BENEFICIATION OF AGRI-INDUSTRY EFFLUENTS

Extraction of Anti-oxidant Phenolics from Apple and Citrus Wastewaters coupled with Fermentation of Residual Sugars to Ethanol or other Value-Added Products

Report to the

WATER RESEARCH COMMISSION

by

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EXECUTIVE SUMMARY

BACKGROUND

Fruit processing industries produce a considerable amount of wastewater which must be treated before the water is discharged. Beneficiation of this wastewater has potential economic and environmental benefits. Fruit processing wastewater contains phenolic compounds and polyphenols that have antioxidant activity and other valuable properties. These, and other compounds in the wastewater, have many commercial applications and thus it is worth investigating methods for their recovery. Several techniques have been developed for the extraction of phenolics from aqueous systems and some of these can be applied to the recovery of these antioxidants from fruit processing wastewater. In addition, fruit processing wastewaters contain residual fruit sugars and some lignocellulosic particles. Fermentation of this material to give a valuable product such as 2,3-butanediol or ethanol, not only reduces the COD of the discharge water but also provides a secondary income stream.

RATIONALE

The antioxidant activity of phenolics and hence their health-related and commercial value varies, depending on their structure and source and can be measured using a variety of assays. In view of the large volumes of fruit processing wastewaters, the feasibility of wastewater beneficiation depends largely on the concentrations of valuable by-products present and the efficiency of the extraction processes that can be applied.

OBJECTIVES AND AIMS

AIM 1: Characterisation of complex wastes from fruit industries

Fruit waste samples were collected from fruit processing industries in the Western Cape, South Africa and characterised to ascertain their physical and chemical characteristics and their potential usefulness as sources of antioxidants and sugars.

AIM 2: Develop and customize new extraction processes for obtaining antioxidants

A range of methods were investigated and developed for the extraction of polyphenolic compounds from fruit processing wastewaters and for the determination of the antioxidant activities of these polyphenolics. The efficiency of these processes was evaluated for its potential application to large volume wastewaters.

AIM 3: Investigate and optimize fermentation of residuals after extraction

Fermentations of the residual fruit wastewaters, before and after extraction of phenolics, by bacteria and the yeast *Saccharomyces cerevisiae* were investigated with a view toward the production of value-added metabolites.

AIM 4: Investigate commercialization aspects

An integrated process was proposed whereby wastewater is transferred through sequential unit operations allowing for the extraction of antioxidants and the production of metabolites, finally resulting in clean water suitable for reuse.

METHODOLOGY

Fruit wastewaters were characterised using HPLC and chemical analyses. Phenolics were quantified using the Folin Ciocalteau test and antioxidant activity was determined by the DPPH radical scavenging assay, the Trolox equivalent antioxidant capacity (TEAC) assay, the Ferric reducing antioxidant power (FRAP) assay and the β-carotene linoleic acid model system. Extraction of phenolics was investigated by means of solvent extraction, adsorption onto a solid phase, supported liquid membrane extraction and supercritical fluid extraction. Residual sugars in the wastewater were fermented to ethanol and metabolites such as 2,3-butanediol through the action of bacteria or the yeast *Saccharomyces cerevisiae* under aerobic or micro-aerobic growth conditions. Solid waste was treated using heat, acid hydrolysis or enzymatic digestion to release additional sugars for fermentation. Concentration of the wastewaters using reverse osmosis was also investigated.

RESULTS AND DISCUSSION

Aim 1

Apple and citrus wastewaters were obtained and were characterised. They were found to be unexpectedly dilute but did contain phenolics and sugars. Significant levels of antioxidant activity were observed.

Aim 2

The use of solvents allowed the extraction of up to 47.7% of total antioxidants present in the effluents when using ethyl acetate, and up to 40.18% when using hexane. Solid phase extraction using adsorbent cartridges gave extraction efficiencies between 44.1 and 56.9% whereas the bulk adsorbent PVPP gave up to 39.58% adsorption over a range of phenolics and up to 80% adsorption of gallic acid in a model wastewater system. Activated carbon effectively adsorbed a range of phenolics with complete adsorption of gallic acid. Elution of

phenolics with sodium hydroxide solution, ethyl acetate and ethanol was also investigated but yields were relatively low, not exceeding 25% recovery.

Gallic acid was successfully extracted into toluene using a supported liquid membrane but solvent contamination of the aqueous phase was observed. Supercritical fluid extraction using ethanol as co-solvent was investigated and gallic acid was extracted at an efficiency of 7.7%.

Aim 3

Fermentation of the residual wastewaters was investigated using bacteria and yeast. The production of 2,3-butanediol was used as an indicator of the potential to produce value-added metabolites and optical density was used as an indicator of the potential for production of biomass. Solid phase extraction by PVPP produced the most successful fermentation medium because the presence of solid fruit particles remaining in the wastewater after this treatment added to the available carbon in the dilute wastewaters. Ethanol was produced by *S. cerevisiae* both from sugars remaining in the fruit wastewater and from sugars released from solid fruit pulp after pretreatment.

Aim 4

An integrated process was proposed whereby fruit processing wastewater is first concentrated through reverse osmosis and is then treated with a solid phase adsorbent for extraction of phenolics. The resulting wastewater is enriched with sugars released from solid fruit pulp waste through the use of enzymatic digestion and these sugars are then converted to ethanol through the action of *S. cerevisiae*. Ethanol produced in this fermentation is used to elute phenolic antioxidants from the solid phase adsorbent. The antioxidant extract obtained could potentially be used in cosmetic or nutraceutical applications.

GENERAL CONCLUSIONS

This report supports the contention that the wastewaters are potentially useful as sources of antioxidants, but very efficient methods of recovery are needed for value addition in the case of such dilute wastewaters.

The use of solvents to extract antioxidants, and the necessity for removal of solvents after extraction is costly and time consuming, and traces of solvent limit possible applications for the antioxidant extract.

Solid phase extraction using commercial cartridges for the separation and recovery of neutral and acidic phenolic compounds was not found to be effective, primarily because of the lack of selectivity in recovery of chosen fractions. Extraction efficiencies using the solid sorbents PVPP, Amberlite XAD4, and activated carbon were high for gallic acid but low for chlorogenic acid. Extraction efficiencies for real wastewaters varied according to the specific phenolics present. Elution of bound phenolics using ethanol and ethyl acetate was possible but the recovery efficiencies were low and more research is warranted.

Gallic acid, a valuable phenolic antioxidant present in silage water, was used as a model phenolic substrate and was successfully extracted using a supported liquid membrane (SLM) with toluene as co-solvent. The technique is relatively simple and easy to operate once the parameters such as solvent and feed rates have been determined. SLM technology offers a better alternative compared to traditional solvent extraction methods but further research would be useful as the tested solvent, toluene, would not be sustainable from an environmental perspective. A wider variety of milder solvents should be tested.

The use of supercritical fluid extraction gave extraction efficiency comparable with that reported by other researchers for determining the solubility of gallic acid in supercritical-CO_2 but this solubility is low. The study confirms the possibility of using supercritical fluid extraction as an alternative to conventional extraction methods but efficiency would be improved by using phenolics which have a higher solubility in supercritical-CO_2.

Overall, the methods of extraction have proved technically feasible but when considering an economically viable process, the absolute yields of antioxidants must be considered. The wastewaters studied contain very low concentrations of phenolic compounds, leading to low recovery of antioxidants despite reasonable extraction efficiencies. To make the process economically feasible, significant pre-concentration of the wastewaters would be required. This would allow extraction from much smaller volumes of wastewater, reducing the volume of solid sorbents or liquid solvents required, leading to lower costs per unit antioxidant recovered.

The sugar concentration in the treated wastewaters is low and there is evidence that natural fermentation has already occurred. Batch fermentations would not be an efficient strategy with such a dilute carbon source and continuous fermentation could be considered. This would be feasible if the product is easy to separate from the fermentation broth. Where the target product is biomass, continuous fermentation with a settling tank or physical separation to collect the biomass would be best. This may then result in an overflow stream of water or

dilute metabolites with the concurrent gradual build up of biomass over time. Alternatively, if a metabolite is the desired product, it should be one that is easy to separate. One example would be the production of a compound that would be retained on an ion-exchange column. The fermentation outflow could pass through the column bed and the product would gradually accumulate over time and be harvested when efficient.

Aerobic fermentation of effluents after solid phase extraction using PVPP was successful and growth was observed. Sugars and acids were utilised for 2,3-butanediol formation in some cases, in both aerobic and micro-aerobic fermentations. For ethanol production by bacteria, PVPP-extracted wastewater could be used and the fermentation must be micro-aerobic.

The yeast *S. cerevisiae* was able to grow and produce ethanol in a synthetic model apple wastewater, concentrated 6 fold, as could reasonably be expected from reverse osmosis concentration (Jesus et al. 2007). Gallic acid in the concentration range of 0.02 to 0.1 g/l was found to have no effect on the growth of *S. cerevisiae* or on ethanol production. However, higher concentrations of gallic acid, 0.1 to 0.3 g/l, enhanced ethanol production, increasing the final ethanol concentration from 3.77 g/l to 6.16 g/l. This is important because enriching apple wastewater with solid apple waste could have greater effects than anticipated if, alongside the fermentation of additional sugars released from the solid waste, released phenolics also contributed to increases in ethanol production.

Solid apple waste which has been treated using steam explosion, acid digestion or enzyme digestion, contains free sugars which are available for fermentation to ethanol. The absolute concentration of sugars after pretreatment is dependent on the levels of sugar in the apples initially. Comparative release of sugars showed that where the sugar level in the apples is high, physical disruption using heat releases the most sugar while acid digestion causes a decrease in available sugar. Where the sugar concentration is lower, treatment with enzymes is most effective in releasing sugar. Enzymatic pretreatment was also effective in increasing the concentration of sugars from sugar-rich solid apple waste and it is therefore recommended as the pretreatment best suited to a range of apples from different cultivars and at different stages of ripening.

Concentration of the wastewater using reverse osmosis is possible and a 2.76 fold concentration was achieved. Increasing processing time may increase the concentration factor, and alternative membranes may prove more resistant to microbial degradation with prolonged use.

Based on these findings, the beneficiation of the agri-industry wastewaters investigated is proposed as a process comprised of a number of unit operations (see Figure A). Fruit waste and wastewater would progress through sequential operations, releasing purified water producing value-added products.

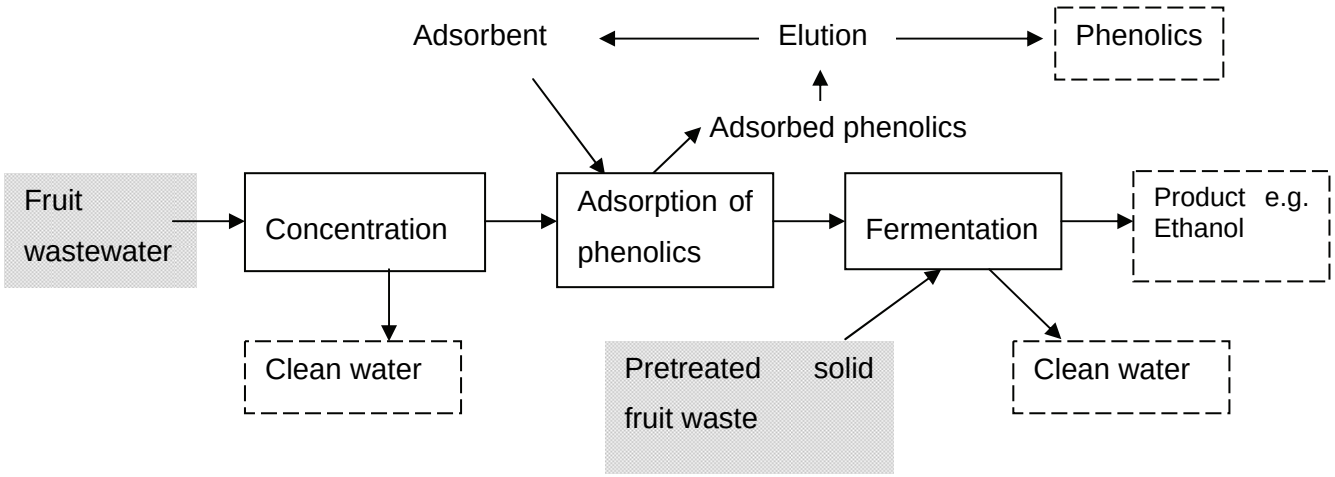

Figure A: Proposed process flow sheet, illustrating the recovery of clean water, antioxidant phenolics and value added products from fruit wastewater

RECOMMENDATIONS FOR FUTURE RESEARCH

It is recommended that membrane-based concentration of the wastewaters be utilised as the first step in treatment. Additional investigations into membrane concentration should be considered. Particularly, it would be recommended to include a greater diversity of membranes, including polyamide membranes which would be more resistant to microbial digestion. The greater the degree of concentration achieved in the first step, the smaller the volumes of wastewater to be treated in subsequent steps. Smaller volumes require less processing space and less energy in the form of electricity for pumping and heating.

Of the adsorbents tested, it is recommended that either PVPP or activated carbon be used for the adsorption of phenolics. With PVPP, the reject from the membrane concentration could be passed through a bed of adsorbent while the emerging water is monitored for phenolics. With activated carbon, a batch process would be preferable, where tanks would be filled with the reject from membrane concentration and allowed to interact for up to four hours. The material in the tanks would be allowed to settle and the treated water would be decanted. Activated carbon tanks could be reused until the decanted water showed increasing levels of phenolics.

Ethanol is the recommended solvent for elution from either PVPP or activated carbon. More work is required to improve elution efficiencies, however, as the current investigation showed that large volumes of ethanol would be used to extract significant percentages of bound phenolics. Extracts could be concentrated by distillation of the ethanol (preferably under vacuum to avoid the use of high temperatures) and the ethanol would then be reused. Eluting a more concentrated antioxidant solution with a smaller volume of ethanol would be preferable.

Supported liquid membrane extraction should be investigated using a less toxic solvent than toluene to extract phenolics since toluene is prohibited in products that will be used as food additives.

The addition of solid fruit waste to the wastewater was found to be a useful approach to increasing the carbon content of the water. Enzymatic pretreatment of the solid waste is recommended as this method releases sugars from solid apple waste with high or low sugar concentrations and is therefore well suited to a range of apples from different cultivars and at different stages of ripening.

While fermentation of the sugars to ethanol by *Saccharomyces cerevisiae* was shown to be possible, for cost efficient recovery, a goal concentration of 40 g/l ethanol in solution would be needed; below this level, the distillation costs involved in recovering the ethanol, would be prohibitive. Simultaneous saccharification and fermentation, and fed batch processing, would be worthy of further investigation to shorten the reaction time, as both enzyme digestion and ethanol production are time consuming unit processes. Combination of these unit operations allows the processes to run concurrently. Fed batch additions of solid waste allow gradual release of sugars, bypassing any inhibition of fermentation caused by high substrate concentrations. It is recommended that both of these processes be investigated to improve the ethanol yield in fermentation.

A distillation unit operation will be necessary in the proposed process, both for concentration of ethanol after fermentation and for concentration of phenolic extracts but through optimisation of all unit processes, the cost of distillation should be minimised and balanced by income received from value-added products.

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LIST OF ABBREVIATIONS

ABTS	2,2'-azonobis (3-ethybenzothiazoline-6-sulphonate)
AC	Activated carbon
AW1	Apple wastewater 1
AW2	Apple wastewater 2
B-CLAM	β-carotene linoleic acid model system
BHT	Butylated hydroxytoluene
BOD	Biological oxygen demand
^{o}Brix	Measure of the sugar content of an aqueous solution: 1^{o} Brix is defined as 1 g sucrose in 100 g solute
CO_2	Carbon dioxide
COD	Chemical oxygen demand
CW1	Citrus wastewater 1
CW2	Citrus wastewater 2
DNS	3,5-dinitrosalicylic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
FC	Folin Ciocalteau
FRAP	Ferric reducing antioxidant power assay
GC	Gas chromatography
GC-MS	Gas chromatography linked to mass spectroscopy
HAT	Hydrogen atom transfer
HFSLM	Hollow fibre supported liquid membrane
HPLC	High performance liquid chromatography
IMBM	Institute for Microbial Biotechnology and Metagenomics
L	litre
MF	Micro-filtration
NB4	Bacterial isolate received from IMBM
NF	Nano-filtration
OD	Optical density
PCET	Proton-coupled electron transfer
PVPP	Polyvinylpolypyrollidine
RO	Reverse osmosis
RP-HPLC	Reverse phase high performance liquid chromatography
SEM	Standard error of the mean
SFE	Supercritical fluid extraction

SLM	Supported liquid membrane
SPE	Solid phase extraction
SSF	Simultaneous saccharification and fermentation
SW	Silage water
TEAC	Trolox equivalent antioxidant capacity assay
TH141	Bacterial isolate received from IMBM
TP	Total phenolics
UF	Ultra-filtration
UWC	University of the Western Cape
%RSA	Percentage radical scavenging activity

1 INTRODUCTION AND OBJECTIVES

1.1 Agri-Industry Wastewaters

Agri-industrial wastewaters are produced in large quantities from the processing of fruits and vegetables and have high organic loads (Bardiya et al*.,* 1996). The agri-food industry also produces large amounts of solid waste, often known as pomace, which is mixed with the wastewaters (Widmer et al*.,* 2010; Zheng and Shetty, 1998). In South Africa the citrus, deciduous fruit (apples, peaches, grapes, apricots), olive, winery and distillery industries are the major producers of agri-industrial wastes. The fruit juice industry produces waste during all of its process stages – unloading, washing, sorting, pressing, juice extraction and packaging – and comprises a large portion of agri-industrial waste (Sigge et al*.*, 2006). In this research the wastewaters from citrus and apple juicing industries were used to test the viability of their treatment and beneficiation.

1.1.1 Citrus and citrus processing waste

Citrus is the most abundant fruit crop in the world, and includes predominantly oranges as well as lemons, grapefruits, mandarins, clementines, tangerines and limes. The main purpose of processing citrus fruit is to obtain juice but other uses of citrus include canning, production of marmalade, and the extraction of flavonoids and essential oils. The citrus industry in South Africa produces 1.6 million tons of fruit annually and during the juicing process a total of 1.5 million litres of wastewater is produced. Globally, industrial citrus wastes are greater than 15 million tons, which can be explained by the fact that about 50% of the original fruit remains after processing (Marín et al*.,* 2007).

Citrus fruits contain the flavonoids: hesperidin, narirutin, naringin and eriocitrin (Schieber et al*.,* 2001; Scordino et al*.,* 2007; Xu et al*.,* 2008) and also contain high amounts of vitamin C, folate, other vitamins, potassium and other minerals (Economis and Clay, 2010). The wastes from the juicing of citrus include peels, membranes, seeds, pulp and washing waters (Tripodo et al*.,* 2004). These wastes are rich in sugars and fibres and also contain flavonoids, carotenoids, anthocyanins, other polyphenols, pectins, hemicelluloses and cellulose. The citrus oils and flavonoids as well as the high COD and BOD of citrus processing wastewaters make them an environmental hazard (De Gregorio et al*.,* 2002; Scordino et al*.,* 2007; Sudha et al*.,* 2007; Tripodo et al*.,* 2004).

1.1.2 Apples and apple processing waste

Apples contain 85.3% water, 0.3% protein, 0.4% lipids, 11.8% carbohydrates, 0.6% organic acids, 2.3% fibre (including lignin) and a variety of minerals (particularly potassium), vitamins

(particularly vitamin C) and amino acids. The carbohydrates in apples are predominantly sugars (glucose, fructose and sucrose) and starch (Besler, 1999). Many of these components will be present in the wastewater and pomace generated from the processing of apples in the canning, juicing and distillery industries. The production of apple juice occurs mainly between February and June, and in South Africa this process results in 6.8 million litres of wastewater annually (Sigge et al., 2006).

Apple pomace and apple processing wastewater contain high concentrations of pectin and phenolic compounds, and have acidic pH's (pH 3.5-5) (Arvanitoyannis and Varzakas, 2008; D'Abrosca et al., 2007; Lu and Foo, 2000; Nawirska and Kwasniewska, 2005; Schieber et al., 2001; Schieber et al., 2003; Sigge et al., 2006). The composition of apple processing wastes varies greatly depending on the apple variety, how ripe the fruit was at the time it was processed, and the juicing process itself (D'Abrosca et al., 2007; Kennedy et al., 1999). For example, as apples ripen, there is an increase in the amount of soluble pectin compared to insoluble pectin in apple pomace (Kennedy et al., 1999). Apple pomace consists of the peel, core, calyx, seed, stem and soft tissue of the fruit (Kennedy et al., 1999) and so contains a higher lignin content than the wastewater stream, which contains higher amounts of sugar, starch and cellulose. The peels of apples are a good source of polyphenols including catechins, hydroxycinnamates, phloretin glycosides, quercetin glycosides and procyanidins (Schieber et al., 2001; Schieber et al., 2003).

1.2 Benefits of Treating Agri-Industry Waste and Wastewater

Wastewater treatment and waste management is a growing global concern and there is a pressing need to develop environmentally friendly methods for the production of clean water from waste streams. Wastewater treatment can also be economically beneficial to industries as there are many compounds in the wastewater that can be re-used and recycled (Dobson and Burgess, 2007).

1.2.1 Agri-industry waste

Currently worldwide some solid agri-industrial waste is placed in landfills or incinerated which poses environmental and public health concerns and is costly (Isci and Demirer, 2007, Zhu et al., 2009). Other solid agri-industry wastes, including fruit processing pomace, are applied to agricultural land as soil amendment (Clarke et al., 2008) which is less wasteful and improves soil fertility but run-off from fields may be high in nitrogen and phosphates if incorrectly managed (Champagne, 2007, Sigge et al., 2006). Apple and citrus wastes are often used as animal feed (Widmer et al., 2010), an environmentally sound option but with limited economic benefit.

1.2.2 Agri-industry wastewater

For fruit processing industries, fresh water is used not only in washing and extraction of fruit juice but often as a convenient transport medium too (Mannapperuma, 2005). Water may be recirculated within this hydraulic transport system but gradual build up of soluble and insoluble material increases the risk of microbial growth, necessitating either chemical sanitising or continuous dilution of process water with fresh water. Where the latter strategy is employed, a large volume of water is discharged into the sewer system. This water is usually combined with other, more concentrated waste streams generated at a much lower rate. The net result is a large volume of wastewater that is comfortably within the limits for municipal discharge.

Considering the increasing global demand for water and specifically the need on the African continent (Dobson and Burgess, 2007, Showers, 2002) this presents an opportunity to conserve water. Schutte and Pretorius (1997) noted that an increase in population caused both direct and indirect increases in water consumption as the growing population used increasing amounts of food products each of which have an associated water consumption and wastewater stream. Kamara and Sally (2003) projected that by 2025 South Africa will be a water-scarce country. Methods of minimising water usage and wastewater production in the citrus industry have been proposed (Thevendiraraj et al., 2003) and should be considered. Alongside minimising potable water usage, the extractive treatment of fruit wastewater should be investigated to generate both clean water for recycle and value-added products.

1.3 Value-Added Products

In addition to the environmental and sociological impacts of wastewater treatment, wastewaters are a potential source of value-added products which can off-set the costs involved in the treatment process (Widmer et al., 2010). Dietary fibre, pectins, natural sweeteners, essential oils and antioxidants are some of the products that can be obtained from apple and citrus processing wastewaters. The sugars and other carbohydrates in the wastewaters can be used for the growth of biomass, fermentation to alcohols, production of biogas, production of fertilisers, enzyme production or used in animal feed (Arvanitoyannis and Varzakas, 2008; Kennedy et al., 1999; Schieber et al., 2003; Zheng and Shetty, 1998).

Dietary fibre includes cellulose, hemicelluloses, lignins, gums and pectins, and is found in plant cell walls (Nawirska and Kwasniewska, 2005; Sudha et al., 2007). It is important for

human digestive processes because it acts as a bulking agent, aiding intestinal mobility and adding water content to faecal matter (Nawirska and Kwasniewska, 2005; Sudha et al., 2007). Dietary fibre, therefore, has significant health benefits including the prevention of diseases such as constipation, haemorrhoids, hypercholesterolemia and colorectal cancer (Marín et al., 2007). Dietary fibre also has physical and chemical properties that make it a valuable product, for example its antioxidant activity, lipid-holding capacity and water-holding capacity (Marín et al., 2007). Apple pomace, the residue after the juicing process, is a rich source of dietary fibre and has particularly high concentrations of pectin (Sudha et al., 2007). Pectins have been extracted from apple pomace and orange peels and used commercially as dietary fibre in food supplements and as gelling agents, thickeners, texturisers, emulsifiers and stabilisers in food (El-Nawawi and Shehata, 1987; Schieber et al., 2003; Sudha et al., 2007).

A second type of food additive that can be obtained from fruit processing wastewaters is natural sweeteners and sugars. Apple and citrus processing wastes contain glucose, fructose and sucrose which can be used as food-additives. For example, a sugar-syrup extracted from orange pulp wash can be used as a sweetener (Scordino et al., 2007).

Essential oils from fruit are commonly extracted for commercial purposes (De Gregorio et al., 2002; Scordino et al., 2007; Vaher and Koel, 2003). Orange essential oil was obtained from orange peels using supercritical carbon dioxide extraction (Mira et al., 1999). This orange essential oil is used commercially to add aroma and flavour to carbonated drinks, ice-creams, air-fresheners and perfumes. The essential oil, *d*-limonene, found in citrus fruit also has antimicrobial properties and has been extracted from the skins of oranges and lemons (Mira et al., 1999; Yi et al., 2008). Essential oils are used in aromatherapy, dermatology and cosmetics (Cakir, 2004). Oil extracts from fruit processing wastes can be used to treat gastric ulcers and have regenerative, anti-inflammatory and photo-protective properties (Cakir, 2004).

Apple and orange processing wastes are often used as animal feed, but due to the low protein content, this is not the best use of these by-products (Carroad and Wilke, 1978; Villas-Bôas et al., 2002; Zheng and Shetty, 1998). Also, the water content in apple pomace and citrus pulp is high and needs to be reduced before these wastes can be used as animal feed (Tripodo et al., 2004). This can be done by pressing and drying in an industrial dryer (Carroad and Wilke, 1978).

Fruit processing wastes can also be used to grow biomass by providing a source of carbohydrates and other nutrients required for microbial growth. Zheng and Shetty (1998) describe the growth of various commercially important fungal species (*Trichoderma* species, a *Penicillium* species, and a *Rhizopus* species) on apple processing wastes. The micro-organisms grown on the fruit processing waste can then be used to produce industrially important enzymes and valuable secondary metabolites, some of which are described in Section 1.5 (De Gregorio et al*.,* 2002; Zheng and Shetty, 1998).

Deciduous and citrus fruits have many health benefits that can be attributed to their high antioxidant content. Many of these antioxidants, including citric acid, phenolic acids, flavonoids, anthocyanins and other polyphenols are present in fruit processing wastewater (Arvanitoyannis and Varzakas, 2008; Schieber et al*.,* 2003; Zheng and Shetty, 1998).

1.4 Antioxidants

Medical research has shown that consumption of fruit as part of the human diet has important health benefits, such as reduced risk of heart disease, strokes, cancer, inflammation, arthritis, immune system decline, brain dysfunction, cataracts and other age-related diseases (Lu and Foo, 2000; Schieber et al*.,* 2003; Wijngaard et al*.,* 2009). These health benefits can mainly be attributed to the micronutrients present in the fruit, such as carotenoids, polyphenolics, tocopherols, vitamin C and others, that possess antioxidant properties (Adil et al*.,* 2007; Lu and Foo, 2000; Schieber et al*.,* 2003). Antioxidants can be defined as substances that neutralise free radicals and thus prevent or retard oxidative damage that is linked to degenerative diseases (Adil et al*.,* 2007; D'Abrosca et al*.,* 2007).

It is interesting to note that there is a growing trend toward natural versus synthetic antioxidant compounds, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which are thought to be carcinogenic and interfere with metabolic enzymes (Peschel et al*.,* 2006; Schieber et al*.,* 2003; Tamano et al*.,* 1998). The health benefits and the increased search for natural antioxidants makes the micronutrients present in fruit processing waste of considerable interest and hence the use of fruit processing waste as a source of antioxidants could be of significant economic advantage to industries (Wijngaard et al*.,* 2009). A study performed by Yi et al., (2008) showed that orange peels (*Pericarpium Citri Reticulatae)*, which constitute waste from citrus processing, exhibit high levels of antioxidant, as well as anti-microbial, activity due to the presence of polyphenols.

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, whereas

polyphenols are phenolic compounds characterised by the presence of more than one aromatic ring (Balasundram et al., 2006). Polyphenols include phenol acids and flavonoids, which are found both in fruit and in fruit processing wastewater, and are responsible for the majority of the antioxidant activity in plant extracts (Siddhuraju, 2007). Research has shown that polyphenols have anti-allergenic, antiviral (Chávez et al., 2006), antibacterial (Vaquero et al., 2007), antithrombotic, cardioprotective and vasodilatory effects (Kris-Etherton et al., 2002). Phenolic compounds are secondary metabolites and derivatives of the pentose phosphate, shikimate and phenylpropanoid pathways (Balasundram et al., 2006). They are amongst the most widely distributed phytochemicals in the plant kingdom, where they play an important role in growth and reproduction (Balasundram et al., 2006; Soobrattee et al., 2005). Phenolics contribute to the colour and flavour of fruits, which is important for attracting pollinating agents, and they also provide protection against pathogens and predators (Balasundram et al., 2006; D'Abrosca et al., 2007; Gomis et al., 2001; Soobrattee et al., 2005). The concentration of phenolic compounds varies depending on the physiological stage of the fruit (D'Abrosca et al., 2007; Gomis et al., 2001), and also varies depending on the type of fruit (Balasundram et al., 2006). Table 1.1 details the levels of phenolics found in types of fruit cultivated in the Western Cape. Apples and apple pomace, in particular, have one of the highest antioxidant activities compared to other fruit and vegetables (D'Abrosca et al., 2007). In apples, phenolics are found mainly in the skins of the fruit and so are present in apple pulp from the juice industry (Lu and Foo, 2000; Naczk and Shahidi, 2004). Many of the antioxidant compounds in fruit are also present in the effluents generated from fruit processing (Adil et al., 2007; Schieber et al., 2003).

Table 1.1 The concentration of phenolics in a variety of fruits grown in the Western Cape (selected from Balasundram et al., 2006).

Fruit	Total phenolics content	Reference
Apple	296.3 ± 6.4[a]	Sun et al. (2002)
Blackberry	417-555[a] 26.7-452.7[a] (*Rubus* species)	Sellappan et al. (2002) Deighton et al. (2000)
Blueberry	270-930[a] (rabbiteye) 261-585[a] (Southern highbush) 171-961[a] (*Vaccinium* species)	Sellappan et al. (2002) Moyer et al. (2002)
Cherry	105.4 ± 27.0[b]	Karakaya et al. (2001)
Guava	126.4 ± 6.0[a] (pink) 247.3 ± 4.5[a] (white)	Luximon-Ramma et al. (2003)
Peach	84.6 ± 0.7[a]	Sun et al. (2002)
Persimmon	1.45[c]	Gorinstein et al. (1999)
Plums	174-375[a] 143.5 ± 40.6[b] (black)	Kim el al. (2003) Karakaya et al. (2001)
Prunes	184 ± 85.5[a]	Donovan et al. (1998)
Raisins	399.4 ± 57.6[b]	Karakaya et al. (2001)
Raspberry	114-178[a]	de Ancos et al. (2000)
Red grape	220.6 ± 61.2[c] 201 ± 2.9[a]	Karakaya et al. (2001) Sun et al. (2002)
Strawberry	161-290[a] 160 ± 1.2[a]	Heinonen et al. (1998) Sun et al. (2002)

[a] Gallic acid equivalents/100 g fresh weight

[b]Catechin equivalents/100 g fresh weight

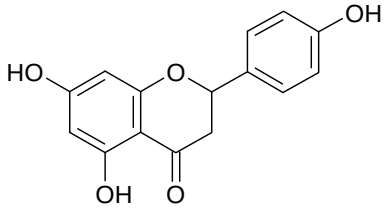
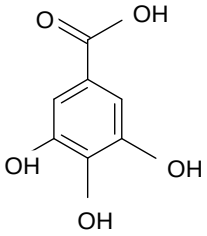
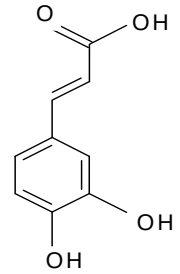
[c]Chlorogenic acid equivalents/100 g fresh weight

Phenolic compounds are already used in industry as antioxidants as well as chemical intermediates, disinfectants, stabilisers and tanning agents. In cosmetology, there has been a recent increase in the incorporation of phenolic compounds during preparation. Peschel et al., (2006) performed suitability tests on the incorporation of phenolic compounds extracted from industrial vegetable and fruit wastes in crème formulations. They concluded that phenolic compounds represented a potential source of antioxidants for use in dermatology. Anthocyanins are being applied in the food, cosmetic and pharmaceutical industries as substitutes for synthetic colorants (Luque-Rodríguez et al., 2007). Capsules of anthocyanins

are now being prepared for use in health rejuvenation and prophylaxis against cold viruses (Petri et al., 1997).

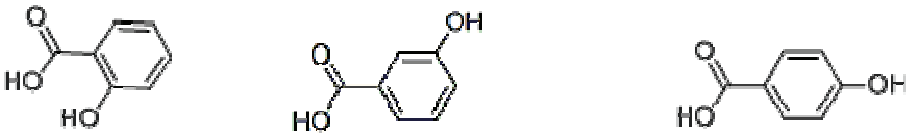
The major phenolic compounds found in fruit and hence fruit processing wastes are listed in Table 1.2. Phenolic acids can be classified into two groups, namely, hydroxybenzoic acids and hydroxycinnamic acids. Hydroxybenzoic acids consist of an aromatic ring and an -OH group to which one carboxylic acid group is attached whereas the aromatic ring in hydroxycinnamic acids have a 3-carbon side chain (Table 1.2) (Balasundram et al., 2006). Flavonoids consist of two aromatic rings linked by a 3-carbon heterocyclic ring (Balasundram et al., 2006). The variations in the substitution patterns on the ring structures results in the different classes of flavonoids, namely: flavonols, flavones, flavanones, isoflavones, flavanols, and anthocyanins (Adil et al., 2007; Wijngaard et al., 2009).

Table 1.2 Examples of some phenolic compounds found in fruit and fruit processing waste (Balasundram et al., 2006).

Class	**Example**	**Structure**
Flavonoids	Naringenin	
Hydroxybenzoic acids	Gallic acid	
Hydroxycinnamic acids	Caffeic acid	

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 (Balasundram et al., 2006). The antioxidant activity of phenolic acids depends on the number and position of hydroxyl groups (-OH) in relation to the carboxyl groups (-COOH) (Podsędek, 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 1.1 However, when the -OH moiety is at the *meta*- (**2**) position, hydroxybenzoic acids exhibit antioxidant activity.



(**1**) (**2**) (**3**)

Figure 1.1: Structures 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 (Balasundram 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*.,* 1996). Furthermore, the antioxidant activity of flavonoids in general, is enhanced by their ability to chelate metals (Ozsoy et al*.,* 2008; Tsao and Yang, 2003). 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).

1.5 Utilisation of Sugars in Fruit Processing Wastewater

After the extraction of phenolics from fruit processing wastewater there are several options for the further remediation of the wastewater to produce clean water and additional economically beneficial products.

1.5.1 Fermentation of residual fruit processing wastewater

The sugars and carbohydrates in fruit processing wastewater are good substrates for fermentation to bioethanol and other alcohols, which can be sold or used for energy (Abbasi and Abbasi, 2010). The most popular organism for bioethanol production is *Saccharomyces cerevisiae*. The carbohydrates in the wastewater, such as cellulose and hemicellulose, first need to be hydrolysed into sugars before fermentation (Prasad et al., 2007; Ruane et al., 2010). This can be carried out by a variety of bacterial or fungal species, many of which have been used together with *S. cerevisiae* in a simultaneous saccharification and fermentation (SSF) system (Sánchez, 2009).

Fruit waste has been studied successfully as a substrate for ethanol fermentation by *S. cerevisiae* (Akin-Osanaiye et al., 2005). In that work, the fruit peels and seeds were removed and only the pulp was used for fermentation but apple wastewater contains material from the skin and seeds of the fruit and can therefore be expected to contain phenolics. Phenolics are known to affect the growth of micro-organisms but the effects of specific phenolics vary based on their concentration. Buikema, McGuiniss and Cairns (1979) reported toxicity in the range 0.084 to 555 mg/l for micro-organisms in aquatic ecosystems while Chen et al. (2010) noted inhibition in the growth *Bacillus subtilis* for concentrations between 437-934 mg/l but stimulation of growth by low concentrations (less than 100 mg/l) of resorcinol and hydroquinone. Figueiredo et al. (2008) studied the effect of wine phenolics on *Oenococcus oeni* and *Lactobacillus hilgardii* and observed inhibition by many phenolic aldehydes but not by catechin, a phenolic compound found in apple waste. A review of the effect of phenolics on lactic acid bacteria (Rodríguez et al., 2009) reported that gallic acid and catechin were among the least toxic phenolics studied by Landete et al. (2007) while Reguant et al. (2000) found that gallic acid and catechin stimulated growth of *O. oeni* and that gallic acid had an inhibitory effect on acetic acid formation from citric acid, allowing manipulation of malolactic fermentation. *L. hilgardii* growth was stimulated by gallic acid and catechin when present in concentrations normal to wine (Alberto et al, 2001). This was also true of *L. collinoides* whose early growth was stimulated by chlorogenic and gallic acids but not of *L. brevis* on which these phenolics had no effect at the chosen concentrations of 100, 500 and 1000 mg/l (Stead, 1994). The effect of phenolics present in the wastewaters used in this study must be assessed.

1.5.2 Other uses of sugar in fruit processing wastewater

The addition of nitrogen to the wastewater facilitates the utilisation of residual carbon nutrients to generate biomass. The biomass may be used for the production of fertiliser, biogas, bioethanol or other high value secondary metabolites, depending on the organism

used for fermentation (Arvanitoyannis and Varzakas, 2008). A full analysis of the residual water can be performed in order to determine if the wastewater has been adequately remediated to be released into the environment.

Biohydrogen, also used a source of energy, is released from a process known as dark fermentation, for which fruit processing wastewater is a viable substrate (Guo et al., 2010). All biological methods for hydrogen production are controlled by hydrogen producing enzymes, such as hydrogenase and nitrogenase (Kothari et al., 2010). Temperature, pH and substrate concentration are important factors in dark fermentation, because they affect hydrogen production and bacterial growth (Feng et al., 2010; Guo et al., 2010; Yu et al., 2002).

