly because the technique is environmentally friendly, and uses mild conditions of temperature and pressure, thereby minimising degradation and loss of antioxidant activity of the phenolic antioxidants.

The stability of different antioxidants in varying sytems is of importance in trying to characterise and determine how effective a particular antioxidant will be in the environment where it can potentially be utilised. Thus, for future work, it is recommended that investigations on the stability of the antioxidants in the wastewaters as well as in organic solvents be determined.

Finally, it was noted that the low concentration of phenolic compounds was the major drawback in making the recovery of antioxidants from the studied fruit processing wastewaters economically feasible. Thus, it is recommended that pre-concentration of the wastewaters be done in order to concentrate phenolic compounds before extraction. Alternatively, richer sources of phenolic compounds such as the silage water could be used to recover phenolic compounds. In the present study, the silage water from deciduous fruit processing contained the highest concentration of phenolic compounds and thus had a high potential for the recovery of phenolic antioxidants, making the process potentially economically feasible. Once more detailed optimisation studies have been completed on the basis of the preliminary study, a full feasibility study will be possible and a more informed decision on which technique to employ can be made.

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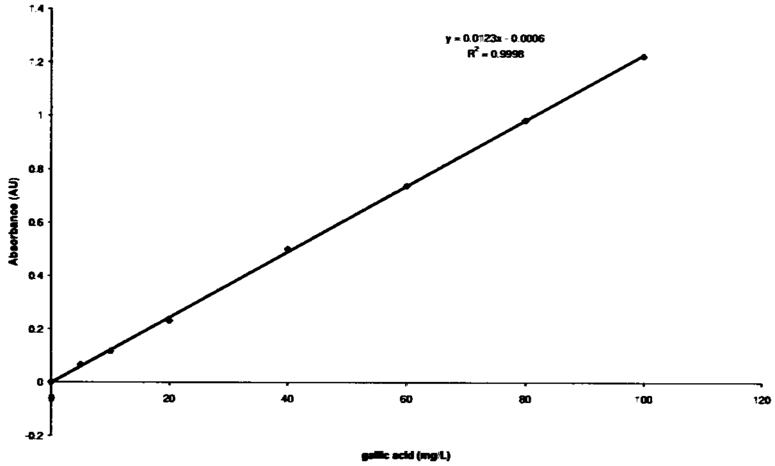
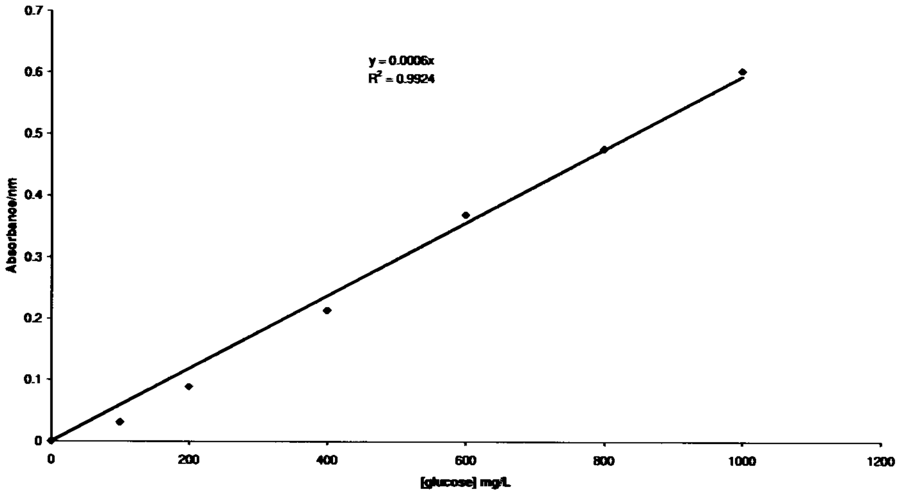
Appendix

Appendix list

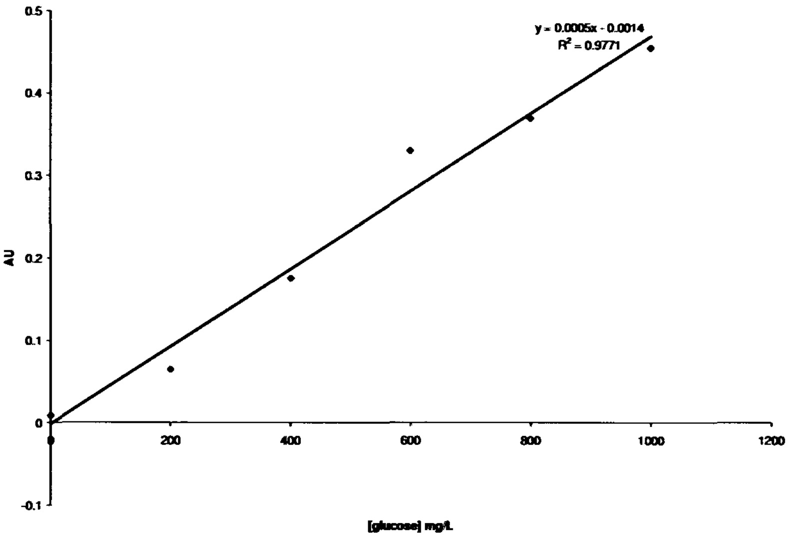
Appendix A-1: Gallic acid standard for the determination of total phenolics using the Folin Ciocalteau assay



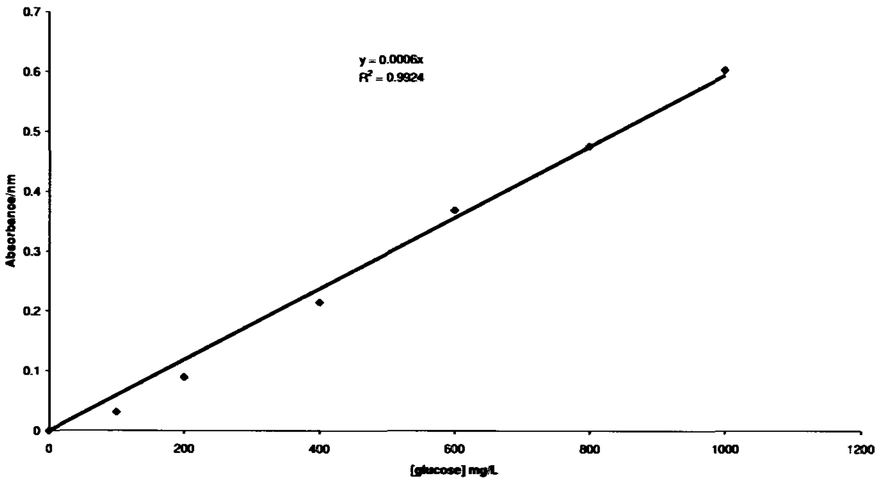
Appendix A-2: Glucose standard curve for the determination of reducing sugars



Appendix A-3: Glucose acid standard curve for the quantification of total carbohydrates



Appendix A-4: Gallic acid standard curve for the quantification of reducing power in the ferric reducing power assay



Appendix B-1: Gallic acid standard curve for the quantification of gallic acid extracted using the supercritical fluid extraction