Anaerobic digestion of agri-industrial wastes releases methane, or biogas which can be converted to methanol or used as energy, to reduce the energy costs of the industry (Kothari et al., 2010). Anaerobic digestion, also known as biomethanation is complex, involving a mixture of bacterial species and several enzymatic stages. The first stage involves the hydrolysis of polymers, such as lipids, proteins and carbohydrates into smaller molecules, such as fatty acids, amino acids and glucose. In the next stage, acidogenesis, these products are metabolised and fermented to produce organic acids, hydrogen and carbon dioxide. In acetogenesis, the organic acids are converted to acetic acid, hydrogen and carbon dioxide. Finally, methanogenic bacteria produce methane from acetic acid (Ruane et al., 2010). Anaerobic digestion is already practised in fruit processing industries in South Africa (Sigge et al, 2006) and will not be investigated in this report.

1.5.3 Fermentation using solid fruit processing waste

Solid waste from the fruit processing industry is currently used as animal feed or to fertilise soil but it may be more profitable to convert this waste into a value-added product through fermentation. Various types of solid fruit waste have been investigated as feedstocks for biofuels production. Fruit peel such as mango (Somda et al., 2011), banana (Tewari et al., 1986), waste from dragonfruit (Hii et al., 2010), bitter kola (Akin-Osanaiye et al., 2006), kinnow citrus (Sharma et al., 2007) and apple (Kumar et al., 2008) have all been studied with a view toward releasing the sugars for ethanol fermentation.

Solid fruit waste requires a pretreatment step before it can be converted to ethanol using the yeast *Saccharomyces cerevisiae* as fruit peels and pulp consist of complex carbohydrates and lignocellulose. Pretreatment using heat or acid hydrolysis disrupts cellulose structure and partially solubilises hemicellulose, releasing fermentable sugar from the lignocellulosic

feedstock. It also disrupts the lignin component, generating aliphatic acids such as levulinic acid and derivatives of furan such as furfural and 5 hydroxymethylfurfural, which inhibit the growth of micro-organisms (Larsson et al., 2000 and 2001). Saccharification using enzymes or whole cells, offers milder conditions but also has drawbacks including longer processing time and expense (when purchasing pure enzymes). When using whole cells, sugars are used in maintaining the growth of the saccharification culture and this results in sugar loss leaving less sugar available for the fermentation culture. An effective pretreatment is performed under conditions that avoid degradation of pentose from hemicelluloses, or glucose from cellulose, and limit formation of degradation products that inhibit the growth of fermentative micro-organisms. Pretreatments should also limit energy, chemical, and/or enzyme usage in order to limit the cost of the pretreatment itself (Mosier et al., 2005).

When considering enzymatic pretreatment, it must be considered that whereas starch is a storage compound consisting of glucose linked via α-1,4 and α-1,6 glycosidic linkages (amylose and amylopectin), present in plants as an energy source, cellulose is a structural wall component that provides rigidity to the plant. It is a highly crystalline and compact substrate, composed of glucose linked β-1,4 glycosidic bonds, which makes it resistant to enzymatic hydrolysis (Gray et al., 2006). In this sense, it is not surprising that lignocellulosic substrates are more resistant to biological attack than starch. It is estimated, on a protein weight basis, that 40-100 times more enzyme is required to degrade cellulose than is used to hydrolyse starch in order to produce equivalent amounts of ethanol. Since the cost of enzyme production is not substantially different (Merino and Cherry, 2007), the overall cost of enzyme use is higher for ligno-cellulosic ethanol conversion.

1.6 Concentration of Fruit Wastewater using Membranes

When the agri-industry wastewater falls into the category of high volume, dilute wastewater as described in section 1.2.1, concentration of the wastewater would immediately lower the volume of waste to be treated by removing a portion of the wastewater as a purified permeate, suitable for reuse. The remaining concentrated waste could then be utilised for phenolic extraction and fermentation. Membrane technology is a technique widely used in fractionation, purification and concentration processes and is commonly used in the water treatment industry. In this industry, the most commonly used membrane systems are micro-filtration (MF), ultra-filtration (UF), nano-filtration (NF) and reverse osmosis (RO). In each process, pressure is applied to a solution which is constrained by a membrane. Each membrane type has a different pore size and selectivity and this directs the application. MF and UF are used to remove larger particles, visible to the human eye, and water treated with

a UF unit is clear and colourless, containing no suspended solids. NF is used to remove smaller particles from liquors (Nazaroff and Alvarez-Cohen, 2001) while RO removes dissolved substances.

Under the normal process of osmosis, water molecules move from an area of lower concentration to an area of higher concentration but through the use of pressure and a semi-permeable membrane, this process may be reversed. In RO filtration, water molecules (the permeate) are forced through a semi-permeable membrane from an area of higher solute concentration to an area of lower solute concentration. The dissolved matter (the reject) is left on the pressurised side of the membrane and concentration of the solute increases. Reverse osmosis membranes have the ability to remove dissolved matter from a solution and separate particles in the size range of 0.0001 to 0.001μm at pressures between 20 bar and 100 bar. RO membranes are commonly used in the desalination of brackish and sea water (brine) where they have a rejection of 97% to 99% salt (Rautenbach and Albrecht, 1982).

Applied to fruit wastewater, the permeate would be clean water available for reuse or discharge. The reject would be a concentrated stream of sugars and phenolics which would serve as the starting material for the extraction step. RO membranes are most commonly constructed using cellulose acetate, polyamide and thin film composites. Polyamide membranes have the advantage of allowing high flow and excellent fructose rejection of 99.7% (Aqua-trex, 2009). Cellulose acetate membranes provide lower flow rates and a lower rejection of 98.5% but have the advantage that the rejection of the membrane increases as the concentration increases, thus the concentration is not directly proportional to the rejection of the membrane (Aqua-trex, 2009).

Filtration has been used to concentrate grape pomace for recovery of antioxidants (Diaz-Reinoso et al., 2010) giving phenolic contents of 3 to 6.6 times higher in the reject than in the feed solution. Reverse osmosis and nano-filtration membranes were shown to provide high rejections of sugars (77-94%) and phenolics (70-94%). In this work, fouling of the membranes was observed as the viscosity of the solution increased. As the solution becomes more concentrated, a boundary layer of the solution forms on the surface of the membrane, reducing the flux.

Fouling was also observed in the concentration of orange juice (Jesus et al. 2007). Here, final 5.8 fold concentration was achieved, to give 36^{o} Brix. When a sucrose solution was concentrated to mimic waste leaching liquid from the citrus juicing industry, an increase from

10^{o} Brix to a final value of 20 to 25^{o} Brix was achieved (Garcia et al., 2002). The reduced concentrating ability of the system was attributed to high osmotic pressure.

The performance of a membrane is characterised by its selectivity and the permeate flux. The selectivity of the membrane, commonly referred to as rejection, is the ability of the membrane to remove the undesired particles from the water and is calculated using the following expression:

$$\mathbf{R= (C_F-C_p)/C_F}$$ (Nazaroff and Alvarez-Cohen, 2001)

$\mathbf{C_F}$ = concentration of solute in the feed
$\mathbf{C_p}$ = concentration of solute in the permeate

The permeate flux is the rate at which the clean water flows out of the membrane per unit surface area of membrane. This can be calculated using the following expression:

$$\mathbf{J= (K/t)\ (\Delta P-\Delta \pi) = (Q_p/A)}$$

K = Constant for a system incorporating the contact area, thickness and permeability of the membrane and viscosity of the solvent
T = Time
Δ**P** = Differential pressure across the membrane
$\Delta\boldsymbol{\pi}$ = Osmotic pressure across the membrane
$\mathbf{Q_p}$ = Rate of clean water recovery in the permeate stream
A = Contact area (Britz and Robinson, 2008; Nazaroff and Alvarez-Cohen, 2001)

Another means of monitoring the performance of the membrane is the percentage recovery of water:

$$\mathbf{Y\%=Q_P/Q_F}$$

$\mathbf{Q_p}$ Rate of clean water recovery in the permeate stream
$\mathbf{Q_F}$ Feed rate

1.7 Aims of the Current Study

The aims of the current study can be summarised:

1.7.1 Characterisation of complex wastes from fruit industries

Waste samples from fruit processing industries in the Western Cape were collected and characterised to ascertain their physical characteristics such as pH and chemical oxygen demand. They were analysed to determine their potential as sources of antioxidants through assessing their total phenolics as well as their radical scavenging ability and ability to inhibit oxidation of β-carotene. The level of sugars was also assessed to determine the potential to grow micro-organisms in the wastewaters for production of value-added metabolites.

1.7.2 Develop and customize new extraction processes for obtaining antioxidants

This research focuses on the extraction of polyphenolic compounds from fruit processing wastewaters and the determination of the antioxidant activities of these polyphenolics. Existing techniques such as solvent extraction were tested and compared to new extraction processes such as extraction using a supported liquid membrane (SLM). Adsorption of phenolics onto solid supports such as C18, PVPP, Amberlite resin and activated carbon was also tested. Here, adsorption of phenolics is common practice but elution of the phenolics to give an anti-oxidant extract would be a new application. In addition, extraction into supercritical CO_2 was also tested as a new application. The anti-oxidant properties of the extracts were assessed for total phenolics content and radical scavenging ability.

1.7.3 Investigate and optimize fermentation of residuals after extraction

Fermentation of residual fruit wastewaters by bacteria and the yeast *Saccharomyces cerevisiae* were investigated with a view toward the production of value-added metabolites such as 2,3-butanediol and ethanol.

1.7.4 Investigate commercialization aspects

In the recommendations, an integrated process was proposed whereby wastewater moves through sequential unit operations allowing for the extraction of antioxidants and the production of metabolites, finally resulting in clean water suitable for reuse.

2 EXPERIMENTAL PROCEDURES

2.1 Chemical analyses of wastewaters

Detailed characterisation of wastewaters is necessary to establish their composition, indicating the presence and concentration of potentially valuable compounds and providing the information needed to choose appropriate treatment and extraction techniques. Wastewaters were characterised through use of standard chemical tests routinely performed to determine water quality. These tests include pH, conductivity, level of solids (dissolved, suspended and total) and chemical oxygen demand which is a measure of the organic load carried by the water. In this work, the wastewaters were not only viewed as potential pollutants but also as a source of valuable phenolics and sugars. As such, the wastewaters were also characterised to determine the available levels of phenolics and carbohydrates. This gave an indication of the viability and economic benefit of extracting the phenolic antioxidants and utilising the carbohydrates in fermentation.

2.1.1 Physical analysis

The pH and conductivity were measured using pH and conductivity meters, respectively, which were appropriately calibrated.

Total solids were determined by evaporating 100 ml of well mixed wastewater samples in an oven at 90^{o}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 by filtering wastewater samples prior to drying in the same manner as for total solids. Suspended solids were determined as the difference between the total and dissolved solids.

2.1.2 Chemical oxygen demand

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 placed in a digestion block for 2 hours at 150^{o}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.

2.1.3 Analysis of carbohydrates

The concentration of reducing sugars was determined using a modified protocol for the dinitrosalicyclic acid (DNS) method according to Frost (2004). The DNS reagent (3 ml), containing 1 g of 3,5-dinitrosalicylic acid (DNS), 1.6 g NaOH and 30 g of sodium sulphite in a litre of water was added to 3 ml of sample and incubated at 90^{o}C for 15 minutes. Thereafter, 40% potassium sodium tartrate (1 ml) was added, cooled at room temperature and the absorbance read at 575 nm. Distilled water was used as a reagent blank and glucose in the range of 0-1000 mg/l was used as a standard for this method.

For the quantification of carbohydrates, the phenol-sulphuric acid assay was conducted according to Mecozzi et al. (2002), with modifications. To 0.5 ml of sample, 5% phenol (0.5 ml) was added followed by concentrated sulphuric acid (2.5 ml). This was incubated at 30^{o}C for 20 minutes and then cooled at room temperature for 30 minutes. The absorbance was read at 490 nm. Distilled water was used as the reagent blank and the absorbance of samples 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.

2.1.4 Quantification of phenolics

A wide range of analytical methods are available for the detection and quantification of phenolic compounds including spectrometry, chromatography and colorimetric assays. All have advantages and disadvantages and for this reason, multiple methods were used to gain a better understanding of the concentration of phenolics in the wastewaters.

2.1.4.1 The Folin Ciocalteau assay

The Folin Ciocalteau (FC) assay is a common method for quantification of total phenols which relies on the reduction properties of phenols. The FC reagent turns blue when reduced and total phenols are measured using a spectrophotometer at 725-735 nm (Naczk and Shahidi, 2004; Robards, 2003). A disadvantage of the FC assay is that all reducing agents are detected and this might lead to overestimated results when used for the detection of phenols in wastewater (Becker et al., 2004). Fruit wastewater contains reducing agents such as ascorbic acid which will interfere with the quantification of phenolics. Despite this potential complication, the FC assay has been widely used. Hagen et al. (2007) and McCann et al. (2007) used the FC assay to measure the total phenolics in apples (838 mg/100 g fresh weight), and fresh apple wastes (690 mg/100 g), 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.

In this work, the method of Yesil-Celiktas et al. (2007) was used in which a 400 µl volume of sample was added into a 4 ml cuvette followed by an equal volume of FC reagent and 20% sodium carbonate. This was stored in the dark for 90 minutes after which the absorbance was read at 765 nm. Gallic acid was used as a standard for this method.

2.1.4.2 Chromatography

High performance liquid chromatography (HPLC) (Francisco and Resurreccion, 2009), gas chromatography (GC) and gas chromatography linked to mass spectrometry (GC-MS) (Torrecilla et al., 2007) have been used in the quantification of phenols. These allow for the profiling and identification of individual phenolic compounds (Robards, 2003). The advantage that these methods have over spectrophotometric assays, such as the FC assay, is the specificity of the analysis. Individual phenolics are identified and there is less likelihood of overestimating the concentration of a particular compound. The disadvantage of such techniques is that quantification is only possible for those phenolics for which standards curves have been run. This may lead to underestimation of phenolics especially in a complex natural mixture such as a wastewater.

HPLC is currently the most popular of these methods for both the separation and quantification of phenols. Naczk and Shahidi (2004) summarises the modern HPLC methods for various food phenolics, including details of the sample preparation and the types of mobile and stationary phases used. The separation of different classes of phenols has been improved by using reverse phase-HPCL (RP-HPLC) (Naczk and Shahidi, 2004). A commonly used RP-HPLC system is a C_{18} stationary phase and a binary mobile phase consisting of an aqueous component and a less polar organic solvent such as acetonitrile or methanol (Robards, 2003). De Marco et al. (2007) used RP-HPLC to identify the phenolic compounds present in olive mill wastewater by comparing the retention times with those of standard solutions. The study showed that hydroxytyrosol was the most abundant phenol, and that the wastewater also contained tyrosol, caffeic acid, vanillic acid, verbascoside, luteolin-7-glucoside, decarboxymethyl oleuropein aglycon, ligstroside and luteolin (De Marco et al., 2007).

The limited volatility of phenols has restricted the use of GC for their separation and identification (Robards, 2003). However, GC and GC-MS can be used of the samples are sufficiently derivatised beforehand (Naczk and Shahidi, 2004; Robards, 2003). For the

improvement of resolution, high temperature columns, electronic pressure controllers, and detectors can be used (Naczk and Shahidi, 2004).

In this work, HPLC was performed on filtered and centrifuged wastewater samples. A Merck Hitachi L-7000 series chromatograph was used. Monophenolics were separated by isocratic elution using a mobile phase of 80:20:2.5 deionised H_2O:methanol:acetic acid. UV detection at 280 nm was used to quantify compounds by comparison to the retention times of external standards (Ting, 2004).

2.2 Determination of antioxidant activity

Phenolics present in wastewaters are valued for their antioxidant properties. It is difficult to evaluate antioxidant activity directly, particularly if only one method is used, and thus the evaluation of the antioxidant action of phenolic compounds should be based on the identification of different mechanisms of action of phenolic compounds under variable conditions. This gives an indication on the multifunctional properties of antioxidants in both physiologically and food-related oxidative processes (Becker et al., 2004). The stereoelectronic effects of phenols and phenoxyl radicals are largely responsible for their reactivity with radicals. The reaction mechanisms by which the hydrogen atom of a phenol is transferred to a radical can be divided into two distinct pathways, namely the hydrogen atom transfer (HAT) and the proton-coupled electron transfer (PCET) (Katarina, 2007). The HAT mechanism is demonstrated in assays such as DPPH radical scavenging assay while the PCET mechanism is illustrated in assays such as the Trolox Equivalent Antioxidant Capacity (TEAC) assay and Ferric Reducing Power assay (FRAP) (Devi and Arumughan, 2007; Ferreira et al., 2007).

2.2.1 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). In this assay the ability of a compound to scavenge the DPPH free radical is indicated by a colour change (deep purple to brown) that is measured at 515 nm (Devi and Arumughan, 2007). The antioxidant activity can then be expressed as an EC50 value (the antioxidant concentration required to reduce the initial concentration of DPPH by 50% at steady state) or as the percentage radical scavenging activity (%RSA; 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) (Devi and Arumughan, 2007).

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., 2009). 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).

Teczan et al. (2009) used the DPPH assay to measure the radical scavenging ability of two orange juices, and a pomegranate juice. The EC50 for the orange juices were 0.18 and 0.29 mg/l respectively, and the %RSA in pomegranate juices ranged from 10 to 67% (Tezcan et al., 2009).

The following method was followed: 0.5 ml of sample was added to 3.5 ml methanolic DPPH and the reaction monitored over time at 515 nm until steady state was reached. The blank for the assay consisted of absolute methanol (Devi and Arumughan, 2007). Radical scavenging activity (RSA) was calculated using the following formula:

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

2.2.2 The trolox equivalent antioxidant capacity (TEAC) assay

The trolox equivalent antioxidant capacity (TEAC) assay, also known as the 2,2'-azonobis (3-ethybenzothiazoline-6-sulphonate) (ABTS) assay, is a common analytical method for the determination of 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 colour change being the 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 (Magalhães et al., 2007).

The TEAC assay is inexpensive, rapid, and easy to carry out. The reaction is also consistent despite variations in pH and therefore can be used to study pH effects on antioxidant activity (Zulueta et al., 2009). It must be considered though that the free radical generated from ABTS salt is not stable for long periods of time and the method must be followed consistently. Without standardisation of the methods, comparison of results from other laboratories would be difficult (Zulueta et al., 2009).

The TEAC assay has been used to measure the antioxidant activity of orange juice and milk beverages, 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 TEAC assay (Pellegrini et al*.,* 2003).

The TEAC assay involved the generation of the ABTS radical 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 radical solution (3 ml) was added to 30 µl of sample, or trolox standard, and readings were taken at 1 minute intervals for 15 minutes at 734 nm (Magalhães et al*.,* 2007).

2.2.3 The ferric reducing power (FRAP) assay

The ferric reducing power assay measures the ability of an antioxidant to reduce the ferric tripyridyltriazine (Fe3+-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 700 nm, giving an indication of the reducing power of the antioxidant (Ferreira et al., 2007; Kubola and Siriamornpun, 2008). The most common way of expressing reducing power is to measure the reducing power of different concentrations of a known antioxidant, such as gallic acid, and express the 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).

Yi et al, 2008 demonstrated the use of the FRAP assay to measure to determine the antioxidant activity of a new citrus cultivar and its flavonoids. The results indicated that all samples exhibited some degree of reducing power (Yi et al*.,* 2008). The nectars of a variety fruit were also analysed for total antioxidant activity using the FRAP assay and the results obtained included 6.54, 8.0, 5.68 and 5.19 moles of antioxidant/ml of nectar for orange, sour cherry, apricot and peach nectars (Tosun and Ustun, 2003).

The FRAP assay was performed according to Ferreira et al. (2007), in which 1 ml of sample was added to 2.5 ml potassium phosphate buffer and 2.5 ml of potassium ferricyanide. This was incubated at 50^{o}C for 20 minutes. Trichloracetic acid (10%) was added immediately afterwards to stop the reaction. A 2.5 ml volume 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 and the absorbance read at 700 nm.

2.2.4 β-carotene linoleic acid model system

This assay (the β-CLAMS assay) was used to determine the ability of the wastewaters to inhibit lipid peroxidation. β-carotene (2 mg) was dissolved in 10 ml chloroform. A 1 ml sample was taken and the chloroform removed *in vacuo*. 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. Aliquots (3 ml) of this mixture were added to 1 ml of sample. Absorbencies at 490 nm were taken immediately and at 15 minute intervals for 2 hours (Liu et al., 2007b).

2.3 Techniques for the Extraction of Phenolics

Various techniques have been developed for the extraction of phenolic compounds, and many of these have been applied to fruit and fruit processing waste. The extraction of phenolics from fruit processing waste is an important step in recovering these valuable compounds for commercial application.

2.3.1 Solvent extraction

Solvent extraction is the most widely used technique for the extraction of phenolic compounds from biological materials. Solvents that are not miscible with water can be used to extract phenolic compounds from aqueous samples such as fruit processing wastewaters (Abad-García et al., 2007). The principle behind solvent extraction 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.

Solvent extraction is relatively inexpensive and simple to operate. It does, however, require large volumes of solvent, the disposal of which poses a threat to the environment. Solvent extraction is also time consuming and requires large quantities of energy. A major restriction

of this technique is the incorporation of the extracted antioxidants into food which limits the choice of solvents to those that can be used without health concerns (Adil et al., 2007).

Abad-Garcia (2007) used solvent extraction to extract a range of phenolic compounds from a variety of fruit juices. A unique gradient program was used to isolate several classes of phenolics, including hydroquinones, hydroxybenzoic acids, flavan-3-oles, hydroxycinnamic acids, coumarins, flavanones, flavones, dihydrochalcones and flavonols (Abad-García et al., 2007). Similarly, Chirnos et al. (2007) used solvent extraction for the extraction of phenolic acids, anthocyanins, flavanols and other antioxidants from mashua (a tuber).

In this work, two solvents were tested: 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).

Wastewaters (100 ml) were extracted three times with equal volumes of ethyl acetate or hexane and the total phenolics were determined by the FC method before and after treatment. 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^{o}C and the residual dried extract was redissolved in a minimal volume 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.

2.3.2 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 (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., 2004).

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., 2004). The membrane surface area and thickness provide rapid transportation of phenolic compounds and leakages and contamination is easily contained. Also, the donor and acceptor phases are more easily recoverable when compared with traditional liquid-liquid extraction. Hollow fibre systems must however, 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).

Selection of an organic solvent to be used is based on two of the most important properties to be considered when choosing a solvent for extraction of a particular solute: boiling pint and water solubility (Table 2.1).

Table 2.1: Properties of organic solvents to be considered for use in supported liquid membranes (SLM)

Solvent	Boiling point (^{o}C)	Solubility in water (mg/l)
Amyl acetate	149.1	800
Toluene	110.6	50
Methylisobutylketone	117.4	1700
1-decanol	231	37
Ethyl acetate	77.1	8300

Amyl acetate and 1-decanol have very high boiling points, and thus the removal of these solvents after extraction would be difficult. Toluene and methylisobutylketone, on the other hand, have boiling points that make their removal relatively simple. The solubility of ethyl acetate in water is 83 g/l, which is problematic since an ideal solvent should have minimal solubility in water and be easily removed *in vacuo*. Solvents chosen for this work were toluene and methylisobutylketone for their favourable extraction properties, and ethyl acetate for its non-toxic nature which allows for wider application of extracts obtained.

The wastewater with the highest total phenolics content, in this case SW, was selected and used for the membrane extraction experiments. Membrane extraction was done according

to González-Muñoz et al., (2003). A Microdyne polypropylene hollow fibre membrane module (MD20CP2N) was used as contactor, which had the following properties:

Membrane specifications	n = 40
	ID = 1.8 MM, OD = 2.0 MM
Pre size	0.2μm
Total membrane surface area	A_m = 0.1 m^2
Lumen cross-section area	A_l = 2.5 X 10^{-6} m^2
Shell cross section area	A_s = 1.8 x 10^{-4} m^2
Membrane length	L = 0.5 m

A schematic diagram of the experimental procedure for the membrane extraction system is shown in Figure 2.1. Reservoirs of wastewater and 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. 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 rig was operated at ambient temperatures and pressures in a fume hood to minimise the risk of inhalation of toxic organic solvents. Flow rates (and corresponding Reynolds numbers) were set so as to ensure minimal boundary layer resistance to mass transfer in the aqueous and organic phases.

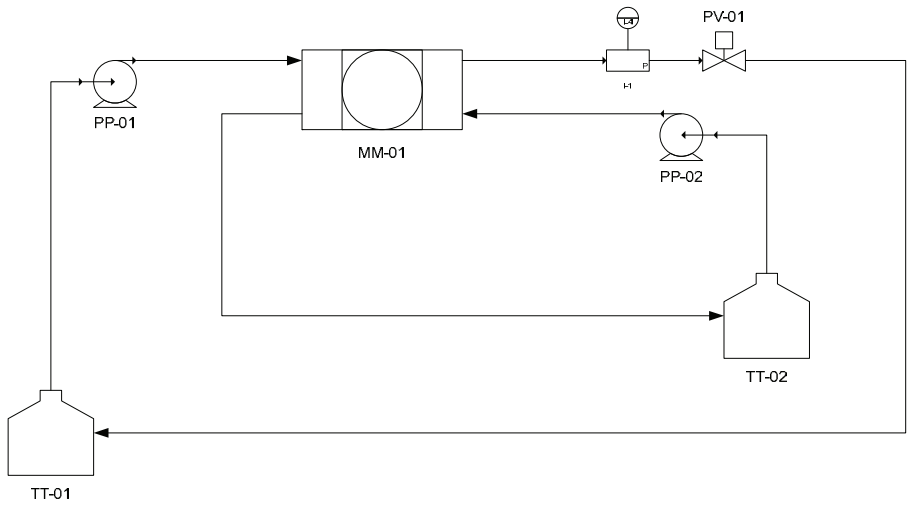


Figure 2.1: Schematic diagram of the membrane extraction system

The distribution confidents of the phenolic compounds between the aqueous and organic phase were determined before performing SLM. Six dilutions of wastewater (0-100%) were mixed with an equal volume (20 ml) of organic solvent, shaken for 30 minutes and left to settle. This allowed for concentrations of solutes in the two phases to reach equilibrium. After separation, concentrations of solutes in the organic phase were determined by first removing the organic solvent *in vacuo* and redissolving the residue in an equal volume of water. The corresponding concentrations of solutes in the aqueous phase were determined using the FC assay, with the resulting ratio so determined being the effective distribution coefficient.

2.3.3 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 and the desired solute is adsorbed by the column. Unwanted components are then washed away by one solvent, followed by addition of a different solvent which elutes the desired solute from the stationary phase.

Adsorption is the selective accumulation of a chemical at the interface between two phases. The substance that adsorbs is called the adsorbate and if it binds at a solid/liquid interface, the solid is called the adsorbent. Different types of adsorption may occur depending on the nature of adsorbent and adsorbate. In exchange adsorption, adsorbent ions are concentrated at the surface as a result of electrostatic attraction to charged sites at the surface. Physical adsorption occurs principally as a result of Van der Waals forces while chemisorptions involve a chemical interaction with the surface. Molecules are not free to move on the surface and elution from the surface requires a chemical reaction. For the selective adsorption and subsequent desorption of phenolics, a balance must be reached between rapid, selective adsorption and gentle desorption conditions.

In calculations, the mass of material adsorbed per unit adsorbent is called the adsorption density and is commonly represented in equations as q. The mass of material adsorbed per unit area is the specific surface area. At chemical equilibrium, an expression of the adsorption density as a function of the adsorbate concentration gives the adsorption isotherm. The isotherms that are used most frequently to characterize adsorption in environmental systems are known as linear, Langmuir and Freundlich isotherms.

The adsorbents chosen for this study were Sep-Pak C18 cartridges, polyvinylpolypyrrolidone (PVPP), Amberlite XAD4 and activated carbon. Methods of extraction are detailed for each solid phase:

2.3.3.1 Extraction of total phenolics using Sep-Pak cartridges

Sep-Pak cartridges are 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 need to be established so as to elute as much of the desired solute as possible, thereby reducing losses during recovery (Michalkiewicz et al., 2008). The mode of adsorption is Van der Waal hydrophobic interactions between the ring structures of the polyphenolics and the hydrophobic 18 carbon chains of the adsorbent.

The procedure of Suárez et al. (1996) was followed. This is illustrated in Figure 2.2. Each Sep-Pak cartridge was preconditioned for neutral phenolics by passing through 8 ml methanol followed by 4 ml distilled water. The wastewater sample was adjusted to pH 7 using 1 M NaOH and was passed through the preconditioned column. This was followed by a washing stage using 10 ml distilled water. The adsorbed phenolic compounds were eluted with 12 ml methanol.

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

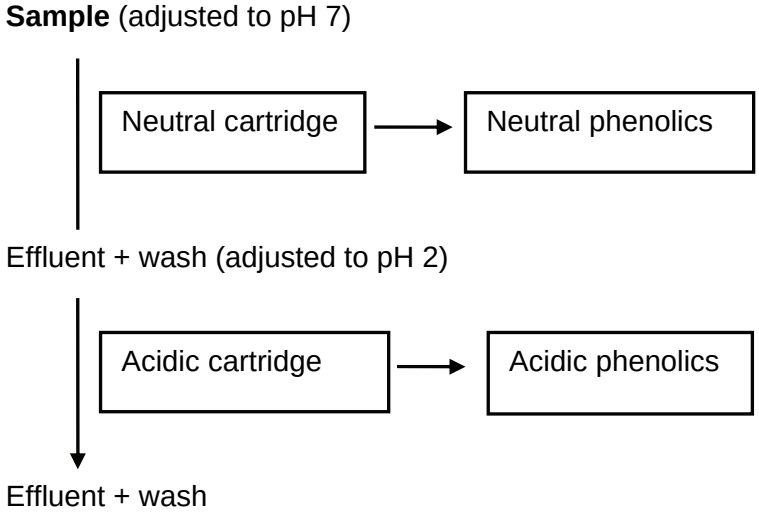


Figure 2.2: Experimental procedure for solid phase extraction.

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 FC assay. The antioxidant activity of the neutral and acidic phenolics was determined by the DPPH radical scavenging assay. The residual effluent was used for fermentation studies.

2.3.3.2 Extraction of total phenolics using PVPP

PVPP is a water-insoluble cross-linked polymer of vinyl pyrrolidone which is customarily used in the treatment of several beverages to remove small quantities of unwanted substances. PVPP extraction is simple and is already used in the apple juice (Sarioglu, 2007), beer (Mitchell et al., 2005) and wine (Laborde et al., 2006) industries and should therefore find ready acceptance in this application. In the apple juice industry, PVPP is used in the stabilisation of apple juices, thereby enhancing the 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 that compromise acceptability of commercial juice. The development of a colloidal haze is linked to the presence of low molecular weight polyphenolic compounds in the apple juice and therefore, to reduce the formation of haze, small quantities of PVPP are added to the apple juice to absorb phenolic compounds. After a sufficient length of time, PVPP with the absorbed phenolic compounds is removed (Sarioglu, 2007). PVPP is used in a similar way to reduce haziness in beer and stabilise its colour (Mitchell et al., 2005) and it is also in wine making to absorb phenolics (Laborde et al., 2006). PVPP is even used in the processing of citrus to remove the bitter flavour caused by naringin (O'Reilly and Merrow, 1998). The mechanism

of adsorption is hydrogen bond formation between carbonyl groups on the PVPP and the phenolic hydrogens. A hydrogen bond is stronger than a Van der Waal hydrophobic interaction and stronger binding is therefore expected.

The method for solid phase extraction using PVPP was adapted from O'Reilly and Merrow (1998). Polyvinylpolypyrrolidone (PVPP) was mixed with wastewater at a ratio of 5 g/L of wastewater for 10 minutes to allow for 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 0.001 M NaOH. Total phenolics were determined before and after PVPP extraction using the FC assay. The residual wastewater (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. The residual wastewater and water wash was used for fermentation studies.

2.3.3.3 Extraction of total phenolics using Amberlite XAD4 and Activated carbon

Amberlite XAD4 is a porous spherical polymer based on highly crosslinked, macroreticular polystyrene. The internal surface area is 750 m^2/g and this polymer is recommended for removal of small molecular weight molecules such as phenolic aromatic hydrocarbons (Rohm and Haas product data sheet http://www.amberlyst.com/xad.htm). Adsorption is by means of hydrophobic interactions in which the aromatic hydrocarbons preferentially associate with the hydrophobic pores in the polymer.

Activated carbon also provides pores allowing for adsorption of molecules by Van der Waals forces. Activated carbon can be prepared from many sources and by different methods, leading to different pore structures. It is estimated that the surface area available for adsorption is 100 m^2 per gram. Activated carbon is a low cost adsorbent and is currently used in the apple juice industry to adsorb phenolics (Appletiser, pers com)

To determine the adsorption kinetics for Amberlite XAD4 and activated carbon, each adsorbent was added in the ratio of 1 or 2 g adsorbent to 100 ml solution (Roostaie et al., 2004) and the mixtures placed on a shaker at 200 rpm. Samples (2 ml) were removed at regular time intervals and analysed using the FC method until equilibrium was reached between the concentration of gallic acid on the adsorbent and the concentration in solution.

To determine the effect of concentration on adsorption by the solid adsorbents, varying concentrations of gallic acid were prepared in the range 0 to 100 mg/l for each adsorbent in

triplicate. Each adsorbent was added in the ratio 1 g:100 ml and the mixtures left on a 200 rpm shaker for 24 hours to ensure equilibrium adsorption. Samples were analysed using the FC method.

2.3.3.4 Desorption using organic solvents

Use of adsorbents for the removal of phenolics is well established in the fruit processing industry but elution of the phenolics is often not pursued. Elution of phenolics would serve a dual purpose to fruit processing industries. Firstly, elution would allow reuse of the solid adsorbents and increase the sustainability of the process. Secondly, the eluant is expected to be high in antioxidant phenolics. This concentrated antioxidant solution could be utilised to generate further income from what would otherwise be considered a waste product.

Dilute sodium hydroxide may be used to elute phenolics from PVPP but use of a strong base may have adverse effects on the antioxidants and may complicate downstream processing if the eluant is desired as a food additive. Elution using a less harsh solution would be preferable for subsequent use of the extract as well as for recycling the adsorbent. Ethanol and ethyl acetate were tested as possible solvents for elution of phenolics.

0.2 g of each adsorbent was added to 40 ml (5 g/l) synthetic wastewaters in triplicate. Samples were agitated at 200 rpm on a rotary shaker at room temperature until equilibrium had been reached (30 minutes for PVPP and 4 hours for activated carbon). The adsorbents were allowed to settle and the remaining liquid was decanted. The liquid portion was analysed for the presence of sugars and phenolics using HPLC and the adsorbent was packed into plastic pipette columns. The columns were washed twice with 5 ml of distilled water and the effluents analysed for phenolics. Thereafter they were washed with 1.5 ml volumes of solvent and each 1.5 ml fraction was analysed for the presence of phenolics using HPLC.

2.3.4 Supercritical fluid extraction of phenolic compounds

Among recent developments in extraction techniques, supercritical fluid extraction (SFE) 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 2.3. 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). The basic principle of

SFE extraction is that the solubility of a given compound (solute) in a solvent is dependent on both temperature and pressure. Carbon dioxide is the most commonly used supercritical fluid for the extraction of phenolic compounds (Shi et al., 2005).

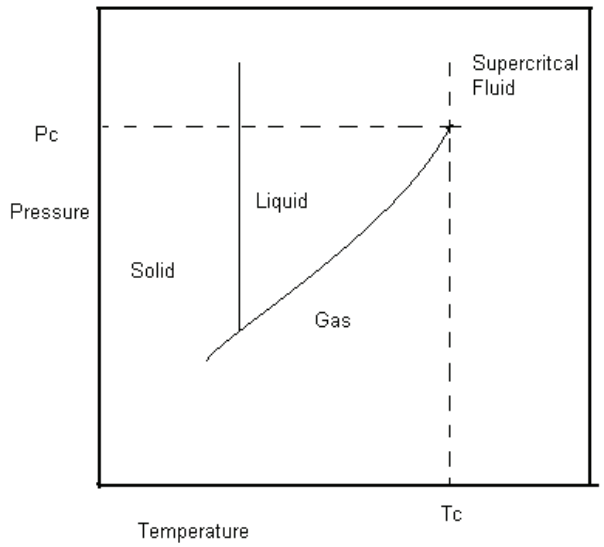


Figure 2.3 Typical phase diagram for a pure compound (Herrero et al., 2006)

Advantages of SFE include:

- The extent of the supercritical fluid to dissolve a solute is controlled by temperature and pressure, thus these variables can be manipulated to increase the solvating power of the supercritical fluid.
- The supercritical fluid, usually carbon dioxide can easily be recovered after extraction by lowering the temperature and pressure (Rodriguez et al., 2008)
- There is no need for a concentration step because only a small volume of organic solvent is normally used (Mendiola et al., 2007; Palma and Taylor, 1999)
- Degradation reactions that occur using other extraction techniques are avoided with SFE due to the absence of light and air
- Compounds that degrade easily with heat can be extracted with minimal damage due to the low temperatures used in SFE (Beyer and Biziuk, 2008)
- SFE can possibly be directly coupled to analytical chromatographic techniques such as gas chromatography and supercritical fluid chromatography (Herrero et al., 2006)

SFE is employed in the extraction of compounds of interest from natural materials for eventual use in food, perfumery, pharmaceutical and nutraceutical industries (Waldron, 2007). Several phenolic compounds and antioxidants have been identified using SFE

(Cavero et al., 2006; Vaher and Koel, 2003). The major industrial application of SFE is in the decaffeination of tea and coffee (Mendiola et al., 2007). The method is also used in the brewing industry, for the extraction of essential oil and aroma spices, in the petrochemical industry, in the pharmaceutical industry and for the purification of contaminated soils (Daintree et al., 2008; Mendiola et al., 2007).

From the chemical characterisation of wastewaters, gallic acid in SW was found in sufficient quantity to make this wastewater a potential source for antioxidant recovery. Supercritical fluid extraction was therefore used on SW to determine the solubility and extraction efficiency of gallic acid.

2.3.4.1 Solubility of gallic acid in supercritical CO_2

The solubility of gallic acid was measured using the apparatus shown in Figure 2.4. CO_2 gas cylinders were provided by BOC and stored at room temperature.

Figure 2.4: Laboratory-scale experimental supercritical fluid extraction system

The 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, which results in an increase in solubility. The employed apparatus was specially designed to operate at pressures up to 200 bar and temperatures up to 120^{o}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. The pressure was monitored by two pressure gauges and a digital controller, and the system was closed above 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 and the extract was redissolved in 10 ml Millipore water. The dissolved extract was analysed 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^{o}C of the desired value.

Four experimental systems were set up and are summarised in Table 2.2. Experiment 1 was designed according to data collected from a literature review and experiments 2 to 4 were performed in order to see the effect of different parameters on the solubility and extraction of gallic acid, thereby optimising the extraction conditions.

Table 2.2: Experimental designs for supercritical fluid extraction, with operating conditions

Experiment	Temperature (^{o}C)	Pressure (bar)	Volume of ethanol used (ml)	Time (hours)
1	40	180	10	2
2	25	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 decreased from 40^{o}C in experiment 1 to 25^{o}C in experiment 2, under the same pressure, time and co-solvent volume. The better temperature was 25^{o}C, and the rest of the experiments were therefore performed at this temperature. The effect of the volume of co-solvent added was studied in experiment 3, and 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 with concentration range of 0.0005 to 0.0125 g/l was obtained at 270 nm. This was used to determine the amount of gallic acid extracted.

2.4 Fermentation of Wastewaters

After removal of phenolics, carbohydrates remaining in the wastewater were used as substrates for fermentation. Fermentation may be pursued either through the action of

yeasts or bacteria. The yeast *Saccharomyces cerevisiae* is commonly used industrially, notably in wine and beer production, to produce ethanol from simple sugars. It is able to tolerate relatively high sugar concentrations and produce high ethanol yields without inhibition of growth (Casey and Ingledew, 1986). One disadvantage of ethanol production by *S. cerevisiae* is its inability to grow or produce ethanol from xylose which is a limiting factor in the use of this yeast to produce ethanol from lignocellulosic sources. Jeffries and Jin (2004) have reviewed research into the modification of *S. cerevisiae* to enable xylose fermentation. Targets for modification include increasing the uptake of pentoses and introducing bacterial xylose isomerase (Jeffries and Jin, 2004). Kotter et al. (1990) introduced genes for xylose reductase and xylitol dehydrogenase from *Pichia stipitis* into *S. cerevisiae* to allow xylose fermentation. Ho et al. (1998) focused on overexpressing xylulokinase from *S. cerevisiae* with the XYL1 and XYL2 genes from *P. stipitus* in *Saccharomyces* sp. 1400 which further improved yields. Strains have been improved through random mutagenesis and directed evolution leading to successful strains such as TMB3400 (Wahlbom et al., 2003). These strains are not yet commercially available but companies such as Taurus Energy are working toward licensing their technology (http://www.taurusenergy.eu). Fortunately, in fruit wastewaters, high xylose content is not anticipated and *Saccharomyces cerevisiae* has therefore been selected as the model organism for ethanol production.

Bacteria may also be used to ferment sugars to useful metabolites and for this work, two bacterial cultures were chosen from the culture collection of the Institute of Microbial Biotechnology and Metagenomics. These cultures, designated NB4 and TH141, were isolated from natural environments and were chosen for their ability to grow quickly and prolifically in simple media using a variety of carbon sources including xylose and arabinose. They reach high optical densities and are therefore good model organisms for biomass investigations. Additionally, they produce a relatively narrow range of by-product metabolites, both producing 2,3-butanediol in high concentration. 2,3-butanediol is not a metabolite commonly found in the wastewaters initially and its increase is therefore easily noted as a model of product formation. Industrially, 2,3-butanediol is converted to 1,3-butadiene and used in the manufacture of pharmaceuticals, plastics and rubber (Mallonee and Speckman, 1988)

2.4.1 Fermentation using *Saccharomyces cerevisiae*

S. cerevisiae was cultured from a commercial baker's yeast sachet. Pre-cultures were prepared in 10 ml malt extract medium (17 g/l malt extract, 5 g/l peptone) in closed 50 ml centrifuge tubes and incubated overnight vertically on a rotary shaker at 160 rpm at 30^{o}C. A

5% inoculum of pre-culture was then used in each experimental fermentation. Experiments were conducted in closed 50 ml centrifuge tubes containing 20 ml synthetic or sampled wastewater. Growth (optical density at 600nm) and metabolite profiles (HPLC) were determined over 18 or 24 hours

2.4.2 Fermentation using bacterial strains NB4 and TH141

All bacterial fermentations were performed in duplicate or triplicate. Purified distilled water from a Millipore Elix 3 system was used for all analyses. Chemicals and reagents were of analytical grade. Bacterial fermentations were performed under micro-aerobic and aerobic conditions at 30^{o}C. Micro-aerobic growth was monitored in closed 15 ml centrifuge tubes containing 10 ml culture medium. These tubes were incubated vertically on a rotary shaker allowing some mixing but limited aeration. Aerobic cultures were grown in 10 ml of culture medium contained in 50 ml Erlenmeyer flasks. Flasks were covered with tinfoil and incubated on a rotary shaker at 160 rpm allowing good aeration of the culture medium. Samples were taken at intervals and were used to determine optical density at 600 nm and metabolite profile by HPLC.

The culture medium used in preliminary bacterial fermentations was a synthetic wastewater consisting of fructose and glucose in defined concentrations. Experimental fermentations were performed using the fruit wastewaters after before and extraction of phenolics.

2.4.3 Analysis of sugars and metabolites

To determine the levels of specific sugars and metabolites, an HPLC method was used on a Merck Hitachi L-7000 series machine. Samples were injected onto a Biorad Aminex 87-H column and eluted isocratically with 10 mM H_2SO_4 as mobile phase. Dual detection was by UV at 210 nm and Refractive Index. At a flow rate of 0.6 ml/min, analyses were completed in 40 min and it was possible to separate compounds of interest. A number of standards were run and standard curves were prepared for expected compounds including glucose, fructose, sucrose, citrate, pyruvate, lactate, formate, succinate, acetate, 2,3-butanediol and ethanol.

2.4.4 Fermentation using apple wastewater enriched by enzymatically digested waste pomace

Supplementation of the sugars available in apple wastewater with solid apple waste was investigated. For incorporation of solid waste, pretreatment is necessary to release sugars stored in the pomace. Pretreatment methods for complex natural feedstocks include steam explosion and acid hydrolysis. The use of enzymatic digestion is also growing either as part

of a two step process or, more commonly, in simultaneous saccharification and fermentation unit processes (Öhgren et al, 2006).

In this work, treatment in an autoclave at 132^{o}C was used to mimic steam explosion and treatment in an autoclave in the presence of dilute sulphuric acid was used as an equivalent to acid hydrolysis. A commercial enzyme preparation was used to investigate enzyme digestion. The process flow diagram is shown in Figure 2.5.

Three apples (with peels) were placed into a commercial juicer to extract juice from the apples and to obtain the residual apple pomace: peels; pips and core. This apple waste was then washed with distilled water to ensure that no free sugars remained in the pomace. A representative 40 g/l (wet weight) pomace slurry was prepared. (method developed by Professor Brett Pletsche et al., pers comm.)

Four pretreatment options were investigated: In the control sample, the 40 g/l pomace slurry was used without further treatment. To mimic heat treatment, the apple pomace slurry was treated with high-pressure saturated steam at a temperature of 132ºC for 15 minutes in an autoclave. This process causes physical disruption of the lignocellulose releasing shorter chain polysaccharides. To mimic digestion by dilute acid, 0.1 ml concentrated sulphuric acid was added to 19.9 ml of the apple pomace slurry to give 0.5% sulphuric acid. This acid slurry was then treated by autoclaving at 132ºC for 15 minutes. This process causes physical and chemical disruption of the lignocellulose, releasing shorter chain polysaccharides and solubilising much of the hemi-cellulose fraction.

Enzyme pretreatment was tested by making use of the commercial enzyme solution Novazym Biocip® Membrane which is marketed for cleaning membranes used in fruit processing industries. The declared enzyme composition is: polygalacturonase, cellulase, gluco-amylase with an activity of 2500 PECTU/ml. A dilute enzyme solution was prepared containing 2 units/ml in 50 mM sodium phosphate buffer (pH 6.5). 2.5 ml of this enzyme solution was added to 15 ml apple pomace slurry which was adjusted to pH 6.5 using 2.5 ml concentrated sodium phosphate buffer. The resulting mixture was incubated at 37^{o}C for 72 hours and shaken at 160 rpm. During enzyme treatment, cellulose and pectin are hydrolysed and the concentrations of glucose, fructose, xylose, arabinose and galactose increase.

Sugars were quantified using HPLC as described in section 2.4.3 and identification of sugars was by means of retention time compared to standards. Xylose and mannose standards are

only separated by 0.01 min and could not reliably be identified. The calibration curve for all monosaccharides tested was similar and the peak was therefore identified as "xylose/mannose" using an average calibration value to determine sugar concentration.

After being treated to release sugars, the preparations were fermented using *S. cerevisiae.* Fermentations were performed as described in 2.4.1 and samples were taken every 12 hours for 72 hours.

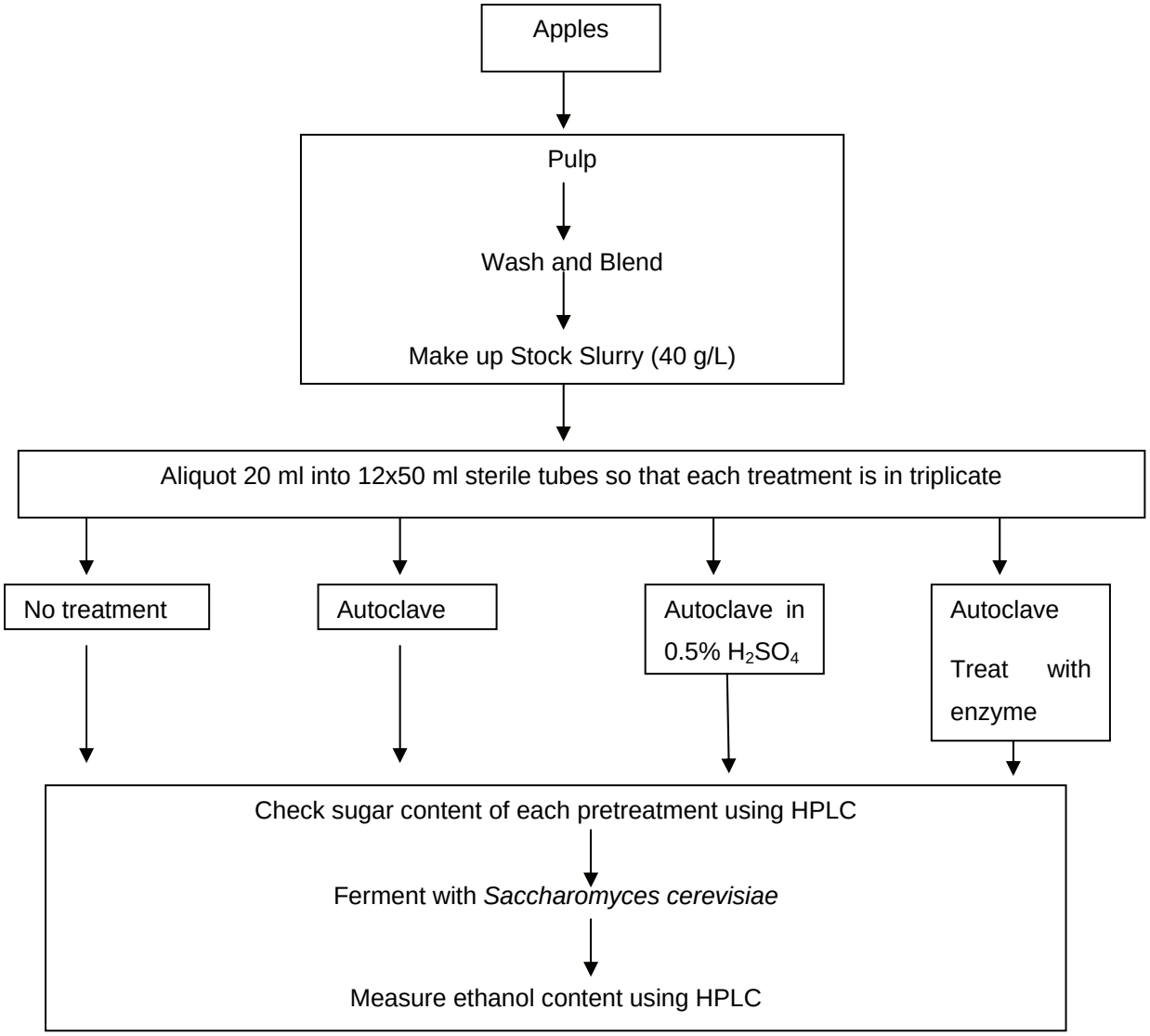


Figure 2.5: Process flow block diagram of experimental protocol for pretreatment studies

2.5 Membrane Concentration

Fruit wastewater contains not only solid tiny particles of fruit but also dissolved solids such as sugars and phenolic compounds. While micro-filtration and ultra-filtration would remove the particulate matter, the resulting permeate would not be pure enough for reuse in the fruit processing plant. For reuse, dissolved solids must also be removed and this requires the

use of reverse osmosis. A reverse osmosis water purification apparatus was tested for potential application in purification of fruit wastewater and production of a concentrated waste solution for efficient extraction of phenolics and fermentation of sugars.

The experimental apparatus is shown in Figure 2.6. The apparatus was run in batch mode in which permeate was removed continuously and the reject was reintroduced into the feed tank. A booster pump was used to ensure positive pressure in the positive displacement (PD) pump to avoid cavitation, the formation of vapour bubbles in the pump caused by the pressure dropping to below the vapour pressure of the feed liquid. Cavitations impair the performance of the pump: they cause vibrations, reduce the flow through the pump and create a loss in the efficiency of the pump (Perry,1997).

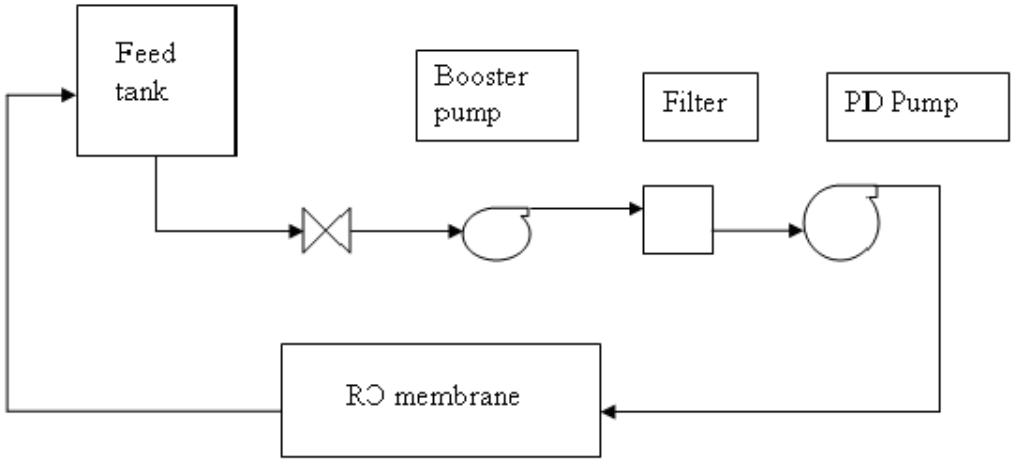


Figure 2.6: Reverse Osmosis membrane filtration unit

A solution of 6 litres of sugar at the desired concentration was prepared and a sample taken. The operating pressure was set to 40 bar by controlling the back pressure valve. The system was allowed to run for a set time after which the amount of permeate was measured using a measuring cylinder and samples of permeate and reject were taken. Samples were analysed for sugar concentration using the method described in section 2.4.3.

3 RESULTS AND DISCUSSION

3.1 Characterisation of Complex Wastes from Fruit Industries

3.1.1 Sampling of wastewaters

Several fruit processing industries in the Western Cape, South Africa were contacted and in April 2006 and March 2009, during the processing season, wastewaters were collected. All companies were reluctant to disclose the production processes for proprietary reasons and the exact point of sampling cannot therefore be disclosed. At some companies, the research team was permitted to enter the processing plant and take samples but at other companies, samples were taken by employees and provided without detail of location.

Two samples of apple wastewater were collected in 2006 and are named AW1 and AW2. AW1 was collected at a point after which primary treatment had been done. AW2 was a mixture of residual liquid after apple pressing and cleaning water from the plant. In 2009, two further apple wastewaters were collected for supplementary tests. AW3 was collected from the processing plant and AW4 was collected at the point of discharge into the municipal sewage system.

Two samples of citrus wastewater were collected and are named CW1 and CW2. CW1 was collected from the combined total wastewater stream. It contained pulping process wastewater, cleaning and rinsing waters, and miscellaneous factory wastewater. This wastewater is discarded directly into the municipal sewage system. CW2 was collected from a wastewater tank and contained undiluted wastewater generated during the juicing process. After the wastewater has been collected in the holding tank, it is discharged to a set dam where it is chemically treated to decrease the COD.

Silage water (SW) was also tested. This wastewater is seepage from solid fruit waste. Fruit waste is collected in a trench and allowed to dry before being used as animal feed. The liquid that leaches from the solid waste is a concentrated juice which requires treatment before discharge. Silage water was collected for a study by Stellenbosch University and samples were provided by kind donation of Prof J Gorgens.

3.1.2 Testing wastewaters

For long term storage, wastewater samples were kept at -20^{o}C and for short term storage at 4^{o}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. Common analyses were performed according to American Public Health Association's (APHA) Standard Methods for the Examination of Water and Wastewater (1998). Methods are described in Section 2.

3.1.3 Results and discussion of chemical analyses

Table 3.1 summarises the chemical analyses conducted on industrial wastewaters from apple, citrus and soft deciduous processing plants. The parameters measured are important in terms of the environmental impact of disposing the wastewaters as well as the recovery of valuable compounds.

Table 3.1: Chemical analysis of agri-industrial wastewaters where AW1, AW2, AW3 and AW4 are apple processing wastewaters, CW1 and CW2 are citrus processing wastewaters and SW is silage water

Sample	AW1	AW2	AW3	AW4	CW1	CW2	SW
pH	6.4	5.1	5.2	4.4	3.6	11.0	3.9
Total Solids (g/l)	1.52	2.44	-	-	5.1	4.9	6.87
Dissolved Solids (g/l)	0.70	1.72	-	-	4.7	4.46	5.42
Suspended Solids (g/l)	0.83	0.72	-	-	0.4	0.44	1.45
Conductivity ($mScm^{-1}$)	0.80	1.00	-	-	0.30	2.34	4.22
Chemical Oxygen demand (mg/l)	328	2200	2937	319	3170	5760	4750
Total phenolics (GAE mg/l)	7.6	42.5	25.9	1.8	18.6	137.0	399.5
Reducing sugars (mg/l)	126.5	215.3	-	-	230.3	396.3	234.8
Total carbohydrates (mg/l)	388.6	688.6	-	-	5793.6	2828.5	7000

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. Laleh et al. (2006) showed that the stability of anthocyanins in citrus fruits and their by-products is dependent on pH with most anthocyanins being more stable at low pH. An increase in pH caused greater destruction of anthocyanins. In addition, pH 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, AW2, AW3 and AW4 having pH values of between 6.4 and 4.4, while the citrus processing wastewater sample CW1 and the silage water SW

had pH values of 3.6 and 3.9, respectively. 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 pH 11.0. It is likely that this wastewater contained waste produced during peeling of the fruit using a caustic solution (Mannapperuma, 1996).

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 of the fruit processing wastewater samples characterised in this study contained a higher proportion of dissolved solids than suspended solids. These suspended and dissolved solids would have arisen during the processes of washing, 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 was generally low (0.3 to 4.22 mS/cm) for a wastewater. In comparison, typical values for olive wastewater range from 25.3 to 36.6 mS/cm (Achak et al., 2009).

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 COD values of wastewaters used in this study ranged

from 319 mg/l in AW4 to 5760 mg/l in CW2 (Table 3.1). Compared to other industrial wastewaters, such as olive mill wastewaters which often have COD values greater than 10 000 mg/l (Garcin, 2005), these are low strength effluents but the COD levels of some samples are still high enough to require treatment before discharge into municipal sewage systems. The apple wastewater AW1 had already undergone primary treatment as evidenced by its COD value of 328 mg/l. AW2, AW3 and the citrus wastewaters CW1 and CW2 had values of 2200 mg/l, 2937 mg/l, 3170 mg/l and 5760 mg/l respectively and would require treatment to reduce COD. The same is true of the silage water sample, SW, which had a COD value of 4750 mg/l. Removal of part of the COD in the wastewater samples would be advantageous not only in the production of additional income but also in reducing the cost of treatment of the wastewaters before discharge or recycle.

A wide range of total phenolics concentrations was found in the wastewaters studied, ranging from 1.81 to 399.52 mg/l GAE (Table 3.1). Much research has been conducted on the characterisation and potential use of citrus and deciduous fruits to obtain phenolic antioxidants but there is limited data on the use of fruit processing wastewaters for the production of antioxidants. The wastewaters studied in this work 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 (Marín-Martinez et al., 2009; Minhalma et al., 2006).

The apple processing wastewater samples, AW1, AW2 and AW3 had total phenolics contents of 7.61, 42.45 and 25.91 mg/l GAE, respectively. According to Schieber et al. (2003), apple pomace tested had a 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 remain in the pomace while the fruit juice contains most of the phenolics. Relatively low proportions would be expected to be present in the wastewaters. Wastewater discharged from the factory (AW4) contained only trace phenolics at 1.81 mg/l GAE.

Citrus processing wastewater samples, CW1 and CW2, contained low amounts of total phenolics, namely 18.6 and 137 mg/l respectively, compared to quantities present in the juice (569 mg/l GAE) and pulp (4871 mg/kg) of sour orange (Ersus and Cam, 2007). The distribution of phenolic compounds in fruits is higher in the pulp than in the juice (Marinova et al., 2005) and only low amounts of phenolic compounds are present in the wastewaters.

The total phenolics content in SW was higher (399.52 mg/l) than the apple and citrus processing wastewaters which was expected because SW comprised the seepage liquid that arises from the storage of residues of various deciduous fruits such as peaches, pears and plums and therefore more closely resembles a waste juice than a wastewater. All of these fruit types are known to contain relatively high concentrations of total phenolics (Balasundram et al*.,* 2006).

To determine the potential for use of the fruit processing wastewaters as a fermentation medium or for conversion to biomass fertiliser, it was necessary to measure the concentrations of sugars and total carbohydrates present. Values ranging from 126.5 to 396.3 mg/l were measured for the reducing sugars in the wastewater samples (Table 3.1). A study conducted by Scordino et al. (2007) found that 1185.6 mg/l of total sugars were recovered from orange pulp wash but the levels here are much lower. There are a number of causes for this. AW1 had already undergone primary treatment and the low level of reducing sugars is consistent with the lower COD. AW2, AW4 and CW1 were mixed waste streams including washing water and here the available sugars have been diluted. CW2 and SW are samples which have been stored for some time in a holding tank or open ditch and here it is likely that available sugars have already been fermented by the microbial population present in the wastewater. Of the wastewaters, CW2 showed the greatest potential for use as a fermentation substrate but the sugar level, 0.396 g/l, is still very low. This will be further investigated in Section 3.3.

The fruit processing wastewaters contain complex carbohydrates, such as pectin, as well as simple sugars, which contribute to the chemical oxygen demand of the wastewaters. The total carbohydrates concentration measured in the wastewaters ranged from 388.6 mg/l in AW1 to 7000 mg/l in SW (Table 3.1). The determination of total carbohydrates by acid hydrolysis (described in Section 2.1.3) is complicated by the fact that absorbance at 490 nm 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 adjusted by subtracting the absorbance of the wastewater sample at 490 nm from the absorbance of the reaction mixture. Xi et al. (2010) studied possible interferences to the phenol-sulphuric acid assay when quantifying carbohydrates in tea and found that polyphenols were a major interfering material because of presence of multiple hydroxyl groups. Vitamin C also affected the absorbance, resulting in a change of 3.64% at a concentration of 10 µg/ml (Xi et al., 2010). Because of the presence of polyphenols and ascorbic acid in the wastewaters, the total carbohydrate will be overestimated but certainly,

the presence of complex carbohydrates is indicated in the wastewaters, particularly in CW1 and SW.

3.1.4 Results and discussion of antioxidant assays

The determination of the antioxidant capacity of a wastewater cannot be achieved comprehensively and consistently by any single method because of the complex nature of phytochemicals (Du et al., 2009). Multiple reaction characteristics and mechanisms are involved, namely the hydrogen atom transfer (HAT) and the proton-coupled electron transfer (PCET) (Katarina, 2007). It is generally agreed that at least two different assays are required to assess antioxidants accurately in mixed or complex systems such as fruit processing wastewaters (Erkan et al., 2008). For this reason, assays such as DPPH radical scavenging assay are performed to demonstrate the HAT mechanism while the PCET mechanism is illustrated in assays such as the Trolox Equivalent Antioxidant Capacity (TEAC) assay and Ferric Reducing Power assay (FRAP) (Devi and Arumughan, 2007; Ferreira et al., 2007).

In this work the DPPH and TEAC assays were used to determine whether the wastewater samples are effective free radical scavengers and provide information on the electron donating ability of the wastewater samples. These assays use synthetic free radicals that are not found in any biological systems and it was therefore also necessary 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; and the β-CLAMS assay determines the potential of the antioxidants to inhibit β-carotene bleaching (Alén-Ruiz et al., 2009).

3.1.4.1 DPPH and TEAC assays

The total free radical scavenging capacities of the fruit processing wastewaters was measured by their ability to scavenge the commercially available stable free radicals DPPH (Figure 3.1) and $ABTS^{+}$ (TEAC assay) (Figure 3.2). In both cases, SW exhibited more radical scavenging activity than the rest of the wastewaters as shown by a more rapid decrease in absorbance which is an indication of the rate of scavenging. Silage water completely decolourised DPPH, indicating strong hydrogen donating ability. This correlates with higher total phenolics in SW compared to the other wastewaters (Table 3.1).

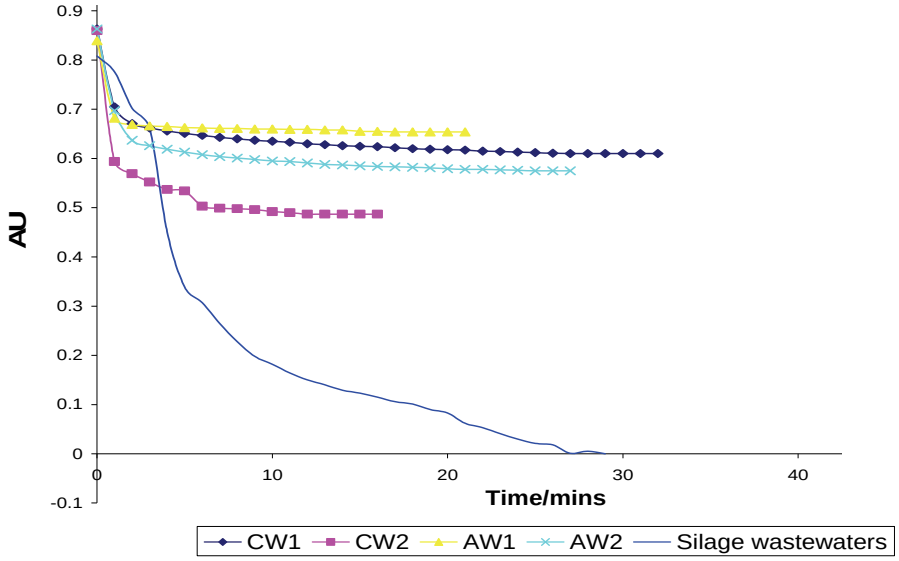


Figure 3.1: DPPH radical scavenging assay for wastewater samples

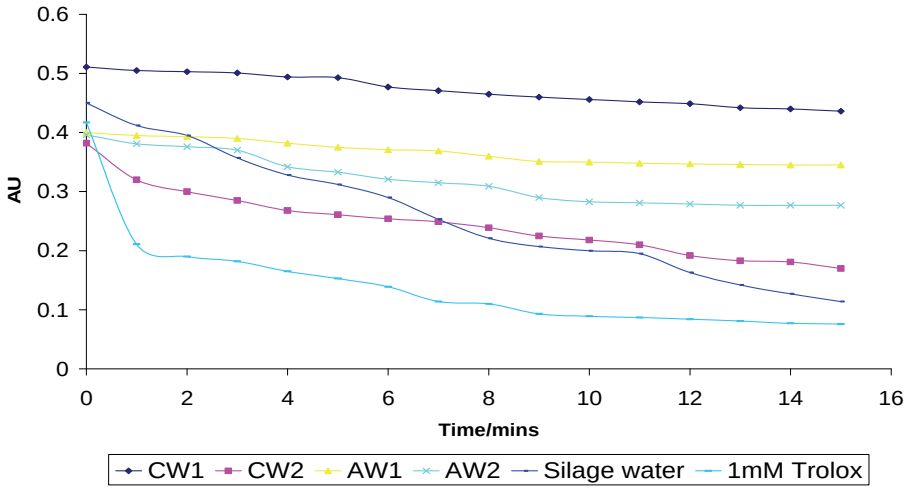


Figure 3.2: Trolox equivalents antioxidant activity (TEAC) assay for wastewater samples

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). Using the DPPH assay, the % radical scavenging activities (RSA) of the wastewaters were calculated and Table 3.2 shows values ranging from 22.05% in AW1 to 100% in the silage water, SW. 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 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 cannot be made, these values give an indication of the potential of the wastewaters to exhibit radical scavenging activity that is comparable to whole fruits.

The TEAC assay has been recently proposed as a standardised method to measure antioxidant capacity of food products and dietary supplements (Magalhães et al., 2007). Table 3.2 shows the total phenolics contents and % radical scavenging activity measured using the TEAC assay. 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.

Table 3.2: Total phenolics and radical scavenging activities of wastewaters

Sample	Total phenolics (GAE mg/l)	%RSA $_{DPPH}$	%RSA $_{TEAC}$
AW1	7.6	22.1	13.8
AW2	42.5	29.3	30.3
CW1	18.6	29.3	21.6
CW2	137	43.4	55.5
SW	399.5	100.0	74.7

3.1.4.2 FRAP assays

The reducing ability of antioxidants present in the fruit processing wastewaters was determined by the FRAP assay. Figure 3.3 shows the reducing power of the wastewaters and ascorbic acid control. This assay measures the ability of the antioxidant to reduce the ferric tripyridyltriazine (Fe^{3+} – TPTZ) to the Fe^{2+} form (Katalinic et al., 2005). The reducing properties are generally associated with the presence of reducing entities which have been shown to exert antioxidant action by breaking the free radical chain through donating a hydrogen atom (Farhoosh et al., 2007), a useful property for a protective food additive. Silage water had 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. The FRAP assay Jayaprakasha et al. (2008) 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 0.418GAE. These values are higher than the reducing power of the citrus wastewater samples studied here, as would be expected.

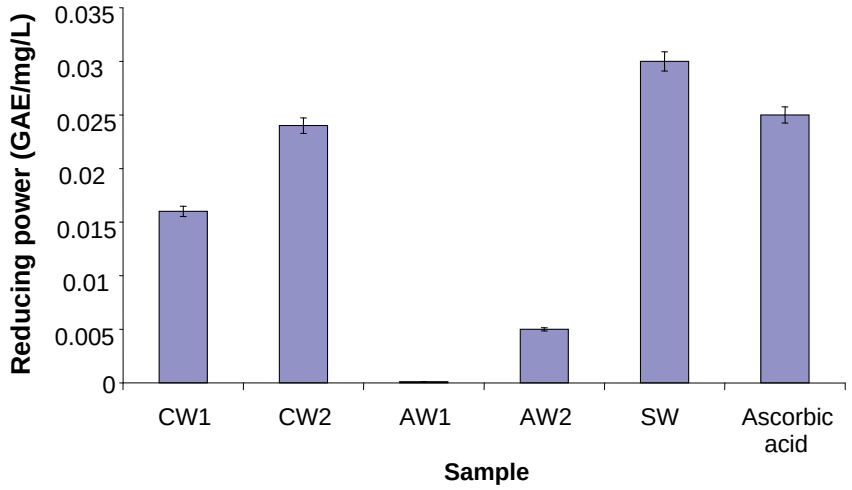


Figure 3.3: Ferric reducing antioxidant power (FRAP) assay for wastewater samples

3.1.4.3 β-CLAMS 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 lipid peroxides. In this system, one of the hydrogen atoms from one of the methylene groups of linoleic acid is withdrawn due to auto-oxidation, leaving the free radical of the acid (Liu et al., 2007b; Reyes-Caudillo et al., 2008). This will attack β-carotene molecules, saturating the double bonds, resulting in loss of characteristic orange colour (Liu et al., 2007a). This assay therefore reveals the wastewaters' ability to stabilise the reactive oxygen species and some lipidic radicals responsible for the oxidation of linolenic acid (Reyes-Caudillo et al., 2008).

Figure 4.4 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, SW exhibited the highest inhibition of β-carotene of 86%, followed by CW1 and CW2 (49.2 and 69.04%, respectively). 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.

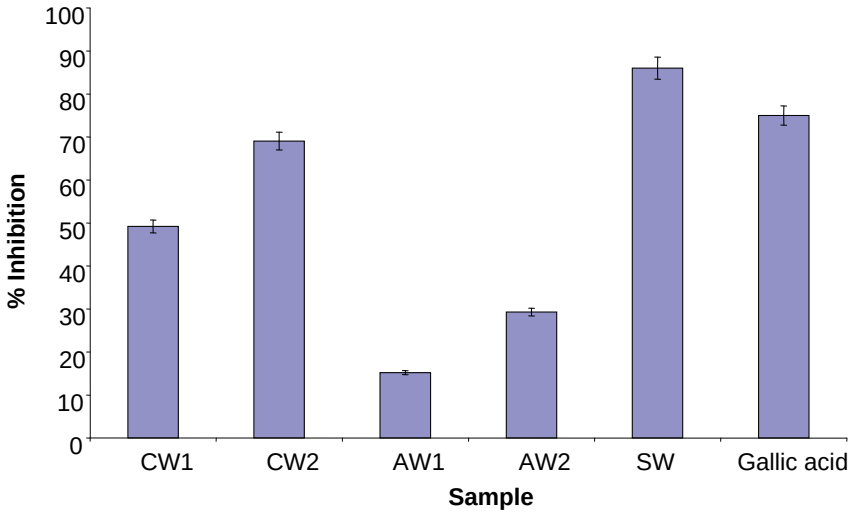


Figure 3.4: β-carotene linoleic acid model system (β-CLAMS) fruit processing wastewaters

3.1.5 HPLC assays

The results of the antioxidant assays discussed above showed overall capacities for radical scavenging, reducing power and the lipid peroxidation inhibition in the fruit processing wastewater samples but 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 280 nm because all aromatic polyphenolic compounds absorb at 280nm, whereas at this wavelength, aliphatic compounds such as sugars and organic acids do not absorb (Alén-Ruiz et al., 2009).

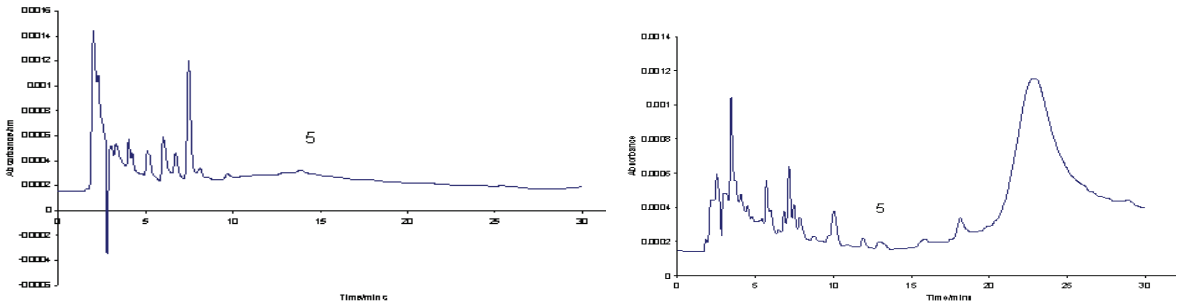
HPLC allows for analysis of phenolic compounds with high precision, sensitivity and within a reasonable time. Resveratrol, gallic acid, catechin, caffeic acid, chlorogenic acid, epicatechin and p-coumaric acid were used as standards. The standards were chosen based on the

results published in literature where gallic acid, caffeic acid, chlorogenic acid, p-coumaric acid were identified in orange juices (Kelebek et al., 2009) and resveratrol, catechin and epicatechin were also detected in deciduous fruits (Lu and Foo, 2000; Suárez et al., 1996). The choice of standards was limited by the availability of the standards at the time the analysis was conducted. Individual phenolics were identified by their retention time when compared to pure standards and spiking experiments were conducted where appropriate (Table 3.3). The concentration of identified phenolics was calculated by plotting a standard curve of concentration against peak area for each of the standards.

Table 3.3: HPLC standards and their retention times

Standard	Peak number	Retention time/mins
Resveratrol	1	2.95
Gallic acid	2	4.16
Catechin	3	7.38
Caffeic acid	4	12.9
Chlorogenic acid	5	13.43
Epicatechin	6	14.53
p-coumaric acid	7	26.43

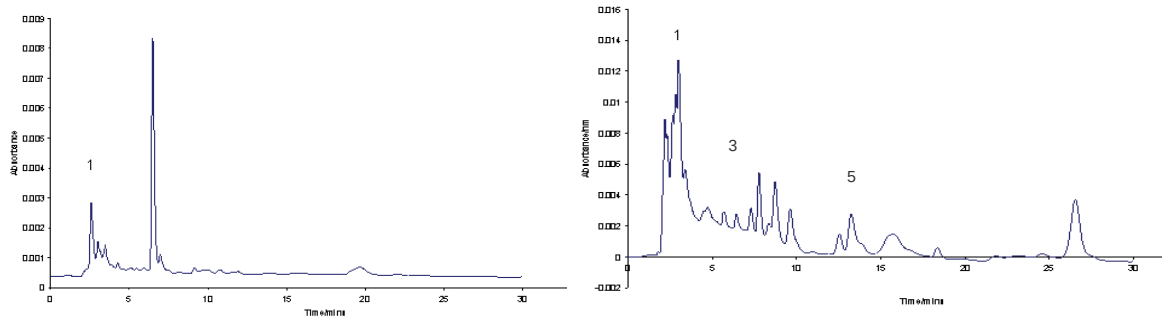
Figures 3.5 to 3.7 show the HPLC profiles of the fruit processing wastewater samples. Individual phenolic compounds identified in the wastewaters are summarised in Table 3.4.



(A) (B)

Figure 3.5: HPLC chromatogram for apple processing wastewater AW1 (A) and AW2 (B).

The peak with retention time of 13.43 minutes corresponds to chlorogenic acid.



(A) (B)

Figure 3.6: 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.

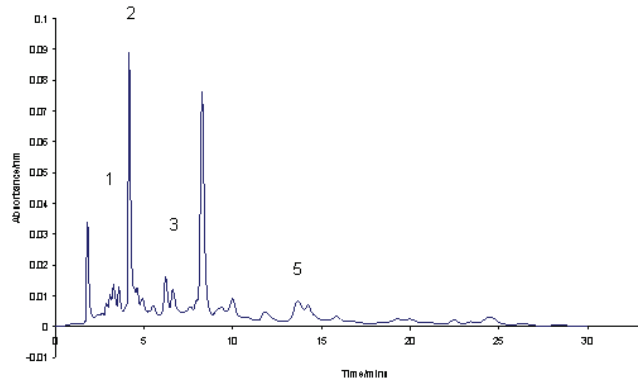


Figure 3.7: HPLC chromatogram of silage water.

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.

Table 3.4: Concentrations of respective phenolic compounds in fruit processing wastewaters as determined by HPLC analysis. Caffeic acid, epicatechin and *p*-coumaric acid were not identified in any sample and were therefore omitted

	Phenolic compound in mg/l			
Sample	**Resveratrol**	**Gallic acid**	**Catechin**	**Chlorogenic 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

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 and 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. There were a few unidentified peaks in the analysis of CW1 and many relatively large peaks in the analysis of CW2 which did not correspond to any available standards. These phenolics contribute to the observed total phenolics values of 18.7 mg/l for CW1 and 137 mg/l for CW2 (Table 3.1). The low concentrations of phenolic compounds identified in CW1 are understandable as this wastewater was diluted with water collected from cleaning the production equipment. 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. Other phenolic compounds that would be expected 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 detected in the silage water at concentrations of 0.6 and 8.1 mg/l respectively.

Overall, all major phenolic compounds were separated with good resolution. Chlorogenic acid and resveratrol were found to be common in most of the wastewaters. There were unidentified peaks in the samples, indicating the need to acquire more pure standards to allow identification by comparison of the retention time. Alternatively, peaks could be identified by mass spectroscopy. Identification of phenolic compounds in natural extracts by liquid chromatography with mass spectroscopy detection is becoming more common because of the complexity of phenolic mixtures in such extracts (García-Villalba et al., 2009).

3.1.6 Summary of Antioxidant Characteristics

A summary of the antioxidant activities of the wastewaters studied is given in Table 3.5.

Table 3.5: Comparison of antioxidant characteristics measured in fruit processing wastewaters

	Total phenolics	**Radical scavenging ability**	**Radical scavenging ability**	**Reducing ability**	**Ability to inhibit lipid peroxidation**	**Specific phenolics**
Assay	**FC**	**DPPH**	**TEAC**	**FRAP**	**β-Clams**	**HPLC**
Unit	**GAE mg/l**	**%**	**%**	**GAE mg/l**	**%**	**mg/l**
AW1	7.6	22.1	13.8	0.005	15.2	0.3
AW2	42.5	29.3	30.3	0.0001	29.3	0.33
CW1	18.6	29.3	21.6	0.016	49.2	0
CW2	137.0	43.4	55.5	0.023	69.04	1.96
SW	399.5	100.0	74.7	0.03	86	120.63

Silage water had the highest total phenolics content of the wastewaters tested. It was very effective at scavenging radicals in aqueous environments (FC and TEAC assays), methanolic environments (DPPH) and lipophilic environments (β-Clams). Silage water is a concentrated waste water, not diluted by other wastewaters generated in fruit processing and cleaning of factories and therefore has a high total phenolics content. It contains phenolics from different fruit types and can therefore be expected to contain a range of different phenolics, as can be seen in Table 3.4. It also contains phenolics derived from both pomace and peel and will therefore have hydrophilic and hydrophobic phenolics which are able to scavenge radicals in environments of different polarity. The very high radical scavenging ability noted in the DPPH assay (100%) is not unexpected considering the high proportion of gallic acid identified in this waste (Table 3.4). Gallic acid has an increased solubility in methanol when compared to pure water (Daneshfar et al., 2008) contributing to the favourable antioxidant capacity measured.

CW2 is also a relatively concentrated wastestream, containing only fruit processing wastewater without washing water. CW2 was noted for its high pH which indicated addition of alkaline processing water from peel removal. Orange peel contains higher levels of phenolics than the corresponding peeled fruit (Gorenstein et al., 2001) resulting in the high antioxidant activity of CW2. Flavenoids present in citrus fruit and specifically in citrus peel such as naringin and hesperidin (Bocco et al., 1998) have multiple phenyl rings which may

increase their ability to associate with hydrophobic β-carotene allowing greater antioxidant activity against lipid peroxidation.

CW1 and AW2 are processing wastewaters that have been diluted with water from washing the processing plant. Their total phenolics content is therefore lower than in CW2 or SW. Phenolics found in citrus waste have been noted to have antioxidant activity against lipid peroxidation, specifically giving anti-carcinogenic and anti-inflammatory effects (Ghasemi et al., 2009) and it is clear that CW1 has a greater ability to prevent bleaching of β-carotene than the phenolics in AW2. The antioxidant assays which are conducted in aqueous environments, TEAC and FC, give higher antioxidant values for AW2. Chlorogenic acid, which was identified in both apple wastewaters, is easily detected by the FC method (Apak, 2007). AW1 is dilute and has already been treated to lower COD. Low levels of phenolics remain and the antioxidant capacity is low.

The antioxidant capacity of raw wastewaters is of interest in showing the potential of each wastewater to yield a product with high antioxidant capacity. It is limited however in that comparative results are strongly biased in favour of more concentrated wastewaters. A dilute wastewater may seem to have insignificant potential as an antioxidant. If the antioxidants in this wastewater were extracted and concentrated through use of a suitable technique though, they may provide a powerful antioxidant extract. Different extraction techniques were investigated to determine the antioxidant capabilities of extracts of each wastewater.

3.2 Extraction of Phenolics from Fruit Wastewaters

Recovery of antioxidants from by-products of food processing plants has gained importance with the move to replace synthetic antioxidants with natural ones. Natural antioxidants have beneficial health implications and improved solubilities in food systems (Adil et al., 2007). In order to make use of the phenolic antioxidants present in 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 Luque de Castro, 2005). Several different sample preparation and extraction techniques are available for the recovery of phenolic compounds as discussed in Chapter 1. In this work, five different extraction techniques were employed for the recovery of phenolic antioxidants from the fruit processing wastewaters collected and analysed. These techniques were: 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 discussed in the following sections.

3.2.1 Solvent extraction

3.2.1.1 Extraction efficiency of different solvents

Table 3.6 shows the total phenolics contents (measured using the FC 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.

Table 3.6: The total phenolics contents of the fruit processing wastewaters and amounts obtained after extraction with ethyl acetate or hexane

	Total phenolics content (GAE mg/l)		
	In original wastewater	**After ethyl acetate extraction**	**After hexane extraction**
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 extracts obtained using ethyl acetate showed higher concentrations of total phenolics than the hexane extracts showing that ethyl acetate was a better solvent than hexane for the extraction of phenolic compounds from all fruit processing wastewaters except AW1. For AW1, the ethyl acetate extract contained 0.93 mg/l total phenolics while 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.

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. The result was expressed as a percentage. The extraction efficiencies of the ethyl acetate and hexane extracts are shown in Figure 3.8. Generally, ethyl acetate exhibited higher extraction efficiencies compared with hexane in all the wastewater samples studied except in AW1. Apple wastewater AW2 gave the highest

extraction efficiency of 47.73% and SW gave the lowest extraction efficiency of 20.29% using ethyl acetate as the solvent. The citrus wastewaters CW1 and CW2 gave extraction efficiencies of 20.65% and 37.45% respectively, 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 exhibited low 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. This can be explained by the fact that ethyl acetate is a more polar solvent than hexane, and compounds with similar polarities, such as phenolic acids, have higher solubilities in this solvent than in non-polar solvents (Mohsen and Ammar, 2009). The silage water, which had gallic acid as its major phenolic compound, as previously shown in Table 3.4, 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, such as hexane, is very poor. These results emphasize the fact that the extraction of phenolic compounds depends on, amongst other factors, the polarity of the phenolic compounds in the wastewater and the type of solvent being used.

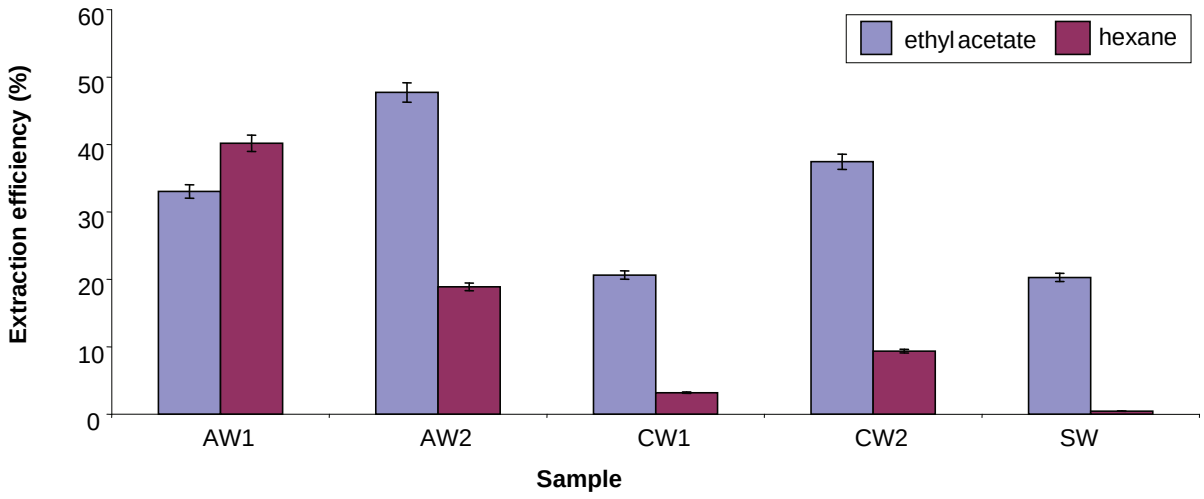


Figure 3.8: Extraction efficiencies of the solvents ethyl acetate and hexane, used for the extraction of phenolic compounds from fruit processing wastewaters. Extraction efficiency is calculated as the ratio of the amount of phenolic compounds obtained by solvent extraction, to the amount of phenolic compounds in the same volume of wastewater sample before extraction, and expressing the result as a percentage. Error bars indicate standard error of the mean (SEM) of duplicate samples.

3.2.1.2 Antioxidant activity of the extracts obtained after solvent extraction

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 3.7. Generally, the ethyl acetate extracts exhibited better scavenging activity than the hexane extracts. As previously mentioned, ethyl acetate is the more polar solvent and will therefore extract more polar phenolics than hexane. These phenolics are more suited to scavenge radicals in the methanolic system provided by the DPPH assay. Miladi and Damak (2008) studied the antioxidant activities of ethyl acetate and hexane extracts of *Aloe vera* and found higher DPPH assay results for the ethyl acetate extracts while the hexane extracts were better able to inhibit decolourisation of non-polar β-carotene (Miladi and Damak, 2008)

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 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 3.7: Antioxidant activity of ethyl acetate and hexane extracts using the DPPH assay

Sample	Original wastewater		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	15.13 ± 0.1	3.34 ± 0.2

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

In order to determine the potency of the phenolic antioxidants in the extracts in scavenging 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 4.7. Here the higher the ratio, the higher the scavenging effect and potency of the phenolic antioxidants in the extract. From Table 3.8, 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 the DPPH free radical with a value of 0.63.

Table 3.8: Ratios of the radical scavenging activity (%RSA) to the concentration of total phenolics (TP) for fruit processing wastewater samples after solvent extraction using ethyl acetate or hexane

	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

Generally, the ethyl acetate extracts exhibited higher ratios compared to the hexane extracts, except in the case of SW where the ratio was 1.71 for the ethyl acetate extract and 4.53 for the hexane extract. This observation 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*.,* 2003). The results obtained in the present study also demonstrate 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.

3.2.1.3 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 investigate other extraction techniques such as solid phase extraction which uses relatively small amounts of solvent.

3.2.2 Solid Phase Extraction

3.2.2.1 Extraction by C18 Sep-Pak® cartridges

Solid phase extraction (SPE) is particularly well adapted to multi-residue analysis including compounds with a wide variety of polarity or characterised by various physico-chemical properties (Pietrogrande and Basaglia, 2007). Solid phase extraction prevents interferences among phenolics by fractionating them into neutral and acidic phenolics.

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. Table 3.9 summarises the total phenolics contents after extraction and the corresponding extraction efficiencies. 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. The results reported in a study 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. 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).

Table 3.9: Total phenolics content of wastewaters before and after solid phase extraction (SPE) and extraction efficiencies

Sample	TP before extraction (mg/l GAE)	TP after SPE (mg/l GAE)	Extraction efficiency (%)
AW1	11.1± 0.5	5.80± 0.3	52.1± 0.3
AW2	23.5± 0.4	12.20± 0.2	51.9± 0.1
CW1	41.7± 0.3	20.70± 0.1	49.5± 0.7
CW2	98.8± 0.5	43.59± 0.3	44.1± 0.5
SW	1014.2± 3.3	577.49± 0.1	56.9± 0.9

Figures 3.9 to 3.13 show the HPLC chromatograms of neutral and acidic phenolics separated using C18 Sep-Pak cartridges. Fractionation capacity and efficiency for SW was not very good as is seen in Figures 3.9 A and B where gallic acid was present in the neutral fraction instead of the acidic fraction. Catechin was also identified in both the neutral and acidic fractions for Silage wastewater. Extraction efficiency for Silage water was 56.9% as shown in Table 3.9.

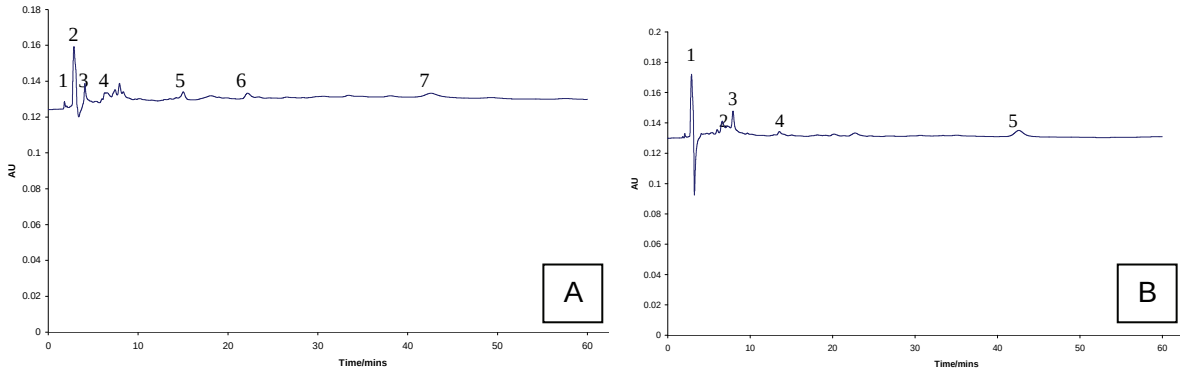


Figure 3.9: HPLC chromatogram of Silage water. A: neutral phenolics after solid phase extraction (SPE), 3 – gallic acid, 4 – catechin, B: acidic phenolics, 3 – catechin, 4 – chlorogenic acid

Figures 3.10 A and B show the neutral and acidic fractions of CW2. Here the fractionation capacity of this wastewater was better compared to SW since only catechin was identified in the neutral fraction whilst the phenolic acids, gallic acid and chlorogenic acid were identified in the acidic fraction. Although CW2 had the lowest extraction efficiency (44.1%), there was a marked improvement in resolution on the HPLC for the neutral and acidic phenolics.

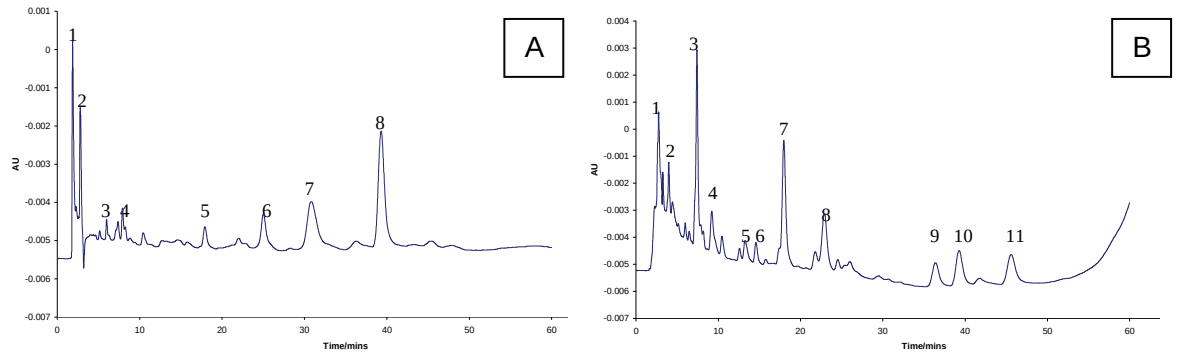


Figure 3.10: HPLC chromatogram for CW2, A: neutral phenolics; 4 – catechin, B: acidic phenolics; 2 – gallic acid, 5 – chlorogenic acid.

Figure 3.11 to 3.13 show the neutral and acidic fractions of CW1, AW1 and AW2. It was not expected that gallic acid would be found in the neutral fraction of CW1. The presence of gallic acid in the neutral fraction can be attributed to its high polarity, making dissociation during the elution stage difficult. The higher carbon loading and pore diameter of cartridges 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). Resveratrol was detected in both the neutral and acidic fractions of AW1 instead of being found only in the neutral fraction, which implies that the neutral phenolics cartridge could not retain resveratrol strongly enough.

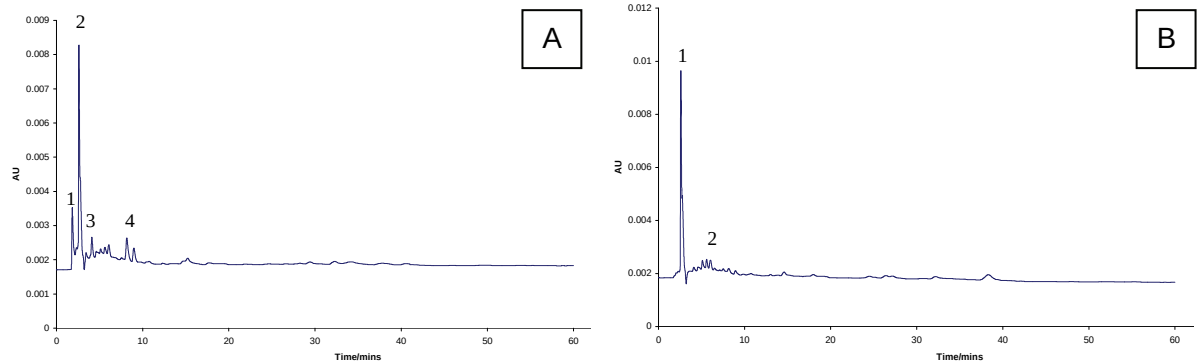


Figure 3.11: HPLC chromatogram for CW1, A: neutral phenolics; 3 – gallic acid; B: acidic phenolics

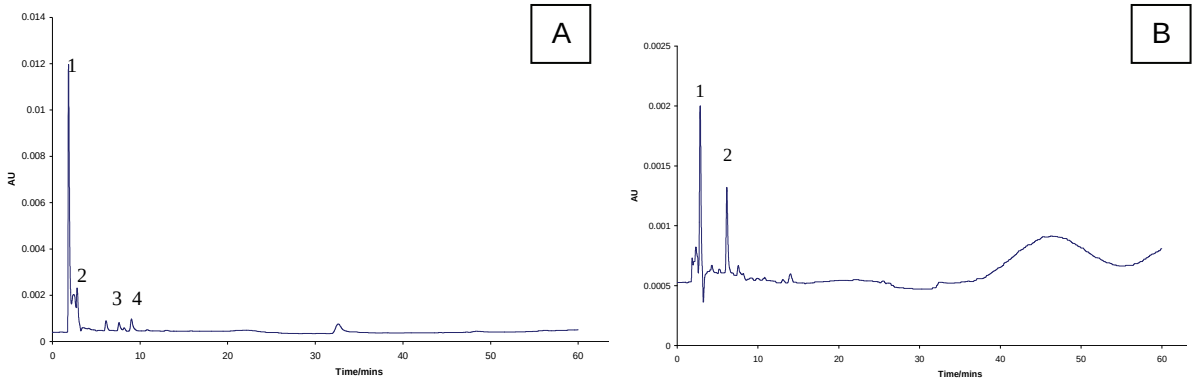


Figure 3.12: HPLC chromatogram for AW1, A: neutral phenolics; B: acidic phenolics.

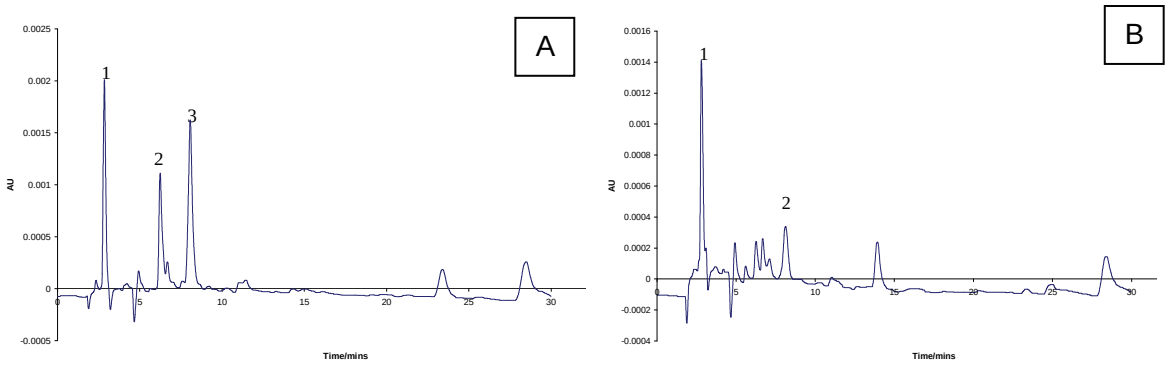


Figure 3.13: HPLC chromatogram for AW2, A: neutral phenolics; 1 – resveratrol; B: acidic phenolics; 1 – resveratrol.

The discrepancies observed in SPE may be explained by the physical characteristics of the packing material as well as the samples. The mechanism of retention of phenolic compounds by C18 cartridges is dependent on Van der Waals forces, hydrogen bonds or dipole-dipole interactions. All these interactions are weak and non-covalent, thus can be easily disrupted (Michalkiewicz et al*.,* 2008). Carbon loading plays an important role in the retention of phenolic acids, as a linear relationship between carbon loading and capacity factor has been described (Gomis et al*.,* 2001). In SPE, compounds of interest are usually absorbed at the top of the column packing material, not throughout, and this may contribute to inefficiencies in the extraction process. Furthermore, the types of solvent and sorbent have been shown to be two of the most influential variables for the extraction and separation of analytes, respectively (Dopico-García et al*.,* 2007). In addition, the water washing stage can lead to losses of some neutral phenolics such as catechin. A study done on extraction of phenolic compounds from apple musts and ciders using Waters Sep-Pak cartridges showed a high percentage of chlorogenic acid remaining in the neutral fraction. The authors concluded that cartridges from Waters had low fractionation ability (Suarez et al., 1996).

After SPE, the antioxidant activities of the neutral and acidic fractions were evaluated using the DPPH assay. The aim of this step was to ascertain the contribution of the individual fractions to the antioxidant activity. In all samples, the neutral fraction exhibited greater antioxidant activity compared to the acidic fraction as shown in Figure 3.14. In CW2 and Silage water, the combined antioxidant activities of the neutral and acidic fractions were greater than 100%. Antioxidant activity of the wastewater extracts depends on the synergistic action of the constituents of the different fractions, with the most important being the neutral fraction. The relative contribution of the phenolic fraction to the antioxidant activity was as follows: neutral fraction › acidic fraction. Significant antioxidant activity (89.5%) was also observed in the neutral fractions of cranberry extracts (Tumbas et al., 2007).

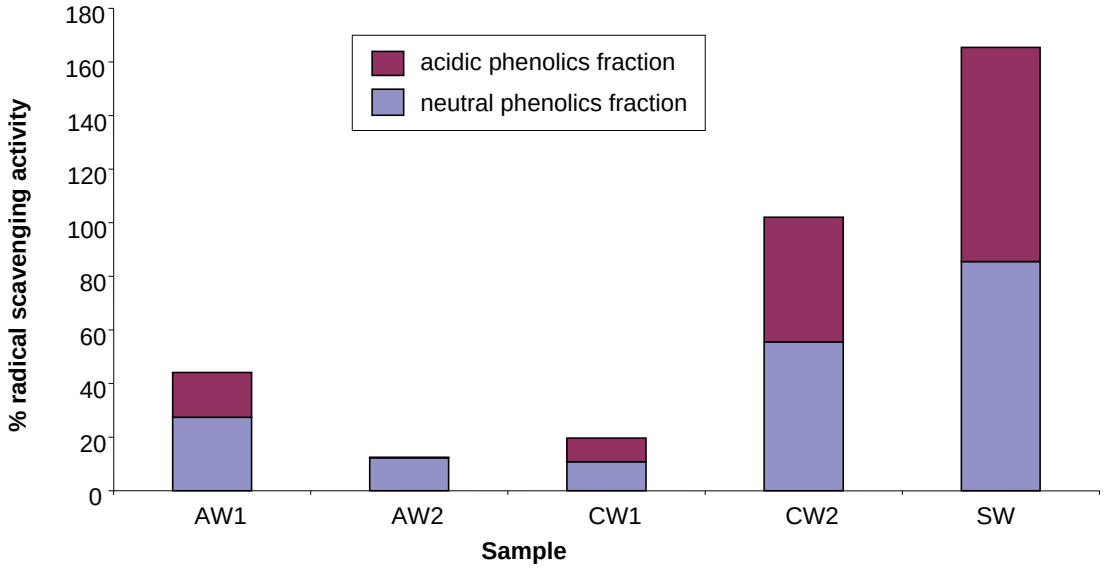


Figure 3.14: % DPPH Radical scavenging assay for neutral and acidic phenolics after solid phase extraction (SPE).

3.2.2.2 Extraction using PVPP

PVPP is used in the apple juice industry to reduce the concentration of polyphenolics and stabilise clear apple juice to prevent haze formation (Gökmen and Serpen, 2002). The aim is not to remove all phenolics but to improve the visual quality of the juice and it is to be expected that PVPP will show differential binding for different classes of phenolics. If PVPP is to be used to extract antioxidant phenolics so that they may be eluted later in a smaller volume of liquid, it is important to ascertain which phenolics will be retained in this treatment and which can later be removed from the solid matrix. Wastewaters were combined with PVPP for 10 minutes and the PVPP was harvested by filtration. The solid material was

washed with water and the adsorbed phenolics were eluted using a sodium hydroxide solution.

Figures 3.15 and 3.16 show the AW1 extract obtained after extracting with PVPP and washing with sodium hydroxide, and then water, respectively. Catechin was found in both the sodium hydroxide eluant and the water wash. Chlorogenic acid, which had been the only compound identified in the wastewater, was not identified after PVPP extraction indicating either that it did not bind to the PVPP or it remained bound and was not eluted. Catechin, which had not been identified in the wastewater, was now identified. This may be attributed to the concentration of analytes that occurs during extraction.

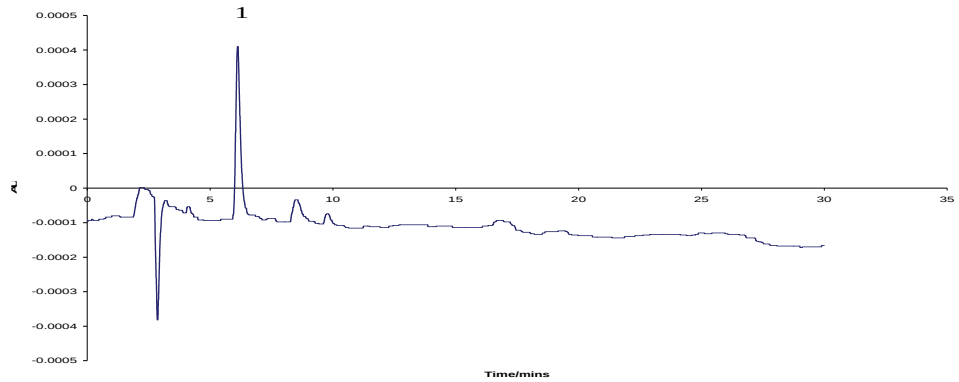


Figure 3.15: AW1 sodium hydroxide eluant after binding of phenolic compounds to PVPP; 1 – catechin

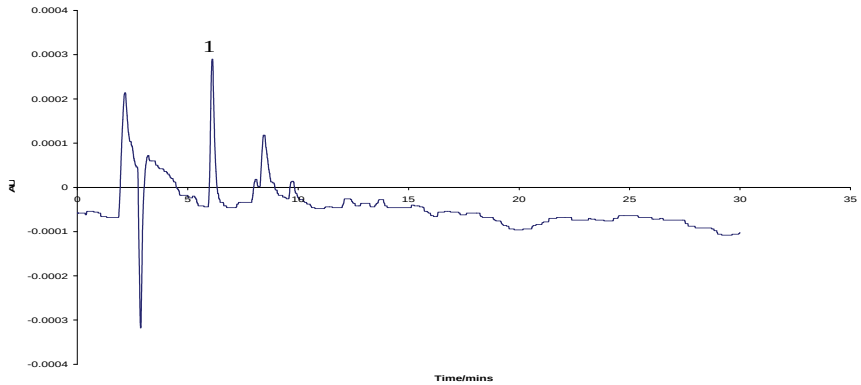


Figure 3.16: AW1 water wash after binding of phenolic compounds to PVPP; 1 – catechin

Figures 3.17 and 3.18 are the chromatograms for the sodium hydroxide eluant and water wash, respectively, for AW2. No peaks were identified in either of these extracts. Chlorogenic acid had been identified in the wastewater but again, was not observed in the water wash or sodium hydroxide elution.

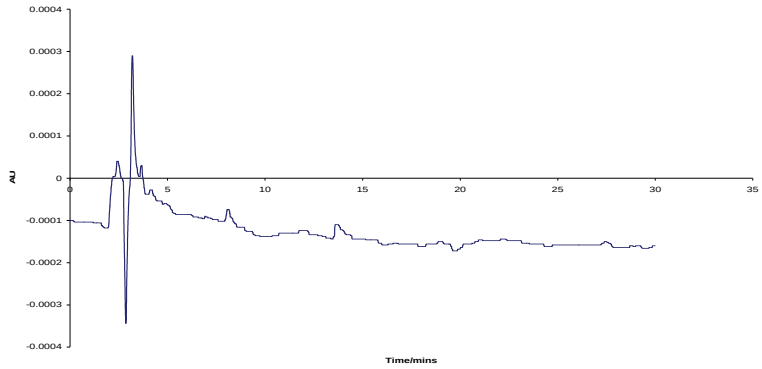


Figure 3.17: AW2 sodium hydroxide eluant

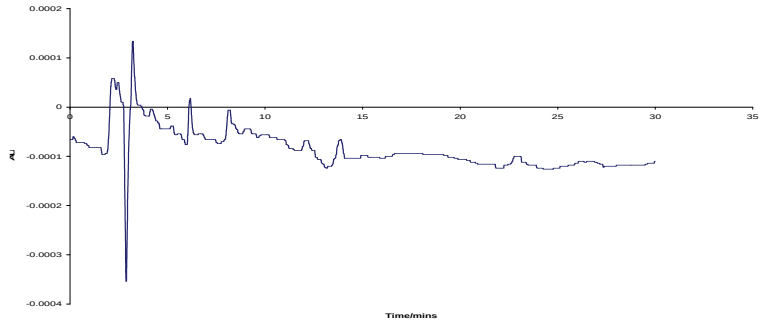


Figure 3.18: AW2 water wash after binding of phenolic compounds to PVPP

Figures 3.19 and 3.20 illustrate the chromatograms for CW1 obtained after PVPP extraction and the water wash, respectively. Gallic acid, initially not identified in CW1, was identified in both eluant and water wash. Resveratrol was not identified in any of the chromatograms.

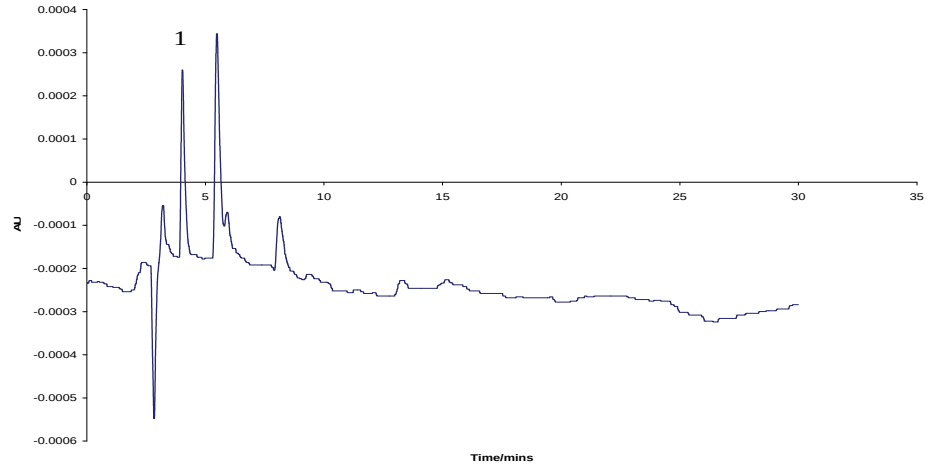


Figure 3.19: CW1 sodium hydroxide eluant; 1 – gallic acid

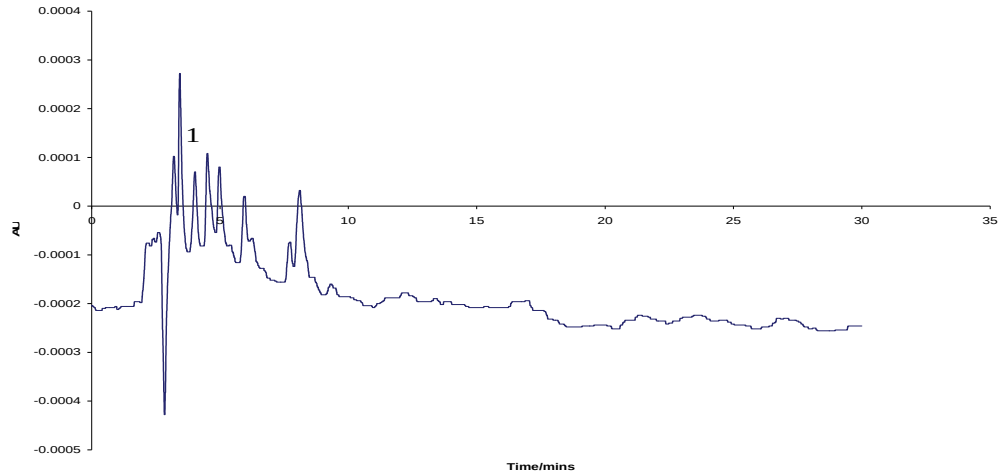


Figure 3.20: CW1 water wash after binding of phenolic compounds to PVPP 1-gallic acid

Figures 3.21 and 3.22 show the chromatograms of CW2 for the sodium hydroxide eluant and the water wash, respectively. Catechin was the only phenolic compound extracted that could be identified, and resveratrol and chlorogenic acid were not identified in the extracts.

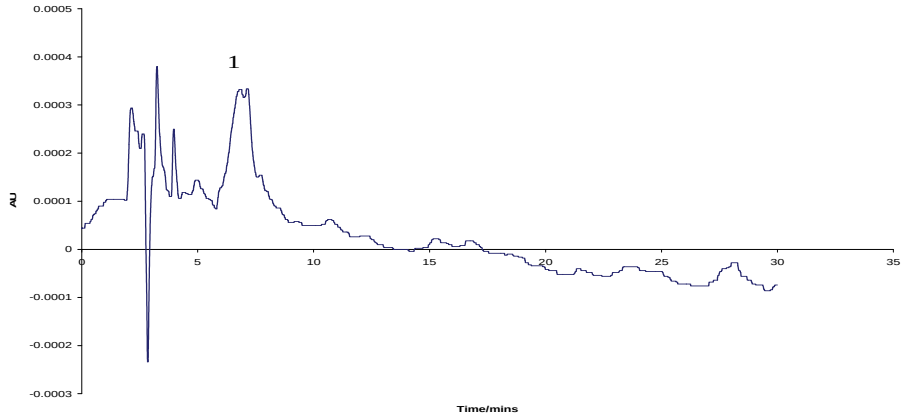


Figure 3.21: CW2 sodium hydroxide eluant; 1 – catechin

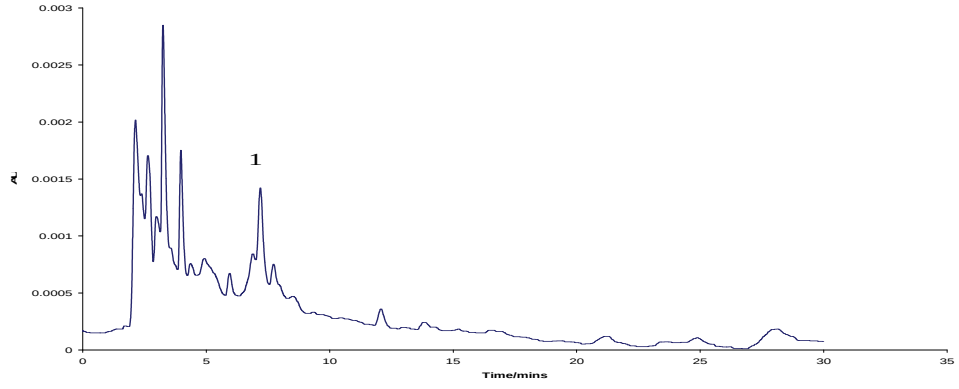


Figure 3.22: CW2 water wash after binding of phenolic compounds to PVPP; 1 – catechin

Figure 3.23 and 3.24 show the HPLC chromatograms for SW of the sodium hydroxide eluant and water wash, respectively. Gallic acid, the major phenolic compound in SW, was identified in the extract. Chlorogenic acid was again not identified.

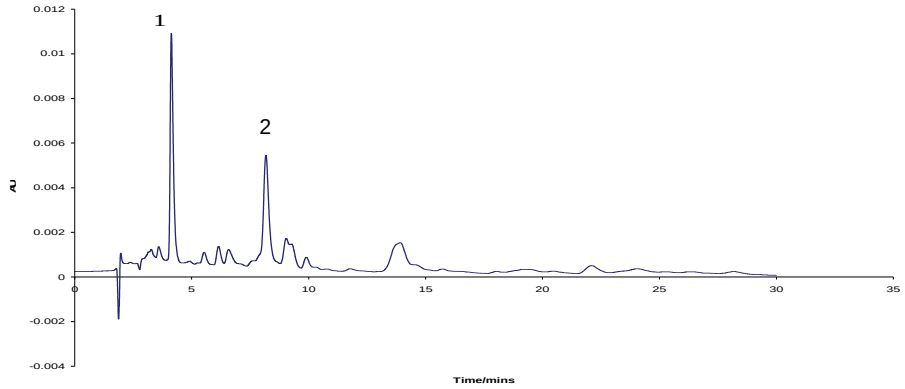


Figure 3.23: Silage water sodium hydroxide eluant; 1 – gallic acid, 2 – catechin

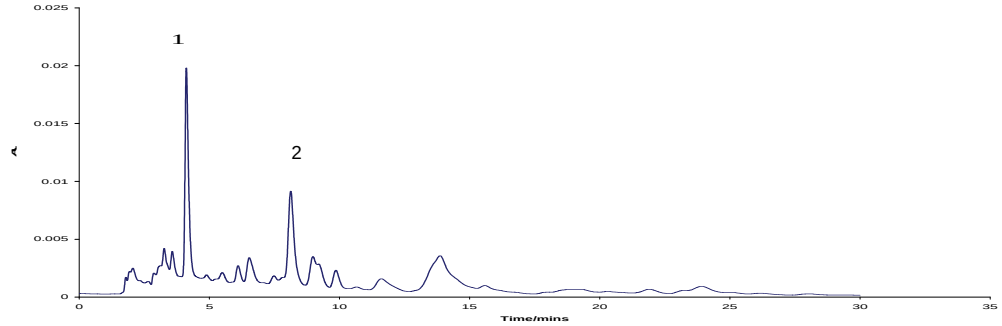


Figure 3.24: Silage water wash after binding of phenolic compounds to PVPP; 1 – gallic acid, 2 – catechin

Table 3.10 shows the extraction efficiencies calculated after extraction with PVPP.

Table 3.10: Total phenolics content of wastewaters and extraction efficiencies after PVPP extraction

Sample	**TP before extraction**	**TP in sodium hydroxide eluant**	**Extraction efficiency (%)**	**%DPPH RSA**
AW1	0.82 ± 0.1	-	-	5.10
AW2	5.37 ± 0.3	1.96 ± 0.2	36.49	0.65
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

The extraction efficiency was low, ranging from 6.2 to 39.6%. In part, low efficiencies can be due to loss of PVPP during the extraction process. For the recovery of PVPP with bound phenolics, vacuum filtration using cheese cloth was used but the pores of the cheese cloth are large enough to allow small particles of PVPP to pass through. Centrifugation may be a more efficient alternative to recover PVPP. A greater factor in the extraction efficiency, however, is binding of phenolics to the PVPP. Gallic acid and catechin were both identified in the eluant but chlorogenic acid was not detected. The implication is either that chlorogenic acid did not bind to PVPP or that it was not eluted with dilute sodium hydroxide solution.

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 π-bond overlap (delocalised electrons), polar and hydrophobic interactions (Sarioglu, 2007). Phenolics with different polarities and hydrophobicities will display different binding characteristics. A significant factor in adsorption of phenolics is the pH of the solution. For adsorption to PVPP, a pH of 3.5 is optimal but at alkaline pH values, phenolics are present in ionised form and do not bind (Andersen and Sowers 1968). SW naturally has a pH close to optimal for adsorption (Table 3.1) but CW2 is strongly alkaline and this results in poor adsorption.

Regeneration of PVPP involves using sodium hydroxide to disrupt the hydrogen bonds and other hydrophobic interactions binding the phenolic compounds to PVPP. Sodium hydroxide will have a stronger affinity for the unbound PVPP before it starts displacing phenolic compounds bound to PVPP. Because the aim of elution in this context is not regeneration of PVPP but collection of a concentrated antioxidant extract, minimal amounts of sodium hydroxide were used. It may be that the quantities were insufficient to saturate the unbound PVPP before displacing phenolic compounds leading to their recovery and that some phenolic compounds remained bound. A more detailed investigation into binding and elution of specific phenolics was warranted and is described in section 3.2.2.5.

Radical scavenging activities of the extracts were also very low as shown in Table 3.10. AW2 exhibited the lowest scavenging capacity of 0.65% while SW had the highest value of 32.71%. This trend was expected because of the low extraction efficiencies of the technique.

Figure 3.25 shows the distribution of the phenolic compounds during PVPP extraction. In all cases, most of the phenolic compounds remained in the stripped wastewater, which is the

residual that is obtained after extracting and filtering of PVPP bound with phenolic compounds.

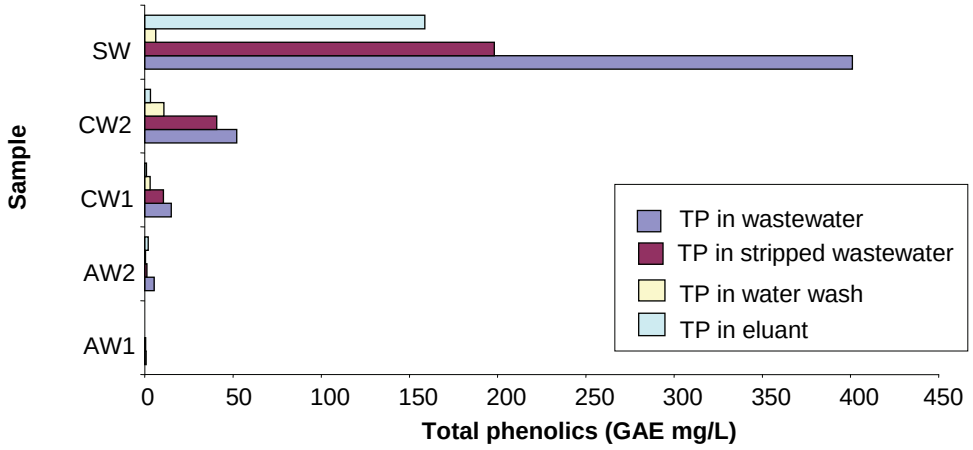


Figure 3.25: Comparison of the distribution of phenolic compounds during PVPP extraction

It was also observed that the sum of the phenolic compounds in the eluant, stripped and water wash do not add up to the total phenolics content of the wastewater. This suggests that some of the phenolic compounds remained bound to the PVPP and were not eluted during the recovery stage. It is possible that low extraction efficiencies could be due to the short exposure time (10 minutes) of the PVPP to the wastewaters. The kinetics of PVPP binding was therefore investigated.

3.2.2.3 Adsorption kinetics

The adsorption kinetics of PVPP were determined and compared to those of Amberlite XAD4 and activated carbon. Synthetic wastewater was prepared for the study of adsorption kinetics and gallic acid was used as the model phenolic at a concentration of 100 mg/l. Adsorption results are given in Tables 3.11 and 3.12 and Figure 3.26

Table 3.11: Adsorption kinetics of a solution of gallic acid treated using Amberlite and PVPP

	Gallic acid concentration in mg/l			
	Amberlite		PVPP	
Time/min	1 g/100 ml	2 g/100 ml	1 g/100 ml	2 g/100 ml
0	94.8	95.1	92.9	97.1
30	52.6	41.6	23	19.5
60	44.9	37.7	21.9	18.4
380	31.7	33.0	22.7	19.1
1460	26.5	30.8	23	19.4
1780	25.7	31.4	23.1	19.5
2100	25.9	31.6	22.9	19.3

Table 3.12: Adsorption kinetics of a solution of gallic acid treated using 1 g/100 ml activated carbon

Time/min	Gallic acid concentration in mg/l
0	99.5
30	49.5
90	37.8
264	14.8
330	15.5
1500	14.7

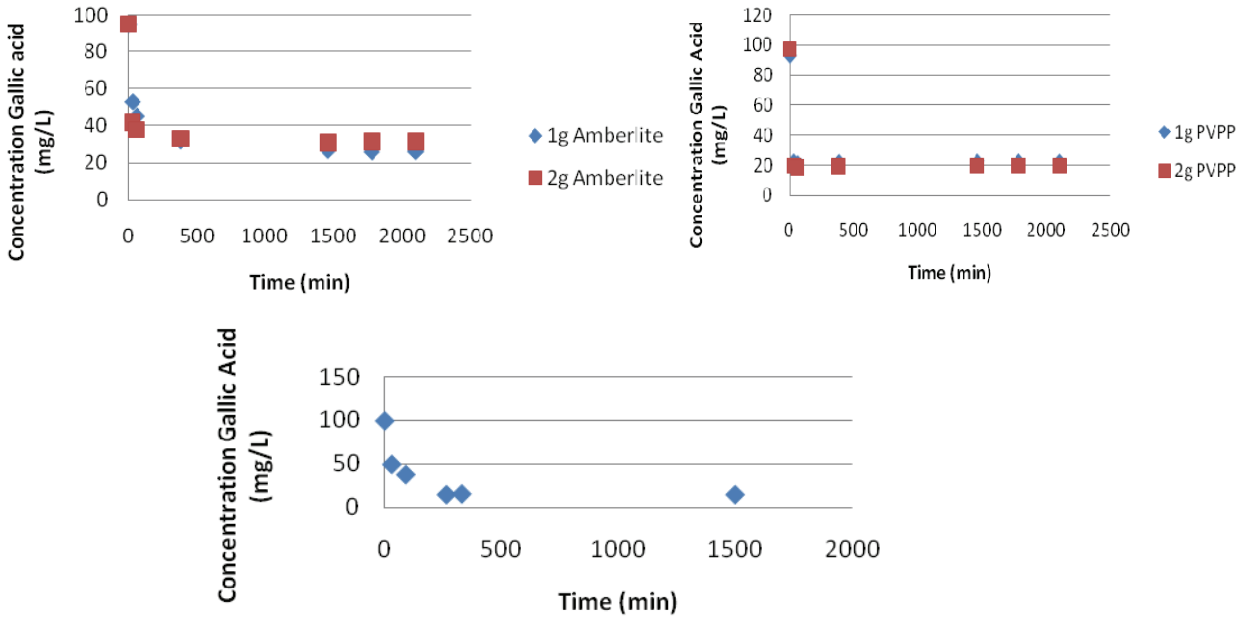


Figure 3.26: Adsorption of gallic acid onto adsorbent over time, Amberlite XAD4 (A), PVPP (B), activated carbon (C).

Using Amberlite® resin as the adsorbent, it was found that there was a large decrease in the gallic acid concentration within the first 60 minutes. The concentration then decreased slightly over the next 5 hours after which, it was found that the concentration of gallic acid in the solution was stable, indicating that adsorption equilibrium was reached. When the concentration of Amberlite® resin was 2 g per 100 ml, adsorption proceeded at a more rapid rate than for 1 g/100 ml but equilibrium was reached in both cases.

Using PVPP as the adsorbent resulted in a large decrease in the gallic acid concentration within the first 30 minutes. After 30 minutes it was found that the concentration of gallic acid in the solution was stable and adsorption equilibrium was reached. Adsorption of gallic acid onto PVPP is rapid and it is unlikely that contact time was a factor in the relatively low adsorption of phenolics from real wastewaters by PVPP.

Activated carbon resulted in a gradual decrease in the gallic acid concentration over the first 4 hours. After 4 hours, the concentration of gallic acid in the solution stabilised as adsorption equilibrium was reached.

3.2.2.4 Effect of concentration on adsorption

The effect of concentration of gallic acid (in the range 0-100 mg/l) on adsorption was tested and results are shown in Tables 3.13 and 3.14 and Figure 3.27

Table 3.13: Concentration of gallic acid in solution before and after 24 hour adsorption using Amberlite, PVPP or activated carbon

Initial concentration in mg/l	Final concentration in mg/l		
	Amberlite	PVPP	Activated carbon
0	0	0	0
10	1.1	4.4	1.6
20	4.5	7.3	0.4
40	8.6	8.03	0.4
60	17.5	12.5	1.0
100	18.7	17.2	2.4

Table 3.14: Percentage gallic acid adsorbed by Amberlite, PVPP or activated carbon for different initial gallic acid concentrations

Initial concentration in mg/l	Amberlite	PVPP	Activated carbon
0	0	0	0
10	88.8	56.5	83.8
20	77.4	63.5	97.9
40	78.6	79.9	99.1
60	70.9	79.1	98.4
100	81.3	82.8	97.6

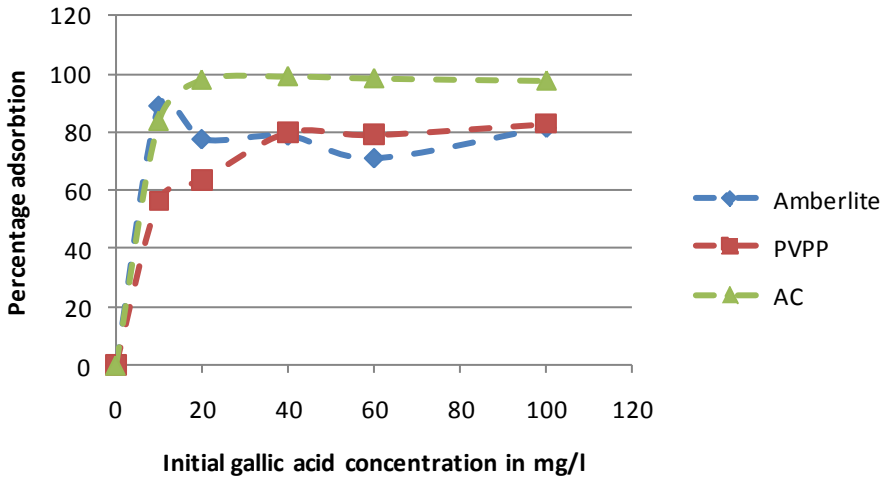


Figure 3.27: Percentage gallic acid adsorption by different adsorbents at different initial gallic acid concentrations

Using Amberlite® as an adsorbent, it was found that the same percentage gallic acid was adsorbed for low and high gallic acid concentrations. This means that no concentration step would be needed to concentrate the phenolics in the solution before adsorption, adsorption would be equally effective at low concentration. The average percent gallic acid adsorbed was found to be 80%. Using PVPP as an adsorbent, lower percentages of gallic acid were adsorbed for dilute solutions. As the concentration of gallic acid in the solution increased, so did the percentage adsorbed up to a value of 80% from 40 mg/l gallic acid. Both AW1 and CW1 had total phenolics concentrations of under 20 mg/l GAE (Table 3.1) and this would have contributed to the poor extraction efficiency from these wastewaters (Table 3.10). For efficient extraction of gallic acid from these wastewaters, a concentration step would be needed. Attention should also be paid to the pH of the wastewater as a high pH, such as in CW2, will result in low adsorption. For optimal adsorption, the pH of the wastewater should be adjusted either through addition of acid or through mixing wastewater streams to dilute the effect of alkaline peel wash wastewater.

When activated carbon was used as an adsorbent, it was found that all gallic acid was adsorbed for initial concentrations above 20 mg/l and 80% was adsorbed at 10 mg/l gallic acid. A concentration step of even 2 fold would improve the extraction of phenolics in all but the most dilute wastewater solutions. Activated carbon is the most effective adsorbent of those considered, effectively removing all phenolics in the solution.

3.2.2.5 Elution of phenolics using ethanol and ethyl acetate

If an antioxidant extract is to be included in a food or cosmetic preparation, the nature of the eluant used is important. Ethanol and ethyl acetate would be preferable to sodium hydroxide and were therefore tested. Instead of using real wastewaters with undefined concentrations of a wide range of phenolics which may have synergistic adsorption effects, a synthetic wastewater was prepared using three phenolics previously identified in the wastewater samples (Table 3.4). The synthetic wastewater contained 100 mg/l gallic acid, 8 mg/l catechin and 10 mg/l chlorogenic acid. Sugars would be present in real wastewaters and may affect adsorption and elution. Glucose and fructose were therefore added at concentrations similar to those determined by HPLC analysis of CW1: 60 mg/l glucose and 150 mg/l fructose (Table 3.20). A 10 × concentrated synthetic wastewater was also prepared to test the effect of concentrating the wastewater.

The adsorption of each phenolic compound to PVPP and AC were determined and the results are shown in Table 3.15. The adsorbents were washed with 10 ml deionised water and the % adsorption was calculated as the % phenolics not recovered in the filtrate or water

wash. Desorption efficiency using ethanol and ethyl acetate was then determined and the results are shown in Table 3.16 and Figures 3.28 to 3.31.

Table 3.15: Adsorption by PVPP and activated carbon (AC) of three phenolic compounds from dilute and concentrated synthetic wastewater prior to desorption with ethanol and ethyl acetate.

				Phenolic/mg		
Absorbent	**Eluant**	**Dilute/conc**		**Gallic acid**	**Catechin**	**Chlorogenic acid**
PVPP	Ethanol	Dilute	Initial	5.25	0.60	0.26
			Filtrate	1.29	0	0.27
			Wash	0.18	0	0.02
			%Adsorbed	72.18	100	0
	Ethanol	Conc	Initial	54.79	10.44	4.54
			Filtrate	22.90	0.16	1.79
			Wash	5.01	0	0.63
			%Adsorbed	49.86	98.43	46.56
	Ethyl	Dilute	Initial	5.25	0.60	0.26
	acetate		Filtrate	1.40	0.56	0.25
			Wash	0.23	0	0.03
			%Adsorbed	68.96	5.67	0
	Ethyl	Conc	Initial	54.79	10.44	4.54
	acetate		Filtrate	24.75	7.92	3.44
			Wash	5.51	0	0.64
			%Adsorbed	44.77	24.09	9.98
AC	Ethanol	Dilute	Initial	5.69	0.16	0.28
			Filtrate	0.51	0	0.01
			Wash	0	0	0
			%Adsorbed	90.97	100	95.08
	Ethanol	Conc	Initial	54.88	3.13	4.05
			Filtrate	35.53	0.88	2.05
			Wash	0.5	0	0.01
			%Adsorbed	34.35	71.97	49.31
	Ethyl	Dilute	Initial	5.69	0.16	0.28
	acetate		Filtrate	0.47	0	0.01
			Wash	0	0	0
			%Adsorbed	91.69	100	94.62
	Ethyl	Conc	Initial	54.88	3.13	4.05
	acetate		Filtrate	32.19	0.57	1.66
			Wash	0.49	0.01	0.01
			%Adsorbed	40.46	81.61	58.94

A large portion of the gallic acid from the dilute synthetic wastewater was adsorbed by both PVPP and AC, although AC adsorbed more (91-92%) than PVPP (69-72%). A smaller percentage of the gallic acid from the concentrated wastewater was absorbed by PVPP (45-50%) and AC (34-41%), which is probably due to the saturation of the adsorbents.

There was a large variation in the amount of catechin absorbed by PVPP from both the dilute and concentrated samples, which was unexpected. The AC successfully absorbed all of the catechin from the dilute wastewater and between 72 and 82% of the catechin from the concentrated wastewater, indicating that AC was a good absorbent for this phenolic compound.

PVPP was not a good absorbent for chlorogenic acid, adsorbing no chlorogenic acid in the dilute wastewater. The concentrated wastewater showed absorption of between 10 and 47% after 2 washes of 5 ml water each but the second wash still had a concentration of 2.25 g/l chlorogenic acid, showing that the adsorption was weak and that the chlorogenic acid was being removed steadily by the water. Andersen and Sowers (1968) reported that chlorogenic acid adsorption to PVPP required higher ratios of solid to phenolic. When they used 25 g PVPP per litre of chlorogenic acid solution (10 mg/l), 79% of the chlorogenic acid was adsorbed and 97% adsorption was only achieved using 150 g PVPP per litre of solution (Anderson and Sowers, 1968). In this experiment, only 5 g PVPP was used per litre of solution and other phenolics were included which bound preferentially to this PVPP. It is not unexpected to discover that under these conditions, the chlorogenic acid did not bind to the PVPP. AC absorbed almost of the chlorogenic acid in the dilute wastewater (95%), and 50 to 59% of the chlorogenic acid in the concentrated wastewater, and so was a better absorbent for this phenolic compound than PVPP.

Table 3.16: Percent desorption of the phenolic compounds that adsorbed to PVPP and activated carbon (AC) from dilute and concentrated synthetic wastewater, using ethanol and ethyl acetate.

Absorbent	Eluant	Dilute /Conc	Aliquot no.	Gallic acid	Catechin	Chlorogenic acid
PVPP	Ethanol	Dilute	1	0.57	0.28	0
			2	0.59	0	0
			3	0	0	0
			4	0.51	0	0
			5	0.63	0.15	0
		Conc	1	2.85	2.35	8.07
			2	3.31	0.07	0.64
			3	2.57	0.86	0.84
			4	2.40	0	0.07
			5	2.52	0.57	0
	Ethyl acetate	Dilute	1	2.78	0.31	0
			2	1.07	0	0
			3	1.25	0	0
			4	1.08	0	0
			5	0.97	0	0
		Conc	1	3.59	0	17.55
			2	5.90	0	10.58
			3	4.86	0	8.28
			4	4.12	0	7.15
			5	3.59	0	0.04
AC	Ethanol	Dilute	1	0	4.40	0
			2	0.01	1.30	0.04
			3	0.01	0.12	0
			4	0.01	0	0
		Conc	1	0.92	1.87	0.50
			2	2.54	6.26	1.88
			3	3.20	4.18	1.08
			4	2.78	1.97	0.42
	Ethyl acetate	Dilute	1	0	3.55	0
			2	0.01	5.60	0
			3	0	5.65	0
			4	0.01	2.37	0
		Conc	1	0.49	1.29	0.04
			2	2.20	6.07	0
			3	3.44	6.59	0
			4	3.57	5.73	0

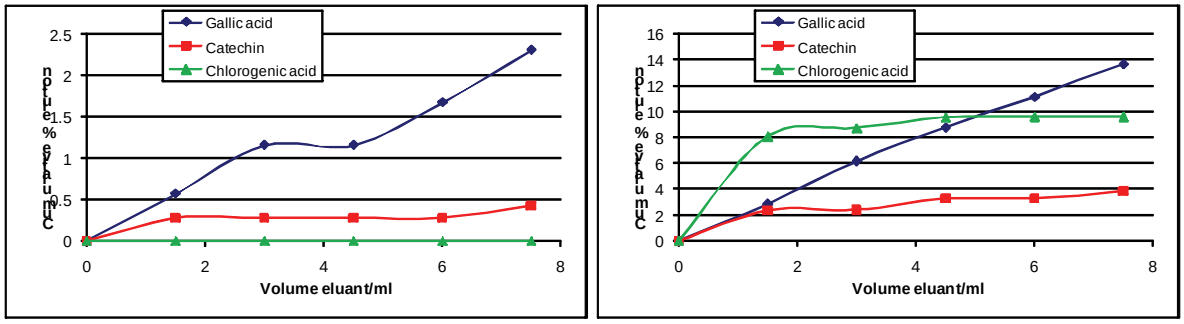


Figure 3.28: Elution of phenolic solution from PVPP using ethanol: dilute wastewater (A), concentrated wastewater (B)

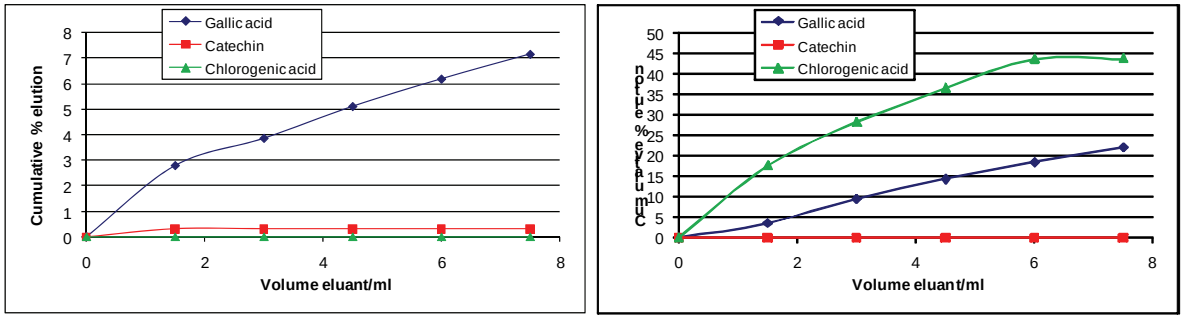
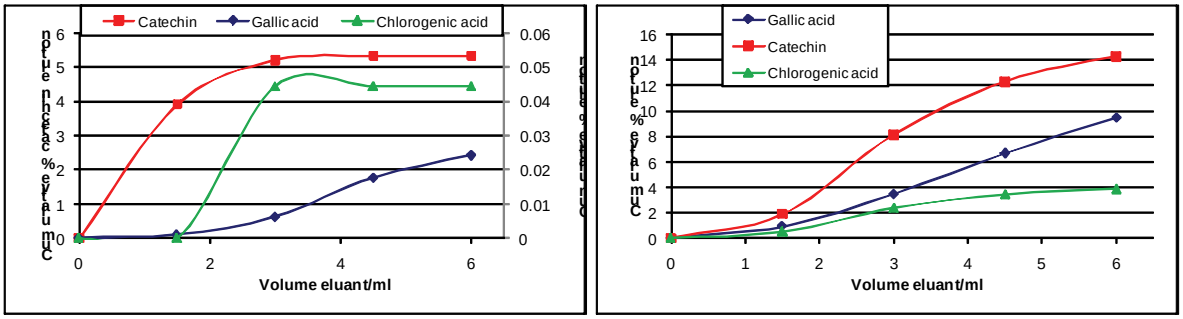


Figure 3.29: Elution of phenolic solution from PVPP using ethyl acetate: dilute wastewater (A), concentrated wastewater (B)

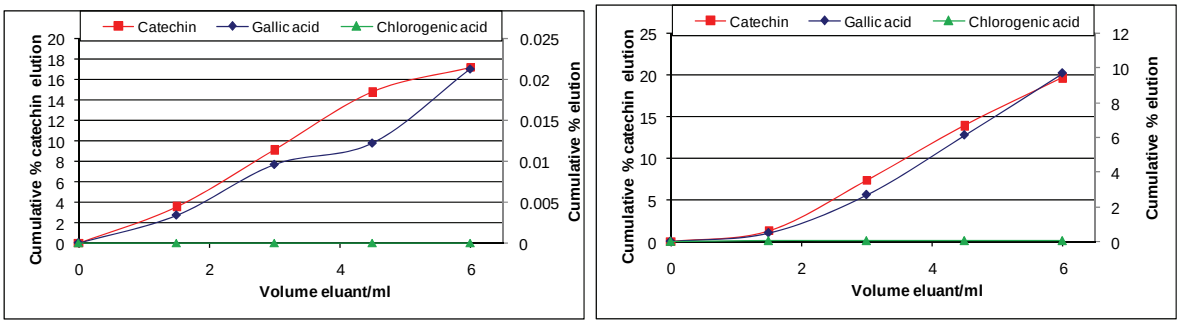
Ethanol eluted 2.5% (dilute) and 14% (concentrated) of the gallic acid bound to PVPP. Only trace amounts of catechin were eluted from the PVPP treated with the dilute phenolic solution and up to 4% was eluted from the column treated with the concentrated solution. Chlorogenic acid did not associate strongly with PVPP and was partially removed using water washes. Ethanol quickly removed 10% of the remaining chlorogenic acid.

Similarly, ethyl acetate eluted 7% (dilute solution) and 22% (concentrated solution) of bound gallic acid and trace catechin. Ethyl acetate removed 44% of the chlorogenic acid bound to the column treated with the concentrated solution but, as before, the association of chlorogenic acid with PVPP was weak which aided elution.



A B

Figure 3.30: Elution of phenolic solution from activated carbon using ethanol: dilute wastewater (A), concentrated wastewater (B)



A B

Figure 3.31: Elution of phenolic solution from activated carbon using ethyl acetate: dilute wastewater (A), concentrated wastewater (B)

Ethanol eluted 5% (dilute) and 14% (concentrated) of bound catechin but only 4% chlorogenic acid (concentrated) and 9.5% gallic acid (concentrated) from activated carbon. Ethyl acetate eluted 17% (dilute) and 19% (concentrated) catechin from activated carbon and again, low amounts of gallic acid (10% from concentrated solution) and no chlorogenic acid.

In no case, was the elution complete. For elution of gallic acid from PVPP with ethanol, the cumulative percentage elution curve was still increasing after 7.5 ml and it is likely that more gallic acid would have been eluted. This identifies ethanol as a possible eluant although not an ideal one as it would be necessary to use a relatively large volume of ethanol to recover the phenolics, reducing the efficiency of the process. An ideal eluant would elute all of the chosen phenolics in a small volume of solvent. Ethyl acetate showed more promise by eluting 7 and 22% of the gallic acid (cumulative % still increasing after 7.5 ml) and almost 45% of the chlorogenic acid.

Activated carbon formed a stronger association with the phenolics than PVPP. When the dilute solution was used, elution was very difficult but with a concentrated solution, a portion of the phenolics were less tightly bound and eluted with ethanol. Cumulated percentage elution was still increasing after 6 ml.

Catechin and gallic acid elution was still increasing after elution with 6 ml ethyl acetate showing that ethyl acetate is also a possible solvent for the extraction of these phenolics from activated carbon.

3.2.2.6 Summary of the adsorption and elution of phenolic compounds from fruit processing wastewaters using solid adsorbents

Of the solid adsorbents tested, PVPP provided the most rapid adsorption with equilibrium reached within 30 minutes. Amberlite XAD4 reached equilibrium within 60 minutes whereas activated carbon required between 1 and 4 hours. PVPP and Amberlite XAD4 would be suitable for either continuous use in a column or batch processes in which components are mixed and allowed to settle. Activated carbon would achieve better results in a batch. A continuous column could be used if the residence time was greater than 1 hour or if recycling was used.

Amberlite XAD4 adsorbed 80% of available gallic acid over the full concentration range of 10-100 mg/l whereas PVPP adsorbed 80% gallic acid only in the concentration range of 40-100 mg/l and lower amounts in more dilute solutions. Activated carbon adsorbed all gallic acid in the range of 20-100 mg/l and 80% at 10 mg/l. For optimal adsorption, it would therefore be best to use activated carbon. The use of a concentration step for dilute wastewaters, to increase the concentration of phenolics from approximately 10 mg/l to 30 mg/l or higher, would give better results. In this experiment, 1 g of adsorbent was used for 100 ml of solution giving a maximum adsorption of 10 mg gallic acid/g adsorbent. At this ratio, no saturation of the adsorbent was observed.

Saturation was observed when gallic acid levels were raised to 275 mg/g adsorbent. Here, only 34-41% of available gallic acid was adsorbed by activated carbon and 45-50% by PVPP. PVPP has a greater capacity for gallic acid and could be used to extract gallic acid from larger volumes of wastewater before being removed for harvesting phenolics. Of the other phenolics tested, PVPP adsorbed variable amounts of catechin and no chlorogenic acid whereas activated carbon adsorbed both, making it the more diverse adsorbent.

Both PVPP and activated carbon are used in the fruit processing industry as adsorbents for phenolics and the results for adsorption clearly demonstrate their effectiveness in this application. It is in the elution of phenolics from the adsorbents that opportunities exist though. Currently, activated carbon is not re-used and phenolic-saturated activated carbon is a waste product generated by fruit processing industries. PVPP can be regenerated through use of a sodium hydroxide wash and this does allow reuse of the adsorbent but the process generates a sodium hydroxide wastestream which must be treated. Both processes would be made more sustainable if the phenolics could be eluted from the adsorbents using solvents that are not environmentally harmful. If the phenolic solution could additionally then be used in food or cosmetic applications, a wastestream could be converted to an income stream.

Elution of bound phenolics using ethanol was possible with up to 14% bound gallic acid being eluted from PVPP after the equivalent of 37.5 ml solvent per g adsorbent was applied. 14% catechin and 9.5% gallic acid were eluted from activated carbon after the application of 30 ml solvent/g adsorbent. Cumulative elution percentages were still increasing at this point and it is probable that more phenolics would have been eluted in subsequent aliquots.

Ethyl acetate was more efficient, allowing elution of 22% bound gallic acid from PVPP after use of 37.5 ml solvent/g adsorbent and 20% catechin and 10% gallic acid from activated carbon after the equivalent of 30 ml solvent/g adsorbent. Again, cumulative elution curves were increasing and it is probably that these percentages would rise with application of more solvent.

Use of a relatively mild solvent to remove phenolics is advantageous because the phenolic solution obtained may then be used in antioxidant applications. The phenolic solution can be concentrated through evaporation of the solvents, an option not available when using non-volatile eluants such as the sodium hydroxide solution used previously. For subsequent use, ethanol would be the recommended eluant of those tested, despite being somewhat less efficient, as it is least toxic and is already used as a solvent in medicinal applications and cosmetic products.

Of the adsorbents, Amberlite XAD4 would not be recommended because it adsorbed the least gallic acid. PVPP was the best adsorbent in terms of rapid adsorption, allowing for either continuous or batch applications, as well as being able to absorb higher percentages of phenolics from concentrated solutions. Higher percentages of bound phenolics could also be eluted from this adsorbent. Activated carbon had the advantage of being able to adsorb

a larger range of phenolics with slightly lower capacity and less efficient elution but it has a clear cost advantage. The choice of adsorbent would depend on the constraints of the process (whether continuous or batch was required) and the financial implications.

3.2.3 Supported liquid membrane extraction

The low molecular weight phenolic antioxidants that occur in agri-industrial wastewaters have potential as value added compounds. Gallic acid in particular, was found in silage water in concentrations that may enable its extraction. The focus of work described in this section is a membrane assisted solvent extraction process for the recovery of low molecular weight phenolic compounds, particularly gallic acid, from SW. This technique is based on a principle similar to that of liquid-liquid extraction but the aqueous and organic phases are not mixed but are separated by a hydrophobic membrane, which is non permeable to liquids at low pressures. The presence of a membrane separating the aqueous and organic phases does result in slower mass transfer rates because of the additional mass transfer resistance offered by the pores of the membrane (González-Muñoz et al., 2003) but this can be alleviated by increasing the surface area per unit volume by using hollow fibre modules such as those used in this work.

Experiments to determine the distribution coefficiencies of ethyl acetate, toluene and MIBK were performed and the results are shown in Table 3.17. 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 solute that can be extracted under normal conditions. Amyl alcohol was also considered as a possible solvent but amyl alcohol has a boiling point of 149.1^{o}C at atmospheric pressure and requires a vacuum of 11 mbar to lower that boiling point to 40^{o}C (Buchi Rotovapor documentation available at http://www.buchi.com). By comparison, ethyl acetate boils in a less stringent vacuum of 240 mbar at 40^{o}C. Removing amyl alcohol from the extracts would require use of elevated temperatures but since phenolic compounds are sensitive to high temperatures, this would have led to loss of antioxidant activity.

Table 3.17: Distribution coefficiencies of various organic solvents

Organic solvent	Distribution coefficient
Ethyl acetate	0.5569
Toluene	0.6556
MIBK	0.6014

Distribution coefficiencies of all the studied solvents show that reasonable quantities of phenolic compounds can be extracted. Toluene had the highest distribution coefficient so it was used for the extraction of phenolic compounds in SW. Gallic acid is the major phenolic compound in SW and it was monitored at various time intervals in the feed and solvent streams as illustrated in Figures 3.32 and 3.33.

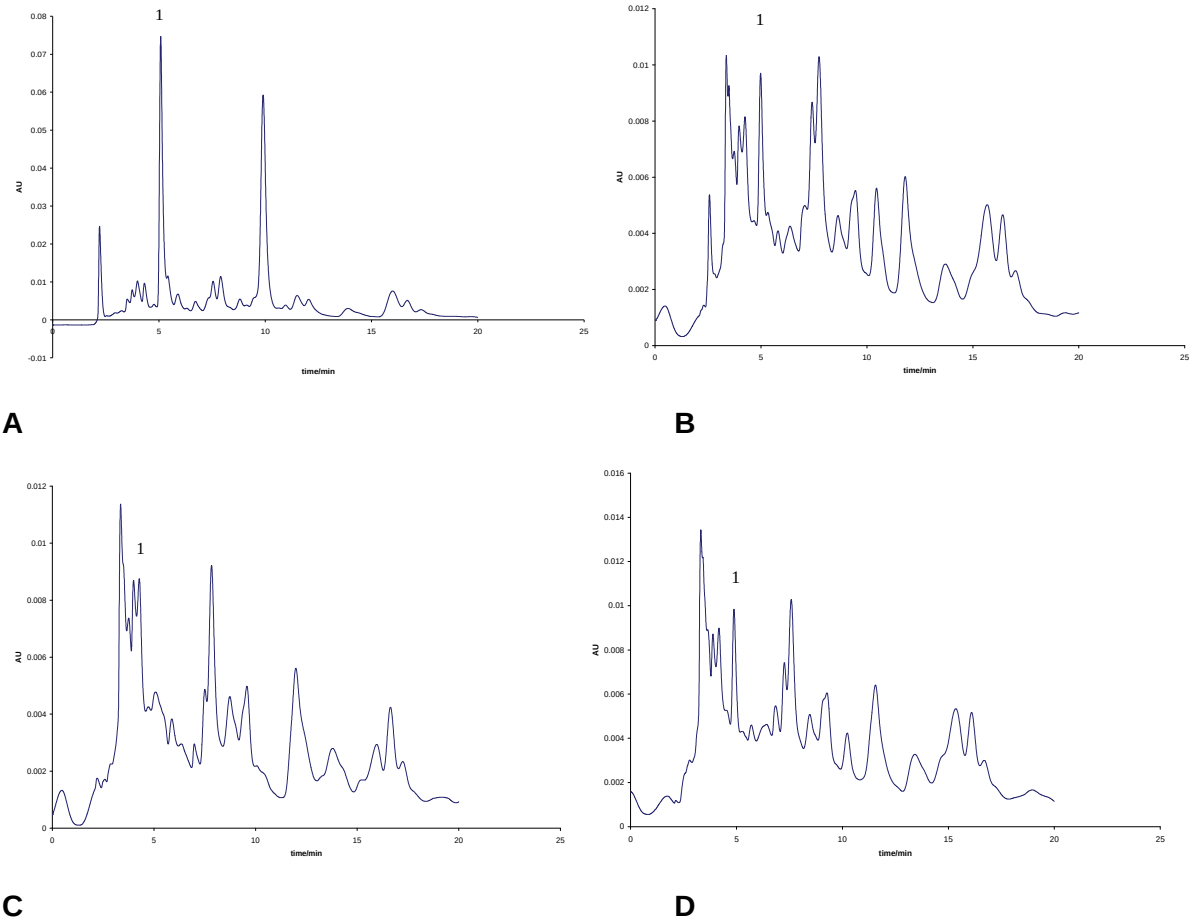


Figure 3.32: HPLC chromatogram of Silage water in the feed stream at 0 (A), 30 (B), 60 (C) and 90 (D) minutes; 1 – gallic acid**.**

Figure 3.32 shows the chromatograms of SW during extraction at 30, 60 and 90 minutes. There was a significant decrease in the concentration of gallic acid in the first 30 minutes followed by a general decrease in gallic acid concentration until 90 minutes. Equilibrium is reached within this second period indicating a relatively rapid extraction.

Figure 3.33 illustrates the profiles of phenolics from SW in the solvent stream. The concentration of gallic acid did not change significantly after 30 minutes, suggesting the system had reached a state of equilibrium. This is consistent with profiles of the feed stream.

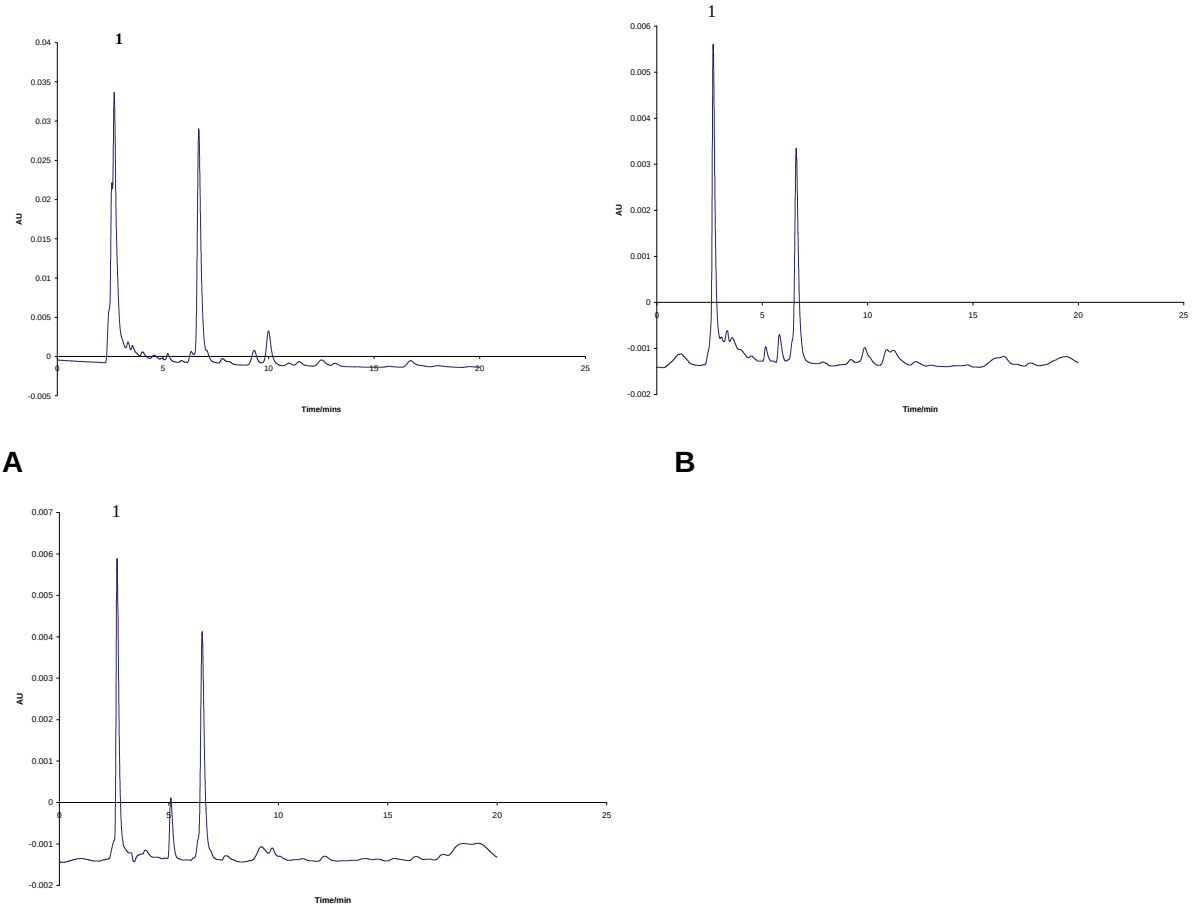


Figure 3.33: HPLC chromatogram of Silage water at 30 (A), 60 (B) and 90 (C) minutes in the solvent stream

Concentrations of gallic acid during the course of the extraction are shown in Table 3.18. The concentration in the aqueous phase decreased from 101.4 mg/L at the start of the extraction, to 13.19 mg/L after 30 minutes and further to 11.41 mg/L after 90 minutes. 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 3.18. 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.

Table 3.18: Concentration of gallic acid in the feed and solvent streams at various time 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
30	13.19	7.66
60	11.85	7.98
90	11.41	45.23

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%. When hydroxytyrosol was extracted from olive mill wastewaters using this system, the extraction efficiency was 64% (Garcin, 2005). A direct comparison cannot be made since the composition of the wastewaters is different and while gallic acid and hydroxytyrosol have similar chemical structures, they will not necessarily behave identically during extraction. The SLM technique does show promise however and with further optimisation, higher efficiencies may be possible.

Gonzalez-Munoz et al. (2003) found that since the membrane used was hydrophobic, it was necessary to have a slight overpressure of the aqueous phase (60-80 kPa) to stabilise the interface in the pores so as to avoid bulk mixing of the two phases. However, in this work, pressures of 10 to 30 kPa were used and it is likely that this led to bulk mixing of the SW sample and toluene. Higher pressures may improve the transfer of gallic acid. Temperature also has a direct effect on the physico-chemical properties of the system, that is; density, viscosity, interfacial tension and mutual solubility. Temperature has no effect on the distribution coefficient but does have an indirect influence on the overall transfer of solute. Gonzalez-Munoz et al., (2003) observed an increase of 60% in overall mass transfer coefficient as temperature increased to 40^{o}C. In this study, extraction was done at room temperature and this may not necessarily be the most suitable temperature to ensure the maximum extraction of phenolic compounds.

Consideration of the pKa values of the compounds of interest may also improve extraction efficiencies. Gallic acid has a carbonyl group with a pKa of 4.5 and three hydroxyl groups with pKa values of 10 or higher. Silage water, the aqueous phase, has a pH of 3.9 and at this pH, gallic acid will be neutral and able to cross the membrane. By increasing the pH of

the acceptor phase (toluene) to neutral or basic pH the gallic acid becomes ionised in the acceptor phase and is effectively trapped (Jung et al., 2002). The concentration gradient is thereby maintained and in this way, extraction efficiencies could be increased above the theoretical maximum implied by the distribution coefficient (Jönsson and Mathiasson, 1999).

In terms of process design, it must be remembered that the removal of organic solvent for the recovery of solutes by vacuum distillation is energy-intensive and is much more likely to be a rate-limiting factor, compared to the extraction process.

3.2.4 Supercritical fluid extraction

The design of supercritical processes requires knowledge of the solute solubility in the supercritical phase and the ability to model and predict it efficiently (Chafer et al., 2007). The present work investigated the solubility of gallic acid in supercritical CO_2 with ethanol as a 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 at various temperatures and a constant pressure of 180 bars. Different volumes of ethanol were used in order to observe the influence of the amount of co-solvent added on gallic acid solubility.

The experiments are detailed in Table 3.19 and the results are shown in Figure 3.34.

Table 3.19: Comparative efficiencies after supercritical fluid extraction under different conditions

Trial	Temperature (^oC)	Volume of ethanol used (ml)	Time (hours)	Efficiency
1	40	10	2	1.85%
2	25	10	2	4.02%
3	25	20	2	5.20%
4	25	20	3	6.09%

The solubility and extraction of gallic acid increased with a decrease in temperature, from 1.85% in trial 1 to 4.02% in trial 2 when the temperature was changed from 40^{o}C to 25^{o}C. This data corresponds with results obtained by Chafer et al., (2007) who studied the solubility of natural gallic acid in supercritical CO_2 with ethanol as a co-solvent. Low solubility 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. The effect of temperature on solute solubility in supercritical CO_2 is complex and cross-over phenomena have been observed. At pressures above the cross-over pressure, the solubility decreases with increasing temperature.

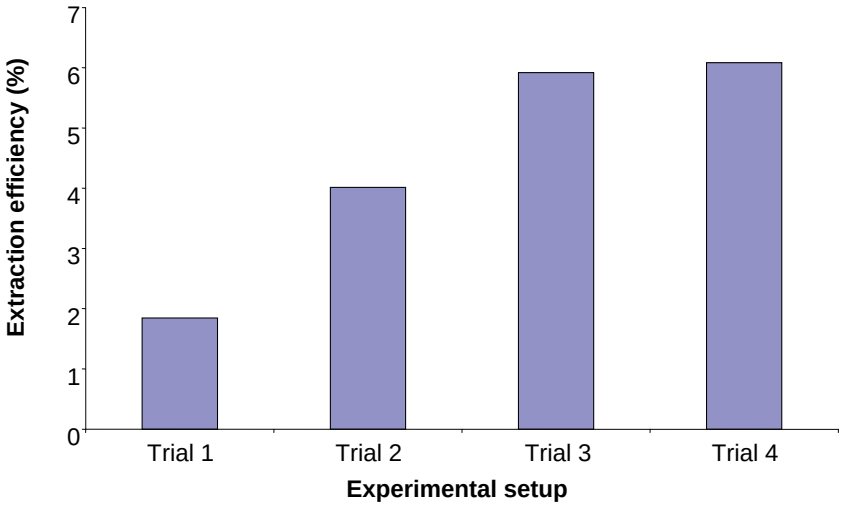


Figure 3.34: Extraction efficiencies of gallic acid in CO_2 and ethanol

The effect of the co-solvent was studied in trials 2 and 3 when the volume of ethanol was increased from 10 ml to 20 ml, respectively. Trial 3 showed an increase in extraction efficiency from 4.02% in experiment 2 to 5.2%. Carbon dioxide, a low polarity solvent, is not effective in extracting more polar compounds such as gallic acid. Modifiers are highly polar compounds like ethanol that, when added, can produce substantial changes of the solvent properties of supercritical CO_2. Thus, increases in extraction with increased volumes of ethanol were expected. The significance of the co-solvent on solubility of gallic acid is noted in a study done by Cortesi et al. (1999) where no co-solvent was added. The solubility of gallic acid was not reported because of the very low solubility data which was close to experimental error.

The effect of the extraction time was studied in trial 4 and compared with trial 3. The extraction time was increased from 2 hours in trial 3 to 3 hours in trial 4. However, only a slight increase from 5.2% in experiment 3 to 6.09% in experiment 4 was observed indicating that extraction time does 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 this investigation could not go beyond pressures of 200 bars, thus the effect of pressure could not be determined. Overall, the greatest factor resulting in low extraction is most likely the high polarity of gallic acid with is to be dissolved in a non-polar solvent such as CO_2. Extraction of less polar compounds should be more feasible.

3.2.5 Comparison of extraction techniques

The aim of the work done in this study was to obtain food or cosmetic additives in the form of phenolic extracts from agri-industrial wastewaters presumed to have high concentrations of naturally occurring phenolic compounds. Various extraction techniques were employed to extract phenolic compounds from five different wastewaters. The antioxidant activity of the extracted products was then assessed using the DPPH assay. This section makes a comparison of the extraction techniques employed and assesses the potential of the extracts as food additives.

Various factors need to be considered when determining the feasibility and potential of obtaining phenolic antioxidants from agri-industrial wastewaters at a commercial scale. The nature in which the final product will be marketed to the consumer determines the preceding steps and costs that will be involved. Phenolic compounds obtained through extraction from agri-industrial wastewaters can be utilised in two ways. Firstly, crude extracts containing total phenolics can be directly incorporated into food or pharmaceutical products. Commercially available extracts include green tea herbal extracts and dietary supplements, for example, apple cider vinegar capsules. Secondly, the crude extract containing total phenolics can be further purified and individual phenolic compounds separated thereby producing pure phenolic compounds. These products are more expensive compared to the crude extracts because of the extra separation and purification steps involved. Thus, these factors, and others, such as labour costs and costs of equipment set-up and maintenance, need to be taken into consideration when determining the most suitable extraction process.

Table 3.20 gives a summary of the extraction efficiencies obtained for the various techniques employed. It also gives cost estimates of the techniques at a large scale. Typically, phenolic compounds are extracted from foods or wastewaters by solvent extraction. In this research, the extraction efficiencies ranged from 20% to 48% using ethyl acetate and from 0.5% to 40% using hexane. Nevertheless, solvent extraction consumes large volumes of organic solvents that are hazardous for use and are expensive. According to the work done

here, for every 1000 L of wastewater, 3000 L of organic solvent will be required to obtain an extract of phenolic compounds. There are very clear implications here regarding sustainability. At a large scale, residues of these organic solvents would require costly disposal strategies (Abad-Garcia et al., 2007). Extractions would be followed by a time consuming purification step, purification being essential for the complete removal of all of the organic solvent which would be harmful when consumed, even in trace amounts. Thus, at large scale, this technique will not be economically feasible or sustainable and because of the presence of organic solvent in the extract, the use of extracts as food additives is not recommended.

Concern over solvent residues in extracted products has catalysed 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 consumption compared with solvent extraction (Ahn et al., 2007). In this research solid phase extraction using Sep-Pak cartridges took 15 minutes to complete, while using only 10 ml of methanol for elution. The low solvent volumes used makes this method attractive for obtaining extracts that can be applied in foods. Also, solid phase extraction resulted in better extraction when compared to solvent extraction (ranging from 44.1% to 56.9%). However, cartridge-based solid phase extraction is generally recognised as a pre concentration technique. Most polar compounds, including phenolic compounds have a low affinity for most 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 as the sorbents are costly. During solid phase extraction with Sep-Pak cartridges, pH of the wastewaters had to be adjusted between 2 and 7 to facilitate elution of neutral and acidic phenolic compounds. This means an additional neutralisation stage will be necessary if extracts are to be considered for application as food additives or nutraceuticals. Because of poor retention abilities for polar compounds, C_{18} cartridges are not the most suitable cartridges to extract trace levels of phenolic compounds that occur in wastewaters.

PVPP was found to adsorb phenolics from synthetic solutions, adsorbing up to 83% of the gallic acid, 100% of the catechin and 47% of the chlorogenic acid. Elution of bound phenolics was found to be possible with ethanol, ethyl acetate or dilute sodium hydroxide. The extraction efficiency for specified phenolics from synthetic wastewaters was higher using ethyl acetate, reaching a maximum of 53.8% for gallic acid, 23.4% for catechin and 68.9% for chlorogenic acid. Ethanol was also able to elute phenolics from PVPP treated

with synthetic wastewater. Maximum extraction efficiencies were 36.6% for gallic acid, 2.3% for catechin and 12.36% for chlorogenic acid.

Sodium hydroxide extraction of phenolics from PVPP treated with real wastewaters ranged from 0% to 39.58% for wastewater samples. Adsorption is pH dependent and, as shown in the synthetic wastewaters, is also selective with some phenolics having a higher affinity for PVPP than others. Extraction efficiencies from real wastewaters are relatively low compared to solvent extraction and C18 solid phase extraction. However, extraction with PVPP has great potential for obtaining phenolic antioxidants for a number of reasons.

Firstly, the beverage-processing industries already use PVPP for the removal of unwanted phenolic compounds that lead to haze formation and use of PVPP in wastewater treatment would therefore be a natural addition. Secondly, in normal production after phenolic compounds bind to PVPP, the main concern of beverage manufacturers is to regenerate PVPP for reuse. This currently involves the use of harsh chemicals such as sodium hydroxide. In this study, the maximum achievable recovery of total phenolics using sodium hydroxide was 39.58% but ethyl acetate and ethanol show potential as alternative solvents for desorption. Further investigation into the optimum conditions necessary for recovery of phenolics will increase the extraction efficiency. The ability to use a current wastestream as a source of value added products while enabling recycling of valuable sorbent without generation of harmful waste, should be an attractive proposition. Thirdly, extraction of phenolic compounds with PVPP is a relatively inexpensive method. In this study, only 5 g of PVPP was required for every 1 L of wastewater.

Activated carbon was also found to adsorb phenolics from synthetic solutions, adsorbing up to 99% of the gallic acid, 100% of the catechin and 95% of the chlorogenic acid. Elution of bound phenolics was found to be possible with ethanol or ethyl acetate. The extraction efficiency for gallic acid and catechin from synthetic wastewaters was higher using ethyl acetate, reaching a maximum of 20.8% for gallic acid and 91.6% for catechin. Only traces of chlorogenic acid were eluted using ethyl acetate. Ethanol was also able to elute phenolics from activated carbon treated with synthetic wastewater. Maximum extraction efficiencies were 17.2% for gallic acid, 55.1% for catechin and 10.2% for chlorogenic acid.

Extraction using SLM was used to recover phenolic compounds from SW. The recovery of gallic acid, the major phenolic compound in SW, was of particular interest. Extraction with SLM led to a recovery of 44.6% which is comparable to other extraction techniques. The unique benefit of SLM extraction is that under normal circumstances, the aqueous and

organic phases are not mixed since they are separated by a hydrophobic membrane. This implies that no additional steps are required to remove organic solvents. Organic solvent removal is a major drawback of solvent extraction and even in bulk solid phase extraction, elution is by means of solvents which will subsequently have to be removed. The SLM system design is simple and energy costs are minimised due to the use of small pumps.

Unfortunately, a major problem noted in this study was the mixing of the aqueous and organic phases and this was due to several reasons. The membrane module used had previously been used for the extraction of olive mill wastewaters that are characterised by high concentrations of phenolic compounds and total solids. This resulted in the system being clogged and the system became blocked several times during extraction. Furthermore, the use of toluene as a solvent may have affected the membrane module. Toluene itself is environmentally hazardous and its use in wastewater treatment cannot be recommended. However, if a suitable membrane module and solvent are used, SLM would be an excellent option for the recovery of phenolic compounds that can be incorporated into food preparations.

Supercritical fluid extraction was then used to determine the solubility and consequently, the extraction efficiency of gallic acid. This gave an indication of how much gallic acid could be extracted from SW and to determine if further research should be done on the use of SFE for the recovery of phenolic compounds. The extraction efficiency was only 6%. However, a direct comparison cannot be made with the other extraction techniques employed in this study since only pure gallic acid was used, not a natural mixture of different phenolics. On the other hand, considering the cost of setting up and operating a SFE system, this technique may not be economically feasible without significant improvement in extraction efficiency. 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 cost of the extracted products coupled with relatively low maintenance costs of SFE will make this the method of choice.

Table 3.20: Comparison of all extraction techniques tested for the extraction of phenolics compounds from fruit processing wastewaters

Extraction method	Sample	Extraction Efficiency (%)		Scale used	Scalability	Cost Estimate (R)	Comments
Solvent		Ethyl acetate	Hexane	100 ml wastewater + 300 ml solvent	Industrial >1000 L	R600 000-2 million (Solvents bought from a chemical supplier such as Sigma)	-average efficiency for ethyl acetate (20-48%) -low to average efficiency for hexane (0-40%) -disposal of residual organic solvents is hazardous and costly -technique is time-consuming and requires large space
	AW1	33.04	40.18				
	AW2	47.7	18.9				
	CW1	20.65	3.21				
	CW2	37.45	9.37				
	SW	20.29	0.46				
Solid phase (Sep-Pak cartridges)	AW1	52.1		20 ml per cartridge	Analytical $\leq$ 1 L	R200 000 (Promo Chrom Technologies)	-average to high efficiency (44-57%) -normally used as a small scale pre-concentration step, at large scale, quantities of sorbents required are expensive, making the process uneconomical
	AW2	51.9					
	CW1	49.5					
	CW2	44.1					
	SW	56.9					
PVPP with sodium hydroxide elution	AW1	-		5 g PVPP + 1 L wastewater	Industrial >1000 L	R20 000 (Solid sorbent bought from a chemical supplier such as Sigma)	-low to average overall efficiency (6-40%) -technique is relatively simple -cheap, PVPP can be regenerated up to 20 times -technique has great potential for use in industry
	AW2	36.49					
	CW1	6.53					
	CW2	6.18					
	SW	39.58					
PVPP with ethanol elution	Solution:	Concentrated	Dilute				
	Gallic acid	36.6	8.9				
	Catechin	0.9	2.3				
	Chlorogenic acid	12.36	11.4				
PVPP with ethyl acetate elution	Gallic acid	53.8	26.6				
	Catechin	4.4	23.4				
	Chlorogenic acid	22.5	68.7				
Activated carbon with ethanol elution	Solution:	Concentrated	Dilute	5 g activated carbon + 1 L	Industrial >1000 L	R3 000 (Solid sorbent bought	-low efficiency for gallic and chlorogenic acid (0-21%)
	Gallic acid	17.2	0.1				

Extraction method	Sample	Extraction Efficiency (%)		Scale used	Scalability	Cost Estimate (R)	Comments
	Catechin	55.1	28.4	wastewater		from a chemical supplier such as Sigma)	-high efficiency for catechin (28-92%) -technique is relatively simple -very cheap but usually not regenerated, more sustainable if regenerating with ethanol or ethyl acetate -technique has great potential for use in industry
	Chlorogenic acid	10.2	0.23				
Activated carbon with ethyl acetate elution	Gallic acid	20.8	0.1				
	Catechin	85.68	91.6				
	Chlorogenic acid	0.13	0.0				
Supported Liquid Membrane	SW	44.60		1 L wastewater + 1 L solvent	Industrial >1000 L	100 000 (Membrana)	-average efficiency (44%) -relatively inexpensive compared to traditional techniques
Supercritical fluid	Gallic acid	7.70		2 g Gallic acid + 20 ml solvent	Industrial >1000 L	800 000-1.5 million (Super Critical Fluid Technologies)	-low efficiency (8%) -initial set up costs are high - maintenance costs are low - uses no/minimal organic solvent

3.3 Fermentation of Fruit Wastewaters

The wastewaters characterised in section 3.1 (Table 3.1) had both phenolic components and residual sugar. During the extraction of phenolic compounds, residual sugar may be extracted along with the phenolics or may remain in the treated wastewater. Where the latter holds, there is potential to use these fermentable substrates for product or biomass formation.

The choice of extraction process has an effect on the subsequent potential for fermentation. Where the extraction introduces substances into the wastewater that inhibit the growth of micro-organisms, such as toxic organic solvents, fermentation will not be possible. Where solvents are not involved, concentration or dilution effects become important. The levels of fermentable carbohydrates must be high enough to sustain microbial growth. Fermentation to a value added product is also only economically feasible if that compound is produced is sufficient concentration to justify the expense of purification. The possibility of using treated wastewater as a fermentation substrate is investigated in this section.

3.3.1 Sample Analysis

Phenolic compounds were extracted using the following methods:

- Solvent extractions with ethyl acetate and hexane
- Solid phase extraction using C18 Sep-Pak cartridges
- Solid phase extraction using PVPP as a sorbent
- Supported liquid membrane extraction using toluene

Supercritical fluid extraction of gallic acid from water was also demonstrated but a synthetic wastewater was used for this procedure so no sugars were present in the wastewater after extraction.

Analysis of the wastewaters after solvent extraction and supported liquid membrane extraction showed the presence of solvent in the wastewater after treatment. Ethyl acetate is soluble up to 8 g/100 ml in water at room temperature so after solvent extraction, a concentration of 8% ethyl acetate should be expected in the aqueous phase after separation. Removal of this residual solvent was attempted by heating under reduced pressure in a rotary evaporator but even after treating for one hour, the ethyl acetate concentration was still so high that this peak masked all other peaks on the HPLC trace. Toluene and hexane are less soluble, with 470 mg/l and 13 mg/l expected in the aqueous phases respectively, but are highly toxic and inhibit microbial growth. Fermentation of wastewaters from which the phenolics had been removed through contact with solvents, either directly in solvent extraction or indirectly in supported

membrane extraction, was not possible because of the toxicity of the residual solvents in the aqueous phase.

The wastewaters treated using solid phase extraction with C18 cartridges (SPE) and PVPP were analysed and their metabolite profiles before and after phenolics extraction are shown in Table 3.21 and 3.22.

Table 3.21: Concentration of sugars (in mg/l) in wastewater solutions before and after treatment to extract phenolics

Sample	**Sugar**	**AW1**	**AW2**	**CW1**	**CW2**	**SW**
Initial (DNS)	reducing sugars	126.45	215.33	230.3	396.3	234.8
Initial (HPLC)	glucose	0	0	60.0	0	0
	fructose	0	0	151.4	0	0
After SPE	glucose	0	0	68.5	0	0
	fructose	0	0	98.5	0	0
After PVPP	glucose	0	0	96.15	0	-
	fructose	0	0	141.07	0	-
Water wash after PVPP	glucose	52.47	6.86	87.94	57.35	-
	fructose	0	0	29.99	24.55	-

Table 3.22: Concentrations of fermentations products in wastewater solutions (in µg/l)

Metabolite	Treatment	AW1	AW2	CW1	CW2	SW
Lactate	Initial	0	0	0.283	0.095	0
	After SPE	0	0	0.137	0.061	0
	After PVPP	0	0	0.085	0	-
	PVPP water wash	0	0	0.023	0	-
Formate	Initial	0	0	0	0	13
	After Isolute	0	0	0	0	182
	After PVPP	0	0	0	0	-
	PVPP water wash	0	0	0	0	-
Acetate	Initial	0.33	1.12	0.31	0.38	0
	After Isolute	0.22	0.65	0.17	0.25	0
	After PVPP	0.36	1.08	0.36	0.39	-
	PVPP water wash	0.085	0.16	0.085	0.081	-
Ethanol	Initial	0	0.022	0.167	0.011	0.0119
	After Isolute	0	0.017	0.079	0.0142	0.0332
	After PVPP	0	0.024	0.132	0.014	-
	PVPP water wash	0	0	0.027	0	-

Sugar concentrations in the wastewaters were very low with a maximum carbon load of 211.4 mg/l for CW1 and a minimum of 33 µg/l for AW1. The wastewaters are dilute and are not sterile. The presence of metabolic products such as organic acids and ethanol show that fermentation has already occurred naturally by a mixture of cultures present in the environment. This is particularly apparent in the silage water which has been completely fermented to formate and ethanol. It is likely that silage water had a relatively high sugar content initially, the level of phenolics is more than double that of the next most concentrated wastewater and the carbohydrates likewise would have been higher, but through the action of natural fungi, bacteria and yeasts, the available nutrients were utilised. Lignocellulose particles would have been digested to release sugars. These may have been partially fermented to organic acids such as acetic and formic acid and to ethanol. Other bacteria could have further metabolised the acetic acid and some of the ethanol leaving only formic acid and carbon dioxide. The importance of single culture fermentation is demonstrated here if the goal of fermentation is a saleable product. Mixed cultures are very effective at degrading complex carbon sources to reduce COD but when a specific product is desired, control must be exercised over the fermentation to allow maximum yield and minimal waste.

No remaining carbon sources were available for fermentation in the silage water and it was therefore not included in the fermentation experiments. Of the other wastewaters, despite the DNS results which showed the presence of reducing sugars, only CW1 had residual monosaccharides that could be quantified by HPLC. The total concentration of these sugars was 0.21 g/l which is very dilute. For comparison, laboratory media for the culture of micro-organisms varies in sugar content from 1-20 g/l in rich media (German Resource Centre for Biological Material – Deutsche Sammlung von Mikroorganismen und Zellkulturen [DSMZ]). Even defined media which are designed for the culture of specific micro-organisms, provide between 1 and 10 g/l glucose (German Resource Centre for Biological Material). Industrially, much higher sugar concentrations are used such as 110 g/l for ethanol production by yeasts (Gong et al, 1993); 80 g/l for butanediol production by co-culture (Yu et al., 1985) and 100 g/l ribitol for production of L-ribose using recombinant *Escherichia coli* (Woodyer et al., 2008).

Of the available carbon in the initial solutions, 65% of the sugars, acetate and ethanol were retained in the wastewater after SPE and 50% of the lactate remained. PVPP extraction removed only lactate (62%) from the wastewaters, allowing the sugars, acetate and ethanol to remain in the aqueous phase. In this respect, PVPP would be the preferable treatment for removal of phenolics as it provides a higher carbon content for subsequent fermentation.

3.3.2 Preliminary bacterial fermentations

In bacterial culture media, a rich source of proteins and vitamins is often supplied in the form of yeast extract, peptone or tryptone. In rich media, this is often as high as 10 to 20 g/l yeast extract and 10 g/l peptone (DSMZ medium 852, 85, 452). In defined media, much lower amounts of yeast extract are added – in the range of 0.5 g/l (DSMZ medium 963, 986). These media instead include a range of salts and vitamins known to be essential for the growth of the micro-organism of choice.

Before attempting bacterial fermentations with the treated wastewaters, it was necessary to perform preliminary fermentations to determine whether or not yeast extract would be required for growth and if so, the level of yeast extract required. For fermentations, two bacterial cultures were used: NB4 and TH141. Growth was determined by increase in optical density at 600 nm. A sugar solution containing 60 mg/l glucose and 150 mg/l fructose was prepared so as to mimic the sugar concentrations in CW1. Both aerobic and micro-aerobic fermentations were performed using the sugar solution with 0; 0.5 and 5 g/l yeast extract.

The growth curves are shown in Figure 3.35. Aerobic cultures are designated “a” and duplicate curves are shown for each culture. Cultures were monitored until the optical density began to decline.

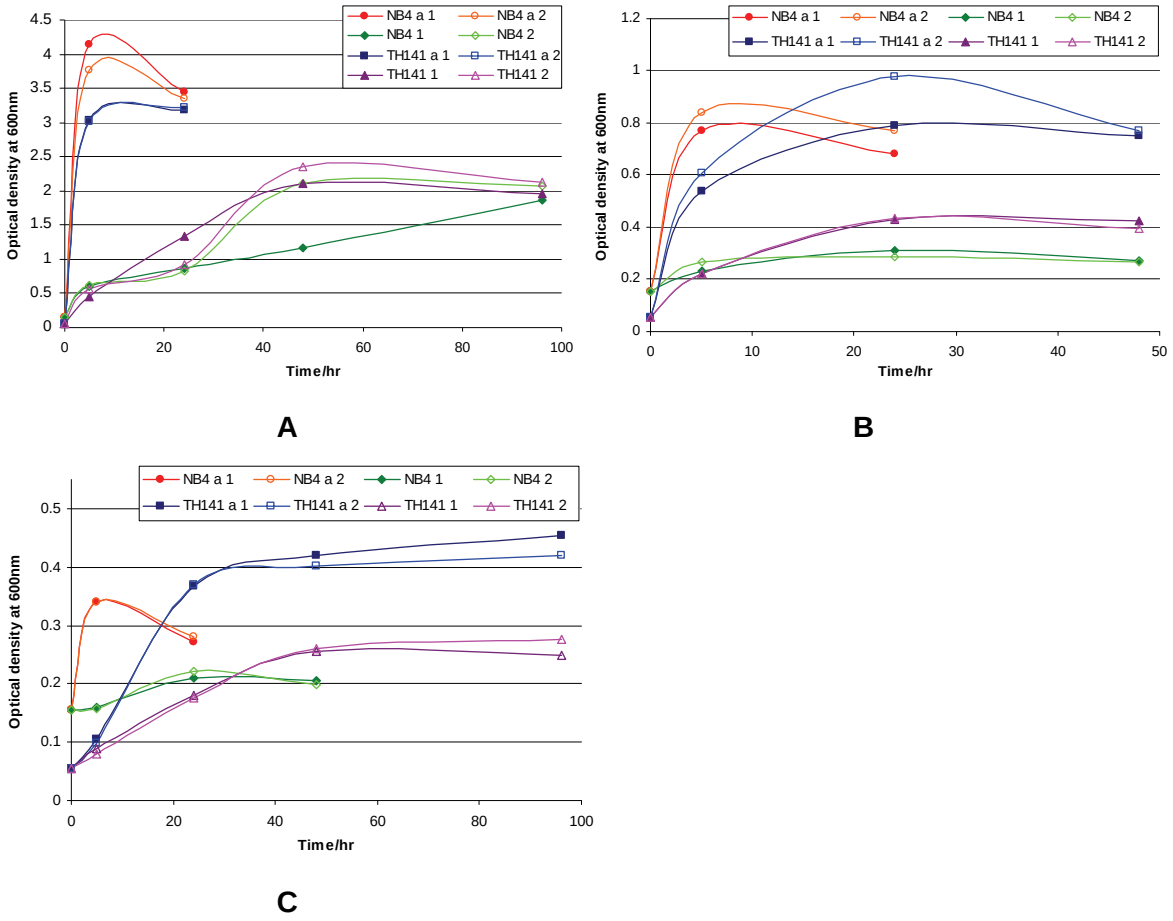


Figure 3.35: Growth of cultures in a sugar solution with added yeast extract: 5 g/l (A), 0.5 g/l (B) and 0 g/l (C)

Cultures that grew in media with added yeast extract reached higher levels of optical density than cultures grown in media with sugar alone. The difference in growth between 5 g/l and 0.5 g/l yeast extract was significant, with much higher optical densities observed for 5 g/l yeast extract. Growth in the sugar solution alone did occur though. This is encouraging as the addition of yeast extract even at the lower concentration, would add cost to the fermentation. It is preferable to avoid addition of further nutrients to wastewaters if possible. This condition, with no added yeast extract, was therefore chosen for the fermentations on the treated waste.

Cultures grown aerobically reached higher optical densities than those grown micro-aerobically. Where biomass is the desired product, aerobic growth should be encouraged.

HPLC analysis of the samples showed evidence of growth in the form of production of metabolites such as acetic acid. Product formation in the form of 2,3-butanediol was also seen. Figures 3.36 and 3.37 show the HPLC traces from the TH141 cultures grown without yeast extract.

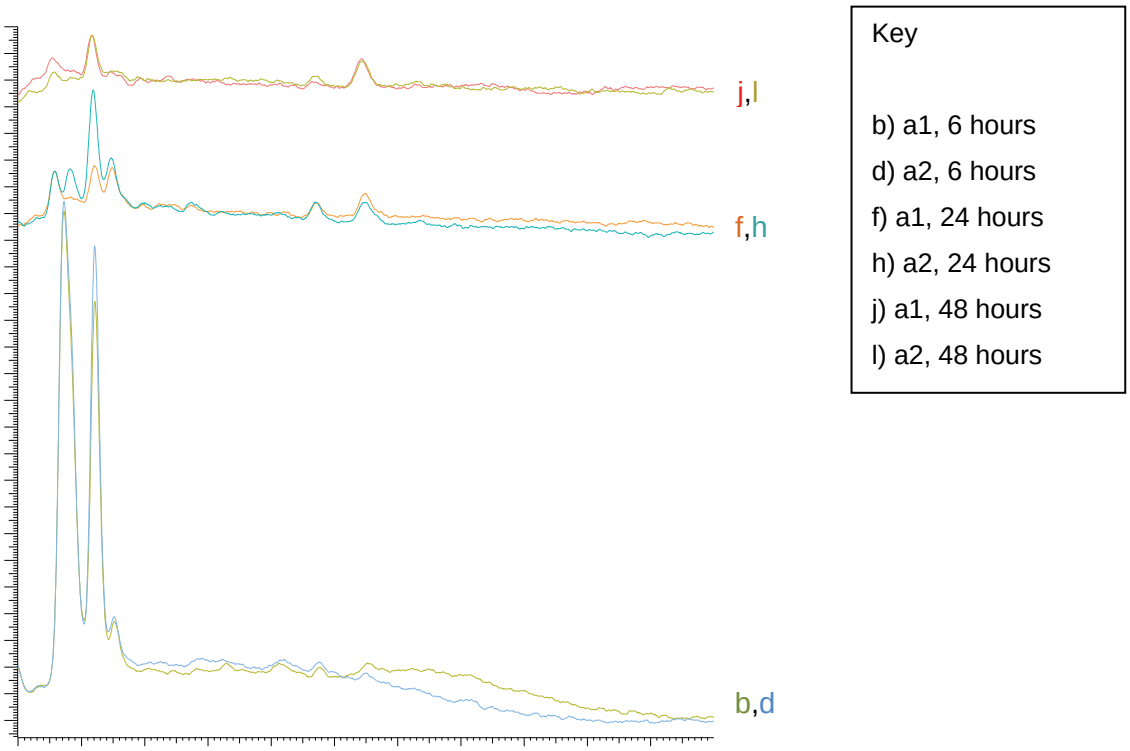


Figure 3.36: HPLC traces for aerobic growth of TH141 in a sugar solution without yeast extract

The peaks at 9.36 min (glucose) and 10.3 min (fructose) decreased with time as the sugars were utilised for biomass and metabolite production. In some cases, acetate (16 min) increased in the early stages of the fermentation but it was subsequently further utilised. 2,3-butanediol concentration (peaks at 17.5 and 19 min) increased with time.

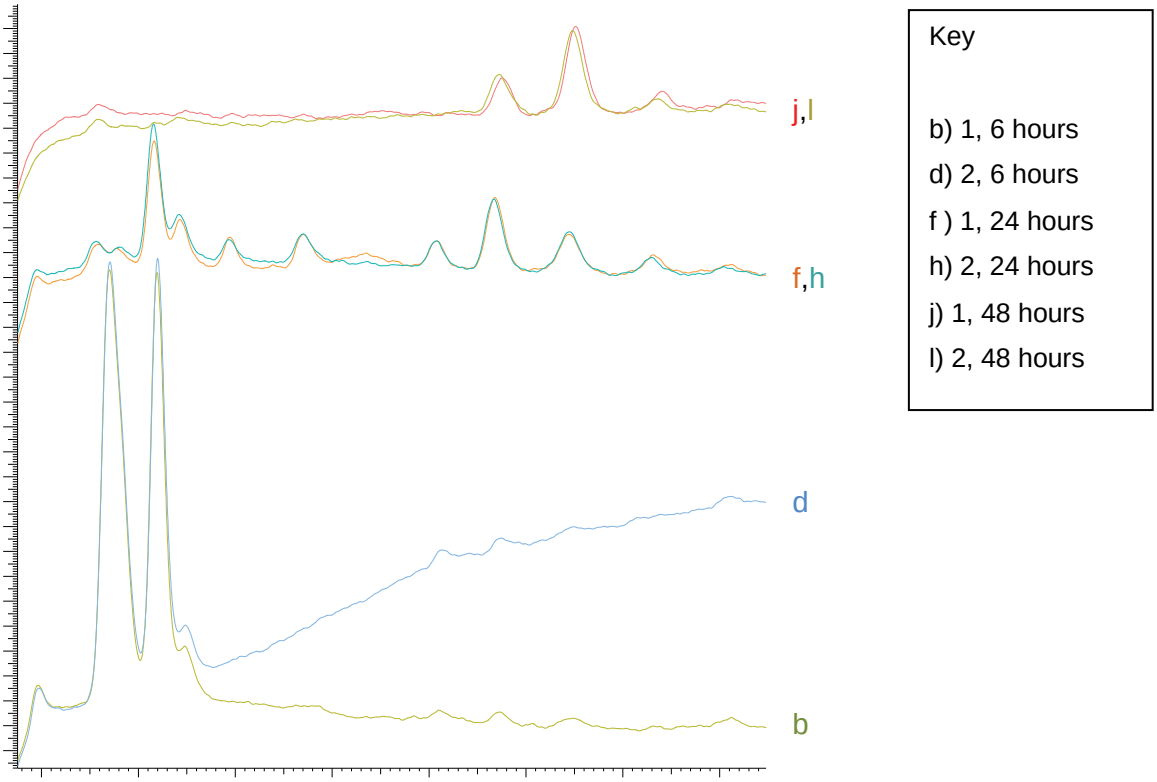


Figure 3.37: HPLC traces for micro-aerobic growth of TH141 in a sugar solution without yeast extract

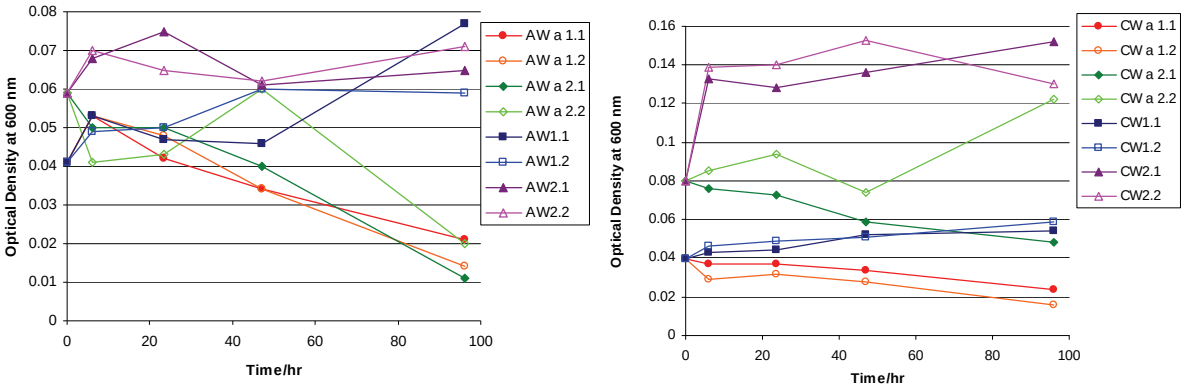
Micro-aerobic growth allowed for greater production of 2,3-butanediol in the culture medium. Where a product such as 2,3-butanediol is desired, micro-aerobic growth should be encouraged.

Of the two cultures, NB4 grew rapidly, converting sugars into biomass and end-product metabolites. TH141 grew at a slower rate and it was possible to see the temporary increase of acetate as an intermediate carbon source. Many of the treated wastes contain only acetate as any sugar initially present has been fermented. TH141 has been seen here to utilise acetate and has therefore been chosen as the bacterium to be used for the treated wastewater fermentations as it may be able to utilise acetate as sole carbon source in the treated wastewater.

3.3.3 Bacterial fermentations with treated wastewater solutions

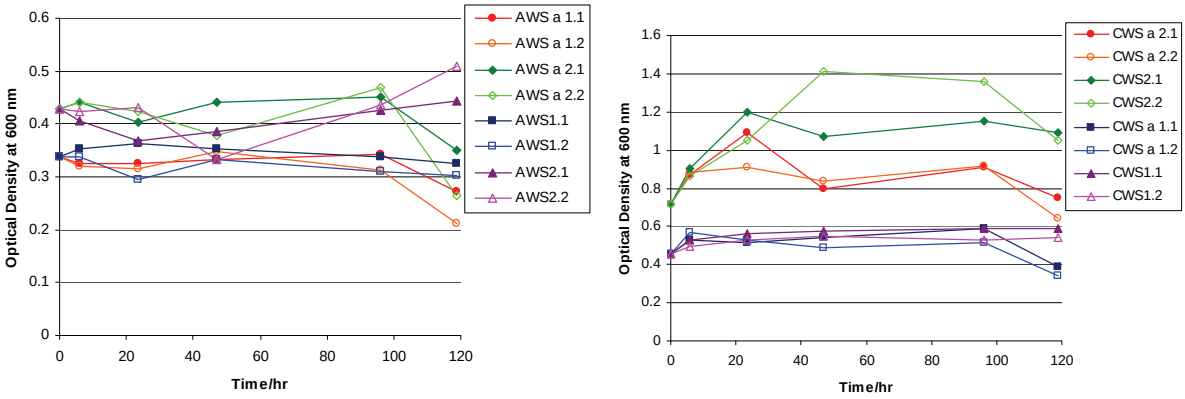
TH141 was inoculated into autoclaved aliquots of the treated wastewater solutions detailed in Table 3.21. For the PVPP extractions, both the stripped water (water decanted from the PVPP phase) and the first water wash contained sugar. These were therefore mixed in a 4:1 (stripped:water wash) ratio and the resulting solution was used. The growth curves are shown

in figures 3.38 to 3.39. Aerobic cultures are designated “a” and duplicate curves are shown for each culture.



A B

Figure 3.38: Growth curves for TH141 after cartridge-based solid phase extraction: AW1 and AW2 (A); CW1 and CW2 (B)



A B

Figure 3.39: Growth curves for TH141 after PVPP extraction: AW1 and AW2 (A); CW1 and CW2 (B)

Growth curves in treated wastewaters are more difficult to interpret than the curves in simple sugar solutions as the treated wastewaters have faint to strong colour initially. The apple wastewaters were visually colourless while the citrus wastewaters were light brown (CW1) and medium brown (CW2). SPE through cartridges removed particulate matter from the wastewaters but in PVPP extraction, the water phase was decanted from the PVPP phase and particulates therefore remained in the treated wastewater. Particulates form a valuable source of additional carbon, creating a richer medium for growth and fermentation, and it is preferable that they are not removed. This does, however, complicate readings of optical density. An increase in bacterial cell mass causes an increase in optical density in a clear solution. Conversely, bacterial digestion of particulate matter causes a decrease in optical density.

Under these conditions, growth may be indicated by an increase in optical density caused by biomass, or a decrease in optical density as the bacteria digest particulates and colour compounds. Fortunately, changes in optical density are not the only way to determine whether or not a culture is growing. HPLC analysis of the changes in metabolite levels during the course of a fermentation provides a very sensitive indicator of bacterial growth and metabolism. These changes are summarised in Table 3.23.

Table 3.23: Change in metabolites during fermentations in wastewater

Sample	No	Aerobic	Micro-aerobic
AW1 SPE	1	No change	Slight increase in acetate and 2,3-butanediol
	2	No change	Acetate and 2,3-butanediol increased slightly then decreased
AW2 SPE	1	Acetate decreased slightly	No change
	2	No change	Acetate and 2,3-butanediol increased slightly
CW1 SPE	1	Ethanol evaporated, no other change	Acetate increased
	2	Ethanol evaporated, no other change	Acetate increased
CW2 SPE	1	No change	No change
	2	No change	No change
AW1 PVPP	1	Sugars, citrate and lactate decreased, 2,3-butanediol increased	Citrate decreased, 2,3-butanediol increased
	2	Sugars, citrate and lactate decreased, 2,3-butanediol increased	Citrate decreased
AW2 PVPP	1	Slight increase in citrate	No change
	2	Slight increase in citrate	No change
CW1 PVPP	1	Increase in succinate, formate, acetate, 2,3-butanediol, ethanol (evaporation also seen)	Increase in acetate, ethanol and 2,3-butanediol; decrease in lactate
	2	Increase in succinate, formate, acetate, 2,3-butanediol, ethanol (evaporation also seen)	Increase in acetate, ethanol and 2,3-butanediol; decrease in lactate
CW2 PVPP	1	Acetate decreased, citrate increased and additional unidentified metabolite	Acetate decreased, increase of 2 unknown peaks
	2	Acetate decreased, citrate increased and additional unidentified metabolite	Acetate decreased, increase of 2 unknown peaks

The HPLC spectra for the aerobic fermentations of CW1 (flask 1) after PVPP extraction are shown in Figure 3.40. The UV traces are shown below the RI traces. The UV and RI instruments are connected in series and there is therefore a 40 second lag between peaks on the UV and on the RI.

A peak at 10.2 min increases (unknown metabolite) as well as the succinate peak at 12.5 min (UV spectra). The acetate peak at 15.5 min (UV spectra) also increases. The 2,3-butanediol peak at 17.5 min (RI spectra) increases. Ethanol which runs at 20.9 min on the RI spectra, decreases which is likely due to evaporation in the aerobic flasks.

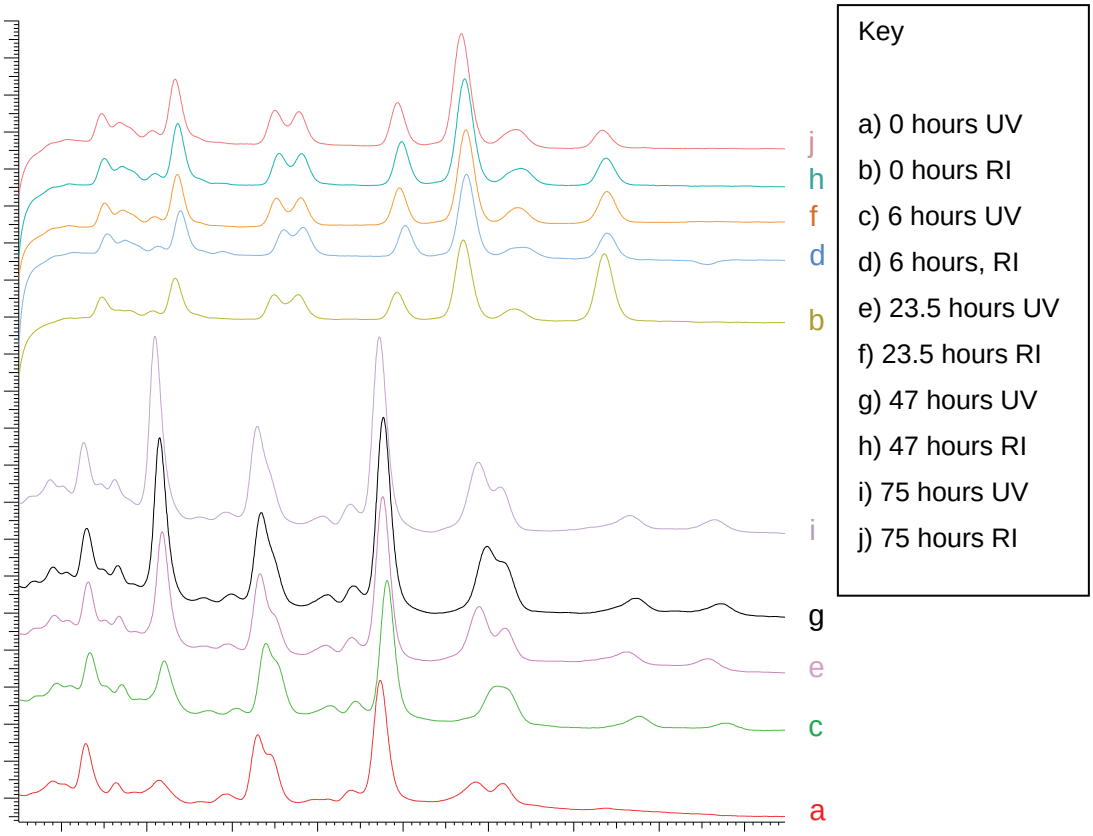


Figure 3.40: HPLC spectra for CW1 (flask 1) after PVPP extraction under aerobic conditions

This stands in contrast to the HPLC spectra for CW1 (flask 1) after SPE which are shown in Figure 3.41. Here, the only peak that changes is the ethanol peak on the RI traces and as above that is likely to be caused by evaporation.

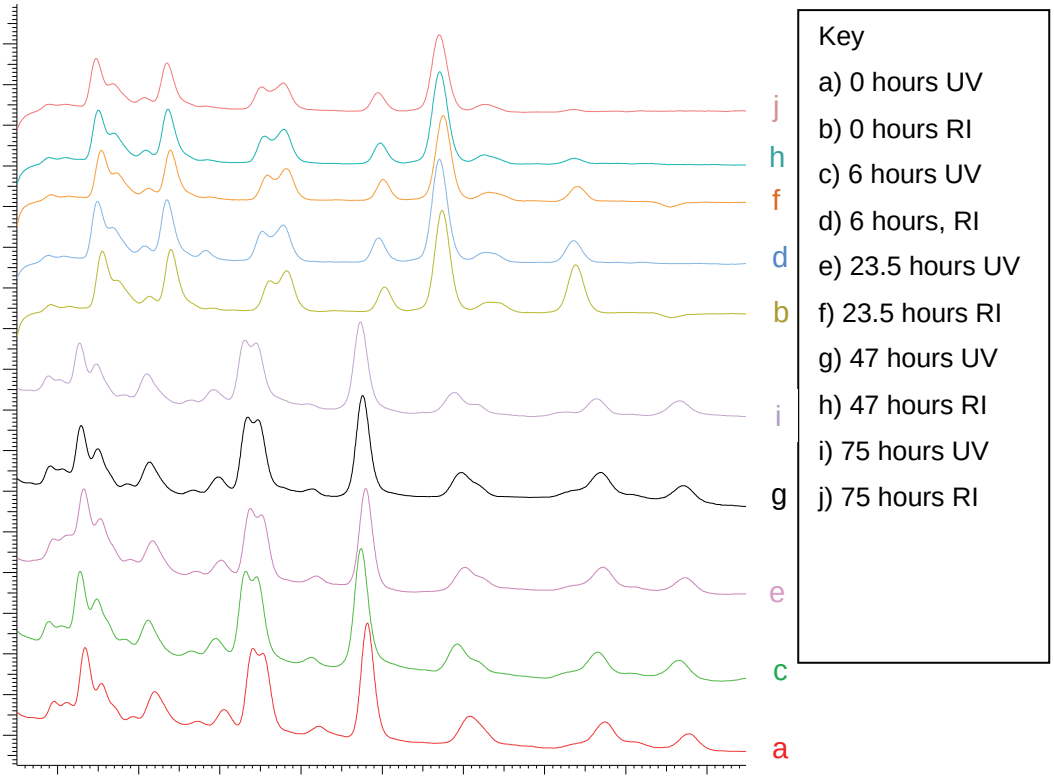


Figure 3.41: HPLC spectra for CW1 (flask 1) after cartridge-based solid phase extraction under aerobic conditions

The HPLC spectra for all PVPP fermentations after 119 hours is shown in Figure 3.42. In this figure it is possible to see the relative yields of products from each of the fermentations. The 2,3-butanediol peak is largest in the CW1 fermentations, both aerobic and micro-aerobic. Ethanol is highest in the micro-aerobic CW1 fermentation. Acetate (16 min on RI) is highest in the AW2 fermentations and succinate (13 min on RI) and lactate (13.6 min) are seen in the citrus wastewater fermentations.

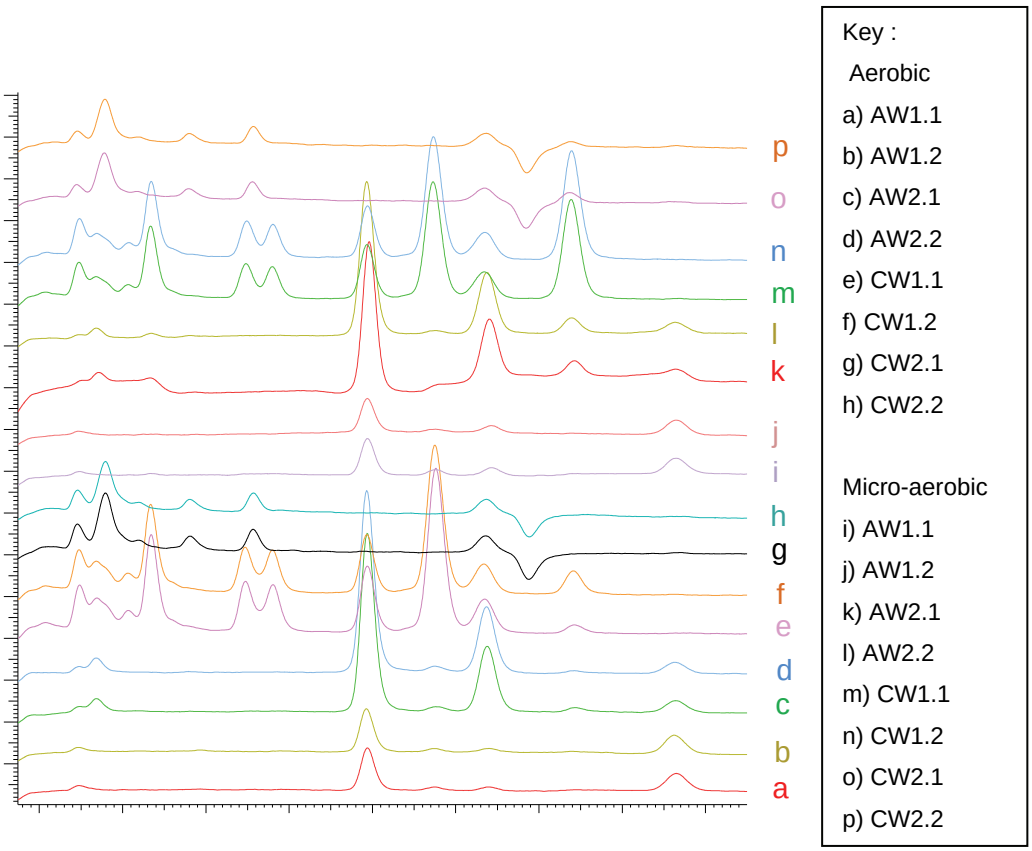


Figure 3.42: HPLC spectra after 119 hours of fermentation in each of the PVPP treated wastewaters

The highest concentration of 2,3-butanediol produced by TH141 after SPE was 25 mg/l from CW1 grown micro-aerobically. This is a yield of 12% of the available carbon. PVPP treatment allowed for higher 2,3-butanediol production. In PVPP extraction, solid fruit particles remaining in the wastewater are decanted with the treated wastewater and are therefore added to the available carbon. The highest concentrations of 2,3-butanediol produced after PVPP extraction were 139 mg/l from CW1 grown micro-aerobically and 259 mg/l from CW1 grown aerobically. These yields are 66% and 123% respectively showing that portions of the complex carbohydrates present in the wastewater were also metabolised. Again, PVPP is shown to be the preferred treatment for extraction of phenolics because of the greater carbon content remaining in the wastewater for subsequent fermentation.

3.3.4 Fermentations with untreated wastewater solutions

The previous fermentations were conducted to assess fermentation after extraction of phenolics. The advantage of this strategy is the removal of phenolics before fermentation as phenolics could inhibit growth of microbial cultures. The analysis of wastewaters has shown that the phenolic content of the citrus and apple wastewaters is low, ranging from 1.8 to 137 mg/l GAE (Table 3.1) and at this level, the phenolics may have no inhibitory effect on growth.

As detailed in section 1.5.1, the effect of specific phenolics on bacterial and yeast cultures is concentration dependent, with low concentrations often stimulating growth while high concentrations resulted in inhibition. The growth of *Lactobacillus hilgardii* was stimulated by gallic acid additions up to 200 mg/l and catechin up to 400 mg/l (Alberto et al. 2001). *L. collinoides* was stimulated in the early growth phases and reached higher cell densities in the presence of up to 1000 mg/l chlorogenic acid and gallic acid (Stead, 1994). *Oenococcus oeni* showed stimulation of growth in the presence of up to 1000 mg/l gallic acid and 100 mg/l catechin (Reguant et al., 2000). The presence of phenolics at levels under 1000 mg/l, as seen in all wastewaters tested, may have no inhibitory effect on growth and may even stimulate growth and ethanol production. The effect of phenolics in the untreated wastewaters was therefore assessed both for bacterial cultures and for *Saccharomyces cerevisiae*

3.3.4.1 Bacterial fermentations with untreated wastewater solutions

Aerobic and micro-aerobic fermentations were performed using TH141 and the untreated wastewaters AW1, AW2, CW1 and CW2. The optical density (at 600 nm) of each culture was determined immediately after inoculation and after 7 hours of growth. Growth in the wastewaters was compared to growth in a salts medium (ISP 9) containing 1% glucose. To allow for direct comparison, salts were added to wastewaters to the same level as in the blank. Glucose was also added to all wastewaters to give a final concentration of 1%.

ISP 9 contains: $(NH_4)_2SO_4$ 2.64 g/l; KH_2PO_4 2.38 g/l; $K_2HPO_4.3H_2O$ 5.65 g/l; $MgSO_4.7H_2O$ 1.00 g/l; $CuSO_4.5H_2O$ 6.4 mg/l; $FeSO_4.7H_2O$ 1.1 mg/l; $MnCl_2.4H_2O$ 7.9 mg/l; $ZnSO_4.7H_2O$ 1.5 mg/l. pH 6.8 to 7.0

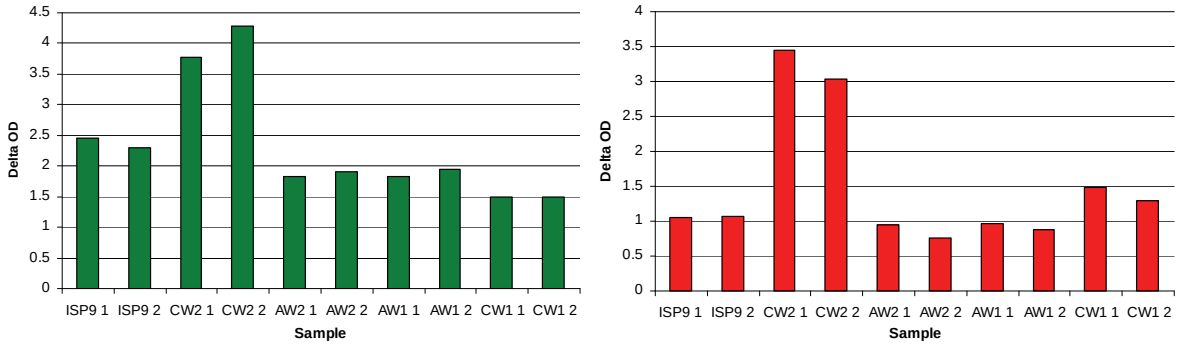
Results are shown in Table 3.24 and 3.25. Figure 3.43 depicts the change in optical density for each culture.

Table 3.24: Aerobic growth of TH141 in untreated wastewater solutions

Optical Density [600 nm]	0 Hours	7 Hours	Difference
ISP 9	0.048	2.50	2.452
	0.048	2.34	2.292
AW1	0.218	2.04	1.83
	0.218	2.16	1.95
AW2	0.312	2.14	1.828
	0.312	2.22	1.908
CW1	0.210	1.708	1.490
	0.210	1.712	1.494
CW2	2.00	5.78	3.78
	2.00	6.28	4.28

Table 3.25: Micro-aerobic growth of TH141 in untreated wastewater solutions

Optical Density [600 nm]	0 Hours	7 Hours	Difference
ISP 9	0.048	1.10	1.052
	0.048	1.12	1.072
AW1	0.210	1.69	1.48
	0.210	1.51	1.30
AW2	0.312	1.26	0.948
	0.312	1.07	0.758
CW1	0.218	1.19	0.972
	0.218	1.09	0.872
CW2	2.00	5.44	3.44
	2.00	5.04	3.04



A **B**

Figure 3.43: Difference in optical density for growth of TH141 on untreated wastewaters: aerobic (A) and micro-aerobic (B)

There was very little difference in the growth of cultures in ISP9 buffer compared to untreated wastewaters showing that the phenolics in the sample were not inhibiting growth. This is confirmed by the finding that CW2 showed increased growth when compared to the other samples. This is the wastewater with the highest phenolics content (Table 3.1) and even at this level, the phenolics are not inhibitory and may have even stimulated growth. CW2 has the greatest density of particulate matter and it is likely that the bacteria were using the particulates as an additional carbon source for growth. It is also possible the bacteria were able to use the phenolics themselves as an additional carbon source. This theory could be tested by growing the bacteria using phenolics as sole carbon source should this application be deemed profitable.

3.3.4.2 Yeast fermentations with untreated wastewater solutions

Fresh apple wastewater samples were obtained in March 2009 (AW3 and AW4) and the sugar concentration in the more concentrated solution was determined by HPLC to be 0.7 g/l glucose and 1.6 g/l fructose (Table 3.1). The phenolic content was determined to be 11.03 mg/l GAE (FC method). Synthetic wastewater was prepared containing 6 fold higher levels of sugar to mimic apple wastewater concentrated by reverse osmosis (Jesus et al., 2007). The synthetic wastewater contained 4.25 g/l glucose and 9.75 g/l fructose for a total sugar concentration of 14 g/l. Table 3.26 and Figure 3.44 show the growth and metabolite profile of *S. cerevisiae* in this wastewater.

Table 3.26: Growth and metabolism of *Saccharomyces cerevisiae* in synthetic wastewater (with no added phenolics)

Time/ hours	Optical density at 600 nm	Concentration in g/l		
		Glucose	Fructose	Ethanol
0	0.72	4.25	9.75	0
1.67	1.02	3.53	6.69	0.81
3.00	1.35	2.60	5.97	1.34
4.67	1.47	1.91	5.41	2.08
6.00	1.77	1.14	4.85	2.19
7.58	1.86	0.84	4.60	2.37
24.00	-	0.03	1.10	4.31

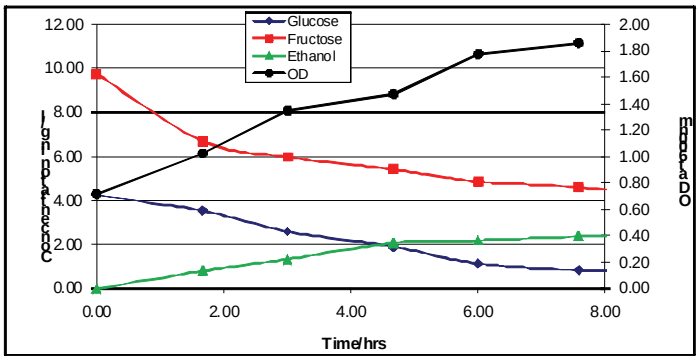


Figure 3.44: Growth and metabolism of *Saccharomyces cerevisiae* in synthetic wastewater

The culture had not yet reached stationary phase after 8 hours and not all sugar had been metabolised. After 24 hours, the glucose was depleted and 90% of the fructose had been utilised. The ethanol concentration had also increased greatly. It was therefore decided to run overnight (18 hour) fermentations to allow time for metabolism of the sugar and for ethanol production.

The growth and metabolism of *S. cerevisiae* in synthetic wastewater with added phenolics are shown in Table 3.27 and Figure 3.45. Gallic acid was chosen as the representative phenolic compound and was added to span the range of 0-100 mg/l. These concentrations are lower than those tested in other studies where levels of gallic acid of 1000 mg/l are typically included (Reguant et al., 2000; Stead, 1994) but these are the highest levels that could be expected in apple wastewater even if it was concentrated up to 10 fold using reverse osmosis membranes.

Table 3.27: Effect of gallic acid on metabolism of *Saccharomyces cerevisiae* in synthetic wastewater (0-100 mg/l)

Gallic acid in g/l	**Concentration in g/l**		
	Glucose	**Fructose**	**Ethanol**
0	0.93	4.73	2.53
0.02	0.58	406	3.14
0.04	1.08	4.87	2.68
0.06	0.64	4.31	3.36
0.08	0.63	4.13	3.40
0.10	0.71	4.35	3.51

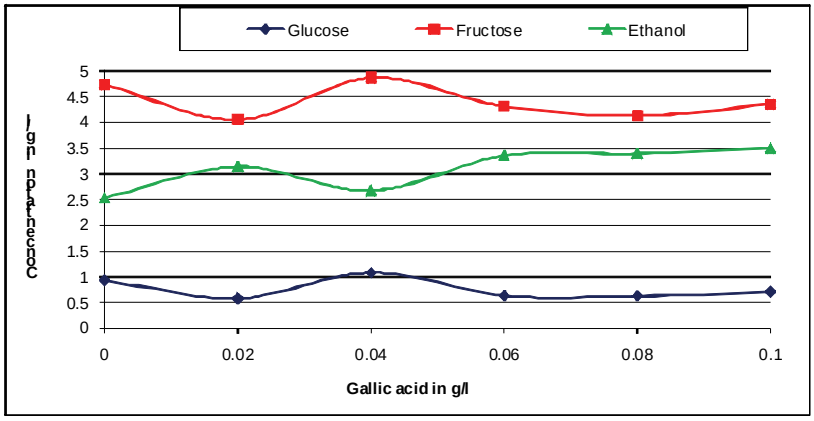


Figure 3.45: Effect of gallic acid on metabolism of *Saccharomyces cerevisiae* in synthetic wastewater

The levels of glucose and fructose metabolised and the concentrations of ethanol produced were very similar for all gallic acid concentrations tested. Gallic acid had no effect on the metabolism and ethanol production of *S. cerevisiae* in the range of gallic acid concentrations expected from wastewater concentrated up to 10 fold. This result is consistent with Kubo et al. (2002) who designed inhibitors for *S. cerevisiae* using alkyl derivatives of gallic acid. No inhibition was seen for up to 3.2 g/l gallic acid although inhibition was noted as chain length increased, up to a maximum for nonyl gallate. Rauha et al. (2000) also found no inhibition by 1 g/l gallic acid.

Higher gallic acid concentrations were also tested to mimic wastewater solutions to which solid apple waste had been added. In this case, phenolic concentration could be significantly higher than seen in wastewaters sampled. Table 3.28 and Figure 3.46 show these results

Table 3.28: Effect of gallic acid on metabolism of *Saccharomyces cerevisiae* in synthetic wastewater (0-300 mg/l)

Gallic acid in g/l	Concentration in g/l		
	Glucose	Fructose	Ethanol
0	0.21	2.76	3.77
0.06	0.40	3.34	3.68
0.12	0.16	2.42	5.00
0.18	0.43	3.65	4.97
0.24	0.65	4.14	5.19
0.30	0.41	3.53	6.16

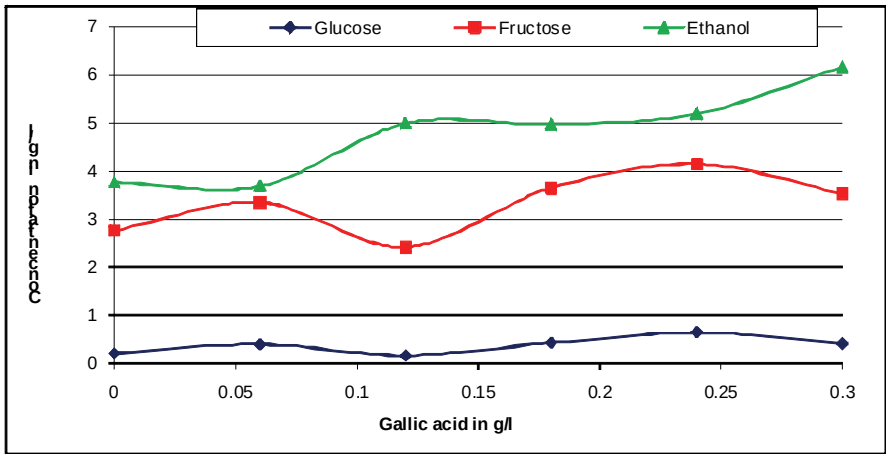


Figure 3.46: Effect of higher gallic acid concentrations on metabolism of *Saccharomyces cerevisiae* in synthetic wastewater

Rather than inhibiting the production of ethanol by *S. cerevisiae*, higher concentrations of gallic acid seemed to stimulate ethanol production. Glucose metabolism was unchanged and slightly less fructose was utilized but ethanol production increased from 3.77 g/l to 6.16 g/l. Lee and co-workers studied the effect on *S. cerevisiae* of inhibitory compounds released during lignocellulose hydrolysis and found that levels of 1 g/l coumaric acid or syringaldehyde did not inhibit growth or ethanol production but conversely resulted in higher ethanol yields than control fermentations without added phenolics (Lee et al., 2011).

The level of gallic acid was tested at the end of the fermentation to determine whether or not it had been metabolised. All gallic acid added to the 0.06 g/l experiment was still present after fermentation but the 0.12 g/l fermentation had 0.65 mg/l less gallic acid than theoretical. This increased to 4.50 mg/l for the 0.18 g/l fermentation and to 13.29 mg/l for the 0.24 g/l fermentation. It is not yet clear why moderate levels of phenolics (up to 1 g/l) stimulate ethanol production. It may be that the phenolics are acting as antioxidants, protecting the cells or moderating redox potentials during fermentation. It is also possible that the gallic acid in this experiment was being utilised as a carbon source for ethanol production, resulting in the decrease in concentration. This finding warrants additional investigation.

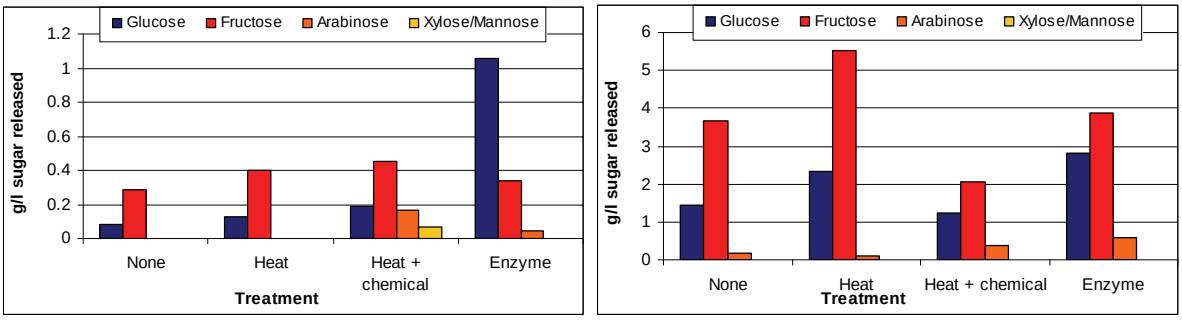
Regardless of the cause, the observation that ethanol production increases as a result of the addition of phenolics, is promising when considering the option of supplementing the wastewater with solid fruit waste.

3.3.5 Fermentations using solid fruit waste

Solid fruit waste was prepared using the solid pomace that remained after juicing apples. This was washed to remove sugars and treated using heat, acid or enzymes to release bound carbohydrates. These were quantified and the resulting solutions were used in fermentation experiments with *S. cerevisiae*.

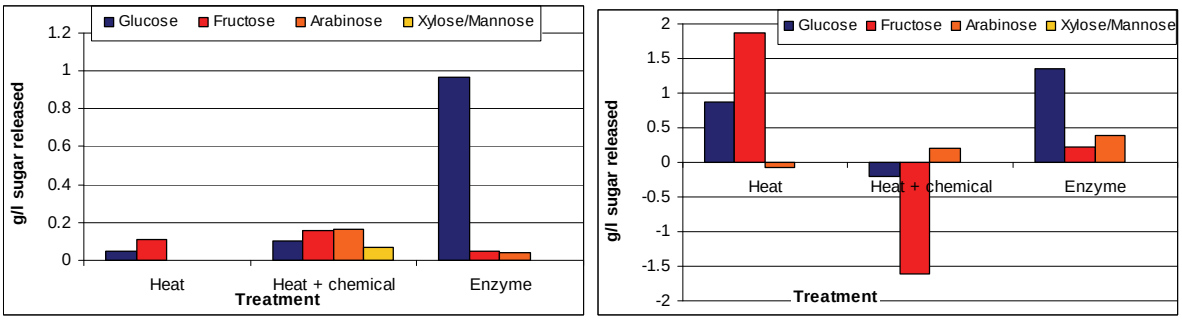
3.3.5.1 Effect of pre-treatment

Pretreatment experiments were performed in September and again in December using apples available in supermarkets at that time. Variability was seen in the raw material, especially in the untreated sample. Apples purchased in December left more residual sugar in the washed pulp than apples purchased in September. The effect of different treatments is shown in Figure 3.47. Figure 3.48 shows the net effect of different treatments after subtracting the sugar found in the untreated pulp.



A **B**

Figure 3.47: Effect of pretreatment on sugar release from apples purchased in September (A) and December (B)



A **B**

Figure 3.48: Net effect of pretreatment on sugar release from apples purchased in September (A) and December (B)

Of the sugars identified, glucose and fructose were released in the highest amount. Both of these sugars were identified in apple wastewaters tested (Table 3.12) and residual levels

remained in the washed pulp with fructose being present at approximately double the concentration of glucose.

Heat treatment (steam explosion) released additional glucose and fructose but did not release other sugars. This is consistent with the understanding that steam explosion provides physical disruption of the pulp, releasing trapped sugars. Acid treatment in conjunction with heat did release other monosaccharides. Xylose (or mannose) was measured as was arabinose. Here acid is chemically attacking available lignocellulose, releasing sugars. The disadvantage of acid treatment is shown in the results of the second experiment. The apples purchased in December had more free sugar available in the pulp. After acid treatment, the pulp slurry was left with less free sugar than the original preparation. The amount of glucose and fructose oxidised by the sulphuric acid exceeded the amount of sugar released by acid hydrolysis.

Enzyme pretreatment proved successful in both experiments. Glucose, fructose and arabinose concentrations increased and sugar initially present in the pulp was not destroyed. In both experiments, glucose increased most after incubation with the enzyme cocktail, showing the effectiveness of the cellulose and gluco-amylase components. Galactose (and galacturonic acid) were not detected after enzyme pretreatment despite these forming the major component of pectin. It is possible that the pectin was hydrolysed to polysaccharides and oligosaccharides but not to monosaccharides which would have been detected by the HPLC. In another study, galacturonic acid was detected after precipitation of the water soluble fraction using ethanol (polysaccharide portion) or extraction into ethanol (oligosaccharide portion) followed by acid hydrolysis and detection of the resulting monosaccharides (Missang et al., 1993). This study also noted the increase of arabinose which was attributed to the release of arabinose-rich pectic side chains by the action of polygalacturonase.

3.3.5.2 Fermentation of pre-treated samples using *Saccharomyces cerevisiae*

Optical density changes are shown in Figure 3.49. The optical density of all cultures increased within the first 12 hours. In the control fermentation and when the pretreatment was heat or enzyme digestion, cell density then stabilised whereas for the heat and chemical pretreatment fermentations, cell density continued to increase until 24 hours and then stabilised.

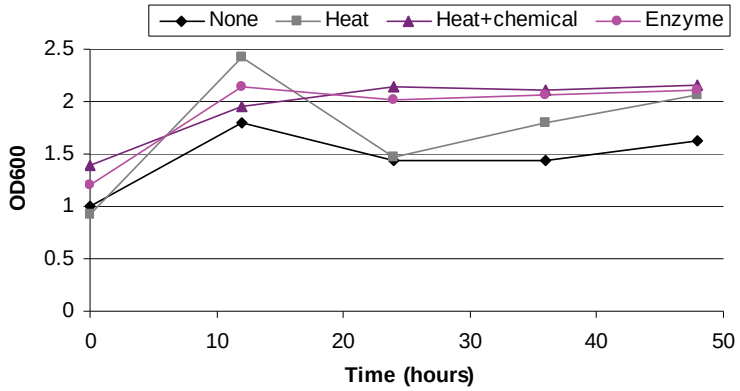


Figure 3.49: Effect of different apple pomace pretreatment methods on the growth of *Saccharomyces cerevisiae*

HPLC analysis of the concentration of sugars remaining and of ethanol produced is shown in Figure 3.50. A component of the starter culture medium obscured the glucose peak in the HPLC spectra and glucose concentration could therefore not be determined.

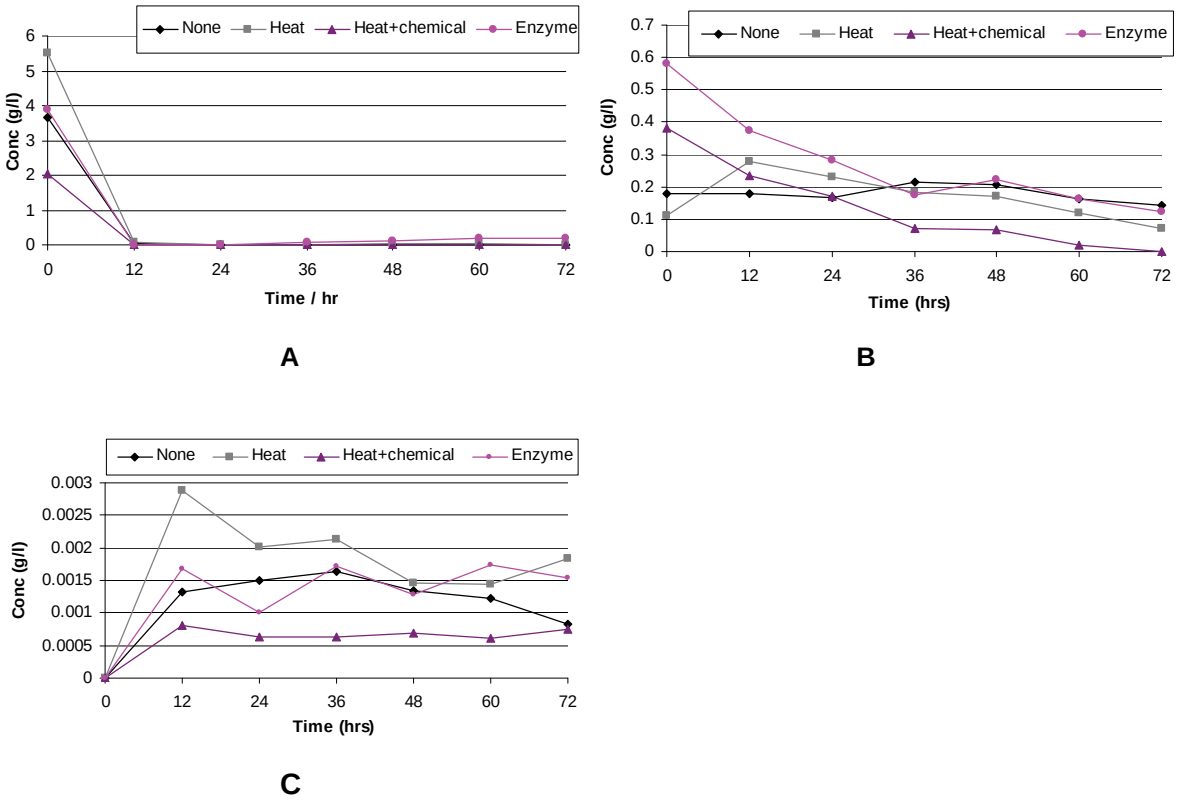


Figure 3.50: Concentration of sugars and ethanol during fermentation: fructose (A), arabinose (B), ethanol (C)

All available fructose in all fermentations was consumed within 12 hours. Arabinose was metabolised more gradually and trace amounts remained at the end of the fermentation

period. Ethanol was produced within the first 12-24 hours of the fermentation, after which concentrations stabilised or decreased. The highest concentration of ethanol was produced by the heat (steam explosion) pretreatment which is not unexpected as this pretreatment had the highest concentration of free monosaccharides at the beginning of the fermentation.

3.4 Concentration of fruit wastewater using reverse osmosis membranes

One of the primary challenges identified in both extraction and fermentation unit operations was the dilute nature of the wastewaters. Concentration of the wastewaters was recommended as an initial step and this is explored using the reverse osmosis membrane rig described in section 2.5.

A salt rejection analysis was performed to test the initial efficiency of the membrane. A 2 000 mg/l NaCl solution was pumped through the apparatus and the conductivity of the permeate and reject were used to determine the rejection of the NaCl by the membrane.

The feed water conductivity was 2 000 µs/cm and the permeate conductivity was 460 µs/cm. The rejection (R) was 77% as calculated according to the formula: $(R)= (C_F-C_p)/C_F$
where:
$\mathbf{C_F}$ = concentration of solute in the feed
$\mathbf{C_p}$ = concentration of solute in the permeate

The observed value of 77% salt rejection was less than the expected 99% salt rejection and new membranes were ordered for future analyses.

The results of the first experimental run are given in Table 3.29. Sucrose was used as the sugar and the apparatus was run at a pressure of 40 bar and speed of 50 Hz for 2 hours. The diameter of the tubular RO membrane was 12.7 mm and the length was 1.085 m giving a membrane area of 0.043 m^2.

Table 3.29: Concentration of a sugar solution using reverse osmosis: membrane 1

Run #	Sample	Concentration in g/l	Volume in l
1	Feed	1.20	6
	Permeate	0.62	1.4
	Concentrate	2.5	
2	Feed	1.22	6
	Permeate	0.64	1.2
	Concentrate	2.4	
3	Feed	1.23	6
	Permeate	2.36	1.2
	Concentrate	0.61	

Rejection of the membrane(R) is defined as $R= (C_F-C_p)/C_F$

Run 1	**Run 2**	**Run 3**
R= (1.206-0.625)/1.206	R = (1.206-0.644)/1.206	R = (1.227-0.646)/1.227
= 0.48 = 48%	= 0.47 = 47%	= 0.501 = 50%

R = Rejection of the membrane

C_F = concentration of solute in the feed

C_p = concentration of solute in the permeate

Permeate flux (J) is defined as $J = (Q_p/A)$

Run 1	**Run 2**	**Run 3**
J= [(1.4 l/2 h)/0.043 m^2]	J= [(1.2 l/2 h)/0.043 m^2]	J= [(1.2 l/2 h)/0.043 m^2]
=16.3 l/m^2.h	= 13.9 l/m^2.h	= 13.9 l/m^2.h

J = Permeate flux

Q_p = Rate of clean water recovery in the permeate stream

A = Contact area

The membrane had a salt rejection of 77% and a sugar rejection of only 50% under recycling, batch conditions. The sugar solution was concentrated by a factor of 2 from the feed to the concentrate which is less than the 6% achieved by Jesus and Leite (Jesus et al., 2007).

A new membrane was obtained and tested as above. The feed water conductivity was 2 020 µs/cm and the permeate conductivity was 10.1 µs/cm. This resulted in a rejection of 99.5% which falls within expectations. The results of the experimental run performed using this membrane are given in Table 3.30

Table 3.30: Concentration of a sugar solution using reverse osmosis: membrane 2

Sample	**Concentration in g/l**	**Volume in l**	**Rejection**	**Permeate flux***
Feed water	1.22	6		
Permeate 1	0.10			
Permeate 2	0.07	2.6	94%	30.23 $l/m^2.h$
Concentrate	3.37			

*Membrane 2 has the same dimensions as membrane 1.

The membrane had a salt rejection of 99.5% and a sugar rejection of 94%. A 2.76 fold concentration of sugar was achieved which compares well to the results obtained by Garcia and Gozálvez (Garcia et al., 2002).

Increasing the processing time above 2 hours may increase the concentration of sugar in the reject. It would also be advisable to use polyamide membranes instead of cellulose acetate as the feed liquid is not expected to be sterile and over time, organisms present in the feed may begin to digest the cellulose acetate membrane.

4 CONCLUSIONS

4.1 Characterisation of Complex Wastes from Fruit Industries

Fruit processing wastewaters were obtained from sources within the fruit processing industry and were characterised both in terms of their physical characteristics and their chemical characteristics. Wastewaters were found to vary widely according to the industry in which they were produced and the point of collection. Apple wastewaters were mildly to moderately acidic (pH 6.4 to 4.4). Where the wastes had been treated, COD values were low, 319 to 328 mg/l and no sugars were detected using HPLC.

Apple wastewaters collected from the processing plant consisted of waste from pressing and washing operations. Here, the COD was higher, 2200 to 2937 mg/l, and in the fresh sample AW3, 2.3 g/l sugars were measured using HPLC. Converting the measured amount of sugars to theoretical COD (Van Haandel and Van der Lubbe, 2007) accounts for 2.461 g/l of the total 2.937 g/l COD measured. Simple sugars are rapidly metabolised by bacteria or yeast and conversion of the sugars to biomass, metabolites or ethanol is possible. The challenge then rests in the downstream processing or collection of products.

Citrus wastewaters collected were more concentrated than the apple wastewaters tested. The combined wastestream was acidic with a pH of 3.6 and a waste collection tank held waste with a pH of 11.0, indicating that it had received waste from alkaline treatment to remove peels from fruit. The measured COD values were high at 3170 to 5760 mg/l but measured sugars were low at 211 mg/l for the combined wastestream. This indicates that carbon is present in more complex structures, less suitable for immediate fermentation. Carbon may be present as pectins and it is likely that flavonoid composition will also be high, particularly in waste generated in process of removing citrus peels.

Silage water was collected as a leachate from collection of solid waste and was found to have a low pH (3.9) and high COD (4750 mg/l). This is not a consistent wastewater stream, it is a byproduct of the collection of solid waste before disposal. The factory manager estimated production at 12000 kilolitres of silage water waste per year (Wimpie Steenkamp, pers comm. to Johann Gorgens) and while this wastewater will vary in composition during the course of the year, the quantities are sufficient to warrant investigation into extraction strategies. Silage water must be treated at the factory before being released to reduce the levels of COD and raise the pH. Extraction of useful compounds would increase

sustainability and decrease the cost of treating the waste and this provides an added incentive to the use of this wastewater.

4.2 Determination of Antioxidant Properties of Fruit Wastewaters

A variety of antioxidant assays were performed to provide a broad understanding of the antioxidant capacity of these wastewaters (Table 3.5). Silage water showed good antioxidant activity both in hydrophilic and hydrophobic environments with a total phenolics content of 399.5 GAE mg/l and 100% decolourisation of DPPH as well as 86% ability to inhibit lipid peroxidation. CW2 also showed strong antioxidant activity, particularly toward inhibition of lipid peroxidation (69.04%).

CW1 and AW2 were more dilute wastewaters and their antioxidant activity was not as strong. CW1 did show the ability to inhibit lipid peroxidation (49.2%) while AW2 had strong activity in aqueous environments (30.3% radical scavenging activity as measured by TEAC analysis). AW1 was very dilute and little antioxidant activity was observed. Concentration of this wastewater would be required to produce an antioxidant extract. Very efficient methods of recovery are needed for value addition in the case of such dilute wastewaters.

4.3 Development of New Extraction Techniques for Extracting Antioxidants

Extraction of phenolics was tested using solvents, both in direct contact with the wastewater and through supported liquid membrane technology. Adsorption to a solid material was also tested using C18 resin (Sep-Pak), PVPP and activated carbon. Finally, extraction into supercritical fluid was tested for a gallic acid solution. The results are summarised in Table 4.1

Table 4.1: Summarised comparison of all extraction techniques tested for the extraction of phenolics compounds from fruit processing wastewaters

Extraction method	Sample	Extraction Efficiency (%)		Comments
Solvent		Ethyl acetate	Hexane	-average efficiency for ethyl acetate (20-48%) -low to average efficiency for hexane (0-40%) -able to run at industrial scale -high cost for solvents and equipment -disposal of residual organic solvents is hazardous and costly -technique is time-consuming and requires large space
	AW1	33.04	40.18	
	AW2	47.7	18.9	
	CW1	20.65	3.21	
	CW2	37.45	9.37	
	SW	20.29	0.46	
Solid phase (Sep-Pak cartridges)	AW1	52.1		-average to high efficiency (44-57%) -normally used as a small scale pre-concentration step, at large scale, quantities of sorbents required are expensive making the process uneconomical -average cost
	AW2	51.9		
	CW1	49.5		
	CW2	44.1		
	SW	56.9		
PVPP with sodium hydroxide elution	AW1	-		-low to average overall efficiency (6-40%) -able to run at industrial scale -technique is relatively simple -low cost, PVPP can be regenerated up to 20 times -technique has great potential for use in industry
	AW2	36.49		
	CW1	6.53		
	CW2	6.18		
	SW	39.58		
PVPP with ethanol elution	Solution:	Concentrated	Dilute	
	Gallic acid	36.6	8.9	
	Catechin	0.9	2.3	
	Chlorogenic acid	12.36	11.4	
PVPP with ethyl acetate elution	Gallic acid	53.8	26.6	
	Catechin	4.4	23.4	

	Chlorogenic acid	22.5	68.7	
Activated carbon with ethanol elution	Solution:	Concentrated	Dilute	-low efficiency for gallic and chlorogenic acid (0-21%) -high efficiency for catechin (28-92%) -able to run at industrial scale -technique is relatively simple -very low cost but usually not regenerated, more sustainable if regenerating with ethanol or ethyl acetate -technique has great potential for use in industry
	Gallic acid	17.2	0.1	
	Catechin	55.1	28.4	
	Chlorogenic acid	10.2	0.23	
Activated carbon with ethyl acetate elution	Gallic acid	20.8	0.1	
	Catechin	85.68	91.6	
	Chlorogenic acid	0.13	0.0	
Supported Liquid Membrane	SW	44.60		-average efficiency (44%) -able to run at industrial scale -average cost
Supercritical fluid	Gallic acid	7.70		-low efficiency (8%) -able to run at industrial scale -initial set up costs are high - maintenance costs are low -uses no/minimal organic solvent

The use of solvents to extract antioxidants allowed extraction of up to 47.70% of antioxidants when using ethyl acetate and up to 40.18% using hexane. After extraction, these solvents would be removed through evaporation to leave a solvent-free antioxidant extract. Removal of solvents is costly and time consuming and while solvents would be recycled, production of some hazardous waste is anticipated. Additionally, traces of solvent remained in the aqueous phase after extraction and the water would then need to be treated to remove all solvent before release. This would be costly and has an adverse impact on the sustainability of the process.

When solvents are separated from the wastewater by a membrane, problems of solvent contamination of the aqueous phase should be avoided. Gallic acid, a valuable phenolic antioxidant, was extracted from silage water using a supported liquid membrane (SLM) at an extraction efficiency of 44.6% using toluene as co-solvent. The technique is relatively simple and easy to operate once the parameters such as solvent and feed rates have been determined.

SLM offers a better alternative compared to traditional solvent extraction methods and further research would be useful. The use of toluene should be avoided because of the toxicity of this solvent and other, less harmful solvents should be tested. Filtration was also necessary before the wastewater could be extracted using SLM, thus an additional filtration step would be necessary to minimise system blockage. For both methods involving solvents, it should be noted that individual phenolics have different solubilities in solvents with different polarities. A solvent that provides good extraction efficiencies for a chlorogenic acid-rich apple wastewater will not necessarily give a good yield of flavonoid phenolics from citrus waste or of gallic acid from silage water. The solvent must be chosen to suit the solubilities of the major phenolics in each wastewater.

Solid phase extraction with Sep-Pak cartridges for the separation and recovery of neutral and acidic phenolic compounds was not very effective since some neutral phenolic compounds were detected in the acidic fraction. Extraction efficiency ranged from 44.1% to 56.9%. Neutral phenolics exhibited higher antioxidant activity compared to acidic phenolics.

Extraction efficiencies using the solid sorbent PVPP ranged from 0% in AW1 to 39.58% in SW. Extracts obtained from PVPP exhibited relatively low radical scavenging activity in the range of 0.65% to 32.71%. When synthetic wastewaters were tested, PVPP adsorbed 80% of gallic acid when the concentration was between 40 and 100 mg/l and reached equilibrium with 30 minutes. PVPP was able to adsorb 45-50% of gallic acid when exposed to 275 mg/g

adsorbent implying a saturation level of approximately 125 mg/g. PVPP did not adsorb chlorogenic acid and adsorbed variable amounts of catechin indicating that it is a selective adsorbent, a useful property when preparing extracts high in a chosen phenolic compound.

Amberlite XAD4 adsorbed 80% of gallic acid with equilibrium reached within one hour making it less suited for use than PVPP. Activated carbon adsorbed all gallic acid in the range of 20-100 mg/l although it was slow to reach equilibrium. Batch processing would be recommended for this adsorbent. Activated carbon was able to adsorb 34-41% of gallic acid when exposed to 275 mg/g adsorbent implying a saturation level of approximately 95 mg/g. It was able to adsorb a range of phenolics and release phenolics when washed with solvent.

Elution of bound phenolics using ethanol and ethyl acetate was possible but the efficiency was low. Of the two, ethyl acetate was more efficient, finally eluting 22% gallic acid from PVPP and 20% catechin, 10% gallic acid from activated carbon. Ethanol would be the better choice for a phenolic antioxidant extract however as ethanol is already used in commercial preparations. Much more investigation is needed on the elution/recovery of phenolics. Batch elution should be investigated, since if phenolics are adsorbed inside pores, it may be possible to remove higher concentrations of phenolics if the adsorbents are suspended in solvents and allowed to reach equilibrium. Further, elution with a more hydrophobic phase might be investigated.

The optimum operating conditions for the extraction of gallic acid using supercritical fluid extraction were 25^{o}C using 20 ml ethanol co-solvent for 3 hours at 180 bars with an extraction efficiency of 6%. The low extraction efficiency is comparable to that reported by other researchers for determining the solubility of gallic acid in supercritical-CO_2 and this technology cannot therefore be recommended for extraction from wastewaters high in gallic acid. The study shows the potential of supercritical fluid extraction as an alternative to conventional extraction methods but only if a co-solvent is found to increase the extraction efficiency. More research should be done to improve extraction efficiencies for gallic acid and to investigate extraction of other phenolics.

Overall, the methods of extraction have proved technically feasible but when considering an economically viable process, the absolute yields of antioxidants must be considered. Some of the wastewaters studied contain very low concentrations of phenolic compounds, leading to low recovery of antioxidants despite extraction efficiency of close to 50%. Extraction of 40% of phenolics present in concentrated silage water by PVPP yields a concentrated

antioxidant extract at low cost but extraction of 48% of phenolics present in dilute apple wastewater through use of large volumes of ethyl acetate which must be removed by distillation, yields only a small amount of antioxidants relative to the cost of the process. For extraction to be economically feasible, one of two conditions must be met.

Where extraction is into another liquid phase (solvent or supercritical fluid), the volumes must be minimised. Significant pre-concentration of the wastewaters would be required to give a small volume of a concentrated wastewater. This would allow extraction from much smaller volumes of wastewater, reducing the volume of liquid solvents required and reducing downstream processing costs, leading to lower costs per unit antioxidant recovered.

Where extraction is onto a solid support, the support must be able to bind antioxidants selectively and quickly from dilute solutions. Batch adsorption would be appropriate for wastes that are produced intermittently such as silage water and CW2. In these applications, either PVPP or activated carbon would be recommended. For wastewaters that are produced in larger amounts and more consistently such as AW2 and CW1 which include process water, continuous adsorption would be more appropriate and PVPP would be the recommended adsorbent. Wastewater would pass through a bed of PVPP before being discharged and phenolics would be extracted.

In both cases, the solid adsorbent would be replaced periodically and the bound phenolics would be eluted. Ethyl acetate showed higher elution efficiency than ethanol and it is cheaper than absolute ethanol. Ethanol, however, could be produced in the fermentation stage of wastewater treatment so may be available on site. In either case, it must be remembered that while ethanol and ethyl acetate are far less toxic than solvents such as toluene, they are nonetheless harmful in high doses. Eluants would need to be concentrated for use in cosmetic applications and for food applications, the solvent would need to be removed from the antioxidant extract. Additionally, if the solid adsorbant is reused after elution, care must also be taken to remove any residual solvent from the adsorbant before reintroducing it to the treatment process. Ethanol is miscible with water and ethyl acetate is soluble in water up to 8.3 g/100 ml and any solvent remaining in the adsorbent bed would contaminate the first batches of wastewater treated. These concerns should be amply compensated for by the reduced costs of treating wastewaters, the added benefit of a product stream and the benefit of a more sustainable process.

4.4 Fermentation of Fruit Wastewaters

The sugar concentration in the treated wastewaters is low and there is evidence that natural fermentation has already occurred. This is to be expected in wastewaters that are drawn from storage tanks such as CW2 and SW. Wastewater has accumulated in the processing plant and micro-organisms have begun to metabolise any available sugars. Actively growing bacteria can deplete a solution of sugars within hours and yeasts can do so in a day and it follows that wastewater that has been stored for more than a few hours will likely have begun to ferment. Wastewaters that are not drawn from storage tanks will be less susceptible to prior fermentation and free sugars were present in CW1. For laboratory experiments, wastewaters were autoclaved before testing to eliminate the effect of naturally present micro-organisms but in a processing plant, wastewaters will not be sterile and should be expected to contain micro-organisms capable of growth. The availability of sugars will be inversely proportional to the time which has elapsed between wastewater production and onset of controlled fermentation with a chosen micro-organism for a chosen product. The implication here for research is that samples must be frozen as soon as possible after collection and should be frozen in aliquots to prevent repeated thawing and refreezing if a multiple step treatment process is envisioned. The implication for application in the fruit processing industry is that treatment should be either continuous or frequent, if a batching application is chosen.

When choosing a micro-organism the non-sterile nature of the wastewater should also be considered. Micro-organisms chosen for the fermentation should be fast growing to enable them to compete against micro-organisms already present. It would be beneficial for the chosen culture to be able to utilise not only sugars but also other more complex carbon sources for optimal production. Ability to utilise partially fermented nutrients in the wastewater, such as acetate, would also increase production yield.

Dilute wastestreams lend themselves to continuous fermentation in which waste is continuously added to the fermentation vessel and treated wastewater is removed. This would be feasible if the product is easy to separate from the fermentation broth. Where the product is biomass, continuous fermentation with a settling tank or physical separation to collect the biomass would be best. This may then result in an overflow stream of water with greatly reduced COD with the concurrent gradual build up of biomass over time. If a metabolite is the desired product, it should be one that is easy to separate. One example would be the production of a compound that would be retained on an ion-exchange column. The fermentation outflow could pass through the column bed and the product would gradually accumulate over time and be harvested when efficient. Where the metabolite

requires more intensive processing to separate, such as ethanol, concentration of the wastestream is essential to minimise the volume which must be processed downstream.

For cost efficient recovery, Öhgren et al. (2006) set a goal concentration of 40 g/l ethanol in solution – below this level, the distillation costs involved in recovering the ethanol, would be prohibitive. Fruit wastewaters samples had sugar concentrations of between 33 µg/l and 2.3 g/l (glucose and fructose as determined by HPLC). *S. cerevisiae* can produce a maximum of 0.51 g ethanol per g sugar (Öhgren et al., 2006) and the minimum sugar concentration for production of 40 g/l ethanol is therefore 78.43 g/l sugar. A minimum concentration factor of 35 fold is therefore needed if fruit wastewater is to be used for ethanol production. Using reverse osmosis, 2.76 fold concentration was achieved. Recommendations for increasing the concentration of sugar in the wastewater are given in Section 5

In this work, bacterial fermentations were performed using unconcentrated waste and the results are therefore understandably disappointing. Aerobic fermentation after SPE using cartridges was not successful and micro-aerobic fermentation only allowed minimal product formation. The sugar concentration here was not sufficient to support growth and metabolism. Fermentation after PVPP extraction was more successful because this method of extraction allows solid particles of fruit waste to remain in the treated solution, providing an additional source of carbon. In AW1, sugars and acids were utilised for 2,3-butanediol formation. In AW2, no growth was observed and this is attributed to the lack of free sugars and relatively high acetate concentration which may have been inhibitory. In CW1, both acids and alcohols were produced by the bacterial culture TH141. The growth curves show a slight decrease in optical density over the course of the aerobic fermentation. It is likely therefore that the bacteria were not only utilising the simple nutrients in the solution but were also digesting particulate matter and utilising this for metabolism. In other work, TH141 has been grown on plates in which the sole carbon source was grass, bran or xylan and it has therefore shown the ability to digest lignocellulose components

In CW2, acetate was consumed and two unknown peaks were seen to increase in area. Under aerobic growth, the optical density of the medium decreased in the later stages of the fermentation suggesting that the bacteria were utilising the particulates and colour compounds in the medium. TH141 was not able to produce the range of metabolites produced in CW1. This suggests that the particulates were not a rich source of available carbon for entry into the glycolytic and citric acid cycles. It may be that the two unknown peaks were break-down products from these particulates or colour compounds which were

able to act in a redox capacity, allowing the utilisation of acetate in the wastewater, but which were not available as fermentable carbon.

The yeast *S. cerevisiae* was able to grow and produce ethanol in a synthetic wastewater constituted as synthetic model apple wastewater, concentrated 6 fold. Gallic acid in the concentration range of 0.02 to 0.1 g/l, was found to have no effect on the growth of *Saccharomyces cerevisiae* or on ethanol production. Higher concentrations of gallic acid, 0.1 to 0.3 g/l, enhanced ethanol production, increasing the final ethanol concentration from 3.77 g/l to 6.16 g/l. Enriching apple wastewater with solid apple waste could have greater effects than anticipated if, alongside the fermentation of additional sugars released from the solid waste, released phenolics also contributed to increases in ethanol production.

Solid apple waste which has been treated using steam explosion, acid digestion or enzyme digestion, contains free sugars which are available for fermentation to ethanol. The absolute concentration of sugars after pretreatment is dependent on the levels of sugar in the apples initially. Comparative release of sugars showed that where the sugar level in the apples is high, physical disruption using heat releases the most sugar while acid digestion causes a decrease in available sugar. Where the sugar concentration is lower, treatment with enzymes is most effective in releasing sugar. Enzymatic pretreatment was also effective in increasing the concentration of sugars from sugar-rich solid apple waste and it is therefore recommended as the pretreatment best suited to a range of apples from different cultivars and at different stages of ripening.

4.5 Concentration of Fruit Wastewaters using Reverse Osmosis

Concentration of the wastewater using reverse osmosis is possible and a 2.76 fold concentration was achieved. Increasing processing time may increase the concentration factor. Instead of using a cellulose acetate membrane, investigation of polyamide membranes is recommended as these should prove more resistant to microbial degradation with prolonged use.

5 RECOMMENDATIONS – FROM LABORATORY BENCH TO COMMERCIAL PROCESS

Beneficiation of agri-industry wastewaters is envisioned as a process comprised of a number of unit operations. Fruit waste and wastewater would progress through sequential operations, releasing purified water and producing value-added products.

The process flow envisioned is shown in Figure 5.1:

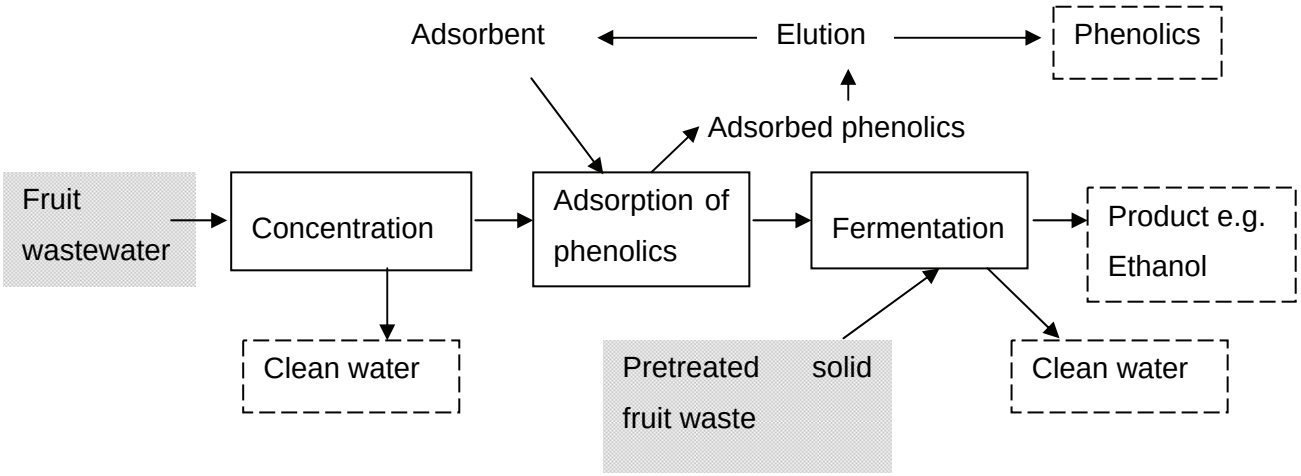


Figure 5.1: Process flow sheet, illustrating the recovery of clean water, antioxidant phenolics and value added products from fruit wastewater

5.1 Fruit Wastewater

Fruit wastewaters samples contained between 7.6 and 137 mg/l phenolics (expressed as GAE) and had sugar concentrations of between 33 µg/l and 2.3 g/l (glucose and fructose as determined by HPLC). These concentrations are very low and any process of phenolic extraction or fermentation based on such a dilute wastestream would be inefficient. Selection of wastestreams is therefore of primary importance. Concentrated wastestreams must be identified within the processing plant and these must not be mixed with dilute wastewater from washing operations. Storage time must also be minimised to prevent uncontrolled fermentation by natural organisms.

5.2 Concentration

Concentration of 2.76 fold is possible through use of a reverse osmosis membrane. This is not sufficient to provide economically viable phenolics extraction or fermentation and further

studies should therefore be performed to optimise the concentration process. Additionally, other investigations into membrane concentration should be considered, particularly, testing a greater diversity of membranes, including polyamide membranes which would be more resistant to microbial digestion. The greater the degree of concentration achieved in the first step, the smaller the volumes of wastewater to be treated in subsequent steps. Smaller volumes require less processing space and less energy in the form of electricity for pumping and heating.

5.3 Adsorption of Phenolics

Of the adsorbents tested, it is recommended that either PVPP or activated carbon be used for the absorption of phenolics. Both of these adsorbents are currently used in the fruit processing industry to adsorb phenolics and thereby prevent haze formation. PVPP was the best adsorbent in terms of rapid adsorption, allowing for either continuous or batch applications, as well as being able to absorb higher percentages of phenolics from concentrated solutions. Higher percentages of bound phenolics could also be eluted from this adsorbent. Activated carbon had the advantage of being able to adsorb a larger range of phenolics with slightly lower capacity and less efficient elution but it has a clear cost advantage. The choice of adsorbent would depend on the constraints of the process (whether continuous or batch was required) and the financial implications.

With PVPP, the reject from the membrane concentration could be passed through a bed of adsorbent while the emerging water is monitored for phenolics. When the level of phenolics is seen to rise (after approximately 125 mg of phenolics have been treated per gram of adsorbent), the PVPP would be replaced with fresh or regenerated PVPP. With activated carbon, a batch process would be preferable where tanks would be filled with the reject from membrane concentration and allowed to interact for up to four hours. The material in the tanks would be allowed to settle and the treated water would be decanted. Activated carbon tanks could be reused until the decanted water showed increasing levels of phenolics.

While the above methods of antioxidant extraction are currently recommended, future investigations of supported liquid membrane extraction and supercritical fluid extraction may show that one of these techniques proves more advantageous. Supported liquid membrane extraction should be investigated using a less toxic solvent to extract phenolics since toluene is prohibited in products that will be used as food additives. Solvent-water mixtures such as methanol-water or ethanol-water can be used. Furthermore, it is recommended that other membrane modules such as the Liqui-Cell membrane be used since Microdyne module used in this work is too porous and leads to mixing of phases. Recovery of phenolic

compounds using supercritical fluid extraction 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. It is recommended that the solubilities of less polar phenolic compounds be investigated as supercritical fluid may still prove effective at extracting other antioxidant phenolics from wastewater solutions.

5.4 Elution of Phenolics

Ethanol or ethyl acetate would be recommended as solvents for elution from either PVPP or activated carbon. Where sugars in fruit wastewaters are utilised for ethanol production using yeasts or bacteria, ethanol produced in this way should be used for elution. More work should be done to improve elution efficiencies however as the current investigation showed that large volumes of ethanol would be used to extract significant percentages of bound phenolics. Extracts could be concentrated by distillation of the ethanol (preferably under vacuum to avoid the use of high temperatures) and the ethanol would then be reused. Eluting a more concentrated antioxidant solution with a smaller volume of ethanol would be preferable.

5.5 Pretreatment of Solid Fruit Waste

Enzymatic pretreatment is recommended as this method releases sugars from solid apple waste with high or low sugar concentrations and is therefore best suited to a range of apples from different cultivars and at different stages of ripening.

After enzymatic treatment, sugar levels had increased by 1 to 2 g/l. The concept has been proved but optimisation of enzyme hydrolysis must still be performed. Increasing the proportion of solids in the pretreatment slurry should be investigated so as to obtain greater increases in sugar concentrations in smaller volumes. Optimal enzyme concentration should also be determined. Increases in enzyme concentration will likely increase sugar yield in a given time period but will also increase costs. The optimal loading of solids and enzyme to produce the highest possible sugar release must be balanced against the economy of the process to determine the optimal ratios.

5.6 Fermentation to Produce Products such as Ethanol

In the current work, 2,3-butanediol, was produced from CW1 after PVPP extraction using the bacterial culture TH141. Both aerobic and micro-aerobic fermentations produced 2,3-butanediol and either growth condition could be chosen. 2,3-butanediol is purified either by distillation or, because the boiling point is high at 180^{o}C making distillation expensive, by extraction into a solvent with a lower boiling point such as propanol (Sun et al., 2009)

For ethanol production by bacteria, CW1 after PVPP extraction is the recommended wastewater and fermentation must be micro-aerobic. Ethanol is purified by distillation. Both bacterial fermentations were performed on treated wastewaters which had not been concentrated in any way. Yields were therefore low but will increase as wastewater concentration increases.

In the current work, the *Saccharomyces cerevisiae* culture utilised 12.87 g/l sugar to produce 4.31 g/l ethanol (Table 3.26) in an overnight fermentation. This is a yield of 0.33 g ethanol per g sugar which is less than the theoretical maximum of 0.51 g/g and indicates that further improvements could be made. While the main challenge in lignocellulose fermentation by *S. cerevisiae* is engineering a yeast strain able to co-ferment glucose and xylose, Öhgren et al. (2006) did employ two strategies that are of interested to this work: simultaneous saccharification and fermentation and fed batch processing. Simultaneous saccharification and fermentation shorten the reaction time as both enzyme digestion and ethanol production are time consuming unit processes. Enzyme digestion proceeded for 72 hours in this work and fermentation for 24 hours after this. Combination of unit operations allows the processes to run concurrently.

Fed batch additions of solid waste allow gradual release of sugars, bypassing any inhibition of fermentation caused by high substrate concentrations. Adding solid material in a step-wise fashion also allows the total solids content to be kept lower than if all solid had been added initially and makes mixing of the solution easier. Fresh material is also added occasionally allowing optimal use of enzymes through-out the SSF process. It is recommended that both of these processes be investigated to improve the ethanol yield in fermentation.

If ethanol produced is to be used as a biofuel, it must be purified through distillation. Distillation unit processes add expense, primarily in the heating step. For biofuel applications, ethanol must contain low concentrations of water and if this is the desired application, distillation costs must be considered. A preferable application of ethanol produced is in the elution of phenolics from the solid phase adsorbent or extraction through supported liquid membranes. For this application, ethanol-water mixtures may be acceptable. A distillation unit operation will be necessary in the proposed process, both for concentration of ethanol after fermentation and for concentration of phenolic extracts but through optimisation of all unit processes, the cost of distillation should be minimised and balanced by income received from value-added products.

5.7 Concluding Remarks

The beneficiation of agri-industrial wastewaters such as fruit processing wastewater is possible. Phenolics are present in the wastewaters and these phenolics have antioxidant properties. It is possible to extract the antioxidants in a sustainable fashion to allow the creation of an added product stream as well as a resulting wastewater which has had much of the phenolics load removed, thereby reducing the cost of treating this wastewater before discharge or reuse.

There are also sugars present in the wastewater and these sugars may be utilised by micro-organisms without any need to add other components to the water. Solid fruit waste can be used to supplement the sugars available in the wastewater through use of enzymatic pretreatment. Gallic acid, which may be released into the wastewater as a result of enzymatic digestion, was not found to decrease fermentation yields but on the contrary, stimulated ethanol production, possibly even being converted to ethanol.

The major obstacle to application of the technologies detailed in this report is the dilute nature of the wastewater. Two strategies are apparent to overcome this obstacle – selection of wastestreams and concentration of wastewaters. The first and easiest strategy is for fruit processing industries to investigate their processes with a view toward identifying the most concentrated sources of waste. These source points will be different for each processing plant and for understandable reasons, sufficient knowledge of processes is limited to the staff of the fruit processing plant. The onus is therefore on the technical staff to identify suitable wastewaters. This will require a fundamental shift in thinking. Currently, the aim of wastewater treatment is to achieve a wastewater stream that is sufficiently low in COD to allow discharge. Mixing of concentrated wastestreams with the washing and processing water is therefore beneficial as this reduces and sometimes eliminates the need for costly treatment to reduce COD.

If wastewaters are instead viewed as sources of valuable products – antioxidants and fermentation metabolites – it becomes economically beneficial to keep wastestreams separate. Washing and processing water which is not mixed with more concentrated waste may require minimal or no treatment to yield water that is suitable for discharge or even reuse and this should provide savings. The concentrated wastewater would then be redirected into processes that generate other products and other income. It may still be necessary to concentrate the wastewater before antioxidant extraction and fermentation and certainly, reducing the volumes of wastewater to be treated can only increase the economy of subsequent steps.

Beneficiation of wastewater to produce clean water, antioxidant extracts and fermentation products should be considered for application in the fruit processing industry.

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7 APPENDIX:

Table A: List of all methods used in project K5/1937

Aim	Method	Purpose
Chemical analysis of wastewaters	pH	Determine pH of wastewaters
	Conductivity	Determine conductivity of wastewaters
	COD	Determine chemical oxygen demand of wastewaters
	Total solids	Determine total mass of solids present in the wastewaters
	Dissolved solids	Determine mass of dissolved solids present in the wastewaters
	Suspended solids	Determine mass of insoluble solids present in the wastewaters
	DNS assay	Quantification of reducing sugars present in the wastewaters
	Phenol-sulphuric acid assay	Quantification of total carbohydrates present in the wastewaters
	Folin Ciocalteau assay	Quantification of total phenolics present in the sample
	HPLC	Quantification of specific individual phenolics, sugars or metabolites present in the sample
Determination of antioxidant activity	DPPH assay	Radical scavenging assay for indicating hydrogen donating ability of an antioxidant
	TEAC assay	Radical scavenging assay for indicating the electron transfer ability of antioxidant compounds
	FRAP assay	Measuring the ability of an antioxidant to reduce ferric tripyridyltriazine to the ferrous form
	β-CLAMS assay	Measuring the ability of an antioxidant to inhibit lipid peroxidation
Techniques for the extraction of phenolics	Solvent extraction	Extraction of phenolics into an organic solvent through direct contact of organic and aqueous phases
	Supported liquid membrane extraction	Extraction of phenolics into an organic solvent through indirect contact via a porous membrane
	C18 Sep-Pak cartridge-based solid phase extraction	Adsorption of phenolics onto a C18 matrix by passing a solution through a pre-conditioned cartridge. Elution from the cartridge gives the antioxidant extract

	PVPP extraction	Adsorption of phenolics onto PVPP resin by mixing solid and aqueous phases and allowing PVPP to settle out. Elution from the resin gives the antioxidant extract
	Amberlite XAD4 extraction	Adsorption of phenolics onto Amberlite resin by mixing solid and aqueous phases and allowing Amberlite to settle out. Elution from the resin gives the antioxidant extract
	Activated carbon extraction	Adsorption of phenolics onto activated carbon by mixing solid and aqueous phases and allowing activated carbon to settle out. Elution from the activated carbon gives the antioxidant extract
	Supercritical fluid extraction	Extraction of phenolics into supercritical CO_2. Evaporation of CO_2 leaves the antioxidant extract
Fermentation of wastewaters	Micro-aerobic fermentation by *Saccharomyces cerevisiae*	Conversion of sugars to ethanol by the yeast *Saccharomyces cerevisiae*
	Aerobic fermentation by TH141 and NB4	Conversion of sugars to CO_2 and harvestable biomass to lower the COD of wastewaters. Fermentation is performed by bacterial cultures TH141 and NB4
	Micro-aerobic fermentation by TH141 and NB4	Conversion of sugars to 2,3-butanediol and ethanol by bacterial cultures TH141 and NB4
	Pretreatment of apple pomace	Supplementation of wastewaters with solid apple pomace made fermentable by pretreatments: heat, chemical or enzymatic
Membrane concentration	Reverse osmosis membrane concentration	Concentration of wastewaters using a reverse osmosis membrane